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## Antimicrobial management of intra-abdominal infections: Literature's guidelines

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### Abstract

Antimicrobial management of severe intra-abdominal infections (IAIs) involves a delicate balance of optimizing empirical therapy, which has been shown to improve clinical outcomes, while simultaneously reducing unnecessary antimicrobial use. Two sets of guidelines for the management of intra-abdominal infections were recently published. In 2010, the Surgical Infection Society and the Infectious Diseases Society of America (SIS-IDSA) created guidelines for the diagnosis and management of complicated IAIs. The new SIS-IDSA guidelines replace those previously published in 2002 and 2003. The World Society of Emergency Surgery (WSES) guidelines represent additional contributions, made by specialists worldwide, to the debate regarding proper antimicrobial drug methodology. These guidelines represent the conclusions of the consensus conference held in Bologna, Italy, in July 2010 during the first congress of the WSES.

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**Key words:** Intra-abdominal infections; Antimicrobial therapy; Literature's guidelines

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### INTRODUCTION

Intra-abdominal infections (IAIs) encompass a variety of pathological conditions, ranging from uncomplicated appendicitis to fecal peritonitis. Cases of IAI are further subcategorized as being either uncomplicated or complicated<sup>[1]</sup>.

In the event of an uncomplicated case of IAI, the infection only involves a single organ and does not extend to the peritoneum. Patients with such infections can be treated with either surgical resection or antibiotics. When the infection is effectively resolved by surgical excision, 24-h perioperative prophylaxis is typically sufficient. Patients with IAIs, including acute diverticulitis and certain forms of acute appendicitis, may be treated non-operatively by means of antimicrobial therapy.

In the event of complicated IAI, the infectious process proceeds beyond the organ, causing either localized or diffuse peritonitis. The treatment of patients with complicated IAIs involves both source control and antibiotic therapy.

Antimicrobial therapy plays an integral role in the management of IAIs, especially in critically ill patients who require immediate empiric antibiotic therapy. An insufficient or otherwise inadequate antimicrobial regimen is one of the variables most strongly associated with unfavorable outcomes<sup>[2,3]</sup>.

Various studies have demonstrated that inappropriate

antimicrobial use is common. Excessive antimicrobial use has contributed to the emergence and spread of drug-resistant microorganisms and has simultaneously increased overall treatment costs<sup>[4-9]</sup>.

An antimicrobial-based approach to treating IAIs always involves a delicate balance between the optimization of empirical therapy, which has been shown to improve clinical outcomes, and the reduction of excessive antimicrobial use, which has been proven to increase the rate of emergence of antimicrobial-resistant strains.

The threat of antimicrobial resistance has been identified as one of the major challenges in the management of complicated IAIs.

In the past few decades, an increased prevalence of infections caused by antibiotic-resistant pathogens, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* species, carbapenem-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*), extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) and *Klebsiella* species, and multidrug-resistant *Acinetobacter* species, has been observed, especially in IAIs.

To resolve the medical community's tendency to over-use antibiotics, a set of guidelines outlining the proper use of antimicrobial therapy has been implemented, which contains specific directions for addressing IAIs.

Two different sets of guidelines outlining the clinical management of IAIs were recently published.

In 2010, the Surgical Infection Society (SIS) and the Infectious Diseases Society of America (IDSA) instituted standardized guidelines for the diagnosis and management of complicated IAIs<sup>[10]</sup>.

The new SIS and IDSA guidelines replace those previously published in 2002 and 2003.

The World Society of Emergency Surgery (WSES) guidelines<sup>[11]</sup> represent an additional contribution to the debate by specialists worldwide. These guidelines represent the conclusions reached by the consensus conference held in Bologna, Italy, in July 2010, during the first congress of the WSES; in attendance at this event were surgeons, infectious disease specialists, pharmacologists, radiologists and intensivists, all of whom wished to define and streamline a standardized set of recommendations for the early treatment and management of IAIs<sup>[11]</sup>.

## **GUIDELINES BY SIS AND IDSA: ANTIMICROBIAL MANAGEMENT FOR COMPLICATED INTRA-ABDOMINAL INFECTIONS**

In the SIS and IDSA guidelines, selection of the appropriate antimicrobial regimen is based primarily on the "risk factor" of the potential failure of the treatment in question.

"High risk" describes patients with an increased likelihood of treatment failure and a greater potential severity of infection according to clinical assessment criteria. Such patients include those with anatomically unfavorable infections or health care-related infections<sup>[10]</sup>.

Clinical factors predicting failure of treatment for IAIs include: (1) delay in the initial stages of intervention (24 h); (2) high severity of illness (Acute Physiology and Chronic Health Evaluation II score > 15); (3) advanced age of patient; (4) comorbidity involving organ dysfunction; (5) low albumin levels; (6) poor nutritional status; (7) peritoneal involvement or diffuse peritonitis; (8) inability to achieve adequate debridement or control of drainage; (9) presence of malignancy; and (10) health care-related infection.

Health care-related infections refer to a spectrum of adult patients treated in acute care hospitals or monitored in chronic care settings. These patients increase their risk of infection due to the emergence of multidrug-resistant bacteria. Health care-related infections have higher risks of complication and mortality than community-acquired disease.

Guidelines developed by the SIS and the IDSA have recommended various single-agent and combination regimens for patients with different levels of risk.

### **Extra-biliary community-acquired intra-abdominal infections**

In the treatment of patients with community-acquired IAIs, empiric antimicrobial therapy should protect against common gram-negative and anaerobic enteric bacteria.

The SIS and IDSA guidelines classify community-acquired IAIs as being mild, moderate, or severe on the basis of the patient's assessed risk factors.

For high severity infections, those cases for which adequate empirical therapy helps reduce the rate of mortality, regimens having a broader spectrum of antimicrobial activity are recommended.

For adult patients with mild-to-moderate community-acquired infections, the SIS-IDSA guidelines recommend the use of ticarcillin-clavulanate, cefoxitin, ertapenem, moxifloxacin, or tigecycline as single-agent therapies; the guidelines also advocate combinations of metronidazole with cefazolin, cefuroxime, ceftriaxone, cefotaxime, or levofloxacin, as opposed to single agents featuring broader antimicrobial activity.

The empiric use of antimicrobial regimens with broad-spectrum activity against gram-negative organisms, which include meropenem, imipenem-cilastatin, doripenem, piperacillin-tazobactam as single-agent therapies, or ciprofloxacin, levofloxacin ceftazidime, cefepime each combined with metronidazole, is recommended by the SIS-IDSA guidelines for treating high-severity community-acquired IAIs.

(Due to the increasing resistance of *E. coli* to fluoroquinolones, local population susceptibility profiles and, if available, isolate susceptibilities should be reviewed).

The SIS-IDSA guidelines do not recommend the routine use of agents effective against enterococci in community-acquired infections, even if infections caused by these organisms may be associated with poorer clinical outcomes<sup>[10]</sup>.

Additionally, antifungal protection is not required for community-acquired infections.

According to the guidelines, Aminoglycosides should be reserved for patients allergic to  $\beta$ -lactam agents and, even in these cases, they are “last resort” options that should be used only when quinolone-based regimens are unavailable. That said, depending on the local susceptibility patterns of nosocomial gram-negative bacilli, Aminoglycosides may be a reasonable choice for the empiric or definitive treatment of certain patients with health care-related IAIs.

#### **Health care-associated intra-abdominal infections**

Health care-related infections are commonly caused by more resistant strains, which may include the non-fermenting gram-negative *P. aeruginosa*, *Acinetobacter* species, *E. coli*, *Enterobacter* species, *Proteus* species, methicillin resistant *Staphylococcus aureus*, enterococci, *Candida* species, and extended spectrum  $\beta$ -lactamase-producing *Klebsiella*. For these infections, given that adequate empiric therapy appears to be a crucial factor affecting postoperative complications and mortality rates, complex multidrug regimens are recommended.

According to the SIS-IDSa guidelines, antibiotic selection should always be tailored to address the nosocomial microorganisms known to be present at the facilities in which the patient developed the infection.

#### **Biliary intra-abdominal infections**

For patients with complicated biliary IAIs, selection of a specific antimicrobial therapy should be based on the origin of the infection (community versus health care), on the severity of illness, and on the presence or absence of a biliary-enteric anastomosis.

For biliary infections, anaerobic therapy is not recommended unless a biliary-enteric anastomosis is present.

Regarding community-acquired biliary infections, antimicrobial activity against enterococci is not required because such strains have not proven to be pathogenic. For certain immunosuppressed patients, however, particularly for those who have undergone extensive hepatic-related procedures or liver transplants, enterococcal infections can be clinically significant and may require treatment.

For community-acquired acute cholecystitis of mild-to-moderate severity, the SIS and IDSA guidelines recommend treatment regimens of cefazolin, cefuroxime, or ceftriaxone. On the other hand, for community-acquired acute cholecystitis causing severe physiologic disturbance, advanced age, and/or immunocompromise, the IDSA guidelines recommend Imipenem-cilastatin, meropenem, doripenem, piperacillin-tazobactam as single-agent therapies, or ciprofloxacin, levofloxacin, cefepime, each in combination with metronidazole. Contrastingly, for acute cholangitis of any severity grade following bilio-enteric anastomosis, the SIS-IDSa guidelines recommend Imipenem-cilastatin, meropenem, doripenem, piperacillin-tazobactam as single-agent therapies, or ciprofloxacin, levofloxacin, cefepime, each in combination with metronidazole. For health care-related biliary infection of any severity grade, the IDSA guidelines recommend Imi-

penem-cilastatin, meropenem, doripenem, piperacillin-tazobactam, or ciprofloxacin, levofloxacin, cefepime, each in combination with metronidazole, supplementing them with vancomycin.

(Due to the increasing resistance of *E. coli* to fluoroquinolones, local population susceptibility profiles and, if available, isolate susceptibilities should be assessed and systematically reviewed).

## **GUIDELINES BY WSES: ANTIMICROBIAL MANAGEMENT FOR INTRA-ABDOMINAL INFECTIONS**

Patients with IAIs are classified by SIS-IDSa guidelines into low risk and high risk.

However the definition of “risk” in IAIs remains too vague. Dividing patients with IAIs into lower and higher risk categories may be not easy, and attempting to assess a patient’s risk of treatment failure may be not sufficient to optimize an antimicrobial treatment plan.

In order to stratify the patients with IAIs, WSES guidelines stratify patients with IAIs according to the specific risk for antimicrobial resistant bacteria and to the clinical patient’s severity.

In order to better identify the pathogens present and evaluate the associated resistance patterns, infections are classified as being either community- or hospital-acquired.

In the past two decades, the incidence rate of hospital-acquired infections caused by resistant microorganisms has risen significantly, a finding that is probably correlated with higher levels of antibiotic exposure and an increasing number of patients with one or more predisposing conditions such as recent exposure to antibiotics, high severity of illness, advanced age, comorbidity, degree of organ dysfunction, low albumin level, poor nutritional status, immunocompromise, and the presence of malignancy.

In the last years, the level of resistance has become significant also in the community acquired infections. The main resistance problem in IAIs is represented by ESBL producers Enterobacteriaceae, even today frequently found in community acquired infections<sup>[12,13]</sup>.

The available therapeutic options for the treatment of ESBL-associated infections are limited by drug resistance conferred by the ESBLs<sup>[14,15]</sup>.

The third generation cephalosporins, recommended by SIS-IDSa for high risk patients in association with metronidazole, should not be used to treat suspected infections with ESBL producing organisms because clinical outcome is poor even in the presence of apparent susceptibility<sup>[14]</sup>.

Also cefepime should not be used as the first line therapy against ESBL-producing organisms<sup>[14]</sup>.

Piperacillin-tazobactam, recommended by SIS-IDSa guidelines for high risk patients, is not regarded as suitable first line therapy for serious infections caused by ESBL producer<sup>[14]</sup>.

Ciprofloxacin has been a potential antimicrobial option for the treatment of infections caused by ESBL-producing enterobacteriaceae; however, in recent years, the usage of ciprofloxacin has risen, and ESBL-producing isolates resistant to fluoroquinolones has increased over time also in *E. coli*<sup>[14]</sup>.

For two decades Carbapenems have been the antibiotics of first choice for ESBLs.

The increased carbapenem consumption has been associated to increasing of carbapenem-resistant bacterial species<sup>[13]</sup>.

The rapid spread of carbapenemases in *Klebsiella pneumoniae* (*K. pneumoniae*)<sup>[16]</sup> emphasizes the concept that the usage of carbapenems should be optimized in terms of indication and exposure.

Therefore, group 2 carbapenems should be used in community acquired IAIs only in critically ill patients where inadequate antimicrobial therapy may have a significant impact on the patients mortality, independently by the site of infection.

The choice of the antimicrobial regimen poses serious problems for the management of critically ill patients. In patients with severe sepsis or septic shock an early correct empirical antimicrobial therapy has a significant impact on the outcome, independently by the site of infection.

It is confirmed by a recent prospective observational study, involving 180 consecutive patients with secondary generalized peritonitis, by Riché *et al*<sup>[17]</sup> that demonstrated, a significantly higher mortality rate in septic shock (35% vs 8% for patients without shock).

Recently published international guidelines outlining the proper management of severe sepsis and septic shock (Surviving Sepsis Campaign)<sup>[3]</sup> recommend the intravenous administration of antibiotics within the first hour following diagnosis; the use of broad-spectrum agents that can effectively penetrate the presumed site of infection; and the daily reassessment of the antimicrobial regimen in order to optimize treatment efficacy, prevent the development of drug resistance, avoid drug-induced toxicity, and minimize the overall cost of hospitalization.

For years, antibiotics have typically been used as single-agent therapies; only once microbiological cultures and susceptibility tests had been performed were more potent compounds then administered. The traditional approach, however, may no longer be appropriate for critically ill patients in the current context of increasing antibiotic resistance.

Increasing rates of antibiotic resistance and a better understanding of the inflammatory process together prompted the medical community to begin advocating the use of broad-spectrum regimens initially when treating critically ill patients.

This two-stage approach, consisting of aggressive initial therapy followed by a less intense follow-up treatment, allows for the immediate and effective treatment of serious infections while simultaneously avoiding the overuse of antibiotics, potential microbial resistance, and excessive hospitalization costs.

### Community-acquired intra-abdominal infections

Empirical antibiotic treatment of community-acquired IAIs should be conducted in accordance with the most frequently isolated germs and the local trends of antibiotic resistance. The major pathogens involved in community-acquired IAIs are enterobacteriaceae, streptococci, and anaerobes. The primary problems with resistance stem from ESBL-producing enterobacteriaceae, which are frequently found in community-acquired infections<sup>[12,13]</sup>.

Many factors can increase the risk of ESBL selection, but prior exposure to antibiotics (mainly third generation cephalosporins) and comorbidities that continuously require antibiotic treatment regimens, are among the most significant predisposing criteria<sup>[18]</sup>.

In the event of community-acquired IAIs, antimicrobial therapy for enterococci should be considered on a patient-by-patient basis, mainly for critically ill and immunocompromised patients as well as patients with valvular heart disease or prosthetic implants.

Community-acquired IAIs may be treated with either single or multiple antimicrobial regimens depending on the patient's condition as well as the predominant risk factors for specific microorganisms and resistance patterns. For stable, non-critical patients presenting with no ESBL-associated risk factors, amoxicillin/clavulanate and ciprofloxacin plus metronidazole regimens are recommended. Contrastingly, for critically ill patients presenting with no ESBL-associated risk factors, treatments of piperacillin/tazobactam are recommended.

On the other hand, for stable, non-critical patients presenting with ESBL-associated risk factors, ertapenem or tigecycline treatments are recommended. Contrastingly, for critically ill patients presenting with ESBL-associated risk factors, meropenem or imipenem plus fluconazole regimens (the latter in the event of risk factors for *Candida*) are recommended.

Antimicrobial regimens recommended by WSES<sup>[11]</sup> for treating extra-biliary community-acquired IAIs was summarized in Table 1.

### Biliary intra-abdominal infections

Antibiotics are always recommended when treating complicated cholecystitis and advanced uncomplicated cholecystitis.

The most important factors for antimicrobial drug selection in biliary infections are the following: antimicrobial activity against causative bacteria, the clinical condition of the patient in question, and the biliary levels of the antimicrobial agents.

An antibiotic's in-bile efficacy as well as the manner in which it is ultimately secreted into the bile are also important selection criteria when choosing an appropriate drug regimen.

The microorganisms that are most often isolated in biliary infections are the gram-negative aerobes, *E. coli* and *K. pneumoniae*, and several anaerobes, especially *Bacteroides fragilis*. Activity against enterococci is not typically required since their pathogenicity in biliary tract infections remains unclear<sup>[19,20]</sup>.

**Table 1** Antimicrobial regimens recommended by the World Society of Emergency Surgery recommendations for treating extra-biliary community-acquired intra-abdominal infections

	Antimicrobial agents	Dosage
In stable, non-critical patients		
With no ESBL-associated risk factors	Amoxicillin/clavulanate	2.2 g every 6 h (2-h infusion time)
	Ciprofloxacin	400 mg every 8 h (30-min infusion time)
With ESBL-associated risk factors	+	
	Metronidazole	500 mg every 6 h (1-h infusion time)
	Ertapenem	1 g every 24 h (2-h infusion time)
	Tigecycline	100 mg LD then 50 mg every 12 h (2-h infusion time)
In critically ill patients presenting		
With no ESBL-associated risk factors	Piperacillin/tazobactam	9 g LD then 18 g per day <i>via</i> continuous infusion or 4.5 g every 6 h (4-h infusion time)
	Meropenem	500 mg every 6 h (6-h infusion time)
With ESBL-associated risk factors	or	
	Imipenem	500 mg every 4 h (3-h infusion time)
	+	
	Fluconazole	600 mg LD then 400 mg every 24 h (2-h infusion time)

ESBL: Extended-spectrum  $\beta$ -lactamase; LD: Loading dose.

**Table 2** Antimicrobial regimens recommended by the World Society of Emergency Surgery recommendations for treating biliary intra-abdominal infections

	Antimicrobial agents	Dosage
In stable, non-critical patients		
With no ESBL-associated risk factors	Amoxicillin/clavulanate	2.2 g every 6 h (2-h infusion time)
	Ciprofloxacin	400 mg every 8 h (30-min infusion time)
With ESBL-associated risk factors	+	
	Metronidazole	500 mg every 6 h (1-h infusion time)
	Tigecycline	100 mg LD then 50 mg every 12 h (2-h infusion time)
In critically ill patients		
With no ESBL-associated risk factors	Piperacillin/tazobactam	9 g LD then 18 g per day <i>via</i> continuous infusion or 4.5 g every 6 h (4-h infusion time)
	Piperacillin	8 g LD then 16 g/d <i>via</i> continuous infusion or 4 every 6 h (4-h infusion time)
With ESBL-associated risk factors	+	
	Tigecycline	100 mg LD then 50 mg every 12 h (2-h infusion time)
	+/-	
	Fluconazole	600 mg LD then 400 mg every 24 h (2-h infusion time)

ESBL: Extended-spectrum  $\beta$ -lactamase; LD: Loading dose.

The efficacy of antibiotics in treating biliary infections depends largely on the drugs' resulting biliary concentrations<sup>[21-23]</sup>.

However, there are no clinical or experimental data available from which to infer the antimicrobial dosage that would safely maximize biliary duct penetration, and as such, no standardized recommendations have been established.

For stable, non-critical patients presenting with no ESBL-associated risk factors, amoxicillin/clavulanate or ciprofloxacin plus metronidazole regimens are recommended.

For stable, non-critical patients presenting with ESBL-associated risk factors, Tigecycline is recommended.

For critically ill patients presenting with no ESBL-associated risk factors, Piperacillin/tazobactam is recommended.

For critically ill patients presenting with ESBL-associated risk factors, tygecycline plus piperacillin (plus flu-

conazole in the event of risk factors for *Candida*) is the recommended drug regimen.

Antimicrobial regimens recommended by WSES<sup>[11]</sup> for treating biliary IAIs was summarized in Table 2.

### **Hospital-acquired intra-abdominal infections**

Hospital-acquired IAIs are, by definition, infections that were not present upon hospital admission but become evident at least 48 h following admission in patients hospitalized for a reason other than IAIs.

The threat of antimicrobial resistance has been identified as one of the major challenges in the management of complicated IAIs.

Hospital-acquired infections are commonly caused by more resistant strains, and for these infections, complex multi-drug regimens are usually recommended.

The use of anti-enterococcal drugs in empirical antibiotic regimens to treat nosocomial IAIs is always warranted if directed against *Enterococcus faecalis*.

**Table 3** Antimicrobial regimens recommended by the World Society of Emergency Surgery recommendations for hospital-acquired intra-abdominal infections

	Antimicrobial agents	Dosage
In stable, non-critical patients	Piperacillin +	8 g LD then 16 g/d <i>via</i> continuous infusion or 4 every 6 h (4-h infusion time)
	Tigecycline +	100 mg LD then 50 mg every 12 h (2-h infusion time)
	Fluconazole	600 mg LD then 400 mg every 24 h (2-h infusion time)
In critically ill patients	Piperacillin +	8 g LD then 16 g/d <i>via</i> continuous infusion or 4 every 6 h (4-h infusion time)
	Tigecycline +	100 mg LD then 50 mg every 12 h (2-h infusion time)
	Echinocandin Caspofungin	(loading dose of 70 mg, then 50 mg daily)
	Anidulafungin	(loading dose of 200 mg, then 100 mg daily)
	Micafungin	(100 mg daily)
	Meropenem or	500 mg every 6 h (6-h infusion time)
	Imipenem or	500 mg every 4 h (3-h infusion time)
	Doripenem +	500 mg every 8 h (4-h infusion time)
	Teicoplanin +	1.6 g <i>via</i> continuous infusion or 400 mg every 6 h (4-h infusion time)
	Echinocandin Caspofungin	(loading dose of 70 mg, then 50 mg daily)
	Anidulafungin	(loading dose of 200 mg, then 100 mg daily)
	Micafungin	(100 mg daily)

LD: Loading dose.

The recently published IDSA guidelines for the treatment of invasive candidiasis don't explicitly address candidal peritonitis<sup>[24]</sup>. However, the use of echinocandins is generally favored as a first-line empirical therapy in treating critically ill patients, while fluconazole is typically used for patients with less severe conditions. Consequently, by applying these trends to the context of IAIs one might suggest the prescription of echinocandins as a first-line treatment for cases of severe nosocomial IAIs.

For stable, non-critical patients presenting with risk factors for multidrug-resistant pathogens, fluconazole and tigecycline plus piperacillin are recommended.

In critically ill patients presenting with risk factors presenting for multidrug-resistant pathogens meropenem, imipenem/cilastatin, and doripenem (plus an echinocandin and Teicoplanin) or Tigecycline (plus an Echinocandin and Piperacillin) are recommended.

Antimicrobial regimens recommended by WSES<sup>[11]</sup> for hospital-acquired IAIs was summarized in Table 3.

## CONCLUSION

Proper empiric antimicrobial therapy has an enormous effect on the morbidity and mortality rates of patients suffering from IAIs, especially those who are critically ill. Inappropriate antibiotic treatments of IAIs may result in poor patient outcome. Furthermore, the selection of an appropriate antimicrobial agent has become a significant challenge due to the emerging resistances of target organisms to commonly prescribed antibiotics.

To more effectively customize antimicrobial treatment

regimens, guidelines outlining the proper therapeutic protocol for administering antimicrobial drugs have been developed to help clinicians to better and more efficiently treat IAIs.

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## Evaluation of inflammatory activity in Crohn's disease and ulcerative colitis

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### Abstract

Crohn's disease and ulcerative colitis evolve with a relapsing and remitting course. Determination of inflammatory state is crucial for the assessment of disease activity and for tailoring therapy. However, no simple diagnostic test for monitoring intestinal inflammation is available. Noninvasive markers give only indirect assessments of disease activity. Histopathological or endoscopical examinations accurately assess inflammatory activity, but they are invasive, time consuming and expensive and therefore are unsuitable for routine use. Imaging procedures are not applicable for ulcerative colitis. The usefulness of ultrasound and Doppler imag-

ing in assessing disease activity is still a matter of discussion for Crohn's disease, and an increased interest in computed tomography enterograph (CTE) has been seen, mainly because it can delineate the extent and severity of bowel wall inflammation, besides detecting extraluminal findings. Until now, the available data concerning the accuracy of magnetic resonance enterography in detecting disease activity is less than CTE. Due to this, clinical activity indices are still commonly used for both diseases.

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**Key words:** Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Diagnostic test; Therapy; Inflammatory markers

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### INTRODUCTION

Inflammatory bowel disease (IBD) comprises two major disease entities: Crohn's disease (CD) and ulcerative colitis (UC). Its etiology is not completely understood, but it is characterized by chronic inflammation of the gastrointestinal tract. Treatment is generally effective in relieving symptoms, but is not curative. Typically, these diseases evolve with

a relapsing and remitting course. Exacerbations are characterized by diarrhea, abdominal pain and rectal bleeding.

Determination of inflammatory activity is crucial for the assessment of disease activity and for tailoring the therapy. Ideally, a disease marker must be disease specific, mirror the presence of activity, be easily applicable in clinical practice and identify patients at risk for relapse. However, no such disease markers have been described so far.

Numerous clinical activity indices and other noninvasive markers are used in IBD, but they all give only indirect assessments of disease activity and none are accurate in evaluating inflammatory activity as found by histopathological or endoscopic examination. On the other hand, endoscopic evaluation is difficult to perform, invasive, time consuming and expensive, and hence is unsuitable for routine use. In the biological era, mucosal healing in CD is associated with a longer duration of remission and fewer hospitalizations, so endoscopic evaluation becomes essential<sup>[1]</sup>. It is clear that treatment must have an impact in the natural course of the disease. In the pre-biological and pre-immunosuppressive era, more than 80% of patients with CD required some kind of surgery during their lifetimes and approximately 75% of patients displayed new lesions on endoscopy 1 year after surgery<sup>[2-4]</sup>. In UC, ultimately, up to a third of patients with extensive disease will require a colectomy at some point during the disease's course<sup>[4]</sup>.

Identifying whether patients are in a relapsing or remission phase is important to offer an adequate therapy and since intestinal symptoms are a frequent cause for referrals to gastroenterologists, it is crucial to distinguish between non-inflammatory functional problems such as irritable bowel syndrome (IBS) and IBD. IBD is characterized by unpredictable flare-ups of symptoms that impair the patient's quality of life. So, markers of inflammation are important in the follow-up of patients, especially during periods of low disease activity, when it is essential to detect sub-clinical intestinal inflammation and to predict relapsing disease. This could promote a refinement of therapy to the actual needs of each case.

This article aims to make a critical review of clinical, endoscopic, laboratory and image markers of disease with respect to their ability of establishing disease activity.

## CLINICAL ACTIVITY INDEX

The instrument most commonly used to quantify disease activity in CD has been the Crohn's disease activity index (CDAI). It is a scoring system derived from the sum of products from a list of 8 items that combines subjective symptoms, objective findings on examination and laboratory testing (Table 1)<sup>[5]</sup>. Index values of 150 or below are associated with non-active disease. Values between 151 and 220 indicate mild activity, and between 221 and 450 indicate moderate to severe activity. Values over 450 indicate extremely severe disease. However, reproducibility of the CDAI is limited by a great deal of inter-observer variation and, in fibrostenotic disease, it may reflect poorly on bowel inflammation as a cause of symptoms because it induces subjective measurements<sup>[6]</sup>. Other disease activ-

**Table 1** Crohn's disease activity index items and weighting factors

Item (daily sum per week)	Weighting factor
Number of liquid or very soft stools	2
Abdominal pain score in one week (rating, 0-3)	5
General well-being (rating, 1-4)	7
Sum of physical findings per week	
Arthritis/arthralgia	
Mucocutaneous lesions (e.g., erythema nodosum, aphthous ulcers)	
Iritis/uveiti	
Anal disease (fissure, fistula, etc.)	
External fistula (enterocutaneous, vesicle, vaginal, etc.)	
Fever over 37.8 °C	20
Antidiarrheal use (e.g., diphenoxylate)	30
Abdominal mass (no = 0, equivocal = 2, yes = 5)	10
47 minus hematocrit (males) or 42 minus hematocrit (females)	6
1-x (1-body weight divided by a standard weight)	1

ity indices include van Hees index, the Cape Town index, Oxford index and Talsted index, but none result in a better approach to identify relapses<sup>[7-10]</sup>.

In order to evaluate the overall state of well-being of patients, with a focus on various domains, the Inflammatory Bowel Disease Questionnaire is the most widely accepted disease-specific instrument and measures separate domains for bowel, social, systemic and emotional function<sup>[11]</sup>.

In UC, the clinical activity index most commonly used to define the severity of disease was established by Truelove and Witts<sup>[12]</sup>. It defines mild and severe disease activity, with moderate activity being present when there are intermediate symptoms. In the mild form there are fewer than four stools daily, with or without blood, with no systemic repercussion and a normal erythrocyte sedimentation rate (ESR). In moderate one, the number of stools daily is greater than four but with minimal systemic repercussion. In the severe form, there are more than six stools daily with blood and with evidence of systemic repercussion, as shown by fever, tachycardia, anemia, or an ESR greater than 30. Clinical remission was defined as 1 or 2 stools per day without blood, absence of fever and tachycardia, a normal hemoglobin or its tendency towards reference values, a normal erythrocyte sedimentation rate and weight gain. It is simple and easy to use, but lacks precision, especially in the definition of more severe cases. It is not clear how many systemic features are required, and, furthermore, an attack can be followed by fever, tachycardia or anemia, which would characterize it as severe, but the patient may look well. Not unrarely, patients can present moderately severe symptoms and that the original index did not predict. Additionally, neither the Truelove and Witts Severity Index nor the definitions, as clinical remission or improvement, have been validated, and also, not being quantitative, no disease severity score is generated. Various attempts to create a numerical index have been made, such as the St. Marks Index, the Clinical Activity Index (also known as the Rachmilewitz Index), the Physi-

cian Global Assessment, and the Lichtiger Index. None have been validated and there is no evidence that any of them are better than Truelove and Witts<sup>[13-16]</sup>.

Although not mentioned in the original classification of Truelove and Witts, the term fulminant is used to describe a particularly severe form and is defined by more than 10 evacuations per day, continuous presence of blood in stool, temperature above 37.5 °C, heart rate over 90 beats/min, erythrocyte sedimentation rate above 30 mm and a need for transfusion.

## NON-INVASIVE MARKERS

A great deal of research has been devoted to the search for a laboratory marker of disease activity in IBD in past decades. The reasons for this are firstly to overcome the subjectivity of symptoms by means of an objective evaluation and, secondly to avoid endoscopic and imaging procedures, which may be invasive, expensive and time-consuming<sup>[17]</sup>. With the introduction of newer biological therapies, there might be potential for laboratory markers in selecting responders along with their role in monitoring therapy<sup>[17]</sup>. Differential diagnosis with IBS and the follow-up of patients in periods of low disease activity, in order to detect sub-clinical intestinal inflammation and to predict disease relapses, are other important roles of these markers<sup>[18]</sup>.

### Serology markers

The acute phase response indicators ESR and C-reactive protein (CRP) have long been used as markers of inflammation and, consequently, of disease activity in IBD. CRP is the most studied among them and is considered to have the best performance. Produced by hepatocytes in low rates under normal circumstances, it rises rapidly in situations of systemic inflammation, under the influence of interleukin-6, tumoral necrosis factor- $\alpha$  and interleukin 1 $\beta$ <sup>[19]</sup>. It correlates well with clinical, endoscopic, radiological and cross-sectional activity markers in IBD, especially in CD, but not in UC<sup>[17,18,20,21]</sup>. CRP has the advantage of an early rise after onset of inflammation and a rapid decrease after its resolution, due to its short half-life of 19 h. In CD, ESR is hampered by its lack of specificity, slow increase and late decrease<sup>[17]</sup>. In UC there is a good correlation between ESR and disease activity, however it is not useful in distal proctitis because of the small area of inflammation involved<sup>[22,23]</sup>. In CD, ESR may correlate with colonic CD involvement<sup>[24]</sup>. Both polymerase chain reaction and ESR relate to systemic host responses but not with intestinal inflammation and, as a consequence, have no predictive value for the course of the disease<sup>[18]</sup>.

Leukocytosis, commonly found during disease activity, may be the consequence of a number of inflammatory conditions and stressful situations. It may also increase or decrease as a consequence of therapy (corticosteroids, azathioprine and 6-mercaptopurine). Thrombocytosis may occur in inflammatory states, but the range of normal values is too wide to allow for good sensitivity or specificity. Decreased levels of serum albumin may be found during activity of CD, but malnutrition, malabsorption, as well

as intestinal protein loss may also lead to albumin level reductions<sup>[17]</sup>.

Other classical acute phase proteins that can be detected in the serum of IBD patients are  $\alpha$ 1-acid glycoprotein (orosomucoid), fibrinogen, serum amyloid A,  $\beta$ 2-microglobulin,  $\alpha$ 2-globulin, and  $\alpha$ 1-antitrypsin. The levels of circulating orosomucoid correlate with disease activity of IBD as assessed by standard indices. Furthermore, circulating orosomucoid levels correlate with the protein loss into the gut, but its five day half-life in serum limits its usefulness as an indicator of improvement in disease activity<sup>[25]</sup>. Most of these acute phase markers have been sparsely studied and do not show advantages over CRP in detecting and monitoring inflammation in IBD<sup>[17,26]</sup>.

The search for an etiologic agent involved in the initiation of the immune-mediated bowel injury of IBD has led to the discovery of immune markers present in the sera of patients with CD and UC. The DNase-sensitive anti-neutrophil cytoplasmic antibody, with perinuclear highlighting (p-ANCA) on immunofluorescence, directed to a nuclear histone has been shown repeatedly to be present in the sera of 60% of UC and 20% of CD patients, with 5% of non-IBD patients being p-ANCA-positive. Anti-*Saccharomyces cerevisiae* (*S. cerevisiae*) IgA and IgG antibodies (ASCA), directed against a specific oligomannosidic epitope present on the cell wall of the yeast appears to represent an immune response to the antigens on the *S. cerevisiae* itself, or a cross reaction to an unidentified antigen present on the cell wall of a luminal bacteria<sup>[27-30]</sup>. ASCA is expressed in 60% of CD, 10% of UC, and 5% of non-IBD patients. Other microbial antigens recently identified to be involved in the IBD immune response [*Escherichia coli* outer membrane porin C (OmpC), the *Pseudomonas fluorescens* CD-related protein (I2), and anti-CBiR1 (anti-flagellin)] are present in 50% of CD patients and uncommon or not detected in the UC and non-IBD population. The role of these antigens in the diagnosis of IBD, in the differential diagnosis between CD and UC and in disease stratification and course are promising. The role of these emerging antigens as indicators of disease activity has not been established<sup>[26,30-33]</sup>.

Recently, it was observed that elevated serum levels of antibodies specific for certain carbohydrate structures might have a relationship with CD<sup>[34]</sup>. Malickova *et al.*<sup>[35]</sup> evaluated anti-chitobiose carbohydrate antibody, anti-laminaribiose, carbohydrate antibodies, and anti-mannobiose carbohydrate antibodies in Central European patients with IBD and concluded that a panel of anti-carbohydrate antibodies might provide additional help in distinguishing IBD from non-IBD disease patterns. However, anti-carbohydrate assays are not helpful for predicting CD behavior<sup>[35]</sup>. Another study, conducted by Rieder *et al.*<sup>[36]</sup>, showed the clinical value of serum anti-glycan antibodies for the prediction of a more complicated disease course in adult patients with CD.

The relationship between pro-inflammatory cytokine serum levels and IBD activity has been demonstrated. More recently, correlation between cytokines and endoscopically determined mucosal inflammation was demonstrated, suggesting the potential role of these markers in determining

disease activity<sup>[37-42]</sup>. IL-6 in active CD and IL-10 in recovery of CD have demonstrated a good correlation, which has been reproducible between studies<sup>[43]</sup>.

### Stool test

A number of reasons have led to the development of fecal markers of inflammation in IBD in addition to, or in substitution of, serum markers. As they are derived from stools, they may be of easy access. Also, they may have a higher specificity than serum markers, since they may reflect intestinal rather than systemic inflammation, a result of the close contact of stools with intestinal mucosa and of the possibility that it may wash out molecules related to inflammation or damage. Finally, they may avoid endoscopic examinations, since they are related to mucosal inflammation<sup>[17,18]</sup>.

Stool markers cannot be considered specific for IBD, since they can be increased in situations of mucosal inflammation, irrespective of an infectious or non-infectious etiology. Markers expressed by phagocytes may be more specific for inflammation, while markers found in epithelial cells may be more sensitive and can increase in conditions of non-inflammatory stress<sup>[18]</sup>.

Fecal occult blood (FOB) and  $\alpha$ -1 antitrypsin are markers of mucosal damage and/or disturbed barrier function. FOB determination lacks specificity for IBD and cannot be related to disease activity<sup>[44]</sup>.  $\alpha$ -1 antitrypsin is considered a sensitive but non-specific parameter reflecting enteric inflammation in IBD and has been replaced by other fecal markers<sup>[45]</sup>.

Substances related to phagocyte influx and activation comprises another group of IBD fecal markers with pathophysiological rationale. They appear as a result of leukocyte degranulation consequent to the activation of innate immunity which, in IBD, relates to phagocyte gathering and cytokine production in areas of inflammation. One interesting and already classic application of this rationale is the use indium-111-labelled granulocyte scintigraphy<sup>[46,47]</sup>. However, this technique is expensive, involves long-term stool sampling, exposure to radiation, and may not be applicable in clinical routine. This is why leukocyte degranulation markers have been studied. Even in situations of milder inflammation, products from activated phagocytes within the mucosa may spill over into the lumen and remain stable in single random stool samples, making them a more sensitive, cheaper and easier alternative to indium-111-labelled granulocyte scintigraphy<sup>[48]</sup>.

Lactoferrin, polymorphonuclear elastase, eosinophil cationic protein (ECP), eosinophilic protein X (EPX), myeloperoxidase and lysozyme are among the leukocyte degranulation markers better evaluated so far. ECP and EPX are eosinophil degranulation markers that have been described in IBD, but are considered inferior to other markers and more indicative of pathological processes that involve eosinophils<sup>[49-51]</sup>. Lactoferrin, polymorphonuclear (PMN) elastase, myeloperoxidase, and human neutrophil lipocalin are neutrophil degranulation markers detected in the stool. Lactoferrin is the most accurate among them, but it may be present in cells other than granulocytes (i.e., epithelial cells) and may have anti-inflammatory action. Also,

its pathogenetic link to IBD has not yet been elucidated.

Recently, a group of molecules with pro-inflammatory activity have been described as part of the innate immune system. The innate immunity starts our primary host defense by recognizing invading microorganisms through pathogen-associated molecular patterns (PAMPs). Activated or damaged cells can secrete the damage-associated molecular pattern proteins (DAMPs). The precise mechanism by which microorganisms activate inflammation in IBD is only partially known, but it seems that PAMPs and DAMPs have an important interaction. There are probably multiple positive feedback loops between both molecules and their overlapping receptors may amplify inflammatory processes. As DAMPs are related to the initiation of cell stress and inflammation and are found in areas of intestine affected by IBD, they are considered good candidates as markers of disease activity. S100A8, S100A9 and S100A12 are recently described DAMPs that are ligands to pattern recognition receptors, such as toll-like receptors 4 and receptors for advanced glycation end products and directly related with the amplification inflammatory processes<sup>[52-54]</sup>.

The complex S100A8/S100A9 was named calprotectin, and a strong correlation between it and indium-111-labelled granulocyte scintigraphy, the gold standard method for detecting inflammatory activity in IBD, has been demonstrated<sup>[47]</sup>. Calprotectin is commercially available and an assay for S100A12 is under development<sup>[55]</sup>. Calprotectin can be used in disease monitoring, showing a closer correlation to endoscopic and histological evidence of inflammation than clinical indices, and detecting inflammatory activity before the appearance of clinical signs<sup>[56-58]</sup>. However, calprotectin seems more predictive of relapse in UC than in CD<sup>[58,59]</sup>. Rapid, qualitative or semi-quantitative tests were developed and seem promising for discrimination of IBD from IBS. A recent meta-analysis involving 13 studies with 670 adults and 371 children and teenagers showed that fecal calprotectin is a useful screening tool for identifying patients who are most likely to need endoscopy for suspected inflammatory bowel disease<sup>[60]</sup>.

The performance of the fecal markers lactoferrin, PMN elastase and calprotectin, along with CRP and clinical indices, compared to endoscopic measures of inflammation has been evaluated. The three fecal markers are able to define disease activity both in UC and CD, and distinguish both IBDs from IBS in some situations depending on the marker, even in the absence of activity. None of the three markers seem superior in their ability to reflect endoscopic inflammation, but all three are superior to CRP in their diagnostic accuracy<sup>[19]</sup>.

Abnormalities in intestinal permeability using urinary concentration of sugar probes can be used as a predictor of imminent relapse of clinically inactive CD. Large sugar molecules (i.e., lactulose) and small molecules (i.e., mannitol), both with near 100% elimination in urine, are mixed in a drink and measured in urine as an index of tight junction function. Tight junctions are dynamic structures that respond to many stimuli and are particularly sensitive to cytokines in situations of inflammatory stress. Studies have shown that, in patients with CD in clinical remission, an increased intestinal permeability can predict the risk of

Table 2 Simple endoscopic score for Crohn's disease

Variable	Values			
	0	1	2	3
Size of ulcers	None	Aphthous ulcers (0.1 to 0.5 cm)	Large ulcers (0.5 to 2.0 cm)	Very large ulcers (> 2 cm)
Ulcerated surface	None	< 10%	10%-30%	> 30%
Affected surface	Unaffected surface	< 50%	50%-75%	> 75%
Presence of narrowing	None	Single, can be passed	Multiple, can be passed	Cannot be passed

relapse<sup>[61-63]</sup>. In all studies, the frequency of relapses was significantly different between those with normal and abnormal intestinal permeability tests.

## ENDOSCOPY

Endoscopy is usually useful to diagnose CD involving terminal ileum and colon and to distinguish it from UC. It is also important to determine the extent and severity of the disease, to assess response to treatment and to screen for dysplasia. Additionally, endoscopy allows for direct visualization of the mucosa and acquisition of biopsies, becoming the primary diagnostic tool.

An endoscopic scoring system has been developed and validated for monitoring activity in CD, and to assess severity of ileal and colonic disease. However, it is time consuming and complicated, due to the analysis of multiple aspects of lesions. It is named Crohn's disease endoscopic index of severity (CDEIS) and it is based upon the presence of four types of lesions: superficial ulcers, deep ulcers, ulcerated stenosis or non-ulcerated stenosis, all of which should be recorded in five different segments: terminal ileum, ascending colon, transverse colon, descending and sigmoid colon, and rectum<sup>[64]</sup>. The combination of values allows the calculation of a severity score, which ranges from between 0 and 30. Unfortunately, in a subsequent study, the same authors demonstrated that the use of endoscopy and the CDEIS to guide therapeutic decisions with regard to corticosteroid therapy was not helpful clinically<sup>[65]</sup>.

Years later, Dapermo *et al*<sup>[66]</sup> proposed a simplified model based on ulcer size, ulcerated surface, affected surface and narrowing of lumen present in the ileum, right colon, transverse colon, left colon and rectum, with a score ranging from 0 to 3 (Table 2). Reproducibility of these parameters was confirmed and it was highly correlated with both CDEIS and CDAI.

However, it must be asked if it is really necessary to establish an endoscopic scoring system, as objective as it is to evidentiate healing of mucosal lesions, which has become an important end point in clinical trials of CD treatment<sup>[67-69]</sup>. It remains to be defined if standardization of endoscopic evaluation can be useful in guiding therapy. Still of concern are possible pitfalls of the SES-CD index of activity including the presence of fistulas, for which endoscopy is not the best diagnostic test, and underestimation of stenosis and overestimation of non-specific lesions because of inexperience with endoscopy in patients with IBD.

In UC, endoscopy is necessary for diagnosis and for

determining disease extent. In order to evaluate the clinical disease activity, various endoscopic indices have been elaborated, such as Baron Score, Rachmilewitz Endoscopic Index, Mayo Score and Sutherland Mucosal Appearance Assessment<sup>[13,70-72]</sup>. All were based in granulation scattering, vascular pattern, vulnerability of mucosa and mucosal damage (mucus, fibrin, exudates, erosions and ulcer). However, no standardized model has been established. In an attempt to determine whether or not any endoscopic indices could be established as a standard, Hirai *et al*<sup>[73]</sup> compared the Baron score with the Rachmilewitz Endoscopic Index and demonstrated that both were almost equally useful for evaluating disease activity. In another study, inter- and intraobserver agreement were evaluated, using Matt's, Mayo Score, Baron, and Blackstone indices<sup>[74]</sup>. Two hundred and seventy nine endoscopic pictures of inflammatory lesions from 93 UC patients were displayed twice to 4 expert and 4 trainee endoscopists, with a one month interval. The Matt's and Mayo indices showed a good degree of concordance for expert endoscopists in terms of inter- and intraobserver agreements, but this was not so evident with the Baron and Blackstone indices. For trainee endoscopists, all weighted kappa values for inter- and intraobserver scores using established indices were lower than for the experts. In 2007, D'Haens *et al*<sup>[75]</sup> published a study that reviewed activity indices and efficacy end points for clinical trials of medical therapy in adults with UC and recommended that absence of friability, blood, erosions, and ulcers in all visualized segments are required components of genuine endoscopic healing.

## IMAGING TECHNIQUES

### Abdominal and doppler ultrasound

Transabdominal ultrasound is a very well established tool to examine the liver, hepatobiliary-pancreatic tree and urogenital tract; however, its use for imaging the intestinal tract has been considered more difficult. In the past two decades, improvements in technology, specially new high frequency probes, highly sensitive color and power Doppler units and development of new contrast agents, along with an increasing experience with sonographic findings in intestinal diseases, have all contributed to establishing the role of ultrasound as a clinically important, non-invasive, radiation free and widely available imaging modality for evaluation of these patients<sup>[76,77]</sup>.

Ultrasound has been successfully used as the imaging method of choice in screening patients with clinically suspected CD; it may be the first diagnostic tool employed

for young patients and can be used in the preliminary diagnostic work-up prior to further invasive tests. Another important application of bowel ultrasound is in the follow-up of patients already diagnosed with CD, in whom it may be useful to assess the site and extent of the lesions and to ensure early detection of intra-abdominal complications<sup>[78]</sup>.

Although it has an important role in the evaluation of CD patients, the usefulness of ultrasound and Doppler imaging in assessing disease activity is still a matter of discussion. Several studies attempted to correlate ultrasound and Doppler findings with clinical and biochemical activity, but the published results are controversial<sup>[78]</sup>.

Bowel wall thickening, bowel wall stratification and length of bowel wall involvement were all tested as sign of disease activity. Of these, only the degree of bowel wall thickening showed a significant, but weak, correlation with clinical CDAI and biochemical (erythrocyte sedimentation rate, C-reactive protein) parameters, and can be viewed as an indirect sign of disease activity<sup>[79]</sup>. Although a sensitivity of 80% has been reported for the cut-off value of 4 mm for the maximum thickness of bowel wall, the specificity of this finding alone is low due to the difficulty in differentiating inflammation from fibrosis<sup>[80,81]</sup>.

As neovascularization and hyperemia of the bowel wall are well established findings in active CD, much effort was also made trying to correlate Doppler sonography of the superior mesenteric artery and power Doppler study of the bowel wall with other markers of activity.

Regarding Doppler sonography of superior mesenteric artery, some authors state that the available results concerning this association are conflicting, but the disagreement seems to be due to crucial differences in methodology, especially in the adopted Doppler parameters<sup>[79,82]</sup>. Van Ostayen *et al.*<sup>[83-86]</sup> showed that superior mesenteric artery flow was the most reliable parameter to characterize disease activity and that the cut-off value of 500 mL/min had a sensibility between 80% and 83% and a specificity of 87% for this diagnosis. The association between increased superior mesenteric artery flow and disease activity was also supported by others<sup>[87-89]</sup>.

Intestinal wall vascularity has been studied for more than a decade and the results were consistent with a correlation between blood vessel density assessed by power Doppler sonography and the degree of local inflammation assessed by endoscopy or clinical and biochemical evaluation<sup>[90,91]</sup>. In this field, newer techniques such as harmonic imaging and the administration of echo-enhancing contrast agents have further improved the sensitivity and accuracy of power Doppler evaluation of the bowel wall in detecting inflammatory activity by showing increased perfusion in the affected bowel<sup>[92-96]</sup>. It has also been demonstrated that the assessment of intramural blood flow by means of power Doppler and intravenous contrast agents may discriminate inflammatory stenosis which are hypervascularized, of those cicatricially transformed, and characterized by fibrosis and hypovascularized scar tissue<sup>[97]</sup>.

Although sometimes helpful in evaluating the extent of the disease, the role of transabdominal ultrasound in UC is much less important than in CD, mostly due to the fact

that the disease affects only the mucosa, resulting in very subtle echographic findings, which are difficult to evaluate<sup>[76]</sup>. The mesenteric blood flow in inferior mesenteric artery, although seemingly related to clinical endoscopic disease activity, is technically much more difficult to measure by Doppler than it is in the superior mesenteric artery due to its smaller diameter<sup>[98]</sup>.

### Computed tomography and magnetic resonance enterography

Computed tomography (CT) in its conventional form has played a significant role in the evaluation of complications and extraenteric manifestations of CD, such as fistulas and abscesses, but it has a limited role for depicting bowel wall and luminal abnormalities. CT enterography (CTE) is a modification of the conventional CT technique, optimized for the evaluation of small bowel. This technique utilizes multidetector CT scanners with high spatial and temporal resolutions, thin sections, multiplanar reconstructions and large volumes of ingested neutral enteric contrast material, combined with the use of intravenously administered iodinated contrast, in order to permit visualization of the small bowel wall, mucosa and lumen<sup>[99]</sup>. Then, apart from detecting extraluminal findings, CTE can delineate the extent and severity of bowel wall inflammation<sup>[100]</sup>.

CTE findings of bowel wall thickening, mural stratification, mural hyperenhancement, increased attenuation in the perienteric fat and engorged vasa recta correlate with mucosal and mural inflammation and so, with active CD<sup>[99,101]</sup>.

Mural thickening refers to wall thickness of greater than 3 mm in a well distended bowel loop. It is the most frequently observed CT finding in CD, present in up to 82% of patients<sup>[102]</sup>.

Mural stratification is a distinction of the bowel wall layers on CT after intravenous contrast injection; mucosa and muscular/serosa layers show contrast enhancement and interposed submucosa has a decreased attenuation, giving the wall a trilaminar appearance<sup>[103]</sup>.

Mural hyperenhancement describes a segmental hyperattenuation of a distended bowel loop when compared to adjacent normal loops. This finding correlates significantly with histologic findings of active CD, being the most sensitive CTE finding of disease activity<sup>[103]</sup>. It has also been observed that the degree of bowel wall enhancement correlates with the severity of inflammation<sup>[104,105]</sup>.

Increased attenuation of mesenteric fat can be due to edema or engorged vasa recta, vessels that penetrate the bowel wall perpendicular to the bowel lumen; these two findings combined are the most specific sign of disease activity and correlate with the levels of C reactive protein<sup>[105]</sup>.

CTE can also depict signs of chronic manifestations of CD, such as submucosal fat deposition, sacculations and fibrofatty proliferation<sup>[101]</sup>. The presence of intramural fat indicates past or chronic inflammation. Sacculations result from the chronic inflammatory process, leading to fibrosis and asymmetric shortening of the mesenteric border of the wall (Table 3)<sup>[106]</sup>.

Many authors addressed the positive correlation between CTE findings and clinical and biochemical markers

**Table 3** Computed tomography enterography findings of Crohn's disease activity and chronic disease

Disease activity	Chronic disease
Bowel wall thickening	Submucosal fat deposition
Mural stratification	Sacculations
Mural hyperenhancement	Fibrofatty proliferation
Increased attenuation in the perienteric fat	
Engorged vasa recta	

of disease activity, such as CDAI and C-reactive protein and erythrocyte sedimentation rate, respectively, but the clinical relevance of these images is still a matter of discussion<sup>[105,107]</sup>. Higgins *et al*<sup>[108]</sup> reviewed the CTE scans and clinical data of 67 patients with CD presenting abdominal pain and a clinical suspicion of either small bowel inflammation or stricture. The authors showed that CTE can detect strictures not clinically suspected, rule out strictures that were radiologically insignificant and change the perceived likelihood of steroid benefit in up to 61% of cases. The CTE ability to detect small bowel strictures can be particularly helpful when considering using endoscopic capsules, which may themselves precipitate small bowel obstruction.

CTE has a major disadvantage: the use of ionizing radiation. The increased spatial resolution of CT with new multidetector CT scanners carries along with it a greater dose of ionizing radiation. In fact, effective doses of radiation are up to five times higher with CTE when compared with small bowel follow through<sup>[109]</sup>. Considering that many patients will undergo various examinations through their lifetime, efforts should be made to minimize the number of CT examinations, decrease CT dose or considering another diagnostic imaging modality, such as magnetic resonance enterography (MRE).

MR imaging also experienced the same technical advances seen in CT in the last ten years. In a similar way, the improvement in spatial and temporal resolution of images, combined with the use of large volumes of oral contrast agents to provide bowel distention, allows the evaluation of bowel wall contrast enhancement, wall thickening and edema; findings useful for the assessment of CD activity<sup>[110]</sup>.

The preference of MRE *vs* CTE has been geographical and based on expertise and public policy. With increasing awareness of radiation exposure risks, there has been a more global interest in implementing techniques that reduce or eliminate radiation exposure. Owing to this excellent soft tissue contrast, direct multiplanar imaging capabilities and lack of ionizing radiation, MRE is well suited to play an important role in the evaluation of small bowel disorders<sup>[111]</sup>.

Until now, the available data concerning accuracy of MRE in detecting disease activity is less than CTE, but early results are encouraging, showing a similar sensitivity and diagnostic effectiveness<sup>[112-114]</sup>, although image quality is still better with CT. Motion artifacts from small bowel motility are more severe with MRE<sup>[112]</sup>, but halting peristalsis by administering 1 mg of glucagon intramuscularly

before contrast-enhanced imaging reduces blurring and artifacts related to bowel motility<sup>[115]</sup>.

As they are imaging methods designed to assess small bowel, both CTE and MRE are not suited for evaluating UC.

In conclusion, in the last few years, a great deal of research and the development of diagnostic tools have been devoted to the task of diagnosing IBD, predicting its course and determining activity. Many of these tools show promising results, but a lack of specificity remains a problem that precludes routine use in clinical practice. Advances in molecular medicine towards a better understanding of genetic and other etiologic factors in IBD may result in better performance of markers of disease. Endoscopy displays direct evidence of mucosal injury. However, it is time consuming, invasive, expensive and, a good deal of endoscopic evaluation criteria lack validation, making it difficult to adopt endoscopic methods for routinely monitoring the course of IBD. Imaging techniques are useful as markers in CD, but they lack applicability in UC. Considering disease markers pitfalls, clinical activity indices still have their place in IBD monitoring. In conclusion, there is not yet an ideal marker, and determination of activity depends on clinical ability to manage information given by the available complementary exams.

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## DNA methylation and microRNAs in cancer

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### Abstract

DNA methylation is a type of epigenetic modification in the human genome, which means that gene expression is regulated without altering the DNA sequence. Methylation and the relationship between methylation and cancer have been the focus of molecular biology researches. Methylation represses gene expression and can influence embryogenesis and tumorigenesis. In different tissues and at different stages of life, the level of methylation of DNA varies, implying a fundamental but distinct role for methylation. When genes are repressed by abnormal methylation, the resulting effects can include instability of that gene and inactivation of a tumor suppressor gene. MicroRNAs have some aspects in common with this regulation of gene expression. Here we reviewed the influence of gene methylation on cancer and analyzed the methods used to profile methylation. We also assessed the correlation between methylation and other epigenetic modifications and microRNAs. About 55 845 research papers have been published about methylation, and one-fifth of these are about the appearance of methylation in cancer. We conclude that methylation does play a role in some cancer types.

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**Key words:** Methylation gene expression; Transcriptional control; Cancer; MicroRNA; Gastric cancer

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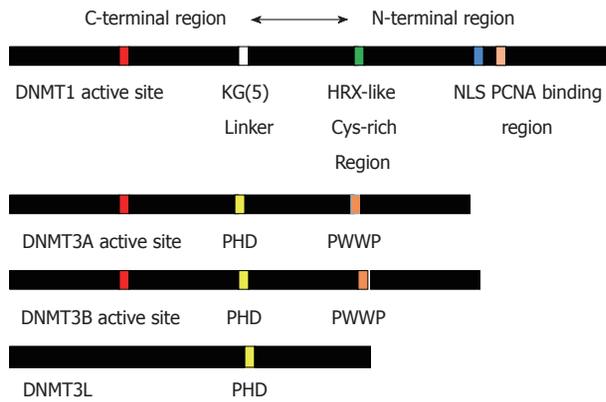
### INTRODUCTION

The occurrence of many diseases, such as cancer, diabetes mellitus and scleroderma, is related to the dysregulated gene expression resulting from epigenetic and genetic abnormalities. Genetic abnormalities can be caused by point mutations, gene amplification, or changes in the promoter (typically caused by chromosomal rearrangements)<sup>[1]</sup>, whereas epigenetic modifications include methylation, deacetylation of genes, and methylation of histone proteins, resulting in the accurate regulation of gene expression, without changing the DNA sequence, in response to changes in the environment and to meet the demands of differentiation<sup>[2]</sup>. In fact, epigenetic modifications are as important as genetic modifications in regulating gene expression and controlling the onset of disease<sup>[3]</sup>. DNA methylation, as a crucial component of epigenetics, plays an important role in cell differentiation and embryogenesis and is also heritable. Since it was first described by Feinberg and Vogelstein in 1983, our knowledge of methylation has grown at a dramatic rate<sup>[4]</sup>. The recent development of methods to profile and study methylation in chromosomes has led to a deeper understanding of this process along with clinical applications to aid in the prognosis and treatment of disease<sup>[5]</sup>.

### DNA METHYLATION

#### Definition of methylation

In 1999, the modern conception of epigenetics was put forward by Jones *et al*<sup>[6]</sup>. Epigenetics is defined as the reg-



**Figure 1** DNA methylation machinery. NLS: Nuclear localization signal; PCNA: Proliferating cell nuclear antigen.

ulation of gene expression without changing the genetic sequence. It is a flexible, heritable process that regulates some genes<sup>[7]</sup>. DNA methylation and the modification of histones including acetylation and deacetylation are important components of epigenetics; however, the most common modification is DNA methylation.

DNA methylation is a type of covalent modification in which a methyl group is added to a cytosine in the genome *via* S-adenosylmethionine; this process occurs as an enzymatic reaction after DNA replication<sup>[8]</sup>. In mammalian cells, methylation of DNA is typically restricted to the 5-position of the pyrimidine ring of cytosine residues that are located in CpG dinucleotides<sup>[9]</sup>. CpG dinucleotides are frequently clustered into CpG islands, regions that are rich in CpG sites. These islands are generally about 0.5-3 kb, occur on average every 100 kb in the genome, and are found in approximately half of all genes in humans<sup>[10]</sup>. Methylation of other CpGs seems to have no biological functions<sup>[11]</sup>.

There are four types of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMTs control the degree of methylation of the genome: DNMT1 is responsible for the maintenance of methylation, and DNMT3A and DNMT3B carry out *de novo* methylation. The DNMT3L does not have enzymatic activity, but it does regulate the activity of the other methyltransferases<sup>[12,13]</sup>. DNMT1 is considered to be the maintenance methyltransferase because of its high activity and preference for hemimethylated DNA during DNA replication. All of the active DNA methyltransferases contain an active site motif in the C-terminal region (red box), whereas DNMT3L does not. DNMT1 contains other functional regions required for its interaction with proliferating cell nuclear antigen, which is adjacent to the nuclear localization signal. The N-terminal region of DNMT1 also contains a cysteine-rich HRX-like region and a lysine-glycine repeat [KG(5)] region. DNMT3A, DNMT3B, and DNMT3L contain a plant homeodomain; DNMT3A and DNMT3B contain a PWWP domain. These two domains are required for targeting DNMT3A and DNMT3B to pericentromeric heterochromatin and contribute to protein-protein interactions by recognizing histone modifications<sup>[14]</sup> (Figure 1).

### Normal and abnormal levels of methylation

The level of methylation changes during the growth of human beings and the development of diseases and, in different tissues, methylation varies substantially. Normally, about 50% of the CpG islands, which customarily are located in the promoter region of housekeeping genes, are unmethylated and thus are active. When those CpGs become methylated, the corresponding gene is silenced. There are, however, CpGs that are located elsewhere in genes and that do not influence transcription when they are methylated. DNA methylation is replicated with a high fidelity in mammalian cells and is almost at a stable state in a specified cell. It is regarded as having tissue and organ specificity<sup>[15]</sup>.

Once the rhythm of methylation is disturbed, many diseases develop<sup>[16,17]</sup> (Table 1). Customarily, the abnormal condition includes two aspects: Hypermethylation and hypomethylation<sup>[14]</sup>.

## DNA METHYLATION IN TRANSCRIPTIONAL CONTROL

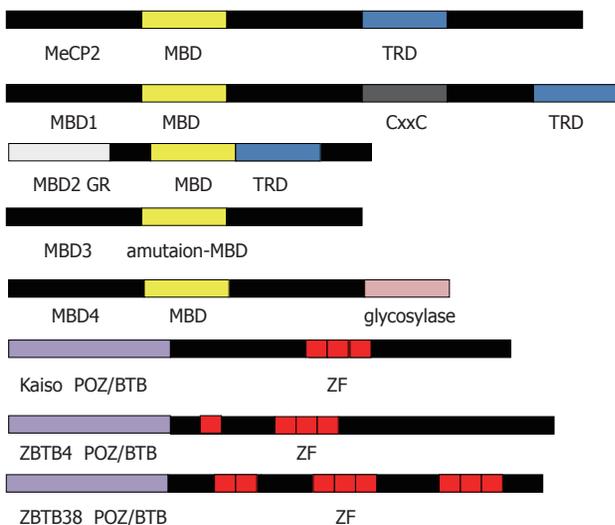
### The role of methylation in transcriptional control

When methylated, chromosomes become stabilized, and their activity is decreased. Gene expression is repressed by methylation in two separate mechanisms<sup>[18,19]</sup>. In direct inhibition, the methylated chromosome prevents the approach of the transcriptase, holding back transcription. The second method is indirect inhibition, in which two types of protein, methylation-binding proteins (MBDs) and histone deacetylase (HDAC) are recruited to the chromosome (Figure 2). MBD proteins display homology within their MBD domains, whereas the transcription repression domains (TRDs) described for MeCP2, MBD1 and MBD2 are nonhomologous. In addition to its MBD domain, MBD1 is able to bind unmethylated DNA *via* its third CxxC zinc-finger motif. MBD2 features a characteristic stretch of glycine and arginine residues in the MBD domain, which when mutated prevent the binding of MBD to methylated CpGs in mammals<sup>[20]</sup>. MBD3 is not able to bind methylated CpGs in mammals because of a mutation in the MBD domain. MBD4, a thymine glycosylase, contains a C-terminal glycosylase domain used for excision-based DNA repair. Three members of the Kaiso protein family which also influence the transcription, have been described so far. Kaiso, ZBTB4 and ZBTB38 share a triple zinc-finger domain and a BTB/POZ domain, which in the case of ZBTB4 contains a 60-amino-acid insertion. Furthermore, ZBTB4 and ZBTB38 contain, respectively, three and seven additional zinc-finger domains and have juxtaposed MBD and TRD domains<sup>[21]</sup>.

MBDs can also prevent the approach of transcription factor (TF) and cofactors, so that they cannot bind the promoter of the gene, thus stopping the transcription. The HDAC is also recruited to the region of methylated DNA, where it affects the activity of the promoter and deacetylates of the lysine of histone3/histone4 charged, and it then reacts with the negatively charged DNA. As a result, the chromosome becomes more tightly packed,

Table 1 Methylation and diseases		
AML	<i>hPer3</i> gene	Hypermethylation
Fragile X syndrome	Loss of FMR1/FMR2 function	Promoter methylation
ATR-X syndrome	Loss of ATRX function	Hypomethylation of certain repeat and satellite sequences
Immunodeficiency, centromeric region instability, and facial anomaly syndrome	DNMT3b mutation	Centromeric DNA hypomethylation
Beckwith-Wiedeman syndrome	Disruption of the imprinted IGF2/CDKN1C loci on 11p15.5	Loss of genomic imprinting
Williams syndrome	Loss of WSTF function	Condensed chromatin structures
Rubinstein-Taybi syndrome	Mutations in the gene encoding CREB-binding protein	Reduced histone H3 acetylation
Prader-Willi syndrome	Disruption of the imprinted SNRF/SNRPN locus on 15q11-13	Disruption of genomic imprinting
Coffin-Lowry syndrome	Mutation in RSK genes	Disrupted chromatin remodeling <i>via</i> activation of CREB-binding protein

CREB: cAMP-response element-binding protein; WSTF: Williams syndrome transcription factor.



**Figure 2** Proteins that bind the methylated DNA. MBD: Methylation-binding protein; TRD: Transcription repression domains.

blocking access to those proteins that are needed to start transcription. Genes with unmethylated (open lollipops), active CpG island promoters (Pro), have TFs (the radial pattern) at the transcription initiation site. Transcripts initiated here proceeding through the downstream elements even though they are methylated (closed lollipops) and presumably are coated with methyl CpG binding domain proteins (MBDs, the cylindrical cast) and HDACs (the trigon). The enhancer is functional because the silencer and insulator are methylated and, thus, not occupied by their respective cognate proteins. Methylation here is permissive for expression. For a permanently silenced gene such as an imprinted gene or a gene on the inactive X chromosome, the promoter is methylated, leading to binding of MBDs, HDACs, other transcriptional suppressors, and chromatin compaction. The TFs, which normally regulate gene expression, are not able to access the promoter. Figure 3 also shows how lack of methylation in a silencer or insulator can lead to binding of the cognate proteins, e.g., GCF2 (GC binding factor 2, the cube) or CTCF (CTC binding factor, the oblong), thus preventing the enhancer

from functioning<sup>[22]</sup> (Figure 3).

From what has been described above, we know that methylation and deacetylation work in conjunction to regulate gene expression, but methylation is the triggering event. Under some conditions, however, deacetylation appears first, followed by methylation<sup>[23]</sup>. The role of each process in the regulation of gene expression needs to be studied in more detail.

### How methylation is maintained and induced

Although methylation is known to be important and has been the focus of research, we are still not clear why abnormal methylation occurs. Because methylation is catalyzed by DNMTs, changes in these enzymes may have some association. When DNMT1 is removed, the level of methylation of the whole genome is reduced by 3%, and when DNMT3 is removed, it is reduced by 4%. When they are both removed, the level is lowered by 98%<sup>[14,24]</sup>. Thus, DNMTs are important for methylation. Their activity can, however, be influenced by many factors, such as ray, temperature. Because cells near the body surface are more easily influenced by the surrounding environment, the methylation of skin cells often becomes dysregulated. In addition, infection can lead to abnormal methylation. Infection with *Helicobacter pylori* (*H. pylori*) in the stomach can cause cancers, accompanied by changes in DNA methylation. Smoking is also a risk factor, as it can cause many genes to gain methylation<sup>[25]</sup>. One study has also shown that eating foods lacking in folic acid, which is the carrier of the one carbon unit, leads to more-complex methylation patterns and increases the likelihood of cancer<sup>[26]</sup>.

## DNA METHYLATION AND MICRORNAS IN CANCER

It is well accepted that cancer is a result of many events, including genetic and epigenetic and others. Since 1983, epigenetics has attracted the most attention of researchers, who have focused in particular on methylation. The appropriate DNA methylation within CpG dinucleotide islands plays a significant role in the regulation of gene

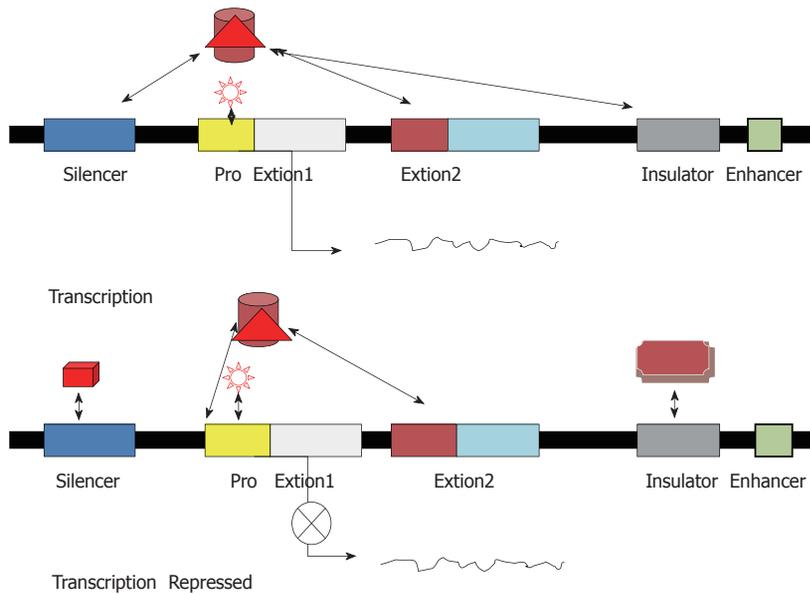


Figure 3 How methylation represses the gene expression..

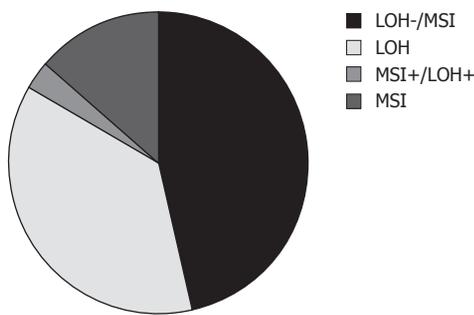


Figure 4 Factors for cancer development. LOH: Heterozygosity; MSI: Microsatellite instability.

expression. Abnormal patterns in DNA methylation often result in many diseases<sup>[27]</sup>. Either as a result of DNMT overexpression or the occurrence of aberrant hypermethylation of tumor cell-specific promoters, the pattern of cell cycle, apoptosis and DNA repair changes following the aberrances of differentiation and adhesion of cells which is often a hallmark of diseases<sup>[28]</sup>. It is reported that 48% of tumors had evidence of loss of heterozygosity (LOH), whereas 14% of tumors had microsatellite instability (MSI), including a minority of tumors (3%) that overlapped with LOH. Nearly one-third of tumors (38%) have neither MSI nor LOH<sup>[1]</sup> (Figure 4).

Four abnormal aspects, inactivation of mismatch repair, instability of chromosomes, hypomethylation of oncogenes, and hypermethylation of tumor suppressor genes, can be observed when abnormal DNA methylation appears. The four aspects often cooperate to cause cancers to happen. The first genes found to be methylated were p16, TSP-1, and IGF2. In colorectal cancer, there is a significant degree of methylation at the previously identified CIMP-associated loci (MINT-1, -2, -31; p16; p14; MLH1), as well as in six new tumor suppressors or gene markers (PTEN, TIMP3, RUNX3, HIC1, APC, and RARβ2), in combina-

tion with the chromosomal instability and microsatellites instability<sup>[1]</sup>.

In addition, it has been proposed that hypomethylation patterns of the genome exist and, more specifically, that hypomethylation and hypermethylation cooperate in cancer development<sup>[29]</sup>. Transcriptional silencing *via* DNA hypermethylation can often be associated with poor clinical outcome in several malignancies, indicating that hypermethylation of tumor-suppressor genes or hypomethylation of tumor genes are related to cancer<sup>[30,31]</sup>.

In addition, methylation can be a marker to judge whether the cancer has been transferred to other tissues. HIN-1, CDH13, RIL, RASSF1A, and RARβ2 were frequently methylated in both primary and metastatic tissues, whereas the methylation status of HIN-1, CDH13, RIL, and RARβ2 isolated from lymph nodes was correlated with that in primary tumors in breast cancer<sup>[32]</sup>.

Recently, small interfering RNAs (siRNAs) have been found to participate in gene regulation together with methylation. siRNAs are RNAs that consist of 21-25 nucleotides. They make up nearly 2% of the genome and can be found either in the host gene or other genes. siRNAs can induce the methylation of the promoter, thus silencing the gene. This factor has been conserved during evolution and has complicated functions. There are at least three kinds of small RNAs, siRNA, miRNA, and others. It has been known that some small RNA can repress gene expression *via* a complex of proteins named the RNA-induced silencing complex, thus regulating gene expression after transcription. miRNA-223 is located in the X chromosome, and its expression is controlled by the upper sequence CCAAT box. Two kinds of proteins, NFI-A and C/EBPα, participate in the process. Abnormal expression of miRNA223 can lead to acute lymphocytic leukemia and acute myelocytic leukemia (AML). The fusion gene *AML1/ETO* will cause down-regulation of the miRNA223 *via* methylation or recruiting of related

enzymes. When miRNA223 is silenced, diseases appear. In the primary carcinoma of liver, miRNA223 is notably down-regulated, reflecting the coordination of methylation and miRNA. The influence of methylation on the expression of miRNAs should be further studied and will be a hotspot of research in the next few years<sup>[33-37]</sup>.

## DNA METHYLATION AND MICRORNAS IN GASTRIC CANER

The roles of DNA methylation and miRNA in gastric cancer have been extensively studied recently. Many genes are methylated specifically in gastric cancer, as are miRNAs and siRNAs. Because oncogenes and tumor suppressor genes can have important roles in cancer and because methylation can repress gene expression, the level of methylation of a specific gene in gastric cancer may reflect whether that gene is an oncogene or tumor suppressor gene. Weak gene expression and loss of gene expression because of promoter hypermethylation may be a cancer-specific event<sup>[38]</sup>.

Ulcer-healing genes (*TFF1*, *TFF2*, *CDH1* and *PPARG*) are methylated in earlier gastric carcinoma, and methylation of hsa-miR-124 is involved in cervical cancer<sup>[39-41]</sup>. The gene encoding BMP3 has been found to cause gastric carcinoma in Chinese population<sup>[42]</sup>. If combined with other aspects, the situation may be more severe. For example, epigenetic inactivation of GATA-4 and GATA-5 by methylation of CpG islands is an early frequent event during gastric carcinogenesis and is significantly correlated with *H. pylori* infection<sup>[43]</sup>. If expression of DNMT is abnormal, methylation will be abnormal, leading to a disease state. It has also been reported that methylation can have an additive effect with other chromosomal abnormalities. This can result in a positive feedback loop that progresses to a disease state<sup>[11]</sup>. Infection with *H. pylori* induces IRX1 promoter methylation and downregulation of the promoter activity as well as a significant reduction in gene expression. Gene silencing of the IRX1 tumor suppressor by promoter CpG methylation, combined with LOH, has been identified in human gastric cancer<sup>[44]</sup>. SLC19A3 was epigenetically down-regulated in gastric cancer, and *via* the technique of quantitative real time polymerase chain reaction (RT-qPCR), it has been shown that aberrant SLC19A3 promoter hypermethylation in plasma may be a novel biomarker for breast and gastric cancer diagnosis<sup>[45]</sup>. Promoter hypermethylation of p16, Runx3, DAPK and CHFR is frequent in gastric cancer. DAPK and CHFR promoter hypermethylation may be important for evaluating the differentiation grade and lymph node status in patients with gastric cancer. Silencing of HIC1 and TOB1 expression is a common occurrence in gastric cancer and may contribute to the development and progression of the disease<sup>[46]</sup>.

Methylation silencing of *miRNA* genes, in addition to that of protein-coding genes, may contribute to the formation of a field defect for gastric cancers<sup>[47]</sup>. Down-regulation of miR-212 may be related to gastric carcinogenesis through its target genes, such as *MECP2*<sup>[48]</sup>.

Hypermethylation of hsa-miR-124a is present in gastric cancer<sup>[49]</sup>. miRNA-34b and miRNA-34c are novel tumor suppressors that are frequently silenced by DNA methylation in gastric cancer, and methylation of miR-34b/c is involved in an epigenetic field defect and that the methylation might be a predictive marker of gastric cancer risk<sup>[50]</sup>. The transformation from gastritis to lymphoma of mucosa-associated lymphoid tissue is epigenetically regulated by miR-203 promoter methylation, and ABL1 is a novel target for the treatment of this malignancy<sup>[51]</sup>. miR-10b methylation may be a useful molecular biomarker for assessing the risk of gastric cancer development, and modulation of miR-10b may represent a therapeutic approach for treating gastric cancer<sup>[52]</sup>.

## CLINICAL APPLICATIONS

Based on how methylation works to repress gene expression, several methods can be used to treat the related diseases. Current research has focused on methods to demethylate the gene of interest. Both HDAC inhibition and DNA demethylating agents have shown clinical efficacy respectively; yet a combination of the two agents has a strong synergistic effect on the reactivation of silenced genes and antiproliferative and cytotoxic effects on cancer cells<sup>[30,53]</sup>. The two compounds do not, however, reverse the methylation entirely<sup>[54]</sup>. Both nucleoside analogs and non-nucleoside analogs can be used to demethylate the gene of interest, but with severe side effects<sup>[55,56]</sup>. The first kind of agents includes 5-azacytidine, 5-aza-2-deoxycytidine (5-aza-CdR), 5,6-dihydro-5-azacytidine, and zebularine, among which 5-azacytidine has been clinically shown to reduce the degree of methylation and prolong the survival of the patients. The second includes procaine, mitoxantrone, N-acetyl-procainamide, procainamide, hydralazine, and the main polyphenol compound of green tea, (-)-epigallocatechin-3-gallate. Although the two kinds of agents are effective, their severe side effects cannot be ignored, for they both will make the whole genome hypomethylated which can cause many problems including the development of new diseases<sup>[57]</sup>.

For these reasons, these agents just acts as some assistance ones. To reduce the side effects, small molecules targeting DNMT are being developed. It has been reported that they are exquisitely S-phase specific, which makes them less toxic<sup>[58]</sup>. RG108 and MG98 are among them. They can apparently inhibit methylation with fewer side effects and activate the repressed genes, but clinical trials have not yet been carried out<sup>[59]</sup>.

Because we now have accurate ways to profile the methylation in a genome or in an individual gene, demethylation can be monitored frequently, which will allow the prompt correction of therapeutic agents, giving greater promise to this approach. It is, however, more important to prevent the occurrence of abnormal methylation.

## METHODS IN METHYLATION PROFILING

Methylation profiling can be approached in two ways:

analysis of genome-wide methylation and analysis of single CpGs. Both approaches can be carried out by the same methods: colorimetry, fluorescence, methylation-sensitive restriction endonuclease treatment and PCR.

The first step in the colorimetric method is to hydrolyze the target DNAs into nucleotides using hydrochloric acid and then test the resulting absorbance. Differences in absorbance suggest that there are differences in the level of methylation between the two chromosomes. This is used to test the whole-genome methylation of DNAs. The fluorescence method has something in common with the colorimetric approach. It uses chloroacetaldehyde to treat the chromosomes, so that the chromosomes will increase in fluorescence, and the fluorescence intensity reflects the level of methylation of the whole genome. The methylation-sensitive restriction endonuclease method is used to analyze single CpGs, and the enzyme pairs used include Hpa II -Msp I and Sma I -XmaI. These enzymes specifically degrade the unmethylated chromosomes into small pieces, whereas the methylated DNA will escape the shearing. The pieces then can be tested *via* PCR or Southern blotting<sup>[60]</sup>.

When treated with bisulfite, methylated cytosines are stable, whereas unmethylated cytosines are modified to uracils. The amplification results thus can indicate whether the CpG is methylated or not<sup>[61]</sup>.

## CONCLUSION

With every new discovery in the epigenetic landscape of tumors, there comes a new opportunity for producing targeted agents to cure cancer. As the understanding of the intricate machinery of tumor growth continues, there is greater hope that methylation-based therapies will prove successful. It is, however, essential to determine the safety of these treatments in the long run as we administer the agents to healthier populations of patients. It is also important to consider the context of all epigenetic processes, as the known treatments for blocking methylation are either non-specific or have severe side effects.

Our understanding of the relationship between DNA methylation and transcriptional control is being deepened but is still far from complete. It may be unrealistic to expect that any unified theory will encompass all the biological consequences of DNA methylation. It must be linked with deacetylation, siRNAs and phosphorylation. The mechanisms by which methylation patterns are generated are still not fully understood. After the human genome project comes into the post-genome period, methylation is of great importance. It is feasible to use the technique of methylation in the future to induce cell differentiation, guide clinical treatment and explore the early stages of cancer<sup>[62]</sup>.

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## Endoscopic ultrasound-guided elastography in the nodal staging of oesophageal cancer

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### Abstract

**AIM:** To assess quantitative endoscopic ultrasound (EUS)-guided elastography in the nodal staging of oesophago-gastric cancers.

**METHODS:** This was a single tertiary centre study assessing 50 patients with established oesophago-gastric cancer undergoing EUS-guided fine needle aspiration biopsy (FNAB) of lymph nodes between July 2007 and July 2009. EUS-guided elastography of lymph nodes was performed before EUS-FNAB. Standard EUS characteristics were also described. Cytological determination of whether a lymph node was malignant or benign was used as the gold standard for this study. Comparisons of elastography and standard EUS characteristics were made between the cytologically benign and malignant nodes. The main outcome measure was the accuracy of elastography in differentiating between benign and malignant lymph nodes in oesophageal cancers.

**RESULTS:** EUS elastography and FNAB were performed

on 53 lymph nodes. Cytological malignancy was found in 23 nodes, one was indeterminate, one was found to be a gastrointestinal stromal tumor and 25 of the nodes were negative for malignancy. On 3 occasions insufficient material was obtained for analysis. The area under the curve for the receiver operating characteristic curve for elastography strain ratio was 0.87 ( $P < 0.0001$ ). Elastography strain ratio had a sensitivity 83%, specificity 96%, positive predictive value 95%, and negative predictive value 86% for distinguishing between malignant and benign nodes. The overall accuracy of elastography strain ratio was 90%. Elastography was more sensitive and specific in determining malignant nodal disease than standard EUS criteria.

**CONCLUSION:** EUS elastography is a promising modality that may complement standard EUS and help guide EUS-FNAB during staging of upper gastrointestinal tract cancer.

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**Key words:** Endoscopic ultrasound; Oesophageal cancer; Lymph nodes; Elastography; Tumour staging

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### INTRODUCTION

Endoscopic ultrasound (EUS) is an integral investigation in the staging of oesophageal and oesophago-gastric junctional tumours<sup>[1,2]</sup>. It provides both an accurate as-

assessment of the tumour (T) stage of a cancer and associated lymphadenopathy (N)<sup>[3-5]</sup>.

EUS lymph node assessment can be challenging. EUS-guided fine needle aspiration biopsy (FNAB) of lymph nodes remains the pre-operative gold standard for determining nodal involvement, with diagnostic accuracy of up to 95% reported in previous studies<sup>[6,7]</sup>. However, EUS-FNAB cannot be undertaken if the needle passes through the primary tumour, and there can be technical difficulties in obtaining lymph node material for analysis.

Using conventional grey-scale B-mode EUS, lymph nodes are characterised by size, shape, density and distinction of the border in an attempt to distinguish between benign and malignant nodes<sup>[8]</sup>. However, the sensitivity and specificity of these features in distinguishing malignant involvement only exceeds 80% when all four of these features are present<sup>[8]</sup>.

Elastography measures the stiffness of a structure and it is known that pathophysiological processes such as malignancy lead to stiffer tissue that deforms less. Over the past 15 years, elastography during ultrasound has been applied to measure tissue elasticity in breast, thyroid and liver disease<sup>[9-13]</sup>. Initial studies assessing nodes with elastography used processing to produce a colour elastography image<sup>[14-16]</sup>. More recent software technology allows quantification of the stiffness in the form of strain. Comparing two different areas of tissue allows calculation of a strain ratio between the two.

There have been several recent reports using EUS elastography to assess pancreatic lesions<sup>[17,18]</sup> as well as lymph nodes<sup>[13,18-20]</sup>. The results from these studies, using first wave software to produce descriptive colour elastograms, are encouraging, suggesting a high sensitivity and specificity for detecting malignant involvement. However, there is limited data on oesophageal cancer nodal staging. This study aimed to compare conventional EUS with quantitative EUS-guided elastography in the assessment of nodal staging in oesophageal and junctional cancers and to assess whether quantitative EUS could accurately distinguish malignant from benign lymph nodes.

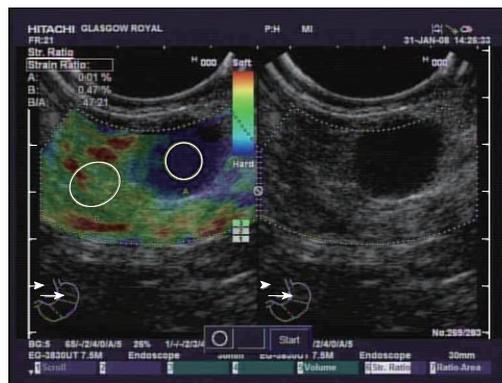
## MATERIALS AND METHODS

### Patients

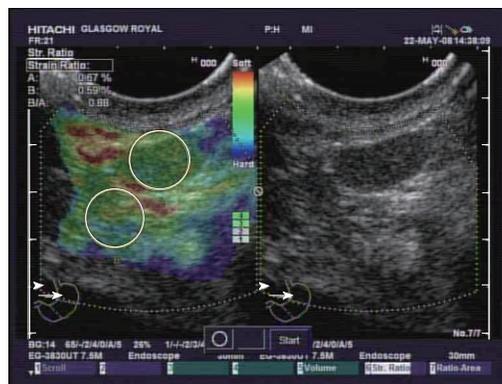
This was a prospective single centre study. Glasgow Royal Infirmary is a West of Scotland tertiary referral centre for EUS staging of upper gastrointestinal tract cancers. All patients who, as part of routine clinical care, were undergoing EUS-FNA lymph node biopsy for staging of upper gastrointestinal tract cancer, during the period July 2007 to July 2009, had elastography of the node prior to sampling.

### Instruments and technique

Initial staging EUS was undertaken by one of two endoscopists (SP/AJS) using a Pentax radial echoendoscope, attached to a Hitachi EUB-8500 ultrasound processor. Standard EUS grey-scale images of suspicious lymph nodes were obtained and conventional characteristics of size, shape, distinction of border and density were re-



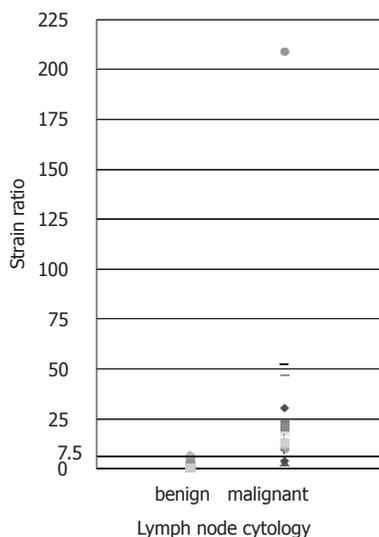
**Figure 1** Endoscopic ultrasound image of a malignant appearing lymph node. The right-hand side of the image displays all 4 of the conventional endoscopic ultrasound criteria characteristics of malignant nodes with regard to size (> 1 cm), shape (round), density (hypodense) and distinction of border (clear edge). The left-hand side of the image is a superimposed elastographic image with strain ratio measurement between an area of the lymph node and a surrounding area of tissue.



**Figure 2** Endoscopic ultrasound elastography of a benign lymph node. The right-hand side of the image displays standard grey-scale endoscopic ultrasound images while on the left is a superimposed elastography image. In the elastography image window the strain ratio measurements of the two areas outlined in the yellow circles is shown as a percentage in the top left-hand corner. The calculated strain ratio is shown as B/A. The elastographic signal is indicated by the bar column in the bottom right of the elastographic image window.

corded. After the decision was made to undertake a FNA biopsy, a Pentax linear array echoendoscope was then used with the same ultrasound processor.

Elastography is a standard function of the Hitachi EUB-8500 ultrasound processor. A conventional grey-scale image was displayed on the right-hand side of the monitor, while the superimposed elastography image was displayed on the left-hand side (Figures 1 and 2). The elastography image and measurements rely on compressions from vascular pulsation and respiratory movement. Measurements were only taken when there was good contact and appropriate compression of the transducer, as indicated on the elastography image on the ultrasound processor. Using the superimposed elastography image, the largest area possible of the node was outlined, as was a similar sized area of surrounding apparently normal tissue. The ultrasound processor measured the strain of each area as a quantitative figure and calculated a strain ratio between the two areas. The strain ratio was recorded a



**Figure 3** Plot of elastography strain ratio for cytologically proven benign or positive lymph nodes. The cut-off line of  $\geq 7.5$  is the optimal strain ratio for discriminating between benign and malignant lymph nodes.

**Table 1** Demographic data of the cytologically confirmed benign and malignant lymph node groups *n* (%)

	Benign node	Malignant node
Gender	M 20:F 5	M 18:F 5
Age (mean, yr)	66.8	67.9
Oesophageal adenocarcinoma	17 (68)	16 (70)
Oesophageal squamous cancer	5 (20)	7 (30)
Gastric adenocarcinoma	2 (8)	0 (0)
Barrett's oesophagus with HGD	1 (4)	0 (0)

HGD: High-grade dysplasia; M: Male; F: Female.

minimum of three times prior to EUS-FNAB. The mean of these recordings was calculated.

EUS-FNAB was performed using a Wilson Cook 22 gauge needle. Three passes were obtained, the samples stored in formalin and sent to the laboratory for later cytological analysis by specialist pathologists who were blinded to the elastography values.

**Statistical analysis**

Cytological determination of whether a lymph node was benign or malignant was used as the gold standard for the purposes of this study, as surgical resection specimens and follow up imaging were not available. Comparison of the demographic variables between cytologically proven benign and malignant nodes was carried out using either contingency table ( $\chi^2$ ) analysis or Mann-Whitney test, as appropriate. In order to assess the intra-observer variation, and hence the reproducibility, for strain ratio, 8 patients had 8 strain ratio values determined and the coefficient of variation was determined. In order to compare the relative sensitivity and specificity of the EUS elastography and 4 conventional EUS criteria (both individually and in combination) with EUS-FNAB for detecting malignant lymph nodes, the area under the receiver operator curve (ROC) was analysed. The area under an

ROC curve (AUC) for a measurement to discriminate between two disease conditions may be viewed as the probability that the measurement would correctly discriminate between two patients, one with and one without disease, each selected randomly from their group (those with and without disease). Note that the *P*-value cited with a single ROC curve is for the rejection of the null hypothesis that the expected value of the strain ratio AUC = 0.50, i.e., non-informative, equivalent to flipping a fair coin; whereas, the other *P*-values for AUCs other than the strain ratio ROC are for rejecting the null hypotheses that those expected values of AUCs are no different than that of the strain ratio AUC. Comparison between ROC curves AUC was undertaken by the statistics software package which uses the DeLong, DeLong, Clarke-Pearson methodology<sup>[21]</sup>. Because of the number of statistical comparisons, a *P* value of  $< 0.01$  was considered to be significant. Analysis was performed with the use of Analyse-It Statistical Package (Analyse-it Software, Ltd. <http://www.analyse-it.com/>; 2009).

**RESULTS**

**Total numbers**

EUS elastography, prior to lymph node EUS-FNAB, was performed in 53 patients undergoing EUS for oesophago-gastric cancer staging. Cytological evidence of malignant disease was found in 23 nodes, while 25 nodes were considered benign (Figure 3). On 3 occasions insufficient node material was sampled to allow cytological analysis and on one occasion the cytological analysis was indeterminate. The pathological analysis of one lesion in a patient with confirmed oesophageal adenocarcinoma revealed a gastrointestinal stromal tumor rather than a lymph node.

**Demographics**

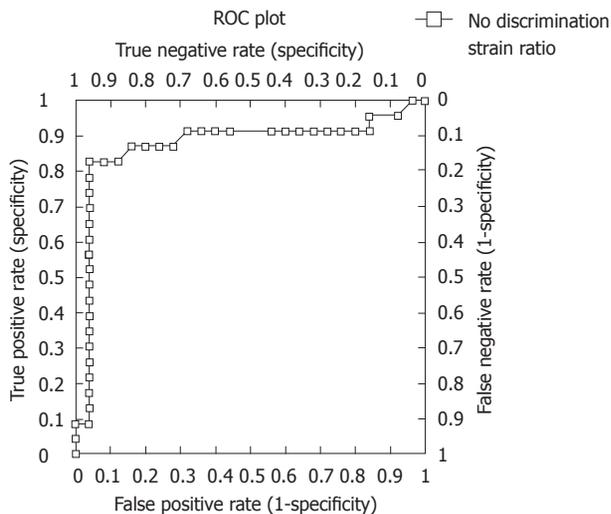
Patient demographics are shown in Table 1. There were no statistically significant differences between the cytologically benign and malignant node groups.

**Elastography strain ratio effectiveness and determination of strain ratio cut-off value**

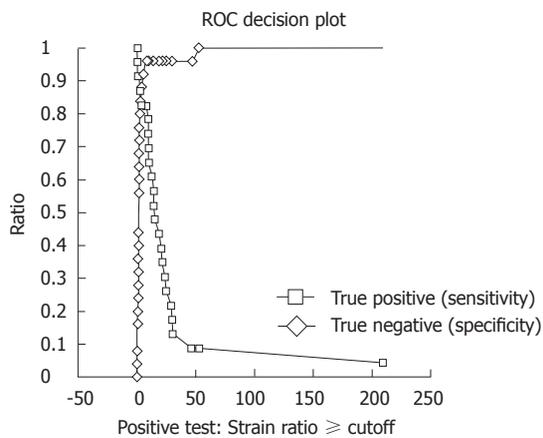
The ROC area under the curve, using elastography strain ratio, was 0.87 (95% CI: 0.75-1.00,  $P < 0.0001$ ) (Figure 4). The strain ratio which was the optimal cut-off point for distinguishing malignant from benign nodes was  $\geq 7.5$  as determined by a ROC sensitivity specificity decision plot (Figure 5), with a strain ratio above this indicating malignant involvement. The likelihood ratio was 20.65 for a strain ratio of 7.5. This gave sensitivity 83%, specificity 96%, positive predictive value (PPV) 95%, and negative predictive value (NPV) 86% (Table 2). The accuracy of elastography with a strain ratio  $\geq 7.5$  was 90%.

**Comparison of elastography strain ratio vs other endoscopic ultrasound methods of distinguishing benign from malignant lymph nodes**

ROC area under the curve comparisons showed that there



**Figure 4** Receiver operating characteristic curve for elastography strain ratio. The receiver operating characteristic area under the curve was 0.87 ( $P < 0.0001$ ).



**Figure 5** Receiver operating characteristic sensitivity, specificity based decision plot to determine the optimal elastography strain ratio cut-off point. The sensitivity and specificity lines cross at strain ratio 7.5.

was a trend for elastography strain ratio to be the best diagnostic test for distinguishing benign from malignant nodes when compared to standard EUS nodal assessment, i.e., size, shape, density, distinction of border and any combination of these four conventional characteristics, although statistical significance was reached for only individual comparisons of size and clear edge (Figure 6 and Table 3).

Elastography strain ratio assessment was more accurate than all other assessed EUS characteristics of lymph nodes, with an accuracy of 90% (Figure 6, Tables 2 and 3).

Elastography strain ratio was favourable compared to other nodal assessments with regard to sensitivity, specificity, PPV and NPV (Table 2).

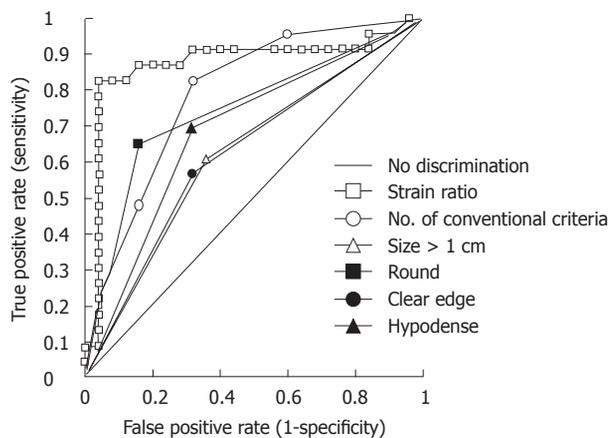
**Intra-observer variation**

The mean coefficient of variation (CV) for all recorded node strain ratio recordings was 44.8%. The mean CV for cytologically malignant nodes was 51.7%, while the mean CV for cytologically benign nodes was 34%.

**Table 2** Comparison of sensitivity, specificity, positive predictive value and negative predictive value for the ability of endoscopic ultrasound-based characteristics to differentiate benign from malignant lymph nodes (% , CI)

	Sensitivity	Specificity	PPV	NPV	Accuracy
Strain ratio	83 (61-95)	96 (80-100)	95 (75-100)	86 (67-96)	90 (77-97)
4/4 conventional criteria present	22 (07-43)	96 (80-100)	83 (36-99)	57 (41-72)	60 (45-72)
3/4 conventional criteria present	48 (27-69)	84 (64-96)	75 (45-92)	64 (45-80)	67 (52-80)
Size > 1 cm	61 (39-80)	64 (43-82)	61 (38-80)	64 (43-82)	62 (47-76)
Shape round	65 (43-84)	84 (64-96)	79 (54-94)	72 (53-87)	75 (60-86)
Clear border	56 (34-77)	68 (47-85)	62 (38-82)	63 (42-81)	62 (47-76)
Hypodense	70 (47-87)	68 (46-85)	67 (45-84)	71 (49-87)	69 (53-81)

PPV: Positive predictive value; NPV: Negative predictive value.



**Figure 6** Receiver operating characteristic curve comparison of elastography strain ratio against conventional endoscopic ultrasound criteria both in combination and individually (size > 1 cm, round, hypodense, clear edge).

**Table 3** The area under the curve for receiver operating curve characteristics for each endoscopic ultrasound-based modality for distinguishing benign from malignant nodes

	ROC AUC	P value <sup>1</sup>
Strain ratio	0.87 (0.75-1.00)	NA
Number of conventional criteria	0.79 (0.66-0.92)	0.2846
Size > 1cm	0.62 (0.48-0.76)	0.0078
Shape round	0.75 (0.62-0.87)	0.1329
Clear edge	0.62 (0.48-0.76)	0.0035
Hypodense	0.69 (0.55-0.82)	0.0100

<sup>1</sup>Comparison with strain ratio. The P value determined relates to the area under the curve (AUC) of an individual characteristic compared to elastography strain ratio receiver operating characteristics (ROC) AUC. NA: Not available.

**DISCUSSION**

This study has shown EUS-guided elastography to be ef-

fective for distinguishing between malignant and benign lymph nodes in patients with oesophago-gastric cancer and that it is more accurate than conventional EUS nodal characteristics.

Elastography of tissue is a new technology that has been successfully applied to several clinical fields. The specific modality of EUS-guided elastography has been developed over the past few years and first generation technology using elastographic colour imaging has shown promising results in the assessment of pancreatic disease<sup>[17,18]</sup>. There has also been encouraging, though limited, data on lymph node assessment<sup>[13,18-20]</sup>.

Conventional EUS lymph node assessment using size, shape, density and distinction of node border does not give adequate sensitivity and specificity to confidently distinguish between malignant and benign nodes. In this study, accuracy of these characteristics is poor (Table 3). This is consistent with previously published data<sup>[8,19,22]</sup>. However, using EUS elastography with a strain ratio cut off of  $\geq 7.5$  for malignancy, we found a sensitivity and specificity of 83% and 96%, respectively. The ROC AUC was highest for strain ratio compared to all of the conventional EUS criteria, either individually or in combination. The strain ratio cut-off point of  $\geq 7.5$  to distinguish malignant nodal involvement was derived from a ROC sensitivity and specificity decision plot. The specificity and the sensitivity using this strain ratio cut-off point were 96% and 83%, respectively.

Out of the 23 cytologically determined malignant cases, there were 4 cases where elastography strain ratio gave a false negative result. This may be explained by vascular or necrotic lymph nodes giving the elastographic appearance of soft tissue<sup>[19,23]</sup>. There were no surgical resection specimens in this study to confirm or refute this hypothesis.

The gold standard for determination of malignant cells within a lymph node at the time of pre-operative staging remains FNAB. However, there are challenges encountered during endosonography such as large peri-tumoural reactive lymph nodal assessment, targeting which suspicious nodes on which to perform FNAB, and avoiding passing the needle through interposed major neurovascular structures. Although EUS-FNAB is considered the gold standard for the purposes of this study, there are previous reports suggesting EUS-FNAB has a false negative rate of 5%-10%<sup>[24,25]</sup>. EUS-FNAB was used as the gold standard due to the lack of availability of routine imaging follow up or surgical resection specimens. This is a limitation of our study. In this study, there was only one patient who had an apparently striking falsely positive strain ratio value of 47.0. Despite radical chemotherapy this patient has not progressed well with rapid deterioration. It is possible that the EUS-FNA was cytologically falsely negative, rather than there being a falsely positive elastography strain ratio value.

Technical challenges were encountered in performing elastography during EUS. The technique relies on respiratory movement and vascular pulsation to generate appropriate compression displacement of the digitalised

radiofrequency echo lines. Obtaining consistent compression recordings was not always possible, leading to some variability in the elastogram produced, as has previously been described by other groups<sup>[26]</sup>. This will have led, in part, to the moderately large intra-observer variation calculated which is a limitation of this technique. It would have been desirable to have a coefficient of variance not exceeding 30%. It is, however, worth noting that the intra-observer variation was only significant when strain ratio values were high and none of the range of values obtained changed the likelihood of a node from being malignant to benign.

The method described relies on comparison of the strain within a node and surrounding tissue. This comparison assumes that the surrounding tissues are normal. It is recognised that the surrounding tissues may have different physical characteristics, which may influence the results. It is also worth noting that, as central necrosis and vascular invasion of a lymph node occur, the strain within the node may start to reduce as these "softer" components of the node are assessed. This is the basis of a fifth colour pattern analysis described by other authors<sup>[18,23]</sup>. However, the use of a strain ratio is an objective measure that is not limited by the inter-observer variability which is inherent in elastogram colour pattern analysis.

A limitation of this study is that it was undertaken within a single centre. However, there are supporting data from a recently reported large European multi-centre trial. Giovannini *et al.*<sup>[23]</sup> report on 38 cases of oesophago-gastric cancer staging EUS-guided elastography lymph node assessments in a larger cohort of lymph node analysis. The elastography sensitivity and specificity of 91.8% and 82.5% recorded from this multicentre study are similar to those presented in this paper. The sensitivity and specificity recorded from the data presented in our paper are also similar to those found when cervical lymph nodes were assessed by sonographic elastography<sup>[19]</sup>.

It is also recognised that there is the potential for selection bias within this study, in that all patients were having EUS-FNAB of nodes which were considered suspicious for malignancy. However, it is proposed that this modality is used in those very situations where EUS-FNAB will have been contemplated but may not be practical.

Patients undergoing staging of oesophago-gastric cancers are often subjected to several staging investigations. EUS-guided elastography offers an additional assessment of any suspicious lymph nodes that can be undertaken at the time of the standard EUS evaluation and is no more invasive. The recent change in the staging system for oesophago-gastric cancers means that the absolute number of involved malignant nodes becomes critical in determining the tumour stage<sup>[27-30]</sup>. Therefore, any modality that improves nodal staging is of critical importance. We believe EUS elastography is complementary to FNA in distinguishing between benign and malignant lymphadenopathy.

In conclusion, EUS elastography has been shown in this study to be superior to standard EUS assessment in characterising benign from malignant lymph nodes in

oesophago-gastric cancer. It appears complementary to current staging investigations and has the potential to improve the staging and management of this disease.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Endoscopic ultrasound (EUS) is an integral investigation in the staging of oesophageal and oesophago-gastric junctional tumours. Determination of lymph node involvement in particular is of critical prognostic importance. At present, staging relies largely on EUS-fine needle aspiration biopsy (FNAB) of suspicious nodes after initial determination of standard EUS characteristics. However, the invasive process of FNAB is not always possible.

### Research frontiers

Elastography has been shown to be useful in distinguishing benign from malignant tissue in the pancreas. However, there are limited data assessing the accuracy of EUS elastography for nodal assessment in upper gastrointestinal malignancy.

### Innovations and breakthroughs

There have been recent reports of the use of elastography to distinguish between benign and malignant tissue, including lymph nodes. Earlier studies used qualitative descriptions of elastogram patterns. More recently, there have been technological advances allowing quantitative assessment of elastography strain ratio which measures, and compares, the stiffness of tissues.

### Applications

This study suggests that quantitative EUS elastography is accurate at distinguishing between malignant and benign lymph nodes in the staging of oesophago-gastric malignancy. Compared to standard EUS characterisation of lymph nodes, elastography is superior in this study. EUS elastography appears complementary to current staging investigations and has the potential to improve the staging and management of this disease.

### Terminology

Elastography is a measure of the stiffness, or strain, of tissue. This can be useful in many disease processes including malignancy where tissue is often "harder" or "stiffer" where there is cancerous tissue present. Measuring elastography relies on applying a mechanical compression and determining how much the tissue deforms. Harder tissue deforms less than soft tissue.

### Peer review

Overall, a very interesting article looking at something that remains a problem without tissue diagnosis. The paper is well written and reads well. The methodology is appropriate, the results presented well and the discussion is appropriate. This paper will add to our understanding and serve as a platform for further studies looking at the characteristics of lymph nodes, not just for the oesophago-gastric lymph nodes but other malignancies as well.

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## Histotype-based prognostic classification of gastric cancer

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### Abstract

**AIM:** To test the efficiency of a recently proposed histotype-based grading system in a consecutive series of gastric cancers.

**METHODS:** Two hundred advanced gastric cancers operated upon in 1980-1987 and followed for a median 159 mo were investigated on hematoxylin-eosin-stained sections to identify low-grade [muconodular, well differentiated tubular, diffuse desmoplastic and high lymphoid response (HLR)], high-grade (anaplastic and mucinous invasive) and intermediate-grade (ordinary cohesive, diffuse and mucinous) cancers, in parallel with a previously investigated series of 292 cases. In addition, immunohistochemical analyses for CD8, CD11 and HLA-DR antigens, pancytokeratin and podoplanin, as well as immunohistochemical and molecular tests for microsatellite DNA instability and *in situ* hybridization

for the Epstein-Barr virus (EBV) *EBER1* gene were performed. Patient survival was assessed with death rates per 100 person-years and with Kaplan-Meier or Cox model estimates.

**RESULTS:** Collectively, the four low-grade histotypes accounted for 22% and the two high-grade histotypes for 7% of the consecutive cancers investigated, while the remaining 71% of cases were intermediate-grade cancers, with highly significant, stage-independent, survival differences among the three tumor grades ( $P = 0.004$  for grade 1 vs 2 and  $P = 0.0019$  for grade 2 vs grade 3), thus confirming the results in the original series. A combined analysis of 492 cases showed an improved prognostic value of histotype-based grading compared with the Lauren classification. In addition, it allowed better characterization of rare histotypes, particularly the three subsets of prognostically different mucinous neoplasms, of which 10 ordinary mucinous cancers showed stage-inclusive survival worse than that of 20 muconodular ( $P = 0.037$ ) and better than that of 21 high-grade ( $P < 0.001$ ) cases. Tumors with high-level microsatellite DNA instability (MSI-H) or EBV infection, together with a third subset negative for both conditions, formed the T8 cell-rich HLR group, the largest group among low-grade histotypes. Coexisting HLR proved to be a factor in improved prognosis in tumors with microsatellite instability ( $P = 0.0015$  vs HLR-/MSI-H tumors) or DR type human leukocyte antigen expression ( $P = 0.033$  vs HLR-/HLA-DR<sup>+</sup> tumors).

**CONCLUSION:** Identification of low- and high-grade histotypes can improve the prognostic assessment of a substantial proportion of gastric cancers in routine diagnostic practice.

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**Key words:** Gastric cancer; High-grade histotype; Low-grade histotype; Lymphoid response; Epstein-Barr virus; Microsatellite instability

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## INTRODUCTION

The difficulty of assessing the prognosis of gastric cancer using histological methods is well known and this is also reflected in the essentially descriptive character of presently used classifications<sup>[1-4]</sup>. However, several histotypes characterized by lower malignant potential have been identified and separated from more common cohesive (so-called “intestinal”) or diffuse tumors. They include lymphocyte-rich cancer<sup>[5-7]</sup>, mucinodular cancer<sup>[8]</sup>, very-well-differentiated tubular cancer with an intestinal<sup>[9]</sup> or gastric<sup>[10]</sup> phenotype, and a low-grade subtype of diffuse desmoplastic cancer<sup>[11]</sup>. Similarly, various kinds of cancer with poor outcome have been identified, from poorly differentiated neuroendocrine carcinoma, small to large cell<sup>[12,13]</sup>, to anaplastic diffuse cancer<sup>[14]</sup> or hepatoid<sup>[14]</sup>, choriocarcinomatous<sup>[15]</sup> and adenosquamous carcinoma<sup>[16]</sup>. In addition, comparative genomic hybridization analysis has shown a clear relationship between the number and severity of genomic alterations and tumor histotype and prognosis<sup>[17,18]</sup>.

The different behavior of these histotypes offered an opportunity to develop a three-grade system of prognostic evaluation, which, when applied to a large tumor series, was highly predictive of patient outcome<sup>[19]</sup>. However, the tumor series used in that study was substantially selected (1) to be representative of all main stages (intramucosal cases apart) and histological types of the disease; and (2) to include uncommon histological subtypes or variants, as well as earlier invasive stages (submucosal or confined to muscularis propria). Therefore, in order to ascertain the effectiveness of the system in routine diagnostic work, a continuous, homogeneous series of advanced cancers needs to be evaluated.

In this study, we retrospectively identified prognostic histotypes according to previously reported criteria<sup>[19]</sup> in a consecutive series of advanced (muscularis propria invasion or beyond) gastric cancers collected at Varese General Hospital during 1980-1987, and we tested such histotypes as potential predictors of patient outcome and compared the results with those of the original Pavia series. During the study, we realized that we needed to investigate further mucinous and lymphocyte-rich can-

cers, due to discrepancies concerning: (1) the impact of histology or stage on mucinous cancer prognosis<sup>[20-23]</sup>; and (2) the contribution of tumor microsatellite instability, Epstein-Barr virus (EBV) infection and DR type human leukocyte antigen (HLA-DR) expression, rather than lymphoid cell response *per se*, to the natural history of lymphocyte-rich neoplasms<sup>[5-7,24-27]</sup>. Therefore, mucinous and lymphocyte-rich tumors from both series were combined to obtain tumor groups large enough to allow appropriate investigation.

## MATERIALS AND METHODS

### Tissue samples

A consecutive series of 200 invasive (T2-T4) gastric cancers were retrieved from the files of the Anatomic Pathology Service, for patients who had undergone potentially curative surgery at Varese General Hospital during 1980-1987. The clinicopathological and follow-up data of all the patients were carefully collected from hospital records, interviews with family doctors, and the Varese Province Tumor Registry. One hundred and eighty-five of the cases had already been the subject of a previous investigation<sup>[2]</sup>. Eight cases were operated on during January 1980-April 1987 and were not considered in the previous study because the available clinical or follow-up documentation was incomplete. This information was retraced, appropriately documented and added to the present study to ensure the continuous pattern of the patient series, together with seven more cases operated on in May-June 1987. The original tumor node metastasis (TNM) stage assessment of each tumor was revised according to the criteria of the 2002, 6th Edition American Joint Committee on Cancer system<sup>[28]</sup>. No antitumor therapy had been given to the patients. For survivors, the follow-up period was prolonged until 2008; a median follow-up of 159 mo was recorded.

### Immunohistochemical analysis

Archival and newly cut paraffin sections were stained with hematoxylin-eosin, Alcian blue-periodic acid Schiff or the immunoperoxidase procedure using antibodies directed against h-MLH1 (G-168.15 clone; Pharmingen, San Diego, CA, United States), hMSH2 (Fe11 clone; Oncogene, Cambridge, MA, United States), hPMS2 (clone A16-4; BD Pharmingen), hMSH6 (clone 44; BD Transduction Laboratories, Lexington, KY, United States), CD8 antigen (C8/144B clone; Dako, Glostrup, Denmark), CD11c antigen (5D11 clone; Novocastra Laboratories, Newcastle, United Kingdom), pancytokeratin (AE1/AE3 clone; Novocastra Laboratories), HLA-DR (LN3 clone; Biotest, Dreieich, Germany) and podoplanin (D2-40 clone; Biocare Medical, Concord, CA, United States) as previously reported<sup>[7,19,29]</sup>.

### Molecular analysis

*In situ* hybridization for the *EBER1* gene of EBV was performed as described previously<sup>[3,7]</sup>. Microsatellite instability was assessed at Bat 25, Bat 26, BAT40, D5S346 and D2S123 loci. Tumors with instability involving at

least two of the five loci were classified as highly instable (MSI-H), while those with only one instable locus were classified as low instable (MSI-L) and included in the MSI negative tumor group together with microsatellite stable cases<sup>[7,19,30]</sup>.

### Morphological analysis

Tumor histotypes were identified as described previously<sup>[8-11,19]</sup>. In particular, very-well-differentiated tubular (VWDT) cancer is characterized by glands with moderately atypical, polarized cells arranged in a monostratified epithelium, low-grade diffuse desmoplastic cancer shows fibroblast-rich desmoplasia surrounding individual (or minute groups of) moderately atypical tumor cells, while muconodular cancer forms extracellular mucin lakes with expansile borders in which isolated signet ring cells or cords of mucin-producing tumor cells are freely floating.

To increase the diagnostic accuracy of lymphocyte-rich tumors, as well as intratumor CD8<sup>+</sup> T cell counts, intraepithelial T8 cells (i.e., cells infiltrating tumor aggregates so as to contact neoplastic cells directly, with the exclusion of purely stromal T8 cells) were also counted<sup>[7,31]</sup>, and an evaluation of dendritic cells was added<sup>[27]</sup>. Thus, in this study, classification of a lymphocyte-rich tumor as high lymphoid response (HLR) required one of the following: (1) a lymphoepithelial type histological pattern with an overwhelming lymphocyte infiltrate dissecting tumor cells; or (2) > 400 intratumor and/or >200 intraepithelial CD8-positive cells in 10 high-power fields (HPFs), coupled with a band of lymphoid cells rich in CD8<sup>+</sup> T cells and CD11c<sup>+</sup> dendritic cells surrounding expansile tumor nodules.

Anaplastic cancers were characterized by small to large, cytokeratin-positive cells with highly atypical nuclei, with or without prominent nucleoli and with or without signs of poor neuroendocrine differentiation, high cellularity, scarce stroma, and high proliferative rates (> 20 mitoses/10 HPFs)<sup>[11,12]</sup>. During characterization of the mucinous infiltrative tumors, it was found that those showing local infiltration of peritumoral tissues in the absence of prominent lymphoinvasion or angioinvasion had a less severe prognosis. Therefore, in this study, infiltrative tumors lacking vascular invasion or with only sporadic lymphoinvasion were added to the grade 2 group, together with ordinary cohesive and diffuse cancers, while only prominently lymphoinvasive (two or more foci per microscopic tumor sections) or angioinvasive cases remained in the grade 3 group together with anaplastic cancers, as in the original classification<sup>[19]</sup>. Cases showing a coexistence of two or more histological patterns were classified according to their prevalent histotype, provided that all the components were low-grade; otherwise, they were classified according to their higher grade component.

A reproducibility test involving two senior pathologists (Solcia E and Capella C) gave a  $\kappa$  value of 0.84 concerning interobserver agreement for five main histotypes (cohesive, diffuse, mucinous, anaplastic and HLR), a  $\kappa$  of 0.81 agreement for nine subtypes (HLR, VWDT, ordinary cohesive, low-grade diffuse desmoplastic, ordinary

**Table 1** Survival analysis of 200 gastric cancers (Varese series) according to tumor node metastasis stage

Stage	n (%)	Death rate	95% CI	Cox survival analysis		
				HR	95% CI	P value
I	40 (20)	1.63	0.81-3.25	1		
II	61 (30.5)	13.67	10.06-18.56	5.64	2.63-12.09	< 0.001 <sup>a</sup>
III	68 (34)	26.04	19.74-34.46	9.49	4.45-20.24	< 0.001 <sup>b</sup>
IV	31 (15.5)	52.66	36.11-76.79	18.90	8.41-42.46	< 0.001 <sup>c</sup>

<sup>a</sup>P = 0.001 vs III + IV; <sup>b</sup>P = 0.015 vs II ; <sup>c</sup>P = 0.004 vs III. CI: Confidence intervals; HR: Hazard ratio.

diffuse, muconodular, ordinary mucinous, invasive mucinous and anaplastic), and a  $\kappa$  of 0.79 agreement for the three histotype-based grades (low, intermediate and high).

From the previously investigated Pavia series of 294 cases<sup>[19]</sup>, 292 cases (two tumors had to be excluded because there was no remaining tumor tissue) were considered for comparative analysis with the Varese series, as well as for a joint reinvestigation of both series looking at mucinous and HLR tumors according to the above criteria. In addition, the prognostic value of the histotype-based grading system was compared with that of the commonly used Lauren classification<sup>[1]</sup>.

### Statistical analysis

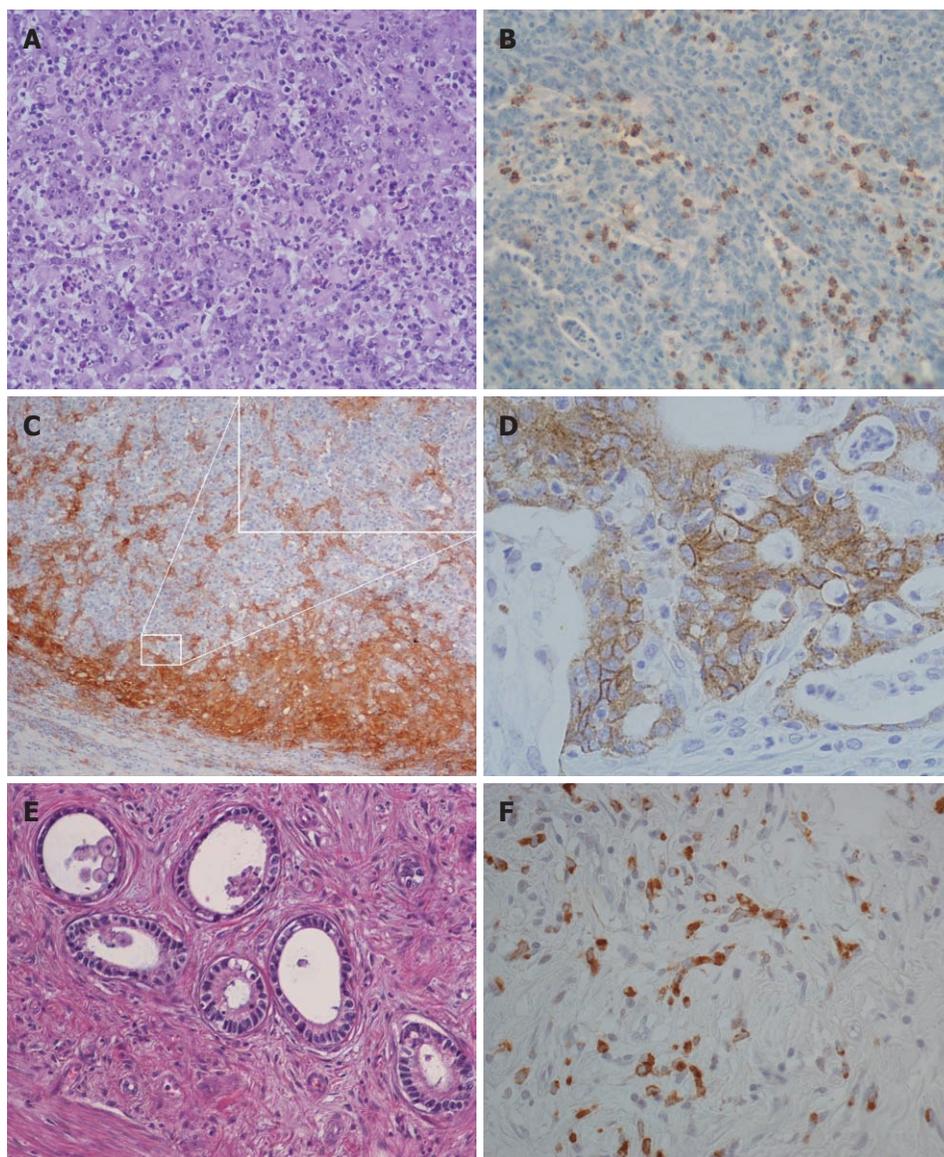
Statistical analysis was performed using Stata version 11 (Stata Corporation, College Station, TX, United States). All tests were two-sided. Categorical variables were described with counts and percentages and compared with the Fisher exact test. Continuous variables were described with median and quartiles, and compared with the Kruskal-Wallis test. Death rates per 100 person-years, with 95% confidence intervals (CIs), and Kaplan-Meier estimates were computed to describe survival. The Cox model was used to assess the prognostic role of the considered variables; both univariate and bivariate models (inclusive of stage) were fitted. The hazard ratio (HR) and 95% CI were reported. Proportional hazard assumptions were satisfied in all cases. The Harrell c statistic was computed to assess model performance (discrimination ability); a value of 0.5 indicating no discrimination and a value of 1 indicating perfect discrimination.

## RESULTS

### Characterization of the Varese continuous series

The Varese consecutive, non-selected series of 200 advanced (T<sub>2</sub> or above) gastric cancers is described in Table 1 according to stage and patient survival. Compared with the original Pavia selected series, which also included a substantial number (44 cases) of deeply submucosal (penetrating T1b) tumors, the present series showed more advanced tumors (Stages III + IV: 49.5% vs 39% of the original series; Stage II: 30.5% vs 25%). A clear step-wise, stage-dependent behavior emerges from the survival analysis.

Of the 200 tumors, 44 (22%) had low-grade, 14 (7%) high-grade and 142 (71%) intermediate-grade histotypes



**Figure 1** Histological and histochemical aspects of high lymphoid response cancers. A: Lymphoepithelioid pattern of an HLR EBV<sup>+</sup> tumor (hematoxylin-eosin,  $\times 200$ ); B: High intratumor T8 cells infiltration in an HLR EBV/MSI case (CD8 immunoperoxidase-hematoxylin,  $\times 200$ ); C: Peritumor dendritic cell rich demarcating band in an HLR MSI-H tumor (CD11c immunoperoxidase-hematoxylin,  $\times 100$ ); in the inset, focal enlargement of image C to show interaction of CD11c-reactive dendritic cells with unreactive neoplastic cells ( $\times 400$ ); D: HLA-DR reactivity of an HLR MSI-H case (immunoperoxidase-hematoxylin,  $\times 400$ ); E: Low-grade very-well-differentiated tubular carcinoma (hematoxylin-eosin,  $\times 200$ ); F: Low-grade desmoplastic tumor with spindle neoplastic cells interspersed among fibroblast rich stroma (CAR5, immunoperoxidase-hematoxylin,  $\times 200$ ).

(Figures 1 and 2). Survival analysis according to histotype-based grade is outlined in Table 2 and Figure 3. The more favorable behavior of grade 1 compared to grade 2 and of grade 2 compared to grade 3 tumors is evident.

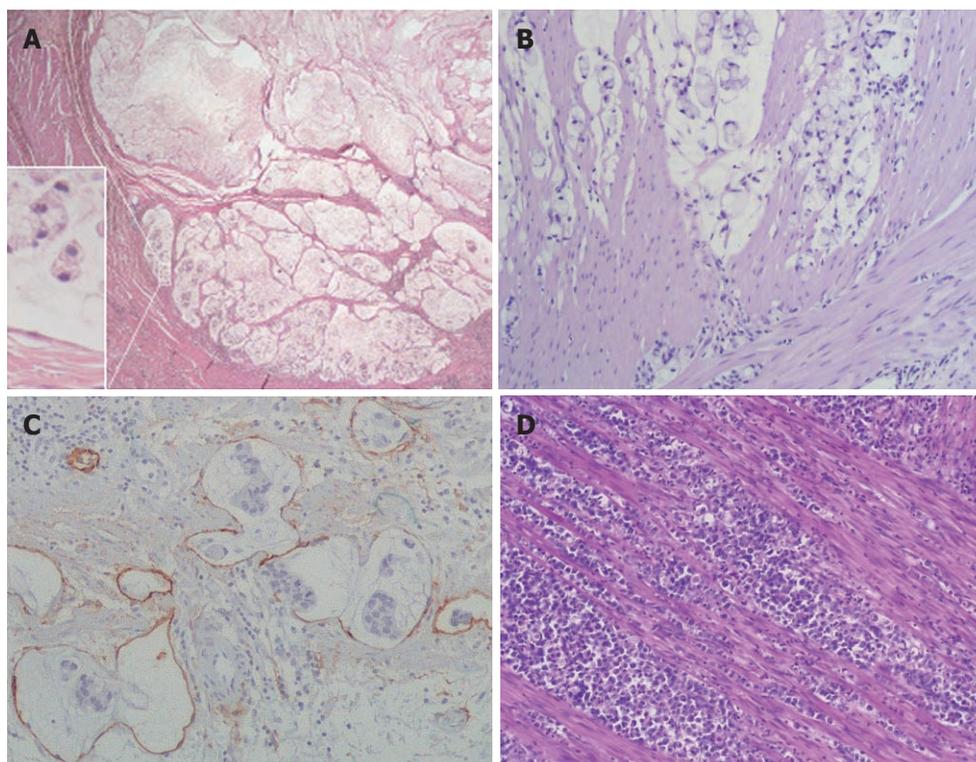
In Table 2, univariate analysis of the Varese series after reclassification according to Lauren<sup>[1]</sup> shows a significantly worse prognosis for the diffuse compared to intestinal and unclassified types [model:  $\chi^2(2\gamma) = 8.67$ ,  $P = 0.013$ ]. However no significant difference was observed among the same cases in the stage-inclusive bivariate analysis (model:  $P = 0.341$  for Lauren classification), while no difference was found by either univariate (model:  $\chi^2(2\gamma) = 4.87$ ,  $P = 0.087$ ) or bivariate (model:  $P = 0.342$ ) analysis among the 292 tumors in the Pavia series. In both the Varese and Pavia series, the Harrell's concor-

dance ( $c$ ) test showed a higher efficiency of the histotype-based grading ( $c = 0.63$  and  $0.73$ , respectively) compared to the Lauren classification ( $c = 0.57$  and  $0.55$ ).

Individual histotypes in the three grades are detailed in Table 3, first column. It appears that, while ordinary cohesive or diffuse and HLR tumors form a substantial group, the number of other histotypes is too low to allow appropriate statistical analysis.

#### **Joint analysis of 492 cases from both Varese and Pavia series**

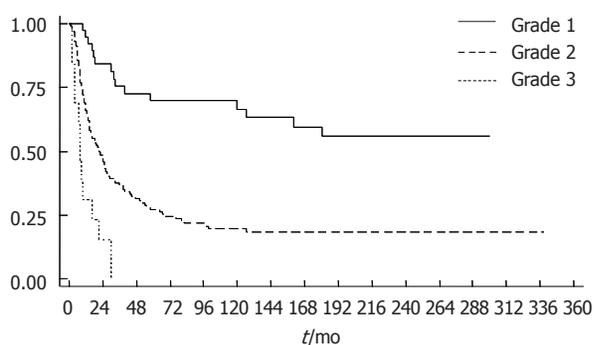
When corresponding tumors of the two series were analyzed jointly (Table 3), the more favorable behavior of grade 1 compared to grade 2 tumors and of the latter compared to grade 3 cases was confirmed. In addition,



**Figure 2 Muconodular, well-differentiated tubular, diffuse desmoplastic and high lymphoid response.** A: Low-grade muconodular cancer with expansile growth (hematoxylin-eosin, × 20); in the inset, note tumor cells floating inside mucin, free of contact with stroma (× 400); B: intermediate-grade, locally infiltrative mucinous cancer (hematoxylin-eosin, × 200); C: massive lymphoinvasion of a high-grade mucinous cancer (podoplanin immunoperoxidase-hematoxylin, × 200); D: Diffuse anaplastic cancer invading the muscularis propria (hematoxylin-eosin, × 200), in the inset the enlargement shows cellular pleomorphism (× 400) .

	n (%)	Death rate	95% CI	Cox survival analysis			
				HR	95% CI	P value	
						Univariate <sup>a</sup>	With stage
Grade							
1	44 (22)	3.35	2.02-5.55	1			
2	142 (71)	17.24	14.16-21.90	3.5	2.02-6.05	< 0.001	0.004
3	14 (7)	95.66	54.33-168.45	9.64	4.41-21.06	< 0.001 <sup>a</sup>	< 0.001 <sup>b</sup>
Lauren type <sup>1</sup>							
Intestinal	116 (58)	8.86	6.93-11.31	1			
Diffuse	50 (25)	21.08	15.20-29.22	1.71	1.13-2.57	0.011	0.362
Unclassified	34 (17)	18.4	12.53-27.03	1.7	1.07-2.68	0.023	0.165

<sup>a</sup>P = 0.001 vs grade 2; <sup>b</sup>P = 0.019 vs grade 2. Harrell's c = 0.63. <sup>1</sup>F: Univariate model:  $\chi^2$  (2γ) = 8.67, P = 0.013. Harrell's c = 0.57. Stage-inclusive model: P = 0.341 for Lauren classification. CI: Confidence intervals; HR: Hazard ratio.



**Figure 3 Kaplan-Meier survival estimate of 200 gastric cancers according to histotype-based grade.**

the resulting number of the rare histotypes was sufficient to allow survival analysis of each histotype. Thus, the prognostic similarity of types belonging to the same grade and their significant difference from those of other grades was assessed.

**Reinvestigation of mucinous and high lymphoid response tumors**

It also appears from Table 3 that mucinous neoplasms, when appropriately reclassified as muconodular, ordinary mucinous and highly invasive mucinous cancers, may form three prognostically different histological subsets, as confirmed by separate Cox univariate and stage-inclusive

**Table 3** Survival analysis of 492 tumors (Varese + Pavia series) according to histotype and grade

	n (%)		Death rate	95% CI	Cox survival analysis			
					Varese series	Joint series	HR <sup>1</sup>	95% CI
	Univariate	With stage						
Grade 1	44 (22)	132 (26.8)	2.54	1.80-3.57	1			
HLR	38 (19)	82 (16.6)	3.67	2.50-5.39	1			
WD tubular	2 (1)	13 (2.6)	0					
Lg diff.desm	2 (1)	17 (3.5)	1.50	0.48-4.64	0.43	0.13-1.42	0.167	0.109
muconodular	2 (1)	20 (4.1)	1.67	0.63-4.44	0.51	0.18-1.46	0.211	0.271
Grade 2	142 (71)	307 (62.4)	15.97	13.96-18.28	4.91	3.40-7.11	< 0.001	< 0.001
Mucinous ord.	4 (2)	10 (2.0)	11.50	5.16-25.59	2.16	0.89-5.24	0.090 <sup>a</sup>	0.987 <sup>b</sup>
Cohesive ord.	95 (47.5)	196 (39.8)	15.28	12.89-18.11	3.43	2.25-5.23	< 0.001	0.001
Diffuse ord.	43 (21.5)	101 (20.6)	18.09	14.36-22.79	3.96	2.53-6.22	< 0.001	< 0.001
Grade 3	14 (7)	53 (10.8)	108.84	82.02-144.43	18.47	11.56-29.50	< 0.001 <sup>c</sup>	< 0.001 <sup>c</sup>
Mucinous Hg.	3 (1.5)	21 (4.3)	100.0	64.07-160.0	11.54	6.32-21.48	< 0.001	< 0.001
Anaplastic	11 (5.5)	32 (6.5)	120.0	80.01-170.0	14.11	8.17-24.39	< 0.001	< 0.001

<sup>1</sup>For grades, based on grade 1; for histotypes, based on high lymphoid response (HLR) type. <sup>a</sup> $P = 0.005$  vs muconodular and  $P < 0.001$  vs mucinous. <sup>b</sup> $P = 0.037$  vs muconodular and  $P < 0.001$  vs mucinous. <sup>c</sup> $P < 0.001$  vs grade 2. Harrell's  $c = 0.69$  for the 3 grades and 0.71 for the 9 histotypes. CI: Confidence intervals; HR: Hazard ratio; WD: Well differentiated; diff.desm: Diffuse desmoplastic; ord.: Ordinary; Hg.: High-grade.

**Table 4** Three subtypes of high lymphoid response tumors and comparison with non-high lymphoid response high-level microsatellite DNA instability cases (Pavia and Varese series)

	n (%)	Death rate	95% CI	HR	95% CI	Cox survival analysis	
						Univariate	With stage
HLR <sup>+</sup>							
EBV <sup>+</sup>	24 (29.6) <sup>1</sup>	7.38	4.09-13.33	2.39	1.03-5.53	0.042	0.817
MSI-H	40 (49.4)	3.01	1.67-5.44	1			
EBV/MSI	17 (21.0)	2.27	0.85-6.06	0.81	0.26-2.53	0.711	0.393
HLR <sup>-</sup>							
MSI-H	38 (48.7) <sup>2</sup>	12.29	8.24-18.33	3.64	1.78-7.47	< 0.001	0.015

<sup>1</sup>One EBV<sup>+</sup>/MSI case omitted; <sup>2</sup>% of all MSI-H cases, HLR<sup>+</sup> or <sup>-</sup>. HLR: High lymphoid response; EBV: Epstein-Barr virus; MSI-H: High-level microsatellite DNA instability.

survival analyses, where ordinary mucinous cancers proved significantly worse than muconodular and better than highly invasive cancers (Table 3). Significant differences were also found between the three groups in terms of TNM stage, T level invasion and lymph node involvement (for all:  $P < 0.001$ , Fisher's exact test) and even diameter ( $P < 0.001$ , Kruskal-Wallis test). In contrast, only a nonsignificant trend (Cox univariate  $P = 0.126$  and stage-inclusive  $P = 0.102$ ) for better survival was noted among mucinous cancers as a whole; for those with cohesive vs diffuse or mixed histological patterns.

No survival difference was found between HLR and the three other low-grade histotypes or, among the HLR cases, between MSI-H and the EBV<sup>+</sup>/MSI subset, while a trend for worse behavior of the EBV<sup>+</sup> compared to the other subsets was noted by univariate analysis, which disappeared with stage-inclusive bivariate analysis (Table 4). EBV<sup>+</sup> tumors also differed from the other two HLR subsets in showing significantly higher proportions of lymphoepithelioid histology (17/24, 71%, vs 5/57, 9%,  $P < 0.001$ , Fisher's exact test) and median intratumor T8 (107.5 vs 58.5 per HPF,  $P < 0.001$ , Kruskal-Wallis test). In contrast, 40 HLR MSI-H cases obviously showed more

favorable behavior than their 38 non-HLR MSI-H counterparts (22 cohesive, 10 mucinous, three diffuse and anaplastic cancers), of which six were low-, 24 intermediate- and eight high-grade.

The proportion of mucinous neoplasms showing MSI-H (10/51; 19.6%) did not differ significantly from that of the whole tumor population (78/492; 15.9%) while remaining significantly lower than that of HLR cases (41/81; 50.6%). Notably, the 10 MSI-H cases were equally distributed among the three grades of mucinous cancers, being grade 1 [14/20 (20%)], grade 2 [1/10 (10%)], and grade 3 [5/21 (23.8%)].

For the combined analysis of HLR and HLA-DR status, 77 of the total 82 HLR tumors from both series had sufficient histological material left to allow reinvestigation, together with 202 randomly selected non-HLR tumors representative of all histotypes and stages. HLA-DR positivity in > 10% of tumor cells was found in 100/279 (35.8%) cases. Positive tumors showed a trend for lower death rate (5.97, 4.39-8.19 vs 9.41, 7.75-11.44) and improved survival (HR: 0.63, 0.44-0.91,  $P = 0.014$ ) compared with HLA-DR<sup>-</sup> cases, a behavior probably accounted for by the HLR<sup>+</sup>/HLA-DR<sup>+</sup> subset, in which

57 tumors showed a significantly lower death rate (3.21, 1.93-5.32 *vs* 11.84, 8.06-17.39) and better survival (HR: 0.35, 0.18-0.66,  $P = 0.001$ ) than their HLR/HLA-DR<sup>+</sup> counterparts.

## DISCUSSION

The present study confirms, in an independent patient series, the effectiveness of a recently proposed histotype-based grading system for the prognostic evaluation of gastric cancer<sup>[19]</sup>, despite substantial differences between the present series and the original one. Indeed, the present series differed in being consecutive rather than selected for uncommon histotypes and in lacking submucosal (T1b) cancers, while including more advanced cases, diagnosed and operated on about a decade earlier in another hospital serving a different territory. All these differences may help to explain the lower prevalence of low-grade cases (22% *vs* 31% in the original series), known to be more frequent in lower stages<sup>[19]</sup>. This is especially true for rare histotypes like muconodular<sup>[8]</sup>, VWD<sup>[9,10]</sup> or low-grade diffuse desmoplastic<sup>[11]</sup> cancers, which were specifically selected in the original series. However, the HLR histotype was confirmed to represent a fairly large (19%) population of low-grade gastric cancers, even after introducing more stringent diagnostic criteria. Thus, our consecutive Varese Hospital series suggests that only about 20% of all invasive (T2 or beyond) gastric cancers may be of low-grade, while < 10% may fit into the high-grade category. However, within these quantitative limits, the histotype-based three-grade system was confirmed to be highly predictive of patient outcome. This conclusion seems especially interesting considering the limited prognostic value of commonly used histological classifications<sup>[1-4]</sup>. Indeed, in this study the histotype-based classification and grading system showed superior Harrell's discriminative power when compared with the Lauren classification and, unlike the latter, outlined stage-independent prognostic differences.

Reinvestigation of the two series taken together increased the number of rare tumor subsets and allowed better characterization of their clinicopathological profile, with special reference to the HLR and mucinous tumors. The presence of three etiological subtypes among HLR tumors<sup>[19]</sup> was also confirmed in the new series. Joint analysis of the 82 HLR cases obtained from the two series allowed us to confirm the distinct clinicopathological pattern of EBV<sup>+</sup> (preferred proximal location in the stomach, higher frequency of lymphoepithelioid pattern, higher intratumor T8 cell counts, and a trend toward worse survival), as already illustrated in previous studies<sup>[6,7,32]</sup>, compared to both the MSI-H and the MSI/EBV<sup>-</sup> subsets, whose behavior was remarkably similar to each other. Among MSI-H tumors, the better prognosis of those associated with HLR compared with those lacking this association is of interest, because it suggests that cytotoxic T8 cell response, more than the MSI status itself, is a crucial factor in defining the behavior of this tumor subset, with potential implications for appropriate therapy<sup>[26]</sup>.

The cause of the T8-cell-rich lymphoid response in the EBV/MSI subset remains to be ascertained. Tumor cell overexpression of highly antigenic molecules like HLA-DR, as suggested in the present investigation, or of mutated p53 protein, as found in a previous study<sup>[19]</sup>, might be among its driving factors. Whatever its origin, it should be pointed out that the favorable prognostic implication of HLR deserves attention in clinical studies because, besides accounting for the better prognosis of MSI-H<sup>+</sup><sup>[5,7,19,26]</sup> and HLA-DR<sup>+</sup> cancers<sup>[24]</sup>, it might interfere with therapies potentially affecting antitumor immune response<sup>[26]</sup>.

In our cumulative series of 492 tumors, the proportion of mucinous cancers showing high MSI was not significantly higher than in the remaining neoplasms; a finding confirming recent observations<sup>[22]</sup>, although at variance with the behavior of mucinous cancers in the colon, where they represent in some series a dominant histotype among MSI-H cases<sup>[31,33]</sup>. Moreover, the few MSI<sup>+</sup> mucinous cancers that we found in the stomach lacked an HLR, and failed to show a preferential concentration in the low-grade, muconodular histotype, or evidence for more favorable survival compared to their MSI counterpart. This further outlines the limited prognostic value of MSI status in the absence of HLR, at least in the stomach. It should also be recalled that, at variance with gut cancers, in other cancers (e.g., breast and lung), MSI has more often been linked to adverse rather than favorable postoperative survival<sup>[25,34]</sup>.

Several studies have shown that mucinous gastric cancers have a worse prognosis<sup>[20-22]</sup>. However, it is uncertain whether a higher stage at diagnosis<sup>[23]</sup>, rather than mucinous type histology, may account for this. In this study we reinvestigated the issue with the help of an improved histological classification separating low-grade muconodular tumors from infiltrative mucinous cancers, among which grade 3 cases showing prominent vascular (especially lympho-) invasion were distinguished from grade 2 "ordinary" cases lacking it. Survival analysis showed that these may represent three prognostically distinct, stage-independent cancer subsets, despite the fact that they also show highly significant differences in stage and size. Thus, for the prognostic assessment of mucinous cancers both stage and histology should be carefully investigated.

In conclusion, our histotype-based grading system for gastric cancers proved to be an effective tool, at least for a minority (about 30%) of neoplasms. For the evaluation of grade 2 ordinary cancers, which form a very large, histologically heterogeneous group with a wide prognostic spectrum (e.g., a 95% CI 14-187 in our joint series), we should rely on common histological parameters (invasive pattern, proliferative rate, structural or cytological atypia, tumor cell phenotype)<sup>[4]</sup> or a variety of promising molecular tools<sup>[17,18,35]</sup>, as well as on carefully assessed stage.

## COMMENTS

### Background

Gastric carcinoma is a very heterogeneous tumor, often characterized by the

coexistence of two or more distinct histological components within the same tumor, and by different host responses in terms of stromal response. A detailed histopathological classification should enable us to identify tumor subtypes that could provide useful prognostic and therapeutic information. The efficacy of a recently proposed classification system in predicting survival needs to be tested in a non-selected series of gastric carcinomas.

### Research frontiers

This new histological classification takes advantage of the cytological, biological and architectural features of tumor cells to identify histological types (histotypes) of tumors with low, intermediate or high malignancy. Lymphoid and stromal reactions, which seem to play an important role in contrasting or favoring tumor growth, and consequently, resulting in a better or worse prognosis, also need to be taken into consideration.

### Innovation and breakthroughs

The proposed three-grade system proved to be highly predictive of patient outcome. It identified low-grade (muconodular, well-differentiated tubular, diffuse desmoplastic and high lymphoid response), intermediate-grade (ordinary cohesive, diffuse and mucinous) and high-grade (anaplastic and mucinous invasive) gastric cancers, with highly significant stage-independent survival differences and had a better prognostic value compared to the Lauren classification.

### Application

A careful histological examination of gastric cancers with the criteria proposed by the histotype-based prognostic classification was shown to be an effective tool in everyday diagnostic practice. Additional studies are necessary to identify histological and molecular parameters that could better characterize the large population of intermediate-grade cancers.

### Peer review

The manuscript fits well with the scope of the journal, and is well written. It addresses a relevant aspect of gastric cancer, the exact histopathological assessment of the tumor and correlation of these data to clinical and molecular alterations.

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## Low-dose steroid pretreatment ameliorates the transient impairment of liver regeneration

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### Abstract

**AIM:** To determine if liver regeneration (LR) could be disturbed following radiofrequency (RF) ablation and whether modification of LR by steroid administration occurs.

**METHODS:** Sham operation, partial hepatectomy (PH), and partial hepatectomy with radiofrequency ablation (PHA) were performed on adult Fisher 344 rats. We investigated the recovery of liver volume, DNA synthetic activities, serum cytokine/chemokine levels and signal transducers and activators of transcription 3 DNA-binding activities in the nucleus after the operations. Additionally, the effects of steroid (dexamethasone) pretreatment in the PH group (S-PH) and the PHA group (S-PHA) were compared.

**RESULTS:** The LR after PHA was impaired, with high serum cytokine/chemokine induction compared to PH, although the ratio of the residual liver weight to body weight was not significantly different. Steroid pretreatment disturbed LR in the S-PH group. On the other hand, low-dose steroid pretreatment improved LR and suppressed tumor necrosis factor (TNF)- $\alpha$  elevation in the S-PHA group, with recovery of STAT3 DNA-binding activity. On the other hand, low-dose steroid pretreatment improved LR and suppressed TNF- $\alpha$  elevation in the S-PHA group, with recovery of STAT3 DNA-binding activity.

**CONCLUSION:** LR is disturbed after RF ablation, with high serum cytokine/chemokine induction. Low-dose steroid administration can improve LR after RF ablation with TNF- $\alpha$  suppression.

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**Key words:** Liver regeneration; Radiofrequency ablation; Steroid; Tumor necrosis factor; Hepatectomy

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### INTRODUCTION

Liver resection is still one of the best curative therapies for primary or secondary liver tumors in most cases with

no extrahepatic metastasis<sup>[1,2]</sup>. Various techniques and devices for liver resection have been employed to improve the perioperative outcome<sup>[3-7]</sup>, although the clamp-crush technique used by a skilled surgeon still has the most favorable outcome according to recent systematic reviews<sup>[8,9]</sup>. Liver surgeons focus on reducing bleeding during liver resection, which leads to shorter operative time. Heat-assist devices such as the harmonic scalpel<sup>[10]</sup>, Ligasure<sup>[11]</sup>, saline-linked monopolar cautery<sup>[12]</sup>, microwave coagulator<sup>[13]</sup>, and radiofrequency (RF) devices<sup>[14]</sup>, can seal vessels and bile ducts to avoid postoperative bleeding and bile leakage. Because these heat-assist devices can achieve firm sealing of vessels and bile ducts, unnecessary ties and clips can be avoided without any adverse events<sup>[15]</sup>. Some randomized trials have indicated the merits and demerits of using heat-assist devices<sup>[4,7]</sup>.

The greatest merit is the sealing effect to reduce perioperative morbidity<sup>[7,16]</sup>. A second merit is enhancement of the surgical margins of tumors located near the cutting surface of the liver<sup>[17]</sup>. On the other hand, a necrotic zone remains in the cutting surface of the residual liver<sup>[14]</sup>. Although cryoablation causes lethal systemic responses with high levels of cytokines<sup>[18,19]</sup>, RF ablation may be safe and result in only minimal release of soluble factors causing systemic responses. However, it is not known whether RF manipulation, and the residual necrotic tissue during liver resection, is beneficial or harmful to liver regeneration (LR). In addition, the molecular events of LR after the use of heating devices for liver resection are also unknown.

LR is regulated by sequential molecular events in which various humoral factors such as tumor necrosis factor (TNF)- $\alpha$  and IL-6 prime and facilitate hepatocyte replication<sup>[20-22]</sup>. Although the humoral factors increase and influence each step of LR in a very short time<sup>[21]</sup>, each cytokine activates subsequent molecular signals to complete LR<sup>[22]</sup>. RF ablation, in particular, excessively increases plasma cytokines such as TNF- $\alpha$  and IL-6 compared to simple liver resection<sup>[23,24]</sup>. Due to superphysiological stimulation of these cytokines, the heat effect of RF ablation may impair liver regeneration<sup>[25]</sup>. On the other hand, fast recovery of liver function in LR after RF ablation has been reported in major clinical hepatectomy<sup>[6]</sup>. Therefore, the exact effect of RF ablation on LR remains unclear.

Steroid administration has been proved to attenuate surgical stress following liver resection<sup>[26,27]</sup>. In addition, steroid pretreatment has been proved to decrease plasma cytokine levels and the therapeutic dose of the steroid does not inhibit hepatocyte proliferation<sup>[28]</sup>. Although previous investigations showed that steroid treatment could ameliorate excessive surgical stress of extended hepatectomy<sup>[26-28]</sup>, the exact benefits of steroid administration in clinical LR are largely unknown. The main aim of this study was to determine whether liver regeneration could be disturbed following RF ablation. The second was to determine the effect of steroid pretreatment on LR after RF ablation.

## MATERIALS AND METHODS

Animal studies were performed in compliance with in-

stitutional and National Research Council guidelines for humane care of laboratory animals.

### Animals

Adult female Fisher 344 rats (250-350 g) were obtained from Charles River Japan (Kanagawa, Japan). They were housed in a climate-controlled (21 °C) room under a 12 h light-dark cycle and were given tap water and standard laboratory chow. All operations were performed between 9:00 a.m. and noon under general (ether) anesthesia using a sterile surgical technique.

### Surgical animal models

**Sham hepatectomy:** The sham hepatectomy consisted of laparotomy and mobilization of the liver.

**Partial hepatectomy:** The two anterior liver lobes were removed as previously described<sup>[29,30]</sup>. In this model, removal of the two anterior lobes (68% of the liver) is known to induce the optimal proliferative response in the remnant liver mass.

**Partial hepatectomy with radiofrequency ablation:** Preceding partial hepatectomy (PH), the two anterior liver lobes were ablated with saline-linked electric bipolar forceps (ERBE Elektromedizin GmbH, Tübingen, Germany). After complete ablation of the two anterior lobes, they were removed the same as PH operation.

### Experimental design

Groups of sham hepatectomy (SH), PH, and partial hepatectomy with RF ablation (PHA) rats were euthanized in batches of six at 1, 3, 5 and 7 d after surgery. A separate experiment was designed to determine the effect of steroid administration. All animals were pretreated with dexamethasone at 30 min prior to the operation. Groups of steroid pretreated PH rats (S-PH) and PHA rats (S-PHA) were euthanized in batches of six at 1 d after surgery. One hour before euthanasia, 5-bromo-2-deoxyuridine (BrdU) was injected intraperitoneally (50  $\mu$ g/kg body weight)<sup>[31]</sup>. When animals were killed, part of the liver tissue was immediately frozen in liquid nitrogen for molecular analysis and part of it was dipped into cold ethanol for immunohistochemical study.

### Blood chemistry and white blood cell counts

Blood samples were analyzed for activity of alanine transaminase (ALT), aspartate transaminase (AST), total protein levels, and albumin (ALB) in a clinical laboratory. White blood cells were counted with an autocalculator in the laboratory.

### Multiple cytokine detection

Serum obtained after euthanasia was kept at -80 °C until submission to a company (Upstate United States Inc., Charlottesville, VA, United States) for analysis. Briefly, multianalyte profiling was performed on a Luminex 100 system and the XY Platform (Luminex Corporation, Austin, TX, United States). Calibration microspheres for

classification and reporter readings as well as sheath fluid were obtained from Luminex Corporation. Acquired fluorescence data were analyzed using MASTERPLEX™ QT (Ver. 1.2; MiraiBio Inc., South San Francisco, CA, United States). Serum concentrations of TNF- $\alpha$ , IL-6, IL-10, and monocyte chemoattractant protein-1 (MCP-1) were measured with an Upstate Beadlyte Mouse Multicytokine Bead master kit (Upstate United States, Inc.)<sup>[31]</sup>. All analyses were performed according to the manufacturers' protocols.

#### **Immunohistochemistry for BrdU and BrdU labeling index**

The proliferative activity in the liver after hepatectomy was determined by measuring incorporation of BrdU as previously described<sup>[31]</sup>. Briefly, a mouse anti-BrdU antibody (X 100 dilution: DAKO A/S, Copenhagen, Denmark) was used as the primary antibody, followed by the ABC method (DAKO Co., Carpinteria, CA). Both labeled and unlabeled hepatocytes were counted in 20 fields in three different sections per time point from five different animals. Data are presented as means  $\pm$  SD from three independent experiments.

#### **Restitution of liver mass**

Growth of the residual liver lobes (right and omental lobes) was calculated as the ratio of residual liver weight/body weight (RLW/BW).

#### **Western blotting analysis**

Western blotting analysis was performed using the Invitrogen NuPAGE® electrophoresis system (Invitrogen, Carlsbad, CA, United States). The samples were homogenized in phosphate buffered saline and kept at -80 °C until use. Briefly, nuclear proteins were extracted using the NE-PER® nuclear and cytoplasmic extraction protocol (Pierce Chemicals, Rockford, IL, United States). A BCA protein assay kit® (Pierce Chemicals) was used to measure the protein concentrations. Proteins (5  $\mu$ g/lane) were separated on NuPAGE 4%-12% Bis-Tris gradient gels (Invitrogen). The gels were transferred to nitrocellulose membranes (Amersham Co., Buckinghamshire, United Kingdom) using an iBlot™ Gel Transfer Device (Invitrogen). Immunodetection of proteins was performed using a Western-Breeze® Chromogenic Immunodetection Kit (Invitrogen). Mouse monoclonal anti-proliferation cell nuclear antigen (PCNA) (Dako Co., Carpinteria, CA, United States) and rabbit polyclonal anti-ALB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as the primary antibodies (1:250). The ECL western blotting analysis system (Amersham Co.) was used to detect signals.

#### **Densitometric analysis**

Scanning densitometry was performed using a Macintosh G4 computer (Apple Computer, Cupertino, CA) and an EPSON GT-9600 scanner (Seiko Epson, Suwa, Japan). The signals were quantified using the NIH Image 1.55 Densitometric Analysis Program<sup>[30]</sup>.

#### **STAT3 DNA-binding activation assay**

STAT3 activation was quantified using a TransAM™STAT3

Kit (Active Motif, Funakoshi Co., Tokyo, Japan)<sup>[32]</sup>. Briefly, 10 g/well of the nuclear cell extract from whole liver tissue (containing an activated transcription factor) was incubated in a 96-well plate on which double-stranded oligonucleotides containing the consensus sequence for the STAT3 DNA-binding site (5'-TTCCCGGAA-3') were immobilized. The primary antibody used to detect STAT3 recognized epitopes on both the alpha and beta forms of STAT3, which are accessible only when STAT3 is activated and bound to its target DNA. After incubation with horseradish peroxidase, absorbance was recorded at 450 nm using a reference wavelength of 655 nm.

#### **Statistical analysis**

The unpaired Student's *t*-test, Welch's *t*-test or one-way analysis of variance (ANOVA) was used as appropriate. Data are given as mean  $\pm$  SD. **Statistical analysis was performed** using the StatView 5.0 program (SAS Institute, Cary, NC, United States) and the difference between the means was considered significant when  $P < 0.05$ .

## **RESULTS**

All rats tolerated the operative procedures well and recovered uneventfully from anesthesia. Samples were collected immediately after each animal was euthanized.

#### **Liver regeneration after ablation**

Although the liver was ablated within a short time and most necrotic tissue was removed in the PHA group, postoperative serum AST and ALT levels in this group at one day after operation were significantly higher than in the PH group (Table 1). On the other hand, the white blood cell counts were not significantly different among the groups (Table 1). Total protein and albumin levels at two, three, and five days after operation in the PHA group were significantly lower than in the PH group.

Serum cytokine and chemokine levels are shown in Figure 1. TNF- $\alpha$  levels in the PHA group at one and two days after operation were significantly higher than in the PH group (Figure 1A). IL-6 (Figure 1B) and MCP-1 (Figure 1D) levels in the PHA group at two, three, and five days after operation were significantly higher than in the PH group. IL-10 levels in the PHA group at two and three days after operation were significantly higher than in the PH group (Figure 1C).

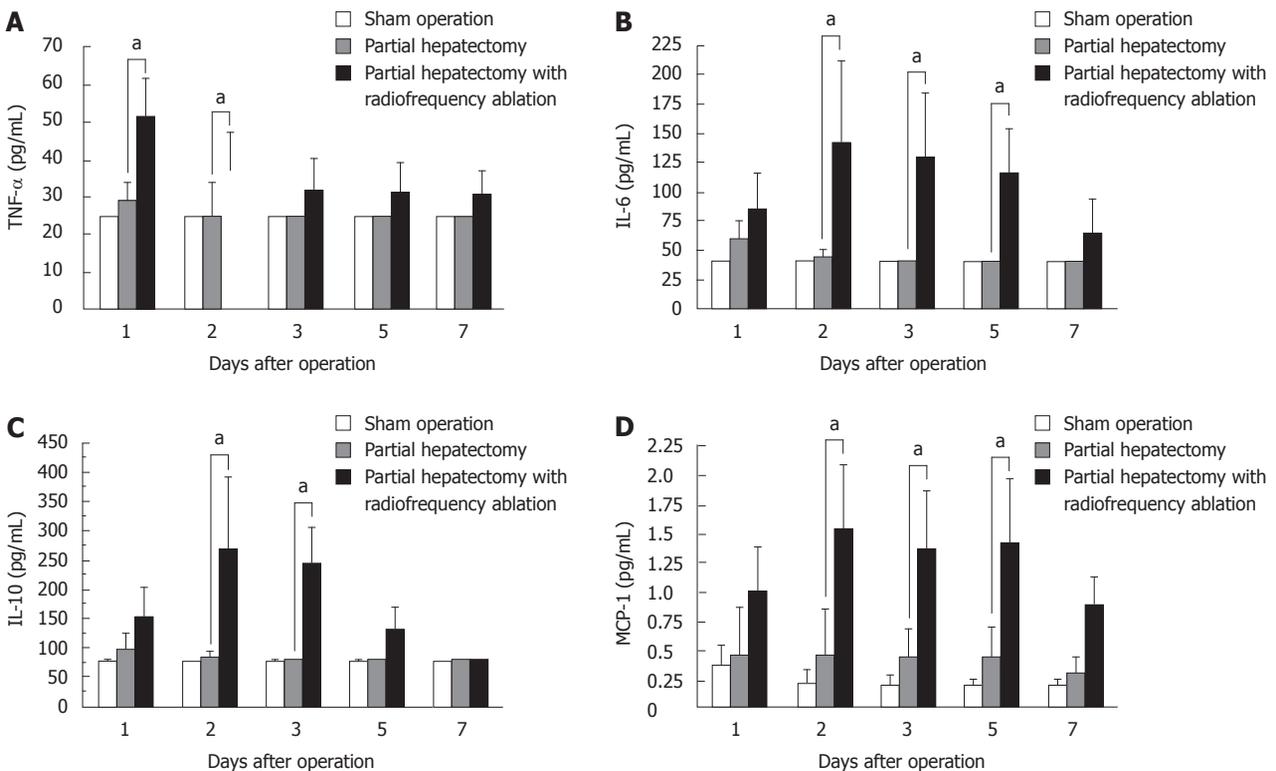
DNA synthetic activity was determined by immunohistochemistry for BrdU (Figure 2A-J) and labeling indices (LIs) (Figure 2K). **The LI at one day in the PHA group was significantly lower than in the PH group** ( $12.17 \pm 3.43$  vs  $29.02 \pm 8.47$ ,  $P = 0.001$ ). On the other hand, the LIs at two days and three days after operation in the PHA group were significantly higher than in the PH group ( $15.85 \pm 4.18$  vs  $7.05 \pm 1.54$ ,  $P < 0.001$ , and  $12.55 \pm 3.14$  vs  $6.03 \pm 2.11$ ,  $P = 0.002$ , respectively). Although DNA synthetic activities between the groups are significantly different, RLW/BW ratio was not greatly significantly different among the groups (Figure 2L).

Protein expression of nuclear PCNA and cytosolic

**Table 1** Alterations of blood cell counts and laboratory tests in partial hepatectomy and partial hepatectomy with radiofrequency ablation models

WBC ( $\times 10^3 \mu\text{L}$ )	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
PH	4.95 $\pm$ 0.74	4.70 $\pm$ 0.81	6.55 $\pm$ 1.26	6.15 $\pm$ 0.81	7.43 $\pm$ 0.99	5.48 $\pm$ 1.14
PHA	5.80 $\pm$ 1.09	5.70 $\pm$ 0.84	7.68 $\pm$ 1.32	6.60 $\pm$ 1.49	7.65 $\pm$ 1.41	6.73 $\pm$ 0.81
<i>P</i> values	NS	NS	NS	NS	NS	NS
TP (g/dL)						
PH	5.55 $\pm$ 0.24	4.71 $\pm$ 0.29	4.58 $\pm$ 0.09	4.87 $\pm$ 0.29	5.18 $\pm$ 0.21	5.43 $\pm$ 0.34
PHA	5.62 $\pm$ 0.17	4.43 $\pm$ 0.25	4.28 $\pm$ 0.17	4.05 $\pm$ 0.13	4.68 $\pm$ 0.24	5.31 $\pm$ 0.36
<i>P</i> values	NS	NS	0.022	0.002	0.019	NS
ALB (g/dL)						
PH	4.10 $\pm$ 0.14	3.73 $\pm$ 0.21	3.45 $\pm$ 0.13	3.58 $\pm$ 0.21	3.80 $\pm$ 0.22	4.08 $\pm$ 0.15
PHA	4.20 $\pm$ 0.18	3.55 $\pm$ 0.21	3.18 $\pm$ 0.17	2.85 $\pm$ 0.21	3.10 $\pm$ 0.22	3.78 $\pm$ 0.28
<i>P</i> values	NS	NS	0.042	0.003	0.004	NS
AST (U/L)						
PH	77.3 $\pm$ 3.3	591.8 $\pm$ 111.8	211.0 $\pm$ 23.6	126.8 $\pm$ 23.7	117.3 $\pm$ 14.5	105.5 $\pm$ 13.4
PHA	79.3 $\pm$ 4.6	2441.8 $\pm$ 501.8	366.5 $\pm$ 136.9	224.0 $\pm$ 110.2	163.5 $\pm$ 59.2	100.8 $\pm$ 11.9
<i>P</i> values	NS	0.001	NS	NS	NS	NS
ALT (U/L)						
PH	48.5 $\pm$ 13.4	734.3 $\pm$ 187.4	207.8 $\pm$ 108.7	71.0 $\pm$ 21.1	59.3 $\pm$ 9.4	47.5 $\pm$ 13.7
PHA	49.3 $\pm$ 9.6	1603.3 $\pm$ 313.7	503.8 $\pm$ 207.8	102.5 $\pm$ 23.1	66.0 $\pm$ 8.9	51.3 $\pm$ 11.1
<i>P</i> values	NS	0.003	0.025	NS	NS	NS

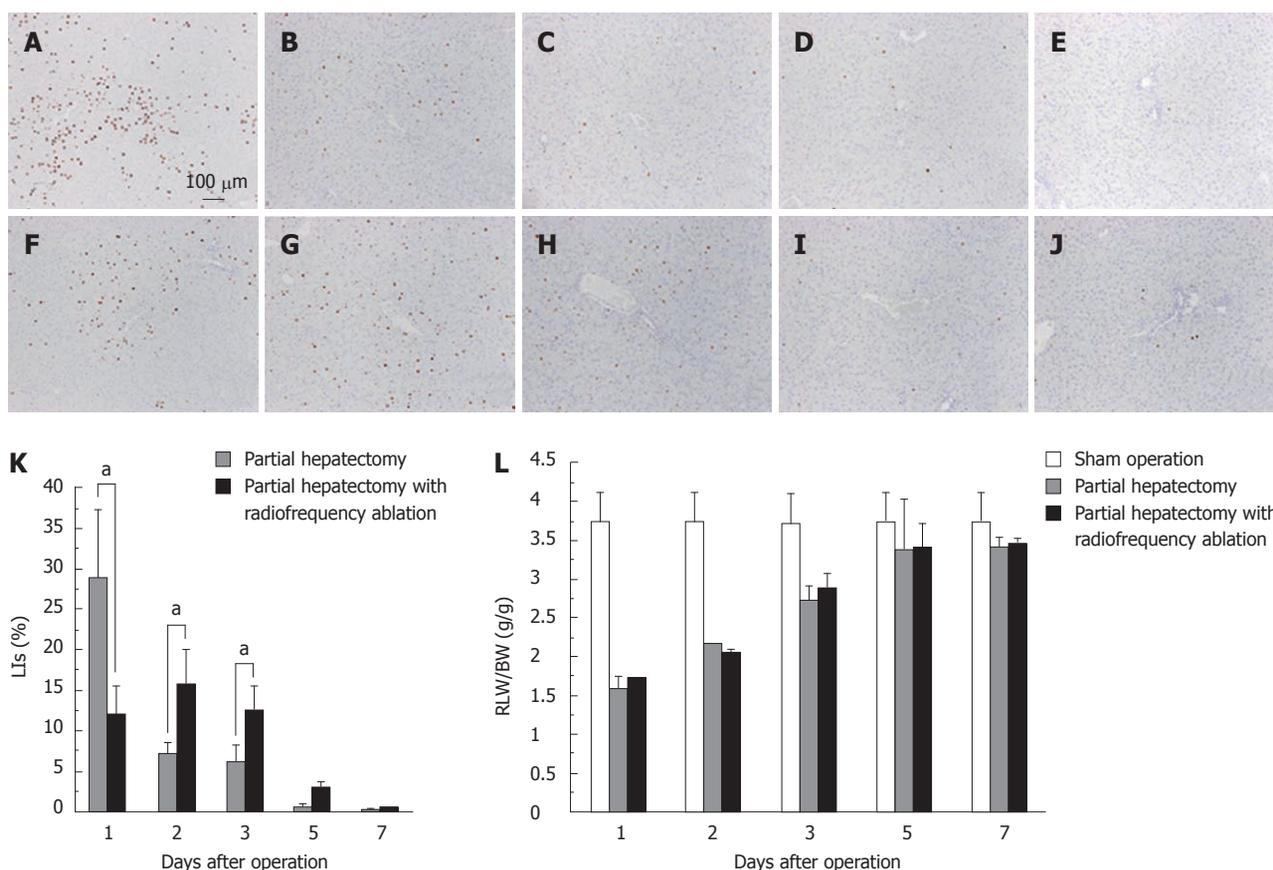
One-way analysis of variance was used for statistical analysis and  $P < 0.05$  is considered to be significant. WBC: White blood cell; PH: Partial hepatectomy; PHA: Partial hepatectomy with radiofrequency ablation; TP: Total protein levels; ALB: Albumin; AST: Aspartate transaminase; ALT: Alanine transaminase; NS: Not Significant.



**Figure 1** Changes in serum tumor necrosis factor- $\alpha$  (A), IL-6 (B), IL-10 (C), and monocyte chemoattractant protein-1 (D) levels after the operations.  $^aP < 0.05$  between groups. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; MCP-1: Monocyte chemoattractant protein-1.

albumin is shown in Figure 3A. Densitometric analysis of the expression of each protein is shown in Figure 3B. The pattern of PCNA expression was similar to the immunohistochemistry for BrdU and LIs. The peak of BrdU expression in the PH group was seen at one day after operation and in the PHA group at two and three

days after operation. ALB expression in the PH group dropped at one and two days after operation and recovered thereafter. In contrast, ALB expression in the PHA group dropped at three and five days after operation. STAT3 DNA-binding activity was also consistent with the results of BrdU, LIs, and PCNA expression (Figure 3C).



**Figure 2** Immunohistochemistry for 5-bromo-2-deoxyuridine in the partial hepatectomy group (A-E) and in the partial hepatectomy with radiofrequency ablation group (F-J) at 1 d (A and F), 2 d (B and G), 3 d (C and H), 5 d (D and I) and 7 d (E and J) after the operations. K: Comparison of labeling indices in the partial hepatectomy group and partial hepatectomy with radiofrequency ablation group; L: The ratio of liver weight/body weight shows restitution of the remnant liver. <sup>a</sup>*P* < 0.05 between groups. LIs: Labeling indices; RLW/BW: Residual liver weight/body weight.

The peak of STAT3 DNA-binding activity in the PH group was seen at one day after operation and in the PHA group at two and three days after operation.

### Response of liver regeneration after ablation with steroid administration

We found that LR was disturbed after RF ablation in hepatectomy, with high cytokine/chemokine induction. Because steroid treatment could block cytokine elevation after hepatectomy, we tested the effects of S-PH and S-PHA groups.

Immunohistochemistry values for BrdU (Figure 4A-P) and LIs (Figure 4Q) at 1 d after steroid administration at different concentrations are shown in Figure 4. BrdU uptake in the S-PH group (Figure 4A-H) gradually decreased when the steroid dose was increased. In contrast, that in the S-PHA group (Figure 4I-P) gradually increased when the steroid dose was increased by 0.04 mg/kg and then it decreased with further increases in dosage. Although the LI at 0.002 mg/kg steroid administration in the S-PH group was significantly higher than in the S-PHA group ( $29.62 \pm 8.28$  vs  $14.87 \pm 4.35$ , *P* = 0.003), the LIs at the other steroid doses were not significantly different between the groups (Figure 4Q). Among the examined cytokines and chemokines, only the TNF- $\alpha$  level at 0.002 mg/kg of steroid in the S-PH group (Figure 4R) was significantly lower than in the S-PHA group ( $27.5 \pm 4.18$  vs  $46.5 \pm 4.18$ ,

*P* = 0.001). In contrast, other levels were not significantly different at any steroid doses (data not shown).

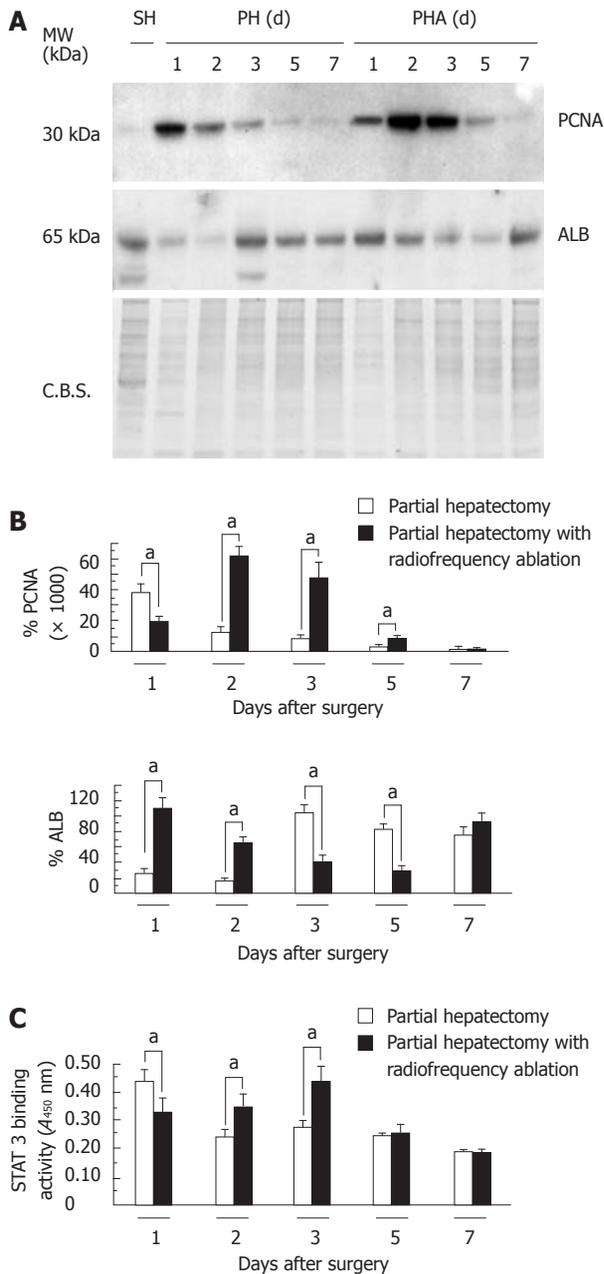
The pattern of PCNA expression in the nucleus (Figure 5A) was similar to that observed in the immunohistochemistry for BrdU and LIs (Figure 4). PCNA expression in the S-PH group gradually decreased when the steroid dose was increased. In contrast, that in the S-PHA group gradually increased when the steroid dose was increased by 0.04 mg/kg and then it decreased with further dosage. STAT 3 DNA-binding activities (Figure 5B) were also consistent with the BrdU staining, LIs and PCNA expression. Only STAT3 DNA-binding activity at 0.002 mg/kg of steroid was significantly different between the groups ( $0.406 \pm 0.042$  vs  $0.298 \pm 0.053$ , *P* = 0.019).

## DISCUSSION

We investigated the influence of RF ablation during hepatectomy on LR. We found that LR after RF ablation was disturbed, with high serum cytokine/chemokine induction. Low-dose steroid administration nearly restored LR after RF ablation during hepatectomy, and STAT3 DNA-binding activity supported this finding.

### Surgical model to investigate influence of radiofrequency ablation on liver regeneration

The method of liver resection should take into account



**Figure 3** Proliferation cell nuclear antigen expression in the nuclear protein and albumin expression in the cytosol after the operations. A: Western blotting analysis for proliferation cell nuclear antigen expression (PCNA); B: Densitometric analysis of each protein signal; C: STAT3 DNA-binding activity in the nuclear protein after the operations. <sup>a</sup>*P* < 0.05 between groups. SH: Sham hepatectomy; PH: Partial hepatectomy; PHA: Partial hepatectomy with radiofrequency ablation; MW: Molecular weight; C.B.S.: Coomassie blue staining; ALB: Albumin.

both perioperative safety and oncological curability. RF ablation is one of the less invasive strategies for small liver tumors<sup>[33]</sup> and can be used for hemostasis during liver resection<sup>[34]</sup>. The one concern is that some necrotic tissue will remain in the residual liver after RF ablation and may affect LR. In addition, thermal energy itself during the operation also may affect it. Most investigations have used large animal models to study the effects of RF ablation after hepatectomy on humoral and oncological activities<sup>[25]</sup>. The use of murine models to investigate RF ablation has been limited<sup>[25,35]</sup>. Large animal models

can be ideal to simulate the human response; however, they are time consuming and it is difficult to examine the molecular details compared to murine models. Although our model did not totally reproduce the human clinical situation, the postoperative course was very similar, as serum transaminases were strongly elevated at one day after operation<sup>[24]</sup>. Furthermore, high serum cytokine levels after ablation have been reported in human studies<sup>[24,36]</sup>. These clinical postoperative alterations of laboratory tests supported the idea that our model could represent the clinical phenomena of hepatectomy using RF ablation. Therefore, our model was suitable to examine the thermal effect of RF ablation after hepatectomy.

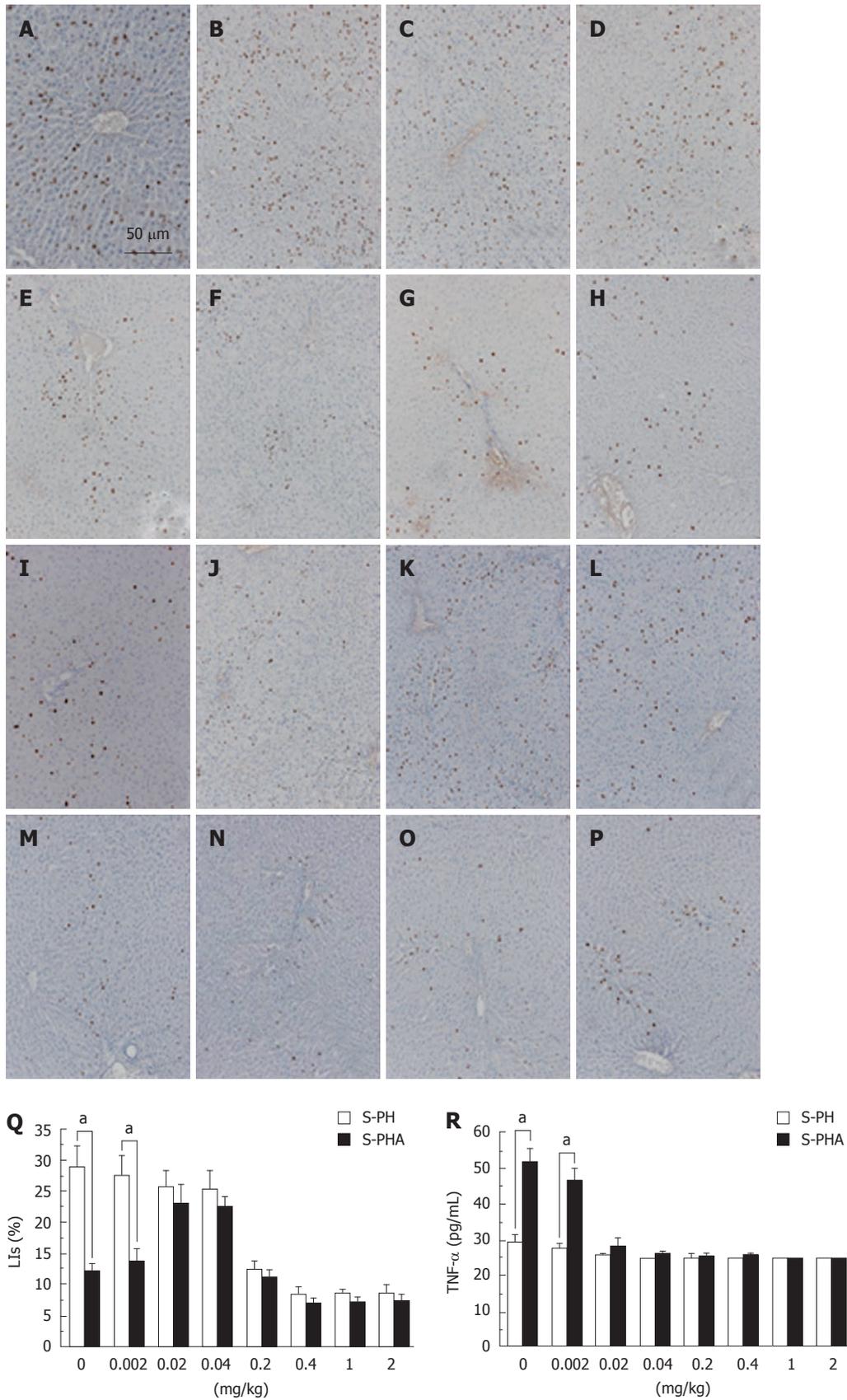
### Effect of radiofrequency ablation on the liver

Effects of RF ablation on cancer cells in the liver could modulate the systemic immune responses, including cytokine/chemokine production and the proliferative activity of the cancer cells<sup>[37,38]</sup>. Necrotic tissue after RF ablation could also modulate the systemic immune response to specific cancer cells<sup>[39]</sup>. On the other hand, Meredith *et al.*<sup>[40]</sup> reported that RF ablation itself did not accelerate tumor growth. In our study, RF ablation delayed the liver regenerative response, which was consistent with a previous study<sup>[25]</sup>. These differently reported proliferative responses may be due to the differences between cancer cells and normal cells. Other reasons could be differences in the amount of necrosis and the duration of the ablation. We could not distinguish between the exact effects of necrosis and ablation, but our results indicated that the heat effect of RF ablation, not necrosis, could delay LR, because most ablated tissues were removed in both groups, and the amounts of necrosis in the PH and the PHA groups were comparable in our model. Serum cytokines such as IL-6, IL-8, and IL-10 are elevated after RF ablation<sup>[24,35,36]</sup>, which is also consistent with our results. Therefore, regardless of how much necrotic tissue was removed after RF ablation, the heat effect during the ablation itself could activate cytokine/chemokine responses.

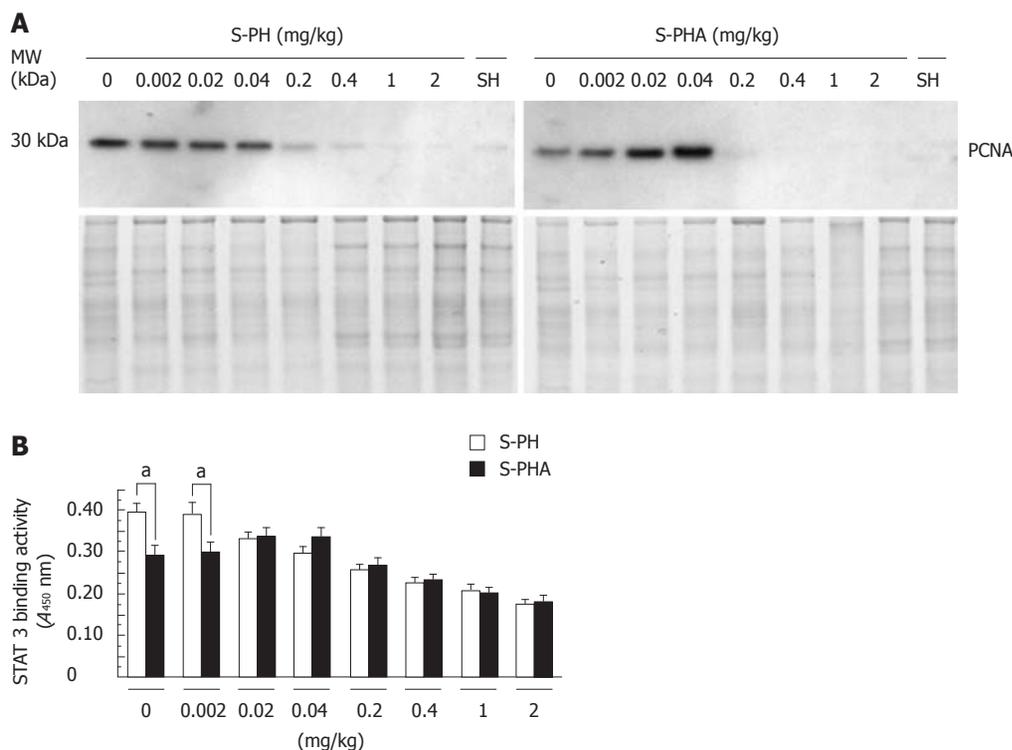
### Cytokine/chemokine signals in liver regeneration

LR after RF ablation was delayed without any difference in the RLW/BW ratio. This indicated that volume recovery after PH did not represent parenchymal cell proliferation itself, which was consistent with a previous report<sup>[41]</sup>. Delayed LR after RF ablation affected the serum albumin level. Albumin production was suppressed when hepatocytes began to proliferate<sup>[42]</sup>. The time lag between DNA synthesis and the serum albumin level could be due to the long half-life of serum albumin. Even though there was no critical event in our model, we need to pay attention to the albumin level, which could decrease in LR after RF ablation and be associated with delayed LR in the clinical setting.

A systemic cytokine/chemokine response was activated by RF ablation even within the short time during operation. We could not determine the specific cytokine/chemokine that disturbed hepatocyte proliferation. Excessively high levels of cytokines such as TNF- $\alpha$  and



**Figure 4** Immunohistochemistry for 5-bromo-2-deoxyuridine staining in the partial hepatectomy with dexamethasone pretreatment group (A-H) and partial hepatectomy after radiofrequency ablation with dexamethasone pretreatment group (I-P) at 1 d after the operations. Animals were pretreated with 0 mg/kg (A and I), 0.002 mg/kg (B and J), 0.02 mg/kg (C and K), 0.04 mg/kg (D and L), 0.2 mg/kg (E and M), 0.4 mg/kg (F and N), 1 mg/kg (G and O), or 2 mg/kg (H and P) dexamethasone. Q: The brown nuclei are positive for 5-bromo-2-deoxyuridine. The labeling indices were calculated from 20 fields in three different sections per treatment for five different animals; R: Serum tumor necrosis factor- $\alpha$  levels at 1 d after the operations. The horizontal axis presents each concentration of dexamethasone pretreatment. <sup>a</sup>*P* < 0.05 between groups. S-PH: Steroid pretreatment in the partial hepatectomy group; S-PHA: Steroid pretreatment in the partial hepatectomy after radiofrequency ablation group; LIs: Labeling indices.



**Figure 5** Proliferation cell nuclear antigen expression in the nuclear protein at 1 d after the operations. A: Western blotting analysis for proliferation cell nuclear antigen (PCNA) expression. Animals were pretreated with various concentrations of dexamethasone 30 min prior to the hepatectomy; B: STAT3 DNA-binding activity in the nucleus at 1 d after the operations. <sup>a</sup>*P* < 0.05 between groups. SH: Sham operation; S-PH: Steroid pretreatment in the partial hepatectomy group; S-PHA: Steroid pretreatment in the partial hepatectomy after radiofrequency ablation group; MW: Molecular weight.

IL-6 are desensitized from growth stimuli<sup>[43]</sup>, although knockout murine models targeting TNF- $\alpha$  receptor and *IL-6* genes have demonstrated that these cytokine signals are necessary to accomplish LR<sup>[44,45]</sup>. The lack of DNA-binding activity of STAT3 in our results supported the finding of growth suppression in the PHA group. Even though the peaks of most cytokine/chemokine levels in the PHA model were between two days and five days after operation, DNA synthesis in PHA continued in these periods. The only distinctive alteration seen was in the TNF- $\alpha$  level, which gradually decreased. In addition, steroid pretreatment in the PHA group showed that only the TNF- $\alpha$  level was different between the PH group and PHA group at one day after operation. Therefore, DNA synthesis after RF ablation could be more affected by TNF- $\alpha$  than by other cytokines/chemokines. Thus, TNF- $\alpha$  activation should be observed within a short time after simple hepatectomy<sup>[21]</sup>. However, it could be prolonged in LR after RF ablation, as shown in our results. Though we could not determine the mechanism of the TNF- $\alpha$  activation after RF ablation, our results strongly suggested that the TNF- $\alpha$  could play a major role in LR after RF ablation. Further study is needed to determine whether TNF- $\alpha$  could be a molecular target to control LR in the clinical setting.

### Steroid administration and liver regeneration

Steroids have been demonstrated to inhibit LR by inhibiting excessive TNF- $\alpha$  and IL-6 production<sup>[46-48]</sup>, although moderate stimulation by TNF- $\alpha$  and IL-6 is necessary

to complete LR<sup>[21]</sup>. Our results also showed that cytokine/chemokine levels decreased gradually depending on steroid administration in the S-PHA group. On the other hand, steroids can inhibit the DNA synthesis of hepatocytes directly<sup>[42]</sup>. The reason why DNA synthesis recovered after low-dose steroid administration in the S-PHA group could be related to the balance between the suppression of excessive cytokine production and the direct inhibition of DNA synthesis. In other words, LR after low-dose steroid administration could recover, escaping from the excessive cytokine production, and be nearly free from the direct inhibition by the steroid. Our results indicated the presence of an optimal threshold of the steroid concentration that facilitated LR when cytokines/chemokines were excessively activated. Therefore, our results strongly suggest that we need to pay careful attention to the clinical steroid concentration because the effects of steroid administration could be altered depending on the clinical condition.

In conclusion, LR was disturbed after RF ablation, with high serum cytokine/chemokine induction. Low-dose steroid administration could improve LR after RF ablation with TNF- $\alpha$  suppression. Further clinical study is needed to confirm that low-dose steroid administration has a clinical benefit for LR after RF ablation.

### ACKNOWLEDGMENTS

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## COMMENTS

### Background

Liver resection is still one of the best curative therapies for primary or secondary liver tumors. Various techniques and devices for liver resection have been employed to improve the perioperative outcome. Radiofrequency (RF) devices can seal vessels and bile ducts to avoid postoperative bleeding and bile leakage. Although some of its merits have been reported, its demerits are largely unknown.

### Research frontiers

The greatest merit of a RF device is the sealing effect to reduce perioperative morbidity. A second merit is enhancement of the surgical margins of tumors located near the cutting surface of the liver. On the other hand, a necrotic zone remains in the cutting surface of the residual liver. The research hotspot is whether RF manipulation, and the residual necrotic tissue during liver resection, is beneficial or harmful to liver regeneration (LR).

### Innovations and breakthroughs

The present study showed LR after RF ablation delayed the regenerative response with high serum cytokine/chemokine induction, and low-dose steroid administration could improve LR after RF ablation with TNF- $\alpha$  suppression. The results indicated that the heat effect of RF ablation, not necrosis, could delay LR, because most ablated tissues were removed. Serum cytokines such as IL-6, IL-8, and IL-10 are elevated after RF ablation, which is consistent with previous studies. Therefore, regardless of how much necrotic tissue was removed after RF ablation, the heat effect during the ablation itself could activate cytokine/chemokine responses.

### Applications

This study provides insights into the mechanism by which RF ablation could activate cytokines/chemokines in LR and found that steroids can be used for controlling LR. The results strongly suggest that they need to pay careful attention to the clinical steroid concentration because the effects of steroid administration could be altered depending on the clinical condition.

### Peer review

The aim of this study focused on liver regeneration after RF ablation is clear and interesting, and these data may provide a basis for RF ablation studies in the future. The impact of this study in this field is moderate.

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## S100A4 silencing blocks invasive ability of esophageal squamous cell carcinoma cells

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### Abstract

**AIM:** To investigate a potential role of S100A4 in esophageal squamous cell carcinoma metastasis (ESCCs).

**METHODS:** Expression of S100A4 and E-cadherin were analyzed in frozen sections from ESCCs (metastasis,  $n = 28$ ; non-metastasis,  $n = 20$ ) by reverse transcription-polymerase chain reaction, quantitative polymerase chain reaction and immunohistochemistry. To explore the influence of S100A4 on esophageal cancer invasion and metastasis, S100A4 was overexpressed or silenced by S100A4 siRNA in TE-13 or Eca-109 cells *in vitro* and *in vivo*.

**RESULTS:** We found the mRNA and protein levels of S100A4 expression in ESCCs was significantly upregulated, and more importantly, that expression of S100A4 and E cadherin are strongly negatively correlated in patients who had metastasis. It was indicated that overexpression of S100A4 in TE-13 and Eca-109 cells downregulates the expression of E-cadherin, leading to

increased cell migration *in vitro*, whereas knockdown of S100A4 inhibited cell migration and upregulation of E-cadherin expression. Moreover, the loss of cell metastatic potential was rescued by overexpression of E-cadherin completely. In addition, nude mice inoculated with S100A4 siRNA-transfected cells exhibited a significantly decreased invasion ability *in vivo*.

**CONCLUSION:** S100A4 may be involved in ESCC progression by regulate E-cadherin expression, vector-based RNA interference targeting S100A4 is a potential therapeutic method for human ESCC.

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**Key words:** Esophagus squamous cell carcinoma; Metastasis; Gene treatment; S100A4; E-cadherin

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Chen D, Zheng XF, Yang ZY, Liu DX, Zhang GY, Jiao XL, Zhao H. S100A4 silencing blocks invasive ability of esophageal squamous cell carcinoma cells. *World J Gastroenterol* 2012; 18(9): 915-922 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i9/915.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i9.915>

### INTRODUCTION

Despite improvements in detection, surgical resection, and (neo-) adjuvant therapy, the overall survival for esophageal squamous cell carcinoma (ESCC), one of the most aggressive carcinomas of the gastrointestinal tract, remains lower than that of other solid tumors due to distant and lymph node metastasis<sup>[1]</sup>. Therefore, efforts are ongoing regarding exploration of novel targets and

strategies for the management of ESCC, and gene targeting therapies in particular are promising. Multiple studies focusing on the effects of various biological factors on the malignant potential of ESCC have been conducted<sup>[2-4]</sup>. One of those factors is E-cadherin as the loss of E-cadherin is an important step in the process of epithelial-to-mesenchymal transition (EMT) in cancer<sup>[5-8]</sup>. In ESCC, loss of E-cadherin expression is associated with tumor invasiveness, metastasis and prognosis<sup>[2,9,10]</sup>. Mechanisms involved in regulation of E-cadherin in ESCC are likely complex and poorly understood.

The S100 family of calcium binding proteins has been shown to be involved in a variety of physiological functions, such as cell proliferation, extracellular signal transduction, intercellular adhesion, and motility as well as cancer metastasis<sup>[11-13]</sup>. Of these, S100A4 (mts1, p9Ka, calvasculin) has been identified as a cytoplasmic protein in normal cells, which is associated with the actin/myosin cytoskeleton in fixed cells<sup>[14]</sup>. Interestingly, elevated levels of S100A4 are closely associated with the process of metastasis in several human solid cancers including gastric cancer<sup>[15,16]</sup>, colorectal adenocarcinoma<sup>[17,18]</sup>, and breast cancer<sup>[19]</sup>. Patients with S100A4 high expression often appear with advanced stage or lymph node metastasis suggesting correlation of the S100A4 expression and the invasion or metastasis of ESCC<sup>[20]</sup>. In this study, we investigated the expression of S100A4 and E-cadherin in ESCC patients and the potential functional relationship in tumor metastasis and proliferation.

## MATERIALS AND METHODS

### Cell lines

EC109 and TE13 were kindly provided by Dr. Zhang (Surgery, the affiliated Hospital of Medical College, Qingdao University, Qingdao, China)<sup>[21]</sup>. All the cells were maintained in 50 mL/L CO<sub>2</sub> atmosphere at 37 °C in RPMI 1640/Ham's F-12 mixed (1:1) medium containing 100 g/L fetal bovine serum.

### Tissue sample collection

A total of 48 cryostat sections of frozen ESCC tissue were enrolled in this study: 28 with lymph node ( $n = 25$ ) or distant metastasis ( $n = 3$ ) and 20 without metastasis. These patients did not receive any preoperative adjuvant radiation or chemotherapy. All research involving human participants was approved in written form by the patients studied and the ethics committee at the affiliated hospital of Tianjin medical university.

### Silencing of S100A4

siRNAs were commercially purchased from Qiagen (Valencia, CA). The sequence of selected regions to be targeted by siRNAs was 5'-AACGAGGTGGACTTCCAAGAG-3' for S100A4 and 5'-AATTCTCCGAACGTGTCTCG T-3' for a nonsilencing siRNA (control). siRNA cloning vector (pGB) was purchased from ABCAM (Shanghai). pGB-S100A4 siRNA and controls were constructed according to the manufacture's instruction. EC109 cells were transfected

with the siRNA plasmids in the presence of Lipofectamine. Stable transfectants were selected with 300 µg/mL G418.

### S100A4 cDNA and E-cadherin cDNA plasmid construction and transfection

The commercial pMD vector (produced by TAKARA) and Homo sapiens S100A4 transcript variant 1 DNA ORF (S100A4 cDNA) and Homo sapiens E-cadherin DNA ORF Clone (E-cadherin cDNA) were purchased from Sino Biological Inc. Beijing; The gene sequence is identical with the Gene Bank Ref. ID sequence: S100A4 cDNA: NM\_002961.2; E-cadherin DNA: NM\_004360.3. The S100A4 cDNA product was then cloned into pMD vector as the manufacture's instruction. The constructs were confirmed by DNA sequencing and restriction enzyme digestion. For transfection studies, EC109 and TE13 cells were plated at a density of  $1 \times 10^6$  cells per well in 6-well plates and incubated for 24 h in complete medium. The cells were then transfected with S100A4 cDNA (E-cadherin) construct by using an lipofectamine transfection kit for 48 h. For controls, the same amount of empty vector was also transfected. Stable transfected TE13 cells (pMD-S100A4 cDNA) were selected with 200 µg/mL G418.

### Real-time quantitative reverse transcription-polymerase chain reaction analysis of archival material or TE-13 and Eca-109 cell lines

Total RNA was extracted from cryostat sections of frozen tissue or TE-13 and Eca-109 cell lines using Trizol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR) was performed using the ABI Prism 7 700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Q-PCR assays were performed in triplicate, and the mean values were used for calculations of mRNA expression. Relative levels were determined using the 2<sup>-ΔΔCt</sup> method<sup>[22]</sup>.

### Reverse transcription-polymerase chain reaction analysis of archival material or TE-13 and Eca-109 cell lines

PCRs were carried out by using forward and reverse primer combinations for S100A4 (forward 5'-TCAGAACTA-AAGGAGCTGCTGACC-3', reverse 5'-TTTCTTCCT-GGGCTGCTTATCTGG-3'), E-cadherin (forward 5'-GGAAGTCAGTTCAGACTCCAGCC-3', reverse 5'-AGGCCTTTTGACTGTAATCACACC-3'), GAPDH (forward 5'-AATCCCATCACCACTTCCAGGAG-3', reverse 5'-GCATTGCTGATGATCTTGAGGCTG-3'). The cDNA was amplified with an initial denaturation at 94 °C for 3 min followed by the sequential cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min for 30 cycles, with final extension at 72 °C for 5 min.

### Western blotting

Whole-cell proteins were isolated, the lysates centrifuged, and the supernatant collected. 30 µg of total protein was loaded per well, separated by 7.5% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and

transferred to polyvinylidene difluoride membranes at 150 mA for 16 h at 4 °C according to the manufacturer's instructions. The membranes were blocked and incubated with primary antibodies. Primary antibodies were as follows: anti-S100A4 (1:200 dilution) and anti-E-cadherin (1:200 dilution; all from Santa Cruz Biotechnology). The immunoblots were detected by using an electrochemiluminescence kit (Amersham, Piscataway, NJ) and exposed to X-OMATAR film.

### Immunohistochemistry staining

The paraffin-embedded sections were stained with primary anti-S100A4 or anti-Ecadherin (Abcam, Cambridge, United Kingdom) antibody and horseradish peroxidase-labeled immunoglobulin (Boshide Biotech Co., Ltd, Wuhan, China). Images were obtained at  $\times 200$  magnification. Stained slides were scored by 2 blinded, independent observers. The results of the immunohistochemical stainings were evaluated by the percentage of positively stained carcinoma cells. Expression of S100A4 was determined as positive when cytoplasmic and/or perinuclear staining was seen in more than 10% of the tumour cells. Expression of S100A4 was considered negative when no cells or less than 10% of the tumour cells were stained. Expression of E-cadherin was determined as described in a previous study<sup>[23]</sup>. Briefly, the tumor cells that stained as strongly as normal epithelial cells were considered to be "preserved expression" (positive), and those which exhibited weaker staining than normal epithelial cells or showed completely negative staining were considered to be "reduced expression" (negative).

### Cell invasion assays

Cell invasion assays were performed using 8- $\mu$ m pore size Transwell Biocoat Control inserts (Becton Dickinson). In brief,  $1 \times 10^4$  cells were seeded on a transwell containing numbers of 8- $\mu$ m pores for invasion assay. The chambers were put into the incubator at 37 °C, 50 mL/L CO<sub>2</sub>. Cells on the top surface of the transwell were removed by scrubbing 24 h after incubation. The cells were fixed by 950 mL/L ethanol, and stained in 30 min by 1 mL/L crystal violet. We counted the number of transmembrane cells under an optical microscope, chose five high power fields by random, and checked each field of vision to evaluate the invasion and metastasis of tumor cells *in vitro*. Such invaded cells were counted and compared among groups. Individual experiments were done in duplicate and repeated four times.

### Invasion study in vivo

All animals were maintained in a sterile environment and cared for within the laboratory animal regulations of the Ministry of Science and Technology of the People's Republic of China (<http://www.most.gov.cn/kytj/kytjz-cwj/200411>). Full details of the study approval by the ethics committee at the affiliated hospital of Tianjin medical university. After growth to subconfluency, transfected (pMD-S100A4 siRNA or mock siRNA) and nontransfected Eca-109 cells, stable transfected TE13 cells (pMD-S100A4

**Table 1** Immunohistochemistry staining of S100A4 and E-cadherin

Groups	n	S100A4			E-cadherin		
		Positive	Negative	P value	Positive	Negative	P value
Metastasis	28	19	9	0.027	10	18	0.036
Non-metastasis	20	7	13		12	8	

cDNA), mock transfected and nontransfected TE13 cells were injected into the pancreas under the envelope near spleen of nude mice ( $n = 8$  for each variant). Twenty-eight days later, the mice were killed following the operation. The number of the seeded tumor naked in the liver and lung is used for assessment of metastases.

### Statistical analysis

All statistical analyses were performed using SPSS 11.0 software. The results were presented as mean  $\pm$  SD of three replicate assays. Differences between various groups were assessed using ANOVA or Dunnett *t*-test. *P* value (of  $< 0.05$ ) was considered to indicate statistical significance.

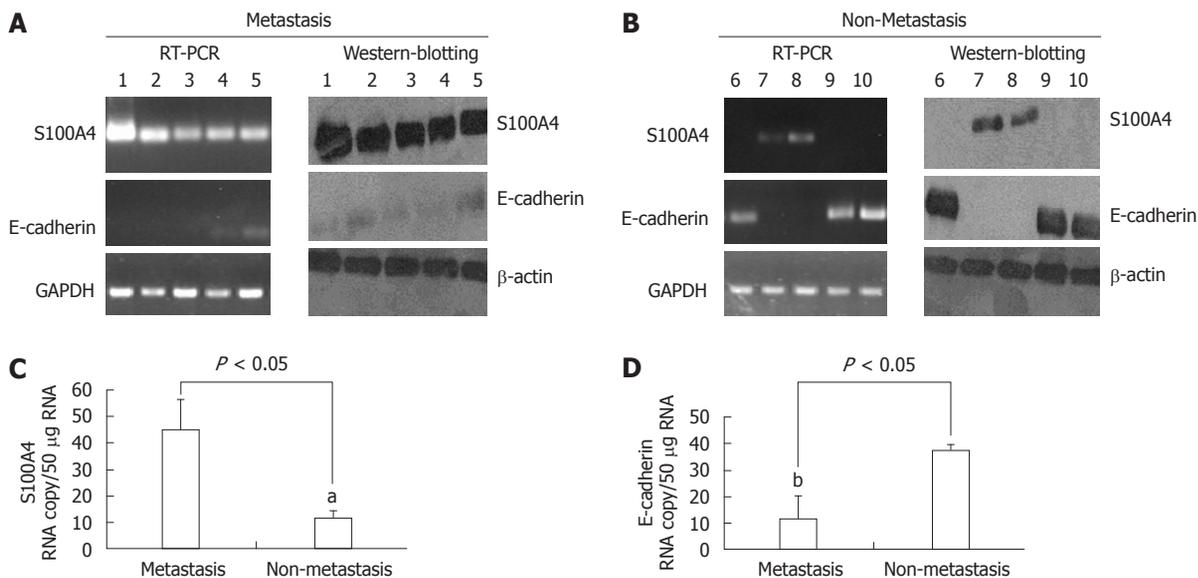
## RESULTS

### Increased expression of S100A4 and E-cadherin is associated with lymph node metastasis

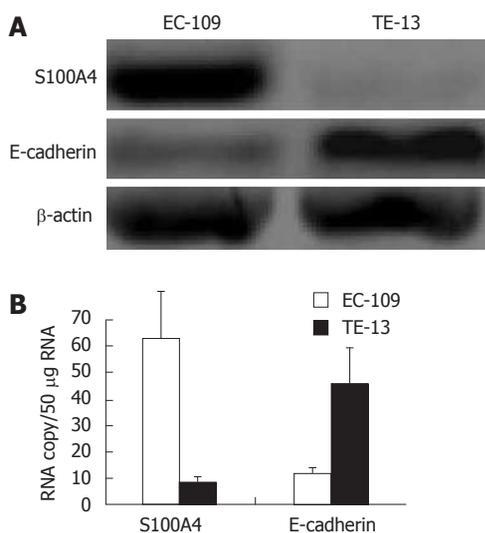
Results of immunohistochemistry (IHC) staining showed that S100A4 was weakly expressed in non-metastasis ESCC, whereas strongly expressed in metastatic ESCC (Table 1,  $P < 0.05$ ). On the contrary, E-cadherin expression was strongly expressed in non-metastatic ESCC, whereas weak E-cadherin expression was detectable in metastatic ESCC (Table 1) Significantly, negative relationship was found between S100A4 and E-cadherin expression. The Western blotting, Q-PCR and reverse transcription-polymerase chain reaction (RT-PCR) analysis has the same results as IHC analysis. A six representative (5 non-metastatic ESCC cases and 5 metastatic ESCC cases) western blotting and RT-PCR results was shown in Figure 1A and B. In the present study, we also detected the levels of transcripts of S100A4 mRNA and E-cadherin mRNA in metastasis (M) tumor samples and non-metastasis tumor (N) samples (expressed as transcript copy number per 50  $\mu$ g of messenger RNA and standardized with  $\beta$ -actin; Figure 1C and D). We found that transcript copy numbers for S100A4 were  $46.3 \pm 9.4$  for M tumor and  $10.5 \pm 3.6$  for N tumor ( $P = 0.036$ ); for E-cadherin, they were  $13.62 \pm 2.3$  for M tumor and  $40.17 \pm 3.91$  for N tumor ( $P = 0.018$ ); A significant negative relationship was observed between S100A4 mRNA and E-cadherin expression ( $P = 0.034$ ). These results above indicates that ESCC metastasis is associated with significantly decreased expression of E-cadherin and increased S100A4 expression.

### S100A4 and E-cadherin expression in EC109 and TE13 cell lines

Western blotting (Figure 2A) and RT-PCR (Figure 2B) analysis shown the levels of S100A4 were significantly



**Figure 1 S100A4 and E-cadherin expression in metastasis and non-metastasis tissue.** A: The representative reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting results for S100A4 and E-cadherin in metastasis tissue; B: The representative RT-PCR and Western blotting results for S100A4 and E-cadherin in non-metastasis tissue; C and D: Levels of transcripts of S100A4 and E-cadherin in M tumor samples in comparison to N tumor (expressed as transcript copy number per 50 µg of messenger RNA and standardized with β-actin). The intensity of each band relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (α-tubulin) band was represented as the mean ± SD, <sup>a</sup>*P* < 0.05 vs metastasis group and <sup>b</sup>*P* < 0.05 vs non-metastasis group.



**Figure 2 Analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines.** A: Western blotting analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines; B: Reverse transcription-polymerase chain reaction analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines. The levels of S100A4 were significantly higher in EC109 cells than that of in TE13 cells, and the levels of E-cadherin were significantly lower in EC109 cells than that of in TE13 cells.

higher in EC109 cells than that of in TE13 cells, and the levels of E-cadherin were significantly lower in EC109 cells than that of in TE13 cells. IHC analysis shown the same result as Western blotting and RT-PCR (data not shown).

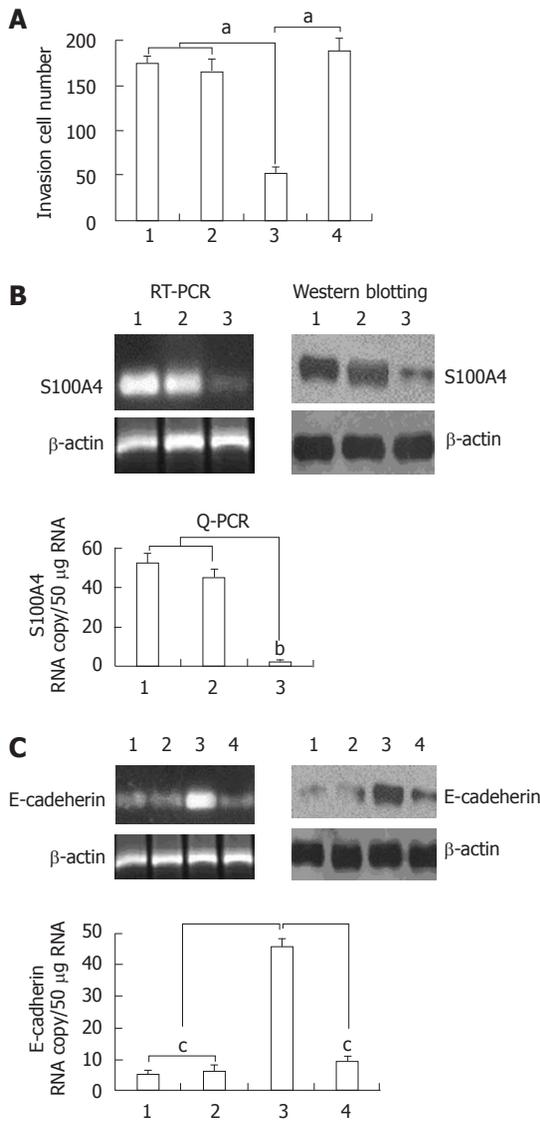
**Knockdown of S100A4 inhibits invasion in EC109 cell by upregulation of E-cadherin**

S100A4 has been implicated in the malignant phenotype of tumor cells, including cell motility, however the bio-

logical function is hardly known. Many studies found that S100A4-induced invasiveness in malignant tumor cells is caused or partially caused by down-regulation of E-cadherin<sup>[24-29]</sup>. Kwak *et al*<sup>[30]</sup> has reported there was no significant association between S100A4 and clinicopathological parameters such as tumor differentiation or TNM stage, and also no correlation between the reactivity and E-cadherin. In the present study, we found that knockdown of S100A4 inhibited invasion in EC109 cells, followed by increased E-cadherin expression in EC109 cells. As shown in Figure 3A, suppression of the S100A4 caused a over 60% reduction (*P* < 0.05) in the number of cells that traversed the membrane versus nonsilencing control or mock control in EC109 cells. Western blotting, RT-PCR and Q-PCR analysis shown E-cadherin mRNA and protein expression was upregulated when the S100A4 expression was knockdown (Figure 3B). When the stable transfectants (S100A4 siRNA) were transfected with S100A4 cDNA for 48h, S100A4 expression was observed to be very high 48 h after transfection (data not shown) to restore the expression of S100A4 in the stable transfected EC109 cells, only to find significantly increased invasion ability in EC109 cells (Figure 3A) followed by the downregulation of E-cadherin expression (Figure 3C). These data suggest that knockdown of S100A4 suppressed the invasion ability of human EC109 cells by upregulation of E-cadherin expression.

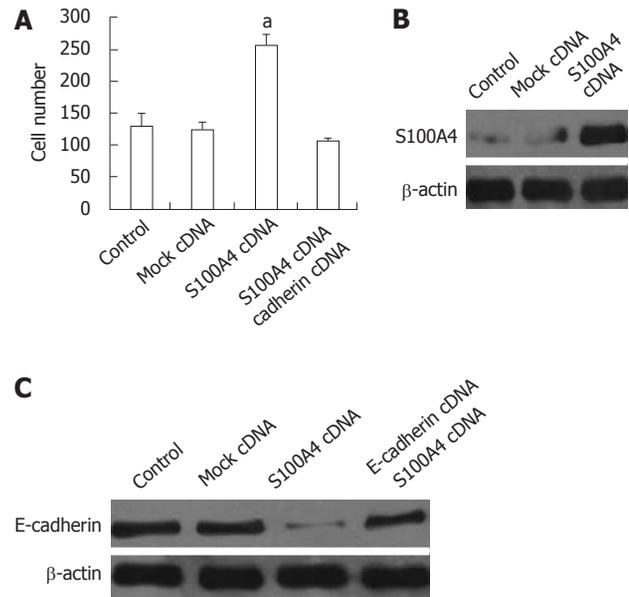
**Overexpression of S100A4 promotes invasion in TE-13 cell by downregulation of E-cadherin**

TE-13 cells stably transfected with pMD-S100A4 cDNA plasmid displayed a significantly increased S100A4 expression as compared with vector control. The overexpression of S100A4 was confirmed by performing Western



**Figure 3 Knockdown of S100A4 inhibits invasive capability of EC109 cells.** A: Transmigration cells of control, mock siRNA, S100A4 siRNA and S100A4 siRNA + S100A4 cDNA cells was calculated from three independent experiments. The indicated cells ( $1 \times 10^4$ ) were seeded on 8-mm porous transwell chambers. After 24 h of plating, transmigration cells were fixed and stained with crystal violet. Transmigration cells were counted for each of the indicated cells. Columns, mean number of cells obtained in three independent experiments; bars, SD; <sup>a</sup> $P < 0.05$  vs control or mock siRNA group; B: Western blotting, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR (Q-PCR) analysis for S100A4 and mRNA and protein expression, <sup>b</sup> $P < 0.05$  vs control or mock siRNA group; C: Western blotting, RT-PCR and Q-PCR analysis for E-cadherin and mRNA and protein expression. <sup>c</sup> $P < 0.05$  vs S100A4 siRNA group. 1: Control; 2: Mock siRNA; 3: S100A4 siRNA; 4: S100A4 siRNA + E-cadherin siRNA.

blotting analysis (Figure 4B). We analyzed the effect of S100A4 overexpression on the invasive ability of TE13 cells. As shown in Figure 4A, overexpression of S100A4 significantly increased the number of invasive cells ( $P < 0.05$ ), followed by the downregulation of E-cadherin (Figure 4C). However, when the stable transfectants (pMD-S100A4 cDNA plasmid) were transfected with pMD-E-cadherin cDNA for 48 h [E-cadherin expression was observed to be very high 48 h after transfection (data not shown)] to restore the expression of E-cadherin in the stable transfected TE-13 cells, only to find significantly



**Figure 4 S100A4 overexpression promotes invasive capability of TE13 cells.** A: Transmigration cells of control, mock cDNA, S100A4 cDNA and S100A4 cDNA + E-cadherin cDNA cells was calculated from three independent experiments. The indicated cells ( $1 \times 10^4$ ) were seeded on 8-mm porous transwell chambers. After 24 h of plating, transmigration cells were fixed and stained with crystal violet. Transmigration cells were counted for each of the indicated cells. Columns, mean number of cells obtained in three independent experiments; bars, SD; <sup>a</sup> $P < 0.05$  vs other 3 groups respectively; B: Western blotting, analysis for S100A4 protein expression; C: Western blotting analysis for E-cadherin protein expression.

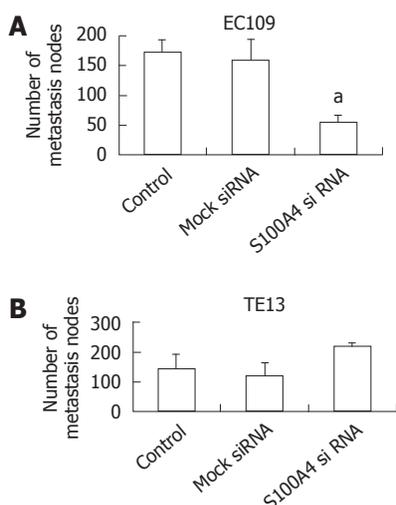
decreased invasion ability in the TE-13 cells (pMD-S100A4 cDNA plasmid-transfected) (Figure 4A). These data suggest that the *S100A4* gene controls the invasion ability of human TE-13 cells by regulation of E-cadherin. These data further support our hypothesis that S100A4 confers the invasive characteristics to cells during human ESCC development.

**Effect of S100A4 on metastasis in vivo model**

EC109 cells (S100A4 siRNA-transfected) or TE-13 cells (S100A4 cDNA-transfected) and their control cells were injected into the pancreas under the envelope near spleen of nude mice ( $n = 6$  for each variant). Twenty-one days later, the mice were killed, autopsy was carried out to remove organs. The number of the seeded tumor naked in the spleen, liver, pancreas and lung is used for assessment of metastases. Less sleep, liver, pancreas and lung metastasis nodes were found in S100A4 siRNA-transfected groups. The total nodes in S100A4 siRNA-transfected groups was significantly fewer than that of in mock siRNA or control ( $P < 0.01$ ) (Figure 5A). However, more seeded nodes were found in S100A4 cDNA transfected groups than that of in mock cDNA or control groups, although the difference was not significant (Figure 5B).

**DISCUSSION**

Metastasis is a complex cascade of events involving a finely tuned interplay between malignant cells and multiple host factors. The transition from benign tumor growth to



**Figure 5** Knockdown of S100A4 inhibits metastasis of xenograft tumors ( $n = 6$  per group). A: The total metastasis nodes in EC109 tumors ( $^*P < 0.05$  vs control or mock siRNA); B: The metastasis nodes in TE13 tumors.

malignancy is manifested by the ability of tumor cells to traverse tissue barriers and invade surrounding tissues<sup>[31]</sup>. Among a multitude of factors playing a role, the small calcium-binding protein S100A4 has been found to add to the invasive and metastatic capacity of cancer cells<sup>[32,33]</sup>. Recent studies have shown S100A4 is up-regulated in many types of epithelial cancers, including esophageal squamous cell<sup>[17-20]</sup>. S100A4S plays an important role in tumor progression and invasion. However, the exact molecular function or mechanism by which S100A4 exerts its putative metastasis-promoting effects has not been fully elucidated, and the protein is most likely involved in several aspects of tumor progression<sup>[34,35]</sup>.

EMT is a crucial process during morphogenesis of multi-cellular organisms. EMT not only is a normal developmental process but also plays a role in tumor invasion and metastasis<sup>[36]</sup>. Currently, EMT is thought to be a key step for cancer metastasis<sup>[37]</sup>. One of the key features of EMT is the down-regulation of the expression of the cell adhesion molecule E-cadherin, a critical event in tumor invasion. Many reports have found E-cadherin expression was significantly reduced in ESCCs, and lower expression of E-cadherin followed by increased lymph node metastasis and poor procession<sup>[2,38-40]</sup>. Several studies have recently described a direct interaction and/or reciprocal influence between S100A4 and E-cadherin<sup>[24-30]</sup>.

In the present study, we found that expression of S100A4 was significantly associated with nodal metastasis in ESCC. The ESCC tissue with a high S100A4 expression had a weak E-cadherin expression, and the expression of S100A4 was significantly associated with decreased E-cadherin. It is suggested S100A4 promotes migration and invasion may correlate with the downregulation of E-cadherin expression.

Several studies found knockdown of S100A4 inhibits invasion and proliferation in carcinoma cells, and overexpression of S100A4 promotes invasion and proliferation<sup>[41-44]</sup>. To test the significance of S100A4 expression

in ESCC, we transfected the S100A4 siRNA into EC109 cells to knockdown of S100A4. After transfection, the invasive ability of EC109 cells decreased dramatically. Our results were consequent with the resent report<sup>[45]</sup>.

We also observed that *S100A4* gene suppression significantly increased the expression of E-cadherin. When we restored the expression of S100A4 in the stable transfected EC109 cells, only to find significantly increased invasion ability in EC109 cells followed by the downregulation of E-cadherin expression. To prove that overexpression of S100A4 promotes invasion again, the TE-13 cells (less S100A4 expression) was transfected with S100A4 cDNA, only to find significantly increased the invasion ability of TE-13 cells, followed by the downregulation of E-cadherin. However, when the S100A4 cDNA transfected TE13 cells were transfected with E-cadherin cDNA to restore the expression of E-cadherin in the TE-13 cells, only to find significantly decreased invasion ability in the TE-13 cells. These data suggest that the *S100A4* gene controls the invasion ability of ESCC by regulation of E-cadherin.

*In vivo*, we found S100A4 knockdown can synergistically reduce the metastatic burden using a EC109 pseudometastatic model in immunodeficient mice, and the effects reached statistical significance. We also observed that overexpression of S100A4 increased the metastatic burden in a TE13 pseudometastatic model.

In summary, we demonstrated that the *S100A4* gene controls invasion ability of human ESCC cells through the regulation of *E-cadherin* gene. We suggest that control of invasion and metastasis through suppression of the *S100A4* gene may contribute to a novel therapeutic approach against ESCC. This approach could be realized through development of specific S100A4 inhibitors or use of a gene therapy approach.

## COMMENTS

### Background

Esophagus squamous cell carcinoma (ESCC) is one of the most deadly malignances because of its high frequency of metastasis. S100A4 possesses a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility and invasion. In the study, the authors explored a potential role of S100A4 in ESCC metastasis.

### Research frontiers

S100A4 was overexpressed or silenced by S100A4 siRNA in TE-13 or Eca-109 cells *in vitro* and *in vivo*, and to explore the influence of S100A4 on esophageal cancer invasion and metastasis.

### Innovations and breakthroughs

It has reported S100A4 may play an important role in promoting metastasis and the early step of tumorigenesis of human cancers. Furthermore, S100A4 silencing could suppress invasion and metastasis in many cancer cells.

### Applications

S100A4 may be involved in ESCC progression, RNA interference (RNAi) targeting S100A4 is a potential therapeutic method for human ESCCs.

### Terminology

S100A4, also known as mts1, p9Ka, FSP1, CAPL, calvasculin, pEL98, metastasin, 18A2, and 42A, was cloned in the 1980s and early 1990s from various cell systems. S100A4 is localized in the nucleus, cytoplasm, and extracellular space and possesses a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility and invasion.

**Peer review**

The study investigates the role of S100A4 oncoprotein in esophageal cancer samples and cell lines. The study clearly shows that the oncoprotein mediates tumor invasion by down-regulating CDH1.

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## High-fat-induced intestinal permeability dysfunction associated with altered fecal bile acids

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### Abstract

**AIM:** To investigate whether high-fat-feeding is associated with increased intestinal permeability *via* alterations in bile acid metabolism.

**METHODS:** Male C57Bl/6J mice were fed on a high-fat ( $n = 26$ ) or low-fat diet ( $n = 24$ ) for 15 wk. Intestinal permeability was measured from duodenum, jejunum, ileum and colon in an Ussing chamber system using 4 kDa FITC-labeled dextran as an indicator. Fecal bile acids were analyzed with gas chromatography. Segments of jejunum and colon were analyzed for the expression of farnesoid X receptor (FXR) and tumor necrosis factor (TNF).

**RESULTS:** Intestinal permeability was significantly increased by high-fat feeding in jejunum (median 0.334 for control *vs* 0.393 for high-fat,  $P = 0.03$ ) and colon (0.335 for control *vs* 0.433 for high-fat,  $P = 0.01$ ), but not in duodenum or ileum. The concentration of nearly all identified bile acids was significantly increased by high-fat feeding ( $P < 0.001$ ). The proportion of ursodeoxycholic acid (UDCA) in all bile acids was decreased

( $1.4\% \pm 0.1\%$  in high-fat *vs*  $2.8\% \pm 0.3\%$  in controls,  $P < 0.01$ ) and correlated inversely with intestinal permeability ( $r = -0.72$ ,  $P = 0.01$ ). High-fat feeding also increased jejunal FXR expression, as well as TNF expression along the intestine, especially in the colon.

**CONCLUSION:** High-fat-feeding increased intestinal permeability, perhaps by a mechanism related to bile acid metabolism, namely a decreased proportion of fecal UDCA and increased FXR expression.

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**Key words:** Bile acids; Bile salts; Diet-induced obesity; Farnesoid X-activated receptor; Intestinal permeability; Ursodeoxycholic acid

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### INTRODUCTION

Intestinal permeability is a term for the paracellular and transcellular translocation of large molecules foreign to the body. Paracellular permeability is mediated by tight-junction proteins, which prevent uncontrolled transport through the epithelium<sup>[1]</sup>. Deterioration of tight-junction proteins may permit translocation of bacterial lipopolysaccharides into the serum, i.e., endotoxemia, which may then cause inflammation. In obesity, low-grade inflammation is a risk factor for type 2 diabetes and cardiovascular diseases<sup>[2,3]</sup>. Impaired gut barrier function is related to several disease states such as steatohepatitis, fatty liver dis-

ease and diabetes<sup>[4,6]</sup>. Furthermore, it may form the link between obesity and its related disorders<sup>[7-12]</sup>.

High dietary fat content may in part lead to barrier dysfunction, supported by cross-sectional data indicating that a diet high in energy and fat is associated with endotoxemia<sup>[13]</sup>. Dietary fat affects bile acid metabolism, because the absorption of fat requires an increase in bile flow. Consequently, a high-fat diet elevates the fecal concentration of bile acids<sup>[14,15]</sup>. In mice, an orally fed secondary bile acid deoxycholic acid (DCA) induces intestinal inflammation<sup>[16]</sup>, and *in vitro* bile acids provoke permeability of intestinal cell monolayers<sup>[17,18]</sup>. In contrast, the most hydrophilic bile acid, ursodeoxycholic acid (UDCA), may counteract increased intestinal permeability<sup>[19]</sup>.

Proteins involved in bile acid absorption are mediated by the intestinal farnesoid X receptor (FXR), also known as the bile acid receptor, which is highly expressed in intestinal and liver tissues<sup>[20]</sup>. Mice deficient in FXR show compromised barrier function and localization of neutrophils in their intestinal villi<sup>[21]</sup>.

The aim of this study was to investigate whether high-fat feeding is associated with increased intestinal permeability *via* alterations in bile acid metabolism.

## MATERIALS AND METHODS

### Animals

Male C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany) and acclimatized for a week prior to the experiment. At 6 wk of age, they were randomized into two groups equal in weight: High-fat ( $n = 26$ ; 60E% fat, D12492; Research Diets, New Brunswick, NJ, United States) and control ( $n = 24$ ; 10E% fat, D12450B; Research Diets). Diets were paired for fiber and protein content. Mice were housed six per cage in a standard animal laboratory with a dark/light cycle of 12/12 h, with free access to food and water. After 15 wk of feeding, mice were euthanized with a gas mixture of CO<sub>2</sub> (70%) and O<sub>2</sub> (30%) (AGA, Riihimäki, Finland), and cervical dislocation. Animal experiments were approved by the National Animal Experiment Board of Finland.

### Intestinal permeability measurements

Fresh segments of duodenum, jejunum, ileum and proximal colon were collected in duplicate, opened along the mesenteric border, pinned onto 0.3 cm<sup>2</sup> sliders and mounted into an EasyMount Ussing chamber system with a voltage-clamp apparatus (Physiologic Instruments, San Diego, CA, United States). Two intestinal segments were collected in duplicate from each mouse, because only four chambers were available. Tissues were surrounded by 5 mL Ringer solution (120 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaHCO<sub>3</sub>, 1.8 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1.25 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgSO<sub>4</sub>, 10 mmol/L glucose) on each side. The system was water-jacketed to 37 °C and carbonated with a carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>; AGA) gas flow. After an equilibration period of 10 min, solutions were replaced with fresh Ringer, and 4 kDa FITC-dextran (TdB Cons, Uppsala, Sweden) was added

to the luminal side to a final concentration of 2.2 mg/mL. Resistance and short-circuit current were followed for 60 min, after which, serosal fluorescence was detected with a Wallac Victor<sup>2</sup> 1420 Multilabel counter (Perkin-Elmer, Waltham, MA, United States). Intestinal permeability was determined by comparing serosal fluorescence to luminal fluorescence as per mille of translocated dextran.

### Fecal bile acid analysis

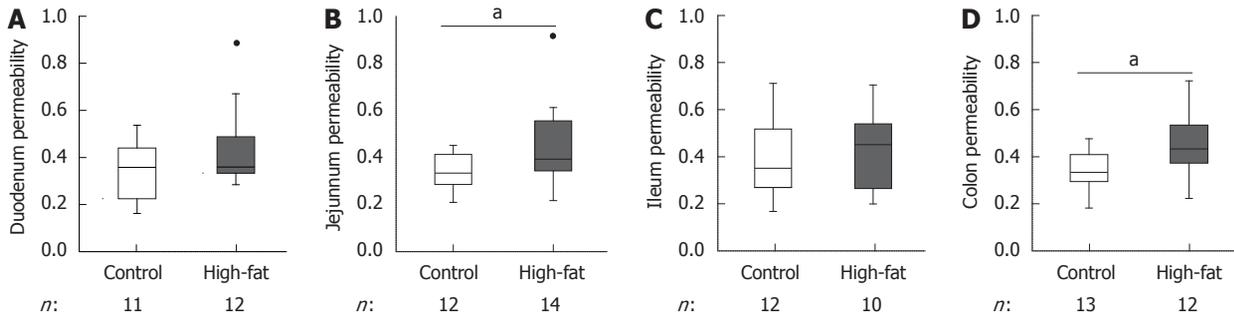
Feces were collected at 13 wk by placing mice individually in metabolic cages for 24 h. Feed and water was provided *ad libitum*. Feces were carefully separated from all other material and frozen at -20 °C. Upon analysis, feces of six mice from each group were dried overnight with nitrogen and pulverized. Bile acids were extracted and measured by gas-liquid chromatography according to the method by Grundy *et al*<sup>[22]</sup> in the laboratory of the Hospital District of Helsinki and Uusimaa. Internal standards were run for isolithocholic acid, lithocholic acid, epideoxycholic acid, DCA, chenodeoxycholic acid, cholic acid and UDCA.

### Farnesoid X receptor and tumor necrosis factor expression assays

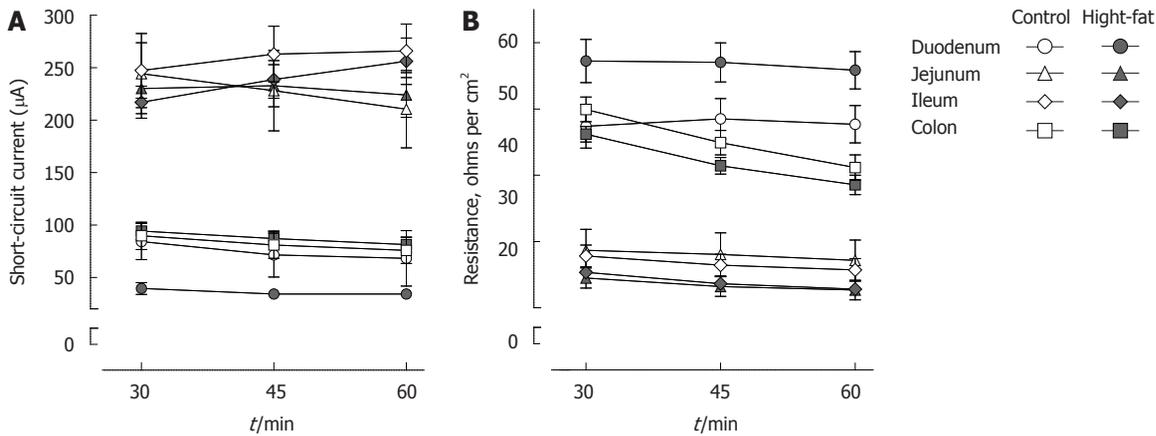
Segments of jejunum were collected from each mouse, snap frozen in liquid nitrogen, and stored at -80 °C. RNA was extracted with TRI Reagent (RT111; Molecular Research Center, Cincinnati, OH, United States) according to the manufacturer's protocol. Tissues were homogenized with Precellus24 (6500 rpm, 2 × 15 s; Bertin Technologies, Montigny-le-Bretonneux, France). Phase separation was performed with chloroform (34 854; Sigma-Aldrich, St Louis, MO, United States), and RNA precipitation with isopropanol (P/7507/15X, Fisher Scientific, United States). RNA concentration was measured with NanoDrop 8000 (Thermo Scientific, Waltham, MA, United States), and converted to cDNA with a SuperScript VILO cDNA synthesis kit (Applied Biosystems, Carlsbad, CA, United States) according to the manufacturer's instructions, using 1 µg RNA in a reaction volume of 20 µL. Reactions for quantitative real time polymerase chain reaction (qPCR) were run using TaqMan chemistry (Applied Biosystems) for FXR (Mm00436420\_m1) and tumor necrosis factor (TNF) (Mm00443258\_m1). Gene expression was normalized to beta-actin and beta-glucuronidase. Reactions were run on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, United States) in triplicate. Skeletal muscle was used as a non-expressing negative control. Gene expression was calculated with Bio-Rad CFX Manager software using the normalized expression  $\Delta\Delta C(t)$  method.

### Statistical analysis

Permeability results were statistically analyzed with a Mann-Whitney *U* test due to the uneven distribution of values around the means. In addition to calculating intestinal permeability for each segment (duodenum, jejunum, ileum and colon), a value for overall intestinal permeability was calculated for each mouse as an average of the permeability in its two measured intestinal segments. Bile acid and gene expression data were analyzed with a *t* test



**Figure 1** Effect of high-fat-feeding on intestinal permeability. Intestinal permeability in duodenum (A), jejunum (B), ileum (C), and colon (D) of high-fat-fed vs control mice. Permeability was measured in an Ussing chamber. Results are shown as per mille translocated dextran. Box plots show median, upper and lower quartiles, and Tukey's whiskers (highest and lowest values, outliers shown as black dots). <sup>a</sup>*P* < 0.05 between high-fat and control.



**Figure 2** Stability of Ussing chamber experiments. Short-circuit current (A) and resistance (B) in Ussing chamber experiments. Bars indicate mean and SEM. *n* = 9-15/group.

between control and high-fat groups. As there was a special focus on UDCA, the concentration of this bile acid relative to total identified bile acids was also calculated. The gene expression data had a setting of four groups, characterized by two factors possibly affecting intestinal FXR and TNF expression: intestinal segment and diet. The independent effects of these two factors were statistically analyzed with a factorial experiment. Groups were also compared with each other individually for both intestinal segments with a *t* test. Equality of variances was tested with Levene's test. All statistical analyses were performed with PASW Statistics version 18.0.2 (IBM, United States). All data are expressed as mean ± SE unless otherwise stated.

## RESULTS

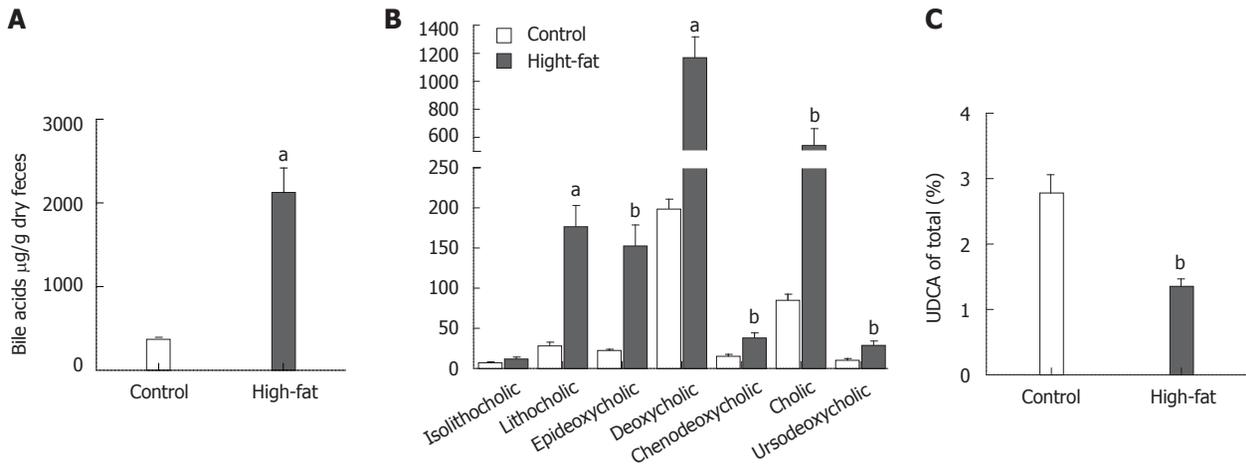
### Intestinal permeability

The weight of the high-fat-fed mice was significantly higher compared to control mice (49.5 ± 0.59 g *vs* 28.6 ± 0.36 g, *P* < 0.001). High-fat-feeding increased intestinal permeability significantly in the jejunum (median 0.334 per mille translocated dextran for control *vs* 0.393 for high-fat, *P* = 0.03) and colon (median 0.335 for control *vs* 0.433 for high-fat, *P* = 0.01), but not in the duodenum (median 0.359 for control *vs* 0.360 for high-fat, *P* = 0.33) or ileum (median 0.351 for control *vs* 0.452 for high-fat,

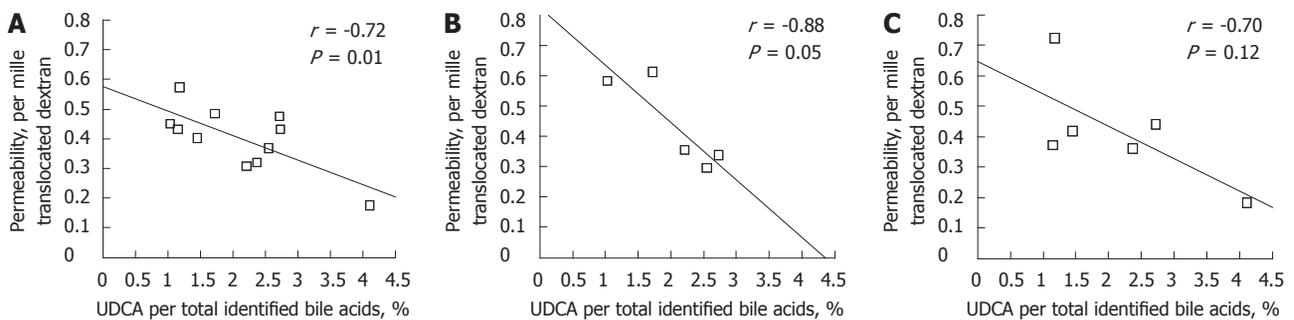
*P* = 0.69, Figure 1). Resistance and short-circuit current were measured during the Ussing chamber experiments to monitor tissue viability. The stability of these values indicates good viability of tissue segments throughout the experiments (Figure 2).

### Fecal bile acids

Feces from high-fat fed mice contained substantially more bile acids compared to feces from control mice (2.13 ± 0.29 mg/g dry feces *vs* 0.37 ± 0.03 mg/g dry feces, *P* < 0.001; Figure 3A). This was reflected as a significantly elevated concentration of each bile acid (*P* < 0.01) except isolithocholic acid (Figure 3B). We were especially interested in the effects of UDCA, which is considered cytoprotective, therefore, we calculated the ratio of UDCA to total bile acids. The proportion of UDCA in feces of high-fat-fed mice was nearly halved compared to that in control mice (1.4% ± 0.1% in high-fat *vs* 2.8% ± 0.3% in controls, *P* < 0.01, Figure 3C). There was also a marked inverse correlation of overall intestinal permeability to the proportion of UDCA in total fecal bile acids (*r* = -0.72, *P* = 0.01, *n* = 11, Figure 4A). The correlation remained significant even after controlling for body weight (partial correlation *r* = -0.64, *P* < 0.05, *n* = 11). A trend for a similar association was seen in the subgroups of jejunal and colonic permeability (*r* = -0.88, *P* = 0.05, *n* = 5 for jejunum and *r* = -0.70, *P* = 0.12, *n* = 6 for colon, Fig-



**Figure 3 Fecal bile acids.** A: Concentration of total measured bile acids; B: Concentration of measured bile acids in feces; C: Proportion of ursodeoxycholic acid (UDCA) in total measured bile acids. Bars indicate mean and SEM.  $n = 6/\text{group}$ ,  $^aP < 0.001$ ,  $^bP < 0.01$  between high-fat and control groups.



**Figure 4 Correlation of proportion of ursodeoxycholic acid with intestinal permeability.** Pearson's correlation of fecal proportion of ursodeoxycholic acid (UDCA) percentage to overall permeability of intestine (A), jejunal permeability (B), and colonic permeability (C). Each dot represents an individual animal.

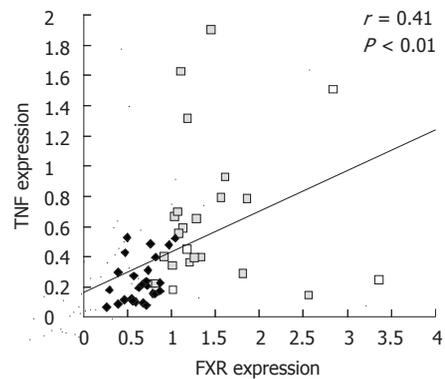
Table 1 Gene expression of farnesoid X receptor and tumor necrosis factor (mean $\pm$ SEM)					
	Control		High-fat		P value
	n	expression	n	expression	
FXR					
Jejunum	14	0.57 $\pm$ 0.051	12	0.74 $\pm$ 0.049	0.03
Colon	10	1.36 $\pm$ 0.234	14	1.49 $\pm$ 0.159	0.63
TNF					
Jejunum	14	0.21 $\pm$ 0.041	12	0.27 $\pm$ 0.040	0.30
Colon	10	0.42 $\pm$ 0.068	14	0.82 $\pm$ 0.148	0.02

FXR: Farnesoid X receptor; TNF: Tumor necrosis factor.

ure 4B and C). In addition, we correlated permeability to the ratio of UDCA to DCA, a cytotoxic bile acid. This was done to prevent bias by the unmeasured muricholic acid, which is a primary bile acid present in murine bile<sup>[23]</sup>, and is left unidentified by methods designed for clinical use. The relation of UDCA to DCA showed a similar halved concentration and inverse correlation to permeability ( $r = -0.70$ ,  $P = 0.02$ ).

### Intestinal farnesoid X receptor and tumor necrosis factor expression

The effect of high-fat-feeding on bile acid metabolism and



**Figure 5 Correlation of tumor necrosis factor and farnesoid X receptor expression.** Pearson's correlation of tumor necrosis factor (TNF) and farnesoid X receptor (FXR) expression. Jejunal values are shown as black diamonds and colonic values as grey squares. Each dot represents an individual animal.

intestinal inflammation was assayed as expression of intestinal FXR and TNF in the jejunum and colon. Jejunal FXR expression was increased 30% by high-fat feeding ( $0.74 \pm 0.049$  for high-fat *vs*  $0.57 \pm 0.051$  for control,  $P = 0.03$ ) and colonic TNF expression was doubled ( $0.82 \pm 0.148$  for high-fat *vs*  $0.42 \pm 0.068$  for control,  $P = 0.02$ ) but no significant differences were seen in jejunal tumor necrosis factor (TNF) ( $P = 0.30$ ) or colonic FXR ( $P = 0.63$ ,

Table 1). FXR and TNF expressions did, however, correlate with each other ( $r = 0.41$ ,  $P < 0.01$ ,  $n = 50$ , Figure 5).

A factorial experiment on the independent effects of diet and intestinal segment on gene expression revealed that overall intestinal TNF expression was elevated by high-fat-feeding ( $P = 0.02$ ). Moreover, both TNF and FXR expressions were higher in colon compared to jejunum ( $P < 0.001$  for both).

## DISCUSSION

The objective of this study was to see whether intestinal permeability was increased by high-fat-feeding *via* a mechanism related to bile acid metabolism. These data showed that permeability of jejunum and colon in high-fat-fed mice was increased. The animals were clearly obese solely due to dietary fat content (60E%), and not by genetic modification. Our results on increased permeability are supported by a similar previous study, which investigated the effects of obesity and a high-fat diet on rat intestinal permeability<sup>[12]</sup>. Also a carbohydrate-free diet containing an extremely high amount of fat (72E%) increased intestinal permeability in mice<sup>[9]</sup>. Our 60E% diet may be considered more physiologically relevant compared to carbohydrate-free diets. We also took care to pair the diets in protein and fiber content. Therefore, the barrier dysfunction in high-fat-feeding was not biased by any fiber-mediated effect. There is no conclusion on whether gut permeability may be affected by obesity alone, without modifications in diet composition<sup>[7,12]</sup>.

In the present study, we found that, in addition to increased fecal bile acid content, high-fat feeding also modulated fecal bile acid profile. Bile acids and bile juice are known to impair barrier function in enterocyte monolayers *in vitro*<sup>[12,17,18]</sup>, as well as recovery from tissue damage *ex vivo*<sup>[24]</sup>, and are elevated in serum of high-fat-fed rats<sup>[12]</sup>. Our data indicate that high-fat-feeding modifies gut permeability by a mechanism related to bile acids, as previously hypothesized<sup>[12]</sup>.

This is, to the best of our knowledge, the first study to show that high-fat-induced intestinal barrier dysfunction is related to increased intestinal FXR expression. Inagaki *et al.*<sup>[21]</sup> have shown in a FXR knockout mouse model that FXR is involved in tight junction integrity. It is unclear, however, what role FXR plays in the pathogenesis of high-fat-induced barrier dysfunction.

Both fecal bile acids and intestinal TNF expression were elevated by high-fat-feeding in the present study. Bile acids are linked to inflammatory pathways, because DCA is able to stimulate the nuclear factor- $\kappa$ B route<sup>[25]</sup>, which regulates TNF expression. A correlation between bile acids and TNF was not observed in our small set of fecal samples. Intestinal FXR and TNF were, however, correlated, which may reflect a link between intestinal bile acid concentration and inflammation. Incubation of jejunum in DCA (0.3 mmol/L) increased prostaglandin E2 in the supernatant, thus indicating a possible role for inflammation in increasing intestinal permeability (data not shown). Orally fed DCA induces intestinal inflammation

in mice<sup>[6]</sup>, which further supports the hypothesis that inflammation is involved in the pathogenesis of gut barrier dysfunction. In this study, we observed increased TNF expression in the intestine. TNF is known to increase intestinal permeability *in vitro*<sup>[26-29]</sup>, and anti-TNF antibodies restore barrier function *in vivo*<sup>[30,31]</sup>. However, these data do not permit us to draw conclusions on whether increased TNF expression, in this study, was a cause or a secondary event in increased intestinal permeability.

We observed a halved proportion of UDCA in fecal bile acids of high-fat-fed mice. Moreover, this proportion of UDCA correlated with increased permeability, even when controlled for body weight. These data suggest a barrier protective effect for UDCA in our study. Our results are in agreement with previous reports showing that, as a hydrophilic bile acid, UDCA does not increase epithelial permeability *in vitro* in comparison to other bile acids<sup>[18]</sup>. On the contrary, it is capable of counteracting intestinal barrier dysfunction<sup>[19]</sup>, protecting against chemically induced colitis<sup>[19,32,33]</sup> and colitis-associated adenocarcinoma<sup>[34]</sup> in rodents. Its role may be especially relevant in comparison to DCA, because UDCA protects mitochondria against DCA-induced reactive oxygen species production<sup>[35]</sup>. It may thus be suggested that, in addition to total bile acid concentration, the bile acid profile is relevant regarding bile-acid-related functions in the intestine.

Intestinal permeability was measured *ex vivo* with the Ussing chamber as the translocation of large dextrans. This direct method is more representative of intestinal permeability than the often used transepithelial resistance and tight-junction protein analysis. Furthermore, the Ussing chamber allows measurement of permeability from selected tissue segments, unlike direct *in vivo* methods.

The 4 kDa FITC-dextrans used here are generally believed to translocate through the paracellular spaces, although there are no reports to confirm this. Permeability to FITC-dextrans does, however, correlate with a decrease in tight-junction protein expression<sup>[10]</sup>. It has also been proposed that translocation of lipopolysaccharides occurs transcellularly *via* chylomicrons<sup>[36]</sup>. The methods used in the present study do not distinguish between these two pathways. Their importance in intestinal permeability needs further elucidation.

We evaluated modifications of bile acid metabolism by analyzing fecal bile acids. It must be noted that fecal bile acids only reflect alterations of bile acid metabolism, and this method does not allow us to draw conclusions about liver bile acid metabolism or bile composition. It is, however, an estimate of colonic bile acid concentrations.

In conclusion, high-fat feeding increases permeability in the jejunum and colon, elevates fecal bile acid concentration, and induces intestinal inflammation in mice. Alterations in bile acid homeostasis, namely UDCA synthesis and intestinal FXR expression, may relate to increased intestinal permeability. Our results show that alterations in bile acid metabolism may be associated with intestinal permeability and should be studied as a possible target in affecting the onset of barrier dysfunction.

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## COMMENTS

### Background

Intestinal permeability has recently been linked to type 2 diabetes, steatosis and steatohepatitis. One proposed cause for increased permeability is a diet high in fat. A high-fat-diet increases excretion of bile. Secondary bile acids are known to increase permeability of epithelial monolayers *in vitro*. However, physiological concentrations of bile juice obtained from healthy rats have failed to increase epithelial monolayer permeability. This may be due to the fact that bile consists of a profile of several bile acids, which differ in cytotoxicity. It has not yet been addressed whether high-fat feeding alters the profile of fecal bile acids, and whether this profile plays a role in the onset of barrier dysfunction.

### Research frontiers

Proposed mechanisms for the dietary induction of increased intestinal permeability have mostly been related to alterations in gut microbiota. Only a few publications have mentioned other luminal factors affecting permeability. The research hotspot in this field is how diet modifies bile acid metabolism and leads to increased intestinal permeability.

### Innovations and breakthroughs

Although bile is considered detrimental to the gut epithelium, physiological concentrations have not increased epithelial monolayer permeability. We show that not only was bile excretion increased by high-fat feeding, but the profile of fecal bile acids had become more cytotoxic than in healthy control mice. The proportion of a hydrophilic bile acid, ursodeoxycholic acid (UDCA), was decreased by high-fat feeding and correlated inversely with intestinal permeability.

### Applications

The prevention of barrier dysfunction may decrease the risk of its associated diseases. The present results suggest that bile acid metabolism is a potential target for the prevention of a barrier dysfunction.

### Terminology

Intestinal permeability refers to how large molecules, such as inflammatory bacterial components, translocate through the intestinal epithelium into the circulation. Translocation may be paracellular, through tight-junction proteins, or transcellular, via chylomicrons. UDCA is a hydrophilic bile acid. Deoxycholic acid is a secondary bile acid produced from cholic acid by intestinal microbes.

### Peer review

The authors tested intestinal permeability in an *ex vivo* model (Ussing chambers; mucosal to serosal flux of FITC-labeled dextran) in mice on a high-fat diet; moreover, analysis of fecal bile acids and expression of mucosal tumor necrosis factor and farnesoid X receptor was performed.

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## Beneficial effect of sulphate-bicarbonate-calcium water on gallstone risk and weight control

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### Abstract

**AIM:** To investigate the effect of drinking sulphate-bicarbonate-calcium thermal water (TW) on risk factors for atherosclerosis and cholesterol gallstone disease.

**METHODS:** Postmenopausal women with functional dyspepsia and/or constipation underwent a 12 d cycle of thermal ( $n = 20$ ) or tap ( $n = 20$ ) water controlled drinking. Gallbladder fasting volume at ultrasound, blood vitamin E, oxysterols (7- $\beta$ -hydroxycholesterol and 7-ketocholesterol), bile acid (BA), triglycerides, total/low density lipoprotein and high density lipoprotein cholesterol were measured at baseline and at the end of the study. Food consumption, stool frequency and body weight were recorded daily.

**RESULTS:** Blood lipids, oxysterols and vitamin E were not affected by either thermal or tap water consumption. Fasting gallbladder volume was significantly ( $P < 0.005$ ) smaller at the end of the study than at baseline in the TW ( $15.7 \pm 1.1$  mL vs  $20.1 \pm 1.7$  mL) but not in the tap water group ( $19.0 \pm 1.4$  mL vs  $19.4 \pm 1.5$  mL). Total serum BA concentration was significantly ( $P < 0.05$ ) higher at the end of the study than at baseline in the TW ( $5.83 \pm 1.24$   $\mu$ mol vs  $4.25 \pm 1.00$   $\mu$ mol) but not in the tap water group ( $3.41 \pm 0.46$   $\mu$ mol vs  $2.91 \pm 0.56$   $\mu$ mol). The increased BA concentration after TW consumption was mainly accounted for by glycochenodeoxycholic acid. The number of pasta ( $P < 0.001$ ), meat ( $P < 0.001$ ) and vegetable ( $P < 0.005$ ) portions consumed during the study and of bowel movements per day ( $P < 0.05$ ) were significantly higher in the TW than in the tap water group. Body weight did not change at the end of the study as compared to baseline in both groups.

**CONCLUSION:** Sulphate-bicarbonate-calcium water consumption has a positive effect on lithogenic risk and intestinal transit and allows maintenance of a stable body weight despite a high food intake.

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**Key words:** Thermal water; Gallstones; Oxidative stress; Body weight; Bile acid

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## INTRODUCTION

Atherosclerosis, coledithiasis and obesity are very frequent and interrelated diseases among postmenopausal women<sup>[1-4]</sup>.

High serum triglycerides and total/low-density lipoprotein (LDL) cholesterol are important risk factors for atherosclerosis, heart attack and stroke<sup>[5]</sup>. Also oxidative stress, particularly the oxidation of LDL, plays a key role in atherogenesis due to the production of reactive oxygen species. New markers based on the detection of lipid peroxidation products by mass spectroscopy, such as oxysterols including 7- $\beta$ -hydroxycholesterol and 7-ketocholesterol, are specific, sensitive and reliable markers of systemic oxidative stress *in vivo*<sup>[6-8]</sup>. In addition, oxysterol coupled to vitamin E measurement in plasma can be used for estimating systemic oxidant stress/antioxidant balance<sup>[8]</sup>.

Cholesterol gallstone disease is very common in postmenopausal women, with incidence ranging from 22% to 30% in Western countries, and this disorder is one of the most common and costly of all digestive diseases<sup>[3]</sup>. Cholesterol gallstone pathogenesis is complex and multifactorial, involving genetic defects and environmental factors<sup>[9-11]</sup>. Changes in bile acid (BA), cholesterol and triglyceride metabolism, gallbladder reduced function and prolonged colonic transit time are critical factors in the pathogenesis of gallstones<sup>[12-15]</sup>.

Obesity and overweight are risk factors for both atherosclerosis and gallstone disease<sup>[16]</sup>. Some data suggest that body weight reduction can be achieved by acceleration of intestinal transit in humans and by BA feeding in animals<sup>[17-21]</sup>.

Thermal water (TW) consumption has been shown to ameliorate blood cholesterol patterns and systemic oxidative stress, and reduce oro-fecal transit time and gallbladder fasting volume<sup>[22-26]</sup>. No data are available on the effect of TW on BA pool composition and plasma oxysterols.

In the present study, we investigated in postmenopausal women the effect of drinking sulphate-bicarbonate-calcium TW on blood cholesterol, triglycerides, oxysterols, vitamin E and BA, and on gallbladder fasting volume, intestinal transit rate and body weight.

## MATERIALS AND METHODS

### Patients

The study protocol was approved by the Ethics Commit-

tee of the University of Rome Sapienza and informed written consent was obtained from all patients.

Forty postmenopausal (at least 1 year) women with functional dyspepsia and/or constipation participated in this study. Patients were divided into 2 groups: (1) TW group were 20 patients enrolled by the medical staff of Chianciano thermal centre (Tuscany, Italy); (2) control (CTRL) group were 20 patients enrolled by the medical staff of the Gastroenterology Division of Department of Clinical Medicine at the Sapienza University (Rome, Italy). Diagnosis of functional dyspepsia and/or constipation was made based on the Roma III criteria<sup>[27,28]</sup>.

Exclusion criteria were a history of liver, pancreatic, gallbladder (including sonographic evidence of gallstones) or other gastrointestinal diseases, lipid disorders, diabetes, severe high blood pressure (diastolic > 110 mmHg, systolic > 180 mmHg), cancer, surgical resection, and thyroid, neurological, muscular, rheumatological and immunological diseases. Patients were also excluded if they were heavy drinkers, heavy smokers or habitual drinkers of more than 3 cups of espresso coffee every day. Individuals enrolled were not receiving estrogen replacement therapy or any medication known to affect lipid metabolism, and were not taking vitamin, mineral, or phytoestrogen supplements. Participants had not consumed diets intended to cause weight loss within 1 year of selection.

Between 8 and 9 a.m. on days 1 and 13 after an overnight fast and before drinking water, all enrolled patients underwent blood sampling and abdominal ultrasonography, and they had daily body weight measurements, according to international standards, using a digital scale that was calibrated, having a capacity of up to 150 kg<sup>[29]</sup>.

Patients in the TW group underwent a 12 d cycle of TW treatment by drinking 500 mL of "Acqua Santa of Chianciano Terme" sulphate-bicarbonate-calcium water, at a temperature of 33 °C, every day in the morning in the fasted state, over a 30 min period. The control group drank Rome tap water at a temperature of 10-12 °C using the same schedule. The chemical composition of the "Acqua Santa of Chianciano Terme" sulphate-bicarbonate-calcium water and of the Rome tap water is reported in Table 1. Each day of the study all patients filled a stool diary<sup>[30]</sup> and a food and beverage frequency daily diary which asked for the number of portions consumed for the following items: Pasta, pizza, meat, fish, vegetables, bread, desserts, soft drinks, fruits, milk, dairy products, legumes and espresso coffee.

### Gallbladder volume and blood analyses

Fasting gallbladder volume was calculated by using the ellipsoid formula on the average of 2 sonographical gallbladder measurements<sup>[31]</sup>.

Plasma and serum were stored at -80 °C. Plasma levels of  $\alpha$ -tocopherol were analyzed by high performance liquid chromatography (HPLC), and 7- $\beta$ -hydroxycholesterol and 7-ketocholesterol were measured from the same sample by mass spectrometry using an isotope dilution method<sup>[8]</sup>. Serum triglycerides, total and high-density lipoprotein (HDL) cholesterol were measured by a colorimetric

**Table 1** Chemical composition of the sulphate-bicarbonate-calcium thermal water and of the Rome tap water, as consumed by the thermal water and by the control group, respectively

	Thermal water (TW)	Tap water (CTRL)
pH	6.8	7.5
Fixed residue at 180 °C (mg/L)	3280	390
Sulphate (mg/L)	1840	15
Bicarbonate (mg/L)	730	-
Calcium (mg/L)	840	98
Magnesium (mg/L)	180	19
Sodium (mg/L)	41	5.5
Chloride (mg/L)	29.4	6.5
Potassium (mg/L)	7	3
Fluoride (mg/L)	2	0.2
Bromide (mg/L)	0.2	-
Carbon dioxide (cc/L)	537	-
Strontium (mg/L)	0.1	-
Iron (µg/L)	0.8	5
Manganese (µg/L)	-	0.3
Nitrate (mg/L)	-	3.8

TW: Thermal water; CTRL: Control.

**Table 2** Demographics and symptoms of the thermal water and the control group (*n* = 20)

	TW	CTRL	<i>P</i> value
Age (yr)	64.0 ± 1.4	61.2 ± 1.8	NS
Weight (kg)	64.4 ± 2.3	61.1 ± 1.5	NS
Height (cm)	161 ± 0.01	160 ± 0.01	NS
BMI (kg/m <sup>2</sup> )	24.9 ± 0.9	24.0 ± 0.6	NS
Diagnosis <i>n</i> (%)			
Dyspepsia only	12 (60)	12 (60)	NS
Constipation only	6 (30)	5 (25)	NS
Constipation + dyspepsia	2 (10)	3 (15)	NS

BMI: Body mass index; TW: Thermal water; CTRL: Control; NS: Not significant.

method. LDL cholesterol was calculated according to the Friedewald Formula<sup>[32]</sup>.

BA standards were obtained from Sigma Aldrich (St. Louis, United States). Total serum BA concentration was determined enzymatically by the 3 $\alpha$ -hydroxysteroid-dehydrogenase assay (Stereognost 3a, Pho, Nycomed, AS, Torsoy, Norway). The qualitative and quantitative BA composition was assessed by an HPLC-electrospray-mass spectrometry method, as previously reported with a slight modification<sup>[33]</sup>. Isolute C18 cartridges were obtained from International Sorbent Technology LTD (Hengoed, United Kingdom). The solid phase extraction cartridge was conditioned with 5 mL of methyl alcohol and 5 mL of water prior to the sample loading. Serum samples were diluted 1:6 (v/v) with 0.1 N solution of NaOH and heated to 64 °C for 30 min. Afterwards, the serum sample was loaded on the conditioned cartridge and then washed with 10 mL of water. The cartridge was then eluted with 5 mL of methyl alcohol. The eluate was dried under vacuum and then reconstituted with the mobile phase (70:30 v/v ammonium acetate buffer/acetonitrile) and injected into the HPLC-electrospray-mass spectrometry instru-

ment. The recovery of all BAs ranged from 80% to 96%. The chromatographic system consisted of a Waters Alliance 2695 HPLC system. The separation was obtained using a 150 mm × 2.00 mm, 4 µm Phenomenex Sinergy Hydro-RP C18 column with a mobile phase consisting of 15 mmol ammonium acetate buffer (pH 5)/acetonitrile. The mobile phase was delivered at a flow rate of 0.150 mL/min, with a total HPLC-electrospray-mass spectrometry run time of 30 min. Mass spectra were obtained with a Quattro LC mass spectrometer (Micromass, United Kingdom) equipped with electrospray source. All BA ions were monitored in a negative mode by the Multiple Reaction Monitoring mode. A seven point calibration curve, ranging from blank to 10 µmol was prepared by spiking BA-free serum with the analytes for serum analysis. Quantification of the analytes in the sample was performed on the peak area, by external calibration. The inter-assay precision and accuracy were determined by analyzing three calibration curves with quality control samples at one-concentration level (1 µmol) on 2 d. The value for the coefficient of variation (%) near the limit of detection was 1%-2%.

### Statistical analysis

Analysis of data was carried out using the “Statistical Package for Social Sciences (SPSS) for Windows (SPSS version 17.0, Chicago, IL, United States). Data are reported as mean ± SE. Intergroup differences between categorical variables were estimated by the  $\chi^2$  test. The non-parametric Kolmogorov-Smirnov test was used to verify the normal distribution of the continuous variables data set. When the data set was normally distributed, the Student *t* test for coupled or uncoupled data was used as appropriate. When the data set was not normally distributed, the variables were analyzed by the Mann-Whitney *U*-test and by the Wilcoxon test as appropriate. A significant level of 0.05 (*P* < 0.05) was chosen to assess the statistical significance.

## RESULTS

### Patient characteristics at enrollment

No intergroup difference was found in terms of age, weight, height, body mass index (BMI) and diagnosis (Table 2). Eight patients in each group had constipation either alone or associated with dyspepsia.

### Blood cholesterol, triglycerides, oxysterols and vitamin E

As shown in Table 3, we did not find any intergroup (TW vs CTRL) difference in serum levels of total, HDL and LDL cholesterol and triglycerides. Plasma 7- $\beta$ -hydroxycholesterol, 7-ketocholesterol,  $\alpha$ -tocopherol,  $\gamma$ -tocopherol and oxysterol to tocopherol ratio, both at baseline and at the end of the study, did not differ between the TW and the CTRL group. In addition, no change in blood lipids or oxidative stress was found at the end of treatment with respect to baseline when each group was considered separately.

Table 3 Serum lipids and plasma markers of oxidative stress in the thermal water and in the control groups

	TW		CTRL	
	Baseline	End	Baseline	End
Total cholesterol (mg/dL)	178.7 ± 5.8	182.4 ± 6.3	181.5 ± 7.6	177.4 ± 6.5
HDL cholesterol (mg/dL)	62.3 ± 4.7	63.7 ± 4.7	56.7 ± 5.0	59.4 ± 6.1
LDL cholesterol (mg/dL)	100.4 ± 8.0	101.9 ± 8.7	103.6 ± 8.5	93.9 ± 7.7
Triglycerides (mg/dL)	79.9 ± 7.5	84.0 ± 10.2	106.1 ± 11.4	120.7 ± 17.9
7β-HC (ng/mL)	51.4 ± 12.3	70.5 ± 15.1	57.0 ± 8.2	45.5 ± 8.9
7-KC (ng/mL)	93.7 ± 32.5	103.8 ± 29.1	38.6 ± 8.4	53.1 ± 25.9
7β-HC + 7-KC (ng/mL)	145.1 ± 44.7	174.3 ± 43.6	95.7 ± 16.2	98.6 ± 34.5
α-TCP (mg/dL)	1.46 ± 0.1	1.43 ± 0.1	1.40 ± 0.1	1.43 ± 0.1
γ-TCP (mg/dL)	0.74 ± 0.1	0.75 ± 0.1	0.67 ± 0.05	0.79 ± 0.1
α-TCP + γ-TCP (mg/dL)	2.20 ± 0.1	2.18 ± 0.1	2.07 ± 0.1	2.22 ± 0.1
7β-HC + 7-KC/α-TCP + γ-TCP	71.1 ± 22.6	65.7 ± 14.3	52.5 ± 9.2	54.1 ± 21.9

HDL: High-density lipoprotein; LDL: Low-density lipoprotein; 7β-HC: 7-beta-hydroxycholesterol; 7-KC: 7-ketocholesterol; α-TCP: Alpha-tocopherol; γ-TCP: Gamma-tocopherol; TW: Thermal water; CTRL: Control.

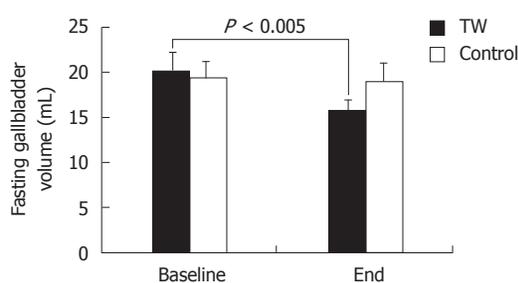


Figure 1 Fasting gallbladder volume at baseline and at the end of the study in the thermal water and in the control group. TW: Thermal water.

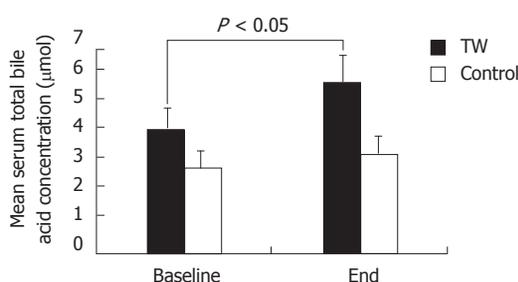


Figure 2 Mean serum total bile acid concentration at baseline and at the end of the study in the thermal water and in the control group. TW: Thermal water.

### Gallbladder volume and serum bile acids

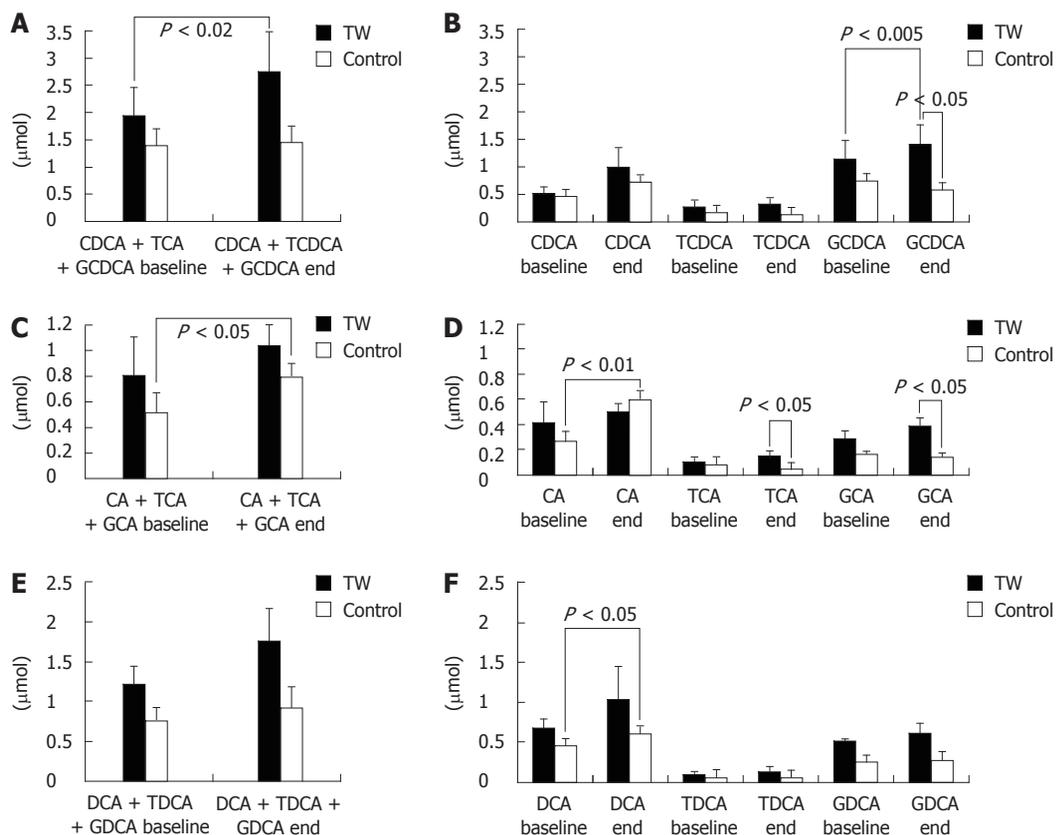
As shown in Figure 1, the mean fasting gallbladder volume did not differ between the TW and the CTRL group both at baseline and at the end of the study. Fasting gallbladder volume was significantly ( $P < 0.005$ ) smaller at the end of the study than at baseline in the TW ( $15.7 \pm 1.1$  mL *vs*  $20.1 \pm 1.7$  mL) but not in the CTRL group ( $19.0 \pm 1.4$  mL *vs*  $19.4 \pm 1.5$  mL).

As shown in Figure 2, although there was a trend for higher baseline values in the TW than in the CTRL group, the mean serum total BA concentration did not significantly differ between the TW and the CTRL group both at baseline and at the end of the study. Serum total BA concentration was significantly ( $P < 0.05$ ) higher at the end of the study than at baseline in the TW ( $5.83$

$\pm 1.24$   $\mu\text{mol}$  *vs*  $4.25 \pm 1.00$   $\mu\text{mol}$ ) but not in the CTRL group ( $3.41 \pm 0.46$   $\mu\text{mol}$  *vs*  $2.91 \pm 0.56$   $\mu\text{mol}$ ).

With regard to the serum BA molecular species, as shown in Figure 3, no intergroup difference was found at baseline. At the end of the study however, the TW as compared to the CTRL group had significantly ( $P < 0.05$ ) higher glycochenodeoxycholic acid (GCDCA) ( $1.41 \pm 0.35$   $\mu\text{mol}$  *vs*  $0.59 \pm 0.07$   $\mu\text{mol}$ , respectively), taurocholic acid (TCA) ( $0.15 \pm 0.04$   $\mu\text{mol}$  *vs*  $0.05 \pm 0.02$   $\mu\text{mol}$ , respectively) and glycocholic acid (GCA) ( $0.39 \pm 0.10$   $\mu\text{mol}$  *vs*  $0.14 \pm 0.02$   $\mu\text{mol}$ , respectively). No other intergroup difference was found at the end of the study.

When the BA molecular species serum concentrations were compared separately in each study group at the end of the study with regard to baseline, in the TW group the mean GCDCA concentration at the end of the study was significantly higher ( $P < 0.005$ ) than at baseline ( $1.41 \pm 0.85$   $\mu\text{mol}$  *vs*  $1.15 \pm 0.35$   $\mu\text{mol}$ ). On the contrary, in the CTRL group there was a trend ( $P = 0.062$ ) for a lower GCDCA concentration at the end with respect to baseline ( $0.59 \pm 0.07$   $\mu\text{mol}$  *vs*  $0.75 \pm 0.21$   $\mu\text{mol}$ ). The mean free cholic acid (CA) concentration was significantly ( $P < 0.01$ ) higher at the end of the study than at baseline in the CTRL ( $0.60 \pm 0.14$   $\mu\text{mol}$  *vs*  $0.27 \pm 0.09$   $\mu\text{mol}$ ) but not in the TW group ( $0.50 \pm 0.12$   $\mu\text{mol}$  *vs*  $0.42 \pm 0.18$   $\mu\text{mol}$ ). The mean free deoxycholic acid (DCA) concentration was significantly ( $P < 0.05$ ) higher at the end of the study than at baseline in the CTRL ( $0.60 \pm 0.16$   $\mu\text{mol}$  *vs*  $0.45 \pm 0.13$   $\mu\text{mol}$ ) but not in the TW group ( $1.03 \pm 0.44$   $\mu\text{mol}$  *vs*  $0.67 \pm 0.16$   $\mu\text{mol}$ ). The other BA molecular species did not change at the end as compared to baseline when each group was considered separately. The sum of free chenodeoxycholic acid (CDCA), GCDCA and taurochenodeoxycholic acid was significantly ( $P < 0.02$ ) higher at the end of the study than at baseline in the TW ( $2.75 \pm 0.70$   $\mu\text{mol}$  *vs*  $1.95 \pm 0.58$   $\mu\text{mol}$ ) but not in the CTRL group ( $1.46 \pm 0.20$   $\mu\text{mol}$  *vs*  $1.40 \pm 0.35$   $\mu\text{mol}$ ). The sum of CA, GCA and TCA was significantly ( $P < 0.05$ ) higher at the end of the study than at baseline in the CTRL ( $0.80 \pm 0.14$   $\mu\text{mol}$  *vs*  $0.52 \pm 0.11$   $\mu\text{mol}$ ) but not in the TW group ( $1.04 \pm 0.20$   $\mu\text{mol}$  *vs*  $0.81 \pm 0.27$   $\mu\text{mol}$ ).



**Figure 3** Mean serum bile acid molecular species concentration at baseline and at the end of the study in the thermal water and in the control group. A: Total chenodeoxycholic acid (CDCA); B: Free CDCA, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA); C: Total cholic acid (CA); D: Free CA, glycocholic acid (GCA) and taurocholic acid (TCA); E: Total deoxycholic acid (DCA); F: Free DCA, glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA). TW: Thermal water.

Table 4 Number of food portions consumed by the thermal water and by the control group subjects during the study period			
	TW	Control	P value
Pasta	16.40 ± 1.22	8.80 ± 1.05	< 0.001
Bread	4.32 ± 1.40	8.90 ± 1.57	0.01
Pizza	0.58 ± 0.23	1.01 ± 0.31	NS
Sweets	9.57 ± 1.87	7.08 ± 1.02	NS
Soft drinks	4.82 ± 1.40	4.30 ± 1.59	NS
Fruits	17.60 ± 1.69	14.59 ± 2.24	NS
Meat	11.52 ± 1.00	5.90 ± 0.51	< 0.001
Fish	4.05 ± 0.75	3.20 ± 0.38	NS
Milk	6.00 ± 1.21	6.89 ± 0.87	NS
Dairy products	6.32 ± 1.23	4.04 ± 0.50	NS
Legumes	0.98 ± 0.36	0.84 ± 0.23	NS
Vegetables	22.80 ± 2.40	13.51 ± 1.08	0.002
Coffee	14.08 ± 2.31	13.32 ± 2.41	NS

TW: Thermal water.

**Meal consumption, bowel movements and body weight**

As shown in Table 4, the number of pasta ( $P < 0.001$ ), meat ( $P < 0.001$ ) and vegetable ( $P < 0.005$ ) portions consumed during the study period was significantly higher by approximately two-fold in the TW than in the CTRL group, while bread consumption was significantly ( $P < 0.05$ ) less frequent, being half the amount in the TW than in the CTRL group. No intergroup difference was found with regard to pizza, dessert, soft drink, fruit, fish, milk,

dairy product, legume and coffee espresso consumption.

During the study period, the TW group had a significantly ( $P < 0.05$ ) higher number of bowel movements per day than the CTRL group ( $1.077 \pm 0.057$  vs  $0.893 \pm 0.055$ , respectively). Body weight did not differ between the TW and the CTRL group both at baseline ( $64.4 \pm 2.4$  kg vs  $61.1 \pm 1.5$  kg, respectively) and at the end of the study ( $64.3 \pm 2.4$  kg vs  $61.3 \pm 1.4$  kg, respectively). In addition, no change in body weight was found at the end of the study with respect to baseline when each group was considered separately.

**DISCUSSION**

The main finding of the present study is that 12 d of sulphate-bicarbonate-calcium TW, but not tap water, administration to gallstone-free postmenopausal women with functional dyspepsia and/or constipation is associated with a reduction of fasting gallbladder volume and an increase in fasting serum BA concentration, especially GCDCA. The effects of sulphate-bicarbonate-calcium TW administration on fasting gallbladder volume and serum BA that we found can be considered protective from gallstone development. In fact, a relatively high fasting gallbladder volume, indicative of a gallbladder motility defect, has been shown to be associated with gallstones. Conversely, a beneficial effect of preserved gallbladder

motility on gallstone recurrence has been demonstrated after extracorporeal shock-wave lithotripsy<sup>[14,34-36]</sup>.

Since CDCA molar percent in serum has been shown to correlate with that in gallbladder bile, the increased concentration of serum GCDCA (the major form of CDCA in humans) that we found after sulphate-bicarbonate-calcium TW administration is likely to reflect bile enrichment with this BA<sup>[37,38]</sup>. Although a direct measurement of the qualitative and quantitative BA composition in bile is the best predictor of gallstone risk, our findings in serum suggest that sulphate-bicarbonate-calcium TW administration can be considered protective from gallstone development. In fact, it has been shown that cholesterol gallstone patients have a lower CDCA and a higher DCA content in gallbladder bile than gallstone-free controls<sup>[39]</sup>. In addition, pharmacological CDCA administration has been used as a litholytic/preventive treatment against gallstones and ameliorates cholesterol solubility in BA<sup>[40]</sup>.

The beneficial effect of sulphate-bicarbonate-calcium TW administration on gallbladder motility has been already demonstrated, but the underlying mechanisms are not clear<sup>[41]</sup>. The effect of sulphate-bicarbonate-calcium TW consumption on the BA pool has never been shown previously and our present data do not allow the clarification of the mechanisms. In fact, limitations of the present study are the lack of measurements of intestinal transit time and of BA hepatic synthesis and fecal losses. However, as indirectly suggested by the higher frequency of bowel movements that we found in the TW than in the CTRL group, it can be hypothesized that TW consumption accelerates intestinal transit. This change in intestinal transit is in agreement with the increased fecal scour score described in pigs ingesting a high mineral sulphated water<sup>[42]</sup> and should be secondary to an osmotic mechanism, although the warm temperature of the TW could also play a role<sup>[41]</sup>. The acceleration of intestinal transit, as well as the improved gallbladder motility, are likely to increase the frequency of BA enterohepatic circulation and fecal losses with a secondary stimulation of primary BA (especially CDCA) hepatic synthesis. The increased frequency of BA enterohepatic circulation and the enrichment of the BA pool with CDCA could then further accelerate colonic transit. The latter hypothesis is in agreement with previously published data showing: (1) a positive correlation between the rate of BA synthesis and colonic transit (the higher the synthesis the faster colonic transit)<sup>[43]</sup>; (2) a positive correlation between serum CDCA and intestinal transit (the higher the concentration the faster intestinal transit)<sup>[14]</sup>; and (3) that CDCA administration accelerates colonic transit in healthy volunteers and in female patients with constipation-predominant irritable bowel syndrome<sup>[43,44]</sup>. The second finding of the present study is that body weight and blood total, HDL and LDL cholesterol, triglycerides, oxysterols and vitamin E were not affected by 12 d of either sulphate-bicarbonate-calcium or tap water consumption. Interestingly, we found that the TW, as compared to the CTRL

group, showed a doubling of frequency of pasta, meat and vegetable consumption during the study period suggesting that drinking sulphate-bicarbonate-calcium TW allows maintenance of stable body weight and blood cardiovascular risk factors under conditions of overfeeding. Again, our present study does not allow clarification of the mechanisms for this unexpected finding. In fact, other than the lack of characterization of BA enterohepatic circulation, we did not assess gastric emptying and energy expenditure. However, the increased food intake in our TW group could be explained by an increased gastrointestinal emptying and more frequent BA enterohepatic circulation, with GCDCA enrichment. In agreement with this hypothesis, both the ingestion of a high mineral sulphated water in pigs<sup>[42]</sup> and the administration of CDCA in humans have been shown to increase food consumption. Furthermore, the increased serum BA concentration that we found during sulphate-bicarbonate-calcium TW consumption could directly avert weight gain, despite increased food consumption. In fact, serum BAs have been recognized as important modulators of whole-body metabolism, by increasing energy expenditure in brown adipose tissue and in muscles, through promotion of intracellular thyroid hormone activation secondary to the activation of the TGR5-signaling pathway<sup>[21]</sup>.

In conclusion, sulphate-bicarbonate-calcium TW consumption in postmenopausal women with functional dyspepsia and/or constipation has a positive effect on the lithogenic risk and intestinal transit and allows maintenance of a stable body weight despite a high food intake. Further studies are needed to confirm these effects of TW in asymptomatic subjects and to prove its potential benefit in weight loss treatments.

## COMMENTS

### Background

Atherosclerosis, gallstones and obesity are very frequent and interrelated diseases, with a very high socioeconomic impact worldwide. High triglycerides, total/low-density lipoprotein cholesterol and increased oxidative stress in blood are important risk factors for atherosclerosis and cardiovascular diseases. In addition to cholesterol and triglyceride metabolism, bile acid (BA) metabolism, gallbladder motility and intestinal motility are critical factors in the pathogenesis of gallstones. Obesity and overweight are risk factors for both atherosclerosis and gallstone disease.

### Research frontiers

Thermal water (TW), and especially sulphate-bicarbonate mineral waters, are used to treat several biliary and digestive tract diseases. In the present study, The authors investigated the effect of drinking sulphate-bicarbonate-calcium TW on risk factors for: (1) atherosclerosis (i.e., cholesterol, triglycerides and markers of oxidative stress in blood); (2) gallstones (i.e., BAs in blood, gallbladder and intestinal motility); and (3) diet and body weight.

### Innovations and breakthroughs

TW drinking has been shown to ameliorate intestinal and gallbladder motility and blood cholesterol and oxidative stress markers. However, in previous studies oxidative stress was evaluated by using methods with poor physiological significance *in vivo*. No data are available on the effect of TW on BA metabolism and body weight. In the present study, for the first time we investigated the effect of drinking sulphate-bicarbonate-calcium TW on BA metabolism and body weight. In addition, the authors evaluated the effect of drinking sulphate-bicarbonate-calcium TW on oxidative stress by measuring sensitive and specific markers of enhanced oxidant stress *in vivo*, such as oxysterols, or antioxidant defense by measuring  $\alpha$ -tocopherol.

### Applications

The results suggest that sulphate-bicarbonate-calcium water consumption has a positive effect on the risk of gallstone development and allows maintenance of a stable atherosclerosis risk and body weight despite a high food intake. This study might be useful in preparation of preventive strategies for atherosclerosis and gallstones in overweight and obese subjects.

### Terminology

Oxysterols are oxidation products of cholesterol, and among them 7- $\beta$ -hydroxycholesterol and 7-ketocholesterol are produced nonenzymatically via a free radical-mediated mechanism and, thus, are very good markers of oxidant stress *in vivo*. BAs are steroid acids found predominantly in the bile and, in lower concentrations, in serum of mammals. Besides their well-established roles in lipid absorption and homeostasis and cholesterol biliary solubilization, BAs also act as metabolically active signaling molecules.

### Peer review

The study is of particular interest to those involved in practical medicine. The authors' data might be used for the prevention of atherosclerosis development and gallstone disease in postmenopausal women and probably for the treatment of these diseases.

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## Duodenal stenting for malignant gastric outlet obstruction: Prospective study

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### Abstract

**AIM:** To evaluate the results of duodenal stenting for palliation of gastroduodenal malignant obstruction by using a gastric outlet obstruction score (GOOS).

**METHODS:** A prospective, non-randomized study was performed at a tertiary center between August 2005 and April 2010. Patients were eligible if they had malignant gastric outlet obstruction (GOO) and were not candidates for surgical treatment. Medical history and patient demographics were collected at baseline. Scheduled interviews were made on the day of the procedure and 15, 30, 90 and 180 d later or unscheduled as necessary.

**RESULTS:** Fifteen patients (6 male, 9 female; median age 61 years) with GOO who had undergone duodenal stenting were evaluated. Ten patients had metastasis

at baseline (66.6%) and 14 were unable to accept oral intake (93.33%), including 7 patients who were using a feeding tube. Laboratory data showed biliary obstruction in eight cases (53.33%); all were submitted to biliary drainage. Two patients developed obstructive symptoms due to tumor ingrowth after 30 d and another due to tumor overgrowth after 180 d. Two cases of stent migration occurred. A good response to treatment was observed, with a mean time of approximately 1 d (19 h) until toleration of a liquid diet and slightly more than 2 d for both soft solids (51 h) and a solid food/normal diet (55 h). The mean time to first failure to maintain liquid intake (GOOS  $\geq$  1) was 93 d. During follow-up, the mean time to first failure to maintain the previously achieved GOOS of 2-3 (solid/semi-solid food), considered technical failure, was 71 d. On the basis of oral intake a GOOS is defined: 0 for no oral intake; 1 for liquids only; 2 for soft solids only; 3 for low-residue or full diet.

**CONCLUSION:** Enteral stenting to alleviate gastroduodenal malignant obstruction improves quality of life in patients with limited life expectancy, which can be evaluated by using a GOO scoring system.

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**Key words:** Enteral stenting; Gastric outlet obstruction scoring system; Gastroduodenal malignancy; Self-expandable metal stent

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## INTRODUCTION

Gastroduodenal strictures may be caused by malignant diseases of the stomach, pancreas and duodenum or by the mass effect of lymphonodal metastasis. Curative resections may not be possible in about 40% of such gastric lesions and in up to 80%-95% of pancreatic lesions, which makes clear the need to develop alternative means to achieve palliation and thus a better quality of life<sup>[1-3]</sup>.

The use of self-expandable metal stents (SEMS) for treatment of gastroduodenal malignancy as a surgical alternative for palliation in patients with high morbidity and limited life expectancy is a feasible, safe and effective method<sup>[4-7]</sup>.

In this study, we aimed to analyze the usefulness of a gastric outlet obstruction score to assess results of duodenal stenting for palliation of gastroduodenal malignant obstruction.

## MATERIALS AND METHODS

A prospective, non-randomized study was performed at a tertiary care center between August 2005 and April 2010. Patients over 18 years of age were eligible if they had gastric outlet obstruction (GOO) and were not candidates for surgical treatment due to high morbidity of the procedure, refusal or poor nutritional status. The main exclusion criteria were multiple lesions with enteral stenosis, suspected intestinal ischemia, impossibility of passing a guide-wire, gastric cancer presenting as *limitis plastica*, and contraindication to gastrointestinal endoscopy. All participants signed an informed consent approved by a review board.

Medical history and patient demographics were collected at baseline, and scheduled interviews were made on the day of the procedure and 15 d, 30 d, 90 d and 180 d later or unscheduled as necessary (Table 1). Patients were withdrawn from the study at the end of 180 d or if death occurred before that time.

The stent used was an uncovered SEMS with a 27 mm diameter (22 mm at the mid-body) and length of 60, 90 or 120 mm preloaded in a 10 Fr delivery system (Wallflex, Boston Scientific Corporation, MA, United States) (Figure 1). All of the procedures were conducted under sedation or general anesthesia, under radiologic guidance with iodine contrast.

## RESULTS

Fifteen patients (6 male, 9 female; median age 61 years) were submitted to duodenal stenting. The procedure was carried out under sedation in six cases, and under general anesthesia in nine patients due to poor clinical status. Most patients had metastasis at baseline (66.60%) and no acceptance of oral intake (93.33%), including seven patients who were using a feeding tube. Three patients had a gastric outlet obstruction score (GOOS)  $\geq 1$  (Figure 2).

Laboratory data showed biliary obstruction in 8 cases (53.33%), all of whom were submitted to biliary drainage (50.00% endoscopic and 50.00% surgical).

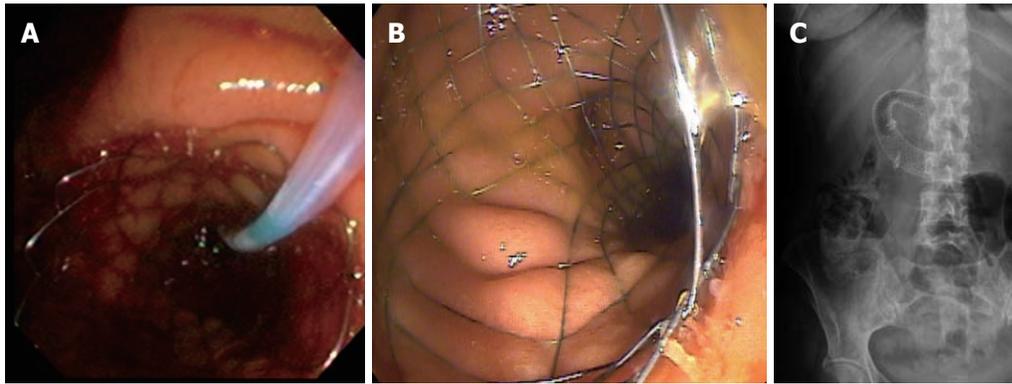
Table 1 Baseline demographic and clinical features of patients

Characteristic	Data (average)
Age (yr)	61.33
Gender	Male (6)/female (9)
Weight (kg)	55.93
Height (m)	1.61
Weight loss (kg)/(mo)	14.53/5.40
Previous chemotherapy/ radiation therapy	Yes (5)/no (10)
Previous biliary drainage	Yes (8)/no (7)
Only local cancer	Yes (5)/no (10)
Malignancy	
Pancreatic adenocarcinoma	9
Gastric adenocarcinoma	3
Cholangiocarcinoma	1
Metastatic disease	2
Location of the obstruction	
Stomach	3
Bulb	7
Second and third portion	5
Tumor extension (cm)	4.93
Biliary stenosis	Bismuth I (2)/bismuth II and III (6)

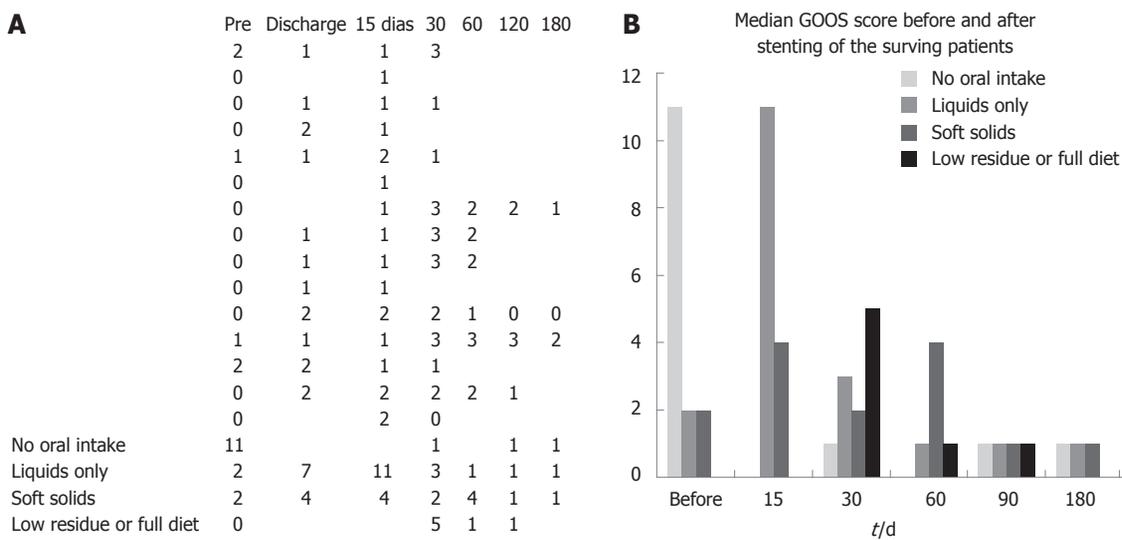
One patient developed obstructive symptoms after 1 mo of stent placement due to tumor ingrowth, which was treated by placing another stent inside the original one, with no recurrence of obstruction. Another patient developed obstructive symptoms after 6 mo due to tumor overgrowth, but it was not possible either to remove the stent or to bridge it because of bleeding and friability. This patient died 9 mo after stent placement.

There were two cases of stent migration. In the first case the patient had a duodenal metastasis from colonic cancer and presented obstructive symptoms after 8 d of stent placement. Two new stents were placed inside the original one, but the patient died 9 d later. The second patient was 53 years old with gastric cancer, liver metastasis and poor nutritional status who developed signs of obstruction within 3 mo after the procedure. Surgical removal of the migrated stent was done without complications and a surgical bypass was performed owing to better nutritional status at that time. One patient was submitted to removal of a foreign body, without complications.

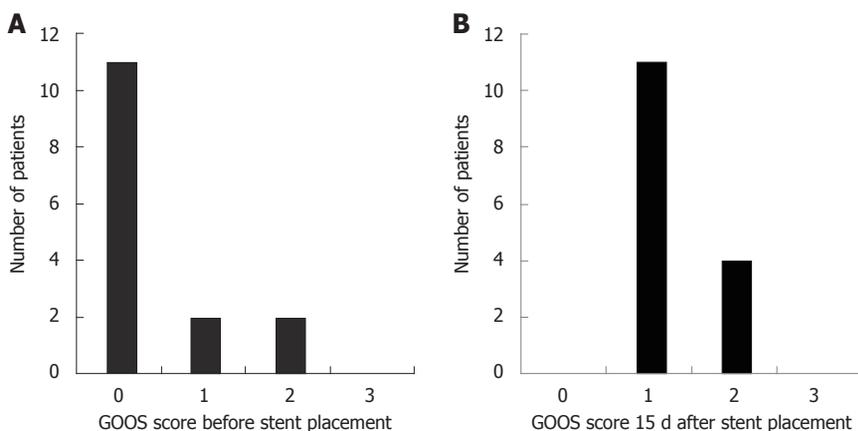
Regarding tolerance to oral diet, a good response to the treatment was observed, with a mean time of approximately 1 d (19 h) till toleration of a liquid diet and slightly more than 2 d for soft solids (51 h) and solid food/normal diet (55 h). These data differ from those of other studies, which showed a faster acceptance of solid food after the procedure, with return to solid food on the same day of the procedure in up to 52% of the cases<sup>[8]</sup>. During the present study, the prescriptions were made by the patients' physicians, mostly surgeons who prescribed liquids on the first day and, if they had good acceptance, prescribed solid food on the second day. The first failure to maintain liquid intake (GOOS  $\geq 1$ ) occurred at a mean of 3.1 mo. During follow-up, the first failure to maintain the previously achieved GOOS of 2-3 (solid/semi-solid food), considered technical failure, occurred at a mean of 2.35 mo (Figure 3).



**Figure 1** Use of self-expandable metal stents in gastroduodenal malignant disease. A and B: Endoscopic view of deployed self-expandable metal stents (SEMS); C: Radiologic view of duodenal SEMS.



**Figure 2** Bar graph showing median gastric outlet obstruction scores before the procedure and after 30 d, 60 d, 90 d and 180 d of stenting of the surviving patients.



**Figure 3** Bar graphs showing the gastric outlet obstruction scores. A: Before stent placement; B: 15 d after stent placement.

## DISCUSSION

Patients with gastroduodenal malignancies usually have limited prognosis with low life expectancy and also a poor quality of life due to inability to swallow at least

semi-solid food. Consequently, there is a high incidence of poor nutritional status and dehydration, which reduce the resources that can be used to palliate this scenario<sup>[9,10]</sup>. Surgical treatment has better results on long-term follow-up but it cannot be offered, initially, to patients with poor

clinical status because of increased morbidity and mortality. Based on the fact that less than 40% of patients who require palliative care are fit to undergo a surgical procedure, the need to achieve this objective with a less invasive, safer and effective method has been made clear<sup>[1,8,11]</sup>. The application of stents to the gastrointestinal tract has addressed this need, with attendant advantages of the surgical procedure although with a lesser long-term patency than desirable, mostly because of growth of tumoral tissue through the mesh (tumor ingrowth) or over the stent (tumor overgrowth) leading to recurrence of obstructive symptoms<sup>[7,12,13]</sup>.

In studies of malignancies it is important to assess each patient's quality of life and performance status, and several scales are used for this purpose such as the World Health Organization performance status (WHO status), the standard Short Form-36 questionnaire and the European Organization for Research and Treatment of Cancer scale. In GOO malignancies, however, the ability to ingest food seems to be the most important factor analyzed in terms of quality of life status, with the GOOS system being used most frequently for assessment<sup>[14]</sup>. A retrospective multicenter study enrolling 62 patients and using GOOS to evaluate the clinical success of enteral stenting, stated that all patients had resumed oral intake, although in 14.5% ( $n = 9$ ) there was no improvement in the score. Some patients had a maximum score prior to stenting, and in all of them relief of symptoms was observed<sup>[15]</sup>.

In a recent prospective study of 101 patients with incurable malignancies of the gastric outlet, three independent predictors of survival were identified: the ability to maintain self-care (WHO status), pain score, and the use of morphinomimetics. A 30-d survival rate of below 10% was found for patients who had all three prognostic indicators (WHO status of 3-4, pain score over 83 at baseline, the use of morphinomimetics stronger than tramadol), suggesting that a less invasive treatment should be considered for this group<sup>[1,15]</sup>.

Analysis of each individual's clinical status, associated diseases, and independent predictors of survival may provide objective data in helping to decide between surgical or endoscopic palliation. Patients with better prognosis and greater life expectancy should obtain more benefit from surgical treatment due to higher long-term patency rates, and patients with shorter life expectancy might benefit more from endoscopic treatment, enjoying a better quality of life, a quick return to oral diet, and less morbidity<sup>[16-18]</sup>.

The cost of gastrojejunostomy (GJJ) *vs* stent placement in GOO was compared in a recent randomized trial that considered both direct and indirect costs of the 2 treatments. The study concluded that, although GJJ had a higher total cost, largely due to longer hospital stays, the difference was small and of lesser importance when deciding on the kind of treatment<sup>[17]</sup>.

The choice of stent is also very important for achieving lower complication rates and higher patency leading

to better quality of life. Plastic stents are associated with higher migration (self-expandable plastic stents) and perforation (non-expandable plastic stents) than SEMs, which are used more often<sup>[19-21]</sup>. Metallic stents may be covered by a membrane made of various plastic materials (covered SEMs) which provide greater resistance to tumor ingrowth but they may lose functionality because of higher migration rates in the colon. Uncovered SEMs have lower migration rates because they are anchored by the tumor, but are associated with higher recurrence of symptoms due to tumor ingrowth; nevertheless, they are used more often than covered metallic stents in colon and gastroduodenal malignant obstruction because overall they bring better results<sup>[18,21-25]</sup>.

A recent randomized prospective study comparing the use of covered SEMs *vs* uncovered SEMs and enrolling 40 patients with gastric cancer in each group, demonstrated a higher stent migration rate within 8 weeks of stent placement in the covered SEMs group (25.8%) than in the uncovered SEMs group (2.8%). At the same time, the restenosis rate related to tumor ingrowth was higher in the uncovered SEMs group (25.0%) than in the covered SEMs (0.0%). In that study a routine endoscopy was performed independent of obstruction symptoms, which could explain the higher migration rates<sup>[25]</sup>.

The evaluation of obstructive biliary signs is crucial in patients with gastroduodenal malignant obstruction because there is an association between them in over 61% of cases<sup>[22,25]</sup>. When jaundice is present it is important to perform imaging exams to determine its cause and help in its characterization, such as excluding other causes like liver failure due to metastasis. Obstructive jaundice may be successfully treated by endoscopic procedures, interventional radiology or surgery with comparable results but with higher morbidity and longer hospitalization periods in the case of surgery. Treatment decision should be made based on the clinical status and tumor staging. Biliary endoscopic drainage can be accomplished with the use of plastic or metallic stents, the latter having lower rates of cholangitis and occlusion and shorter hospitalization stays but being more expensive. Both stents are equally effective in maintaining patency during the first 3 mo. If the patient has a short life expectancy, then plastic stents can be used safely and effectively at a lower cost, but they must be changed every 3 to 6 mo or in suspected cases of cholangitis<sup>[22,25-27]</sup>.

In conclusion, gastroduodenal malignancies are associated with gastric outlet obstruction symptoms and consequently with poor quality of life due to incapacity to swallow solid food, intractable nausea and vomiting, post-prandial epigastric tenderness and pain, and usually presenting with poor nutritional and clinical status that limit the options for treatment<sup>[18-20]</sup>. It has been shown that the use of SEMs for gastroduodenal malignancies is a feasible, safe and effective method, especially in those patients with limited life expectancy or in more critical conditions, allowing improvement not only in nutritional status but also in quality of life. SEMs placement may

serve as a bridge to definitive surgical treatment in high-risk patients<sup>[28,29]</sup>, as was conducted in one patient in the present study. We observed a quick return to an oral diet in our cohort after the procedure, and patency was estimated by the clinical efficacy to maintain an oral diet of solid/semi-solid food (GOOS  $\geq$  1), with a mean time to recurrence of obstructive symptoms of 3.1 mo.

The complications regarding the recurrence of symptoms observed in this study in two cases of stent migration, one case of tumor ingrowth and one of tumor overgrowth are similar to those reported in other publications and can be treated with a high success rate in most cases.

The association of GOO symptoms in gastroduodenal malignancies with biliary obstruction was shown in several published studies; therefore, we performed biliary stenting in eight of our patients (53.33%) prior to duodenal stenting. During the follow-up, three biliary stents were changed but there was no need to implant a new stent. In cases of biliary obstruction after duodenal stenting, biliary stents can be placed through the mesh of the duodenal SEMS to successfully palliate this condition.

Most published studies regarding endoscopic treatment of gastroduodenal malignancies have included only a limited number of patients, thus highlighting the need to perform more comparative studies between this method and surgery and to assess the costs involved.

## COMMENTS

### Background

Use of self-expandable metal stents in gastroduodenal malignancy as a surgical alternative for palliation in patients with high morbidity and limited life expectancy is a feasible, safe and effective method, allowing a quick return to oral intake and low morbidity.

### Research frontiers

Cost analyses comparing treatment of gastric outlet obstruction (GOO) malignancies by surgery or endoscopic procedures shows that any difference, considering direct and indirect costs, is small and should not have influence on patient's treatment.

### Innovations and breakthroughs

A prospective, non-randomized study was performed at a tertiary center between August 2005 and April 2010. Patients were eligible if they had GOO and were not candidates for surgical treatment. Medical history and patient demographics were collected at baseline. Scheduled interviews were made on the day of the procedure and 15, 30, 90 and 180 d later or unscheduled as necessary.

### Applications

Assessment of patient's quality of life and performance status is crucial to provide more accurate information on whether the treatment being offered to the patient is satisfactory. Gastric outlet obstruction score (GOOS) system is an important tool to achieve this goal when the ability to ingest food seems to be an important factor to patient's quality of life in GOO malignancies.

### Peer review

This is a good descriptive study in which authors analyze the results of duodenal stenting, by using a GOOS, for palliation of gastroduodenal malignant obstruction.

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## Original single-incision laparoscopic cholecystectomy for acute inflammation of the gallbladder

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### Abstract

**AIM:** To investigate the safety and feasibility of our original single-incision laparoscopic cholecystectomy (SILC) for acute inflamed gallbladder (AIG).

**METHODS:** One hundred and ten consecutive patients underwent original SILC for gallbladder disease without any selection criteria and 15 and 11 of these were diagnosed with acute cholecystitis and acute gallstone cholangitis, respectively. A retrospective review was performed not only between SILC for AIG and non-AIG, but also between SILC for AIG and traditional laparoscopic cholecystectomy (TLC) for AIG in the same period.

**RESULTS:** Comparison between SILC for AIG and non-AIG revealed that the operative time was longer in SILC for AIG (97.5 min vs 85.0 min,  $P = 0.03$ ). The open conversion rate (2/26 vs 2/84,  $P = 0.24$ ) and complication rate (1/26 vs 3/84,  $P = 1.00$ ) showed no differences, but a need for additional trocars was more frequent in SILC for AIG (5/24 vs 3/82,  $P = 0.01$ ). Comparison between SILC for AIG and TLC for AIG revealed no differences based on statistical analysis.

**CONCLUSION:** Our original SILC technique was ade-

quately safe and feasible for the treatment of acute cholecystitis and acute gallstone cholangitis.

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**Key words:** Single-incision laparoscopic cholecystectomy; Acute cholecystitis; Acute cholangitis

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Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i9/944.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i9.944>

### INTRODUCTION

Single-incision laparoscopic cholecystectomy (SILC) has recently gained popularity, just as laparoscopic cholecystectomy (LC) became popular in the early 1990s. Although LC was initially established as the treatment of choice for symptomatic cholelithiasis, LC for acute inflammation of the gallbladder (AIG), such as that caused by acute cholecystitis and gallstone cholangitis, was considered to be a contraindication. The complication rate for LC was believed to be higher than that for AIG. Ultimately, LC was accepted as a safe procedure for AIG, when it was performed by an expert in laparoscopic techniques<sup>[1]</sup>. As with LC, SILC for AIG is currently considered to be a contraindication because of its technical difficulty and infancy. SILC is developing, and there are a wide variety of operative techniques. The safety and feasibility of these operative techniques also varies; some are adequate for the treatment of AIG, but others are not. In the near

future, SILC will probably be considered an acceptable treatment and the standard operative technique for AIG, effectively eliminating inappropriate operative techniques.

Here, we report our experience with SILC for AIG and explore the safety and feasibility of our original SILC technique.

## MATERIALS AND METHODS

A total of 110 consecutive patients underwent SILC for gallbladder disease from July 2009 to November 2010, without any selection criteria. Of these 110 patients, 15 and 11 were diagnosed with acute cholecystitis and acute gallstone cholangitis, respectively. We performed both SILC and traditional laparoscopic cholecystectomy (TLC) during the same period. There were four staff surgeons in our department, each of whom operated on or supervised the patients, who came to or were referred to their own outpatient clinics. Two of the four staff surgeons performed our original SILC technique routinely, and the other two performed a traditional four-port technique. All SILC operations were performed by staff surgeons only. However, several TLC procedures were performed by young surgical residents under the supervision of a staff surgeon. A surgical resident was considered eligible for performing TLC only if he/she had 2-6 years of experience in general surgery. Staff surgeons performed TLC in cases with severe inflammation or dense adhesions and in cases in which malignancy was suspected. There was no predesigned patient selection bias between the patients in the SILC and TLC groups.

A diagnosis of acute cholecystitis and the presence of acute cholangitis were determined based on the Tokyo guidelines and criteria for acute cholecystitis and cholangitis, as follows. Patients exhibiting one of the local signs of inflammation, such as a Murphy's sign or a mass, or tenderness in the right upper quadrant, as well as one of the systemic signs of inflammation, such as fever or elevated C-reactive protein (CRP) level, were diagnosed as having acute cholecystitis. Patients in whom suspected clinical findings were confirmed by diagnostic imaging were also diagnosed with acute cholecystitis (Table 1)<sup>[2]</sup>. Patients were classified as grade I (mild), grade II (moderate), or grade III (severe), according to the severity grading of the Tokyo guidelines for acute cholecystitis (Table 1)<sup>[2]</sup>. Acute cholangitis was diagnosed if the clinical manifestations of Charcot's triad, namely, fever and/or chills, abdominal pain (right upper quadrant or epigastric), and jaundice, were present. When not all components of the triad were present, then a definite diagnosis could be made if laboratory and imaging data supported the evidence of inflammation, and biliary obstruction was revealed (Table 2)<sup>[3]</sup>. We diagnosed patients with acute cholangitis due to gallstones and/or debris with gallstone cholangitis. Acute cholangitis patients were also classified as grade I, II or III, according to the severity grading of the Tokyo guidelines for acute cholangitis (Table 2)<sup>[3]</sup>.

The general policy for acute cholecystitis in our department is delayed surgery following medical treatment,

**Table 1** Tokyo guideline diagnostic criteria and severity assessment of acute cholecystitis

Diagnosis criteria
A: Local signs of inflammation
Murphy's sign
Right upper quadrant mass/pain/tenderness
B: Systemic signs of inflammation
Fever
Elevated C-reactive protein
Elevated white blood cell count
C: Imaging findings
Sonographic Murphy sign
Thickened gallbladder wall
Enlarged gallbladder
Pericholecystic fluid collection
Sonolucent layer in the gallbladder wall
Definite diagnosis
One item in A and one in B are positive
C confirms the diagnosis when acute cholecystitis is suspected clinically <sup>1</sup>
Severity assessment
Mild (grade I)
Acute cholecystitis does not meet the criteria of severe (grade III) or moderate (grade II) acute cholecystitis or acute cholecystitis in a healthy patient with no organ dysfunction and mild inflammatory changes in the gallbladder, making cholecystectomy a safe and low risk operative procedure
Moderate (grade II)
Elevated WBC count (> 18 000/mm <sup>3</sup> )
Palpable tender mass in the right upper quadrant
Duration of complains > 72 h <sup>2</sup>
Marked local inflammation (biliary peritonitis, pericholecystic abscess, hepatic abscess, gangrenous cholecystitis, emphysematous cholecystitis)
Severe (grade III)
Acute cholecystitis associated with dysfunction of any one of the following organs/systems
Cardiovascular dysfunction (hypotension requiring treatment with dopamine $\geq$ 5 $\mu$ g/kg per minute, or any dose of dobutamine)
Neurological dysfunction (decreased level of consciousness)
Respiratory dysfunction (PaO <sub>2</sub> /FiO <sub>2</sub> ratio < 300)
Renal dysfunction (oliguria, creatinine > 2.0 mg/dL)
Hepatic dysfunction (PT-INR > 1.5)

<sup>1</sup>Acute hepatitis, other acute abdominal disease, and chronic cholecystitis should be excluded; <sup>2</sup>Laparoscopic surgery should be performed within 96 h of the onset of acute cholecystitis. WBC: White blood cell; PT-INR: Prothrombin time and international normalized ratio.

such as antibiotics or percutaneous cholecystotomy. The general policy for acute gallbladder cholangitis in our department is delayed surgery following medical treatment, with endoscopic stone extraction. The timing of surgery depends upon the extent of inflammation, and we typically perform LC after inflammation has decreased considerably.

The definition of AIG in this study was acute cholecystitis, excluding acalculous cholecystitis; acute cholangitis with gallbladder stones or/and debris; and choledocholithiasis. Even if the patient had concomitant gallstone pancreatitis, we defined the condition simply as acute gallstone cholangitis. We defined the operation for AIG as surgery that was performed within 4 mo of the primary acute inflammation.

We performed magnetic resonance cholangiopancrea-

**Table 2 Tokyo guideline diagnosis criteria and severity assessment of acute cholangitis**

Diagnosis criteria (suspected diagnosis and definite diagnosis)
Severity assessment
A: Clinical context and clinical manifestations
History of biliary disease
Fever and/or chills
Jaundice
Abdominal pain (right upper quadrant or upper abdominal)
B: Laboratory data
Evidence of inflammatory response <sup>1</sup>
Abnormal liver function tests <sup>2</sup>
C: Imaging findings
Biliary dilation, or evidence of etiology (stricture, stone, stent, <i>etc.</i> )
Two or more items in A
Charcot's triad (2 + 3 + 4)
Two or more items in A + both items in B + C
Severity assessment
Mild (grade I)
Acute cholangitis that responds to initial medical treatment <sup>3</sup>
Moderate (grade II)
Acute cholangitis that does not respond to initial medical treatment and is not accompanied by organ dysfunction
Severe (grade III)
Acute cholangitis that is associated with the onset of dysfunction at least in any one of the following organs/systems
Cardiovascular system; hypotension requiring dopamine $\geq$ 5 $\mu$ g/kg per minute, or any dose of dobutamine
Nervous system: disturbance of consciousness
Respiratory system: PaO <sub>2</sub> /FiO <sub>2</sub> ratio < 300
Kidney: serum creatinine > 2.0 mg/dL
Liver: PT-INR > 1.5
Hematological system: platelet count < 100 000/ $\mu$ L

Compromised patients, for example, elderly (> 75 years old) and patients with comorbidity, should be monitored closely. <sup>1</sup>Abnormal white blood cell count, increased serum C-reactive protein level, and other changes including inflammation; <sup>2</sup>increased serum alkaline phosphatase,  $\gamma$ -glutamyltransferase, aspartate aminotransferase and alanine aminotransferase levels; <sup>3</sup>general supportive care and antibiotics. PT-INR: Prothrombin time and international normalized ratio.

tography for all patients undergoing LC to gain preoperative information about the anatomy of the biliary tree and the presence of common bile duct stones. Perioperative patient care was identical between patients undergoing TLC and SILC.

A retrospective review of prospectively collected data was performed to investigate the safety and feasibility of SILC for AIG. We compared multiple variables, not only between SILC for AIG and SILC for non-AIG, but also between SILC for AIG and TLC for AIG during the same period. A comparison between SILC for AIG and SILC for non-AIG was performed to reveal the influence of AIG on SILC. In this analysis, operative findings, such as intra-abdominal adhesion and gallbladder thickening, were evaluated by the same hepatopancreatobiliary specialist. Additionally, the comparison between SILC for AIG and TLC for AIG was performed to reveal the influence of the operative method of LC on AIG. In the comparison between SILC for AIG and TLC for AIG, the maximum white blood cell (WBC) count and CRP level during the acute inflammatory phase were categorized as follows: WBC > 14 000/mm<sup>3</sup> or not and CRP level > 10 mg/dL

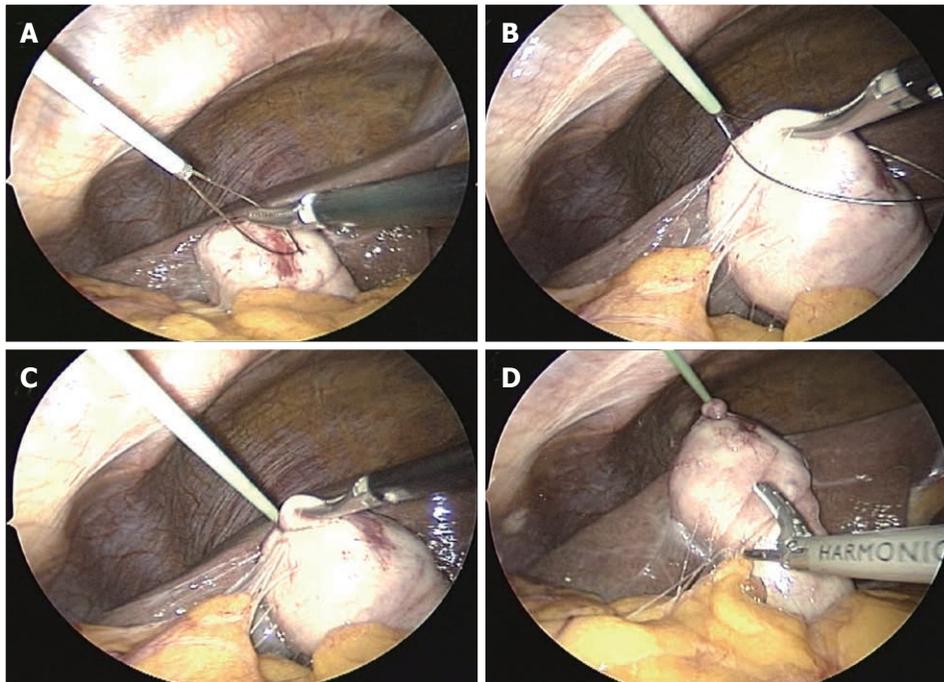
or not. These concrete cutoffs were determined to be indicators of severe inflammation according to the Japanese version of the Tokyo guidelines for acute cholecystitis and acute cholangitis<sup>[4]</sup>.

### Operative technique of our original single-incision laparoscopic cholecystectomy

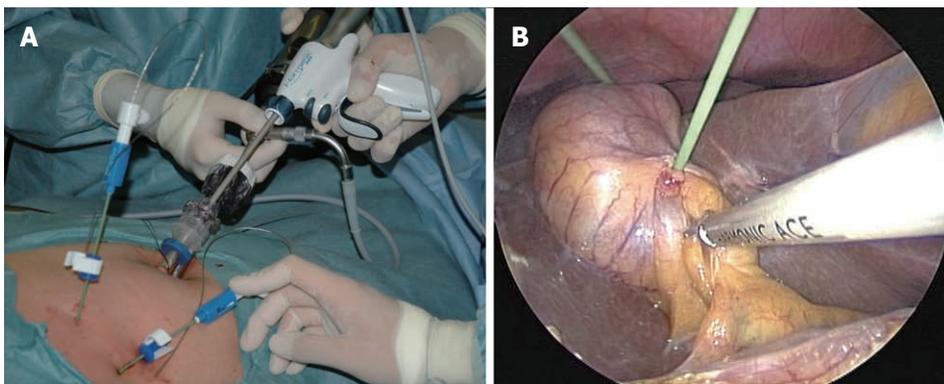
The operative technique and analysis of our original SILC technique have been described in another study<sup>[5]</sup>; here, we describe the procedure briefly as follows. The patients were placed in a low modified lithotomy position; the operator stood between the legs, the laparoscopist stood on the left side, and the second assistant stood on the right. A 10-20-mm skin incision was created by pulling out the umbilicus. After exposing the fascia, a 5-mm, 95-mm-long trocar was placed using an open approach. Pneumo-peritoneum was established, and another 5-mm, 70-mm-long trocar was placed through the same skin incision but through a separate fascial incision, which was created as far as possible above the first trocar. The first trocar was for the 30-degree laparoscope, and the second trocar was for the grasper and laparoscopic coagulating shears (LCSs). After inspection of adhesions and the gallbladder, a 2-mm wire loop retractor (WLR) (Mini Loop Retractor II, Covidien, Tokyo, Japan) was inserted from the right subcostal space, and the body or fundus of the gallbladder was retracted. The WLR was used as follows: (1) the grasper was inserted into the wire, and the tissue needing retraction was grasped; and (2) the wire was wrung, and retraction was performed (Figure 1). If the gallbladder was so distended that it could not be grasped, then bile was aspirated and decompressed using a 16-gauge needle for intraoperative cholangiography (IOC). Both the dissection of the adhesions and exposure of the infundibulum of the gallbladder were performed mainly by LCSs. The second WLR was then inserted obliquely above the first to retract the neck of the gallbladder; this WLR was used as the grasper for retraction in the lateral direction (Figure 2). After visualizing the so-called "critical view of safety," we performed routine IOC, using the catheter insertion technique. Closure of the cystic duct and dissection of the gallbladder from the liver bed were performed in the same manner as for TLC. The cystic duct was closed using a 5-mm laparoscopic clip. The gallbladder was extracted with a specimen bag through the umbilicus. The final appearances of the umbilical incision and the WLR insertion site at 3 mo after surgery were virtually scarless.

### Statistical analysis

All statistical analyses were performed with SPSS II for Windows software (SPSS, Chicago, IL, United States). Parametric summary statistics are presented as mean  $\pm$  SD, whereas nonparametric summary statistics are presented as medians with interquartile ranges. Categorical data were analyzed using the  $\chi^2$  test or Fisher's exact test, as appropriate. The two-sample *t* test was used to test the hypothesis of equality of means, and the Mann-Whitney *U* test was used to test the hypothesis of equality of medians. *P* < 0.05 was considered statistically significant.



**Figure 1** The way to grasp by wire loop retractor. A: Insert the grasper into the loop of wire; B: Grasp the tissue needed for retraction; C: Wring the wire; D: Retract the same as for the grasper in wire loop retractor.



**Figure 2** External and internal view of our original single-incision laparoscopic cholecystectomy. A: External view of the placement of trocars and wire loop retractors; B: Internal view of the original technique.

## RESULTS

A total of 110 patients underwent attempted SILC and 191 patients underwent attempted TLC during the same period. A total of 23.6% (26/110) of SILCs and 28.3% (54/191) of TLCs were diagnosed and operated on as AIG. The comparison of the patients' demographics and operative outcomes between SILC for AIG and SILC for non-AIG are shown in Table 3. Patients' demographics between SILC for AIG and SILC for non-AIG showed no significant differences without ASA scores. SILC for AIG patients included more patients with complicated backgrounds, but there was only one ASA III patient who had severe systemic disease.

In the operative outcomes, intra-abdominal adhesions and gallbladder wall thickening were more frequently seen in SILC for AIG. The operative time was significantly

longer in SILC for AIG (97.5 min *vs* 85 min,  $P = 0.03$ ). The open conversion rate (2/26 *vs* 2/84,  $P = 0.24$ ) and complication rate (1/26 *vs* 3/84,  $P = 1.00$ ) showed no significant differences, but a need for additional trocars was significantly more frequent in SILC for AIG (5/24 *vs* 3/82,  $P = 0.01$ ). There were two cases of open conversion in SILC for AIG. The first case involved gangrenous cholecystitis with a cholecystocholedochal fistula, which we noticed when we dissected the gallbladder from the liver bed, and we converted to an open procedure to repair the fistula. This case also suffered wound infection, which was the only operative complication with SILC for AIG. The second case involved dense adhesions in a patient with severe bronchial asthma; in this case, we converted to laparotomy to shorten the operative time. Additional trocars were required in five cases of SILC for AIG; three required an additional 5-mm trocar in the

**Table 3** Comparison of patients' demographics and operative outcome between dingle-incision laparoscopic cholecystectomy for acute inflamed gallbladder and single-incision laparoscopic cholecystectomy for non-acute inflamed gallbladder

Patient demographics	SILC for AIG	SILC for non-AIG	P value
<i>n</i>	26	84	
Age (yr) median (range)	61.5 (22-81)	56.5 (31-81)	0.06
Sex (male/female)	12/14	42/42	0.82
BMI median (range)	22.0 (18.4-29.4)	22.2 (16.0-30.0)	0.85
ASA score I / II / III	14/11/1	65/19/0	0.02
Previous upper abdominal surgery (yes/no)	2/24	4/80	0.63
Indication for operation	Acute cholecystitis 15 Acute gallstone cholangitis 11	Symptomatic gallstone 65 Cholelithiasis 2 No inflammation 17	
Operative outcome			
Operative time (min)			0.03
Median (range)	97.5 (60-163)	85 (45-195)	
mean (SD)	105.7 (31.9)	91.0 (29.3)	
Intra-abdominal adhesion	8/15/3	52/27/15	0.02
none to mild/moderate/severe			
Gallbladder wall thickening	16/2/8	66/14/4	< 0.01
none to mild/moderate/severe			
IOC completion <sup>1</sup>	23/24	81/82	0.4
Conversion to open cholecystectomy	2	2	0.24
Bile spillage	9	15	0.1
Use of additional port site	5	3	0.01
Complication (total)	1	3	1.00
Wound infection	1	2	
Bile duct injury	0	1	

<sup>1</sup>Excluded open converted cases. BMI: Body mass index; ASA: American Society of Anesthesiologists; SILC: Single-incision laparoscopic cholecystectomy; AIG: Acute inflamed gallbladder; IOC: Intraoperative cholangiography.

right subcostal space, one required an additional 10-mm trocar in the epigastrium to perform intraoperative ultrasonography, and one required two 5-mm trocars in the subcostal spaces due to stone dissemination.

The comparison of patients' demographics and operative outcomes between SILC for AIG and TLC for AIG is shown in Table 4. The two groups were similar with respect to sex, age, body mass index, indication for surgery, preoperative inflammation findings, severity assessment following Tokyo guidelines, and time between onset and operation. In the severity assessment, SILC for AIG included five moderate cases: three showed WBC counts > 18 000/mm<sup>3</sup>, one showed gangrenous cholecystitis, and one acute cholangitis case did not respond to initial medical treatment and required emergency endoscopic stone extraction. Furthermore, SILC for AIG included two severe cholecystitis cases; both cases showed remarkable inflammation findings (WBC > 22 000/mm<sup>3</sup> and CRP > 25 mg/dL), cardiovascular dysfunction, and neurologic dysfunction, and required biliary drainage. Of 54 TLCs for AIG, 39 were performed by surgical residents under the supervision of staff surgeons. However, all SILCs were performed by staff surgeons.

Even when the operative outcome did not reach statistical significance, the operative time of SILC for AIG was 10 min longer than that of TLC for AIG (97.5 min *vs* 87.5 min, *P* = 0.12). The open conversion rate (2/26 *vs* 5/54, *P* = 1.00) and complication rate (1/26 *vs* 7/54, *P* = 0.26) showed no significant differences. All open

conversions in TLC for AIG were performed for unclear anatomic relationships due to severe adhesions. Complications in TLC for AIG were as follows: postoperative hemorrhage in two, fluid collection in two, paralytic ileus in one, intra-abdominal abscess formation in one, and wound infection in one.

## DISCUSSION

At our institution, we performed SILC with very liberal selection criteria. We performed SILC without any contraindications, and we adopted SILC for AIG. The above findings clearly showed that neither acute cholecystitis nor acute gallstone cholangitis were contraindications for our original SILC technique.

LC for AIG was considered to be an absolute contraindication in the early laparoscopic era. The fear of an increased risk of complications, compared with open cholecystectomy, was unfounded based on the results of randomized controlled trials<sup>[6]</sup>. However, the conversion and complication rates of LC for AIG were greater than those of elective LC for other indications<sup>[7,8]</sup>. In the present study, the open conversion rate of SILC for AIG was 7.7% (2/26), which was a favorable result when compared with the results of TLC for AIG<sup>[6-11]</sup>. Open conversion itself is not a complication, but failure of the operative procedure is; surgeons are frequently obliged to convert due to uncertain anatomy, uncontrollable bleeding, and difficulty with manipulating swollen and thick-

**Table 4** Comparison of patient demographics and operative outcome between single-incision laparoscopic cholecystectomy for acute inflamed gallbladder and traditional laparoscopic cholecystectomy for acute inflamed gallbladder

	SILC for AIG	TLC for AIG	P value
Patient demographics			
<i>n</i>	26	54	
Age (yr) median (range)	61.5 (22-81)	61 (25-89)	0.94
Sex (male/female)	14/12	34/20	0.47
BMI median (range)	22.0 (18.4-29.4)	22.8 (15.4-32.0)	0.53
ASA score I / II / III	14/11/1	25/25/4	0.73
Previous upper abdominal surgery (yes/no)	2/24	5/49	0.59
Indication for operation	Acute cholecystitis 14 Acute gallstone cholangitis 11	Acute cholecystitis 29 Acute gallstone cholangitis 25	0.81
Max WBC count in acute phase			
WBC > 14 000	5	13	
WBC < 14 000	21	41	
Max CRP in acute phase			
CRP > 10	6	18	0.44
CRP < 10	20	36	
Severity assessment by Tokyo Guidelines Grade I / II / III	19/5/2	38/13/3	0.85
Day from onset to operation	19 (6-111)	20 (8-104)	0.82
Clinical result			
Operative time (min)			
Median (range)	97.5 (60-163)	87.5 (35-245)	
mean (SD)	105.7 (31.9)	94.7 (34.4)	
Surgeon	26/0	16/39	
Staff surgeon/surgical resident			
IOC completion <sup>1</sup>	23/24	42/49	0.26
Conversion to open cholecystectomy	2	5	1
Bile spillage	9	14	0.44
Complication	1	7	0.26

<sup>1</sup>Excluded open converted cases. ASA: American Society of Anesthesiologists; BMI: Body mass index; WBC: White blood cell; CRP: C-reactive protein; IOC: Intra-operative cholangiography; SILC: Single-incision laparoscopic cholecystectomy; TLC: Traditional laparoscopic cholecystectomy; AIG: Acute inflamed gallbladder.

ened gallbladders. The greater the open conversion rate is, the less safe the operative technique. Thus, our original SILC for AIG was proved to be sufficiently safe given the open conversion rate. The complication rate of our SILC for AIG was 3.8% (1/26), which was also more favorable than the reported complication rates of TLC for AIG<sup>[6-11]</sup>.

With regard to feasibility, even if we considered both open conversion and the requirement for additional trocars to be operative method failures, 73% (19/26) of AIG cases that fulfilled the Tokyo guidelines underwent virtually scarless operations. Considering that the reported open conversion rate in the initial experiences of TLC for AIG was 33.7%, and that it remained at 10%-25% after a decade of experience, our original SILC technique is sufficiently feasible for AIG<sup>[6-11]</sup>.

However, SILC for AIG required additional trocars significantly more frequently than SILC for non-AIG. Looking back over individual cases, we especially needed additional trocars in cases with thickened gallbladder walls. In the early cases, we were inexperienced in handling WLRs and could not grasp the thickened gallbladder walls; consequently, we required additional trocars to grasp and manipulate the inflamed gallbladders. After we gained experience and became familiar with using WLRs, we could grasp even severely thickened gallbladder walls. Of the first 12 cases, four required additional trocars, but only one of the next 12 cases required an additional trocar (excluding two open conversion cases). We are convin-

ced that, after the accumulation of another dozen cases, we will be able to perform SILC for AIG with a lower combined conversion rate (open conversion + requirement for additional trocars).

The analysis of SILC for AIG and TLC and AIG revealed no significant differences based on statistical comparison. However, all SILCs for AIG were performed by hepatopancreatobiliary specialists, and satisfactory operative results depended partly on the surgeons' experiences.

The sufficient safety and feasibility of SILC for AIG achieved in our study were derived from some unique characteristics of our original technique. First, we employed two WLRs, which were sufficient to accomplish retraction, even in severely thickened, inflamed gallbladder walls. Second, we inserted only two trocars into the umbilical incision, which resulted in good handling of the instruments and gallbladder manipulation, without interfering with the other instruments or the laparoscope. Third, almost all dissections were performed by LCS, which allowed us to operate easily in dense fibrosis and tissue with neovascularization secondary to inflammation. Fourth, inserting two WLRs from the subcostal margin and using LCS created a triangulation of devices that allowed us to manipulate the gallbladder, as we did in TLC. All of these characteristics allowed us to employ the same operative technique and anatomical knowledge as in TLC, and ultimately, we could perform SILC without selection criteria.

In this study, we adopted the Tokyo guidelines for the diagnosis and severity assessment of gallbladder inflammation. Many reports about TLC for AIG exist in the literature, but the diagnostic criteria and severity assessment were inconsistent among the studies. Employment of the guidelines, which are based on a systemic literature review and the consensus of experts, allowed us to compare each operative result. SILC is still developing, and it has not yet been standardized. Many original procedures exist, but some may not be suitable to perform in cases of AIG. Comparisons using complication and conversion rates under the same diagnostic criteria and severity assessments should become standards of the ideal operative technique.

There are some limitations to our study. First, we did not perform early operations for acute cholecystitis, even though several prospective, randomized, controlled studies comparing early and delayed LC have concluded that early LC is safe and decreases the length of hospital stay<sup>[12]</sup>. We prefer delayed elective surgery, not only for medical reasons but also for social reasons. In our experience, we struggled with difficult bleeding from inflamed tissue in the early phase of AIG; we also struggled with dense adhesions in the delayed phase of AIG. Meticulous dissection of fibrous tissue and sure exposure of Calot's triangle allowed us to operate safely for AIG, although the operative time was slightly longer. Regarding social reasons, our institution is a tertiary referral hospital with only four hepatopancreatobiliary specialists. Considering the availability of surgical staff, anesthesiologists, and operating rooms, we prefer to delay elective surgery unless a patient needs an emergency cholecystectomy. Similar to our institute, there has been a general reluctance to adopt this approach in the United Kingdom, despite increasing evidence supporting early cholecystectomy; currently, only 20% of surgeons perform cholecystectomies during acute cholecystitis<sup>[13]</sup>.

In this study, we showed sufficient operative results for the safety and feasibility of the operative technique, even though delayed surgery is generally considered technically difficult because of acute inflammation and subsequent fibrosis, dense adhesions, and neovascularization. We are convinced that our original SILC technique can be adapted to early operations for AIG if needed. The second limitation is that we evaluated only 15 cases of SILC for acute cholecystitis and 11 cases of SILC for acute gallbladder cholangitis. These numbers of cases were too small to conclude that our SILC is statistically safe and feasible, and we must continue to analyze cases. Third, the occupied percentages of acute gallstone cholangitis in AIG in this study were 42% in SILC and 46% in TLC, which were greater than the reported prevalence of acute gallstone cholangitis<sup>[14,15]</sup>. This finding may have been because our institution is a tertiary referral hospital and there were many referrals of acute gallstone cholangitis that required endoscopic stone extraction from other institutes.

In conclusion, the significant influence of AIG on SILC in this study was due to the longer operative time

and high rate of requirement for additional trocars. The open conversion rate of SILC for AIG was increased to a similar degree as that of TLC for AIG. In experienced hands, the influence of the operative method seemed to decrease, and SILC for AIG could be satisfactorily performed, comparable to TLC for AIG. Our original SILC technique was adequately safe and feasible for the treatment of AIG, with greater requirements for extra ports than non-AIG cases, and a slightly greater conversion rate. We are convinced that, in the near future, SILC will be one of the principal techniques for the management of AIG, just as TLC for AIG evolved from absolute contraindication to the first-choice standard treatment.

## COMMENTS

### Background

Single-incision laparoscopic cholecystectomy (SILC) has recently gained popularity, just as laparoscopic cholecystectomy (LC) became popular in the early 1990s. Although LC was initially established as the treatment of choice for symptomatic cholelithiasis, LC for acute inflammation of the gallbladder (AIG), such as that caused by acute cholecystitis and gallstone cholangitis, was considered to be a contraindication. The complication rate for LC was believed to be possibly higher than that of AIG. Ultimately, LC was accepted as a safe procedure for AIG, when it is performed by an expert at laparoscopic techniques. As with LC, SILC for AIG is currently considered to be a contraindication because of its technical difficulty and infancy.

### Research frontiers

SILC is developing, and there is a wide variety of operative techniques. There is also variety in the safety and feasibility of these operative techniques; some are adequate for the treatment of AIG, but some are not. In the near future, SILC will be considered an acceptable treatment and the standard operative technique for AIG, effectively eliminating inappropriate operative technique.

### Innovations and breakthroughs

The authors investigated the feasibility and safety of their original SILC technique for AIG. The original SILC technique was proven to be adequately safe and feasible for the treatment of AIG by statistical analysis. The sufficient safety and feasibility of SILC for AIG achieved was derived from some unique characteristics of the technique. First, the authors used two wire loop retractors, which were sufficient to accomplish retraction even in severely thickened, inflamed gallbladder walls. Second, they inserted only two trocars into the umbilical incision, which resulted in good handling of instruments and gallbladder manipulation, without interfering with other instruments or the laparoscope. Third, inserting two wire loop retractor from the subcostal margin and using laparoscopic coagulating shear maintained a triangulation of devices that allowed them to manipulate the gallbladder as they did with traditional laparoscopic cholecystectomy.

### Peer review

This is accepted for publication because this study represent a lot of experience of SILC for AIG.

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## Biliary reflux detection in anomalous union of the pancreatico-biliary duct patients

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### Abstract

**AIM:** To demonstrate the imaging findings of biliopancreatic and pancreatico-biliary reflux in patients with anomalous union of the pancreatico-biliary duct (AUPBD) on gadoxetic acid-enhanced functional magnetic resonance cholangiography (fMRC).

**METHODS:** This study included six consecutive patients (two men and four women; mean age 47.5 years) with AUPBD. All subjects underwent endoscopic retrograde cholangiopancreatography (ERCP); one subject also underwent bile sampling of the common bile duct (CBD) to measure the amylase level because his gadoxetic acid-enhanced fMRC images showed evidence of pancreatico-biliary reflux of pancreatic secretions. Of the five patients with choledochal cysts, four underwent pylorus-preserving pancreaticoduodenectomy.

**RESULTS:** The five cases of choledochal cysts were classified as Todani classification I. In three of the six patients with AUPBD, injected contrast media reached the distal CBD and pancreatic duct on delay images, suggesting biliopancreatic reflux. In two of these six patients, a band-like filling defect was noted in the CBD on pre-fatty meal images, which decreased in size on delayed post-fatty meal images, suggesting pancreatico-biliary reflux of pancreatic secretions, and the bile sampled from the CBD in one patient had an amylase level of 113 000 IU/L. In one of the six patients with AUPBD, contrast media did not reach the distal CBD due to multiple CBD stones.

**CONCLUSION:** Gadoxetic acid-enhanced fMRC successfully demonstrated biliopancreatic reflux of bile and pancreatico-biliary reflux of pancreatic secretions in patients with AUPBD with and without choledochal cysts.

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**Key words:** Bile reflux; Choledochal cyst; Endoscopic retrograde cholangio-pancreatography; Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid; Magnetic resonance imaging

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### INTRODUCTION

Functional hepatocytes uptake a maximum of 50% of

the intravenous (IV) dose of gadoxetic acid (gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid Primovist, Bayer Schering Pharma) administered. Gadoxetic acid is excreted into the bile ducts, allowing visualization of the bile ducts on hepatobiliary phase T1-weighted images. In patients with normal hepatic function, the hepatobiliary phase usually occurs within 20 min of gadoxetic acid administration<sup>[1-3]</sup>.

Hepatocyte-specific agents can be used in a wide range of hepatobiliary applications, and gadoxetic acid-enhanced T1-weighted magnetic resonance cholangiography (MRC) provides additional information to T2-weighted MRC<sup>[4,5]</sup>.

At our institution, we previously evaluated the time sequence of gadoxetic acid-enhanced MRC in 40 normal healthy subjects in 2009; the study was approved by the Korea Food and Drug Administration and our institutional review board. In this previous study, we performed gadoxetic acid-enhanced MRC 60 min after contrast administration and then another 30 min after a fatty meal. In all subjects, we found complete filling of the distal common bile duct (CBD) with contrast on 30-min delayed pre-prandial images, while 50- and 60-min delayed images showed better image quality of bile ducts than early images due to increasing signal-to-noise ratio of extrahepatic bile ducts and bile duct-to-liver contrast-to-noise ratio, and post-prandial images showed gall bladder contraction and more extension of contrast excretion into the small bowel. We found no evidence of contrast media in the pancreatic duct in any subjects (unpublished data). In light of these results, 50- and 60-min delay images after gadoxetic acid administration and 10-, 20- and 30-min delayed images after fatty meal oral uptake were included in the gadoxetic acid-enhanced MRC protocol when requested by the gastroenterologist majoring in pancreato-biliary disorders. In our experience, these images can help verify bile excretion and flow after gallbladder contraction. We were able to evaluate normal or pathological physiology of bile excretion of the liver and bile flow along the biliary tract. These experiences indicated that gadoxetic acid-enhanced functional magnetic resonance cholangiography (fMRC) could reveal the physiology of bile excretion in certain pathologic conditions, including biliopancreatic and pancreato-biliary reflux. In this study, we present six patients with anomalous union of the pancreato-biliary duct (AUPBD) who exhibited biliopancreatic bile reflux and pancreato-biliary pancreatic juice reflux on gadoxetic acid-enhanced fMRC.

The purpose of this study was to investigate the value of gadoxetic acid-enhanced fMRC in the evaluation of AUPBD, with emphasis on the detection of biliopancreatic bile reflux and pancreato-biliary pancreatic reflux.

## MATERIALS AND METHODS

### Patients

Our institutional review board approved this retrospective study and waived the requirement to obtain informed consent. AUPBD was diagnosed radiologically as a long

common channel (> 1.5 cm) or a perpendicular confluence of the CBD and the main pancreatic duct on T2-MRC images and endoscopic retrograde cholangiopancreatography (ERCP). Choledochal cysts were diagnosed when T2-MRC images and ERCP showed typical dilation of the CBD without an obstructive lesion<sup>[6-8]</sup>.

After reviewing 176 patients who underwent gadoxetic acid-enhanced fMRC at our institution for evaluation of biliary pathology due to increased serum bilirubin level, incidental detection of bile duct dilatation on other imaging studies, and right upper quadrant pain between March 2009 and May 2010, we enrolled 6 patients with AUPBD. Of these participants, five had a choledochal cyst. The study group consisted of four women and two men (mean age, 47.5 years; range, 35-64 years), all six of whom underwent ERCP.

We reviewed participant medical records for pertinent clinical features, including medical history, presenting symptoms, results of other imaging studies, and operative records. Of the five patients with both AUPBD and choledochal cysts, four underwent pylorus-preserving pancreaticoduodenectomy. We reviewed these pathology reports and correlated them with imaging findings. One subject underwent bile sampling of the CBD to evaluate the amylase level because his gadoxetic acid-enhanced fMRC showed evidence of pancreato-biliary reflux of pancreatic secretions.

### Imaging techniques

All magnetic resonance imagings (MRIs) were performed on a 3-Tesla MRI machine (Achieva; Philips Medical Systems, Best, the Netherlands). Patients underwent gadoxetic acid-enhanced fMRC with hepatocyte-specific contrast agents; we obtained both contrast-enhanced and un-enhanced fat-saturated 3D gradient-echo T1-weighted images during the arterial, portal venous, and equilibrium phases. We obtained images 50 and 60 min after administration of gadoxetic acid (Primovist, Bayer Schering Pharma, Berlin, Germany) at a dose of 0.025 mmol/kg of body weight at a flow rate of 1 mL/s, followed by a 10-mL saline flush at the same flow rate, using a IV power injector (Spectris Solaris; MedRad, Indianola, PA, United States). Patients then consumed a fatty meal, and post-prandial images were obtained at 10, 20 and 30 min. We used the enhanced-T1 High Resolution Isotropic Volume Examination technique to obtain 3D gradient-echo T1-weighted images. We performed 3D reconstruction using the Maximum Intensity Projection technique 60 min after gadoxetic acid administration and 30 min after the fatty meal. We obtained T2-MRC images as axial and coronal T2 single-shot sequences and maximum intensity projection (MIP) reconstruction images in all patients (Table 1).

### Image review

Two radiologists reviewed the image sequences of all six patients and classified choledochal cysts according to the Todani classification and AUPBD according to Kimura's classification (B-P type: a right angle between the bile duct

Table 1 Protocol for gadoxetic acid-enhanced functional magnetic resonance cholangiography at our institution

	T2-MRC (single shot, SPAIR)			Gadoxetic acid-enhanced MRC (3D-T1-TFE, eTHRIVE)		
	Axial	Coronal	MRCP slab	Axial	Coronal	MIP
TR/TE (ms)	1475/80	2415/235	10 695/920	3/2	3/2	
Flip angle (°)	90	90	90	10	10	
Field of view (mm)	300 × 350	350 × 350	250 × 250	304 × 330	350 × 350	315 × 315
Matrix	276 × 203	256 × 254	256 × 256	220 × 222	292 × 292	292 × 292
Thickness (mm)	4	2	40	4	2.4	
Gap (mm)	1.2	1		2	1.2	

MRC: Magnetic resonance cholangiography; SPAIR: Spectral attenuation with inversion recovery; THRIVE: T1-weighted high-resolution isotropic volume excitation; MIP: Maximum intensity projection; TR: Repetition time; TE: Echo time; MRCP: Magnetic resonance cholangiopancreatography.

Table 2 Study results

Sex	Age	Type of choledochal cyst <sup>1</sup>	Type of AUPBD <sup>2</sup>	Surgery	T2-MRC		Gd-EOB-DTPA-enhanced MRC			
					CBD stone	BP	PB	Travel extent of contrast on pre-prandial images <sup>3</sup>	Travel extent of contrast on post-prandial images <sup>4</sup>	
F	52	Ia	B-P	Yes	No	Yes	NI		MPD	MPD
M	64	Ia	B-P	Yes	No	Yes	NI		CBD	MPD
M	46	None	B-P	No	No	NI	Yes		CHD <sup>5</sup>	CBD
F	35	Ia	P-B	Yes	Yes	NI	NI		CHD	CHD
F	48	Ia	B-P	Yes	No	Yes	NI		CBD	MPD
F	40	Ic	P-B	Yes	No	NI	Yes		CHD <sup>6</sup>	CBD

<sup>1</sup>Todani classification of choledochal cyst; <sup>2</sup>Kimura classification of anomalous union of pancreatico-biliary duct (AUPBD); <sup>3</sup>extent of contrast material on gadoxetic acid-enhanced magnetic resonance cholangiography (MRC) 60 min after administration; <sup>4</sup>Extent of contrast material on gadoxetic acid-enhanced MRC 30 min after a fatty meal; <sup>5</sup>filling defect of contrast material in common bile duct (CBD); <sup>6</sup>filling defect of contrast material in CBD. Biliary amylase level: 133 000 (IU/L). Gd-EOB-DTPA: Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; BP: Biliopancreatic reflux; PB: Pancreatico-biliary reflux; MPD: Main pancreatic duct; CHD: Common hepatic duct; NI: Not identified.

and pancreatic duct or the bile duct inserted into the pancreatic duct; P-B type: an acute angle between the bile duct and pancreatic duct or the pancreatic duct inserted into the bile duct)<sup>[9-11]</sup>.

The radiologists evaluated by consensus the extent of injected contrast media and recorded the presence of biliopancreatic and pancreatico-biliary reflux on all image sequences. The radiologic diagnosis of biliopancreatic bile reflux was made when biliary-excreted contrast media was visible in the main pancreatic duct on gadoxetic acid-enhanced fMRC. The diagnosis of pancreaticobiliary reflux of pancreatic secretions was made when a filling defect was present on pre-prandial images, which decreased in size or was absent on post-fatty meal images. Based on an unpublished study we conducted on normal volunteers, which found that the CBD was filled with excreted gadoxetic acid 30 min after contrast administration and a fatty meal uptake can make more extension of biliary excreted contrast media by contraction of the gallbladder, we therefore considered a filling defect in the distal CBD on 50 and 60 min delayed images as evidence of reflux of pancreatic secretions.

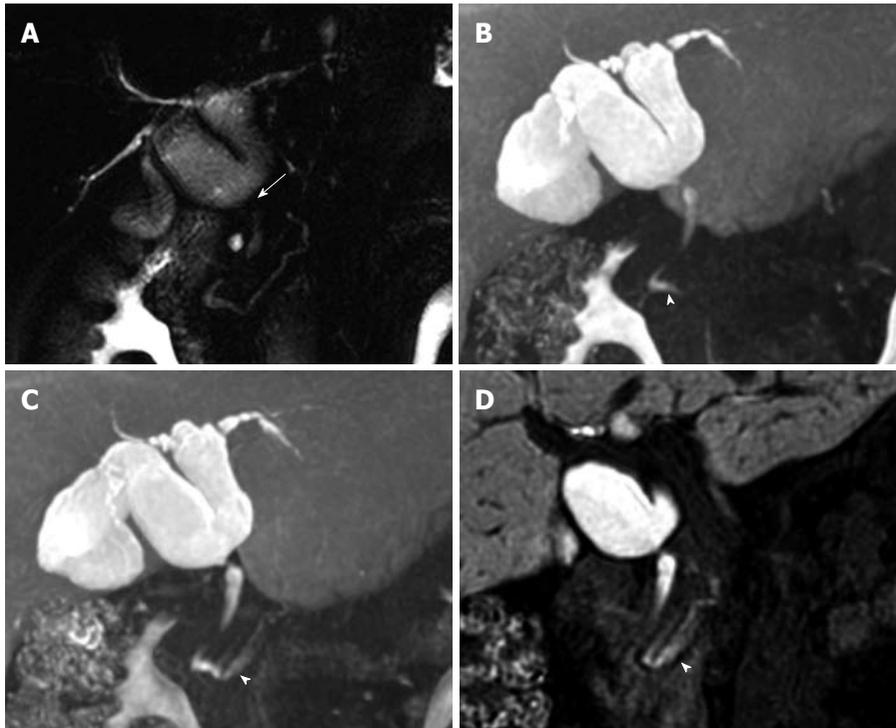
## RESULTS

The results of our study are summarized in Table 2. Of the six patients, four had B-P type AUPBD and two had P-B type AUPBD. All five choledochal cysts were Toda-

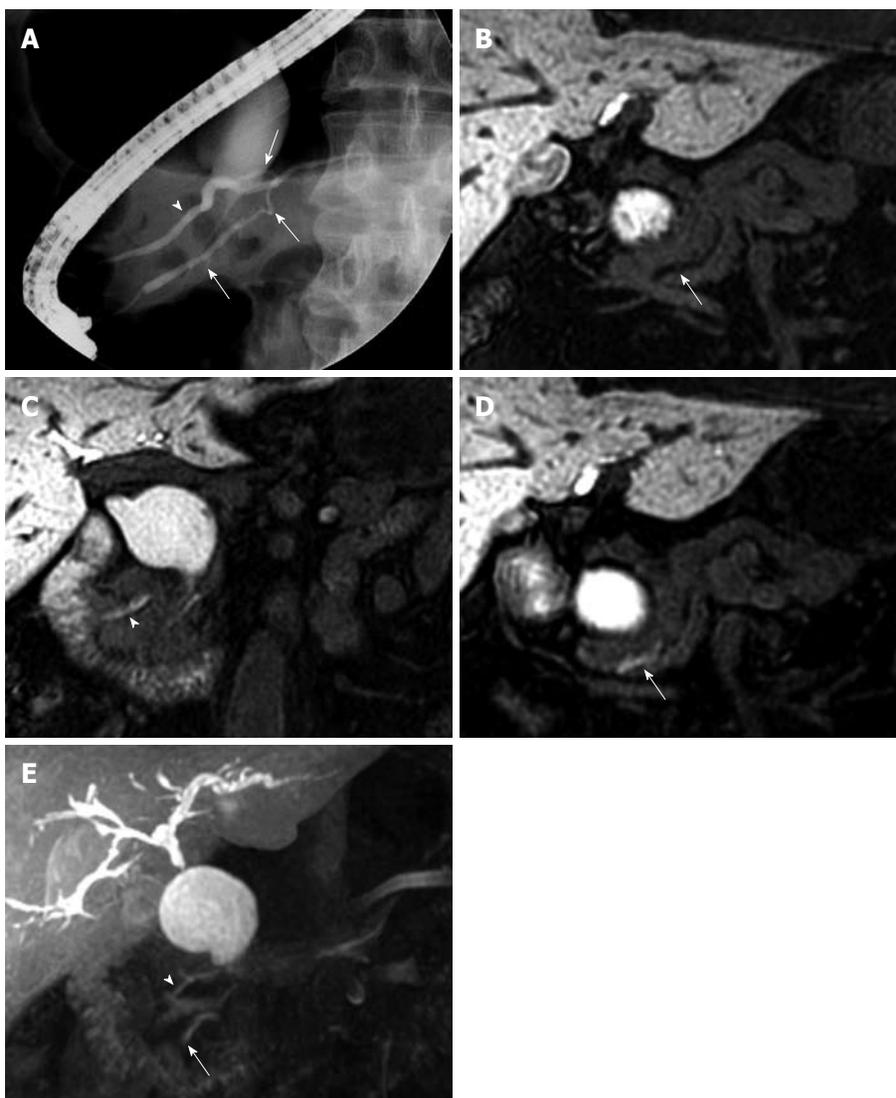
ni classification I (four patients with I a and one patient with I c), with confined dilation of the extrahepatic bile duct.

In three of the six patients with AUPBD, injected contrast media reached the distal CBD and pancreatic duct, suggesting biliopancreatic reflux. We observed extension of contrast to the pancreatic duct on images taken 20 min after a fatty meal in two patients (Figure 1). In one patient, we observed contrast extending to the pancreatic duct on images taken 50 min after contrast administration; later images showed contrast extending to the more distal portion of the pancreatic duct (Figure 2). These three patients all had Todani type I a choledochal cysts and B-P type AUPBD. The one patient with AUPBD without a choledochal cyst had a history of recurrent acute pancreatitis with no history of alcohol abuse.

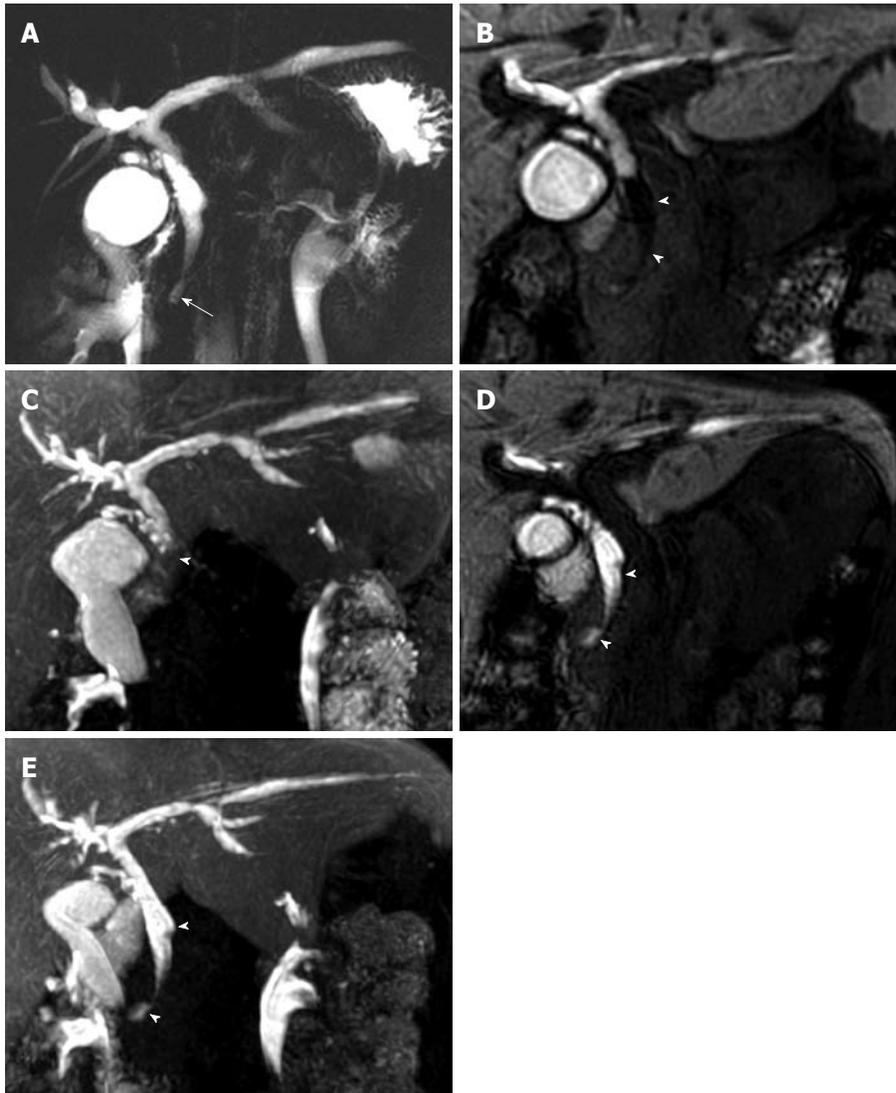
In two patients, we observed a band-like filling defect in the central portion of the distal CBD on pre-fatty meal images. The filling defect decreased in size on post-fatty meal images, which we considered evidence of pancreatico-biliary reflux of pancreatic secretions. One of these two patients had B-P type AUPBD with no combined bile duct dilatation, and the other had P-B type AUPBD with a Todani type I c choledochal cyst. A bile amylase level of 113 000 IU/L was measured from the CBD in the latter patient (Figure 3). In one patient with a choledochal cyst (Todani I a and Kimura P-B), contrast did not reach the distal CBD and did not appear to enter the



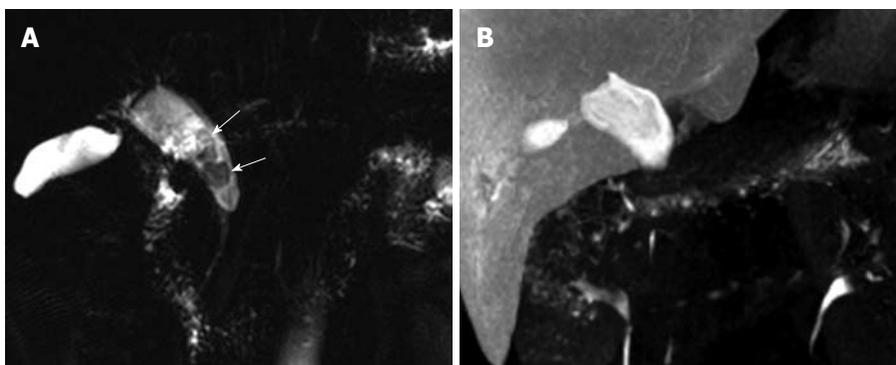
**Figure 1** A 52-year-old woman with anomalous union of the pancreatico-biliary duct and a type I choledochal cyst. A: Fusiform dilation of the common hepatic and cystic ducts with a focal stricture in the common bile duct (arrow) on T2-magnetic resonance cholangiography (MRC); B: Maximum intensity projection (MIP) reconstruction image of 60-min delayed gadopentetate acid-enhanced MRC shows the main pancreatic duct (arrowhead), indicating biliopancreatic reflux; C: MIP reconstruction image of 30-min delayed gadopentetate acid-enhanced MRC after a fatty meal shows progression of contrast media (arrowhead) along the main pancreatic duct; D: Gadopentetate acid-enhanced MRC coronal image taken 30 min after a fatty meal shows visualization of the main pancreatic duct (arrowhead) using contrast material.



**Figure 2** A 64-year-old man with anomalous union of the pancreatico-biliary duct and a type I choledochal cyst. A: Endoscopic retrograde cholangiopancreatography shows cystic dilation of the extrahepatic bile duct with a focal stricture in the distal common bile duct. The end of the long ventral pancreatic duct (duct of Wirsung, arrows) is fused with the dorsal pancreatic duct (duct of Santorini, arrowhead). The common bile duct inserts into the ventral pancreatic duct; B: Sixty-minute delayed gadopentetate acid-enhanced magnetic resonance cholangiography (MRC) coronal image does not visualize the main pancreatic duct (arrow); C and D: Gadopentetate acid-enhanced MRC coronal images taken 30 min after a fatty meal show the duct of Santorini (arrowhead) and duct of Wirsung (arrow), indicating biliopancreatic reflux of contrast material; E: Gadopentetate acid-enhanced MRC maximum intensity projection reconstruction images taken 30 min after a fatty meal show major and minor pancreatic ducts.



**Figure 3** A 40-year-old woman with anomalous union of the pancreatico-biliary duct and a type I c choledochal cyst. A: T2-magnetic resonance cholangiography (MRC) shows a long common channel (arrow), with diffuse bile duct dilation; B: Sixty-minute delayed gadoxetic acid-enhanced MRC coronal image; C: Maximum intensity projection (MIP) reconstruction image show a filling defect (arrow heads) in the central portion of the distal common bile duct (CBD); D: Gadoxetic acid-enhanced MRC coronal images taken 30 min after a fatty meal; E: MIP reconstruction images show a decreased filling defect (arrow heads) in the distal CBD, indicative of pancreatico-biliary reflux.



**Figure 4** A 35-year-old woman with a type I a choledochal cyst and multiple common bile duct stones. A: Fusiform dilatation of the extrahepatic bile duct is seen on T2-magnetic resonance cholangiography (MRC) and multiple nodular filling defects (arrows) are seen in the common bile duct (CBD) which represent CBD stones; B: Contrast media did not pass through the ampulla of Vater until 30 min after fatty meal ingestion and multiple CBD stones are not seen due to masking by contrast media with high signal intensity.

duodenum. We noted multiple CBD stones in this patient (Figure 4).

## DISCUSSION

AUPBD is defined as an anomalous union of the bile and pancreatic ducts outside the duodenal wall proximal to the sphincter of Oddi. The diagnostic criteria for AUPBD include the radiological and anatomical detection of the extramural location of the junction of the

pancreatic and biliary ducts in the duodenal wall, as well as radiological detection of a long common channel (> 1.5 cm). Detection of the extramural location is difficult in AUPBD patients with a short common duct (less than 1 cm in length)<sup>[6]</sup>. The sphincter of Oddi, which regulates the outflow of bile and pancreatic secretions, is deficient in AUPBD due to the long common channel, allowing two-way regurgitation: Pancreatico-biliary reflux of pancreatic secretions and biliopancreatic bile reflux. This reflux can result in various pathological condi-

tions including choledocholithiasis, cholangitis, gallstones, acute pancreatitis, bile duct cancer, gallbladder cancer, and pancreatic ductal carcinoma<sup>[12,13]</sup>. It is known that choledochal cysts are embryologically associated with AUPBD, and their various clinical signs and symptoms have been shown to be closely related to the presence of AUPBD<sup>[14]</sup>.

Various methods have been reported to confirm biliopancreatic reflux, including operative or postoperative T-tube cholangiography, computerized tomography combined with drip infusion cholangiography, histological detection of gallbladder cancer cells in the main pancreatic duct, and bile reflux on the cut surface of the pancreas<sup>[12,15,16]</sup>. Furthermore, pancreatico-biliary reflux of pancreatic secretions has been confirmed by measurement of bile amylase in the bile duct, secretin-stimulated dynamic MRC, and pancreatography *via* the minor duodenal papilla<sup>[15,17]</sup>. However, these methods are limited by their levels of invasiveness, the time required, patient discomfort, and adverse effects of contrast materials<sup>[18-20]</sup>.

Gadoxetic acid is widely used because of its diagnostic efficacy in focal lesions of the liver and its safety<sup>[21-24]</sup>. In our study, there were no serious adverse effects related to the use of contrast material that required medical management. Although our protocol for functional MR cholangiography to detect biliary reflux in AUPBD patients required an additional 90 min and 70 min when compared with non-contrast enhanced T2-MRC and Gd-enhanced T1-MRC, respectively, it is easy to perform and does not augment the risk of contrast agent-related adverse effects.

As previously described, AUPBD was diagnosed by identifying biliopancreatic bile reflux and pancreatico-biliary pancreatic reflux on gadoxetic acid-enhanced fMRC in addition to the detection of a long common channel on T2-MRC or the customary 20-min delayed gadoxetic acid-enhanced MRC. Delayed hepatobiliary MRC (greater than a 30 min delay) after contrast administration, and MRC of gallbladder contraction induced by a fatty meal were required to detect both types of reflux.

Some researchers report that, if the passage of contrast material through the ampulla of Vater takes longer than 30 to 60 min, it can be considered delayed. In comparison, excretion of contrast material past the ampulla in less than 20 to 30 min is considered normal<sup>[4-5]</sup>. There have been many reports on gadoxetic acid-enhanced MRC which showed that bile excretion could be visualized by capturing images during the hepatobiliary phase approximately 20 or 30 min after contrast administration<sup>[4,5,25,26]</sup>. Images taken one hour after contrast administration and again after a fatty meal allow bile to travel further along the normal or anomalous pathway, providing additional information about the patient's biliary system. Since there are no studies which looked into the optimal phase to observe AUPBD, further study is warranted to simplify the study protocol.

Our fMRC protocol requires additional time for delayed images compared with contrast enhanced T1-MRC. However, gadoxetic acid-enhanced fMRC allows the as-

essment of bile excretion and pancreas secretion physiology in addition to visualization of bile duct and pancreatic duct morphology, thus obviating the need for additional imaging studies such as hepatobiliary scan and ERCP.

It is difficult to generalize the results of our study due to its small sample size. Nonetheless, one patient in our study was found to have a filling defect in the distal CBD along with an abnormally high amylase level (113 000 IU/L) in the CBD. One study reported the biliary amylase levels of patients with biliopancreatic disease to range widely from less than 10 to 300 000 IU/L<sup>[27]</sup>. Sai *et al.*<sup>[28]</sup> reported a mean biliary amylase level of 238 IU/L in patients with no reflux of pancreatic secretions into the bile duct, while Horaguchi *et al.*<sup>[27]</sup> adopted 168 IU/L as the upper limit of a normal biliary amylase level. Our previous study of gadoxetic acid-enhanced MRC in 40 normal volunteers found no filling defects in the distal CBD up to one hour after contrast administration or after a fatty meal (unpublished data). Taken together, we used these data to presume pancreatico-biliary reflux in the case of a central filling defect in the CBD that diminished after a fatty meal.

In normal physiology, the pressure in the pancreatic duct exceeds the choledochal pressure, allowing pancreatic secretions to flow into the biliary tract rather than reflux into the pancreatic duct where they can cause biliary complications<sup>[29,30]</sup>. In the case of AUPBD, however, bile can reflux into the pancreatic duct under conditions such as increased pressure in the bile duct due to bile stasis in a choledochal cyst or cholangitis<sup>[31]</sup>.

A study of 2980 patients undergoing ERCP found a 1.7% prevalence of a long common channel. In that study, 13 patients underwent intraoperative cholangiography, 11 of whom were found to have biliopancreatic reflux with an elevated biliary amylase level<sup>[32]</sup>. In our study, all three patients with biliopancreatic reflux were found to have B-P type AUPBD as well as Todani type I a choledochal cysts. We hypothesize that bile duct stasis in the dilated bile duct resulted in elevated choledochal pressure, resulting in biliopancreatic bile reflux. Of the two patients with pancreatico-biliary reflux of pancreatic secretions, one had B-P type AUPBD and the other had P-B type AUPBD.

Our study had several limitations. First, the sample size is too small to allow for generalization. Second, the type of choledochal cysts in our study were limited to Todani classification type I, meaning a cystic or fusiform dilation of the extrahepatic bile duct. Further studies that include larger sample sizes and several types of choledochal cysts are required to generalize the imaging findings of biliopancreatic and pancreatico-biliary reflux, as well as to evaluate the diagnostic accuracy of these findings for AUPBD in patients with choledochal cysts.

In conclusion, gadoxetic acid-enhanced fMRC can show biliopancreatic bile reflux and pancreatico-biliary reflux of pancreatic secretions in patients with AUPBD with and without combined Todani type I choledochal cysts.

## COMMENTS

**Background**

Gadoxetic acid has both properties of extracellular contrast media, which make dynamic study possible, through the organic anion-transporting polypeptide and is excreted into the bile ducts, allowing visualization of the bile ducts on hepatobiliary phase T1-weighted images. In patients with normal hepatic function, the hepatobiliary phase usually occurs within 20 min of gadoxetic acid administration. From a previous study in normal healthy patients, we know that images obtained after a delayed time period and after a fatty meal allow bile to travel further along the normal or anomalous pathway, providing additional information about the patient's biliary system.

**Research frontiers**

Gadoxetic acid has hepatobiliary properties which mediate specific uptake of the agent into the hepatocytes. Gadoxetic acid-enhanced magnetic resonance cholangiography (MRC) can visualize the physiology of bile excretion directly, in contrast to conventional T2-weighted MRC which can visualize fluid filled space by heavily T2-weighted and fat-suppressed images. The usefulness of gadoxetic acid-enhanced MRC was demonstrated in many reports in a wide range of hepatobiliary applications including evaluation of biliary tract anomalies, the diagnosis of acute cholecystitis, assessment of postsurgical anatomy and complications, and to determine whether fluid collections communicate with the biliary tree.

**Innovations and breakthroughs**

The present study clearly showed that biliopancreatic bile reflux and pancreatico-biliary reflux of pancreatic secretions in patients with anomalous union of the pancreatico-biliary duct (AUPBD) could be easily diagnosed using the convenient and safe imaging method of gadoxetic acid-enhanced MR cholangiography.

**Applications**

The present study revealed that biliopancreatic bile reflux and pancreatico-biliary reflux of pancreatic secretions in patients with AUPBD could be diagnosed with gadoxetic acid-enhanced MRC. Further studies which include larger sample sizes and several types of choledochal cysts are required to generalize the imaging findings of biliopancreatic and pancreatico-biliary reflux, as well as to evaluate the diagnostic accuracy of these findings.

**Peer review**

Although gadoxetic acid-enhanced functional MRC is not a new method to observe the bile system, it is reasonable and interesting to use it to detect biliopancreatic and pancreatico-biliary reflux in patients with AUPBD.

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## Synergistic effect of multiple predisposing risk factors on the development of bezoars

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### Abstract

**AIM:** To describe the clinical characteristics of patients with gastric or intestinal bezoars recently treated in our hospital.

**METHODS:** In this study, a retrospective chart review of consecutive patients with gastrointestinal bezoars, who were treated at the Samsun Education and Research Hospital between January 2006 and March 2011, was conducted. Data on demographic characteristics, clinical presentation, history of risk factors, diagnostic procedures, localization of bezoars, treatment interventions, and postoperative morbidity and mortality rates were collected and evaluated.

**RESULTS:** Forty-two patients [26 (61.9%) males and 16 (31.1%) females] with a mean  $\pm$  SD (range) age of  $55.8 \pm 10.5$  (37-74) years were enrolled in this study. Thirty-six patients (85.7%) had one or more predisposing risk factors for gastrointestinal bezoars. The most common predisposing risk factor was a history of previous gastric surgery which was identified in 18 patients (42.8%). Twenty three patients (54.8%) had multiple

predisposing risk factors. Phytobezoars were identified in all patients except one who had a trichobezoar in the stomach. Non-operative endoscopic fragmentation was performed either initially or after unsuccessful medical treatment in 14 patients with gastric bezoars and was completely successful in 10 patients (71.5%). Surgery was the most frequent treatment method in our study, which was required in 28 patients (66.7%). Intestinal obstruction secondary to bezoars was the most common complication ( $n = 18$ , 42.8%) in our study.

**CONCLUSION:** The presence of multiple predisposing factors may create a synergistic effect in the development of bezoars.

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**Key words:** Bezoar; Diospyrobezoars; Persimmon; Phytobezoar; Trichobezoar

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### INTRODUCTION

Bezoars can be defined as masses of indigestible, hard materials formed in the gastrointestinal tract. Etymologically, the word bezoar came from the Persian word "padzahr" meaning to expel poison. In some societies, animal bezoars were formerly considered a useful medicine and possessed certain magical properties<sup>[1]</sup>. In 1854,

Quain reported an intragastric alimentary mass in an autopsy and called it a "bezoar"<sup>[2]</sup>.

Bezoars can be classified as phytobezoars (undigested vegetables), trichobezoars (hairs), lactobezoars (milk) and pharmacobezoars (medications) according to their composition<sup>[3]</sup>. They usually form in the stomach and can pass into the small bowel where they occasionally cause obstruction. Phytobezoars are composed of undigested food fibers, such as cellulose, hemicellulose, lignin and fruit tannin. These fibers occur in fruits and vegetables such as celery, pumpkin, prunes, raisins, leeks, beets and persimmons.

The aim of this study was to describe the clinical characteristics of patients with gastric or intestinal bezoars recently treated in our hospital.

## MATERIALS AND METHODS

A retrospective chart review of consecutive patients with gastrointestinal bezoars, who were treated at the Samsun Education and Research Hospital between January 2006 and March 2011, was conducted. Data on the demographic characteristics, clinical presentations, history of predisposing risk factors, diagnostic procedures, localization of bezoars, treatment interventions, morbidity and mortality rates were collected and evaluated. In addition, the patients were contacted by phone to determine any recurrence of bezoars after treatment.

In this study, previous gastric surgery, excessive consumption of some types of fruit and vegetables, diabetes mellitus, mastication problems, long-term antacid treatment and mental disorders were considered predisposing risk factors in the development of bezoars.

All calculations were performed in Microsoft Office Excel 2007. Continuous variables were summarized as mean  $\pm$  SD or median when appropriate, and categorical variables as frequency and percentage (%).

## RESULTS

### Demographic characteristics and presentation

Forty-two patients [26 (61.9%) males and 16 (31.1%) females] with a mean  $\pm$  SD (range) age of 55.8  $\pm$  10.5 (37-78 years) were enrolled in this study. The peak incidence was in the 6th decade of life (51-60 years). Twelve patients (28.6%) were in the 6th decade.

The most common presenting symptom was abdominal pain which was noted in 40 patients (95.2%). Dyspeptic symptoms other than epigastric pain were found in 32 patients (76.2%). Mild to severe nausea and vomiting were observed in 29 cases (69%). Loss of appetite was found in 19 patients (45.2%) and a significant weight loss history was identified in 5 (11.9%) patients. Some degree of abdominal distention as a sign of intestinal obstruction developed in 18 patients (42.9%). Two patients presented with acute gastric outlet obstruction.

Twelve patients with intestinal or gastric outlet obstruction (47.6%) were admitted to our emergency service. Five patients (11.9%) were referred by gastroenterologists.

Table 1 Distribution of predisposing factors

Predisposing factors	n	%
Single predisposing factor	13	31.0
Only gastric surgery	4	9.5
Only persimmon consumption	3	7.1
Only mastication problems	3	7.1
Only diabetes mellitus	2	4.8
Trichotillomania	1	2.4
Multiple predisposing factors	23	54.7
Gastric surgery + persimmon consumption	3	7.1
Gastric surgery + diabetes mellitus	3	7.1
Gastric surgery + mastication problem	3	7.1
Gastric surgery + mastication problem + persimmon consumption	3	7.1
Gastric surgery + mastication problem + diabetes mellitus	2	4.8
Persimmon consumption + mastication problem	3	7.1
Persimmon consumption + diabetes mellitus	2	4.8
Mastication problem + diabetes mellitus	1	2.4
Diabetes mellitus + antacid drug	1	2.4
Persimmon consumption + antacid drug	1	2.4
Mastication problem + antacid drug + persimmon consumption	1	2.4
No predisposing factor	6	14.3

The remaining patients ( $n = 17$ , 40.4%) were admitted to the general surgery clinic.

During the study period, 257 patients with mechanical bowel obstruction due to various reasons were admitted to our emergency service. Bezoars were the cause of mechanical bowel obstruction in 18 of these patients (7%).

### History of predisposing factors

Thirty-six patients (85.7%) had one or more predisposing risk factors (Table 1). The most common predisposing risk factor was previous gastric surgery which was identified in 18 patients (42.8%). Excessive persimmon consumption was another significant predisposing risk factor in our study. A history of excessive persimmon consumption was observed in 17 patients (40.5%). Mastication problems and diabetes mellitus were identified in 16 (38.1%) and 12 (28.6%) patients, respectively. Twenty-three patients (54.8%) had multiple predisposing risk factors. All predisposing risk factors are summarized in Table 1.

### Diagnostic procedures

Initial diagnosis was made by gastroscopy in 15 patients (35.7%). Abdominal sonography was the first diagnostic method used in 7 patients (16.7%), which was carried out in 12 patients as the first imaging method. Plain abdominal radiography (PAR) showed air-fluid levels in 18 patients (40.5%). The typical bezoar image on PAR, involving a mottled air pattern, was identified in only two patients. Abdominal tomography was carried out in 16 patients and bezoars were revealed in 14 of these patients (87.5%).

### Localization and composition

A single bezoar was found in 38 (90.4%) patients. Four patients (9.6%) had multiple bezoars in different locations.

Bezoars were mainly located in the stomach ( $n = 28$ ). Other locations were the ileum, jejunum and colon ( $n = 14$ ,  $n = 3$  and  $n = 1$ , respectively). Phytobezoars were identified in all patients except one who had a trichobezoar in the stomach. The patient with the trichobezoar was a 43-year-old woman, who had a history of psychiatric problems and trichotillomania.

### Intervention

Medical treatment with various enzymatic agents (including cellulase and cola) was initially tried in 15 cases with small gastric bezoars, however, enzymatic treatment was completely successful in only 4 patients (26.7%). Non-operative endoscopic fragmentation was performed either initially or after unsuccessful medical treatment in 14 patients and was completely successful in 10 patients (71.5%).

Surgery was the most frequent treatment method in our study, which was required in 28 patients (66.7%). Bezoars were removed from the stomach by gastrotomy in 8 patients. Preoperatively diagnosed small bezoars which were located in the distal ileum were carefully milked into the cecum in 8 cases. In 9 cases, it was not possible to milk the bezoars into the large intestine and an enterotomy was required. The patient who had a colonic bezoar in the ascending colon was treated with colotomy. In these 18 patients with intestinal bezoars, the stomach was surgically explored for additional bezoars and additional gastric bezoars were found and extracted *via* gastrotomy in 4 patients.

Coexisting gastric ulcers were identified in 5 (20.8%) of the patients with gastric bezoars. While anti-ulcer medication was prescribed in endoscopically treated patients, ( $n = 3$ ), wedge resection of ulcers was added to the gastrotomy in operated patients ( $n = 2$ ). Histopathological examinations of the ulcers revealed benign findings in all 5 patients.

### Postoperative outcomes and complications

The mean postoperative hospital stay was  $6.1 \pm 1.7$  d (range, 3-12 d) in our study. Postoperative complications developed in 7 (25%) patients (surgical site infection in 3 (10.7%) cases, chest infection in 2 (7.1%) patients and prolonged ileus in 2 (7.1%) patients).

We were only able to contact 32 (76.2%) patients by phone. There were no clinical recurrences in these patients during a median follow-up time of 25 mo (range, 3-63 mo).

## DISCUSSION

A number of predisposing factors may contribute to the risk of bezoar formation. Previous gastric surgery was reported in 20% to 93% of patients with bezoars and the incidence of bezoar formation after gastric surgery ranged from 5% to 12%<sup>[4-8]</sup>. Similar to previous published studies, the most common predisposing risk factor was previous gastric surgery which was identified in 42.8% of the patients in our study. Altered anatomy and physiology of the gastric remnant after vagotomy and partial

gastrectomy are largely responsible for bezoar formation. Vagotomy and partial gastrectomy diminish the ability of the stomach to break up and digest food. Both the quantity and the acidity of the gastric juice are reduced and peptic activity is adversely affected<sup>[9,10]</sup>. Additionally, the antrum has an important role in the mechanical fragmentation of ingested material, and the pylorus prevents large boluses from reaching the small intestine. Resection of the antrum and pylorus may lead to the passage of a non-fragmented, large bolus to the small intestine. The interval between gastric surgery and bezoar detection was 9 mo to 30 years<sup>[4-7]</sup>. In our study, the mean interval between surgery and bezoar detection was  $7.4 \pm 2.3$  years (5-11 years).

Excessive consumption of persimmon was identified in 40.5% of our patients. Persimmon, which grows in many areas in our region and widely consumed, is the fruit of a number of species of trees belonging to the genus *Diospyros*. The word *Diospyros* means "the fruit of the gods" in ancient Greek. Persimmon bezoars are also known as diospyrobezoars. Unripe persimmons contain soluble tannin. Tannin polymerizes in an acidic environment to form a glue-like coagulum, which can affix to other materials in the stomach<sup>[11]</sup>. In 1986, Krausz *et al*<sup>[4]</sup> reported that 91.2% of 113 patients with phytobezoars had a history of persimmon intake. Erzurumlu *et al*<sup>[12]</sup> from our country reported that 17.6% of their 34 patients with bezoars had a history of persimmon or cherry laurel intake.

Mental retardation and trichotillomania are major risk factors for the development of trichobezoars<sup>[13]</sup>. In our study, there was only one patient with trichobezoar who had a history of psychiatric disorders and trichotillomania. The other predisposing factors observed in our study included mastication problems, diabetic gastroparesis and antacid drug use. Consequently, 85.7% of patients had one or more predisposing factors in our study. While about one third of our patients had only one predisposing risk factor, over fifty percent had multiple predisposing risk factors. In our opinion, these results may indicate that the presence of multiple predisposing risk factors creates a synergistic effect in the development of bezoars. On the other hand, 14.3% of the patients in our study had no apparent predisposing risk factors. Erzurumlu *et al*<sup>[12]</sup> reported that only 5.9% of the patients in their study had no apparent predisposing risk factors. Bezoar formation is postulated to be provoked by dietary and eating habits in patients without predisposing factors<sup>[14]</sup>.

Until only a few decades ago, the differential diagnosis of intestinal obstruction secondary to bezoars was difficult before surgery, because the clinical and radiographic findings are similar to those of intestinal obstruction attributable to other causes<sup>[11,15]</sup>. However, findings from recent studies suggest that sonography or computerized tomography (CT) can assist radiologists in diagnosing bezoars before surgery<sup>[6,16]</sup>. In our study, PAR showed air-fluid levels in 18 patients with intestinal obstruction. The typical bezoar image on PAR, involving a mottled air pattern, was identified in only two patients (11.1%). Abdom-

inal CT was carried out in 16 patients and bezoars were revealed in 14 (77.7%) of these patients before surgery. Although sonography was not the preferred imaging modality for the patients with intestinal obstruction in our study, it was carried out in 12 patients with gastric bezoar as the first imaging method and the presence of a bezoar was suspected in 7 (58.3%) of these patients before endoscopy.

Both mechanical and chemical procedures are used in the treatment of gastric bezoars. Bezoars can be endoscopically fragmented into pieces using polypectomy snares, endoscopic forceps, Dormia baskets, endoscopic lithotripsy, electrosurgical knives or YAG laser. However, this technique requires specific equipment and is not complication free. Bleeding, perforation or even migration of bezoar pieces causing intestinal obstruction are potential complications<sup>[17]</sup>. In our study, endoscopic fragmentation was performed either initially or after unsuccessful medical treatment in 14 patients and was completely successful in 10 patients (71.5%). Medical treatment may also be useful in the management of gastric bezoars. Several chemical agents have been tested; these are administered orally, through a nasogastric tube or injected directly into the bezoar *via* endoscopy. However, the development of these techniques usually takes time, is not free of complications such as electrolytic disorders, gastric ulcer and has indistinct results<sup>[17]</sup>. In our study, medical treatment was initially tried in 15 cases with gastric bezoars, but was completely successful in only 4 patients (26.7%).

Although bezoars are the most common type of foreign body lodged in any part of the gastrointestinal tract, the overall incidence of bezoar-induced intestinal obstruction remains relatively low. Epidemiological data show that 2% to 4% of intestinal obstructions are caused by bezoars<sup>[2]</sup>. This figure was 7% in our study. Although intestinal obstruction was reported to be the most frequent clinical presentation of bezoars in the majority of previous studies, it was observed in 42% of the patients in our study. Surgical management of intestinal obstruction secondary to bezoars entails milking the object into the cecum or performing enterotomy for retrieval in difficult cases. In our study, 47% of patients with intestinal obstruction were managed by milking. Enterotomy was performed in 53% of patients with intestinal obstruction. Although therapeutic laparoscopy has been demonstrated to be feasible in the management of intestinal obstruction secondary to bezoars<sup>[18]</sup>, all operations were conducted as open surgery in our study.

Intestinal bezoars are often found in association with gastric bezoars<sup>[6]</sup>. Coexisting gastric bezoars was reported in 17%-21% of patients<sup>[19,21]</sup>. In our study, a coexisting gastric bezoar was found in 22.2% of patients with an intestinal bezoar. Consequently, when an intestinal bezoar is diagnosed, the possible presence of coexisting gastric or intestinal bezoars should be investigated cautiously.

Major complications of bezoars other than intestinal obstruction include gastric ulcer, gastritis, gastric perforation and gastric outlet obstruction. In our study, coexisting

gastric ulcers were identified in 20.8% patients with gastric bezoars. While anti-ulcer medication was prescribed in endoscopically treated patients, wedge resection of ulcers was added to the gastrotomy in operated patients. Two patients with gastric outlet obstruction were treated with gastrotomy and extraction of bezoars.

Although, there was no clinical recurrence of bezoars during a median follow-up time of 25 mo after treatment in our study, Klamer *et al.*<sup>[22]</sup> reported recurrence in approximately 20% of patients with gastric bezoars after initial treatment. Therefore, patients should be instructed to avoid a high fiber diet, persimmons and certain medications to minimize the potential risk of recurrence.

In conclusion, over fifty percent of the patients in our study had multiple predisposing factors for gastrointestinal bezoars. In light of these results, it may be concluded that the presence of multiple predisposing factors create a synergistic effect in the development of bezoars. Intestinal obstruction is the most common complication of bezoars. Although the prevalence of intestinal obstruction secondary to bezoars is quite low, differential diagnosis of intestinal obstruction secondary to adhesions is important in patients with previous abdominal surgery; CT can help to make this differentiation. Therefore, CT should be obtained whenever possible in all patients with bowel obstruction to establish the diagnosis and avoid inappropriate treatment.

## COMMENTS

### Background

Bezoars have become increasingly recognized as a cause of acute mechanical intestinal obstruction. Bezoars are classified according to their composition. The major types are phytobezoars, trichobezoars, and pharmacobezoars. Phytobezoars, composed of undigested vegetable matter, are the most common type of bezoar. Trichobezoars, composed of hair, are often associated with psychiatric problems. *Pharmacobezoars* are composed of ingested medications.

### Research frontiers

Previous studies have shown that different types of predisposing factors may increase the risk of developing bezoars. During data extraction, the authors realized that most of their patients had more than one predisposing factor for bezoar formation. This result was of interest to them and they would like to emphasize this finding. The present study is the first to address the possible synergistic role of multiple predisposing factors in the development of bezoars.

### Innovations and breakthroughs

The results of the present study suggest that the presence of multiple predisposing factors may create a synergistic effect in the development of bezoars.

### Applications

Early recognition of high-risk individuals, who have multiple predisposing factors, may prompt early investigation and the prevention of potential life-threatening sequelae of intestinal obstruction due to bezoars.

### Terminology

Diospyrobezoar is a type of phytobezoar which is caused by unripe persimmons and it is considered to be harder than other types of phytobezoars. Trichotillomania is a disorder where people compulsively pull out their hair.

### Peer review

The manuscript has been written properly and clearly. Case reports are the most common types of articles considering this problem.

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## Genetic characteristics and pathogenicity of human hepatitis E virus in Nanjing, China

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### Abstract

**AIM:** To investigate the genetic characteristics and pathogenicity of hepatitis E virus (HEV) and assess the potential risk factors for sporadic hepatitis E.

**METHODS:** Sixty-two serum samples from the patients with acute hepatitis E were collected, including 23 cases coinfecting with hepatitis B virus. Anti-HEV detection and partial HEV RNA amplification were performed by enzyme immunoassays and reverse transcription-nested polymerase chain reaction (RT-nPCR) method, respectively, and PCR products were sequenced. The isolated human HEV sequences were analyzed phylogenetically.

**RESULTS:** The positive rate of serum HEV RNA were 21.0% (13/62), including 5 cases of liver failure. All the 13 isolates shared a 82.1%-98.0% nucleotide homology with each other and had identities of 74.7%-81.0%, 75.3%-78.6%, 75.3%-80.0% and 82.1%-96.1% with

the corresponding regions of HEV genotypes 1-4, respectively. The human HEV strain GS-NJ-12 shared a 100% nucleotide identity with the swine HEV strain swIM6-43 isolated from Inner Mongolia, China.

**CONCLUSION:** Swine may be a principal risk factor for occurrence of sporadic hepatitis E in eastern China, and genotype 4 HEV can induce acute liver failure.

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**Key words:** Genotype; Hepatitis E virus; Liver failure; Zoonotic transmission; Pathogenicity

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Geng JB, Wang MR, Wang L, Wang J, Yang ZG, Cheng Y, Qiao F, Wang M. Genetic characteristics and pathogenicity of human hepatitis E virus in Nanjing, China. *World J Gastroenterol* 2012; 18(9): 965-970 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i9/965.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i9.965>

### INTRODUCTION

Hepatitis E virus (HEV) is a single-strand, positive-sense RNA, non-enveloped virus which is classified into the *Hep- eiviridae* family with a single serotype and at least 4 known main genotypes of mammalian HEV, one avian HEV and a new HEV genotype have been isolated from rabbits recently<sup>[1]</sup>. Genotype 1 and 2 of mammalian strains are predominant in humans and associated with large waterborne epidemics in endemic regions<sup>[2]</sup>. However, genotype 3 and 4, which were suggested to be zoonotically transmitted between animals and humans, are mainly responsible for sporadic cases of hepatitis E clinically ma-

nifested as icterus, malaise, anorexia, fever, hepatomegaly and pruritus. Additionally, increasing reports suggest that different HEV genotypes are associated with different disease severity. HEV genotype 1 and 2, which have similar epidemiological and sporadic features, can result in acute hepatitis, acute liver failure, and acute-on-chronic liver failure. However, HEV genotype 3 and 4, which were generally considered to cause acute, self-limiting illness followed by a complete recovery, seem to be less virulent in humans than genotype 1 and 2<sup>[3]</sup>, and do not cause severe liver diseases<sup>[4]</sup>. In mainland China, HEV genotype 4 has become the dominant genotype instead of genotype 1 since 2004<sup>[5]</sup>.

Since the first swine HEV strain was isolated in 1997, many strains of HEV have been identified from human and other mammalian reservoirs (swine, wild boar, deer, mongooses, rabbits and rats), and swine was considered to be the principal reservoir of HEV<sup>[6-10]</sup>. Accumulating data indicates that hepatitis E is a zoonotic disease. Transmissions through the consumption of contaminated food products such as pork have provided further direct evidence. Thus, zoonotic transmission of hepatitis E raises an important public health concern over food safety and zoonotic risk<sup>[11]</sup>.

In China, seroepidemiological studies in patients with viral hepatitis have shown a high superinfection rate (32.4%) with two or more types of hepatitis virus; and HEV superinfection in patients with chronic hepatitis B (CHB) accounts for 17.6%<sup>[12]</sup>. HEV could result in severe disease and a poor outcome in patients with pre-existing liver diseases<sup>[13,14]</sup>. However, there were few reports on the association between genetic characteristics and pathogenicity of HEV infection. In addition, whether genotype 3 and 4 HEV could induce liver failure in normal population and patients with chronic liver disease (CLD) is still unclear. This study was designed to investigate the genotype of HEV prevalent in eastern China, the pathogenicity of HEV in patients with or without CLD and the phylogenetic relationship between human and swine HEV.

## MATERIALS AND METHODS

### *Patients and serum samples*

A total of 62 serum samples were collected from the hospitalized patients with hepatitis E during the period from November 2008 to December 2010. The diagnostic criteria of hepatitis E are as follows: the elevation of alanine aminotransferase (ALT) level (> 2ULN); the positive result for anti-HEV IgM or at least 4-fold increase of IgG levels during hospitalization. Patients coinfecting with hepatitis B virus had positive serum HBsAg and HBV DNA. All patients were negative for anti-human immunodeficiency virus, anti-hepatitis A virus, anti-hepatitis C virus antibodies and autoantibodies. As some patients did not seek medical care in the early stages of their illness, the presence of HEV-IgG was used to diagnose acute hepatitis E in this study<sup>[14-16]</sup>. The clinical data of patients with acute liver failure or acute-on-chronic liver failure were recorded.

### *Enzyme immunoassay of serum anti-hepatitis E virus antibodies*

All the serum samples were detected for anti-HEV IgM, anti-HEV IgG, anti-HAV IgM, HBsAg, anti-HCV IgG using commercial enzyme immunoassay (EIA) kits (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, China). All assay procedures were carried out according to the manufacturer's instructions. All anti-HEV antibody positive specimens were confirmed by Wantai EIA kit one more time.

### *RNA extraction and reverse transcription-nested polymerase chain reaction*

All serum samples were tested for presence of HEV RNA by reverse transcription-nested polymerase chain reaction (RT-nPCR). RNA was extracted from 200 µL of serum according to the instructions of TRIzol reagent (Invitrogen). The viral RNA was reverse-transcribed to cDNA for 1h at 42 °C with M-MuLV reverse transcriptase (Promega) and specific external anti-sense primers in a 10 µL reaction volume. Nested PCRs for open reading frame (ORF) 2 and ORF1 were performed to detect HEV sequences using two sets of consensus oligonucleotide primers. The primer sequences and amplification parameters were as described previously<sup>[10]</sup>. The final PCR product was analyzed by 15 g/L agarose gel electrophoresis.

### *Sequencing and phylogenetic analysis*

The target second-round PCR products were purified and double-ends sequenced by ABI model 3730 sequencer. Nucleotide sequences were analyzed with the MEGA 4.0, ALIGNX and Bioedit v7.0.9 software. Phylogenetic trees were constructed by the neighbor-joining method and the interior branch test with the aid of MEGA 4.0 software package. One thousand resamplings of the data were used to calculate percentages of the branches obtained.

Designations and accession numbers of full-length reference sequences representing different genotypes for analysis of HEV ORF1 and ORF2 were retrieved from GenBank as follows: Genotype 1: Abb-2B (AF185822); Bur86 (D10330), Sar-55 (M80581), Uigh179 (D11093), FHF (X98292), Morocco (AY230202), T3-Chad (AY204877); Genotype 2: M1 (M74506); Genotype 3: US2 (AF060669), Osh-205 (AF455784), JBOAR-1Hy04 (AB189070), swArnell (AY115488), HE-JA10 (AB089824), JKN-Sap (AB074918), JMY-HAW (AB074920); Genotype 4: 4a: ChH-S-1 (EF077063), swGX32 (EU366959), JKO-ChiSai98C (AB197673); 4b: swGX40 (EU676172), swDQ (DQ279091); 4c: swJ13-1 (AB097811), HE-JA1 (AB097812), HE-JK4 (AB099347), JSN-SAP-FH02 (AB200239); 4d: T1 (AJ272108), swCH25 (AY594199); 4g: ccc220 (AB108537).

In addition, the reference sequences used for analysis of partial ORF2 regions included subtype 4e: IND-SW1 (AF324501), IND-SW2 (AF324502), IND-SW3 (AF324503); 4d: swIM6-26 (AB550622), swIM6-43 (AB550624); subtype 4f: HE-JA2 (AB082558).

Table 1 Clinical data of patients with hepatic failure

Patient No.	Sex	Age (yr)	TBIL ( $\mu\text{mol/L}$ )	DBIL ( $\mu\text{mol/L}$ )	ALT (U/L)	AST (U/L)	PA (g/L)	ALB (g/L)	CHE (U/L)	PT (s)	PTA (%)
1 II III	Male	23	507.4	442.9	183	390	30	26.1	1.6	19	34.7
2 I II III	Male	61	590.9	481.2	779	787	12	25	1.2	14.5	46.4
3 I	Male	69	179.2	136.7	5056	4754	45	34.6	3.3	16.9	34.2
4 I II III	Female	21	636.4	437.5	355	611	12	31.8	1.1	65.7	4.3
5 I II III	Female	44	993.5	775.4	166	451	29	25	0.9	20.1	31.8
6 I II	Male	45	711.4	603.4	409	209	30	28.4	1.4	16.6	43
7 I II III	Male	57	639.9	494.2	2100	2820	9	33.9	1.7	28.2	18.9
8 I	Female	58	443.1	302.5	689	1493	15	27.8	3.1	21.4	23.8
9 I II III	Male	36	759.8	541.9	319	278	27	29.7	1.7	25.3	18.4
10 I III	Male	79	506.8	428.7	329	548	4	30	1.4	15.7	38.9

I ascites, II dead, III hepatic encephalopathy; patient number 1, 5, 6, 7, 9 and 10 with underlying chronic liver disease, such as chronic hepatitis B, autoimmune hepatitis, alcoholic hepatitis. Patient number 4, 6, 7, 8 and 10 were positive for hepatitis E virus (HEV) RNA. Patient number 1, 2, 4, 6, 8, 9 and 10 were positive for anti-HEV IgM, and patient number 3, 4, 5, 7 and 8 were positive for anti-HEV IgG. All biochemical indices of liver function were recorded at peak level during treatment at our hospital. Because some patients were treated at other hospitals at early period of illness, the biochemical indices of liver function, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) may not represent the actual peak level. TBIL: Total bilirubin; DBIL: Direct bilirubin; PA: Pre-albumin; ALB: Albumin; CHE: Cholinesterase; PTA: Prothrombin time activity; PT: Prothrombin time.

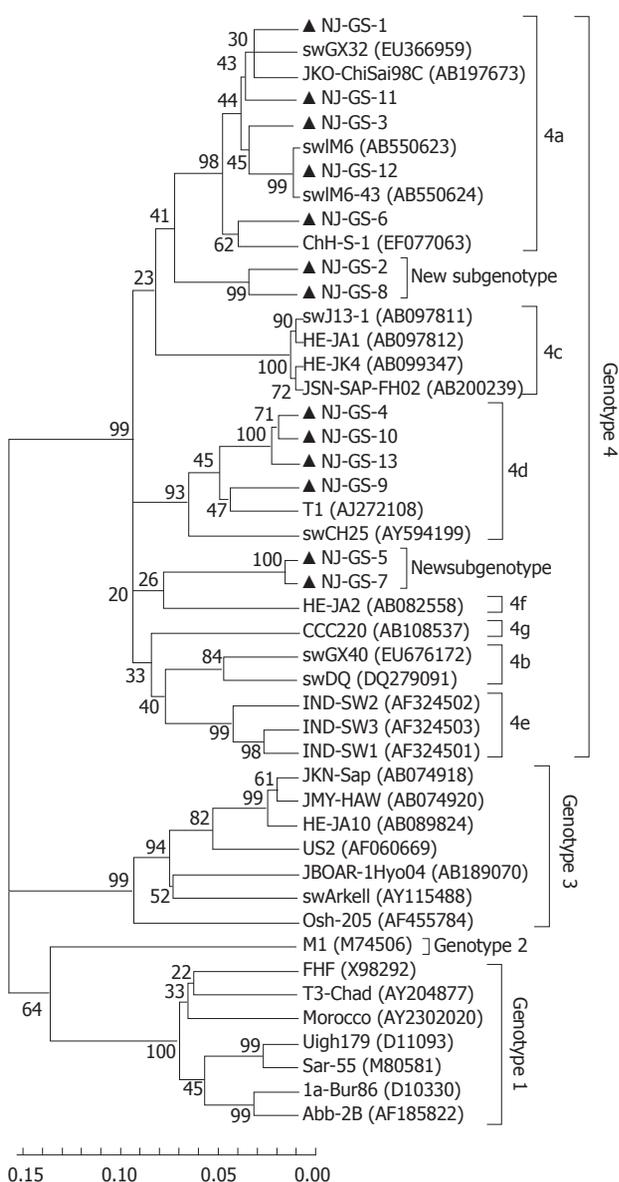


Figure 1 Phylogenetic tree based on 286 bp of open reading frame 2 of hepatitis E virus genotypes 1-4, 13 human sequences. Human hepatitis E virus isolated in this study were signed with solid triangle.

## RESULTS

### Clinical data of patients with liver failure

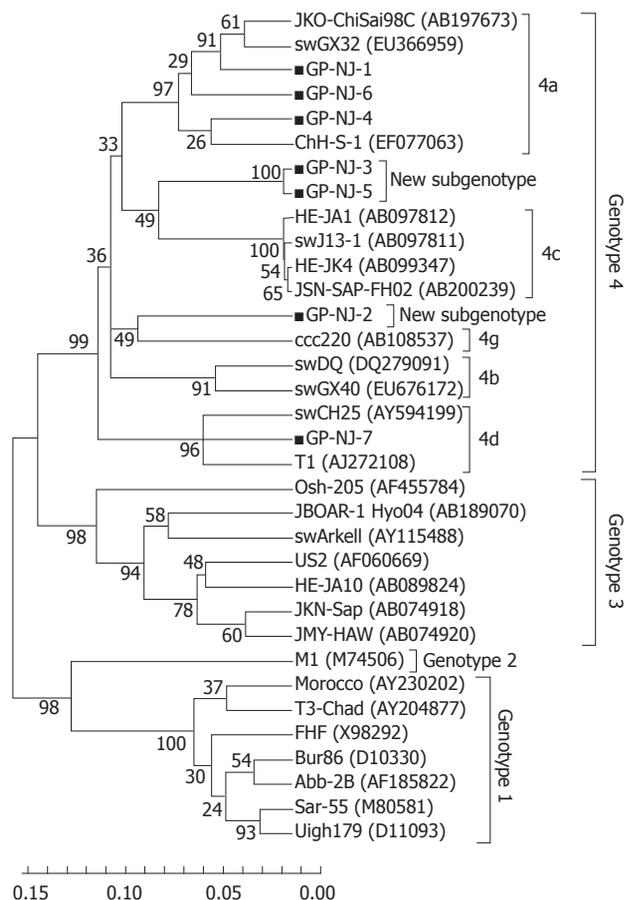
The clinical data of patients with liver failure is summarized in Table 1. Among 62 cases, 10 developed liver failure (4 with acute liver failure and 6 with acute-on-chronic failure), 5 with CLD died of acute-on-chronic liver failure, and one 21-year-old female patient died of acute liver failure.

### Detection of anti-hepatitis E virus and hepatitis E virus RNA

Out of 62 serum samples, 33 were positive for anti-HEV IgM but negative for anti-HEV IgG, while 23 were positive only for anti-HEV IgG, 6 were positive for both IgG and IgM. The overall positivity rate for HEV RNA was 21.0% (13/62).

### Phylogenetic analysis of hepatitis E virus genome isolated from patients

HEV cDNA was amplified from 13 serum samples using primer S4, and the 365nt PCR products of partial ORF2 sequences were determined. The 13 isolated strains were designated as GS-NJ-1 to GS-NJ-13 (GenBank accession numbers JF309208 - JF309220). These 13 isolates shared 82.1%-98.0% nucleotide homology, and had identities of 74.7%-81.0%, 75.3%-78.6%, 75.3%-80.0% and 82.1%-96.1% with the corresponding regions of HEV genotypes 1-4, respectively. Phylogenetic analysis based on partial ORF2 (286 bp) showed that the 13 sequences could be clearly grouped into four main clades (Figure 1), one of which consisted of 4 HEV isolates sharing an 88.4%-97.2% identity with HEV subtype 4d. The second clade included 5 isolates sharing a 90.6%-96.1% identity with HEV subtype 4a. The third clades were formed by GS-NJ-5 and GS-NJ-7 sharing a 98.0% identity with each other and 82.1%-87.3% nucleotide homology with subtypes 4a-4g. The last clade contained 2 isolates sharing a 94.2% identity with each other and 82.5%-89.0% identity with subtypes 4a-4g. Phylogenetic tree showed that both the last two branches were formed individually, separat-



**Figure 2** Phylogenetic tree based on 243 bp of open reading frame 1 of hepatitis E virus genotypes 1-4, 7 human sequences. Human hepatitis E virus isolated in this study were signed with solid square.

ing from the established subgenotypes 4a-4g, which indicated that they may belong to novel subgenotypes. The phylogenetic analysis based on partial ORF1 sequences confirmed the genotyping results (Figure 2).

### Sequence analysis of partial hepatitis E virus ORF1

To exclude the possibility of contamination and confirm the results of phylogenetic analysis based on partial ORF2 sequences, 13 positive samples were amplified using ORF1 primers (P primer) and 11 were positive. Sequence analysis of partial HEV ORF1 fragments showed that there were 7 groups, designated as GP-NJ-1 to GP-NJ-7 with GenBank accession numbers JF309201-JF309207. The 7 strains shared an 82.0%-99.1% sequence identity with each other, and had identities of 73.2%-78.1%, 75.3%-79.0%, 74.0%-82.7% and 80.6%-95.0% with the corresponding regions of HEV genotypes 1-4, respectively. Phylogenetic analysis (Figure 2) confirmed the subtyping result based on partial sequences of ORF2.

## DISCUSSION

In China, a substantial proportion of people had serological evidence of prior HEV exposure but no disease<sup>[17]</sup>. Moreover, according to the national investigation of 2002,

the positive rate of HBsAg was 9.09% in persons over 3 years of age and most of the HBsAg carriers were asymptomatic and under recognized. During the long period of CHB, there was a chance for patients to be sporadically superinfected by HEV. Studies had demonstrated that patients with CLD coinfecting with HEV had more severe liver diseases with higher rates of compensated cirrhosis, hepatic failure, and complications<sup>[13,14]</sup>. In present study, 10 of 62 patients suffered from hepatic failure and 6 patients died (1 died of acute liver failure and 5 died of acute-on-chronic liver failure) (Table 1). Therefore, all susceptible people, especially the patients with CLD should take appropriate strategies to decrease the incidence of HEV infection, such as consumption of boiled water and well-cooked food and hand washing with soap.

As many studies reported, HEV was transmitted primarily by fecal-oral route, usually *via* the consumption of contaminated water or food. However, recent investigations have not consistently found well-defined water sources of HEV, suggesting other possible modes of transmission<sup>[18,19]</sup>. Transmissions through blood transfusions, mother-to-fetus, and person-to-person were also reported<sup>[20,21]</sup>, but these routes of transmission were not thought to be frequent. In this study, a substantial proportion of patients had not consumed any raw meat in the recent past, and no clear source of infection was found, raising the suspicion of transmission through contaminated water or food. It is worth mentioning that in eastern China, where there are many rivers, water are usually contaminated by domestic pig feces and used to fertilize crops without special treatment. As HEV was isolated frequently from swine feces in most parts of the world, people who drink unboiled water or inadequately cooked food may be infected by HEV. Therefore, clinicians should be vigilant and should consider hepatitis E in the differential diagnosis of unexplained jaundice and clinical indications, such as the lack of evidence for other causes of abnormal liver failure, should increase the threshold for testing for HEV infection.

Phylogenetically, sequence analysis of partial ORF2 and ORF1 showed the highest identity with genotype 4, and phylogenetic tree conformed that all HEV strains belonged to genotype 4 and could further subdivided into 4 subgenotypes (Figures 1 and 2). These results indicate that patients in eastern China are currently infected with divergent genotype 4 HEV strains that may be indigenous; genotype 4 has emerged as the predominant genotype in this region with at least 4 subgenotypes. Additionally, further phylogenetic analysis showed a very close relationship and a high nucleotide identity between human and swine HEVs by blasting the partial human HEV sequences in Genbank (Table 2). The GS-NJ-12 strain shared a 100% nucleotide identity with two swine HEVs (swIM6-43 and swIM6-41) isolated from Inner Mongolia, China. Moreover, all swine HEVs showing the highest nucleotide identities comparable with human HEVs were isolated from different areas of mainland China, indicating that sporadic hepatitis E was acquired domestically,

**Table 2** Nucleotide homology between human hepatitis E virus and swine hepatitis E virus

Human HEV	Swine HEV	Identity (%)	Coverage (%)
GP-NJ-1	CHN-XJ-SW36 (FJ775168)	96	100
GP-NJ-2	swCH31 (DQ450072)	96	100
GP-NJ-3	CHN-XJ-SW16 (GQ306000)	99	100
GP-NJ-4	CHN-XJ-SW10 (FJ775167)	93	100
GP-NJ-5	CHN-XJ-SW16 (GQ306000)	99	100
GP-NJ-6	CHN-XJ-SW61 (FJ775170)	93	100
GP-NJ-7	bjsw1 (GU206559)	97	100
GS-NJ-1	CHN-XJ-SW36 (FJ775175)	95	100
GS-NJ-2	swSH02 (DQ450074)	96	99
GS-NJ-3	swIM8-4 (AB550626)	95	97
GS-NJ-4	KMsw-3 (HQ008864)	97	98
GS-NJ-5	CHN-XJ-SW16 (GQ306004)	96	100
GS-NJ-6	CHN-XJ-SW10 (FJ775174)	95	100
GS-NJ-7	CHN-XJ-SW16 (GQ306004)	95	100
GS-NJ-8	swSH02 (DQ450074)	95	98
GS-NJ-9	bjsw1 (GU206559)	97	99
GS-NJ-10	KMsw-3 (HQ008864)	98	98
GS-NJ-11	CHN-SJ-SW36 (FJ775175)	95	100
GS-NJ-12	swIM6-43 (AB550624)	100	97
GS-NJ-13	KMsw-3 (HQ008864)	97	98

HEV: Hepatitis E virus.

and swine may be a principal risk factor for occurrence of sporadic hepatitis E.

Among 62 serum specimens, 13 samples had positive result of HEV RNA using ORF2 primers. The ORF1 degenerate primer (P primer) set amplified 11 of 13 HEV sequences successfully, and 2 specimens positive for S primer were negative for P primer. While both primer sets could amplify human and animal HEV successfully<sup>[6,10,17,22]</sup>, multiple nucleotide substitutions in the primer-binding regions or a few bases mismatch in 3' end of primers, the narrow window of HEV incubation, low HEV RNA titer in sera and "false-positive" results from cross-reactivity with unknown antigens may result in a low detection rate of HEV RNA. Because the window for HEV diagnosis may be narrow, sample collection from the patients in due course is considered to be the key for HEV RNA detection.

Up until now, hepatitis E is diagnosed by detecting viral RNA in serum and/or feces during the incubation period or early acute phase of disease, or, more commonly, by demonstrating IgM anti-HEV or a rising titer of IgG anti-HEV in the serum during the late acute phase or convalescent phase of the illness<sup>[16]</sup>. However, there are still problems with the enzyme immunoassays used to detect current or previous HEV exposure. Commercial assays vary markedly in their sensitivity and specificity<sup>[14,23]</sup>. Recently, the Wantai kit used in this study was proved to be more sensitive than GeneLab kit (98% vs 56%)<sup>[24]</sup>, which has a high specificity for diagnosis of acute infection of HEV<sup>[25,26]</sup>. Detection of rising IgG or RNA was considered diagnostic with a specificity of 100%; the specificity of IgM was found to be 99.4%<sup>[15]</sup>. Yet, based on previous studies, it is still very hard for researchers to detect HEV RNA in all serum specimens positive for

anti-HEV. In present study, because some patients did not seek medical care in the early stages of their illness, and the period of viraemia is short<sup>[3]</sup>, only 5 of 10 cases with hepatic failure were confirmed by the presence of HEV RNA in serum samples. Although some cases negative for HEV RNA might be misdiagnosed as hepatitis E, 5 patients with liver failure (No. 4, 6, 7, 8, 10) were confirmed by detecting serum HEV RNA using S primers (designated as GS-NJ-11, GS-NJ-4, GS-NJ-6, GS-NJ-9, GS-NJ-5) and P primers (designated as GP-NJ-6, GP-NJ-7, GP-NJ-4, GP-NJ-7, GP-NJ-2). Patients 4 and 8 with no underlying liver diseases suffered from acute liver failure, and patient 4 died. Furthermore, 5 (No. 1, 5, 6, 7 and 9) out of 6 patients with CLD died of hepatic failure and relative complications, suggesting that it is imperative to develop a reliable hepatitis E vaccine.

In conclusion, this study presented the first finding that HEV genotype 4 could result in acute liver failure and acute-on-chronic liver failure. Genotype 4 was the dominant strain among the patients involved in this study, and there were at least 4 subgenotypes (4a, 4d and 2 new subgenotypes) prevalent in eastern China. Phylogenetic analysis showed a very close relationship between human and swine HEV, suggesting that swine may be a principal risk factor for occurrence of hepatitis E in eastern China. However, the implication for subgenotype classification and other issues such as the relationship between different genotypes/subtypes and different modes of transmission, pathogenicity, possibility of cross species transmission are still indistinct and remain to be understood.

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## COMMENTS

### Background

Hepatitis E, caused by the hepatitis E virus (HEV), is the most important cause of acute viral hepatitis in adults throughout Asia, the Middle East and Africa where the sanitation conditions are usually substandard. HEV genotypes 1 and 2, which have similar epidemiological and sporadic features, can result in acute hepatitis, acute liver failure, and acute-on-chronic liver failure. However, HEV genotype 3 and 4, which were generally considered to cause acute, self-limiting illness followed by a complete recovery, seems to be less virulent for humans than genotypes 1 and 2 and have not been implicated in causing severe liver diseases. However, there were few reports on the association between genetic characteristics and pathogenicity of HEV infection. In addition, whether genotype 3 and 4 HEV could induce liver failure in normal population and patients with chronic liver disease (CLD) is still unclear.

### Research frontiers

HEV is widespread in swine and is likely to be endemic in many developed/developing countries. There is a very close relationship between human and swine HEV by phylogenetic analysis of partial/full-length genomic sequence. Accumulated evidences support the hypothesis that hepatitis E is a zoonosis, and swine may be a principal risk factor for occurrence of hepatitis E in many areas. HEV infection in healthy individuals are associated with a mortality rate of 0.04%-4%. Some studies have reported a high prevalence of HEV antibodies in patients with CLDs and others have suggested that cirrhotics were prone to HEV infection, and superinfection with HEV in patients with underlying CLD

can cause severe hepatic decompensation, leading to increased morbidity and mortality.

### Innovations and breakthroughs

In China, superinfection with genotype 4 HEV in patients with underlying CLD, especially in patients with chronic hepatitis B, can cause severe hepatic decompensation, leading to increased morbidity and mortality. This study presented the first finding that genotype 4 HEV can induce liver failure in normal population and patients with CLD. The GS-NJ-12 strain isolated from a case of sporadic hepatitis E shared a 100% nucleotide identity with two swine HEVs (swIM6-43 and swIM6-41) isolated from Inner Mongolia, China. Moreover, all swine HEVs showing the highest nucleotide identities comparable with human HEVs were isolated from different areas of mainland China, indicating that sporadic hepatitis E is acquired domestically, and swine may be a principal risk factor for occurrence of sporadic hepatitis E in eastern China.

### Applications

This study found that the dominant genotype of HEV prevalent in eastern China was genotype 4, which can induce liver failure in normal population and patients with CLD and swine may be a principal risk factor for occurrence of sporadic hepatitis E. Therefore, a standardized management of swine in stock farm is the key to prevent HEV transmission from swine to human.

### Peer review

The work investigated the genetic characteristics of the HEV in Eastern China and assessed the potential risk factors. Among acute infected patients, they found a seroprevalence of HEV RNA in 21%. The isolated HEV strains shared a high percentage of nucleotide homology with a HEV strain isolated from swine. The manuscript demonstrates a well performed study which hints one more time the direction that HEV may be acquired by the contact to swines/wild boars.

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## B7-H1 expression is associated with expansion of regulatory T cells in colorectal carcinoma

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### Abstract

**AIM:** To investigate the expression of B7-H1 in human colorectal carcinoma (CRC) to define its regulating effects on T cells in tumor microenvironment.

**METHODS:** One hundred and two paraffin blocks and 33 fresh samples of CRC tissues were subject to this study. Immunohistochemistry was performed for B7-H1 and CD3 staining in CRC tissues. Ficoll-Hypaque density gradient centrifugation was used to isolate peripheral

blood mononuclear cells of fresh CRC tissues; flow cytometry and immunofluorescence staining were used for detection of regulatory T cells. Data was analyzed with statistical software.

**RESULTS:** Costimulatory molecule B7-H1 was found strongly expressed in CRC tissues, localized in tumor cell membrane and cytoplasm, while weak or none expression of B7-H1 was detected in paired normal colorectal tissues. Meanwhile, CD3 positive T cells were found congregated in CRC tumor nest and stroma. Statistic analysis showed that B7-H1 expression level was negatively correlated to the total T cell density in tumor nest ( $P < 0.0001$ ) and tumor stroma ( $P = 0.0200$ ) of 102 cases of CRC tissues. Among the total T cells, a variable amount of regulatory T cells with a clear Foxp3<sup>+</sup> (fork-head box P3) staining could be detected in CRC tissues and patients' blood. Interestingly, in the 33 samples (15 cases of B7-H1<sup>high</sup> CRC tissues and 18 cases of B7-H1<sup>low</sup> CRC tissues) of freshly isolated mononuclear cells from CRC tissues, the percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells were found remarkably higher in B7-H1<sup>high</sup> CRC tissues than in B7-H1<sup>low</sup> CRC tissues ( $P = 0.0024$ ,  $P = 0.0182$ ), indicating that B7-H1 expression was involved in proliferation of regulatory T cell. No significant difference was found in CRC peripheral blood ( $P = 0.0863$ ,  $P = 0.0678$ ). PD-1 is the specific ligand for B7-H1 pathway transferring inhibitory signal to T cell, which is expressed by activated T cell. Our further analysis of PD-1 expression on T cells in CRC tissues showed that conventional T cells (CD4<sup>+</sup>Foxp3<sup>-</sup>/CD8<sup>+</sup>Foxp3<sup>-</sup>), which was thought to contribute to the anti-tumor immune response, highly expressed PD-1; while regulatory T cells (CD4<sup>+</sup>Foxp3<sup>+</sup>/CD8<sup>+</sup>Foxp3<sup>+</sup>) almost failed to express PD-1. The average percentage of PD-1 expression on regulatory T cells was significantly higher than the percentage of PD-1 on conventional T cells (CD4<sup>+</sup>Foxp3<sup>-</sup> T cell,  $P < 0.0001$ ; CD8<sup>+</sup>Foxp3<sup>-</sup> T cell,  $P < 0.0001$ ). The diverse expression of PD-1 might lead to different fate of T cell subsets in B7-H1 over-expression CRC tumor microenvironment.

**CONCLUSION:** B7-H1 expression in tumor cells can inhibit the conventional T cell proliferation in tumor microenvironment through the PD-1 expression on conventional T cells.

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**Key words:** Costimulatory molecule; B7-H1; PD-1; Regulatory T cell; Colorectal carcinoma

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## INTRODUCTION

Tumor genesis is associated with a wide array of both genetic and epigenetic changes. Although host immune surveillance may prevent tumor outgrowth during the earliest stages of tumor growth, locally invasive or metastatic tumors must evade host immunity<sup>[1]</sup>. Immune escape is not merely a passive process of immune evasion but an active process in which tumor cells, stromal cells and immune cells within the tumor microenvironment actively suppress the antitumor immune response. Both myeloid-derived cells and lymphocyte subsets, most notably regulatory T cells (Treg), collaborate with their malignant counterparts to suppress the host immunity<sup>[2,3]</sup>. Recent evidence showed that experimental depletion of Tregs improves immune-mediated tumor clearance and enhances the response to immune-based therapy<sup>[4,5]</sup>. Tregs have been shown to suppress tumor-specific T cell immunity and therefore may contribute to the progression of human tumors<sup>[6,7]</sup>. Furthermore, tumor Tregs are associated with a reduced survival of the patients with ovarian carcinoma and brain tumors<sup>[8,9]</sup>. In contrast, it has been found in Hodgkin lymphoma that decreased number of infiltrating Foxp3<sup>+</sup> cells in conjunction with increased infiltration of cytotoxic T lymphocytes predicts an unfavorable clinical outcome<sup>[10]</sup>. Although previous studies have suggested that tumors could induce CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs from naïve CD4 T cells in the absence of thymus, the cellular and molecular mechanisms for that, however, are still not well understood<sup>[11,12]</sup>. Optimal activation of antigen-specific lymphocytes requires specific antigen recognition by lymphocytes and costimulatory signals<sup>[13]</sup>. Up to date, a cohort of important costimulatory molecules, including B7 family ligands, and those which interact with known or unknown receptors, has been identified, namely B7-1 (CD80), B7-2 (CD86), B7-H1 (PD-L1), B7-DC (PD-L2), B7-H2 (ICOS ligand), B7-H3 and B7-H4 (B7x, B7-S1),

which essentially contribute to the T cell activation and tolerance<sup>[14-19]</sup>. B7-H1 was identified in 1999 as a member of B7 family that was described to negatively regulate T cell function by engagement with PD-1, a CD28 family member receptor. Besides antigen-presenting cells, B7-H1 mRNA was found in a variety of nonlymphoid parenchymal organs, including the heart, placenta, skeletal muscle, and lung<sup>[15]</sup>. B7-H1 was thought to inhibit T cell growth and cytokine production by ligation of the PD-1 receptor<sup>[20]</sup>, which is expressed on activated T and B cells<sup>[15,21]</sup>. Zou *et al*<sup>[22]</sup> reported the presence of B7-H1 protein by immunohistochemistry in a wide range of human cancers. Tumor-associated B7-H1 induced apoptosis of effector T cells and was thought to contribute to immune evasion by cancer. Other studies indicated that blockade of B7-H1 enhanced tumor immunity but had no direct effect on tumor cells<sup>[23-25]</sup>. To make the situation more complicated, B7-H1 can also function as a receptor to transmit signals to T cells and tumor cells<sup>[26,27]</sup>. In summary, B7-H1 can act as both ligand and receptor to execute immuno-regulatory functions. B7-H1 was reported to be involved in the induction of Tregs, dysfunctional dendritic cells that expressed up-regulated B7-H1 may lead to the generation of Tregs, and *in vivo* blocking of B7-H1 signaling abolished the conversion in a tumor-induced Treg conversion model<sup>[28]</sup>. Whether the tumor-associated B7-H1 could affect the Tregs generation in the tumor microenvironment deserves further exploration.

Colorectal carcinoma (CRC) is one of the most frequent malignancies worldwide, its incidence and mortality are especially high in Western developed counties, and it is the second leading cause of cancer-related death<sup>[29]</sup>. CRC is a multi-pathway disease since numerous pathological factors and polygene transformation are involved in its oncogenesis and progression. Within recent decades, varieties of therapeutic strategies including conventional surgery, chemotherapy, radiotherapy and immunotherapy, or even combination of these therapies have been available in the treatment of CRC patients. However, these therapies yielded different outcomes due to different physical conditions of the patients, which shaped the tumor microenvironment with immune suppressions<sup>[30-32]</sup>. Therefore, it is critical for clinicians to perform further analysis of the immune suppression and establish individualized strategy for CRC patients.

In the present study, we performed immunohistochemistry to characterize the B7-H1 expression in human CRC and examined its effect on infiltrating T lymphocytes in tumor tissues. The regulatory T cells were detected in the tumor tissues and peripheral blood of CRC patients, and the relationship between the B7-H1 expression and Tregs population was analyzed. The mechanism of regulatory T cell expansion related to B7-H1-PD-1 signal was also investigated.

## MATERIALS AND METHODS

### Patients

For B7-H1 expression and T cell infiltration analysis, 102

patients with CRC who underwent surgery from May 2004 to December 2007 were included in the present study. No patient received pre-operative chemotherapy or radiotherapy. The paraffin blocks of tumor tissues were assembled from the archival collections of the Department of Pathology, and all 102 specimens were identified as CRC by hematoxylin and eosin (HE) staining. For regulatory T cell and B7-H1 expression analysis, 33 CRC patients who underwent surgery from January 2008 to July 2009 were subjected to this study. No patient received pre-operative chemotherapy or radiotherapy. The freshly removed tumor tissues were identified as colorectal carcinoma by pathologist according to the HE staining. The blood samples were collected from the 33 CRC patients before the surgery by venipuncture. Thirty-three normal tissues from autologous non-malignant portion of colon or rectum were resected surgically for the analysis as well, and used as the normal tissue control.

The specimens were collected from the Third Affiliated Hospital and the Fourth Affiliated Hospital of Soochow University, China, with the approval of the Ethic Committees of these hospitals.

### Immunohistochemistry

Immunohistochemistry was performed using the Dako Elivision™ according to the manufacturer's instructions. Both tumor tissues and non-malignant tissues were fixed with formalin, and embedded in paraffin wax. Before immunohistochemical staining, 3- $\mu$ m-thick consecutive sections were cut by microtome, dewaxed in xylene and rehydrated through graded ethanol solutions. Antigens were retrieved by heating the tissue sections at 100 °C for 30 min with citrate solution (10 mmol/L, pH 6.0, for CD3 antigen retrieval) or ethylenediaminetetraacetic acid solution (1 mmol/L, pH 8.0, for B7-H3 antigen retrieval). Sections were cooled down and immersed in 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity, and then rinsed in phosphate-buffered saline (PBS) for 5 min, blocked with 5% bovine serum albumin (BSA) at room temperature for 15 min, and incubated with primary antibodies against CD3 (Maixin Biotechnology Limited Corporation, Fuzhou, China) and B7-H1<sup>[21]</sup>, respectively, at 4 °C overnight. Negative controls were performed by replacing the specific primary antibody with PBS. The sections were then rinsed in PBS for 5 min for 3 times and were followed by incubation with HRP-labeled goat anti mouse/rabbit secondary antibody (Dako, Glostrup, Denmark). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. The sections were dehydrated, cleared and mounted.

### Evaluation of B7-H1 and CD3 immunohistochemical staining

Two independent observers who blinded to the clinicopathological parameters of the patients assessed the immunohistochemical staining sections. The B7-H1 immunostaining intensities were scored according to a scale as grade 0, negative; grade 1, weakly positive; grade 2, moderately positive; grade 3, strongly positive. The nega-

tive means no tumor cells showing positive immunostaining. Sections were considered as positive when the tumor cells showed cytoplasmic or membranous B7-H1 immunostaining with proper intensities and were extended as grades 1, 2 and 3. The sections of grade 0 and grade 1 were classified as low expression group, and other sections of grade 2 and grade 3 were classified as high expression group.

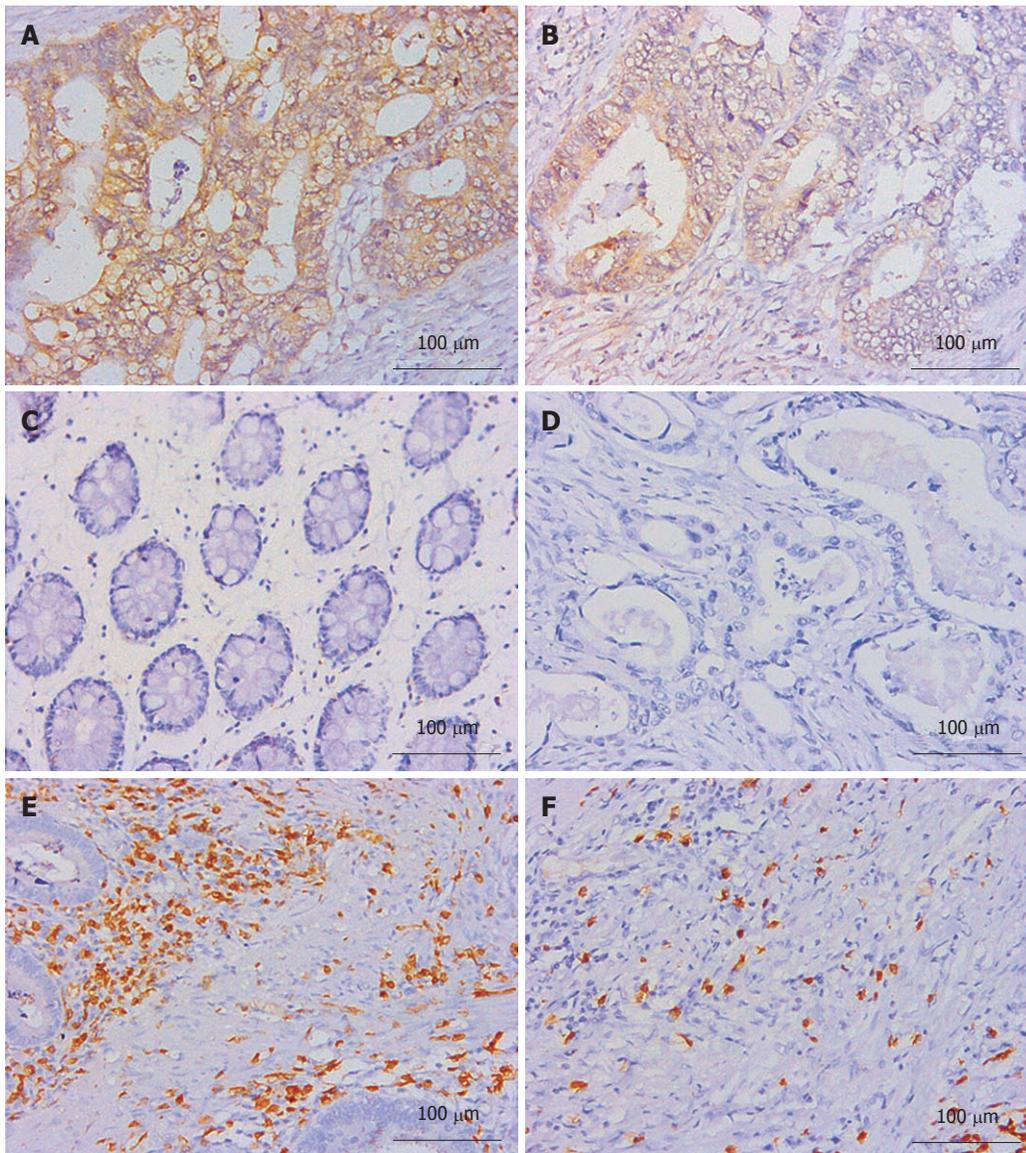
Infiltrating T lymphocytes in both tumor stroma and tumor nest were determined according to the CD3 immunolabeling. First, the infiltrating T lymphocytes in tumor stroma were examined at low magnification ( $\times 40$ ) and categorized according to the density as: grade 0, scanty; grade 1, moderate infiltration; grade 2, abundant infiltration; grade 3, the most abundant infiltration. The group of grade 0 and grade 1 was defined as low infiltration group, and another group of grade 2 and grade 3 was defined as high infiltration group. Second, the infiltrating T lymphocytes in tumor nest were counted as follows: five areas in tumor nest with the most intense infiltrating T lymphocytes were selected at low magnification ( $\times 40$ ), then the infiltrating T lymphocytes were counted and recorded at high power field (HPF,  $\times 200$  magnification). Results from the five areas were averaged and used in the statistical analysis. In the present study, the sections with the infiltrating T lymphocytes in tumor nest of less than 60 per HPF were defined as low infiltration group, and other sections with the infiltrating T lymphocytes in tumor nest of more than 60 per HPF were defined as high infiltration group. The cutoff point of 60 T lymphocytes per HPF for low/high infiltration assessment in tumor nest was set at the median value of the entire sections.

### Cell isolation from fresh tumor tissues and peripheral blood

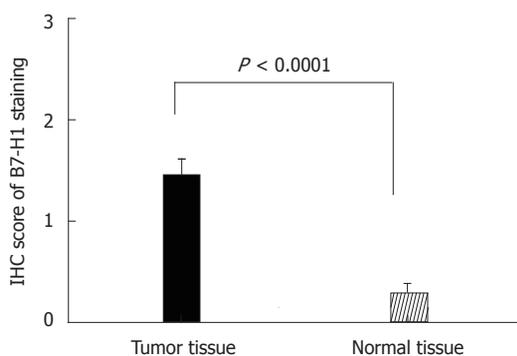
Fresh tumor specimens were gently minced on a wire mesh screen to obtain a cell suspension. The cell suspension was centrifuged over Ficoll-Hypaque (Amersham Biosciences, Sweden) at 1400 r/min for 25 min. After density gradient centrifugation, the mononuclear cells were collected and washed with RPMI 1640 media (Gibco, United States) containing 50 g/mL fetal bovine serum (Hyclone, United States) and 10 g/mL penicillin/streptomycin (Sigma-Aldrich, United States). Peripheral blood mononuclear cells were also isolated with Ficoll-Hypaque density gradient centrifugation, and the isolated mononuclear cells were subjected to the analysis immediately.

### Flow cytometry and intracellular staining

Cells were washed in PBS containing 5 g/mL BSA and incubated with the specific fluorochrome-conjugated antibodies identifying surface molecules on T cells for 30 min at 4 °C. The antibodies included CD4-FITC, CD8-FITC (Beckman Coulter, United States) and PD-1-PE (eBioscience, United States). For intracellular staining, washed cells were fixed with Foxp3 Fixation/Permeabilization Solution (eBioscience, United States) at room temperature, then incubated with PE-cy5 conjugated anti-Foxp3 anti-



**Figure 1** B7-H1 and CD3 immunostaining of colorectal carcinoma tissues. A and B: B7-H1 immunostaining in colorectal carcinoma tissues (A: Magnification 400 ×; B: Magnification 200 ×); C: B7-H1 immunostaining in normal colorectal tissues; D: Negative control in colorectal carcinoma tissues; E and F: CD3 stained infiltrating T lymphocytes (E: Tumor stroma; F: Tumor nest).



**Figure 2** B7-H1 expression level in colorectal carcinoma tissues and adjacent normal tissues from 33 patients evaluated by immunohistochemistry. IHC: Immunohistochemistry.

body (eBioscience, United States) at room temperature in the dark for 30 min. Labeled cells were re-suspended in

0.5 mL cell staining buffer, and then were analyzed with flow cytometry and the Beckman-Coulter's Expo32 Multicom software (Beckman Coulter, United States). Isotype controls were done for each staining.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software package (GraphPad Software, United States). Paired or unpaired Student's *t* test, Wilcoxon signed rank test, and the Pearson  $\chi^2$  test were used where appropriate. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### **B7-H1 expression was found in colorectal carcinoma tissues but not in normal colorectal tissues**

Thirty-three cases of paired CRC tissues and adjacent nor-

Infiltrating T lymphocytes in colorectal carcinoma tissues	Cases	B7-H1 expression		$\chi^2$	P value
		Low	High		
Tumor stroma				9.549	0.0200
Low infiltration (scanty and moderate)	68	24	44		
High infiltration (abundant and the most abundant)	34	23	11		
Tumor nest				17.4	<0.0001
Low infiltration (less than 60 T lymphocytes per HPF)	51	13	38		
High infiltration (more than 60 T lymphocytes per HPF)	51	34	17		

HPF: High power field.

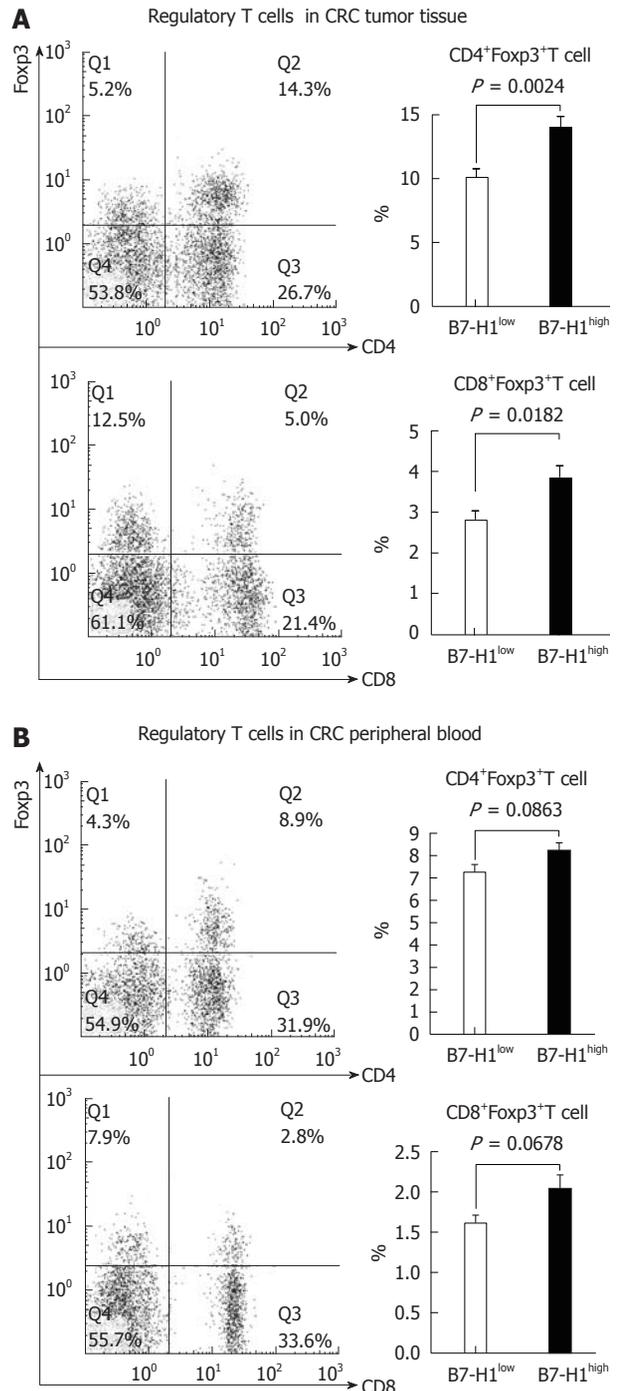
mal tissues resected surgically were used to investigate the B7-H1 expression. As we described in previous works in human gastric carcinoma<sup>[21]</sup>, B7-H1 was also found strongly expressed in tumor cells, localized in the membrane and cytoplasm (Figure 1A and B). Based on the immunohistochemical scores, 33 cases of tumor tissues showed B7-H1 expression (Score 1, 2 or 3), and 15 cases of tumor tissues had high B7-H1 expression (Score 2 or 3). In the normal tissues, 3 cases showed low B7-H1 expression (Score 1), and none showed high B7-H1 expression (Figure 1C). According to the statistical analysis, B7-H1 expression level in CRC tumor tissues was higher than in the adjacent normal colorectal tissues ( $P < 0.0001$ , Figure 2).

**B7-H1 expression level was negatively correlated to the total T cell infiltration density**

CD3 staining was considered as T cell labeling, and the number of positive cells represented the total T cell infiltration density<sup>[30]</sup>. CD3 positive T cells were found congregated in CRC tumor and stroma (Figure 1E and F). One hundred and two cases of paraffin embedded CRC tissues were used to study the B7-H1 expression and the T cell infiltration. As shown in Table 1, tumor cell B7-H1 expression was negatively and significantly correlated to the density of CD3 positive T cells in tumor nest ( $P < 0.0001$ , Table 1) and tumor stroma ( $P = 0.0200$ , Table 1). Thus, the data further implied an important role of B7-H1 in suppressing T cell-based cellular immune surveillance of CRC.

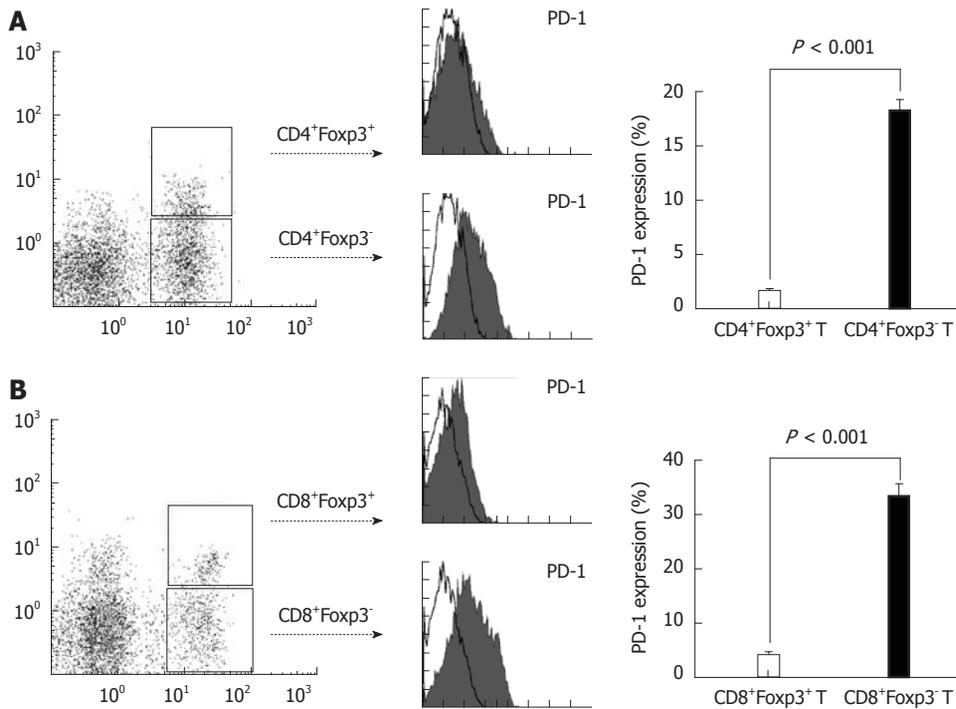
**B7-H1<sup>high</sup> colorectal carcinoma tissues were infiltrated with elevated numbers of CD4<sup>+</sup>/CD8<sup>+</sup> regulatory T cells**

To evaluate the effect of tumor B7-H1 on intratumoral regulatory T cells, we first characterized the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells and CD8<sup>+</sup>Foxp3<sup>+</sup> T cells. Mononuclear cells isolated from resected specimens of CRC patients were stained with multicolor-labeled antibodies and analyzed by flow cytometry. The peripheral blood mononuclear cells were also analyzed in the regulatory cell population. Among the CD4<sup>+</sup> or CD8<sup>+</sup> T cells, a population with a clear Foxp3<sup>+</sup> could be detected (Figure 3).



**Figure 3** An elevated CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>Foxp3<sup>+</sup> T cell amount observed in B7-H1<sup>high</sup> colorectal carcinoma tissues. Mononuclear cells were harvested from fresh tumor tissues (A) and the peripheral blood (B) of the same colorectal carcinoma patient. The percentage of the Foxp3<sup>+</sup> T cells was determined by fluorescence-activated cell sorting analysis. A: The population of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>Foxp3<sup>+</sup> T cells was increased remarkably in B7-H1<sup>high</sup> colorectal carcinoma (CRC) tissues compared with B7-H1<sup>low</sup> CRC tissues; B: In peripheral blood, there was no significant diversity of regulatory T cells between the B7-H1<sup>low</sup> and B7-H1<sup>high</sup> CRC patients.

A variable amount of regulatory T cells was found in CRC tissues and patients' blood. Next, to determine the correlation between the tumor cell B7-H1 expression and regulatory T cells, we divided the 33 CRC specimens into B7-H1<sup>high</sup> group ( $n = 15$ ) and B7-H1<sup>low</sup> group ( $n = 18$ )



**Figure 4** PD-1 expression on CD4<sup>+</sup>Foxp3<sup>+</sup> or CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells and conventional T cells in colorectal carcinoma tissues. Mononuclear cells were harvested from fresh tumor tissues of the same colorectal carcinoma patient. The PD-1 expression was determined by fluorescence-activated cell sorting analysis, gated on CD4<sup>+</sup>Foxp3<sup>-</sup> or CD8<sup>+</sup>Foxp3<sup>-</sup> T cells. A: CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells could hardly express PD-1 on the cell surface, while PD-1 expression level was significantly higher on the conventional CD4<sup>+</sup>T cells; B: CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells almost failed to express PD-1, while PD-1 expression was significantly higher on the conventional CD8<sup>+</sup>T cells as well.

according to the B7-H1 expression in tumor tissues. The results showed that in the CRC tumor tissues, the percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells and CD8<sup>+</sup>Foxp3<sup>+</sup> T cells in B7-H1<sup>high</sup> group were remarkably higher than in the B7-H1<sup>low</sup> group ( $P = 0.0024$ ,  $P = 0.0182$ ). On the contrary, in the CRC peripheral blood, there was no significant difference of the percentages of regulatory T cells between B7-H1<sup>high</sup> group and B7-H1<sup>low</sup> group ( $P = 0.0863$ ,  $P = 0.0678$ ).

**PD-1 expression was decreased on regulatory T cells and increased on conventional T cells in colorectal carcinoma tissues**

PD-1-B7-H1 ligation has been shown to have an inhibitory effect on T cells<sup>[33]</sup>. However, the effect of this pathway on regulatory T cells infiltrated in tumor tissues has not been investigated. The highly expressed B7-H1 on tumor cells could lead to a low amount of total T cell infiltration, but a high amount of regulatory T cell infiltration in the local tissues, which indicated that the PD-1-B7-H1 pathway could be different in the conventional T cells and regulatory T cells. Therefore, we assessed the surface PD-1 expression on CD4<sup>+</sup>Foxp3<sup>+</sup>/CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells and CD4<sup>+</sup>Foxp3<sup>-</sup>/CD8<sup>+</sup>Foxp3<sup>-</sup> conventional T cells in CRC tissues. We found a high percentage of CD4<sup>+</sup>Foxp3<sup>-</sup>/CD8<sup>+</sup>Foxp3<sup>-</sup> conventional T cells expressing PD-1 on the cell surface (Figure 4,  $P < 0.0001$ ). However, the CD4<sup>+</sup>Foxp3<sup>+</sup>/CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells could hardly express PD-1 (Figure 4,  $P < 0.0001$ ), which could allow the survival of regulatory T cells in B7-H1<sup>high</sup> tumor microenvironment.

**DISCUSSION**

B7-H1, with engagement of either of its receptor, PD-1, plays a critical role in suppressing T cell-based immunity and has emerged as an important mediator of tumor-associated immune suppression. B7-H1 was found in several solid tumors and evaluated as a potent prognostic factor. Our previous work has described the immunosuppressive effects of B7-H1 pathway in gastric carcinoma<sup>[33]</sup>. We demonstrated in this study that B7-H1 was over-expressed in human colorectal carcinoma, whereas B7-H1 was not expressed or expressed at a very low level in normal colorectal tissues. Furthermore, we found that the protein levels of B7-H1 in tumor cells were negatively correlated to the densities of total T cells, suggesting that it may encompass a mechanism of immune suppression in antitumor immunity in human CRC. This part of work is consistent with the previous studies of B7-H1 in other solid tumors, which again emphasize the important role of up-regulated B7-H1 expression in human tumors. Tumors have a specific microenvironment that contains many types of immune cells. Although the potent value of B7-H1 in tumor progression was confirmed repeatedly, the mechanism of tumor-associated B7-H1 involved in the regulation of these immune cells is still poorly understood.

Regulatory T cells have been identified in the lung, gastric and esophageal, ovarian, breast and pancreatic tumor specimens, and Hodgkin lymphoma. It has been suggested that CD4<sup>+</sup>CD25<sup>+</sup>Treg cells are involved in the mediation of antitumor immunity by suppressing tumor-specific T

cell immunity, thereby contributing to the growth of human tumors<sup>[6-10]</sup>. How regulatory T cells could survive and expand in the tumor microenvironment remains unclear, and how to regulate the regulatory T cells in microenvironment would be critical to tumor immunotherapy<sup>[34]</sup>. In the present study, we detected the CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cell population in the CRC tumor tissues and peripheral blood. The percentage of regulatory T cells was significantly elevated in B7-H1<sup>high</sup> CRC tissues, but not in the peripheral blood. These results indicated that the up-regulated B7-H1 expression could lead to a local congregation of regulatory T cells, which might promote the tumor progression. B7-H1 expression was positively related to the regulatory T cell expansion, but negatively related the total T cell infiltration in CRC tissues. This suggested that B7-H1 pathway could have different effects and functions on the subgroups of T cells. B7-H1 pathway could participate in the immune suppressions through multi-regulations of T cells.

T cells are a very diverse lymphocyte population with respective functions in tumor microenvironment. Understanding of their polarization toward stimulatory or inhibitory activity is important to know how they work in diseases<sup>[30,35]</sup>. Regulatory T cells expressing the hallmark forkhead transcription factor 3 (Foxp3) are of therapeutic value in cancer immunotherapy due to their potent immunosuppressive effects. The presence of regulatory T cells was determined by the complex tumor microenvironment. How to decrease the number of regulatory T cells and increase the conventional T cells is critical to obtain a desired outcome of immunotherapy. Here, we assessed the PD-1 expression on T cell subsets to explore the mechanism of B7-H1 pathway in regulation of tumor-infiltrated T cells. The data showed a very interesting phenomenon that conventional T cells (CD4<sup>+</sup>Foxp3<sup>-</sup>/CD8<sup>+</sup>Foxp3<sup>-</sup>) expressed PD-1 on the cell surface at a high level, while regulatory T cells (CD4<sup>+</sup>Foxp3<sup>+</sup>/CD8<sup>+</sup>Foxp3<sup>+</sup>) almost failed to express PD-1. Under these circumstances, the ligation of tumor-associated B7-H1 with PD-1 on conventional T cells would lead to the failure of T cell-mediated anti-tumor effect, and inhibit the conventional T cell proliferation and survival. At the same time, the loss of PD-1 expression could allow the existence and expansion of regulatory T cells, which could further inhibit the conventional T cells. B7-H1 expression in tumor cells could collaborate with its regulation outcomes on T cells, suppress the tumor immune response, and shape the tumor immune escape circumstance.

In conclusion, the inhibitory co-stimulatory molecule B7-H1 expression was found in CRC tissues, but not in normal colorectal tissues. B7-H1 expression in tumor cells could inhibit the conventional T cell proliferation in tumor microenvironment through the PD-1 expression on conventional T cells. Regulatory T cells could barely express PD-1, and consequently gain the survival and expansion in a B7-H1<sup>high</sup> microenvironment. Our works also support the efforts to develop immunotherapeutic approaches targeting B7-H1 pathway for the treatment of CRC.

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## COMMENTS

### Background

Inhibitory co-stimulatory molecules from B7-family have been implicated in suppression of tumor immunity. B7-H1 was found to be over-expressed in many human malignancies, and was significantly correlated to the clinicopathological parameters and prognoses of various human tumors. B7-H1 was suggested to play an important role in tumor immune escape, while the full mechanism of tumor-associated B7-H1 pathway needs further studies.

### Research frontiers

B7-H1 expression in tumor tissues was evaluated as a potent target for tumor immune therapy. The mechanism of B7-H1 pathway was considered involving the suppressive effect on tumor-specific T cell immunity and therefore may contribute to the progression of human tumors. Previous studies have suggested that tumors could induce CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) from naive CD4 T cells in the absence of thymus, while the key molecules and pathway are still unknown. In this study, the authors focus on the significance and function of tumor-associated B7-H1 in Tregs' expansion.

### Innovations and breakthroughs

The mechanism of B7-H1 pathway in tumor microenvironment is commonly considered as decreasing and suppressing of antigen-specific T cells. This article reported for the first time that tumor-associated B7-H1 was involved in the expansion of Tregs in colorectal carcinoma (CRC) tissues, which provided a new strategy for the future researches. Furthermore, B7-H1-PD-1 was found not responsible for the Tregs expansion, which offers a presumption of another receptor for B7-H1 on Tregs.

### Applications

Regulatory T cells are of therapeutic value in cancer immunotherapy due to their potent immunosuppressive effects. How to decrease the number of regulatory T cells and increase the conventional T cells is critical to obtain a desired outcome of immunotherapy. This study supports the efforts to develop immunotherapeutic approaches targeting B7-H1 pathway for immune therapy through regulating conventional and regulatory T cells in CRC.

### Peer review

The authors investigated the immunolocalization of the protein B7-H1 in colorectal cancer tissues using a standardized immunohistochemical approach. Furthermore, they compared the B7-H1 expression with the density of FOXP3 and CD3 immune cell infiltrate. They found that B7-H1 expression in tumor cells inhibits the proliferation of T cells, but allows the survival and expansion of T regulatory lymphocytes. The manuscript is interesting, and well done.

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## Interleukin-8 associates with adhesion, migration, invasion and chemosensitivity of human gastric cancer cells

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### Abstract

**AIM:** To investigate the relationship between Interleukin-8 (IL-8) and proliferation, adhesion, migration, invasion and chemosensitivity of gastric cancer (GC) cells.

**METHODS:** The IL-8 cDNA was stably transfected into human GC cell line MKN-45 and selected IL-8-secreting transfectants. The expression of IL-8 in human GC cell line KATO-III was inhibited by RNA interference. The expressions of mRNA and protein of IL-8 in GC cells were detected by real-time reverse transcription-polymerase chain reaction or enzyme-linked immunosorbent assay (ELISA).

**RESULTS:** The overexpression of IL-8 resulted in an increased cell adhesion, migration and invasion, and

a significant resistance to oxaliplatin in MKN-45 cells. Inhibition of IL-8 expression with small interfering RNA decreased the adhesion, migration and invasion functions and oxaliplatin resistance in KATO-III cells. IL-8 increased NF- $\kappa$ B and Akt activities and adhesion molecules ICAM-1, VCAM-1, and CD44 expression in GC cells.

**CONCLUSION:** Overexpression of IL-8 promotes the adhesion, migration, invasion, and chemoresistance of GC cells, indicating that IL-8 is an important therapeutic target in GC.

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**Key words:** Interleukin-8; Gastric cancer; Adhesion; Migration; Invasion

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### INTRODUCTION

Gastric cancer (GC) is still a serious health problem and remains the second most common type of fatal cancer worldwide<sup>[1,2]</sup>. GC is one of the most aggressive tumors and is frequently associated with lymph node metastasis, peritoneal dissemination and hematogenous metastasis.

Interleukin-8 (IL-8), a cytokine of the CXC chemokine family that was originally classified as neutrophil

chemoattractant, is now reported to play an important role in tumor progression and metastasis in a variety of human cancers<sup>[3]</sup>. It has been suggested that tumor cells produce IL-8 as an autocrine growth factor, which promotes tumor growth, tissue invasion and metastatic spread<sup>[4,5]</sup>. Moreover, IL-8 expression correlates with vascularity in gastric carcinoma<sup>[6]</sup>. In human moderately differentiated gastric adenocarcinoma cancer cell line SCG-7901<sup>[7]</sup> and poorly differentiated adenocarcinoma cancer cell line TMK-1<sup>[8]</sup>, constitutive expression of IL-8 has been linked to tumorigenesis and angiogenesis *in vitro* and *in vivo*. However, the exact role of IL-8 in the progressive tumorigenesis of GC remains unclear.

The purpose of this study was to provide evidence for the role of IL-8 in determining the migration, invasion and chemosensitivity of human GC. We found that expression of IL-8 participated in the migration and invasion and was correlated with oxaliplatin resistance of GC cells *in vitro*. This study may provide the basis for the development of new therapies for GC by increasing chemosensitivity and decreasing the proliferation, migration and invasion of the cancer cells.

## MATERIALS AND METHODS

### Cell culture

Human GC cell lines MKN-45, and KATO-III, and human umbilical vein endothelial cells (HUVECs) were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). GC cells were cultured in RPMI-1640 medium (Invitrogen, United States) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen). HUVECs were cultured in Human Endothelial-SFM (Invitrogen). Cells were maintained at 37 °C in a humidified chamber containing 5% CO<sub>2</sub>.

### Stable overexpression of interleukin-8 in MKN-45 cells

The empty plasmid vector pcDNA3.1 (Invitrogen) or the plasmid vector containing IL-8 cDNA was transfected into MKN-45 cells using Lipofectamine 2000 (Invitrogen). Multiple clones were selected in the presence of 0.75 mg/mL G418. IL-8-transfected clones were screened for IL-8 expression. Stably transfected clones were picked and maintained in the medium containing 0.1 mg/mL G418. To avoid clonal variations, six positive clones were pooled for further studies.

### Stable knockdown of interleukin-8 in KATO-III cells

The DNA sequence of RNAi of IL-8 was designed to hybridize and destroy human IL-8 mRNA (accession no. NM\_000584) using the Web-based siRNA target finder and design tool provided at the Ambion website (Ambion). The DNA sequence of RNAi of IL-8 (sense, 5' -ACCACCGGAAGGAACCAUCdTdT-3'; antisense, 5' -GAUGGUUCCUCCGGUGGdTdT-3') was synthesized and cloned into the pSilencer 2.1-U6 neo (Ambion) according to the manufacturer's instructions. The human

specific negative control siRNA was also designed with the sequences as follows: sense: 5'-UUCUCCGAACGU-GUACGUDtT-3'; antisense: 5'-ACGUGACACGUUC-GGAGAAdTdT-3'.

The negative control siRNA vector or the vector containing RNAi sequence of IL-8 was transfected into KATO-III cells using Lipofectamine 2000. Multiple clones were selected in the presence of 0.4 mg/mL G418. IL-8-RNAi clones were screened for IL-8 expression. Stable RNAi clones were picked and maintained in the medium containing 0.1 mg/mL G418. To avoid clonal variations, six positive clones were pooled for further studies.

### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were performed using commercial IL-8 ELISA kits from R and D Systems. Cells ( $1 \times 10^6$  cells per well) were plated in a 6-well plate and incubated at 37 °C for 72 h. An equal volume of cell culture supernatants was collected. The assays were done in triplicate, and the concentration of IL-8 in culture supernatants was determined by comparing their optical density with the standard curve.

### 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cells growing exponentially were plated in 96-well plates at a density of  $1 \times 10^5$  cells per well for 7 d. One hundred microliters of 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution (1 mg/mL) was added to each well daily, and the cells were further incubated at 37 °C for 4 h. The supernatant was discarded and 200 µL dimethyl sulfoxide was added. When the precipitates were completely dissolved, the absorbance at wavelength 595 nm was measured with a micro-ELISA reader.

### Growth inhibition assay

Growth inhibition was measured as previously described<sup>[9]</sup>. Cells were trypsinized and seeded at  $1 \times 10^4$  cells per well in 96-well plates. After 24 h, cells were exposed to oxaliplatin (Sigma, St. Louis, MO) for 72 h, at stepwise concentrations from 0 µg/mL to 10 µg/mL. The cells were quantified as described in MTT assay, to calculate the mean cell growth inhibition.

### Cell adhesion assay

To measure the cell adhesion, monolayer adhesion assay and extracellular matrix component (ECM) adhesion assay were performed. For the monolayer adhesion assay, HUVECs were seeded onto 24-well plates ( $1 \times 10^5$  cells/well) 48 h before adhesion assay. Cells ( $1 \times 10^5$  cells/well) were seeded onto HUVEC monolayers and incubated at 37 °C. After 2 h, non-adherent cells were removed by washing with phosphate-buffered saline (PBS).

For ECM adhesion assay, 96-well plates were coated with human fibronectin (BD Biosciences) at a final concentration of 2 µg/cm<sup>2</sup> overnight at 4 °C. Plates were washed with 1% bovine serum albumin in PBS to block

nonspecific cell adhesion. Cells were seeded and incubated for 2 h. Nonadherent cells were washed up to three times with PBS.

The adherent cells were quantified as described in MTT assay, to calculate the mean cellular adhesion capability. The absorbance at 570 nm was measured by the ELX-800 ELISA plate reader (Bio-Tek Instruments, Winooski, VT). After subtracting background absorbance, results were calculated as the mean cellular adhesion rate.

### Cell migration assay

To measure the cell migration activity, Transwell and wound-healing assays were performed. The Transwell cell migration assay was performed as previously described<sup>[10]</sup> using Transwells (8  $\mu\text{mol/L}$  pore size polycarbonate membrane) obtained from Corning. Cells ( $1 \times 10^5$ ) in 0.5 mL serum-free medium were placed in the upper chamber, whereas the lower chamber was loaded with 0.8 mL medium containing 10% FBS. The total number of cells that migrated into the lower chamber was counted after 24 h of incubation at 37 °C with 5% CO<sub>2</sub>. Nonmigratory cells were removed. Migratory cells were stained with 0.2% crystal violet in 10% ethanol. To quantitate migratory cells, three independent fields of migratory cells per well were photographed under phase contrast microscope. The number of cells per field was counted and averaged.

To carry out the wound-healing assay, cells ( $5 \times 10^5$  cells per well) were plated in 6-well plates. After 24 h, the confluent monolayer cells were scratched manually with a plastic pipette tip, and after being washed with PBS, wounded monolayers of the cells were allowed to heal for 12-24 h. Each migration assay was done for at least three times independently.

### Cell invasion assay

Cell invasion was measured using 8- $\mu\text{m}$  pore BD Bio-Coat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions. Cells ( $2.5 \times 10^5$ ) were added to chambers and incubated for 24 h at 37 °C. Matrigel and noninvasive cells were removed and chambers were stained as described above. To quantitate invasive cells, three independent fields of invasive cells per well were photographed under phase contrast microscope. The number of cells per field was counted and averaged. Each invasion assay was done at least three times independently.

### RNA isolation and reverse transcription polymerase chain reaction

Total RNA was purified from cells using a Trizol reagent (Life Technologies). First-strand cDNA was synthesized using 2.5  $\mu\text{g}$  RNA and AMVretroviridase (Promega). Quantitative real-time polymerase chain reaction (PCR) was performed using the Bio-Rad iCycler iQ real-time PCR system (Bio-Rad) and following primers: IL-8, IL8-L: 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'; IL8-R: 5'-TCTCAGCCCTCTTCAAAAATTCT-3'. As a control, each cDNA sample was simultaneously subjected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using

the primers: GAPDH-L: 5'-CCACCCATGGCAAATTCCATGGCA-3'; GAPDH-R: 5'-TCTAGACGGCAGGTCA GGTCCACC-3'. The threshold cycle (Ct) of each sample was determined, and the relative level of a transcript ( $2^{\Delta\text{Ct}}$ ) was calculated by obtaining  $\Delta\text{Ct}$  (test Ct - GAPDH Ct) and then expressed as arbitrary units ( $1/2^{\Delta\text{Ct}} \times 100$ ) = fold difference.

### Protein isolation and Western blotting

Cells at 80% culture confluence were harvested for Western blotting analysis. The harvested cells were lysed and their protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). The cell lysates (50  $\mu\text{g}$  protein each lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (Hyclone, Logan, UT). The membranes were blocked with 5% (v/v) skim milk and probed with primary antibody at 4 °C overnight. Following washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Primary antibodies were specific for phospho-NF- $\kappa\text{B}$ -p65, NF- $\kappa\text{B}$ -p65, phospho-Akt, Akt, ICAM-1, VCAM-1, CD44, and  $\beta$ -actin (Cell Signaling Technology, Inc., Danvers, MA). The bound antibodies were visualized using an electrochemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

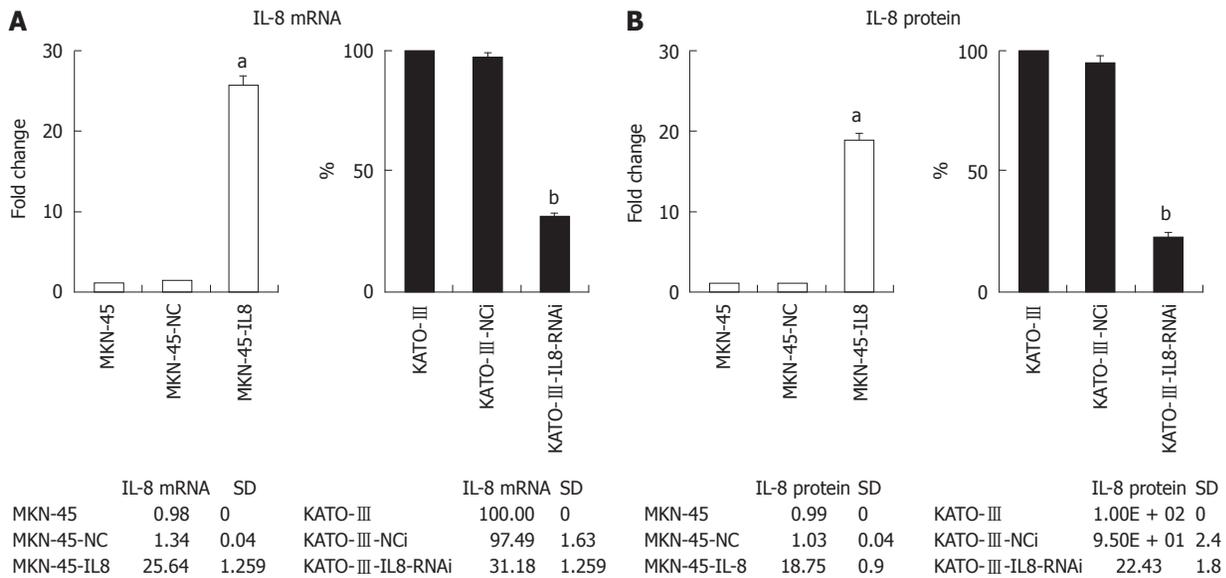
### Statistical analysis

Data were presented as means  $\pm$  SE. Differences of the variables between groups were analyzed by Student's *t* test. Differences were considered significant when  $P < 0.05$ .

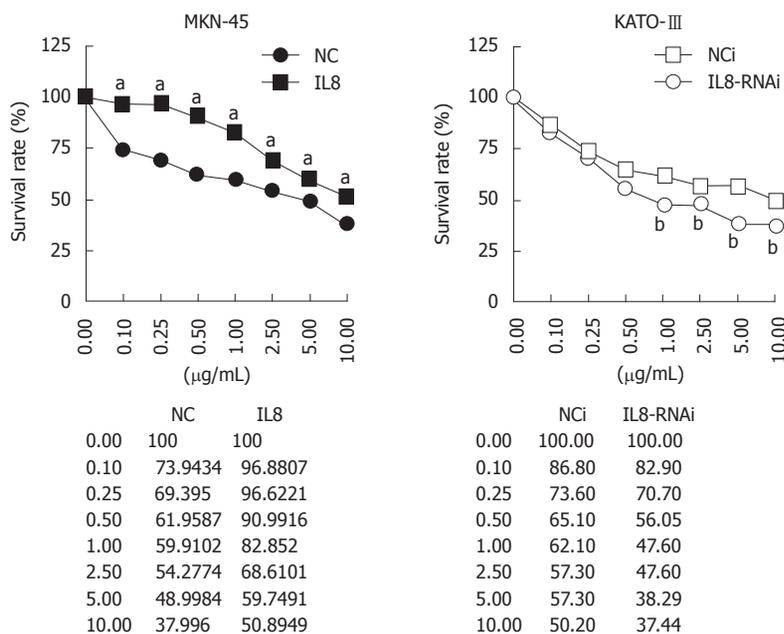
## RESULTS

### Stable overexpression and silencing expression of interleukin-8 in gastric cancer cells

To investigate the effect of IL-8 in GC, the mRNA expression of IL-8 was quantified in three GC cells (Figure 1A). MKN-45 cell lines had lower expression of IL-8 in comparison with KATO-III cells, which is consistent with a previous study by Kitadai *et al*<sup>[11]</sup>. To increase the IL-8 expression, MKN-45 cells were stably transfected with full-length IL-8 expression construct. The RNAi technology was employed in order to stably silence the IL-8 expression in KATO-III cells. The mRNA expression of IL-8 in cells was examined by real-time PCR. The ELISA assay was performed to quantify secreted IL-8 present in the cell culture medium. After transfection and G418 selection, IL-8 mRNA expression level in the stably IL-8 transfected MKN-45 cells (MKN-45-IL8) was 25.6-folds higher than in MKN-45 cells (Figure 1A). As shown in Figure 1B, the secretion levels of IL-8 in MKN-45-IL8 cells were 18.4-folds higher than in MKN-45 cells. Endogenous IL-8 mRNA expression and protein secretion levels were significantly lowered by RNAi in KATO-III cells (KATO-III-IL8-RNAi) when compared with control transfections (KATO-III-NCi) and KATO-III cells (Figure 1).



**Figure 1 Interleukin-8 expression of gastric cancer cells.** A: Real-time polymerase chain reaction determined the level of Interleukin-8 (IL-8) mRNA in gastric cancer (GC) cells. Expression levels were determined by the  $\Delta\Delta C_t$  method using glyceraldehyde-3-phosphate dehydrogenase as endogenous control. Histograms show fold change over the relative expression levels of IL-8 mRNA of control cells. Each bar represents the mean  $\pm$  SD (<sup>a</sup> $P < 0.05$  vs MKN-45, <sup>b</sup> $P < 0.05$  vs KATO-III); B: IL-8 production in GC cells measured by enzyme-linked immunosorbent assay. Histograms show fold change in the relative expression levels of IL-8 protein of control cells. All experiments were repeated three times with similar results. Each bar represents the mean  $\pm$  SD (<sup>a</sup> $P < 0.05$  vs MKN-45, <sup>b</sup> $P < 0.05$  vs KATO-III).



**Figure 2 Growth inhibition assay of gastric cancer cells treated with oxaliplatin.** Survival of gastric cancer cells is tested by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Each bar represents the mean  $\pm$  SD. All experiments were repeated three times with similar results (<sup>a</sup> $P < 0.05$  vs MKN-45-NC, <sup>b</sup> $P < 0.05$  vs KATO-III-NCi).

**Interleukin-8 insignificantly affects proliferation of gastric cancer cells**

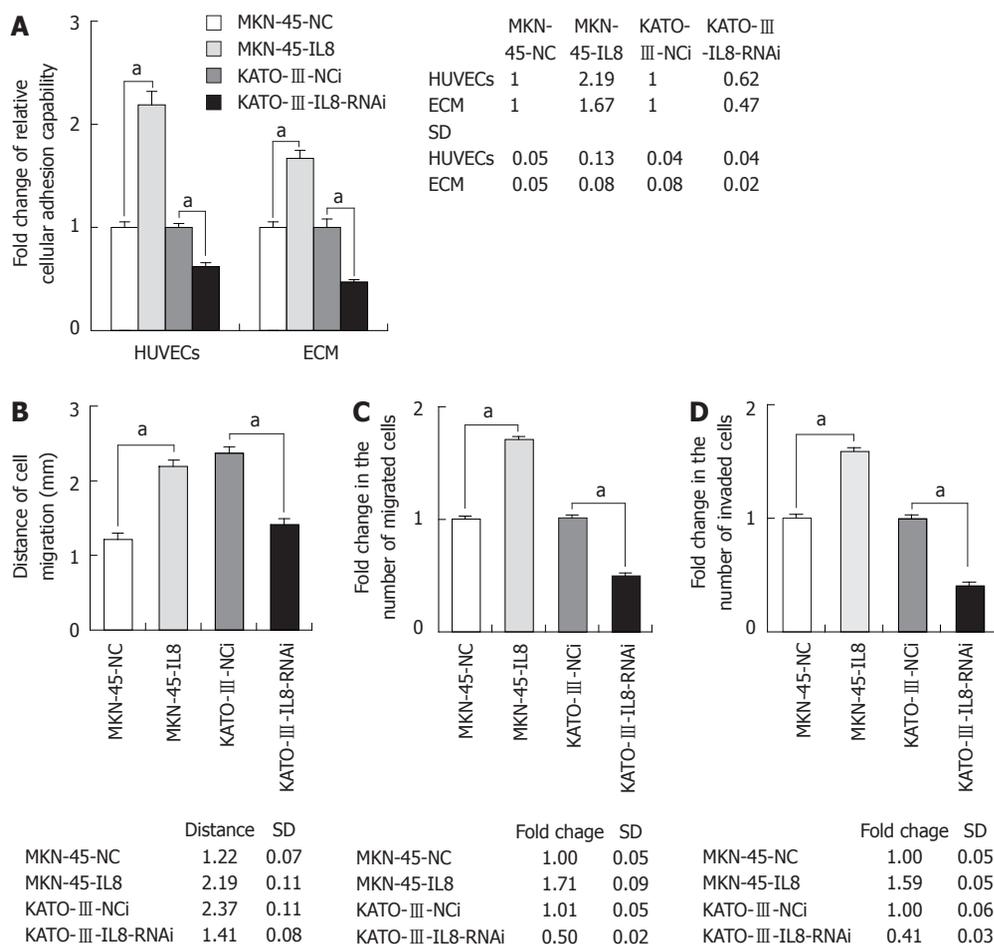
Since IL-8 has been reported to be an autocrine growth factor<sup>[12]</sup>, we examined its role in the growth of human GC cells. Overexpression or silencing expression of IL-8 in GC cells had insignificant effect on cancer cell proliferation as measured by the MTT assay (data not shown).

**Interleukin-8 increases resistance to oxaliplatin in gastric cancer cells**

Previous reports indicated that increased IL-8 was associ-

ated with chemoresistance, such as oxaliplatin<sup>[13-15]</sup>. To investigate whether IL-8 is associated with chemosensitivity in GC cells, the effects of IL-8 expression on oxaliplatin sensitivity in IL-8-overexpressed MKN-45-IL8 cells and IL-8-silenced KATO-III-IL8-RNAi cells were tested using growth inhibition analyses.

As shown in Figure 2, MKN-45-NC and KATO-III-NCi cells were sensitive to oxaliplatin. In MKN-45-IL8 cells, the survival rate significantly increased than in the control cells ( $P < 0.05$ ) after treatment with oxaliplatin at the concentrations from 0.1  $\mu\text{g/mL}$  to 10  $\mu\text{g/mL}$  ( $P <$



**Figure 3 Interleukin-8 involved in gastric cancer cell adhesion, migration and invasion.** A: Effect of Interleukin-8 (IL-8) on gastric cancer (GC) cell adhesion to human endothelial cells and extracellular matrix components. Histograms show fold change in the relative OD value of control cells quantified by cell proliferation assay to calculate the mean cellular adhesion capability; B: Effect of IL-8 on GC cell migration tested by wound-healing assays. Histograms show distances of cell migration; C: Effect of IL-8 on GC cell migration tested by Transwell assays. Histograms show fold change in the number of migrated cells vs the control cells; D: Effect of IL-8 on GC cell invasion. Histograms show fold change in the number of invaded cells vs the control cells. Each bar represents the mean  $\pm$  SD. All experiments were repeated three times with similar results ( $^{\#}P < 0.05$ ). HUVEC: Human umbilical vein endothelial cell.

0.05). On the contrary, when IL-8 expression was suppressed in KATO-III cells using RNAi, there was significant decrease in growth rate after treatment with 1.0-10  $\mu\text{g}/\text{mL}$  oxaliplatin ( $P < 0.05$ ). These results suggest that IL-8 expression in GC cells decreased the sensitivity to the cytotoxic effects of oxaliplatin.

#### Interleukin-8 is involved in gastric cancer cell adhesion

To determine the role of IL-8 expression in GC cell adhesion, adhesion capacity of GC cells to endothelium or extracellular matrix components was evaluated (Figure 3A). The 2-h adhesion capability of GC cells to a monolayer of HUVECs or ECM components was significantly increased in MKN-45-IL8 cells as compared with the control cells. Silencing expression of IL-8 in KATO-III cells significantly reduced the cell adhesion capability ( $P < 0.05$ ).

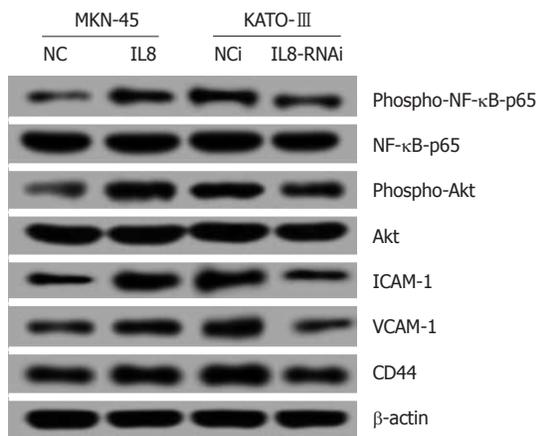
#### Interleukin-8 is involved in gastric cancer cell migration and invasion

To measure the cell migration activity, Transwell and wound-healing assays were performed. As shown in Figure 3B and C, expression of IL-8 could significantly promote

GC cell migration into the cell-free region and migration through cell culture inserts. The invasive potential of GC cells was investigated using *in vitro* Matrigel invasion assay. Cell invasion was similar to cell migration. MKN-45-IL8 cells exhibited significant increase in invasion capacity whereas KATO-III-IL8-RNAi cells exhibited significant decrease (Figure 3D). Therefore, IL-8 overexpression was sufficient to increase the rate of GC cell migration and *in vitro* invasion.

#### Interleukin-8 increases NF- $\kappa$ B and Akt activities, adhesion molecules ICAM-1 and VCAM-1, and CD44 expression in gastric cancer cells

Recent studies have reported that induction of IL-8 signaling increased NF- $\kappa$ B transcriptional activity, and activated the phosphoinositide-3-kinase and cascade. As shown in Figure 4, the levels of phospho-NF- $\kappa$ B-p65 and phospho-Akt were upregulated in IL-8 overexpressed MKN-45-IL8 cells compared with the control cells, and were downregulated in KATO-III-IL8-RNAi cells. These data suggest that constitutive IL-8 expression strongly increased NF- $\kappa$ B and Akt activation.



**Figure 4** Effect of Interleukin-8 on NF-κB and Akt activities and adhesion molecules ICAM-1 and VCAM-1, and CD44 expression in gastric cancer cells. The protein expression levels of phospho-NF-κB-p65, NF-κB-p65, phospho-Akt, Akt, ICAM-1, VCAM-1, and CD44 were determined by Western blotting. All experiments were repeated three times with similar results.

Adhesion molecules play an important role in cell-cell, cell-ECM interactions in cancer invasion and metastasis. As shown in Figure 4, IL-8 increased the protein expression levels of adhesion molecules ICAM-1 and VCAM-1, and CD44 expression in GC cells.

## DISCUSSION

It has been shown that IL-8 modulates proliferation and migration of tumor cells, including melanoma<sup>[16-18]</sup>, prostate cancer<sup>[14,19]</sup>, breast cancer<sup>[20]</sup>, and colon cancer<sup>[13]</sup>. A study confirmed that IL-8 increases angiogenesis of human gastric carcinoma<sup>[6]</sup>. Our goal was to evaluate whether IL-8 is involved in proliferation, adhesion, migration, invasion and the sensitivity to chemotherapeutics in human GC cell lines. We found that IL-8 overexpression in GC cells was associated with increased adhesion, migration, and invasion activity and resistance to oxaliplatin, suggesting that IL-8 is a promising therapeutic target.

In this study, we examined the biological role of IL-8 in adhesion, migration, and invasion of GC cells with either overexpression or knocked down expression of IL-8. The stable IL-8 transfectants and IL-8 RNAi were successfully generated in MGC803 cells. The constitutive expression of IL-8 in MGC803 cells increased cell adhesion, migration and invasion, which was opposed by silencing IL-8 expression in KATO-III cells using RNAi. Our findings suggest that expression of IL-8 plays an important role in modulating cell adhesion, migration, and invasion of GC cells. This observation is supported by the study of Ju *et al.*<sup>[7]</sup>, which showed that recombinant interleukin-8 promoted the adhesion, migration and invasion of GC SCG-7901 cells and up-regulated the expression of matrix metalloproteinase-9, intercellular adhesion molecule-1 and E-cad *in vitro*. However, IL-8 in GC cells had insignificant effect on cell proliferation according to the previous studies<sup>[6,7]</sup>.

The NF-κB and Akt signaling pathway activations are involved in cellular transformation, survival, prolifera-

tion, invasion, angiogenesis, metastasis and inflammation in cancers. It has been reported that NF-κB stimulates IL-8 production, and endogenous IL-8 causes constitutive activation of NF-κB (p65) in colon cancer<sup>[13]</sup> and prostate cancer cells<sup>[14]</sup>. In this study, we found that IL-8 overexpression caused activation of NF-κB and Akt signaling in GC cells.

It is widely accepted that the invasion and metastasis of cancer is dependent on the capacity of cancer cell adhesion and migration<sup>[21]</sup>. In this study, we found that GC cells overexpressing IL-8 produced increased adhesion and migration activity with upregulated ICAM-1, VCAM-1, and CD44. Further investigations are warranted to elucidate the regulative mechanism of IL-8 as a migratory and invasive factor in GC cells.

In conclusion, our studies provide significant evidence that IL-8 expression contributes to GC cell adhesion, migration and invasion, and leads to resistance to oxaliplatin. These findings may help develop novel IL-8-targeted therapies for GC.

## COMMENTS

### Background

Gastric cancer (GC) is still a serious health problem and remains the second most common type of fatal cancer worldwide. Interleukin-8 (IL-8), a cytokine of the CXC chemokine family that was originally classified as neutrophil chemoattractant, is now reported to play an important role in tumor progression and metastasis in a variety of human cancers.

### Research frontiers

The exact role of IL-8 in the progressive tumorigenesis of GC remains unclear. The purpose of this study was to provide evidence for the role and molecular mechanism of IL-8 in determining the migration, invasion and chemosensitivity of human GC.

### Innovations and breakthroughs

This study demonstrated that IL-8 expression is associated with cell adhesion, migration, and invasion in GC. Overexpression of IL-8 promotes invasion phenotype of GC cells with activated NF-κB and Akt and increased expression of adhesion molecules ICAM-1, VCAM-1, and CD44 *in vitro*.

### Applications

The findings help clarify the molecular mechanisms of IL-8 involved in GC invasion and indicate that IL-8 may be an important therapeutic target in GC.

### Peer review

The authors have shown that overexpression of IL-8 *in vitro* promotes the adhesion, migration, invasion, and chemoresistance of some gastric cancer cell lines, thus indicating IL-8 as possible therapeutic target in gastric cancer. The work addresses an interesting topic with the proper methodological approach.

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## Serological and molecular study of hepatitis E virus among illegal blood donors

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### Abstract

**AIM:** To investigate the seroprevalence and molecular characteristics of hepatitis E virus (HEV) in the illegal blood donors (IBDs) of central China in the early 1990s.

**METHODS:** A total of 546 blood samples were collected from the IBDs in Maanshan city, a questionnaire was completed by each subject, detailing the age, sex, and periods of blood or plasma donation. Anhui Province and tested for the anti-HEV antibodies. The seropositive

samples were subjected to nested reverse transcription-polymerase chain reaction and sequencing to analyze HEV partial genome.

**RESULTS:** The prevalence of IgG and IgM HEV antibody in IBDs was 22.7% and 1.8%, and genotype 4 was the dominant circulating HEV type in IBDs. The prevalence of anti-HEV IgG was significantly related to sex (OR = 4.905,  $P = 0.004$ ) and increased with age (OR = 2.78,  $P = 0.022$ ), which ranged from 13.0% in those < 40 years old to 30.6% among older persons aged > 60 years. Moreover, frequency of blood donation was significantly associated with HEV seropositivity (OR = 2.06,  $P = 0.006$ ). HEV partial sequences of ORF2 and obtained 3 sequences in serum samples of 10 IBDs which developed HEV specific IgM.

**CONCLUSION:** This study helps define one of the possible routes of transmission of sporadic HEV infection and provides guidance to screen HEV in the blood donors so as to guarantee safe blood banks in China.

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**Key words:** Molecular; Sero-epidemiology; Hepatitis E; Hepatitis E virus; Commercial blood donors

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## INTRODUCTION

Hepatitis E virus (HEV) infection is an important public-health concern as a major cause of enterically transmitted hepatitis worldwide. Epidemiologic studies have shown that HEV is prevalent in most developing countries, such as southeast Asia, northern and central Africa, India and central America. In addition, a high incidence of sporadic HEV infection has been observed in several industrialized countries, including the United States, European countries and Japan<sup>[1-5]</sup>. Although the ingestion of contaminated drinking water contributes mainly to the spread of HEV, other routes of transmission should be considered, because some studies implicated that blood transfusion was the possible route of sporadic HEV infection in non-endemic developed countries<sup>[6-8]</sup>.

Between 1992 and 1995, illegal blood donation (IBDs) occurred frequently in several provinces in central China, including Henan, Anhui and Shanxi provinces. Although commercial blood donation was eradicated by the Chinese government by the end of 1995, the practice of using contaminated blood collection equipment caused the spread of some viruses, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV)<sup>[9,10]</sup>.

While many studies have reported the prevalence of HEV infection and the HEV genome characteristics in different groups in China, to date, there has been no report on the prevalence of HEV infection among the IBDs. The aim of this study was to investigate whether HEV can be transmitted by the blood transfusion route and analyze the partially conserved nucleotide sequences of HEV strains among the IBDs in Maanshan city in Anhui Province, one of the provinces with the illegal blood collection in the early 1990s.

## MATERIALS AND METHODS

### Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was initiated after the study protocol was approved by the Institutional Review Board (IRB) of China Center for AIDS/STD Control and Prevention and the IRB of Maanshan Center of Disease Control and Prevention.

### Study population

A total of 546 samples were collected between January and August in 2005 from those who donated their blood or plasma frequently from 1992 to 1995. All participants were from Dangtu District in Maanshan city. A questionnaire was completed by each subject, detailing the age, sex, and periods of blood or plasma donation.

### Detection of antibodies against hepatitis E virus

Serum samples were tested by enzyme-linked immunosorbent assay (ELISA) for IgG and IgM with anti-HEV activity described previously<sup>[11,12]</sup>. All serum samples were

assayed at a 1:20 dilution. The absorbance of each sample was read at 450 nm. The cutoff value used for the anti-HEV IgG and IgM assay was 0.152. Then the serum positive for IgM antibody against HEV were tested for HEV RNA. All patients were previously tested for HBsAg (Diasorin, United States) and anti-HCV (third generation assay, Diasorin, United States) by ELISA.

### Extraction of RNA and reverse transcription-polymerase nested chain reaction

Viral RNA was extracted from serum samples using Qia-gen viral RNA kit (Qiagen) according to the manufacturer's instructions. The viral RNA was finally dissolved in 20  $\mu$ L RNase-free water. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using TaKaRa RNA PCR kit (TaKaRa, Japan). The primers and the PCR protocol used were adapted from a previous study<sup>[13]</sup>. The external primers were P1 (5'-CCGACAGAAATTGATTTCGTCCGC-3') and P4 (5'-CCGTAAGTTCGACTGGTCTGTACTC-3'). The internal primers were P2 (5'-GTTGTCTCGCCAATGGCGAGCC-3') and P3 (5'-TCGGCGGCGGTGAGAGAGCCA-3'). The first-round and the second-round amplifications were carried out according to the following cycling program denaturation at 9  $^{\circ}$ C for 45 s, annealing at 52  $^{\circ}$ C for 60 s, extension at 72  $^{\circ}$ C for 60 s, for 35 cycles. The size of the first-round PCR product was 307 bp, and that of the second-round PCR was 236 bp.

### Sequencing and analysis of sequences

The PCR products were visualized on a 10 mL/L agarose gel, excised and purified using a gel extraction kit (Qiagen) and directly sequenced on an ABI model 3730 DNA Auto Sequencer (Shanghai, China). The 236 bp fragment amplified from ORF2 of HEV genome was sequenced for comparison with the corresponding regions of other known human, porcine and avian HEV strains available in the GenBank database (DNASTar). Sequences were aligned using ClustalX v1.8 (<http://www-igbmc.ustrasbg.fr/Bio-Info/ClustalX/Top.html>), and the phylogenetic analysis was performed using the MEGA program, version 3.1 (Pennsylvania State University).

### Statistical analysis

The Pearson  $\chi^2$  test was used to evaluate the difference in the prevalence between groups in the univariate analyses. Odds ratios (OR) with 95% confidence intervals were used to determine whether a variable was associated with HEV infection.

## RESULTS

### Hepatitis E virus seropositivity in illegal blood donors

A total of 546 IBDs in the 1990s were enrolled to this study. The IBDs were aged from 29 to 75 years with a mean of  $51 \pm 9$  years. Among them, 156 (28.6%) were males and 390 (71.4%) were females. While 124 IBDs developed HEV IgG antibody, only 10 IBDs developed HEV IgM antibody, therefore, the prevalence of IgG and

**Table 1** Prevalence of hepatitis E virus IgG seropositivity in 546 commercial blood donors

	HEV positive IgG <i>n</i> (%)	OR (95% CI)	<i>P</i> value
Sex			
Female	76/390 (19.5)	-	0.004
Male	48/156 (30.8)	1.84 (1.20, 2.80)	
Donation frequency			
< 10	47/252 (18.7)	-	
10-20	44/191 (23.0)	1.31 (0.82, 2.07)	0.258
≥ 20	33/103 (32.0)	2.06 (1.22, 3.46)	0.006
Age (yr)			
< 40	7/54 (13.0)	-	
40-50	37/192 (19.3)	1.50 (0.63, 3.60)	0.361
50-60	46/189 (24.3)	2.02 (0.85, 4.80)	0.105
≥ 60	34/111 (30.6)	2.78 (1.14, 6.79)	0.022

HEV: Hepatitis E virus; OR: Odd ratio; CI: Confidence interval.

IgM HEV antibody in this group was 22.7% and 1.8%, respectively.

The prevalence of HEV IgG seropositivity in the 546 IBDs is shown in Table 1. In the male group, 30.8% (48/156) were positive as against 19.5% (76/390) in the female group. The prevalence of HEV IgG seropositivity was significantly higher in men than in women (OR = 4.905,  $P = 0.004$ ). In addition, subjects over 60 years of age had a higher prevalence of HEV IgG seropositivity than those aged < 40 years (OR = 2.780,  $P = 0.022$ ). The frequency of plasma donation was also associated with HEV infection. The odds ratio was 2.06 among those who donated more than 20 times compared with those who donated 10 or fewer times.

The sex specific prevalence of HEV IgG in IBDs with different frequency of donation is shown in Table 2. HEV infection prevalence was significantly correlated with the increasing age in total participants ( $\chi^2 = 2.91$ ,  $P = 0.004$ ) and female participants ( $\chi^2 = 1.97$ ,  $P = 0.048$ ).

We also examined the relationship between HEV infection and HBV or HCV coinfection in these IBDs. The results showed no significant difference in HBsAg positive status (3.2% *vs* 3.1%) and HCV positive status (11.3% *vs* 11.1%) between HEV IgG positive and negative IBDs (Table 3).

#### Phylogenetic analysis of hepatitis E virus strains

We detected HEV partial sequences of ORF2 and obtained 3 sequences in serum samples of 10 IBDs which developed HEV specific IgM. Sequence analysis demonstrated that these 3 strains were 83.3%-93.6% identical to each other. When compared with the HEV reference isolates, the strains were closely related to Chinese strain T1 with an 82.6%-89.4% nucleotide homology, and demonstrated a 91.1% sequence homology to a Japanese strain JAK-Sai. The nucleotide homology of other Japanese HEV strains with the strains from IBDs ranged from 78.6% to 85.4%. The homology of strains from Burma, Mexico and the United States was 79.2%-80.2%, 80.5%-81.4% and 77.1%-78.0%, respectively.

In the phylogenetic tree generated, the Bur82 strain

**Table 2** Sex specific prevalence of hepatitis E virus IgG seropositivity in commercial blood donors with different frequency of donation *n* (%)

Frequency	Hepatitis E virus positive IgG		<i>P</i> value
	Male	Female	
< 10	15/54 (27.8)	32/198 (16.2)	0.052
10-20	18/57 (31.5)	28/137 (20.4)	0.097
≥ 20	15/45 (33.3)	16/55 (29.1)	0.648

**Table 3** Relationship between hepatitis E virus infection and hepatitis B virus or hepatitis C virus co-infection in commercial blood donors *n* (%)

Characteristics	HEV positive IgG ( <i>n</i> = 124)	HEV negative IgG ( <i>n</i> = 422)	<i>P</i> value
HBsAg positive	4 (3.2)	13 (3.1)	0.935
HCV antibody positive	14 (11.3)	47 (11.1)	0.962

HEV: Hepatitis E virus.

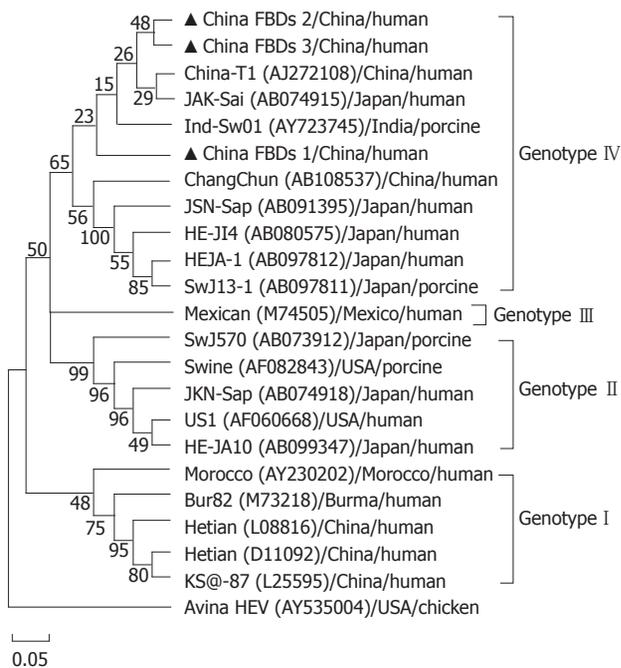
(genotype 1), the Mexican strain (genotype 2), the US1/swine strain (genotype 3), and the Chinese strain T1 (genotype 4) represent major branches. Phylogenetic analyses clearly illustrate that all HEV sequences except avian strain can be divided into these four distinct genotypes and the three HEV sequences isolated in our study were genotype 4. The IBDs 1 sequence we analyzed formed an exclusive cluster, and was bound to a new subgenotype within genotype 4, which was supported by the bootstrap values obtained from 1000 replicates resampling analysis (Figure 1).

## DISCUSSION

HCV and HIV infections were prevalent among the IBDs who donated blood in the early 1990s in China. However, data on HEV infection in this population have been unavailable so far. To our knowledge, the present study is the first seroepidemiological and molecular study on HEV infection in this unique population. Our results demonstrated that HEV infection had been introduced into this population in this area and that the prevalence was much higher (22.5%) than that in the normal population (4.76%) in this area<sup>[14]</sup>.

Our data indicated that 30.7% of males were HEV positive compared to 19.5% of females and the difference was statistically significant (OR = 1.84,  $P = 0.004$ ). In addition, we found that males with a history of blood transfusion had a high HEV seropositivity than females, suggesting that male IBDs are more likely to get infected by HEV than female donors. Our study also showed that HEV seropositivity increased with age of the first donating blood, consistent with previous studies demonstrating that age may be an important risk associated with HEV in other populations<sup>[15,16]</sup>.

Although no statistically significant association was observed between HEV seropositivity and blood-borne hep-



**Figure 1** A phylogenetic tree is constructed using the neighbor-joining method based on the 236-nt ORF2 sequences of four genotypes hepatitis E virus isolates. The isolates were named by the accession number in parentheses, the name of the country of origin and species from which it was isolated. Bootstrap values were indicated for the major nodes as a percentage of the data obtained from 300 replicates. Bar, 0.05 substitutions per site. Percent bootstrap support was indicated at the respective nodes. An avian hepatitis E virus strain is included as outgroup. The isolates identified in this study were marked with solid triangle.

atitis viruses such as HBV and HCV, our findings show that more frequent blood/plasma donation increased the risk of HEV infection, providing evidence that HEV can be transmitted by viremic blood units and have similar or overlapping routes of transmission with HCV<sup>[17]</sup>.

HEV IgM antibody is known as a marker of the early seroconversion period. In this study, HEV IgM antibody was detected in 10 samples in the population. We also tested the presence of serum HEV RNA in the IBDs. HEV has been classified into four genotypes based on the full sequence heterogeneity. These include genotypes 1 (mainly prevalent in Asia and Africa), 2 (mainly prevalent in Mexico, Nigeria), 3 (mainly prevalent in the US, Japan, Argentina, and Europe), and 4 (mainly prevalent in Taiwan, Japan, and mainland China)<sup>[18]</sup>. More recently, HEV genotype 4 has been isolated from various regions of China, ranging from the south (Guangzhou and Shanghai), the centre (Henan province) to the north (Liaoning Province and Beijing), and has been found to be responsible for a significant proportion of cases of sporadic acute hepatitis in China<sup>[19-21]</sup>. Schlauder *et al.*<sup>[22]</sup> reported that the analysis of small regions of HEV genome yields evolutionary distances similar to those produced from the full-length HEV genome. Therefore, we amplified and sequenced three HEV partial sequences in the serum samples of 10 IBDs positive for HEV IgM antibody. The three sequences share an 81.4%-88.1% identity

at the nucleotide level with each other, and 79.2%-80.2%, 80.5%-81.4%, 77.1%-78.0% and 83.3%-93.6% identity with HEV genotypes 1-4, respectively. Clearly, they belong to genotype 4 and resemble HEV genotype 4 sequences but form some new subgenotypes. These results indicate that there is great genetic variability in HEV genome of genotype 4, even within a certain region or population investigated in China. Previous data showed that a substantial proportion of voluntary blood donors (3/200 or 1.5%) was positive for HEV RNA and the isolates all had a high nucleotide sequence identity (> 90%) with swine HEV, which means that HEV may be zoonotically transmitted from viremic animals to humans<sup>[23]</sup>, however, in the present study, three isolates only shared a 77.5%-86.0% identity with swine HEV.

In conclusion, we report the first molecular and sero-epidemiological study on HEV infection in the IBDs in China in the 1990s. The results demonstrate that HEV is widely spread in this population and confined to genotype 4. Males, individuals aged beyond 60 years, and people who donated blood more than 20 times showed a higher rate of previous HEV infection. Our study may help define one of the possible routes of transmission of sporadic HEV infection in this population and provide guidance to screen HEV in the donors to guarantee safe blood banks in China.

## COMMENTS

### Background

Hepatitis E virus (HEV) infection is an important public-health concern as a major cause of enterically transmitted hepatitis worldwide. Epidemiologic studies have shown that HEV is prevalent in most developing countries and some industrialized countries. Although commercial blood donation was eradicated by Chinese government by the end of 1995, the practice of using contaminated blood collection equipment caused the spread of some viruses such as hepatitis C virus and hepatitis I virus, but there has been no report on the prevalence of HEV infection in the illegal blood donors (IBDs).

### Innovations and breakthroughs

This is a first serological and molecular study on HEV infection in IBDs. The results showed that the prevalence of HEV IgG antibody was higher (22.5%) in the IBDs than in general population, and the risks of HEV infection in IBDs were age, gender and times of donation. Additionally, phylogenetic analysis showed that 3 HEV strains isolated from IBDs belong to genotype 4. Therefore, the present study indicated that HEV is widely spread in the IBDs and the new possible modes of transmission of sporadic HEV infection in IBDs should be defined.

### Applications

As it was indicated in this study that HEV is widely spread among the IBDs, and age, gender and times of blood donation are the risk factors of HEV infection. Therefore, HEV should be detected regularly among the IBDs for the safety of transfusion.

### Terminology

Hepatitis E virus (HEV): Hepatitis E Virus 1 has a particle diameter of 32-34 nm, a buoyant density of 1.29 g/mL in KTar/Gly gradient, and is very labile. Serologically related smaller (27-30 nm) particles are often found in feces of patients with hepatitis E and are presumed to represent degraded viral particles. HEV has a single-stranded polyadenylated RNA genome of approximately 8 kb. Based on its physicochemical properties, it is presumed to be a calicivirus-like virus.

### Peer review

The molecular and sero-epidemiological study on HEV infection is presented in a proper way and this study could help in defining one of the possible modes of transmission of sporadic HEV infection in Chinese population and guide the

screening of HEV in the donors to guarantee safe blood banks. The manuscript is well presented and of interest and the design of this study is appropriate. The study was done well and their results can contribute to knowledge of this topic.

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## Prophylaxis of chronic kidney disease after liver transplantation - experience from west China

Zhen-Yong Shao, Lu-Nan Yan, Wen-Tao Wang, Bo Li, Tian-Fu Wen, Jia-Yin Yang, Ming-Qing Xu, Ji-Chun Zhao, Yong-Gang Wei

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### Abstract

**AIM:** To evaluate the prophylaxis of chronic kidney disease (CKD) after liver transplantation (LT) with low-dose calcineurin inhibitor (CNI) and mycophenolate mofetil (MMF).

**METHODS:** From March 1999 to December 2009, a total of 572 patients (478 males and 94 females) underwent LT enrolled in the study. Initial immunosuppression was by triple-drug regimens that included a CNI, MMF, and prednisone. The initial dose of CNI was 0.05-0.10 mg/kg per day for tacrolimus (TAC) and 5-10 mg/kg per d for cyclosporine A (CSA) respectively, and was gradually reduced based on a stable graft function. The serum trough level of CNI was 6-8 ng/mL for TAC and 120-150 ng/mL for CSA 3-mo post-operation, 4-6 ng/mL for TAC and 80-120 ng/mL for CSA 1-year after transplantation

was expected with stable liver function. MMF was personalized between 1.0-1.5 g/d. Glomerular filtration rate (GFR) was estimated by an abbreviated Modification of Diet in Renal Disease formula. Risk factors of CKD were examined by univariate and multivariate logistic regression.

**RESULTS:** With a definition of GFR < 60 mL/min per 1.73 m<sup>2</sup>, the incidence of CKD was 17.3% 5-year after LT. There were 68.3% (293 of 429 cases) patients managed to control their TAC trough concentrations within 8 ng/mL and 58.0% (83 of 143 cases) patients' CSA trough concentrations within 150 ng/mL. Of the 450 recipients followed-up over 1 year, 55.5% (183 of 330 cases) of which were treated with TAC had a trough concentration ≤ 6 ng/mL while 65.8% (79 of 120 cases) of which were treated with CSA had a concentration ≤ 120 ng/mL. The incidence of CKD in the groups of lower CNI trough concentrations was significantly lower than the groups with CNI concentrations above the ideal range. Patients with CKD had much higher CNI trough concentrations than that of patients without CKD. MMF was adopted in 359 patients (62.8%). Patients administrated with MMF had a relatively low CNI trough concentrations but with no significant difference. The graft function remained stable during follow-up. No difference was found between different groups of CNI trough concentrations. Pre-LT renal dysfunction, ages, acute kidney injury, high blood trough concentrations of CNI in 3 mo (TAC > 8 ng/mL, CSA > 150 ng/mL) and hypertension after operation were associated with CKD progression, while male gender and adoption of MMF were protection factors.

**CONCLUSION:** Low dose of CNI combined with MMF managed to prevent CKD after LT with stable graft function.

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**Key words:** Liver transplantation; Chronic kidney disease; Calcineurin inhibitor; Mycophenolate mofetil; Risk factor

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## INTRODUCTION

Since the introduction of calcineurin inhibitor (CNI) in the 1980s, its use in clinical solid organ has greatly increased transplant recipient survival rates and reduced graft rejection rates<sup>[1]</sup>. More and more patients with end stage liver disease are benefiting from liver transplantation (LT) in the last three decades. However, although the number of long-term surviving recipients has increased, many of them suffer from chronic complications. Chronic kidney disease (CKD) is one such complication that has severely affected the quality of life and survival of organ recipients<sup>[2-7]</sup>. Cohen *et al*<sup>[8]</sup> reported that 27.5% of 191 patients had progressive renal dysfunction [glomerular filtration rate (GFR) < 40 mL/min] 5 years after LT. Ojo *et al*<sup>[9]</sup> found that GFR < 29 mL/min was in up to 18% of patients by 5 years post LT, and that chronic renal failure elevated the risk of death after transplantation (relative risk 4.55). Long duration of CNI-taken is one of the many factors adversely affecting renal function after transplantation<sup>[2,4,7]</sup>. Its nephrotoxicity is seen by kidney biopsy, which includes severe tubular atrophy, interstitial fibrosis and focal hyalinosis of small renal arteries and arterioles<sup>[10,11]</sup>. Lee *et al*<sup>[12]</sup> pointed out that rapid progression of kidney disease was associated with CNI nephrotoxicity which significantly increased the risk by a factor of 4.24.

There are two main strategies for CNI induced CKD, one is CNI withdrawal and conversion to a non-nephrotoxic immunosuppressant, such as sirolimus, mycophenolate mofetil (MMF) and azathioprine; the other is dose reduction in combination with an auxiliary immunosuppressant<sup>[4,6,7,13,14]</sup>. Shenoy *et al*<sup>[13]</sup> found no significant improvement in renal function after 12 mo' follow-up in a prospective trial of CNI withdrawal and replacement with sirolimus for renal insufficiency in liver transplant recipients. Cantarovich *et al*<sup>[15]</sup>, on the other hand, found significant improvement in the renal function of long-term liver-transplant recipients with renal dysfunction by introducing MMF and tapering cyclosporine A (CSA) to a very low dose (50 mg/d), however, this strategy may increase the risk of acute rejection (AR). No agreement has been reached on this issue. Since it has been proven that

the nephrotoxicity was associated with the dosage and duration of CNI, we can expect that administration of initial low-dose CNI and maintaining low blood concentrations after would prevent the progression of renal dysfunction. However, such a conclusion cannot be drawn yet because most researches were based on patients who had pre-existing renal dysfunction. Moreover, there is no consensus on the minimum CNI dose which is considered to be safe for LT recipients.

In our center, a protocol of combining CNI [tacrolimus (TAC) or CSA] with MMF was adopted after LT. The CNI initial dose and blood concentrations after were kept at a relatively low level. The purpose of this study was to delineate the risk factors for developing CKD, and more important, to find out whether the strategy of combination low-dose CNI and MMF can make a successfully prophylaxis of CKD after LT.

## MATERIALS AND METHODS

### Study population

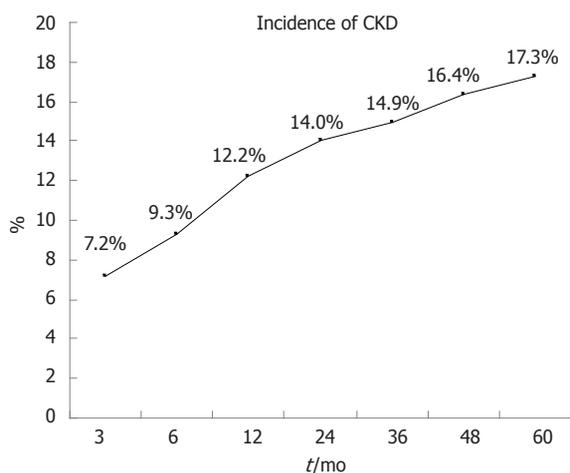
Data from the clinical records of 772 consecutive Chinese patients who underwent LT from March 1999 until December 2009 were retrospectively analysed. Patients were monitored till August 2010 or to their death. Recipients with a short follow-up (less than 3 mo), died within 3 mo after transplantation and younger than 18 years old were excluded. All the liver grafts were from brain-dead donors or living donors. Living and deceased donations were voluntary in all cases, approved by the West China Hospital Ethics Committee, and in accordance with the ethical guidelines of the Declaration of Helsinki.

### Evaluation of kidney function

Renal function was assessed by estimated glomerular filtration rate (eGFR) using the abbreviated Modification of Diet in Renal Disease formula:  $eGFR = 186 \times \text{creatinine (mg/dL)}^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female})$ . Acute kidney injury (AKI) was defined as more than 25% decrease of GFR in the first post-operative week compared with the pre-operative level by the RIFLE (risk, injury, failure, loss and end-stage renal failure) criteria<sup>[16]</sup>. CKD was defined as GFR < 60 mL/min per 1.73 m<sup>2</sup> for at least 3 consecutive months. Hepatorenal syndrome was defined as Salerno *et al*<sup>[17]</sup> reported: cirrhosis with ascites, serum creatinine > 1.5 mg/dL, no improvement of serum creatinine after at least 2 d with diuretic withdrawal and volume expansion with albumin, no current or recent treatment with nephrotoxic drugs, absence of parenchyma kidney disease. Renal dysfunction before LT was also defined as eGFR < 60 mL/min per 1.73 m<sup>2</sup>.

### Definitions of other clinical parameters

According to the latest guideline of prevention and treatment of plasma lipid abnormality for Chinese adults, hypercholesterolemia was defined as total plasma cholesterol  $\geq 6.22$  mmol/L, hypertriglyceridemia as triglyceride  $\geq 2.26$  mmol/L<sup>[18]</sup>. Diabetes mellitus (DM) was diagnosed if



**Figure 1** Incidence of chronic kidney disease 5 years after liver transplantation. The estimated glomerular filtration rate (eGFR) was calculated by the abbreviated Modification of Diet in Renal Disease formula after each visit of a patient. And once met the criterion of chronic kidney disease (CKD) (eGFR < 60 mL/min per 1.73 m<sup>2</sup>), they were registered into the CKD group. Seventeen point three percents of the whole population (99 cases) developed CKD during the 5-year's follow-up.

random blood glucose  $\geq$  11.1 mmol/L or fasting plasma glucose  $\geq$  7.0 mmol/L. AR was confirmed either by liver biopsy or recovery from high-dose methylprednisolone. If chronic rejection (CR) was suspected, liver biopsy was also carried out. Hypertension was defined as a systolic blood pressure over 140 mmHg or diastolic pressure over 90 mmHg twice at different time. Mayo end-stage liver disease (MELD) scores were calculated for each patient.

### Immunosuppressive protocols

Initial immunosuppression was by triple-drug regimens that included a CNI (TAC or CSA), MMF and prednisone. The initial dose of CNI was 0.05-0.10 mg/kg per day for TAC and 5-10 mg/kg per day for CSA respectively. MMF was personalized between 1.0-1.5 g/d. At the early phase in our center, patients were administrated with MMF only when they were diagnosed hypertension and DM; however, all recipients in the late period were administrated with it unless severe gastrointestinal side effects or myelosuppression happened. Prednisone was generally discontinued within 3 mo after transplantation.

### Adjustment of calcineurin inhibitor dose during follow-up

Observations of clinical indices including CNI trough concentrations were checked daily for the first week and weekly for the next three in the first month post-operation, monthly within 3-mo and every three months thereafter. The ideal serum trough level of CNI was 6-8 ng/mL for TAC and 120-150 ng/mL for CSA 3-mo post-operation. Liver function was monitored intensely while adjusting the CNI dose. If AR happened, prior dosage was restarted, together with the prednisone increase or high-dose methylprednisolone administration. Dose reduction was more carefully and slowly carried out. A trough level of 4-6 ng/mL for TAC and 80-120 ng/mL

for CSA one year after transplantation was expected with stable liver function.

### Statistical analysis

SPSS 17.0 statistical software (SPSS Company, Chicago, IL) was used to analyse the relevant data. Numerical data are presented as the mean  $\pm$  SD or as the median. Continuous data were compared using the independent *t*-test if data were normally distributed, or using the rank-sum test if data were non-normally distributed. Categorical data were compared using the  $\chi^2$  test. Univariate logistic regression analysis was used to discover risk factors for CKD. Variables reaching statistical significance were then included for multivariate analysis. Results were reported as odds ratios with 95% confidence intervals.

## RESULTS

### Patients population

The medical records of 572 patients [male:female 478:94, mean age 44 (20-69) years old] meeting the inclusion criteria were reviewed retrospectively. Mean follow-up duration was 28 (3-125) mo. Pre-operation baseline included DM in 36 (6.29%) patients, hypertension in 13 (2.27%) patients, renal dysfunction in 54 (9.44%) patients, and hepatorenal syndrome in 27 (4.72%) patients, 19 (3.32%) were given hemodialysis therapy within a 2-wk period before surgery. The main indications for LT were tumors and end stage liver diseases, with 268 (46.9%) and 276 (48.2%) patients respectively. More than 80% patients were found to be hepatitis B virus (HBV) related. The deceased donor transplantation rate was 75.5%.

### Incidence of chronic kidney disease

The eGFR was calculated after each visit of a patient. And once met the criterion of CKD, they were registered into the CKD group. As shown in Figure 1, 17.3% of the whole population (99 cases) developed CKD during the 5-year's follow-up.

Our analysis of the difference in over 20 clinical indices between patients with and without CKD showed that the CKD group had older age, higher MELD scores, more female, more patients with pre-operative renal dysfunction, more with hepatorenal syndrome and more received pre-operative hemodialysis. There was also a between-group difference in immunosuppression protocols, TAC and MMF was preferred in the non-CKD group. Of the 85 cases of AKI (14.9%), 32 progressed to CKD. In addition, the CKD group had more patients with post-operative DM, hypertension and hypertriglyceridemia (Table 1).

### Subgroups of calcineurin inhibitor trough concentrations

The CNI trough concentrations were recorded in each visit too. Mean concentrations of both TAC and CSA were calculated at different time points. A decreasing trend was discovered with lengthening of follow-up time (Figure 2).

As we mentioned before, an ideal concentration was expected at 3-mo and 1-year post LT. The results showed

**Table 1** Clinical features between chronic kidney disease and non-chronic kidney diseases recipients *n* (%)

Clinical features	CKD group ( <i>n</i> = 99)	Non-CKD group ( <i>n</i> = 473)	<i>P</i> value
Age (median years)	49	42	0.001
Sex (male/female)	70/29	408/65	0.001
Donor type (DDLT/LDLT)	82/17	350/123	NS
Indications for LT			
Cirrhosis	38 (38.4)	160 (33.8)	NS
Chronic active hepatitis	13 (13.1)	39 (8.2)	NS
Tumors	34 (34.3)	234 (49.5)	0.006
Others	14 (14.1)	40 (8.5)	NS
HBV infection	80 (80.8)	401 (85.0)	NS
Complications pre-LT			
DM	10 (10.1)	26 (5.5)	NS
Renal dysfunction	22 (22.2)	32 (6.8)	0.001
HRS	11 (11.1)	16 (3.4)	0.003
Hemodialysis	9 (9.1)	10 (2.1)	0.002
Hypertension	5 (5.1)	8 (1.7)	NS
MELD scores	14	11	0.001
CNI type (TAC/CSA)	64/35	365/108	0.009
MMF adoption	49 (49.5)	310 (65.5)	0.003
Complications post-LT			
DM	27 (27.3)	85 (18.0)	0.034
Hypertension	19 (19.2)	35 (7.4)	0.001
Hypercholesterolemia	19 (19.2)	57 (12.1)	NS
Hypertriglyceridemia	26 (26.3)	81 (17.1)	0.034
AKI	32 (32.3)	53 (11.2)	0.001
AR	18 (18.2)	56 (11.8)	NS
CR	4 (4.0)	11 (2.3)	NS
Graft failure	6 (6.1)	11 (2.3)	NS
Re-transplantation	5 (5.1)	8 (1.7)	NS

CKD: Chronic kidney disease; Age: Age at transplantation; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; NS: No significance; LT: Liver transplantation; HBV: Hepatitis B Virus; DM: Diabetes mellitus; HRS: Hepatorenal syndrome; MELD: Mayo end-stage liver disease; CNI: Calcineurin Inhibitor; TAC: Tacrolimus; CSA: Cyclosporine A; MMF: Mycophenolate mofetil; AKI: Acute kidney injury; AR: Acute rejection; CR: Chronic rejection.

that there were 68.3% (293 of 429 cases) patients managed to control their TAC trough concentrations within 8 ng/mL and 58.0% (83 of 143 cases) patients' CSA trough concentrations within 150 ng/mL. Of the 450 recipients followed-up over 1 year, 55.5% (183 of 330 cases) of which were treated with TAC had a trough concentration  $\leq$  6 ng/mL while 65.8% (79 of 120 cases) of which were treated with CSA had a concentration  $\leq$  120 ng/mL. The incidence of CKD in the groups of lower CNI trough concentrations was significantly lower than the groups with CNI concentrations above the ideal range (Table 2). At the same time, we compared the CNI trough concentrations between patients with and without CKD. As shown in Figure 2A and B, the CKD group had much higher CNI trough concentrations than that of patients without CKD.

Also, recipients were grouped by whether MMF was used. We found its adoption in 359 patients (62.8%). It was used in 49.5% of the CKD group and 65.5% of the non-CKD group ( $P = 0.003$ ). Although patients administered with MMF had a relatively low CNI trough concentrations, but no significant difference was found

between groups (Figure 2C and D).

To assess the impact of CNI concentrations on the chronic complications and graft function post transplantation, patients were still grouped according to the CNI trough concentrations 3-mo post transplantation. The analysis showed between-group differences in these parameters were without statistical significance (Table 3).

### Risk factors for chronic kidney disease progression

Together with the different CNI concentrations and whether uses of MMF, over twenty parameters were examined by univariate logistic analysis to identify the risk factors of CKD (Table 4). All the factors with statistical significance were chosen for multivariate logistic analysis, and seven of them were singled out. Age at LT, pre-operative renal dysfunction, AKI, high CNI concentration 3 mo after LT (TAC > 8 ng/mL or CSA > 150 ng/mL), post-operative hypertension were risk factors of CKD; male, use of MMF were protective factors of CKD (Table 5).

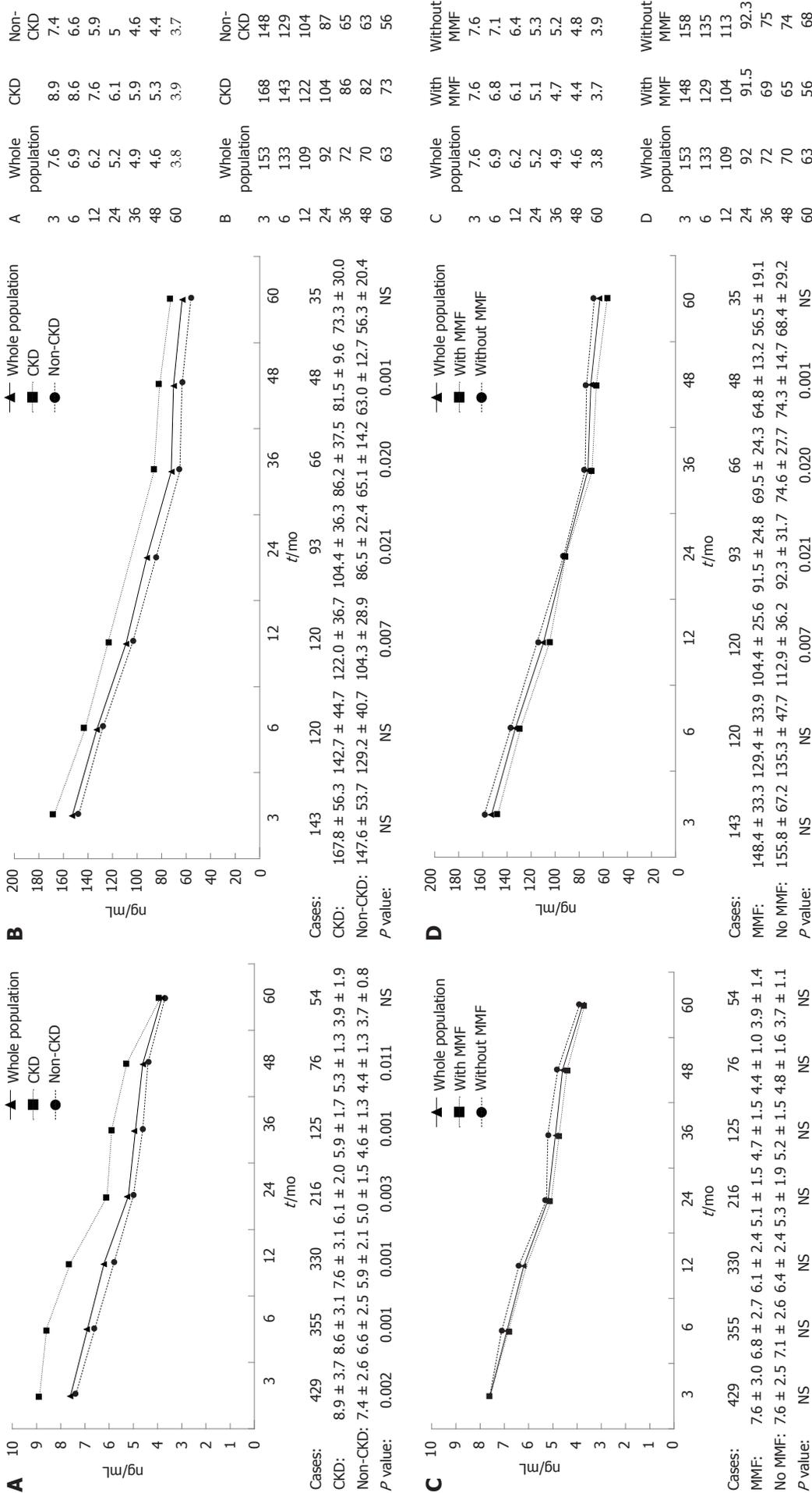
## DISCUSSION

The incidence of CKD increases with survival time after LT. As its nephrotoxicity proved by more researches, every center realized the importance of CNI dose adjustment. But how and when to adjust it still remains a question. There are two mainly strategies for CNI induced CKD, one is CNI withdrawal and conversion to a non-nephrotoxic immunosuppressant, such as sirolimus, MMF and azathioprine; the other is CNI dose reduction in combination with an auxiliary immunosuppressant<sup>[4,6,7,13,14]</sup>. Both were used. However, because of the CNI withdrawal time and dose reduction level differed among different centers, no agreement was reached.

Unlike the strategies mentioned above, in our center, an initial low CNI dose was administered. And by intensely monitoring of the graft function and gradually CNI dose reduction, a low blood concentration of CNI was maintained thereafter. The results displayed that over half the patients managed to maintain a CNI level within the target range in 3-mo and 1-year post LT and graft function remained stable compared with the high CNI level group. Moreover, groups of CNI concentrations within target range had significantly lower CKD incidence than the rest.

It was reported that CKD incidence varied to a great distance. Data from different centers varies partly because of different definitions of CKD<sup>[2-7]</sup>. We defined CKD as GFR < 60 mL/min per 1.73 m<sup>2</sup> in this research, a level at which the prevalence of complications of CKD begins to increase<sup>[19,20]</sup>. By this criterion, the incidence of CKD 5 years after LT was 17.3%, lower than many reports. Five factors have been incriminated as etiologic factors of CKD as demonstrated by the multivariate logistic analysis.

An important risk factor for CKD was CNI trough concentrations 3 mo after LT (OR = 2.935). Dose of CNI varies between different centers. Nevertheless, there is a consensus that the CNI concentration should be as low as



**Figure 2 Calcineurin inhibitor trough concentrations between different groups.** We compared the mean trough concentrations of tacrolimus (TAC) and cyclosporine A (CSA) at different time points between patients with and without chronic kidney disease (CKD) (A and B), between patients combined mycophenolate mofetil (MMF) use and no use (C and D). A: Trough concentrations of TAC grouped by CKD and non-CKD at different time points. Apart from 5 years after liver transplantation (LT), the CKD people had higher TAC trough concentrations than non-CKD people with statistical significance; B: Trough concentrations of CSA grouped by CKD and non-CKD at different time points. The CKD people had higher CSA trough concentrations than non-CKD people with statistical significance in 1, 2, 3, 4 years after LT; C and D: Trough concentrations of TAC (C) and CSA (D) grouped by combination with and without MMF at different time points. Patients with MMF combination had a lower calcineurin inhibitor trough concentrations but most without statistical significance. NS: Not significant.

**Table 2 Chronic kidney disease incidence between groups 3 mo and one year after liver transplantation**

Groups	Cases (CKD incidence %)	P value
Three months after LT		
CSA trough concentrations		
> 150 ng/mL	20/60 (32.3)	0.036
≤ 150 ng/mL	15/83 (18.1)	
TAC trough concentrations		
> 8 ng/mL	32/136 (23.5)	0.001
≤ 8 ng/mL	32/293 (10.9)	
One year after LT		
CSA trough concentrations		
> 120 ng/mL	19/41 (46.3)	< 0.001
≤ 120 ng/mL	12/79 (15.2)	
TAC trough concentrations		
> 6 ng/mL	36/147 (24.5)	< 0.001
≤ 6 ng/mL	17/183 (9.3)	

Patients were divided by the calcineurin inhibitor types and trough concentrations 3 mo post transplantation. Groups with ideal trough concentrations [cyclosporine A (CSA) trough concentrations ≤ 150 ng/mL, tacrolimus (TAC) trough concentrations ≤ 8 ng/mL] had much lower chronic kidney disease (CKD) incidence. Patients followed-up over 1-year (*n* = 450) were divided by the calcineurin inhibitor types and trough concentrations at one year post transplantation. Groups with ideal trough concentrations (CSA trough concentrations ≤ 120 ng/mL, TAC trough concentrations ≤ 6 ng/mL) had much lower CKD incidence. LT: Liver transplantation.

**Table 3 Chronic complications and graft function between different groups of calcineurin inhibitor trough concentrations**

Complications	Cases (CKD incidence %)	Group 1 (%)	Group 2 (%)	P value
DM	112 (19.6)	17.6	23.5	NS
Hypertriglyceridemia	107 (18.7)	18.1	19.9	NS
Hypercholesterolemia	76 (13.3)	12.8	14.3	NS
Hypertension	54 (9.4)	8.2	11.7	NS
AR	74 (12.9)	11.7	15.3	NS
CR	15 (2.6)	2.1	3.8	NS
Graft failure	17 (3.0)	2.7	3.6	NS
Re-transplantation	13 (2.3)	2.1	2.6	NS

Patients were divided according to the trough concentrations 3 mo after liver transplantation. Group 1: 376 cases, tacrolimus (TAC) ≤ 8 ng/mL or cyclosporine A (CSA) ≤ 150 ng/mL; Group 2: 196 cases, TAC > 8 ng/mL or CSA > 150 ng/mL. No statistical significance was found between groups. DM: Diabetes mellitus; AR: Acute rejection; CR: Chronic rejection; CKD: Chronic kidney disease.

possible to avoid CKD. Morard *et al*<sup>[21]</sup> identified trough levels of CSA ≥ 150 ng/mL or TAC ≥ 10 ng/mL at 1 year and CSA ≥ 100 ng/mL or TAC ≥ 8 ng/mL at 5 years as independent risk factors for impaired renal function. However, no agreement has yet been reached on what is the minimum and safe CNI dose for LT recipients.

Pre-LT baseline renal function has a major impact on that post-transplantation<sup>[3,6,7,22]</sup>. In this study, both renal dysfunction pre-operation and AKI post-operation proved to be important risk factors for CKD. Velidedeoglu *et al*<sup>[23]</sup> suggested that a combination of events during the first postoperative week after LT serve as a physiologic “stress test” for the kidneys. Patients who failed the test (peak creatinine > 2 mg/dL) were at increased risk of

**Table 4 The risk factors of chronic kidney disease by univariate logistic regression**

Clinical factors	P value	OR	95% CI
Factors before LT			
Gender (female = 0, male = 1)	0.001	0.385	0.232-0.638
Ages at LT	0.001	1.048	1.025-1.072
Liver cirrhosis	0.303	1.590	0.658-3.840
End stage liver disease	0.014	1.740	1.120-2.703
HBV infection	0.327	0.756	0.432-1.323
DM	0.091	1.932	0.900-4.147
Hypertension	0.052	3.092	0.990-9.659
Renal dysfunction	0.001	3.937	2.173-7.134
HRS	0.002	3.570	1.603-7.953
Hemodialysis	0.001	4.630	1.830-11.716
TB	0.014	1.001	1.000-1.003
MELD	0.000	1.049	1.023-1.075
Factors after LT			
Re-operation	0.454	1.324	0.635-2.761
Use of TAC	0.010	0.541	0.340-0.861
Use of MMF	0.003	0.520	0.336-0.805
CNI trough concentrations	0.001	2.528	1.627-3.927
AKI	0.001	3.785	2.275-6.296
Hypertension	0.001	2.972	1.619-5.455
DM	0.035	1.712	1.037-2.824
AR	0.509	1.107	0.820-1.494
CR	0.338	0.565	0.176-1.814
Graft failure	0.055	2.710	0.978-7.510
Re-transplantation	0.052	3.092	0.990-9.659
Hypertriglyceridemia	0.036	1.724	1.038-2.863
Hypercholesterolemia	0.059	1.733	0.979-3.070

OR: Odds ratios; CI: Confidence intervals; LT: Liver transplantation; HBV: Hepatitis B virus; DM: Diabetes mellitus; HRS: Hepatorenal syndrome; TB: Total serum bilirubin at baseline; TAC: Tacrolimus; MMF: Mycophenolate mofetil; AKI: Acute kidney injury; AR: Acute rejection; CR: Chronic rejection.

**Table 5 The risk factors of chronic kidney disease by multivariate logistic regression**

Clinical factors	P value	OR	95% CI
Age at LT	0.001	1.048	1.020-1.076
Pre-operative renal dysfunction	0.049	2.300	1.005-5.260
AKI	0.001	4.435	2.404-8.182
CNI trough concentration	0.001	3.233	1.923-5.438
Post-operative hypertension	0.035	2.230	1.059-4.696
Female	0.018	0.464	0.245-0.877
Use of MMF	0.002	0.435	0.255-0.741

OR: Odds ratios; CI: Confidence intervals; LT: Liver transplantation; AKI: Acute kidney injury; CNI: Calcineurin inhibitor; MMF: Mycophenolate mofetil.

chronic renal disease. Although both pre-operative renal dysfunction and AKI were considered to be reversible, with persistently nephrotoxication of CNI, the chance of recovery for kidney function is small and the injury could become irreversible and chronic finally. Induction therapy with both lymphocyte-depleting and non lymphocyte-depleting antibodies and delayed introduction of CNI (3-7 d) may preserve or ameliorate renal function in LT recipients with pre-transplant renal dysfunction without increasing the risk of rejection or compromising patient and graft survival<sup>[24-26]</sup>.

Hypertension commonly causes renal disease in general population, so it was not surprising that post-operative hypertension became risk factor of CKD. Once confirmed, recipients should receive active treatment. Administration of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers were recommended, for they could theoretically protect these patients from both the acute hemodynamic component and chronic vascular and tubulointerstitial injury. The benefit of the former class of agents is conferred predominantly *via* reduction in CNI-induced afferent arteriolar vasoconstriction and that of the latter is conferred *via* the inhibition of angiotensin II effects of transforming growth factor- $\beta$  and other profibrotic mediators<sup>[27]</sup>.

Our finding showed use of MMF as a protective factor of CKD. And as an accessory immunosuppressant, MMF does not have nephrotoxicity and could reduce the CNI dosage. Strategies to alleviate CNI nephrotoxicity include use of MMF with CNI dose reduction or CNI-withdrawal and conversion to MMF<sup>[4,6,7,13,14,28]</sup>. We found that, with an 1.0-1.5 g/d administration, most people had no severe side effects, which proved to be safe and effective.

Another protective factor of CKD was male gender. In the general population, however, male gender is more closely associated with renal disease progression, but the reason for this inconsistency is unclear<sup>[5,29]</sup>.

HBV infection was not a significant risk factor compared to other causes in this research. This finding was similar to previous studies based on Asian people<sup>[12]</sup>. HCV infection was also not a significant risk factor for CKD progression. In western countries, HCV is the most common cause of liver failure, and it increases CKD risk in liver transplantation primarily because it may cause glomerulonephritis<sup>[9,30]</sup>. Nevertheless, only 7 patients had HCV related cirrhosis in this cohort. Lamivudine combined with individualized low-dose hepatitis B immunoglobulin was used as a prophylaxis against HBV recurrence after LT in our center. Adefovir, which is known to be nephrotoxic, was administered with only a few patients<sup>[31]</sup>. This could partly explain why our center has a relatively low incidence of CKD.

Posttransplantation DM is prevalent among LT recipients. It was reported earlier that the incidence of post-transplantation DM could reach 14.9% in the living donor liver transplantation<sup>[32]</sup>. However, in this study we found no relationship between DM and CKD. The proper explanation maybe that insulin was widely accepted and used among LT recipients. Most people had his blood sugar under the ideal range. Complications of DM were not as popular as usual.

While there is conflicting evidence in the literature on whether a TAC or CSA use is more beneficial<sup>[33,34]</sup>, we found a lower incidence of CKD in the TAC group and identified CSA as a risk factor for CKD by univariate logistic regression analysis. However, the analysis did not take into account a small number of patients ( $n = 20$ ) who switched CNIs during the follow-up. Therefore, the benefit of TAC over CSA remains inconclusive. Randomized, prospective studies with a large number of patients

will be needed to resolve this issue.

In conclusion, many factors have been associated with CKD progression. With few practical and validate strategy, we have shown that administration of low-dose CNI in combination with MMF could lower CKD incidence and did not increase AR rate, which provided as a successful experience for Chinese LT recipients. As mentioned above, there was part of the population left whose CNI concentration was above the target range. So the low dose was not adaptable for everyone. Closely monitoring of liver function during tapering the CNI dose was needed. Dose reduction must be based on a stable liver function. Limitation of this study is that data were collected retrospectively and that GFR was evaluated rather than measured. Study of prospective designed and CKD defined by measured GFR would be more convincing.

## COMMENTS

### Background

Use of calcineurin inhibitor (CNI) has greatly increased liver transplant recipient survival rates and reduced graft rejection rates in recent years. However, long duration of its use may cause chronic complications, like chronic kidney disease (CKD), which has severely affected the quality of life and survival of organ recipients.

### Research frontiers

There are two main strategies for CNI induced CKD, one is CNI withdrawal and conversion to a non-nephrotoxic immunosuppressant, such as sirolimus, mycophenolate mofetil (MMF) and azathioprine; the other is dose reduction combined with an auxiliary immunosuppressant. Both strategies were used, but no agreement has been reached.

### Innovations and breakthroughs

It has been proven that CNI nephrotoxicity was associated with its dosage and duration. However, no consensus was reached on the minimum CNI dose which is considered to be safe for liver transplantation (LT) recipients. Different with other centers, the authors carried out a strategy of initial low-dose CNI and maintaining low blood concentrations after. By closely monitoring of liver function and gradually tapering the CNI dose, the result was favorable with low incidence of CKD and acceptable graft functions.

### Applications

Administration of low-dose CNI in combination with MMF could lower CKD incidence and did not increase acute rejection rate, which provided as a successful experience for Chinese LT recipients. Limitation of this study is that data were collected retrospectively and that glomerular filtration rate (GFR) was evaluated rather than measured. Study of prospective designed and CKD defined by measured GFR is needed in the future.

### Peer review

The research proved a successful strategy to prevent CKD liver transplantation with ample data and strict design, which should be popularized by more centers.

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## Transient small bowel angioedema due to intravenous iodinated contrast media

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### Abstract

Three cases of transient proximal small bowel angioedema induced by intravenous administration of nonionic iodinated contrast media (CM) are presented. Computed tomography (CT) images in the venous phase displayed the proximal small bowel with circumferential thickening of the wall including the duodenum and proximal segment of the jejunum. The bowel wall was normal in non-enhanced images, and normal or inconspicuous in arterial phase enhanced images. In one of the three cases, the bowel wall was thickened in venous phase but disappeared in the 40 s delayed phase images. No filling defect was seen in the lumen of the superior mesenteric artery and vein. No peritoneal effusion or mesentery abnormality was found. Each of these patients reported only mild abdominal discomfort and recovered without specific treatment within a short time. Only one patient suffered mild diarrhea after scanning which had resolved by the following day. The transient anaphylactic small bowel angioedema due to intravenous iodinated contrast media was easily diagnosed based on its characteristic CT findings and clinical symptoms. Differential diagnosis may include inflammatory and ischemic bowel disease, as well as neoplasms. A three-phase CT protocol and good under-

standing of this disorder are fundamentally important in the diagnosis of this condition. The supposed etiology behind the transient anaphylactic reaction to intravenous administration of iodinated CM in small bowel is similar to other CM-induced hypersensitive immediate reactions. The predilection location of transient anaphylactic bowel angioedema is the small intestine, particularly the proximal segment. A speculated cause may be the richer supply of vessels in the small intestine, ample mucous folds and loose connective tissue in the duodenum and the jejunum.

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**Key words:** Bowel angioedema; Bowel thickening; Computed tomography; Contrast media; Small bowel anaphylaxis

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### INTRODUCTION

Anaphylactic reactions to intravenous nonionic iodinated contrast media (CM) range from mild flushing to severe cardiopulmonary arrest and occur in about 1% of patients<sup>[1,2]</sup>. Such anaphylactic reactions can occur in the gastrointestinal tract presenting as bowel angioedema. To our knowledge, only four cases of small bowel angioedema and one case of colon angioedema were previously reported in the literature<sup>[3-5]</sup>. Here we report three cases of proximal small bowel angioedema and discuss their computed tomography (CT) findings, clinical features and outcomes.

## CASE REPORT

### Case one

A 55 year-old man with upper abdominal pain for one month was referred for an upper abdominal multi-phase contrast-enhanced CT examination. The patient drank 400 mL of iso-osmotic mannitol solution 30 min before the study and 100 mL just before the scan. After a conventional plain CT scan, a total of 90 mL nonionic iodinated CM (370 mgI/mL, Ultravist, Bayer Schering Pharma) was administered intravenously at a rate of 3 mL/s using an automated injector. The patient suffered mild abdominal discomfort during the examination and mild diarrhea after scanning. No dermal rash or other disorders were reported. These symptoms had resolved by the following day.

CT images in the venous phase displayed the proximal small bowel with circumferential thickening of the wall of a long segment (Figure 1A), including the descending, horizontal and ascending duodenum. However, the bowel wall was normal in the non-enhanced (Figure 1B) and arterial phase (Figure 1C) images. The fat around the bowel was clear. No filling defect was seen in the lumen of the superior mesenteric artery and vein. No peritoneal effusion or other abnormality was found. Therefore, the patient was clinically diagnosed with bowel angioedema, and follow-up was recommended.

Four months later, the patient received a repeat abdominal enhanced CT scan using another nonionic CM (370 mgI/mL, Iopamidol Injection, Bracco Diagnostics). The proximal small bowel was normal.

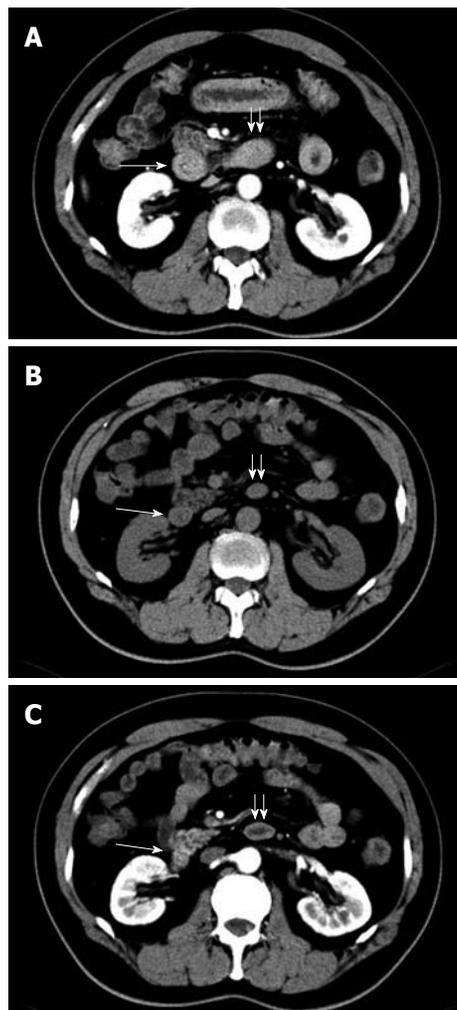
### Case two

A 46 year-old man with multiple colon polyps was referred for a complete abdominal contrast-enhanced CT exam. Prior to the CT scan, 1000 mL oral iso-osmotic mannitol solution was administered to distend the alimentary duct within one hour. The protocol for administering iodinated CM was the same as that in the first case, however, the CM (370 mgI/mL, Iopamidol Injection, Bracco Diagnostics) was different. The patient complained of mild abdominal discomfort after the CT examination. Symptoms resolved about thirty minutes later.

In non-enhanced CT images, no abnormality in the small bowel was found. The proximal segment of the small bowel showed slight thickening in arterial phase (Figure 2A) and marked circumferential thickening in venous phase (Figure 2B) CT images. The affected segment included the descending, horizontal and ascending duodenum. However, no extraluminal, mesenteric, or peritoneal pathological process was found.

### Case three

A 32-year-old woman with a mass in the right kidney was referred for a multi-phase abdominal enhanced CT exam. The examination protocol was the same as that in case one, except the CM was Omnipaque (350 mgI/mL, Iohexol Injection, GE Healthcare). An edematous proximal

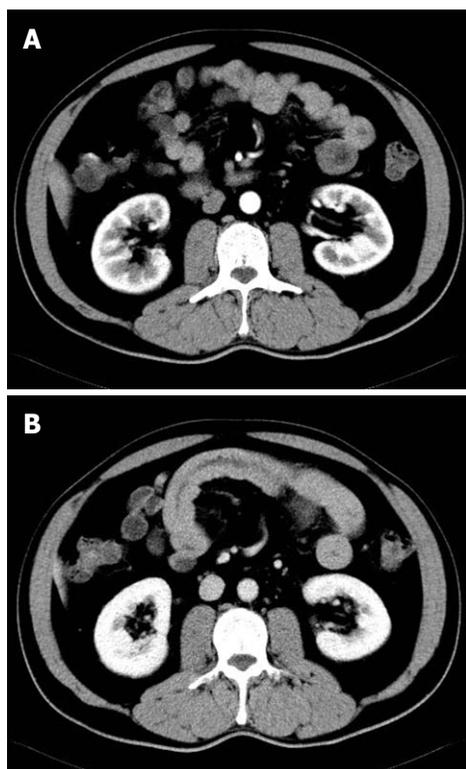


**Figure 1** A 55-year-old man. A: Circumferential thickening of the proximal small bowel in a long segment including the descending duodenum (long arrow) and horizontal duodenum (double short arrows) in venous phase image; B and C: This bowel segment appears normal in unenhanced image (B) and arterial phase image (C).

intestinal segment, including the second to fourth segment of the duodenum, was identified in venous phase CT images (Figure 3A). The intestinal wall was marked by circumferential thickening and the lumen was slightly dilated. Slight bowel edema was found in both arterial phase and 40-s delayed phase images (Figure 3B). However, the bowel segment was normal on the non-enhanced images (Figure 3C). Mild abdominal discomfort was the only symptom in this case and resolved spontaneously.

## DISCUSSION

Segmental bowel wall thickening is usually indicative of inflammatory bowel disease, mesenteric ischemia, or neoplastic disease. In the above-mentioned cases, thickening of the intestinal wall was most prominent on venous phase imaging. However, the bowel wall was normal in non-enhanced images and inconspicuous in arterial phase enhanced images. It is interesting that the bowel wall thickening disappeared in the 40-s delayed phase images in

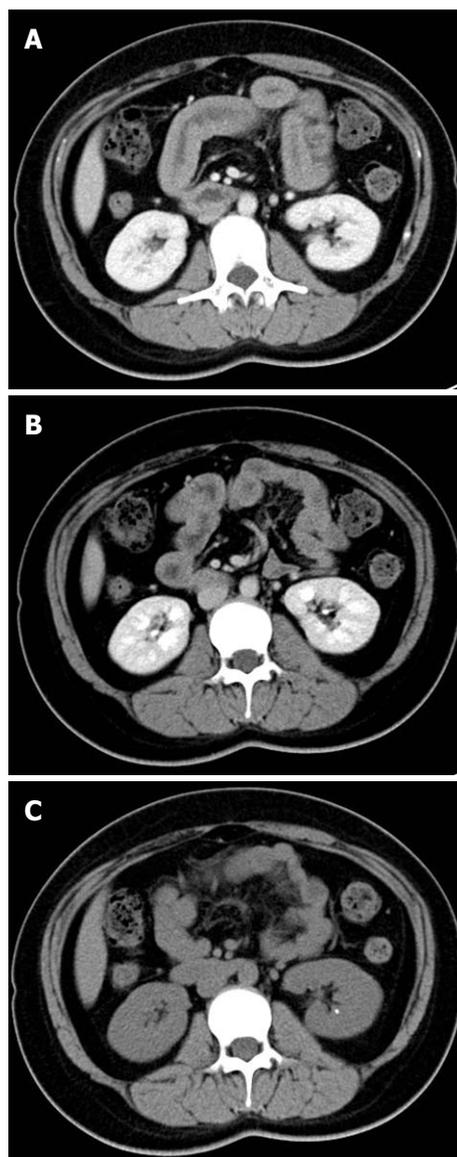


**Figure 2** A 46-year-old man. A: The proximal bowel segment is slightly thickened in arterial phase image; B: The proximal bowel segment shows marked circumferential thickening in venous phase.

the third case. A rapid change in bowel wall thickening may be an exclusive characteristic of anaphylactic small intestinal angioedema due to intravenous iodinated contrast. Although bowel wall angioedema can appear slightly thickened at an initially enhanced CT and be markedly edematous 4 h later, the majority of cases in the literature revealed that circumferential wall thickening of small bowel segments was found only at initial enhanced CT images<sup>[3,4]</sup>. In our cases, peak wall thickening appeared on the venous phase about 65 s after administration of intravenous contrast. Another feature of the CT findings in our cases was that there was no exudation, vascular engorgement, or lymphadenopathy around the thickened bowel segment, which is distinguishable from other pathological processes.

All three patients felt mild abdominal discomfort during scanning with contrast enhancement, but recovered after the CT examination without special treatment. Only one patient complained of mild diarrhea on the day of examination. Such symptoms may be caused by oral administration of iso-osmotic mannitol solution before examination for distending the stomach and small intestine<sup>[6]</sup>. Our findings, in accordance with previous reports, indicate that anaphylactic angioedema of the small bowel induced by iodinated CM was self-limiting and resolved quickly without additional intervention<sup>[4]</sup>.

To our knowledge, there have only been 4 cases reported in the literature with iodinated CM-induced small bowel anaphylactic angioedema. The incidence of CM-induced bowel anaphylactic angioedema is very low com-



**Figure 3** A 32-year-old woman. A: Venous phase image reveals an edematous proximal small bowel segment, including the descending and horizontal duodenum; B: The edematous wall resolved after the 40-s delayed image; C: This same bowel segment presented as normal on the unenhanced image.

pared with pruritus and mild urticaria in affected patients<sup>[1]</sup>. Due to high time resolution, CM-induced bowel anaphylactic angioedema may not be well displayed in CT images before or after the venous phase, about 65 s after administration of iodinated CM. A transient wall thickening without pathological CT findings around the bowel wall and/or obvious clinical symptoms may miss the diagnosis of CM-induced bowel anaphylactic angioedema. Occasionally, it may be misdiagnosed as inflammatory or ischemic bowel disease, especially if only the venous phase protocol is used for multi-detector CT enterography<sup>[7]</sup>. Based on the above-mentioned possibilities, we presume that such a condition may be clinically underestimated.

The exact etiology of the anaphylactic reactions to intravenous administration of iodinated CM is not completely understood. In our three cases, anaphylactic angioedema of the proximal small intestine was induced

by different brands of nonionic iodinated CM. In case one, the patient was hypersensitive to one type of commercial product, but not to another type. The concentration of iodinated CM we used was relative high (350 or 370 mgI/mL). However, anaphylactic small bowel angioedema can also be induced by iodinated CM at lower concentrations (300 or 282 mgI/mL) according to the literature<sup>[4,5]</sup>. Thus, the relationship between anaphylactic small bowel angioedema and the concentration of iodine in the CM was not assured. We propose that transient anaphylactic small bowel angioedema shares the same underlying etiology as the other non-allergic CM-induced hypersensitive immediate reactions<sup>[8,9]</sup>. With regard to the reason why most cases (including ours) of transient anaphylactic bowel angioedema occur in the small intestine, particularly the proximal segment, the speculated cause may be the richer supply of vessels in the small intestine than in the colon, as well as the ample mucous folds and loose connective tissue in the jejunum.

In conclusion, transient anaphylactic small bowel angioedema due to intravenous iodinated contrast media is easily diagnosed based on its characteristic CT findings. However, the three-phase CT protocol and a good understanding of this disorder are fundamentally important.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

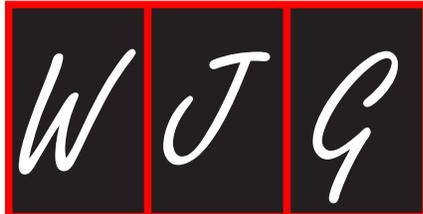
October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States



## GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only

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## Instructions to authors

AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g.  $6.92 \pm 3.86$  vs  $3.61 \pm 1.67$ ,  $P < 0.001$ ; CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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### Acknowledgments

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#### 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. Immunolog-

ic 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]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. 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 dis-

eases. *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/index.htm>

**Patent (list all authors)**

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

**Statistical data**

Write as mean  $\pm$  SD or mean  $\pm$  SE.

**Statistical expression**

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 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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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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## Radiofrequency ablation or percutaneous ethanol injection for the treatment of liver tumors

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### Abstract

The liver is a common location of both primary and secondary malignancies. For unresectable liver cancer, many local ablative therapies have been developed. These include e.g., percutaneous ethanol injection (PEI), percutaneous acetic acid injection, radiofrequency ablation (RFA), cryoablation, microwave ablation, laser-induced thermotherapy, and high-intensity focused ultrasound. RFA has recently gained interest and is the most widely applied thermoablative technique. RFA allows more effective tumor control in fewer treatment sessions compared with PEI, but with a higher rate of complications. However, there are certain circumstances where PEI therapy represents a better strategy to control liver tumors than RFA, especially in situations where RFA is difficult, for example when large vessels surround the tumor. In the context of hepatocellular carcinoma (HCC), both RFA and PEI are feasible and of benefit in non-operable patients. RFA seems superior to PEI in HCC > 2 cm, and the combination of interventions may be of benefit in selected patients. Liver resection is superior to RFA for patients with HCC meeting the Milan criteria, but RFA can be employed in tumors ≤ 3 cm and where there is an increased expected

operative mortality. In addition, some lines of evidence indicate that RFA and PEI can be employed as a bridge to liver transplantation. The use of RFA in colorectal liver metastases is currently limited to unresectable disease and for patients unfit for surgery. The aim of this article is to summarize the current status of RFA in the management of liver tumors and compare it to the cheap and readily available technique of PEI.

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**Key words:** Colorectal liver metastases; Hepatocellular cancer; Liver resection; Percutaneous ethanol injection; Radiofrequency ablation

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the third highest cause of cancer-related death and the incidence appears to be rising<sup>[1,2]</sup>. Partial hepatectomy and liver transplantation are potential curative options, but only a minority (10%-20%) are candidates for these therapies<sup>[3]</sup>. For inoperable cases, sorafenib (Nexavar<sup>®</sup>) is now available, inhibiting tumor-cell proliferation and tumor angiogenesis, as well as increasing the rate of apoptosis. It acts by inhibiting the serine-threonine kinase Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptor  $\beta$ <sup>[4,5]</sup>. Sorafenib increased median survival

and the time to radiological progression by nearly three months (7.9-10.7 mo and 2.8-5.5 mo, respectively) in patients with inoperable HCC<sup>[5]</sup>. The effect of sorafenib has been reproduced<sup>[6]</sup>. Furthermore, sorafenib has been reported to be cost-effective as compared to best supportive care in HCC<sup>[7]</sup>. Its multikinase inhibition, which is effective in RAS-mediated signal transduction pathways, is potentially applicable in other tumors<sup>[8,9]</sup>. Currently, the combination of sorafenib and various other chemotherapeutic agents in HCC is under investigation<sup>[10]</sup>. In the future, sorafenib might become an adjuvant following resection or local ablation of HCC<sup>[11]</sup>.

Liver metastases occur in almost half of cases with colorectal cancer<sup>[12]</sup>. Resection is the treatment of choice for the 20% to 30% that have metastatic disease limited to the liver<sup>[13]</sup>. Novel local treatment, embolization, and not least, neoadjuvant radiochemotherapy have increased the number of resectable cases<sup>[14,15]</sup>. For those with initially unresectable colorectal liver metastases (CLM), downstaging with chemotherapy followed by rescue liver resection is safe and effective<sup>[16]</sup>. The role of chemotherapy in resectable CLM is not entirely certain. The use of perioperative FOLFOX has been associated with improvement of 3-year progression-free survival<sup>[17]</sup>.

Over the years, local ablative methods have been developed for patients that are not surgical candidates because of multifocal disease, an inadequate liver remnant (size, function), or co-morbid conditions. These techniques include percutaneous ethanol injection (PEI), percutaneous acetic acid injection, radiofrequency ablation (RFA), cryoablation, microwave ablation, laser-induced thermotherapy, high-intensity focused ultrasound, and irreversible electroporation<sup>[18]</sup>. RFA has recently received increased attention, and is the most studied of the thermal ablation techniques<sup>[19]</sup>. This article reviews the current role of RFA in the treatment of liver tumors and compares it to PEI, which is a safe, cost-efficient, and readily available alternative to RFA.

## RADIOFREQUENCY ABLATION AND PERCUTANEOUS ETHANOL INJECTION

Modern RFA for the treatment of liver tumors has been available since the early 1990s<sup>[20,21]</sup>. RFA induces thermal injury to the target tissue using electromagnetic energy with a frequency < 900 kHz. An electrode is placed into the lesion and a high-frequency alternating current is delivered. The current causes ionic agitation and frictional heat, leading to irreversible cellular changes, such as protein denaturation and coagulative necrosis of tumor cells. Optimal ablation results are achieved when local temperatures of 60 °C to 100 °C are employed. At temperatures above 100 °C, the increase in the electrical impedance because of tissue carbonization and vaporization limits the amount of energy that can be delivered. Internally cooled-wet electrodes have been developed that combine interstitial saline infusion and intra-electrode cooling, thereby avoiding tissue desiccation around the electrode

tip and maintaining low impedance during treatment, while increasing the ablative zone<sup>[22,23]</sup>. RFA can be performed either percutaneously or intraoperatively with laparoscopy or laparotomy. The percutaneous approach is less invasive and may be associated with reduced postoperative pain, fewer complications, shorter hospital stay, and reduced costs<sup>[24-26]</sup>. However, surgical RFA allows more accurate cancer staging because of the direct macroscopic visualization of tumors and the use of intraoperative ultrasound. In patients with small HCC ( $\leq 3$  cm), percutaneous and surgical RFA had similar complete ablation rates and the overall and disease-free survival were comparable at 1 and 3 years<sup>[25]</sup>. However, for larger tumors (3.1-5 cm), the surgical approach was associated with better overall survival. In addition, surgical RFA may be preferred for HCC occurring in high-risk locations, defined as less than 5 mm from a large vessel or an extrahepatic organ<sup>[27]</sup>.

PEI was described for the first time in 1983<sup>[28]</sup>. PEI is performed under local anesthesia using a 21-gauge needle guided by ultrasound or computed tomography. Ethanol diffuses into the tumor cells and causes dehydration and protein denaturation, resulting in coagulative necrosis. This is followed by microvascular thrombosis and subsequent tumor ischemia. The amount of ethanol required can be calculated using the formula of a sphere, where 0.5 is added to provide extra safety margin:  $V = 4/3 \times \pi \times (r + 0.5)^3$ . The advantages of PEI are its relative safety, simplicity, and low cost. However, PEI is limited to small lesions and requires multiple sessions because of its reduced diffusion capacity over intratumoral septa.

Tumor seeding has occasionally been reported following RFA and PEI, but the risk remains low (< 2%) when an adequate ablation technique is employed and there is no previous biopsy<sup>[29-33]</sup>. Several studies have confirmed the safety of RFA and PEI in patients awaiting liver transplantation<sup>[34-41]</sup>. According to the practice guidelines from the American Association for the Study of Liver Diseases (AASLD), there is level II evidence to support the use of RFA and PEI as a bridge to liver transplantation<sup>[42]</sup>. However, the actual value of local therapies before liver transplantation remains controversial. It has been suggested that bridging therapies such as RFA or PEI should be reserved for patients with a predicted waiting list time of more than 6 mo<sup>[43,44]</sup>.

## RADIOFREQUENCY ABLATION VS LIVER RESECTION

Two recent systematic reviews<sup>[45,46]</sup> showed no significant differences in survival between RFA and resection for HCC within the Milan criteria, although RFA was associated with higher recurrence rates. When RFA was used for tumors outside the Milan criteria, overall and disease-free survival rates were significantly better after resection<sup>[46]</sup>. Zhou *et al.*<sup>[47]</sup> performed a meta-analysis of one randomized controlled trial (RCT) and nine controlled studies comparing RFA and liver resection for HCC

**Table 1 Summary of meta-analyses of radiofrequency ablation vs liver resection and radiofrequency ablation vs percutaneous ethanol injection**

Author	Diagnosis	Treatment	n	Tumor response (OR)	Complications (OR)	Local recurrence (OR)	Overall survival (OR)
Zhou <i>et al</i> <sup>[47]</sup>	HCC	RFA	744	-	0.29 (0.13-0.65)	4.50 (2.45-8.27)	0.60 (0.36-1.01) (5 yr)
		resection	667				
Wu <i>et al</i> <sup>[50]</sup>	CLM	RFA	273	-	0.32 (0.07-1.52)	4.89 (1.73-13.87)	0.41 (0.22-0.90) (5 yr)
		resection	574				
Bouza <i>et al</i> <sup>[63]</sup>	HCC	RFA	396	1.10 (1.04-1.17)	Total: 2.55 (1.8-3.65) Major: 1.85 (0.68-5.01)	0.37 (0.23-0.59)	1.24 (1.05-1.48) (4 yr)
		PEI	391				

PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; OR: Odds ratio; HCC: Hepatocellular carcinoma; CLM: Colorectal liver metastases.

(Table 1). Seven hundred and forty-four patients treated with RFA and 667 treated with resection were included. The 5-year overall survival rate was borderline significant at 5 years [OR 0.60 (0.36-1.01)]. RFA was associated with higher local recurrence [OR 4.50 (2.45-8.27)] and the disease-free survival rates were significantly better for resected patients. A recently published RCT by Huang *et al*<sup>[48]</sup> based on 230 patients fulfilling the Milan criteria, confirmed the superiority of surgical resection. The benefits of liver resection are further supported by data concerning quality of life adjusted survival, which seem to favor resection over RFA, at least in cirrhotic patients<sup>[49]</sup>. However, although current evidence favors liver resection for the treatment of early HCC (within the Milan criteria), percutaneous RFA can be used as a first-line treatment when the tumor is ≤ 3 cm and the expected operative mortality rate is above 3%<sup>[45]</sup>.

Data concerning RFA vs resection for CLM are accumulating. A recent meta-analysis by Wu *et al*<sup>[50]</sup> compared RFA and liver resection for solitary CLM (Table 1). Liver resection was better than RFA in terms of 5-year overall survival, as well as local control of the disease. There were no statistically verified differences regarding the rate of adverse events. According to the study by Gazelle *et al*<sup>[51]</sup>, liver resection is more effective in terms of quality-adjusted life year (QALY) as compared to RFA.

### PERCUTANEOUS ETHANOL INJECTION VS LIVER RESECTION

PEI is suggested to be as safe and effective as resection in early-stage HCC (n = 76)<sup>[27]</sup>. On the other hand, Cho *et al*<sup>[52]</sup> reported that liver resection had a better survival profile as compared to PEI in 230 HCC patients (5-year overall survival 49% vs 19%). Repeated single-session PEI is effective in patients with advanced HCC and a combination of transcatheter arterial chemoembolization (TACE) and PEI results in longer survival<sup>[53]</sup>.

The effectiveness of PEI for the treatment of CLM is unclear. It was reported that in more than 50% of cases of liver metastases < 4 cm, complete necrosis can be obtained by means of PEI<sup>[54]</sup>. Giorgio *et al*<sup>[55]</sup> reported that the survival rates of patients with liver metastases who underwent PEI were 94%, 80%, 80%, and 44% at 12 mo, 24 mo, 36 mo, and 44 mo, respectively. However, Livraghi *et al*<sup>[56]</sup> reported that PEI was ineffective for

the treatment of colorectal metastases. The main difficulty is that liver metastases are typically scirrhous and the alcohol tends to spread into the “soft” adjacent liver parenchyma. HCC is often surrounded by a cirrhotic parenchyma and is more vascularized than CLM, allowing for a better local effect of PEI<sup>[57,58]</sup>.

### RADIOFREQUENCY ABLATION VS PERCUTANEOUS ETHANOL INJECTION IN HEPATOCELLULAR CARCINOMA

Giorgio *et al*<sup>[59]</sup> recently conducted an RCT of percutaneous RFA and PEI in 285 HCC patients with tumors ≤ 3 cm. The primary endpoint was 5-year survival. In the RFA and PEI groups, the 5-year survival rates were 70% and 68%, respectively (not significant). The local recurrence rates were also not significantly different. The overall costs of RFA and PEI were 171 000 Euros and 1359 Euros, respectively (P < 0.0001). According to pooled data from two previous RCTs<sup>[60,61]</sup>, RFA and PEI are equally effective for tumors ≤ 2 cm<sup>[62]</sup>. However, the effect of RFA is more predictable than PEI when considering all tumor sizes. The meta-analysis by Bouza *et al*<sup>[63]</sup> included a total of 396 patients treated by RFA and 391 treated by PEI (Table 1). RFA yielded a better overall survival (1 year, 2 year, 3 year, and 4 year), as well as disease-free survival (1 year, 2 year, and 3 year). The risk of local recurrence was reduced in the RFA group. Total complications were increased for RFA, but major complications were not significantly different. In terms of cost-effectiveness of PEI over RFA, data are still controversial. Brunello *et al*<sup>[64]</sup> reported that mean direct hospital costs were 6540 Euros for RFA treated patients and 4097 Euros for the PEI group (P < 0.001), whereas Seror *et al*<sup>[65]</sup> reported that RFA was the most cost-effective option.

### RADIOFREQUENCY ABLATION-IMPROVEMENT WHEN COMBINED WITH PERCUTANEOUS ETHANOL INJECTION

RFA enables more effective tumor necrosis in fewer treatment sessions, albeit with a higher rate of complications than with PEI therapy. Nevertheless, there are certain circumstances or clinical situations that PEI therapy

offers a better strategy to control liver tumors than RFA, especially in situations where RFA is difficult. PEI can be useful when tumors are in close vicinity (< 1 cm) to vital structures, including biliary ducts, stomach, intestinal loops, and kidney, and whose location make them difficult to treat with thermal ablative techniques<sup>[66]</sup>. In addition, PEI is useful for lesions that remain undetected by ultrasound, such as masses in the hepatic dome or small nodules, or when the tumor is located subcapsularly or exophytically or surrounded by major vessels<sup>[66]</sup>. The efficacy of RFA in HCC can be improved if combined with PEI. The combination of RFA and PEI was shown to be more effective than RFA alone for high risk locations<sup>[67]</sup>. The combination of RFA and PEI can also be used for recurrent HCC (diameter > 3 cm)<sup>[68]</sup>. Furthermore, RFA and PEI can be combined with other locoregional therapies. TACE and RFA were as effective as liver resection for HCC in a recent study, with similar 1-, 3-, and 5-year overall and recurrence-free survival rates<sup>[69]</sup>. The combination of microwave ablation and PEI was safe and effective for the treatment of liver tumors adjacent to the hepatic hilum<sup>[70]</sup>.

## CONCLUSION

Liver resection still remains the gold standard for the treatment of liver tumors. RFA and PEI are feasible and of benefit in non-operable patients, and as bridging therapies before liver transplantation. RFA seems superior to PEI in HCC > 2 cm and the combination of both interventions may be of benefit in selected patients. Liver resection is superior to RFA for patients with HCC meeting the Milan criteria, but RFA can be employed in tumors ≤ 3 cm and where there is increased expected operative mortality. Using RFA as the primary treatment of resectable CLM is still not recommended; however, RFA may have a role in unresectable lesions and for patients unfit for surgery. Newer advancements in the field of local ablative therapies are to be expected, requiring constant evaluation of the different techniques. In the future, local ablative approaches may be combined with systematically administered molecular targeted therapies (e.g., sorafenib) to further improve the outcomes of patients with liver malignancies.

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## Neuroendocrine liver metastases: Contributions of endoscopy and surgery to primary tumor search

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### Abstract

Neuroendocrine tumors (NETs) are diagnosed with increasing frequency and patients often present with liver metastases at the time of diagnosis. Apart from treatment of the metastases, resection of the primary tumor at an early phase is recommended to prevent complications, although it may be difficult to locate, especially in patients with functionally inactive NETs. Small and multifocal tumors in the jejunum and ileum represent a particular challenge. Primary hepatic neuroendocrine carcinoma is extremely rare and is diagnosed only after exclusion of other primary tumors. Therefore, some uncertainty may remain, as small non-hepatic primary tumors may escape detection. Diagnostic work-up in these patients includes biochemical assays and imaging modalities (also comprising specific techniques of scintigraphy and positron emission tomography). This editorial highlights the contributions of endoscopy and operative exploration to the search for the primary tumor. Besides esophago-gastro-duodenoscopy, endoscopic ultrasonography, colonoscopy and bronchoscopy, special endoscopic techniques such as balloon enteroscopy or capsule endoscopy are used with growing experience. Compared with balloon enteroscopy, capsule endoscopy is non-invasive and better tolerated, but it cannot localize a lesion precisely and does not allow biopsy or removal

of lesions. Before proceeding to surgery, a discussion of the findings by a tumor board should be a standard procedure. Improvements in diagnostic tools have created new perspectives for the detection of obscure primary tumors in patients with neuroendocrine liver metastases, and these searches are best coordinated by a multidisciplinary team.

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**Key words:** Neuroendocrine tumor; Neuroendocrine carcinoma; Liver metastasis; Primary tumor; Endoscopy

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### INTRODUCTION

Neuroendocrine tumors (NETs)<sup>[1-9]</sup> are diagnosed with increasing frequency. The overall annual incidence of gastrointestinal NETs was recently reported to be 1.32 per 100 000 in men and 1.33 per 100 000 in women, with 12% of tumors in the stomach, 29% in the small intestine, 38% in the appendix, 13% in the colon, and

8% in the rectum<sup>[10]</sup>. After the lymph nodes, the liver is the second most common site of neuroendocrine metastases; in the study by Hlatky *et al*<sup>[11]</sup>, 39% of patients had liver metastases at the time of diagnosis. Resection of neuroendocrine liver metastases, if feasible, is the treatment of choice. In patients with advanced disease, an aggressive surgical approach, including staged procedures if required, is warranted as part of multimodal treatment<sup>[12-15]</sup>. Although resection of the primary tumor in an early phase is recommended to prevent complications<sup>[16,17]</sup>, the primary tumor may be difficult to locate<sup>[18]</sup>, especially in patients with functionally inactive NETs. If curative resection of liver metastases is not possible, however, it is unclear whether the primary tumor should also be removed. When possible, debulking, leaving < 10% of residual tumor volume, has proven helpful<sup>[19]</sup>. One argument for removing the primary tumor from patients with unresectable liver metastases is that these hepatic foci can be treated by interventional techniques, whereas the primary tumor is best treated by resection. Indeed, recent studies<sup>[20,21]</sup> have suggested that resection of the primary tumors, even from patients with unresectable neuroendocrine liver metastases, may be associated with improved survival.

About 11% to 14% of NET patients have metastatic disease with an unknown primary tumor<sup>[22]</sup>, and these primary tumors may also be multifocal. The most common primary location is the gastrointestinal tract, followed by respiratory and thymic carcinoids<sup>[23]</sup>. Primary tumors in the jejunum and ileum may be small, but they often cause a characteristic fibrosis in the mesentery (desmoplastic reaction), resulting in bowel obstruction, ischemia or perforation in approximately one-third of patients<sup>[24,25]</sup>. Primary hepatic neuroendocrine carcinoma is extremely rare<sup>[26,27]</sup> and is diagnosed only after exclusion of other primary tumors. Therefore, some uncertainty may remain, because small non-hepatic primary tumors may escape detection. As with metastases, the therapy of choice is resection<sup>[28]</sup>.

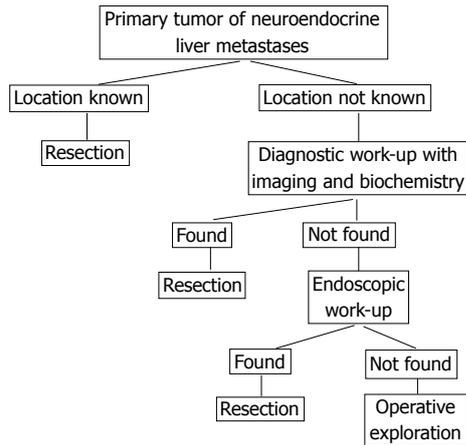
Diagnostic work-up in these patients includes biochemical assays and imaging modalities (Figure 1). Although ultrasonography is an important screening method with easy access and low costs, it remains operator dependent and its capacity for detecting primary tumors, especially in the small intestine, is limited. On contrast-enhanced ultrasonography using microbubbles, NETs of the small intestine and pancreas may present as hypoechoic, isoechoic, and/or hyperechoic liver lesions. Necrotic areas can also be identified, especially after treatment<sup>[29]</sup>. Computed tomography (CT) scans are very helpful in diagnosing NETs, especially larger foci in the mesentery or in the liver, but may not be useful in diagnosing small primary tumors. Magnetic resonance (MR) imaging is performed to determine the extent, anatomical relationships and resectability of the liver metastases but may also contribute to the detection and localization of intestinal primary tumors. On post-gadolinium T1-weighted fat-suppressed images, these primary tumors

can be visualized as nodular masses originating from the bowel wall, or as regional uniform bowel wall thickening with moderately intense enhancement<sup>[30]</sup>. Less is known about the uses of CT/MR enteroclysis. For example, CT enteroclysis in a small number of patients ( $n = 8$ ) had a sensitivity for detection of primary NETs of 50%, but false-positive results were also reported<sup>[31]</sup>. MR enteroclysis is an evolving technique that has shown promise in initial studies<sup>[30]</sup>.

Selective receptor-targeting radiopeptides have been developed for molecular imaging and therapy of NETs that overexpress peptide receptors on their cell membranes. The somatostatin analog <sup>111</sup>In-octreotide can be used to localize NETs expressing somatostatin receptors. Newer modified somatostatin analogs, including Tyr(3)-octreotide and Tyr(3)-octreotate, have been successfully used for diagnosis of these tumors, including the localization of primary tumors, and for radionuclide therapy and have therefore been named “theragnostics”. Radiopeptides targeting other receptors, including radiolabeled analogs of cholecystokinin, gastrin, bombesin, substance P, vasoactive intestinal peptide, and neuropeptide Y, may also be used<sup>[32]</sup>. As scintigraphy detects tumors not seen on CT and *vice versa*, these two imaging techniques can be seen as complementing each other. Imaging by <sup>18</sup>F-Dopa positron emission tomography (PET) is based on the decarboxylase contained in these tumors and is especially useful in patients with negative scintigraphy results. Due to better anatomical allocation of “hot spots”, PET can be combined with CT. Fluorodeoxyglucose (FDG) is taken up primarily by poorly differentiated tumors due to their enhanced glucose metabolism, making FDG-PET useful in depicting and identifying this group of tumors. <sup>68</sup>Ga-DOTATOC PET/CT is presently being investigated and has yielded promising results<sup>[33-35]</sup>. The combination of <sup>68</sup>Ga-DOTATOC with PET/CT was found to have an impact on the therapeutic management of 38% of patients with NET, including identifying the primary tumor for resection in about 8%; moreover, in 50% of these patients, the relevant findings were detected by a single imaging modality (i.e., CT or PET)<sup>[36]</sup>. These findings indicate that CT and PET have comparable sensitivity, provide complementary information and contribute equally to therapeutic decision making<sup>[36]</sup>.

## ENDOSCOPY

Upper and lower gastrointestinal endoscopy should be performed not only to identify the primary tumor, but also to rule out concomitant malignancies, which are present in a higher percentage (about 20%) of NET patients than in the normal population<sup>[37]</sup>. The importance of esophago-gastro-duodenoscopy (EGD) is emphasized by findings showing that the incidence of stomach NETs is increasing. Although one study<sup>[38]</sup> reported a 10-fold increase, this applies mainly to well-differentiated NETs and is probably due to increased rates of screening and associated diagnosis. Endoscopic ultrasonogra-



**Figure 1** Use of endoscopic and operative exploration in the search for primary tumors in patients with neuroendocrine liver metastases.

phy (EUS) is the method of choice for the assessment of tumor size and depth of infiltration<sup>[38]</sup> and is very helpful in the diagnosis of pancreatic NETs. A study of the EUS characteristics of cystic pancreatic NETs found that these tumors constitute 0.67% of all cystic pancreatic lesions and 9.5% of all confirmed pancreatic NETs<sup>[39]</sup>. Primary tumors in the colon can be detected by colonoscopy, and these examinations should include the terminal ileum, as far as possible. Figure 2 shows a resected small primary tumor in the terminal ileum that was detected by colonoscopy extending beyond the ileocecal valve. Intraoperatively, this tumor was not detectable, either by inspection from the serosal side or by palpation; rather, its removal was based on its endoscopic localization.

Bronchoscopy, in combination with thoracic CT or PET/CT, can help identify primary tumors in the bronchi/lungs.

Capsule endoscopy<sup>[40]</sup> is a practical technique, but analysis of the films requires expertise and time. Extraluminal tumor growth may limit this method. A comparison of CT, enteroclysis, nuclear imaging, and video capsule endoscopy (VCE) of the small bowel showed that CT and enteroclysis were unable to detect a primary tumor, whereas nuclear imaging identified abnormalities in the abdominal area in about two-thirds of the patients but was unable to relate this to an intestinal localization<sup>[16]</sup>. In contrast, VCE had a high diagnostic yield (45%) in identifying primary tumors in the small intestine. Although nuclear imaging had a comparable diagnostic yield, it could not differentiate between intestinal and mesenteric localization<sup>[16]</sup>. A study of 300 patients who underwent VCE for obscure bleeding following non-diagnostic EGD and colonoscopy showed that one had a neuroendocrine carcinoma, and nine had other small-bowel masses. Capsule retention occurred in two patients, with one requiring urgent surgery<sup>[41]</sup>.

An alternative for examination of the small bowel is balloon endoscopy, using either a single or double balloon technique<sup>[42,43]</sup>. This method, however, is time-consuming, and complications, including small bowel

perforation, ileus, and pancreatitis, have been reported in 0.8%–4.0% of patients<sup>[44]</sup>. A recent study<sup>[45]</sup> has shown that a single-balloon push-and-pull enteroscopy system was safe, useful, and highly effective in the diagnosis and treatment of several small-bowel diseases. Moreover, double balloon enteroscopy (DBE) in 12 consecutive patients with suspected NETs resulted in the detection of submucosal tumors in the ileum or jejunum in seven patients; in two patients with a submucosal ileum protrusion suspicious for NET, however, laparotomy and intraoperative endoscopy did not confirm that the protrusion was a tumor. The diagnostic yield of DBE for primary tumors in patients with metastatic or suspected NET was 33%, suggesting that DBE should only be performed in selected patients, possibly based on a previous positive work-up<sup>[46]</sup>.

Compared with balloon enteroscopy, capsule endoscopy is non-invasive and better tolerated, but it cannot localize a lesion precisely and does not allow biopsy or removal of lesions. The velocity of the transport of the capsule by peristalsis is irregular and there is no possibility of rinsing and suction; thus, a lesion may be missed. New technologies may expand the role of capsule endoscopy by, for example, controlling capsule movement, enabling this method to be used therapeutically or to obtain tissue biopsies and providing a transcatheter power delivery system<sup>[47]</sup>. A recent meta-analysis<sup>[48]</sup> found that capsule endoscopy and DBE had similar diagnostic yields in patients with obscure gastrointestinal bleeding.

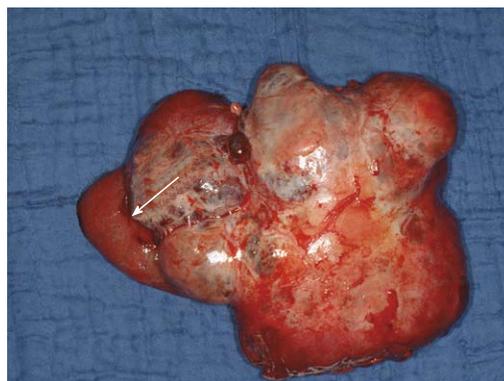
## OPERATIVE EXPLORATION

Intraoperative exploration may be performed as a self-contained procedure or in combination with other surgical goals such as resection of liver metastases or mesenteric masses, or cholecystectomy. Prophylactic removal of the gallbladder from NET patients during abdominal operations is recommended for two reasons. First, anticipating future long-term somatostatin analog therapy of these patients, removal of the gallbladder will prevent gallstone-associated complications associated with this treatment. Second, removal of the gallbladder will prevent gallbladder necrosis from hepatic artery embolization for liver metastases. In a recent study, preoperative identification of the primary tumor failed in 21% of patients, whereas laparoscopic exploration was successful in locating the primary tumor in 87%<sup>[49]</sup>. The presence of a mesenteric mass may indicate a primary tumor in the adjacent small bowel loop. Figure 3 shows the operative specimen from an NET patient with bulky disease in the mesentery; no primary tumor could be found pre- or intraoperatively. The adjacent loop of the ileum was resected *en bloc* with the mesenteric tumor (for reasons of blood supply), and its meticulous histopathological work-up yielded two foci of neuroendocrine carcinoma.

In contrast, if a small primary tumor is present in the small intestine without visible disease in the mesentery, a wide mesenteric dissection would be preferable, given



**Figure 2** Operative specimen of a small primary neuroendocrine tumor (arrow) in the terminal ileum detected by colonoscopy, extending beyond the ileocecal valve. Intraoperatively, this tumor was not detectable, either by inspection from the serosal side or by palpation; it was removed based on its endoscopic localization.



**Figure 3** Operative specimen from a neuroendocrine tumor patient with bulky disease in the mesentery; no primary tumor could be found pre- or intraoperatively. The mesenteric tumor was resected *en bloc*, along with the adjacent loop of the ileum (arrow); meticulous histopathological work-up of this ileal loop yielded two foci of neuroendocrine carcinoma.

the propensity of these tumors to cause mesenteric spread and desmoplastic masses.

During the course of laparoscopic exploration, exteriorization of the bowel and thorough palpation are recommended. Although these tumors may cause dimpling of the serosa, this is not a reliable feature and will not always be visible during laparoscopic exploration. Careful palpation, however, also allows identification of small and multifocal primary tumors.

Intraoperative ultrasonography (IOUS) is very helpful for liver resections and may be used to screen the pancreas, if no primary tumor is found in the intestine or at other locations. IOUS is also useful for measuring the distance between a pancreatic primary tumor and the pancreatic duct<sup>[50]</sup>. The pancreas was recently found to be the most common primary site (35%) for NET liver metastases but, as pancreatic tumors are well visualized on CT, all of them were detected preoperatively by CT<sup>[49]</sup>. Thus, occult primary tumors in the pancreas are highly unlikely in patients with NET liver metastases, limiting the use of EUS/IOUS of the pancreas in patients previously examined by CT.

During the course of the exploration, a biopsy can be taken of the liver metastases if they have not been identified histopathologically. Due to the rarity of these tumors, there have been few studies on the frequency and success of operative exploration, suggesting the need for multicenter studies using a standardized protocol. It is also unclear whether conversion to an open procedure is warranted if no primary tumor is found during laparoscopic exploration. If all laparoscopically visible parts of the abdominal cavity have been thoroughly inspected and the intestine has been exteriorized and meticulously palpated, open access by laparotomy will probably not yield any additional diagnostic benefit, although multiple tight adhesions may be an indication for conversion. To date, however, this has not been studied.

Before proceeding to surgery, a discussion of the findings by a tumor board should be a standard procedure.

In conclusion, improvements in diagnostic tools have

created new perspectives for the detection of obscure primary tumors in patients with neuroendocrine liver metastases, and these searches are best coordinated by a multidisciplinary team.

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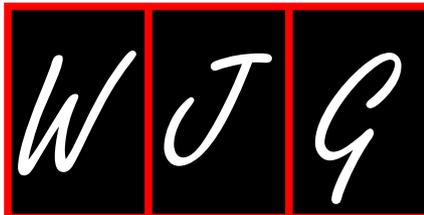
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## Strategy to differentiate autoimmune pancreatitis from pancreas cancer

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### Abstract

Autoimmune pancreatitis (AIP) is a newly described entity of pancreatitis in which the pathogenesis appears to involve autoimmune mechanisms. Based on histological and immunohistochemical examinations of various organs of AIP patients, AIP appears to be a pancreatic lesion reflecting a systemic "IgG4-related sclerosing disease". Clinically, AIP patients and patients with pancreatic cancer share many features, such as preponderance of elderly males, frequent initial symptom of painless jaundice, development of new-onset diabetes mellitus, and elevated levels of serum tumor markers. It is of uppermost importance not to misdiagnose AIP as pancreatic cancer. Since there is currently no diagnostic serological marker for AIP, and approach to the pancreas for histological examination is generally difficult, AIP is diagnosed using a combination of clinical, serological, morphological, and histopathological features. Findings suggesting AIP rather than pancreatic cancer include:

fluctuating obstructive jaundice; elevated serum IgG4 levels; diffuse enlargement of the pancreas; delayed enhancement of the enlarged pancreas and presence of a capsule-like rim on dynamic computed tomography; low apparent diffusion coefficient values on diffusion-weighted magnetic resonance image; irregular narrowing of the main pancreatic duct on endoscopic retrograde cholangiopancreatography; less upstream dilatation of the main pancreatic duct on magnetic resonance cholangiopancreatography, presence of other organ involvement such as bilateral salivary gland swelling, retroperitoneal fibrosis and hilar or intrahepatic sclerosing cholangitis; negative work-up for malignancy including endoscopic ultrasound-guided fine needle aspiration; and steroid responsiveness. Since AIP responds dramatically to steroid therapy, accurate diagnosis of AIP can avoid unnecessary laparotomy or pancreatic resection.

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**Key words:** Autoimmune pancreatitis; Pancreatic cancer; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography

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### INTRODUCTION

Autoimmune pancreatitis (AIP) is a recently described entity of pancreatitis in which the pathogenesis appears

to involve autoimmune mechanisms<sup>[1,2]</sup>. Characteristic histopathological findings in AIP patients in Japan include dense infiltration of T lymphocytes and IgG4-positive plasma cells, storiform fibrosis, and obliterative phlebitis in the pancreas; this form is termed lymphoplasmacytic sclerosing pancreatitis (LPSP)<sup>[1-3]</sup>. Recently, another AIP variant having different histological findings has been described. It is called idiopathic duct-centric pancreatitis (IDCP), and is rare in Japan but more prevalent in Europe and the United States<sup>[4-6]</sup>.

Clinically, AIP patients and those with pancreatic cancer have many features in common, such as painless jaundice, development of new-onset diabetes mellitus (DM), and elevated levels of serum tumor markers. In both populations there is preponderance of elderly males. In North America, about 2.5% of pancreatoduodenectomies were performed in AIP patients following a mistaken diagnosis of pancreatic cancer<sup>[7]</sup>. Since AIP responds extremely well to steroid therapy, it is of utmost importance that it be differentiated from pancreatic cancer to avoid unnecessary laparotomy or pancreatic resection.

Other prominent features of AIP include a variety of extrapancreatic complications. Patients frequently have significantly elevated serum IgG4 levels<sup>[8-10]</sup>. Currently, AIP is recognized as a pancreatic lesion of IgG4-related systemic disease<sup>[2,11]</sup>.

In this review, we will summarize clinicopathological features of AIP and describe a strategy to differentiate it from pancreatic cancer.

## AUTOIMMUNE PANCREATITIS

### Clinical features

AIP occurs predominantly in elderly males<sup>[12]</sup>. Typical presentation with severe abdominal pain and clinically acute pancreatitis is rare; the major presenting complaint is painless obstructive jaundice due to associated sclerosing cholangitis. Failure of pancreatic exocrine or endocrine function is frequently seen. Up to 50% of AIP patients present with glucose intolerance. The diagnoses of DM and AIP are made simultaneously in many cases, but some patients experience exacerbation of preexisting DM with the onset of AIP<sup>[2,11]</sup>.

### Other organ involvement: IgG4-related sclerosing disease

In addition to symptoms resulting from pancreatic involvement, AIP patients often have other complications, such as biliary stricture and thickening of the gallbladder wall, swelling of salivary and lacrimal glands, and a retroperitoneal mass. Histological features in these other anatomical locations include dense fibrosis with abundant infiltration of T lymphocytes and IgG4-positive plasma cells and obliterative phlebitis. We have observed these features in the periportal area of the liver, gastric mucosa, colonic mucosa, dermis, lymph nodes, and bone marrow of AIP patients<sup>[11,13,14]</sup>. Based on histological and immunohistochemical examinations of various organs of AIP

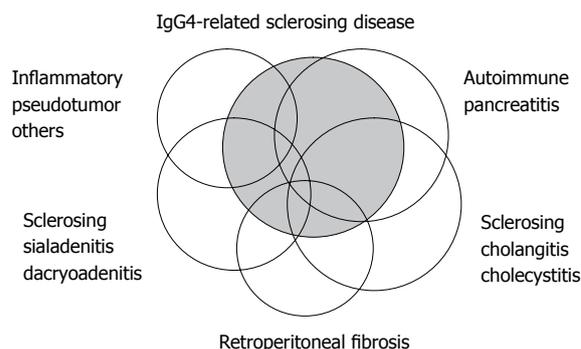


Figure 1 Schematic illustration of IgG4-related sclerosing disease.

patients, we proposed that a novel clinicopathological entity, an “IgG4-related sclerosing disease”<sup>[2,11,13]</sup> should be described.

IgG4-related sclerosing disease is a systemic disease affecting multiple organs with tissue fibrosis and obliterative phlebitis. We suggest that AIP appears to be a pancreatic lesion reflecting a systemic IgG4-related sclerosing disease, which can be manifest elsewhere to varying degree. In some cases, only 1 or 2 organs are clinically involved, while in others, 3 or 4 organs are affected (Figure 1)<sup>[2,11,13]</sup>. These extrapancreatic lesions can be synchronous or metachronous<sup>[15]</sup>.

### Histopathological features

Histological pancreatic findings in AIP patients with LPSP are characterized by dense infiltration of T lymphocytes and IgG4-positive plasma cells and storiform fibrosis. Obliterative phlebitis is frequently detected. The pancreatic duct is narrowed by periductal fibrosis and lymphoplasmacytic infiltration, but the ductal epithelium is usually preserved<sup>[1-3]</sup>.

American and European pathologists have described another unique histological pattern in AIP, which they have termed IDCP<sup>[4]</sup> or AIP with granulocyte epithelial lesion (GEL)<sup>[5]</sup>. Neutrophilic infiltration in the epithelium of pancreatic ducts is a characteristic feature of IDCP; this is not seen in LPSP. Infiltration of IgG4-positive plasma cells and obliterative phlebitis are uncommon in IDCP<sup>[4,5,16]</sup>. IDCP is seen mostly in Western countries, but it appears uncommon in Asia<sup>[6,17]</sup>. LPSP and IDCP are regarded as two distinct subtypes of AIP, and it has been proposed that LPSP be called “type 1 AIP” and IDCP “type 2 AIP”<sup>[6,16,18]</sup>.

### Diagnostic criteria for AIP

Since there is currently no diagnostic serological marker for AIP, and approach to the pancreas for histological examination is generally difficult, AIP is currently diagnosed on the basis of presence of a combination of abnormalities unique to AIP. The Japanese clinical diagnostic criteria for AIP were revised in 2006<sup>[19]</sup>. In 2006, new diagnostic criteria for AIP were proposed in Korea<sup>[20]</sup> and the United States<sup>[21]</sup>. In 2008, Asian diagnostic criteria for AIP were published by Japanese and Korean pancreatologists<sup>[22]</sup>.

**Table 1** Diagnosis of definitive and probable type 1 autoimmune pancreatitis using international consensus diagnostic criteria

Diagnosis	Primary basis for diagnosis	Imaging evidence	Collateral evidence
Definitive type 1 AIP	Histology	Typical/indeterminate	Histologically confirmed LPSP (level 1 H)
	Imaging	Typical indeterminate	Any non-D level 1/level2 Two or more from level 1 (+ level 2 D <sup>1</sup> )
Probable type 1 AIP	Response to steroid	Indeterminate	Level 1 S/OOI + Rt or level 1 D + level 2 S/OOI/H + Rt Level 2 S/OOI/H + Rt

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosing pancreatitis; H: Histology of the pancreas; S: Serology; D: Ductal imaging; OOI: Other organ involvement. <sup>1</sup>Level 2 D is counted as level 1 in this setting.

**Table 2** Level 1 and level 2 criteria for type 1 autoimmune pancreatitis

Criterion	Level 1	Level 2
Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)	Indeterminate (including atypia <sup>2</sup> ): Segmental/focal enlargement with delayed enhancement
Ductal imaging (ERP)	Long (> 1/3 length of the main pancreatic duct or multiple strictures without marked up stream dilatation)	Segmental/focal narrowing without marked upstream dilatation (duct size, < 5 mm)
Serology other organ involvement	IgG4, > 2x upper limit of normal value a or b a: Histology of extrapancreatic organs Any three of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration (2) Storiform fibrosis (3) Obliterative phlebitis (4) Abundant (> 10 cells/HPF) IgG4-positive cells b: Typical radiological evidence At least one of the following: (1) Segmental/multiple proximal (hilar/intrahepatic) or proximal and distal bile duct stricture (2) Retroperitoneal fibrosis	IgG4, 1-2x upper limit of normal value a or b a: Histology of extrapancreatic organs including endoscopic biopsies of bile duct <sup>3</sup> : Both of the following: (1) Marked lymphoplasmacytic infiltration without granulocytic infiltration (2) Abundant (> 10 cells/HPF) IgG-positive cells b: Physical or radiological evidence At least one of the following: (1) Symmetrically enlarged salivary/lachrymal glands (2) Radiological evidence of renal involvement described in association with AIP
Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells	LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells
Response to steroid (Rt) <sup>1</sup>	Diagnostic steroid trial Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestations	

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosing pancreatitis; HPF: High power field; ERP: Endoscopic retrograde pancreatography. <sup>1</sup>Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration; <sup>2</sup>Atypical: Some AIP cases may show low-density mass pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm); <sup>3</sup>Endoscopic biopsy of duodenal papilla is a useful adjunctive method because ampulla often is involved pathologically in AIP.

In 2011, international consensus diagnostic criteria for AIP were proposed<sup>[23]</sup>. According to these, AIP is classified into type 1 and 2. Five cardinal features of AIP are used: imaging of pancreatic parenchyma and ducts; serology; other organ involvement; pancreatic histology; and an optional criterion of response to steroid therapy. Each feature is categorized as a level 1 or 2 finding, depending on the diagnostic reliability. The diagnosis of type 1 and type 2 AIP can be definitive or probable (Tables 1 and 2).

### Treatment and prognosis

A multicenter study for steroid treatment of AIP was performed in Japan in 2009<sup>[24]</sup>, and Japanese consen-

sus guidelines for treatment of AIP were proposed in 2010<sup>[25]</sup>. According to the guidelines, steroid treatment is a standard therapy for AIP, as it is usually effective clinically, serologically, and radiologically in these patients, including for extrapancreatic lesions. It is most important to distinguish AIP from pancreatic cancer before starting steroid therapy. Indications for steroid therapy are symptoms such as obstructive jaundice, abdominal pain, and hydronephrosis. Before beginning steroid therapy, jaundice is usually managed by endoscopic or transhepatic biliary drainage in patients with obstructive jaundice, and the blood glucose level should be controlled with insulin in patients with DM. Initially, oral prednisolone (0.6 mg/



**Figure 2** Dynamic computed tomography of an autoimmune pancreatitis patient showing well-enhanced enlargement of the pancreas.

kg per day) is administered for 2-4 wk, and then the dose is tapered by 5 mg every 1-2 wk while carefully monitoring the patient's symptoms, as well as the biochemical, serological, and imaging findings, to a maintenance dose, a process usually requiring a period of 3-6 mo. Morphological and serological evaluation for effectiveness of steroid therapy is performed 2 wk after initiation. A poor response to steroid therapy should raise the possibility of pancreatic cancer and the need for re-evaluation of the diagnosis.

To prevent relapse, maintenance therapy (2.5-5 mg per day) is recommended for almost all patients for at least 6 mo. In patients showing complete remission 1 year after initial administration of steroids, maintenance therapy can be withdrawn. Maintenance therapy should be continued for a maximum of 3 years. In relapsed cases, re-administration or increasing the dose is effective.

AIP prognosis appears to be good over the short term with steroid therapy. However, long-term outcomes are unclear, because there are many unknown factors<sup>[26]</sup>. Pancreatic stone formation is observed in some relapsing AIP patients because of stenosis of the pancreatic duct system and facilitated pancreatic juice stasis<sup>[27,28]</sup>. AIP occurs predominantly in elderly males, and steroid therapy is immunosuppressive. It is reported that some patients develop malignancies during treatment, but it is unclear whether prolonged AIP is a risk factor for the malignancy<sup>[25,26]</sup>.

## STRATEGY TO DIFFERENTIATE AUTOIMMUNE PANCREATITIS FROM PANCREATIC CANCER

AIP should be included in the differential diagnosis for an elderly man presenting with obstructive jaundice and a pancreatic mass. Before therapy is initiated, it is of the utmost importance to differentiate AIP from pancreatic cancer.

### Obstructive jaundice

Obstructive jaundice induced by bile duct stenosis secondary to pancreatic cancer typically progresses steadily,

whereas the jaundice of AIP in IgG4-related sclerosing disease sometimes fluctuates or, in rare cases, improves spontaneously<sup>[2,11,25]</sup>.

### Serum IgG4 levels

AIP patients frequently have significantly elevated serum IgG4 levels<sup>[29]</sup>. In our series of 39 patients<sup>[30]</sup>, the median level was 301.5 mg/dL, and 30 (77%) had levels greater than 135 mg/dL. On the other hand, the median level was 34.0 mg/dL in 114 pancreatic cancer patients. However, 5 of these had levels  $\geq 135$  mg/mL; therefore, elevation of serum IgG4 levels alone cannot rule out pancreatic cancer. According to Ghazale *et al.*<sup>[31]</sup>, serum IgG4 levels were elevated in 13/135 (10%) of pancreatic cancer patients; however, only 1% had IgG4 levels  $> 280$  mg/dL, compared with 53% of AIP patients.

### Computed tomography imaging

Diffuse enlargement of the pancreas and effacement of the lobular contour of the pancreas, the so-called "sausage-like" appearance, is a typical finding in AIP, and is rarely seen in pancreatic cancer (Figure 2). On delayed-phase of dynamic computed tomography and magnetic resonance imaging (MRI), enhancement of an enlarged pancreas is characteristic of AIP. As fibroinflammatory changes involve the peripancreatic adipose tissue, a capsule-like rim surrounding the pancreas, is specifically detected in some AIP patients<sup>[32-34]</sup>.

### Diffusion weighted MRI

The clinical utility of diffusion weighted MRI (DW-MRI) for differentiating AIP from pancreatic cancer was reported<sup>[35]</sup>. AIP and pancreatic cancer were detected as high signal intensity areas. However, the high signal-intensity areas were found to be diffuse, solitary, and multiple in AIP patients, whereas all patients with pancreatic cancer had solitary areas. Additionally, the apparent diffusion coefficient (ADC) values were significantly lower in AIP than in pancreatic cancer patients or in individuals with a normal pancreas. Morphological differences seen in high signal intensity areas on DW-MRI and ADC values may prove useful to help distinguish AIP from pancreatic cancer.

### Endoscopic retrograde cholangiopancreatography

Irregular narrowing of the main pancreatic duct (MPD) on endoscopic retrograde cholangiopancreatography (ERCP) is a characteristic radiological feature of AIP, and is mandatory for meeting the Japanese diagnostic criteria for AIP<sup>[19]</sup>. In our study<sup>[36,37]</sup>, comparing the ERCPs of AIP and pancreatic head cancer patients, MPD findings that were highly suggestive of the former included no obstruction, skipped lesions, side branch derivation from the narrowed portion, narrowed portion  $> 3$  cm long, and a maximum diameter of  $< 5$  mm upstream (Figure 3). The histopathological differences around the ducts represent the different pancreatographic findings between AIP and pancreatic cancer (PC). Infiltrating cancer cells cause scirrhous changes, destroy ductal epithelium, and



**Figure 3** Endoscopic retrograde cholangiopancreatography of an autoimmune pancreatitis patient showing narrowing of the main pancreatic duct.

frequently obstruct main and branch ducts.

### Magnetic resonance cholangiopancreatography

Since magnetic resonance cholangiopancreatography (MRCP) has become popular as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree, it is becoming preferable to diagnostic ERCP in many cases. However, the narrowest MPD seen on ERCP cannot be visualized by MRCP due to the inferior resolution of MRCP compared with ERCP, so distinguishing between narrowing of the MPD in AIP and stenosis of the MPD in pancreatic cancer is not possible. However, less upstream dilatation of the MPD on MRCP suggests AIP rather than pancreatic cancer. Furthermore, MRCP is useful for judging response to steroid therapy<sup>[37,38]</sup>.

### Other organ involvements

Presence of other organ involvements such as bilateral salivary gland swelling, retroperitoneal fibrosis and hilar or intrahepatic sclerosing cholangitis is highly suggestive of AIP rather than pancreatic cancer.

On 18F-Fluorodeoxyglucose (FDG)-Positron Emission Tomography (PET), pancreatic FDG uptake is observed in both, but abnormal extrapancreatic uptake, such as extensive lymph nodes or swollen salivary glands, is highly suggestive of AIP<sup>[39]</sup>.

### Endoscopic ultrasound-guided fine needle aspiration

In some cases, when diagnosis is difficult, especially when segmental-type AIP is involved, histopathological examination is necessary. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is useful to either diagnose or rule out pancreatic cancer. However, definitive diagnosis of AIP is sometimes difficult by EUS-FNA, because of the small sample size obtained<sup>[40]</sup>. Therefore, EUS-guided core biopsy is recommended<sup>[41]</sup>. Positive IgG4-immunostaining in biopsy specimens taken from the major duodenal papilla supports a diagnosis of AIP<sup>[42]</sup>.

### Steroid responsiveness

There is reversible improvement of AIP with oral steroid therapy. In patients with typical radiological findings

highly suggestive of AIP, a diagnosis cannot be made, according to Japanese criteria<sup>[19]</sup>, if there are no histological features and negative laboratory tests. Although it can be diagnostic, a steroid diagnostic trial is not generally recommended; it should only be performed with extreme caution by pancreatologists in carefully selected patients after obtaining negative results from a thorough work-up for pancreatic cancer, including EUS-FNA<sup>[22,23]</sup>.

## CONCLUSION

For an elderly male presenting with obstructive jaundice and a pancreatic mass, AIP should be considered as a differential diagnosis to avoid performance of unnecessary surgery for presumed pancreatic cancer. As it is sometimes difficult to obtain adequate biopsy material from the pancreas, AIP is currently diagnosed based on careful consideration of a combination of characteristic clinical, serological, morphological, and histopathological features.

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## Botulinum toxin for chronic anal fissure after biliopancreatic diversion for morbid obesity

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### Abstract

**AIM:** To study the effect of botulinum toxin in patients with chronic anal fissure after biliopancreatic diversion (BPD) for severe obesity.

**METHODS:** Fifty-nine symptomatic adults with chronic anal fissure developed after BPD were enrolled in an open label study. The outcome was evaluated clinically and by comparing the pressure of the anal sphincters before and after treatment. All data were analyzed in univariate and multivariate analysis.

**RESULTS:** Two months after treatment, 65.4% of the patients had a healing scar. Only one patient had mild incontinence to flatus that lasted 3 wk after treatment, but this disappeared spontaneously. In the multivariate analysis of the data, two registered months after the treatment, sex ( $P = 0.01$ ), baseline resting anal pressure ( $P = 0.02$ ) and resting anal pressure 2 mo after treatment ( $P < 0.0001$ ) were significantly related to healing rate.

**CONCLUSION:** Botulinum toxin, despite worse results than in non-obese individuals, appears the best alternative to surgery for this group of patients with a high risk of incontinence.

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**Key words:** Botulinum toxin; Anal diseases; Anal fissure; Severe obesity; Bariatric surgery; Biliopancreatic diversion

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### INTRODUCTION

Approximately two-thirds of individuals living in the United States are overweight, and of those, almost half are obese<sup>[1,2]</sup>. The incremented prevalence of obesity is associated with an increase in the frequency of obesity comorbidity<sup>[3,4]</sup>, which is responsible for > 2.5 million deaths per year worldwide<sup>[5-9]</sup>.

In 1991, the US National Institutes of Health established guidelines for the surgical therapy of morbid obesity (body mass index  $\geq 40$  or  $\geq 35$  in the presence of significant comorbidity), now referred to as bariatric surgery<sup>[10]</sup>. The literature on postoperative weight loss and the problems associated with various bariatric surgical procedures is extensive and has been summarized elsewhere.

Bariatric surgery in morbidly obese individuals re-

verses, eliminates, or significantly ameliorates diabetes, hyperlipidemia, hypertension, and obstructive sleep apnea<sup>[11]</sup>. These benefits occur in the majority of patients who undergo surgery. Biliopancreatic diversion (BPD) is a malabsorptive bariatric technique that is successful in achieving long-lasting weight lost in super-obese patients<sup>[12,13]</sup>. In fact, the diarrhea (steatorrhea) that is expected after any malabsorptive technique can sometimes cause significant nutritional changes and anal disease. These patients are frequently referred to a coloproctology clinic due to hemorrhoids, fissures, and anal sepsis and fistula due to changes in quality and quantity of their feces. The aim of this technique in maintaining long-term weight loss is to produce malabsorption for fats and starch, which will lead to an important qualitative change in the patient's feces<sup>[14-16]</sup>. In a recent long-term study of outcome after BPD, Marinari *et al*<sup>[17]</sup> found that 85.5% of patients scored good or better; however, little is known about the direct consequences of changes in defecation and flatulence after BPD. In addition, a change in the equilibrium of intestinal flora and bacterial overgrowth syndrome may also increase malodorous gas and discomfort. Changes in bowel habit may have a direct influence on quality of life reported by patients after BPD. Although many of the possible metabolic side effects can be controlled with vitamin complexes, micronutrients and protein prophylactics, chronic diarrhea can be the cause of different proctological disorders. These lead to an increase in the assistance required by these patients, who therefore need intervention and re-intervention, with a consequential increase in health expenditure.

The most commonly recorded anal disorders seen in this type of patient are hemorrhoids, fissures, abscesses, and anal fistulae mainly due to repeated, continuous diarrhea and changes in qualitative composition that are typical of steatorrhea in malabsorptive processes. The aim of this study was to analyze the results of botulinum toxin treatment in chronic anal fissure after BPD.

## MATERIALS AND METHODS

This was an open label study. We present the results of the treatment with type A botulinum toxin in patients affected by chronic anal fissure developed after BPD for morbid obesity, observed at the Department of Surgery of the University Hospital "A. Gemelli" in Rome. Patients were sent to our Proctological Center after 1 year follow-up after BPD. History and physical examination pre-BPD did not show any anal disorders that existed before the bariatric surgery. The diagnosis was based on the following clinical criteria: (1) evidence of circumscribed ulcer with a large sentinel tag of skin, induration at the edges and exposure of the horizontal fibers of the internal anal sphincter; and (2) symptoms (post-defecation and/or nocturnal pain, bleeding) persisting for > 2 mo.

The exclusion criteria were as follows: acute fissure; fissure associated with different pathologies (i.e., inflammatory bowel disease, human immunodeficiency virus

infection, anal or perianal cancer); previous surgical procedures on the anal canal; the existence of systemic diseases with alteration of the cholinergic transmission that could confound the evaluations; concomitant oral medications that could interfere with the action of type A botulinum neurotoxin, such as aminoglycosides, baclofen, dantrolene or diazepam; known hypersensitivity to any of the components of the formulations of type A toxin; pregnant or breast-feeding women.

The study protocol was reviewed and approved by the Institutional Ethical Committee of the Catholic University of Rome. All patients gave written informed consent to participate in the study.

### Treatment target

In every treated patient, the primary target was the complete healing of the fissure. The healing was documented by inspection, physical examination and reported symptoms. Treatment was considered satisfactory only when a complete cicatrization was obtained.

### Baseline assessment and operative technique

All patients underwent pretreatment evaluation, including clinical inspection and anorectal manometry. In all the patients, age and sex were registered. We divided the patients into four age groups to make the examined population more homogeneous ( $\leq 30$ , 31-40, 41-50 and > 50 years). Specific data relative to the anal diseases, association with other anal or systemic pathologies, allergies, and previous surgery have been registered in every patient. Defecation pattern, fecal consistency, evacuation frequency, and eventual utilization of laxatives or enemas have been particularly emphasized. As regards duration of symptoms, two groups have been identified ( $\leq 7$  mo or > 7 mo). Finally, we divided our population into two groups according to whether they had  $\leq 7$  or > 7 fecal evacuations/wk.

Anorectal manometry was performed at rest and after maximum voluntary contraction, and was compared with the normal range for our laboratory<sup>[18,19]</sup>. The resting anal tone and maximal squeeze pressure (i.e., the maximal voluntary increase above the resting tone) were measured according to a stationary pull-through technique. One and two months after treatment, patients underwent the same examination as performed at baseline.

Type A botulinum toxin was diluted with saline solution; after individuating the internal anal sphincter, the toxin was injected with a 27 gauge needle; during the procedure the patient was lying on the left side. The injection was given in the anterior or posterior midline. During the procedure, no local or systemic sedative was administered. The phials were containing 100 IU of botulinum toxin A (Botox, Allergan, Irvine, CA, United States) were stored at a temperature of -20 °C and diluted in saline solution at the moment of its utilization. As regards the treatment with botulinum toxin, there were three groups according to the number of units injected: low dose (20 IU injected for single treatment); middle dose (30 IU);

and high dose (50 IU). The patients received different doses of botulinum toxin to test which dose may have a lower complication rate and higher healing rate.

### Statistical analysis

Data have been analyzed with statistical standard methods. At the outset, we performed a univariate analysis with all the factors potentially influencing the course of disease using the  $\chi^2$  test or Fisher's exact test for categorical data and the ANOVA test for continuous data divided in more than two groups. Subsequently, we executed a logistic multivariate regression, constructing models with the factors potentially influencing the course of disease, which by univariate analysis had a *P* value < 0.25. Additionally, we inserted age and sex into our multivariate analysis. Data were processed using GraphPad® Prism Software (GraphPad, San Diego, CA, United States). *P* < 0.05 was considered statistically significant, regardless of the test used.

## RESULTS

Demographic data and parameters registered at the first observation are reported in Table 1. Specifically, we observed 21 (35.6%) male patients and 38 (64.4%) females, aged between 21 years and 61 years (average: 40.49 ± 10.63 years). We did not observe prevalence of the considered disease in patients of a particular age: we observed 12 (20.3%) patients aged ≤ 30 years, 19 (32.2%) aged 31-40 years, 15 (25.4%) aged 41-50 years, and 13 (22.0%) aged > 50 years.

The anal fissure was localized posteriorly in 91.5% and anteriorly in 5.1% of the patients. In 31 (52.5%) patients, symptoms started in the 7 mo before the clinical observation and treatment with botulinum toxin. 98.3% of cases (58 patients) were referred with post-defecatory pain, which tended to persist independently from the evacuation in 22 patients (37.3%) and during the night in 17 (28.8%). Bleeding, even mild, was reported in 44 patients (74.6%), and 22.0% complained of mucorrhea. The relaxation of puborectal muscle was documentable at the physical examination in almost all patients (98.3%). Associated anal or systemic pathology due to obesity was observed in only 11.9% and 39.0% of patients, respectively.

As regards defecation characteristics, 50 patients (84.7%) were referred for diarrhea and evacuation of feces with decreased consistency. In 89.8% of cases, the number of weekly evacuations was > 7. Only three patients (5.1%) were referred for forced evacuations, a sense of incomplete evacuation, and continuous utilization of laxatives and evacuative enemas.

In almost all patients, botulinum toxin was injected into the internal anal sphincter, at the anterior midline (56 cases). No patients received local anesthesia and/or systemic sedation, and in all patients, the internal sphincter was easily identified with digital palpation alone (Table 2).

### Healing after treatment with botulinum toxin

We observed 45 and 26 patients at 1 mo and 2 mo, re-

**Table 1** Parameters considered in the analysis of 59 patients who were treated with botulinum toxin for chronic anal fissure and had previously undergone biliopancreatic diversion

	Frequency	Percent (%)
Age (yr)		
≤ 30	12	20.3
31-40	19	32.2
41-50	15	25.4
> 50	13	22.0
Sex		
Female	38	64.4
Male	21	35.6
Duration of symptoms (mo)		
≤ 7	31	52.5
> 7	28	47.5
Postdefecatory anal pain		
Present	58	98.3
Absent	1	1.7
Nocturnal anal pain		
Present	17	28.8
Absent	42	71.2
Anal pain unrelated to defecation		
Present	22	37.3
Absent	37	62.7
Bleeding		
Present	44	74.6
Absent	15	25.4
Mucorrhea		
Present	13	22.0
Absent	46	78.0
Other anal pathologies		
Present	7	11.9
Absent	52	88.1
Extra-anal pathologies		
Present	23	39.0
Absent	36	61.0
Defecation pattern		
Normal	5	8.5
Diarrhea	50	84.7
Constipation	4	6.8
Stool consistency		
Formed	5	8.5
Soft	50	84.7
Hard	4	6.8
Number of evacuations per week		
≤ 7	6	10.2
> 7	53	89.8
Straining		
Present	3	5.1
Absent	56	94.9
Incomplete evacuations		
Present	3	5.1
Absent	56	94.9
Laxative use		
Yes	3	5.1
No	56	94.9
Enemas/suppositories use		
Yes	3	5.1
No	56	94.9
Location of fissure		
Posterior midline	54	91.5
Anterior midline	3	5.1
Others	2	3.4

spectively. One month after treatment with botulinum toxin, healing was observed in 68.9% of patients. Only one patient developed mild incontinence to flatus that

**Table 2 Results of treatment with botulinum toxin A**

	Frequency	Percent (%)
Botox dose		
20 IU	3	5.1
30 IU	11	18.6
50 IU	45	76.3
Site of injection		
Unspecified - internal sphincter	1	1.7
Posterior side internal sphincter	2	3.4
Anterior side internal sphincter	56	94.9
Resting anal tone		
Normal	1	1.7
Increased	58	98.3
Maximal voluntary anal squeeze		
Normal	4	6.8
Increased	53	89.8
Decreased	2	3.4
Pubo-rectal muscle relaxation		
Present	58	98.3
Absent	1	1.7

**Table 3 Manometric data before, and 1 and 2 mo after botulinum toxin treatment (mean ± SD)**

Time	Resting anal pressure (mmHg)	Maximum voluntary anal squeeze pressure (mmHg)
Baseline	107.1 ± 20.0	78.2 ± 17.0
1 mo	84.4 ± 23.6 <sup>b</sup>	70.9 ± 18.3 <sup>a</sup>
2 mo	82.3 ± 22.7 <sup>b</sup>	70.1 ± 19.2

All patients were included in all evaluations. <sup>a</sup>*P* = 0.036, <sup>b</sup>*P* < 0.001 *vs* baseline.

lasted 3 wk after treatment but disappeared spontaneously. Two months after treatment, no patient had incontinence. At the same time, the complete cicatrization of the fissure, with no residual specific symptoms, was evident in 65.4% of patients. Healing persisted for a period of 32.2 ± 33.9 mo (range: 0-141 mo).

**Manometric results**

At the first observation, before treatment, resting anal pressure was 107.1 ± 20.0 mmHg and maximal voluntary squeezing was 78.2 ± 17.0 mmHg. One month after treatment with botulinum toxin, the mean resting pressure and maximum voluntary squeeze pressure were 21.2% (84.4 ± 23.6 mmHg, *P* < 0.0001) and 9.3% (70.9 ± 18.3 mmHg, *P* = 0.03) lower, respectively, than the respective baseline values. Two months after treatment, the mean resting anal pressure was similar to the 1-mo value (*P* = 0.7) and was 23.2% lower than the baseline value (82.3 ± 22.7 mmHg, *P* < 0.0001). The maximum voluntary squeeze pressure did not differ significantly from the 1-mo value (*P* = 0.9) and was 10.4% lower than the baseline value (70.1 ± 19.2 mmHg, *P* = 0.05) (Table 3).

**Univariate analysis**

The considered parameters were compared with the healing rate at 1 mo and 2 mo after treatment with botulinum

**Table 4 Univariate analysis of parameters registered 1 and 2 mo after treatment**

Risk factor	<i>P</i> value <sup>1</sup>	<i>P</i> value <sup>2</sup>
Age	0.4275	0.4034
Sex	0.1147	0.4128
Duration of symptoms	0.4566	0.4018
Post-defecatory anal pain	0.6889	NA
Nocturnal anal pain	0.1008	0.1039
Anal pain unrelated to defecation	0.1507	0.1039
Bleeding	0.4669	0.1585
Mucorrhea	0.5826	0.2081
Other anal pathologies	0.1656	0.1591
Extra-anal associated pathologies	0.3223	0.133
Defecation pattern	0.3825	0.3972
Stool consistency	0.3825	0.3291
Number of evacuations/wk	0.1656	0.4197
Straining	0.2244	0.2615
Sensation of incomplete evacuation	0.2244	0.7323
Laxative use	0.2244	0.2615
Enemas/ suppositories use	0.2244	0.7323
Botulinum toxin dose	0.8151	0.1635
Resting anal tone	0.6889	NA
Maximal voluntary anal squeeze	0.2094	0.6179
Puborectal muscle relaxation	0.3111	NA
MVAS 0 (mmHg)	0.2457	0.7281
MVAS 1 (mmHg)	0.5292	0.4133
MVAS 2	NA	0.8272
RAP 0 (mmHg)	0.284	0.1322
RAP 1 (mmHg)	0.3513	0.0985
RAP 2	NA	0.172
Site of injection	0.0818	0.5815

MVAS 0: Maximal voluntary anal squeeze (pretreatment); MVAS 1: Maximal voluntary anal squeeze (1 mo after the treatment); MVAS 2: Maximal voluntary anal squeeze (2 mo after the treatment); RAP 0: Resting anal pressure (pretreatment); RAP 1: Resting anal pressure (1 mo after treatment); RAP 2: Resting anal pressure (2 mo after treatment). NA: Not applicable. 1: 1 mo after treatment; 2: 2 mo after treatment.

toxin. No demographic parameter had a direct influence on healing rate (Table 4). Both age and sex did not show a statistically significant difference (*P* = 0.42 and *P* = 0.11, respectively). None of the clinical parameters had an influence on the results. Dose and site of injection did not have a significant effect on healing rate.

**Multivariate analysis**

One month after treatment (Table 5), no significant relationship was revealed with any parameter. Two months after treatment, sex (*P* = 0.01), baseline resting anal pressure (*P* = 0.02) and resting anal pressure 2 mo after treatment (*P* < 0.0001) were significantly related to healing rate (Table 6).

**DISCUSSION**

It has been shown that the more aggressive a bariatric technique is, the better are the results in terms of fat loss and maintenance of that fat loss over time. and although there is an improvement in comorbidity, the price paid by the patient, in relation to undesirable and adverse side effects and associated illnesses derived from these aggress-

**Table 5** Multivariate analysis of parameters registered 1 mo after treatment

Risk factor	P value	T ratio	95% CI
Age	0.8286	0.2183	-0.02207 to 0.01780
Sex	0.4231	0.8115	-0.2156 to 0.5011
MVAS 0	0.9669	0.04185	-0.01058 to 0.01102
Site of injection	0.7336	0.3433	-0.2783 to 0.3911
Maximal voluntary anal squeeze	0.3607	0.9273	-0.6433 to 0.2409
Enemas/suppositories use	0.5132	0.6612	-0.5860 to 1.149
Sensation of incomplete evacuations	0.7892	0.2696	-0.8144 to 1.063
Straining	0.8183	0.2317	-0.9509 to 1.195
Number of evacuations/wk	0.4590	0.7496	-0.03187 to 0.01473
Anal pain unrelated to evacuation	0.6136	0.5099	-1.341 to 0.8041
Nocturnal anal pain	0.5195	0.6513	-0.7694 to 1.492
Other anal pathologies	0.4454	0.7727	-0.3460 to 0.7686

MVAS 0: Maximal voluntary anal squeeze (pretreatment).

**Table 6** Multivariate analysis of parameters registered 2 mo after treatment

Risk factor	P value	T Ratio	95% CI
Age	0.3091	1.059	-0.01680 to 0.005750
Sex	0.0111	2.957	0.1110 to 0.7129
Nocturnal pain	0.8100	0.2454	-0.3677 to 0.4619
Bleeding	0.2130	1.310	-0.4982 to 0.1221
Mucorrhea	0.3122	1.052	-0.6795 to 0.2345
Other anal pathologies	0.0712	1.965	-0.8284 to 0.03926
Extra-anal pathologies	0.2659	1.163	-0.4816 to 0.1446
Dose of botulinum toxin	0.0771	1.920	-0.02639 to 0.001555
RAP 0	0.0225	2.588	0.001689 to 0.01872
RAP 1	0.8975	0.1314	-0.008417 to 0.007451
RAP 2	< 0.0001	5.953	-0.03038 to -0.01420

RAP 0: Resting anal pressure (pretreatment); RAP 1: Resting anal pressure (1 mo after treatment); RAP 2: Resting anal pressure (2 mo after treatment).

sive techniques, is high<sup>[13,20]</sup>. The etiology of anal pathology in this group of patients is multifactorial. One trigger-factor present after the intervention (as well as poor dietary habits), is diarrhea (steatorrhea), due to chemical irritation of the liquid feces. BPD produces an increase in the number of bowel movements and a decrease in their consistency due to malabsorption of fat<sup>[21]</sup>. Diarrhea generally appears during the first months after intervention and then it stabilizes between the 18th and 24th months when in general, fat loss has also stabilized.

Fich *et al*<sup>[22]</sup> showed a significant increase in the frequency of phase 3 of the migrating motor complex in patients after Billroth II and Roux-en-Y operations. Change in consistency of bowel motions after BPD was even greater than after Roux-en-Y gastric bypass, with nearly 80% of patients reporting loose stools or diarrhea, even 6 years after the intervention.

A high prevalence of fecal incontinence in both women and men after bariatric surgery has been described<sup>[23-25]</sup>. Women with diarrhea were four times as likely to have fecal incontinence and over half perceived their fecal in-

tinence to be worse after surgery<sup>[26]</sup>. Furthermore, women who perceived their diarrhea to be worse after surgery were significantly more likely to have fecal incontinence. This suggests that fecal incontinence in this population may be due to an underlying weakness in continence mechanisms that is clinically expressed after surgery because of altered stool consistency and delivery or changes in diet. Roberson and colleagues highlighted, for the first time, that fecal incontinence may begin or worsen after bariatric surgery<sup>[14]</sup>.

In the general population, diarrhea, cholecystectomy, stress, and mixed urinary incontinence were risk factors for fecal incontinence in univariate analysis. However, only diarrhea remained a significant risk factor in multivariate analysis. Stool consistency is a critical factor affecting fecal continence. In addition to fat malabsorption resulting from BPD, an alteration in intestinal and colonic bacterial flora after bariatric surgery may also contribute to diarrhea<sup>[27]</sup>.

Bacterial overgrowth is one of the complications related to diarrhea caused by the excluding loop in patients who undergo bariatric surgery. It occurs when a lack of equilibrium between the colonic flora and pathogenic bacteria is present. When untreated, it can evolve into malnutrition, anemia, poor absorption syndrome and can worsen diarrhea<sup>[28,29]</sup>.

Anal fissure is a linear ulceration of the distal part of the anal canal, extending below the dentate line to the anal verge<sup>[30]</sup>. It is a very common pathology affecting the quality of life of subjects who, in most cases, do not have any other significant comorbidity. Most of the fissures are localized in the posterior midline; in 10% of females and 1% of males, they are localized anteriorly<sup>[31,32]</sup>.

The etiology of anal fissure is multifactorial and not completely understood. There is a general agreement that trauma of the anal canal represents the initial insult. Hard bowel movements are the most common antecedent, but diarrhea may also be associated with the onset of fissure symptoms<sup>[31]</sup>. Anal hypertonicity and subsequent decreased blood flow to the anoderm are now recognized as pivotal factors in the pathogenesis of anal fissures. The increased internal sphincter pressure in patients with fissures reduces the blood flow to this area even further. Sphincterotomy reduces the anal canal pressure and improves anodermal blood flow at the posterior midline, resulting in fissure healing, which provides further evidence that abnormal activity in the sphincter contributes to the development of a fissure.

Although anal fissures are generally associated with constipation, in 7%, diarrhea could be the trigger as passing irritant liquid feces is a factor in the deterioration of the situation more often than with the patients who are constipated. This appeared together with the increase in the number of bowel movements following dietetic transgressions. Diarrhea/steatorrhea, due to malabsorption, is one of the common consequences of this type of operation, which can become incapacitating. In the general population, the fundamental pathogenetic element

in anal fissure is high anal pressure; therefore, different therapeutic strategies are needed to resolve the internal sphincter spasm, temporarily or permanently. The most used surgical procedure is partial lateral internal sphincterotomy<sup>[33]</sup>. The procedure has been considered the most effective treatment for anal fissure, although up to 10% of patients have recurrence, and > 35% of patients may experience incontinence. In fact, continence mechanisms can be acutely unbalanced by the execution of sphincterotomy. To avoid the permanent sequences of sphincterotomy, especially in multiparous women with a high risk of incontinence, alternative treatments can be adopted, such as botulinum toxin injection or topical application of nitrates<sup>[31,34]</sup>. The advantage in using these therapeutic approaches is that they cause a significant decrease in anal resting tone, inducing fissure healing, without permanent damages to anal sphincters<sup>[30]</sup>.

We reported the results of observation and treatment with type A botulinum toxin of patients affected by chronic anal fissure who had undergone Scopinaro's BPD. We observed a prevalence of anal fissure in the female patients, but we did not observe any difference between the age groups. One month after treatment with botulinum toxin, healing was observed in 68.9% of patients. Two months after treatment, complete healing of the fissure without any specific symptoms was present in 65.4% of cases. Healing persisted for a mean  $32.2 \pm 33.9$  mo. The number of evacuations per week did not significantly relate to persistence of fissure at 1 mo and 2 mo follow-up. We think that not only the frequency of bowel movements but also the altered composition of feces can play a role in the development of anal disorders in these patients. Mild incontinence to flatus with botulinum treatment was probably related to the diffusion of botulinum toxin to the external anal sphincter. The multivariate analysis of the parameters registered at the first control revealed no statistically significant values, instead, at the second control, sex, baseline resting anal pressure and resting anal pressure 2 mo after treatment had a significant influence on healing, by multivariate analysis. In particular, male sex had a direct influence on healing and high resting anal pressure pre- and post-treatment was associated with persistent anal fissure.

Despite the high dose of botulinum toxin, the healing rate in this study, and the reduction of resting anal canal pressure and maximum anal canal squeeze pressure were lower than in our previous trials<sup>[18,19,30,34,35]</sup>. The results of the present study were different from our previous observation of 100 patients: 50 treated with 50 IU Botox (group I) and 50 with 150 IU Dysport (group II)<sup>[35]</sup>. We considered the difference in group I. One month after treatment, fissure healing was present in 82% of the patients in our previous study and in 68.9% of the patients in the present trial ( $P = 0.15$ ). In patients in previous trials, a 38.7% decrease of resting anal pressure, compared to pretreatment values, was registered; in the present study, we observed a lower decrease of resting anal pressure (21.2%). The reduction of maximal anal squeezing

was 22.4% and 9.3% in the previous and present study, respectively. Two months after treatment, 92% and 65.4% of the patients in the previous and present study, respectively, ( $P = 0.007$ ) had a healing scar. The reduction of resting anal canal pressure was lower than in our previous trial (23.2% *vs* 41.8%). The decrease of maximum anal canal squeeze pressure was lower than in our previous study (10.4% *vs* 20.2%)<sup>[35]</sup>.

We think that the increase of baseline resting anal pressure in bariatric patients is secondary to repeated, continuous diarrhea and changes in qualitative composition that are typical of steatorrhea in malabsorptive processes. The reduction of resting anal pressure and maximal anal squeezing after treatment, lower than in the general population, and the association between persistent high levels of resting anal pressure and a low healing rate can result from chronic irritation of the anal canal by liquid stools. The reduction of resting anal pressure favors healing but the results are worse than in the general population because the anal hypertonicity is not the principal pathogenetic factor of chronic anal fissure in bariatric patients.

In summary, we believe that, in patients who undergo BPD, chronic steatorrhea can be the cause of different anal disorders. It is of utmost importance to be conservative in the treatment of anal disorders for bariatric patients. It is also important to remember that the anatomy of the anal canal should be preserved as much as possible so as to avoid incontinence, because in these patients, at least one of the continence mechanisms is already altered. Botulinum toxin, despite worse results than in non-obese population, appears the best alternative to surgery for this group of patients with high risk of incontinence. The effectiveness of other treatments such as nitrates depends on patient compliance, which may be poor in bariatric patients. Moreover, repeated evacuations and altered fecal composition can decrease the pharmacological effect of nitrates. Botulinum toxin injection has the advantage of a good healing rate, can be repeated if necessary, and the possible incontinence is temporary.

## COMMENTS

### Background

Anal fissure is a split in the distal anal canal. It is a common problem that causes substantial morbidity in people who are otherwise healthy. There is accumulating agreement that bariatric surgery is currently the most efficacious and enduring treatment for clinically severe obesity, and as a result, the number of bariatric surgery procedures performed has risen dramatically in recent years. Biliopancreatic diversion (BPD) by Scopinaro is a malabsorptive technique that is successful in achieving long-lasting weight lost in super-obese patients. The diarrhea that is expected after the procedure can sometimes cause significant nutritional changes and anal disease.

### Research frontiers

Treatment of chronic anal fissure has undergone a transformation in recent years from surgical to medical, both approaches sharing the common goal of reducing spasm of the internal anal sphincter. Surgical sphincterotomy results in a healing rate up to 95%. Despite this, it carries a significant risk of persistent disturbance in anal continence. This is believed to be the first study of the use of botulinum toxin injection for treatment of chronic anal fissure in patients undergoing BPD. This article, unlike others that focus only on the positive effects

of bariatric surgery on improvement of comorbidity in obese patients, highlights the side effects that may affect the quality of life and draws new boundaries in the treatment of chronic anal fissure after BPD.

### Innovations and breakthroughs

Different types of bariatric operations might alter bowel habits as a consequence of the surgical technique used. Disordered bowel habits and anal diseases might influence quality of life after bariatric surgery. There are few papers in the literature devoted to the study of the anal disorders after bariatric surgery. Prospective randomized trials are needed to estimate the true incidence and to establish the etiology of proctological disorders in these patients.

### Applications

The treatment of chronic anal fissure in these high-risk patients should have the minimum risk of fecal incontinence. Botulinum toxin injection has the advantages of an excellent healing rate and can be repeated if necessary. Any incontinence, as a complication, however, is transient.

### Peer review

This is an interesting paper about the usefulness of botulinum toxin A in healing chronic anal fissure after BPD for morbid obesity. The authors report complete healing in 65.4% of patients.

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## 15-PGDH is reduced and induces apoptosis and cell cycle arrest in gastric carcinoma

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### Abstract

**AIM:** To investigate the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in human gastric cancer and its mechanism in apoptosis and cell cycle arrest.

**METHODS:** Expression of 15-PGDH mRNA and protein was examined by immunohistochemistry, immunocytochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting in tissue from human gastric cancer, gastric precancerous state (gastric polyps and atrophic gastritis), normal stomach, and gastric cancer cell lines. The relationship between gastric cancer, gastric precancerous state and 15-PGDH

expression was determined. The association between expression of 15-PGDH and various clinicopathological parameters in gastric cancer was evaluated. Human gastric cancer cell line SGC-7901 was transfected with 15-PGDH expression plasmids. The effect of 15-PGDH on the cell cycle was examined by flow cytometry. The effect of 15-PGDH on apoptosis was examined by transmission electron microscopy, flow cytometry and transferase mediated nick end labeling (TUNEL) assay. Expression of cell cycle (p21, p27, p16 and p53) and apoptosis (*Survivin*, *BCL-2*, *BCL-X<sub>L</sub>*, *BAK* and *BAX*) genes was analyzed by RT-PCR.

**RESULTS:** Expression of 15-PGDH mRNA and protein in human gastric cancer tissues was significantly lower than in normal gastric tissues ( $P < 0.01$ ). Expression in human gastric cancer cell lines MKN-28 and MKN-45 was reduced, and absent in SGC-7901 cells ( $P < 0.05$ ). Reduction of 15-PGDH expression was also found in precancerous tissues, such as gastric polyps and atrophic gastritis ( $P < 0.01$ ). There was a significant difference in expression of 15-PGDH among various gastric cancer pathological types ( $P < 0.05$ ), with or without distant metastasis ( $P < 0.05$ ) and different TNM stage ( $P < 0.01$ ). Flow cytometry demonstrated a significant increase in apoptotic cells in SGC-7901 cells transfected with pcDNA3/15-PGDH plasmid for 24 h and 48 h ( $P < 0.01$ ), and an increased fraction of sub-G1 phase after transfection ( $P < 0.05$ ). TUNEL assay showed an increased apoptotic index in cells overexpressing 15-PGDH ( $P < 0.01$ ). After transfection, expression of proapoptotic genes, such as *BAK* ( $P < 0.05$ ), *BAX* and *p53* ( $P < 0.01$ ), was increased. Expression of antiapoptotic genes was decreased, such as *Survivin*, *BCL-2* and *BCL-X<sub>L</sub>* ( $P < 0.01$ ). Expression of cyclin-dependent kinase inhibitors p21 and p16 ( $P < 0.01$ ) was significantly upregulated in cells overexpressing 15-PGDH.

**CONCLUSION:** Reduction of 15-PGDH is associated with carcinogenesis and development of gastric carcinoma. 15-PGDH induces apoptosis and cell cycle arrest in SGC-7901 cells.

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**Key words:** Gastric carcinoma; 15-hydroxyprostaglandin dehydrogenase; Apoptosis; Cell cycle arrest; Tumor growth

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## INTRODUCTION

Gastric carcinoma is one of the most common malignant tumors in humans and continues to be a major unresolved health problem. New approaches for the management of gastric cancer are needed. NAD<sup>+</sup>-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is the key enzyme responsible for the biological inactivation of prostaglandins (PGs) and related eicosanoids. It catalyzes the oxidation of the 15(S)-hydroxyl group of PGs and lipoxins. The products, 15-keto-metabolites, exhibit greatly reduced biological activities<sup>[1]</sup>. 15-PGDH is widely distributed in various mammalian tissues such as lung, breast, prostate, placenta and gut. The stomach is one of the most active tissues expressing 15-PGDH<sup>[2]</sup>. Recent studies<sup>[3-6]</sup> have shown a reduction of 15-PGDH in some cancers, such as colorectal, breast, prostate and lung. Some studies<sup>[3,4,6,7-9]</sup> have revealed that 15-PGDH may have tumor-suppressive properties. Recently, some studies<sup>[10-15]</sup> have indicated that 15-PGDH is downregulated in gastric cancer and is a suppressor of human gastric cancer. It provides a new target for the chemoprevention and treatment of cancer, especially in gastric carcinoma. However, to the best of our knowledge, there has been no study on 15-PGDH expression in gastric precancerous tissue. The association between 15-PGDH expression and various clinicopathological parameters of gastric cancer needs to be validated. Only a few studies have been published on its mechanism of tumor inhibition. Therefore, more studies on the mechanism of 15-PGDH suppression of gastric cancer are necessary.

In this study, we investigated the expression of 15-PGDH in human gastric cancer and gastric precancerous tissues, and determined the relationship between occurrence, development, infiltration, metastasis, cell differentiation of gastric carcinoma and 15-PGDH expression. We also examined the association of 15-PGDH with gastric cancer cell proliferation, apoptosis and the cell cycle. We studied the role of 15-PGDH reduction in carcinogenesis and de-

velopment of gastric cancer and the possible mechanism of gastric cancer inhibition of 15-PGDH. Our results suggest the use of 15-PGDH in chemoprevention and treatment of gastric cancer.

## MATERIALS AND METHODS

### Human gastric specimens

Human gastric carcinoma specimens ( $n = 30$ ) were obtained from surgical resections, with the approval of the Shanghai First People's Hospital Ethics Committee. The specimens were frozen and stored in liquid nitrogen and 10% formaldehyde solution. Each tumor sample was matched with adjacent tissues (3 cm and 6 cm from the border of tumor) collected during the process. Other gastric tissues, including normal gastric tissues ( $n = 10$ ), gastric polyps ( $n = 10$ ) and chronic atrophic gastritis ( $n = 10$ ), were obtained from gastroscopic biopsy and stored in liquid nitrogen and 10% formaldehyde solution. Specimens were dissected macroscopically by trained pathologists.

### Cell culture

Human gastric carcinoma cell lines MKN-45, MKN-28 and SGC-7901 (obtained from Shanghai Institute of Biochemistry and Cell Biology) were maintained in RPMI-1640 (Gibco, United States) medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 5% CO<sub>2</sub> atmosphere at 37 °C. These cells were plated in six-well plates at about  $2 \times 10^5$  cells/well in duplicate, and grown for 24 h before transfection.

### Expression of wild-type 15-PGDH

The mammalian expression vector pcDNA3 containing the cDNA of the wild-type 15-PGDH and pcDNA3 expression vector were donated by Dr. Tai HH (Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, United States). Both pcDNA3/15-PGDH and pcDNA3 (200 ng) plasmids were transfected into SGC-7901 cells by Lipofectamine 2000 reagent for 24 h and 48 h, according to the manufacturer's directions. Expression of the wild-type 15-PGDH mRNA and protein was monitored by reverse transcriptase polymerase chain reaction (RT-PCR), cellular immunohistochemistry and Western blotting.

### Immunohistochemistry and immunocytochemistry

Paraffin-embedded tissue sections (3 µm) were dried, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide in ion-free water for 30 min. After nonspecific binding sites, tissue slides were blocked with 10% goat serum. Cellular slides were treated by 4% paraformaldehyde for 30 min. Both kinds of slides were incubated at 4 °C overnight with a 1:50 dilution of rabbit polyclonal 15-PGDH antibody (Cayman, United States), followed by a 30-min incubation in horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG (Changdao, China), rinsed with PBS, developed with the DAB kit (DakoCytomation, United States), and then counterstained with haematoxylin. Each slide

**Table 1 Polymerase chain reaction primers**

Target genes	Primer sequence	Size (bp)	Annealing temperature (°C)	
GAPDH	Sense	5'-CCACCCATGGCAAATTCATGGCA-3'	593	62
	Antisense	5'-AACAAAGCCTGGACAAAAT-3'		
15-PGDH	Sense	5'-GCTGGAGTGAATAATGAGA-3'	285	55
	Antisense	5'-GCTGAGCGTGTGAATCCAAC-3'		
Survivin	Sense	5'-GGCATGGGTGCCCCGAGGTT-3'	320	58
	Antisense	5'-AGAGGCCTCAATCCATGGCA-3'		
BCL-2	Sense	5'-GGTGCCACCTGTGGTCCACCT-3'	458	54
	Antisense	5'-CCTCACTGTGGCCAGATAGG-3'		
BAX	Sense	5'-CTGACATGTTTCTGACGGC-3'	289	54
	Antisense	5'-TCAGCCCATCTTCTCCAGA-3'		
BCL-X <sub>L</sub>	Sense	5'-TTGGACAATGGACTGGTTG-3'	765	54
	Antisense	5'-GTAGAGTGGATGGTCAGTG-3'		
BAK	Sense	5'-TGAAAAATGGCTTCGGGGCAAGGC-3'	642	54
	Antisense	5'-TCATGATTGAAGAATCTTCGTACC-3'		
p53	Sense	5'-CCTTCCCAGAAAACCTACCA-3'	371	59
	Antisense	5'-TCATAGGGCACCACCACACT-3'		
p21	Sense	5'-CAGGGGACAGCAGAGGAAGA-3'	335	63
	Antisense	5'-GGGCGCCAGGGTATGTAC-3'		
p27	Sense	5'-ATGTCAAACGTCGAGTGTG-3'	395	58
	Antisense	5'-TCTGTAGTAGAACTCGGGCAA-3'		
p16	Sense	5'-GGGCTCTCACAACACTAGGAA-3'	371	59
	Antisense	5'-CGGAGGAGGTGCTATTAATC-3'		

15-PGDH: 15-hydroxyprostaglandin dehydrogenase.

was scanned at 100 and 400 × magnification. Immunohistochemistry score = intensity score (absent, 0; weak, 1; moderate, 2; strong, 3) × percentage score (< 5%, 0; 5%-25%, 1; 25%-50%, 2; 50%-75%, 3; > 75% of total tumor area, 4).

**Reverse transcriptase polymerase chain reaction analysis**

Total RNA of tissues and gastric cancer cells was extracted with TRIzol (Invitrogen, United States) following the manufacturer’s instructions. cDNA was synthesized from 2 µg total RNA using the M-MLV RT-PCR kit (Promega, United States) in a 20 µL volume, according to the manufacturer’s instructions. Two µL of cDNA, 2 µL each primer (50 pmol/L), 1 µL dNTP mix (10 mmol/L) and 1 µL Taq DNA polymerase (Sangon, China) were used for PCR analysis. The PCR amplification cycles consisted of denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 60 s, annealing for 60 s, extension at 72 °C for 60 s, and final elongation at 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized by UV light. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and shown as the ratio of absorbance values. The primer sequences and annealing temperature are listed in Table 1.

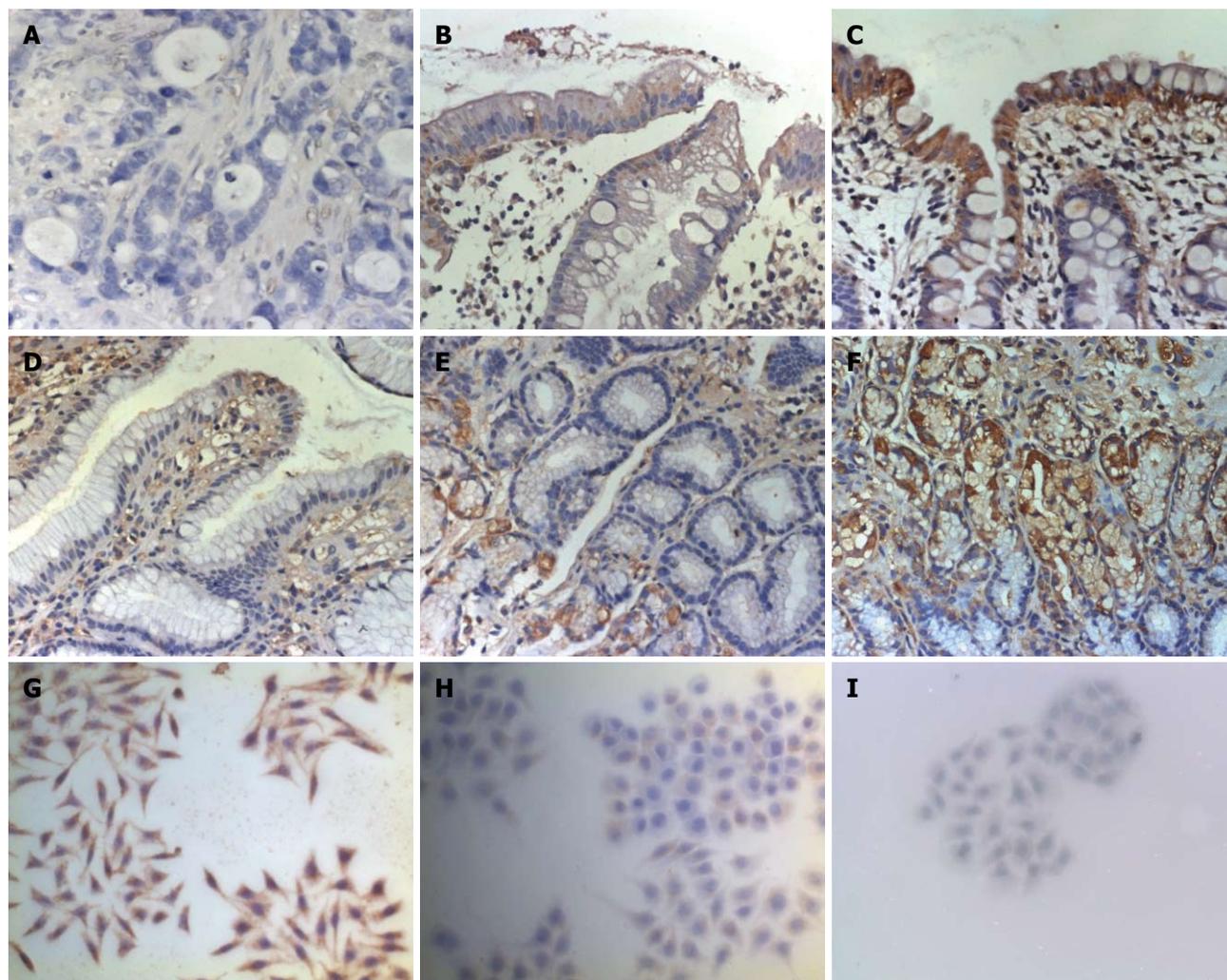
**Western blotting**

Tissues and gastric cancer cells were lysed with lysis buffer containing 0.5% NP-40, 40 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl, and a protease cocktail inhibitor (Complete Mini; Pierce, Rockford, IL, United States). Samples (40 µg protein per lane) were subjected to sodium

dodecyl sulfate-polyacrylamide gel electrophoresis, and then blotted onto polyvinylidene difluoride membranes. Membranes were blocked for 2 h at room temperature with 5% skimmed milk and then probed with 1:200 dilution of rabbit polyclonal 15-PGDH antibody overnight at 4 °C. Membranes were washed and incubated for 1 h at room temperature with anti-rabbit IgG-HRP. Results were visualized by ECL chemiluminescence detection kit (Kangcheng, China). Protein expression was normalized to ACTIN.

**Cell cycle analysis and apoptosis assays**

The effect of 15-PGDH on the cell cycle and apoptosis in SGC-7901 cells was analyzed by flow cytometry. Cells floating in medium combined with the adherent layer were trypsinized and fixed with 2 mL citrate buffer for 1 h. Cells were then incubated with RNase A (1500 µL) and stained with propidium iodide (1500 µL). Samples were immediately analyzed by flow cytometry for cell cycle and apoptosis assays. Cells were observed under transmission electron microscopy (TEM) at Shanghai Medical College of Fudan University. The number of apoptotic cells was counted per 100 cells. Terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay, in which residue of digoxigenin-labeled dUTP was catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II, was performed according to the manufacturer’s instructions (Boster, Wuhan, China). The positive particles of DAB staining were viewed under an optical microscope. The number of apoptotic cells was counted under a microscope (400 ×) and expressed as the apoptotic index (AI = the number of apoptotic bodies/1000 cells).



**Figure 1 Immunohistochemistry image of 15-hydroxyprostaglandin dehydrogenase (400 ×).** A: Gastric cancer (IHC Score  $1.20 \pm 1.13$ ,  $P < 0.01$  vs B, C and F); B and C: Paracancerous tissue 3 cm ( $6.83 \pm 2.78$ ,  $P < 0.01$  vs C and F) and 6 cm ( $10.20 \pm 1.92$ ) distant from tumor; D: Gastric polyps ( $6.00 \pm 2.74$ ,  $P < 0.01$  vs F); E: Atrophic gastritis ( $5.14 \pm 1.57$ ,  $P < 0.05$  vs F); F: Normal gastric tissues ( $11.00 \pm 1.63$ ). G: MKN-28 ( $3.31 \pm 0.92$ ,  $P < 0.05$  vs H and I); H: MKN-45 ( $1.29 \pm 0.48$ ); I: SGC-7901 (absent).

### Statistical analysis

Quantitative results were expressed as mean  $\pm$  SD. Statistical analysis was assessed by Student's *t* test (between two groups) or Student-Newman-Keuls test (among three or more groups), with SAS version 8.02 software.  $P < 0.05$  was considered statistically significant.

## RESULTS

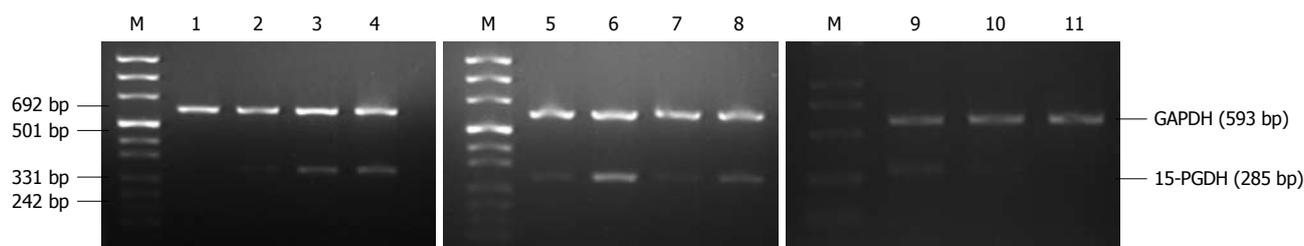
### Downregulation of 15-PGDH expression in gastric cancer, paracancerous and precancerous tissues and gastric cancer cell lines

Immunohistochemistry analysis confirmed that 15-PGDH protein was expressed mainly in the cytoplasm of epithelial, inflammatory and gastric cancer cells in the lamina propria. Of the 30 gastric cancer cases, 15-PGDH expression was undetectable in 10 tumors (33.3%). Immunohistochemistry score of 15-PGDH was decreased in gastric cancer, paracancerous tissues 3 cm and 6 cm from the tumor, gastric polyps and atrophic gastritis compared with normal gastric

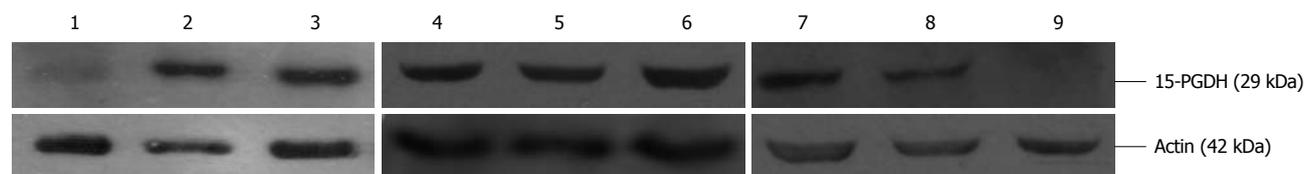
tissues. Immunocytochemical analysis showed that expression of 15-PGDH in various differentiated gastric cell lines was dissimilar. Poorly differentiated gastric cell line SGC-7901 displayed no 15-PGDH, whereas MKN-28 and MKN-45 displayed little 15-PGDH (Figure 1).

RT-PCR analysis showed that expression of 15-PGDH mRNA in gastric cancer, paracancerous tissues, gastric polyps and atrophic gastritis was significantly lower than in normal gastric tissues. We also found loss of 15-PGDH in nine tumors (30%). Expression of 15-PGDH was absent in SGC-7901 cells, and significantly decreased in MKN-45 and MKN-28 cells (Figure 2).

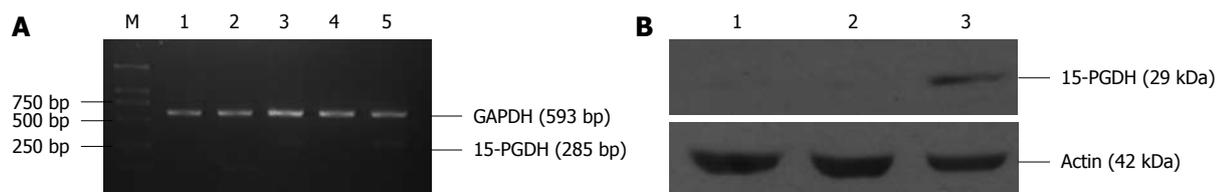
Western blotting demonstrated that 15-PGDH protein expression was absent in nine of 30 gastric cancer cases (30%), and an average 5.7- and 8.3-fold less 15-PGDH expression was found in cancer tissues compared with paracancerous tissues at 3 cm and 6 cm from the tumor. There was a twofold reduction in gastric polyps and 2.1-fold reduction in atrophic gastritis tissues compared with normal gastric mucosa. Expression of 15-PGDH



**Figure 2** Expression of 15-hydroxyprostaglandin dehydrogenase mRNA in gastric cancer tissue, paracancerous tissue, gastric polyps, atrophic gastritis, gastric cancer cell lines and normal gastric tissue by reverse transcriptase polymerase chain reaction. Lane M: DNA marker; 1: Gastric cancer ( $0.21 \pm 0.23$ ,  $P < 0.01$  vs 2, 3 and 4); 2 and 3: Paracancerous tissue 3 cm ( $0.56 \pm 0.45$ ) and 6 cm distant from tumor ( $0.81 \pm 0.43$ ); 4, 6 and 8: Normal gastric tissue ( $0.82 \pm 0.31$ ); 5: Gastric polyps ( $0.44 \pm 0.21$ ); 7: Atrophic gastritis ( $0.48 \pm 0.19$ ); 9: MKN-28 ( $0.31 \pm 0.04$ ,  $P < 0.01$  vs 10 and 11); 10: MKN-45 ( $0.08 \pm 0.01$ ,  $P < 0.01$  vs 11); 11: SGC-7901 (absent). 15-PGDH: 15-hydroxyprostaglandin dehydrogenase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



**Figure 3** Expressions of 15-hydroxyprostaglandin dehydrogenase in gastric cancer tissue, paracancerous tissue, gastric polyps, atrophic gastritis, gastric cancer cell lines and normal gastric tissue by Western blotting. 1-3: Gastric cancer, paracancerous tissue 3 cm and 6 cm from the same case; 4: Gastric polyps; 5: Atrophic gastritis; 6: Normal gastric tissue; 7: MKN-28; 8: MKN-45; 9: SGC-7901. 15-PGDH: 15-hydroxyprostaglandin dehydrogenase.



**Figure 4** Expressions of 15-hydroxyprostaglandin dehydrogenase in SGC-7901 cells before and after transfected by pcDNA3/15-hydroxyprostaglandin dehydrogenase plasmid. A: Reverse transcriptase polymerase chain reaction image of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and GAPDH. Lane M: DNA marker; 1: SGC-7901; 2: pcDNA3 24 h group; 3: pcDNA3/15-PGDH 24 h group; 4: pcDNA3 48 h group; 5: pcDNA3/15-PGDH 48 h group; B: Western blotting image of 15-PGDH and actin. 1: SGC-7901; 2: pcDNA3 36 h group; 3: pcDNA3/15-PGDH 36 h group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

protein in gastric cancer cells was the same as shown by RT-PCR (Figure 3).

**Relationship between expression of 15-PGDH and clinicopathological parameters in gastric cancer**

Expression of 15-PGDH protein was significantly different among the various gastric cancer pathological types ( $P < 0.05$ ). Reduction of 15-PGDH was more distinct in gastric cancer with distant metastasis than in tumor without distant metastasis ( $P < 0.05$  at protein level,  $P < 0.01$  at mRNA level). There was also a significant difference in expression of 15-PGDH among tumors of different TNM stage ( $P < 0.01$  at both protein and mRNA level). More reduced 15-PGDH expression was associated with worse TNM stage (Table 2).

**Over-expression of 15-PGDH induced cell cycle arrest and apoptosis in SGC-7901 cells**

After transfection by pcDNA3/15-PGDH plasmid, SGC-7901 cells were induced to overexpress 15-PGDH (Figure 4). At the same time, an increased fraction of sub-G1 phase ( $57.21\% \pm 0.53\%$  for 24 h transfection

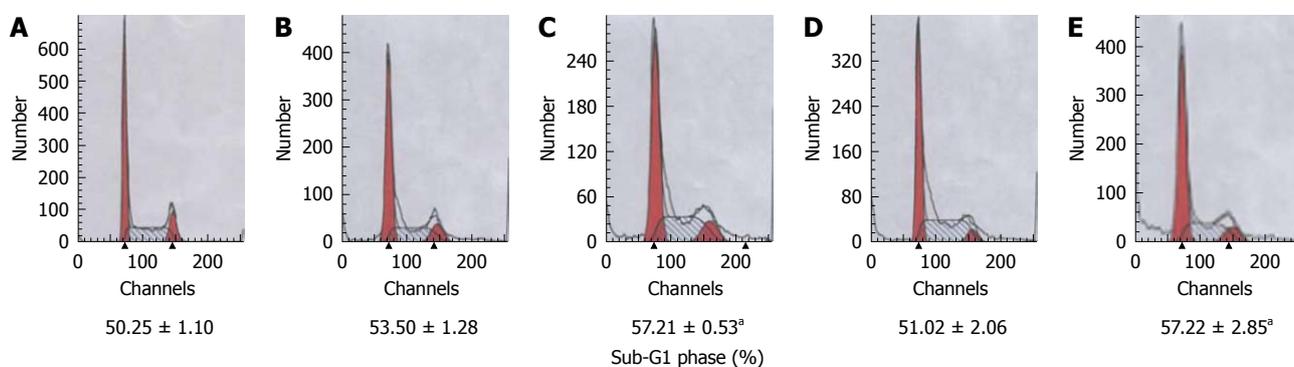
and  $57.22\% \pm 2.85\%$  for 48 h transfection,  $P < 0.05$ ) was found by flow cytometry (Figure 5). It showed that 15-PGDH promoted cell cycle arrest in the sub-G1 phase. To assess the effect of 15-PGDH on induction of cell apoptosis in gastric cancer, we observed SGC-7901 cells under TEM and by flow cytometry, and then performed a TUNEL assay. Under TEM, nuclear and cytoplasmic shrinkage, condensation and margination of chromatin against the nuclear membrane, and formation of apoptotic bodies were observed in SGC-7901 cells that overexpressed 15-PGDH (Figure 6). The proportion of apoptotic cells was significantly increased after transfection for 24 h and 48 h ( $12.33\% \pm 1.15\%$  and  $25.00\% \pm 1.00\%$  vs  $3.33\% \pm 0.58\%$ ,  $P < 0.01$ ). Flow cytometric analysis showed induction of apoptosis ( $10.49\% \pm 0.81\%$  and  $24.02\% \pm 0.37\%$  vs  $4.17\% \pm 0.68\%$ ,  $P < 0.01$ ), which was further confirmed by TUNEL assay (AI:  $25.27\% \pm 1.19\%$  and  $48.37\% \pm 2.67\%$  vs  $6.50\% \pm 0.30\%$ ,  $P < 0.01$ ) (Figure 7). It indicated that cell cycle arrest and increased apoptosis was one mechanism of cancer suppression of 15-PGDH in SGC-7901 cells.

Expression of genes associated with the cell cycle

**Table 2 Relationship between expression of 15-hydroxyprostaglandin dehydrogenase and clinicopathological parameters in gastric cancer**

Clinicopathological parameters	Cases (n = 30)	IHC score	P values	Ratio (15-PGDH/GAPDH mRNA)	P values
Age (yr)			0.847		0.970
< 60	12	1.25 ± 1.22		0.21 ± 0.25	
≥ 60	18	1.67 ± 1.10		0.21 ± 0.22	
Sex			0.092		0.814
Male	23	1.39 ± 1.16		0.20 ± 0.21	
Female	7	0.57 ± 0.79		0.23 ± 0.31	
Location			0.891		0.407
Antrum	16	1.28 ± 1.27		0.20 ± 0.23	
Fundus and corpus	6	1.00 ± 0.82		0.35 ± 0.36	
Cardia	8	1.13 ± 0.99		0.17 ± 0.15	
Size (diameter)			0.578		0.388
< 5 cm	14	1.07 ± 1.21		0.17 ± 0.15	
≥ 5 cm	16	1.31 ± 1.08		0.24 ± 0.28	
Pathological type			0.019		0.138
Well differentiated adenocarcinoma	4	2.50 ± 1.00		0.24 ± 0.10	
Moderately differentiated adenocarcinoma	9	1.44 ± 1.24		0.31 ± 0.25	
Poorly differentiated adenocarcinoma	9	1.11 ± 0.78		0.24 ± 0.27	
Mucinous adenocarcinoma	5	0.60 ± 0.89 <sup>a</sup>		0.06 ± 0.11 <sup>a</sup>	
Signet ring cell carcinoma	3	0.00 ± 0.00 <sup>a</sup>		0.00 ± 0.00 <sup>a</sup>	
Distant metastasis			0.038		0.002
Negative	21	1.48 ± 1.17 <sup>c</sup>		0.27 ± 0.24 <sup>d</sup>	
Positive	9	0.56 ± 0.73		0.06 ± 0.09	
TNM stage			0.007		0.001
I	3	2.67 ± 1.15 <sup>e</sup>		0.43 ± 0.25 <sup>e</sup>	
II	5	1.60 ± 1.52		0.47 ± 0.27 <sup>e</sup>	
III	7	1.57 ± 0.79		0.17 ± 0.15	
IV	15	0.60 ± 0.74		0.09 ± 0.14	

<sup>a</sup>P < 0.05 vs well differentiated adenocarcinoma; <sup>c</sup>P < 0.05, <sup>d</sup>P < 0.01 vs distant metastasis; <sup>e</sup>P < 0.05 vs TNM stage IV. 15-PGDH: 15-hydroxyprostaglandin dehydrogenase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



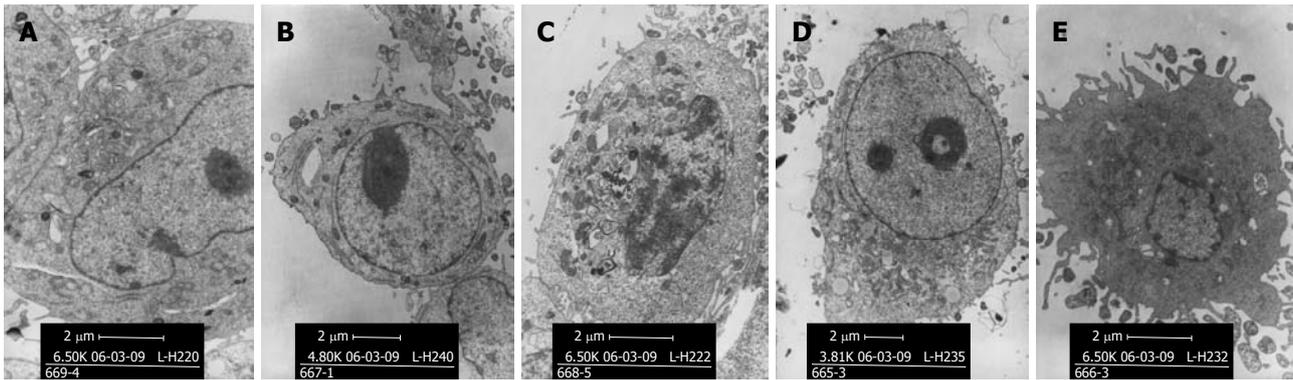
**Figure 5 Flow cytometry results of gastric cancer cell cycle.** A: SGC-7901; B: pcDNA3 24 h group; C: pcDNA3/15-PGDH 24 h group; D: pcDNA3 48 h group; E: pcDNA3/15-PGDH 48 h group. <sup>a</sup>P < 0.05 vs A, B and D.

(p21, p27, p16 and p53) and apoptosis (Survivin, BCL-2, BCL-X<sub>L</sub>, BAK and BAX) was determined by RT-PCR in SGC-7901 cells transfected with pcDNA3/15-PGDH plasmids. p21 (1.75 ± 0.51 for 24 h transfection and 1.76 ± 0.52 for 48 h transfection vs 0.46 ± 0.06 SGC-7901, P < 0.01), p16 (0.33 ± 0.12 and 0.32 ± 0.17 vs absence, P < 0.01) and p53 genes (0.19 ± 0.04 and 0.19 ± 0.06 vs 0.08 ± 0.02, P < 0.01) were significantly upregulated in cells treated with 15-PGDH for 24 h group and 48 h, whereas the level of p27 mRNA did not change (P > 0.05). Expression of the proapoptotic genes, such as BAK (0.92 ± 0.14 and 1.04 ± 0.27 vs 0.52 ± 0.24, P < 0.05) and BAX

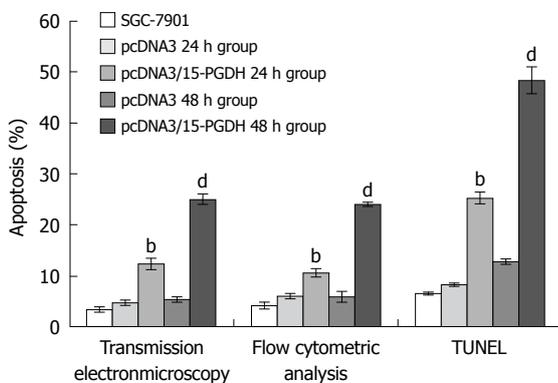
(1.73 ± 0.17 and 1.72 ± 0.07 vs 1.14 ± 0.11, P < 0.01) was significantly increased. The antiapoptotic genes, such as Survivin (0.14 ± 0.06 and 0.13 ± 0.02 vs 0.34 ± 0.06, P < 0.01), BCL-2 (0.02 ± 0.01 and 0.02 ± 0.01 vs 0.08 ± 0.03, P < 0.01) and BCL-X<sub>L</sub> (0.63 ± 0.11 and 0.63 ± 0.08 vs 1.12 ± 0.08, P < 0.01), were significantly down-regulated (Figure 8).

## DISCUSSION

These findings demonstrate that the reduction of 15-PGDH is related to occurrence and development of



**Figure 6** Transmission electron microscopy of gastric cancer cell apoptosis (5000 ×). A: Normal configuration of SGC-7901 cell; B: Normal configuration of cell in pcDNA3 24 h group; C: Apoptotic cell in pcDNA3/15-PGDH 24 h group; D: Normal configuration of cell in pcDNA3 48 h group; E: Apoptotic cell in pcDNA3/15-PGDH 48 h group.



**Figure 7** Induction of apoptosis in SGC-7901 cell line. There was a significant apoptosis in erlotinib pcDNA3/15-hydroxyprostaglandin dehydrogenase (15-PGDH) 24 h and 48 h groups in transmission electron microscopy, flow cytometry analysis and TUNEL assay. <sup>b</sup>*P* < 0.01 vs SGC-7901, pc DNA3 24 h and 48 h group; <sup>d</sup>*P* < 0.01 vs SGC-7901, pcDNA3/15-PGDH 24 h group, pc DNA3 24 h and 48 h group.

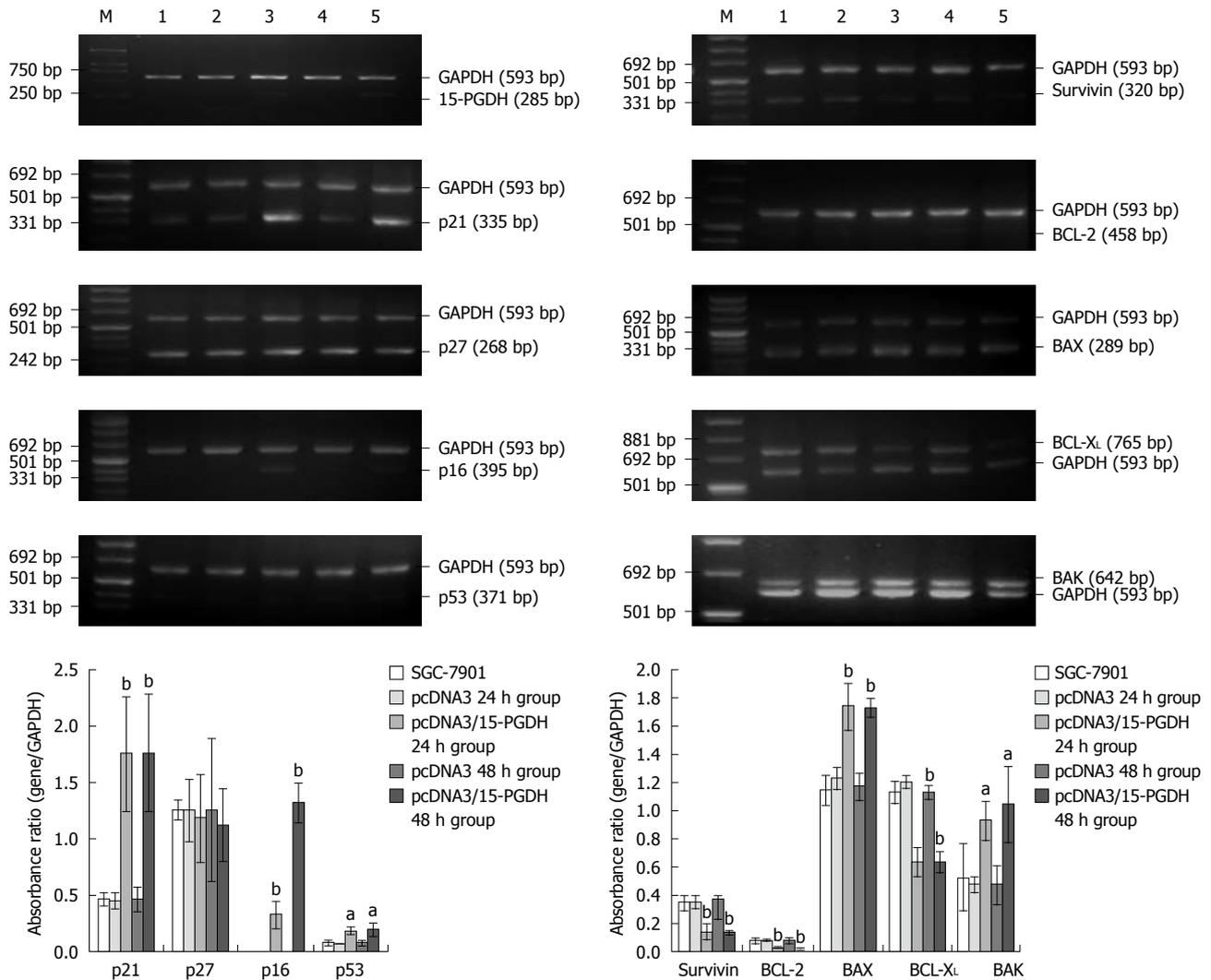
gastric cancer in humans. 15-PGDH also induces cell cycle arrest and apoptosis in gastric cancer cells. It may be a suppressor of gastric cancer through these two pathways.

15-PGDH catalyzes the oxidation of the 15(S)-hydroxyl group of PGs and lipoxins. 15-PGDH is one of the target genes. Some cytokines, factors and cell signaling pathways affect carcinogenesis and tumor progression through 15-PGDH. It shows that epidermal growth factor (EGF) and EGF receptor tyrosine kinase inhibitor<sup>[16,17]</sup>, histone deacetylase inhibitors, transforming growth factor-β (TGF-β)<sup>[18]</sup>, hepatocyte nuclear factor 3β<sup>[7]</sup>, interleukin (IL)-4<sup>[19]</sup>, tumor necrosis factor α<sup>[20]</sup>, IL-1β<sup>[21]</sup>, peroxisome proliferator-activated receptor γ ligands<sup>[22]</sup>, hepatocyte growth factor receptor, Met<sup>[23]</sup>, bile acids<sup>[24]</sup> adjust tumor growth through 15-PGDH. Recent studies have shown an obvious reduction of 15-PGDH in some cancers, for example, colorectal, breast, prostate and lung<sup>[3-6]</sup>. It also has been reported that 10%-80% of gastric cancer exhibits downregulation of 15-PGDH expression<sup>[10,11-14]</sup>. Our finding that 15-PGDH expression is decreased in gastric cancer is consistent with these reports. 15-PGDH protein and mRNA expression is absent

in 33.3% of gastric cancer tissues, and reduced in almost all gastric cancer tissues and cell lines examined. There was also a significant reduction of 15-PGDH expression in paracancerous and precancerous tissues, for example, gastric polyps and atrophic gastritis. Downregulation of 15-PGDH expression was positively correlated with differentiation in gastric cancer tissues, distant metastasis and different TNM stages of gastric cancer. This result is similar to that in previous studies. It has also been reported that expression of 15-PGDH is reduced and associated with tumor differentiation, lymph node metastasis, clinical stage<sup>[11,13]</sup> and prognosis<sup>[10]</sup> in gastric cancer. We verified the relationship between differentiation of gastric cancer cells and 15-PGDH expression *in vitro*. We showed that poorer differentiation in carcinoma was associated with lower 15-PGDH expression. Taken together, reduction of 15-PGDH is related to the carcinogenesis and development of gastric cancer. Evaluation of 15-PGDH expression in tumor and precancerous tissues is a useful diagnostic or prognostic marker for gastric carcinoma.

After determining the relationship between 15-PGDH expression and gastric cancer, we suggest that reduction of 15-PGDH promotes occurrence and development of gastric cancer and that it is an inhibitor of human gastric cancer. Some studies have already demonstrated that 15-PGDH suppresses some tumors. Overexpression of 15-PGDH by transfection with plasmid or adenovirus vectors encoding 15-PGDH reduces occurrence and growth of tumor<sup>[3-5,8-10]</sup>, whereas silencing of 15-PGDH using siRNA enhances cell proliferation and growth of cancer<sup>[3,10]</sup>. 15-PGDH gene knockout increases the colon tumor incidence in the APC+/Min mouse model<sup>[25]</sup>. The antitumor effect in human gastric cancer has only been shown in one study<sup>[15]</sup>. However the mechanism is still not clear.

The mechanism of the antitumor effect of 15-PGDH can be explained by the following hypothesis. 15-PGDH substantially inhibits production of PGE2 and changes the microenvironment to suppress tumor formation by *Ras* gene<sup>[8]</sup>; controls growth of tumor by regulation of cyclooxygenase-2<sup>[21]</sup>; suppresses synthesis, secretion and



**Figure 8** Expressions of 15-PGDH, p21, p27, p16, p53, Survivin, BCL-2, BAX, BAK and BCL-Xl mRNA in SGC-7901 cells transfected by pcDNA3/15-PGDH plasmids for 24 h and 48 h by reverse transcriptase polymerase chain reaction. Lane M: DNA marker; 1: SGC-7901; 2: pcDNA3 24 h group; 3: pcDNA3/15-PGDH 24 h group; 4: pcDNA3 48 h group; 5: pcDNA3/15-PGDH 48 h group. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs SGC-7901, pc DNA3 24 h and 48 h group.

activation of matrix metalloproteinase-2, inhibits cell adhesion to extracellular matrix and reduces CD44 expression, which contributes to the inhibition of the growth, invasion and metastasis of cancer cells<sup>[4,9]</sup>; reduces expression of antiapoptotic protein Bcl-2, which indicates a role for Bcl-2 in mediating or triggering the event of apoptosis<sup>[4]</sup>; inhibits endothelial cell proliferation by 15-oxo-5,8,11,13-(Z,Z,Z,E)-eicosatetraenoic acid, a metabolite of 15-PGDH, suppressing DNA synthesis and implicating a potential antiangiogenic role<sup>[26]</sup>; and attenuates tumor-induced immune suppression and substantially reduces the secretion of immunosuppressive mediators and cytokines such as PGE2, IL-10, IL-13 and IL-6 to regulate the local antitumor immune response<sup>[27]</sup>.

Our present research showed that apoptosis occurred in gastric cancer cells overexpressing 15-PGDH. In SGC-7901 cells transfected with pcDNA3/15-PGDH plasmid, we found by TEM nuclear and cytoplasmic shrinkage, condensation and margination of chromatin against the nuclear membrane, and formation of apoptotic

bodies. Flow cytometric analysis and TUNEL assay showed that the proportion of apoptotic cells was increased by 15-PGDH. Previous research has found the same phenomenon in lung cancer<sup>[4]</sup>. Overexpression of this enzyme induces apoptosis in lung cancer cell line A549. When A549 cells overexpress 15-PGDH by transfection with Ad-15-PGDH, they become apoptotic, as shown by DNA fragmentation, activation of pro-caspase-3 and cleavage of poly ADP ribose polymerase<sup>[4]</sup>. Furthermore, we analyzed genes associated with apoptosis. There was a reduction in expression of antiapoptotic genes (*Survivin*, *BCL-2* and *BCL-Xl*) and increased expression of proapoptotic genes (*BAK*, *BAX* and *p53*). As we know, *Survivin*, *BCL-2* members and *p53* genes are crucial regulators of apoptotic cell death<sup>[28-32]</sup>. *BCL-2* prevents the release of apoptosis-inducing factor and cytochrome c from the mitochondria, which is assumed to be a key event during apoptosis<sup>[30,31]</sup>. Overexpression of 15-PGDH regulates expression of *Survivin*, *BCL-2* members and *p53*, indicating a role in mediating or trig-

gering apoptosis. The results are inconsistent with previous findings, in which apoptosis induced by 15-PGDH is independent of the p53 pathway. 15-PGDH only decreases expression of antiapoptotic protein BCL-2 in lung cancer<sup>[4]</sup>. The mechanism of apoptosis varies in different tumors. In gastric cancer, 15-PGDH induces apoptosis by Survivin, BCL-2 and the p53 pathway.

In our study, we also observed cell cycle arrest in SGC-7901 cells that overexpressed 15-PGDH. We showed an increased accumulation of cells in the sub-G1 phase compared with the control group, and upregulated expression of p21, p16 and p53 without altering p27 expression. p16, p21 and p27 all belong to the cyclin-dependent kinase (CDK) inhibitors<sup>[33-37]</sup>. The product of *p16* gene is an inhibitor of CDK4. Its function is to cyclin E and CDK2 complexes. p27 functions as a negative regulator of G1 progression and is a possible mediator of TGF- $\beta$ -induced G1 phase arrest<sup>[35]</sup>. *p53* is known as a suppressor gene that can adjust the cell cycle<sup>[29]</sup>.

In conclusion, our study provides evidence that loss or reduction of 15-PGDH is related to human gastric cancer. 15-PGDH induces cell cycle arrest and apoptosis of gastric cancer cells *in vitro*, and it may be the mechanism by which it suppresses human gastric cancer and other tumors. Further research is needed to establish the role of 15-PGDH as a target for treatment and chemoprevention of gastric cancer.

## COMMENTS

### Background

Gastric carcinoma is one of the most common malignant tumors in humans and continues to be a major unresolved health problem. New approaches for the management of gastric cancer are needed. Recent studies have shown a reduction of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in some cancers, such as colorectal, breast, prostate and lung. Some studies have revealed that 15-PGDH may have tumor-suppressive properties. It provides a new target for the chemoprevention and treatment of cancer, especially in gastric carcinoma. However, to the best of our knowledge, there has been no study on 15-PGDH expression in gastric precancerous tissue. The association between 15-PGDH expression and various clinicopathological parameters of gastric cancer needs to be validated. Only a few studies have been published on its mechanism of tumor inhibition.

### Research frontiers

NAD<sup>+</sup>-dependent 15-PGDH is the key enzyme responsible for the biological inactivation of prostaglandins (PGs) and related eicosanoids. It may have tumor-suppressive properties. The research hotspot is first study on 15-PGDH expression in gastric precancerous tissue. The association between 15-PGDH expression and various clinicopathological parameters of gastric cancer and its mechanism of tumor inhibition.

### Innovations and breakthroughs

We found that 15-PGDH expression in human gastric cancer was reduced and even absent. Reduction of 15-PGDH expression was also found in precancerous state tissues, e.g., gastric polyps and atrophic gastritis. There was significant difference in expression of 15-PGDH among various pathologic type, with or without distant metastasis and different TNM stage. Apoptosis and cell cycle arrest was found after transfected with pcDNA3/15-PGDH plasmid. In 15-PGDH over-expression cells, expression of the pro-apoptotic genes, such as *BAK* and *p53*, was increased. Expression of anti-apoptotic genes was decreased, e.g., *Survivin*, *BCL-2* and *BCL-X<sub>L</sub>*. Cyclin-dependent kinase inhibitors p21 and p16 expression was significantly up-regulated. The researchers drew a conclusion that reduction of 15-PGDH is associated with carcinogenesis and development of gastric carcinoma. 15-PGDH induces apoptosis and cell cycle arrest in gastric cancer. It will be a novel chemotherapy strategy in gastric cancer.

## Applications

The study results suggest the use of 15-PGDH in chemoprevention and treatment of gastric cancer.

## Terminology

NAD<sup>+</sup>-dependent 15-PGDH is the key enzyme responsible for the biological inactivation of PGs and related eicosanoids. It catalyzes the oxidation of the 15(S)-hydroxyl group of PGs and lipoxins. The products, 15-keto-metabolites, exhibit greatly reduced biological activities.

## Peer review

This is an interesting and well presented study, supported by primary data. It presents the studies on the expression of 15-PGDH in normal and cancerous gastric mucosal tissue, and its effect on apoptosis and cell cycle progression. Further, the authors examined the effect of 15-PGDH overexpression in gastric cell line, SGC-7901, on cell cycle progression and apoptosis. Based on the presented results, it is concluded that the loss or reduction in the mucosal cell 15-PGDH expression is associated with carcinogenesis and the development of gastric carcinoma.

## ACKNOWLEDGMENTS

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## Chronic bile duct hyperplasia is a chronic graft dysfunction following liver transplantation

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### Abstract

**AIM:** To investigate pathological types and influential factors of chronic graft dysfunction (CGD) following liver transplantation (LT) in rats.

**METHODS:** The whole experiment was divided into three groups: (1) normal group ( $n = 12$ ): normal BN rats without any drug or operation; (2) syngeneic transplant group (SGT of BN-BN,  $n = 12$ ): both donors and recipients were BN rats; and (3) allogeneic transplant group (AGT of LEW-BN,  $n = 12$ ): Donors were Lewis and recipients were BN rats. In the AGT group, all recipients were subcutaneously injected by

Cyclosporin A after LT. Survival time was observed for 1 year. All the dying rats were sampled, biliary tract tissues were performed bacterial culture and liver tissues for histological study. Twenty-one day after LT, 8 rats were selected randomly in each group for sampling. Blood samples from caudal veins were collected for measurements of plasma endotoxin, cytokines and metabonomic analysis, and faeces were analyzed for intestinal microflora.

**RESULTS:** During the surgery of LT, no complications of blood vessels or bile duct happened, and all rats in each group were still alive in the next 2 wk. The long term observation revealed that a total of 8 rats in the SGT and AGT groups died of hepatic graft diseases, 5 rats in which died of chronic bile duct hyperplasia. Compared to the SGT and normal groups, survival ratio of rats significantly decreased in the AGT group ( $P < 0.01$ ). Moreover, liver necrosis, liver infection, and severe chronic bile duct hyperplasia were observed in the AGT group by H and E stain. On 21 d after LT, compared with the normal group ( $25.38 \pm 7.09$  ng/L) and SGT group ( $33.12 \pm 10.26$  ng/L), plasma endotoxin in the AGT group was remarkably increased ( $142.86 \pm 30.85$  ng/L) (both  $P < 0.01$ ). Plasma tumor necrosis factor- $\alpha$  and interleukin-6 were also significantly elevated in the AGT group ( $593.6 \pm 171.67$  pg/mL,  $323.8 \pm 68.30$  pg/mL) vs the normal ( $225.5 \pm 72.07$  pg/mL,  $114.6 \pm 36.67$  pg/mL) and SGT groups ( $321.3 \pm 88.47$  pg/mL,  $205.2 \pm 53.06$  pg/mL) ( $P < 0.01$ ). Furthermore, Bacterial cultures of bile duct tissues revealed that the rats close to death from the SGT and AGT groups were strongly positive, while those from the normal group were negative. The analysis of intestinal microflora was performed. Compared to the normal group ( $7.98 \pm 0.92$ ,  $8.90 \pm 1.44$ ) and SGT group ( $8.51 \pm 0.46$ ,  $9.43 \pm 0.69$ ), the numbers of *Enterococcus* and *Enterobacteria* in the AGT group ( $8.76 \pm 1.93$ ,  $10.18 \pm 1.64$ ) were significantly increased (both  $P < 0.01$ ). Meanwhile, compared to the normal group ( $9.62 \pm 1.60$ ,  $9.93 \pm 1.10$ ) and SGT group ( $8.95 \pm 0.04$ ,  $9.02 \pm 1.14$ ), the numbers of *Bifidobacterium* and *Lac-*

*tobacillus* in the AGT group ( $7.83 \pm 0.72$ ,  $8.87 \pm 0.13$ ) were remarkably reduced (both  $P < 0.01$ ). In addition, metabonomics analysis showed that metabolic profiles of plasma in rats in the AGT group were severe deviated from the normal and SGT groups.

**CONCLUSION:** Chronic bile duct hyperplasia is a pathological type of CGD following LT in rats. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function and microflora as well as plasma metabolic profiles.

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**Key words:** Liver transplantation; Chronic graft dysfunction; Chronic bile duct hyperplasia; Metabonomics; Intestinal barrier function

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## INTRODUCTION

Liver transplantation (LT) has become an established therapy for various end-stage liver diseases for more than three decades<sup>[1-3]</sup>. Numerous advances in surgical technique, organ preservation, perioperative anesthesia, postoperative care, and clinical immunosuppression, as well as improved recipient selection and donor management have together significantly increased the survival rates of allograft and improved life quality of patients following LT<sup>[4]</sup>. Currently, approximately 90% of liver transplant patients are alive after 1 year and 75% after 5 years with majority living a full and near-normal life<sup>[3,5]</sup>. However, although early mortality rates after transplantation have fallen dramatically, long-term graft survival has barely improved over the last two decades<sup>[6,7]</sup>. That is to say, the incidence of chronic graft dysfunction (CGD) and the mortality of patients after LT have remained constant, and CGD has become the biggest obstacle for long-term function of allograft and better life quality of patients. Thus, it is essential to investigate the causes and relevant mechanisms of CGD to improve long-term outcomes of patients following LT.

In the early day after LT, common causes of hepatic allograft dysfunction include ischemia and reperfusion injury, infection, technical complications such as hepatic artery thrombosis and recipient diseases<sup>[3,5]</sup>. Thereafter the causes of allograft dysfunction are variable with dis-

ease recurrence and chronic rejection as major causes of graft loss<sup>[3,8]</sup>. Moreover, the biliary tract is still the most common site for postoperative complications. The importance of this condition lies in the fact that the biliary tract complications can be a serious source of morbidity and sometimes mortality<sup>[9,10]</sup>. These complications not only affect allograft survival but also have a major impact on the life quality for a hepatic allograft recipient.

However, so far little is known on biliary tract variation of CGD and its influential factors in recipients following LT. In this study, we want to explore hepatic graft pathology and the relevant mechanisms of CGD from aspects of inflammation, intestinal barrier function, intestinal microflora and metabonomics following LT.

## MATERIALS AND METHODS

### Animals

Specific pathogen-free (SPF) male inbred Lewis and BN rats (weight 220-250 g, 12-15 wk) were purchased from Beijing Vital River Laboratories (Beijing, China). All rats were housed in a SPF lab (Zhejiang Academy of Medical Sciences, China). The rats were caged in 21 °C, 12 h light/dark cycle, and fed with sterilized standard rat chow and water. All animals received humane care and the study was conducted according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

### Experimental design

Sixty rats were raised in SPF animal facility. Twelve inbred BN rats were served as normal controls; 12 inbred Lewis and 12 inbred BN rats as donors; 24 inbred BN rats as recipients. All donors and recipients were randomly performed orthotopic LT under strict sterile conditions. The remaining 36 BN rats were divided into three groups: (1) normal group ( $n = 12$ ): normal BN rats without any drug or operation; (2) syngeneic transplant group (SGT of BN-BN,  $n = 12$ ): both donors and recipients were BN rats; and (3) allogeneic transplant group (AGT of LEW-BN,  $n = 12$ ): donors were Lewis and recipients were BN rats. In the AGT group, all recipients were subcutaneously injected by Cyclosporin A at 1 mg/kg daily in the first 30 d, and then at 2 mg/kg daily for the next 100 d after orthotopic LT. All the recipients in the SGT and AGT groups were received alanine (ALA) daily by gastric perfusion for preoperative 3 d and postoperative 130 d. Survival time was observed for 1 year.

### Surgery procedures

All rats were fasted for 12 h before the operation. The initial anesthesia of rats was performed by intraperitoneal injection of Ketamine Hydrochloride (100 mg/kg) and Atropine (1 mg/kg) (Shanghai No. 1 Biochemical and Pharmaceutical, China), and then ether was inhaled to maintain anesthesia. The profiles of rats with orthotopic LT were established according to the previous techniques<sup>[11,12]</sup>, with slight modifications. Briefly, after the

liver of the donor was dissociated, the graft was perfused with chilled saline containing 25 U/mL heparin *via* the portal vein, and then preserved in cold normal saline for no more than 1 h before being placed in the abdomen of recipient. After the anastomosis of suprahepatic vena cava and portal vein was finished, the graft was reperfused. The common bile duct was reconstructed by tying the duct over a stent. All recipients recovered in a short time, and no further treatment was performed.

### Sample collections

The survival conditions of rats were monitored continuously. When any rat was dying, the following samples were collected under strict sterile condition; the liver was fixed in 40 g/L neutral formaldehyde for later histological study and the biliary tract tissue for bacterial culture. Moreover, on 21 d after the surgery, biliary tract tissues of 4 rats selected randomly in the normal group were collected for bacterial culture. Eight rats were selected randomly in each group for sampling. Blood samples from the caudal veins were gained for measurements of plasma endotoxin, cytokines and analysis of ultra performance liquid chromatography-mass spectrometry (UPLC-MS), and faeces were collected for the determination of intestinal microflora.

### Graft histopathology

The sample from the left lobe of hepatic graft was fixed in 40 g/L neutral formaldehyde and embedded in paraffin, cut into 3  $\mu$ m slices, stained with hematoxylin and eosin (HE), and then observed under light microscopy by a pathologist.

### Plasma endotoxin and cytokines

The blood sample (100  $\mu$ L) was placed in the pyrogen-free heparin-containing tube, and then centrifuged at 3000 *g* for 15 min at 4 °C. Plasma endotoxin of the caudal vein was determined using a quantitative, chromogenic Limulus Amebocyte Lysate assay according to the manufacturer's instruction (Eihua Medical, Shanghai, China). The value was expressed as nanogram per liter of plasma (ng/L).

The levels of plasma tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) were tested with enzyme-linked immunosorbent assay (ELISA) (Groundwork Biotechnology Diagnostic Ltd, United States) according to the protocol of manufacturer. The result was expressed as ng/L.

### Bacterial culture and identification

Biliary tract tissues from the grafts were weighed and placed in a germ-free glass homogenizer containing a nine-fold amount of anaerobic buffer (phosphate buffered saline with 0.5 g cysteine HCl, 0.5 mL tween 80, and 0.5 g agar). They were homogenized and 50  $\mu$ L of 10% homogenate were placed on the agar base of Colombian culture medium within 30 min, incubated for 48 h at 37 °C. Bacterial colonies were evaluated qualitatively ac-

ording to their growth conditions respectively at the end of the culture. In addition, bacterial colonies from biliary tract tissues were identified by the Automatic analyzer of bacteria (Model Viger 60, France) to identify bacterial species according to the report<sup>[13]</sup>.

### Determination of intestinal microflora

Intestinal microflora was studied with 4 selected agar media according to the reports<sup>[14]</sup>. Samples from colorectal contents were placed in sterile tubes, weighed and transferred into other sterile tubes containing appropriate anaerobic buffer (as described above) to approach a 10-fold dilution of samples, and then serial decimal dilutions were taken in the same way from 10<sup>-2</sup> to 10<sup>-8</sup>. Within 30 min of sample collection, bacterial cultures were finished with placing 50  $\mu$ L dilutions on 4 agar media. According to the instructions, TPY agar medium, LBS agar medium, EC medium, and Eosin-Methylene Blue Agar (EMB) were used for Bifidobacterium, Lactobacillus, Enterococcus and Enterobacter, respectively. Anaerobic bacteria were incubated in Anaerobic Box System including AnaeroPack (MGC, Japan) and GENbox anaer (BioMérieux, France), and aerobic bacteria were incubated aerobically for 48 h at 37 °C, respectively. Bacterial colonies on every plate were counted and calculated at the primal weight of samples. The results were expressed as bacterial colony forming units per gram content (log<sub>10</sub> CFU/g).

### UPLC-MS analysis

Blood samples from caudal veins of rats were collected for metabonomics analysis according to the method of UPLC-MS described by Wang *et al.*<sup>[15]</sup> and Yang *et al.*<sup>[16]</sup>. Prior to analysis, blood samples were defrosted at room temperature and mixed with acetonitrile (ACN) at the ratio of 1:3 (v/v), the mixture was vortexed and centrifuged at 10 000 *g* for 10 min. The supernatant was transferred to sample bottles for UPLC separation. Chromatographic separations were performed at a 100 mm  $\times$  2.1 mm ACQUITY-1.7  $\mu$ m C18 column (Waters Co., Milford, United States) using an ACQUITY-Ultra Performance Liquid Chromatography system (Waters). Mass spectrometry was performed on a Premier-Q-ToF (Waters MS Technologies, Milford, United States).

### Partial least squares-discriminate analysis

The UPLC-MS data were analyzed with the SIMCA-P+ 12 Software (Umetrics, Sweden). An ApexTrack-peak detection algorithm was adopted in MarkerLynx V4.1 software (Waters, United States) to measure peaks and align retention times of the peaks for all chromatograms. The results were transferred into a single data matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their relevant intensities. The resulting dataset containing peak numbers (RT-m/z pair), sample names, and ion intensities was analyzed by partial least squares-discriminate analysis (PLS-DA) with the SIMCA-P+ 12 Software.

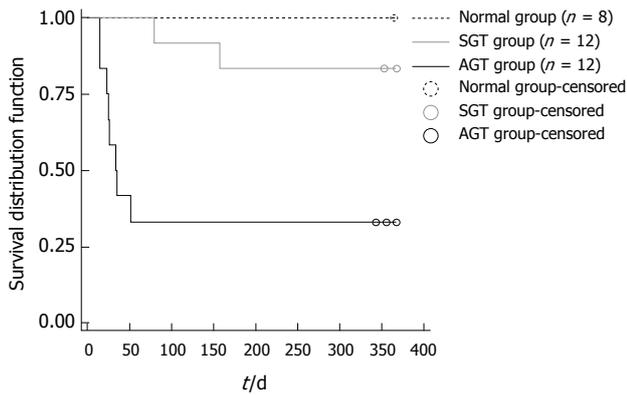


Figure 1 Kaplan-Meier survival curve in the different groups.

### Statistical analysis

All the data were presented as mean  $\pm$  SD. The survival distribution function was evaluated by the Kaplan-Meier survival curve and the others were determined using Students *T*-test. These analysis were performed with the statistical software SAS 9.1.3 (SAS Institute Inc., North Carolina State University, United States), and UPLC-MS data were analyzed by PLS-DA with the SIMCA-P+ 12 Software (Umetrics, Sweden). A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

During the surgery of LT, no complication of blood vessels or bile duct happened, and all rats in each group were still alive in the next 2 wk.

### Survival distribution function

The survival distribution function in each group was shown in Figure 1. A total 8 rats in the normal group were alive for more than one year. Two rats in the SGT group respectively died of hepatic necrosis and chronic bile duct hyperplasia on the 78th and 156th day post-operation. It was worth noting that a total of 8 rats in the AGT group were dead: two died of hepatic necrosis on the 25th and 26th day post-operation, respectively; two died of abdominal infection on the 15th and 23th day post-operation, respectively; the remaining 4 rats died of severe chronic bile duct hyperplasia on the 25th, 34th, 35th and 51th day post-operation, respectively. Compared to the rats in the SGT group and normal group, the survival ratio of rats significantly decreased in the AGT group ( $^aP < 0.01$ ,  $^bP < 0.001$ , respectively).

### Hepatic graft histopathology

As shown in Figure 2, the different characteristics of hepatic graft histology were observed in the rats of different groups. Under light microscope, rats' livers in the normal group showed the normal hepatic structure that the hepatocyte cords were presented the radial distribution around the central vein without the infiltration of inflammatory cells (Figure 2A). The rat dead of chronic

bile duct hyperplasia in the SGT group showed special pathological features that the proliferative epithelial cells of bile duct invaded into the hepatic parenchyma without lymphocyte infiltration (Figure 2B). However, the dead rats in the AGT group were presented unique pathological structures, including liver necrosis, liver infection, and severe chronic bile duct hyperplasia. Specifically, liver necrosis was presented the extensive destruction of hepatic structure, and the necrosis of lots of hepatocytes (Figure 2C). Liver infection was described as the destruction of hepatic structure, the excessive proliferation of ectogenic bacteria, and the infiltration of lots of inflammatory cells (Figure 2D). And severe chronic bile duct hyperplasia was expressed by some special points that the proliferative epithelial cells of bile duct extensively invaded into the hepatic parenchyma, and bile duct lumen was significantly extended with the destruction of hepatic structure (Figure 2E).

### Plasma endotoxin and cytokines

Plasma endotoxin, as a critical medium to aggravate graft injury, is a vital stimulus of CGD following allogeneic LT. As showed in Figure 3, there was no significant difference in plasma endotoxin between the normal group ( $25.38 \pm 7.09$  ng/L) and SGT group ( $33.12 \pm 10.26$  ng/L). By contrast, plasma level of endotoxin in the AGT group was remarkably increased ( $142.86 \pm 30.85$  ng/L) *vs* the other two groups ( $P < 0.01$ ).

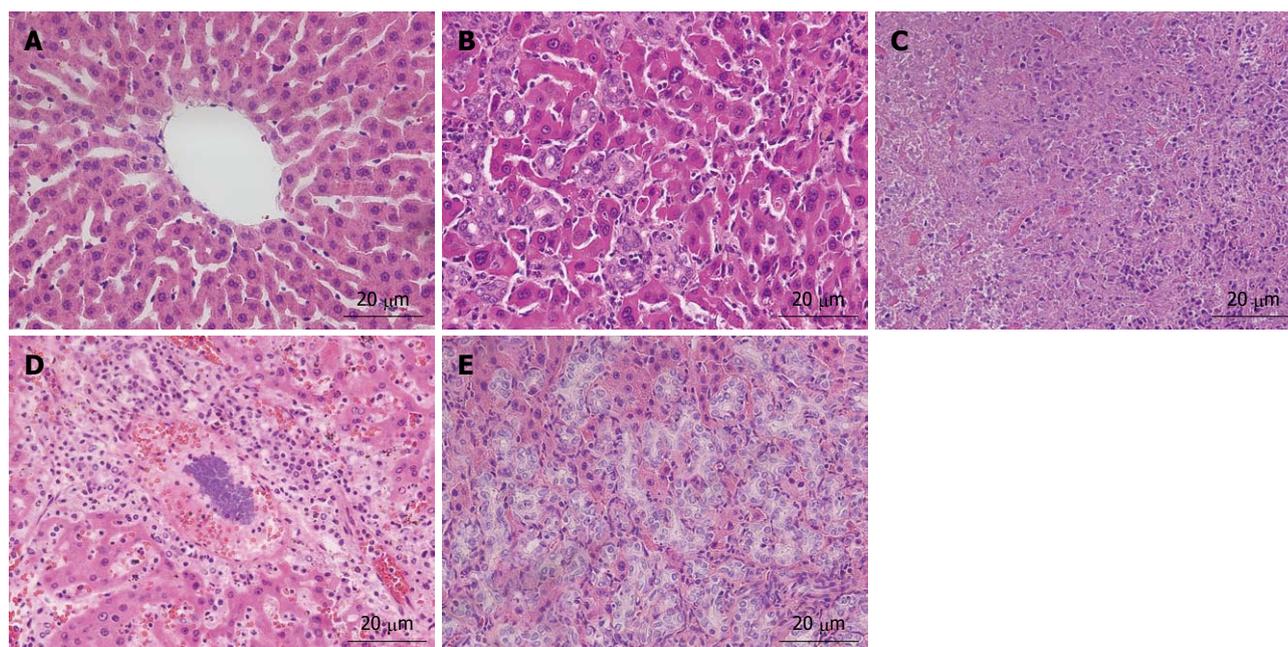
Both TNF- $\alpha$  and IL-6 are important pro-inflammatory cytokines and can directly or indirectly cause graft injury and CGD of recipients after LT. As shown in Figure 3 (pg/mL), compared to the normal group ( $225.5 \pm 72.07$  ng/L,  $114.6 \pm 36.67$  ng/L) and SGT group ( $321.3 \pm 88.47$  ng/L,  $205.2 \pm 53.06$  ng/L), the levels of plasma TNF- $\alpha$  and IL-6 were significantly elevated in the AGT group ( $593.6 \pm 171.67$  ng/L,  $323.8 \pm 68.30$  ng/L) ( $P < 0.01$ ). In addition, plasma levels of both TNF- $\alpha$  and IL-6 in the SGT group were also increased *vs* those in the normal group ( $^aP < 0.05$ ,  $^bP < 0.01$ , respectively).

### Bacterial culture and identification

Bacterial translocation may participate in some physiological and pathological procedures when recipient suffers from CGD. Samples from biliary duct tissues of hepatic graft were cultured and results were summarized in Table 1. There was rare bacterial colony in the 4 rats from the normal group, while bacterial culture of samples from the SGT and AGT groups were strongly positive. Bacterial identification revealed significantly increased aerobic bacteria, such as *Escherichia coli* and *Enterococcus* in the groups of LT. Moreover, *Protens vulgaris*, *Streptococcus agalactiae* and *Protens mirabilis* presented remarkably positive in the AGT group, which suggested that more kinds of bacteria translocated to biliary duct tissues when recipients suffered from CGD following allogeneic LT.

### Intestinal microflora

To determine the alterations of intestinal microflora when recipients suffered from CGD after LT, bacterial



**Figure 2** Light micrograph of hepatic histopathology stained with hematoxylin and eosin (HE, × 400). A: The normal group showed the normal hepatic structure; B: The SGT group revealed the chronic bile duct hyperplasia; C: The AGT group presented hepatic graft necrosis; D: The AGT group showed the infectious hepatic graft; E: The AGT group emerged severe chronic bile duct hyperplasia.

**Table 1** Bacterial culture of biliary duct tissues from hepatic graft in the different groups

Bacterial species	SGT group (n = 2)	AGT group (n = 8)
<i>Escherichia coli</i>	2/2	8/8
<i>Enterococcus</i>	2/2	8/8
<i>Staphylococcus aureus</i>	1/2	0/8
<i>Proteus vulgaris</i>	0/2	8/8
<i>Streptococcus agalactiae</i>	0/2	6/8
<i>Proteus mirabilis</i>	0/2	5/8

There is rare bacterial colony in the normal group (n = 4). SGT group (n = 2): Syngeneic transplant of BN-BN rats; AGT group (n = 8): Allogeneic transplant of LEW-BN rats.

**Table 2** Bacterial counts of colorectal contents in the different groups (log<sub>10</sub> CFU/g, mean ± SD)

Group	Normal group	SGT group	AGT group
<i>Enterococcus</i>	7.98 ± 0.92	8.51 ± 0.46	8.76 ± 1.93 <sup>a,d</sup>
<i>Enterobacteria</i>	8.90 ± 1.44	9.43 ± 0.69	10.18 ± 1.64 <sup>a,d</sup>
<i>Bifidobacterium</i>	9.62 ± 1.60	8.95 ± 0.04	7.83 ± 0.72 <sup>a,d</sup>
<i>Lactobacillus</i>	9.93 ± 1.10	9.02 ± 1.14	8.87 ± 0.13 <sup>a,d</sup>

CFU/g: Colony-forming unit per gram colorectal content; Normal group (n = 8): Normal BN rats without any drug or operations; SGT group (n = 8): Syngeneic transplant of BN-BN rats; AGT group (n = 8): Allogeneic transplant of LEW-BN rats. <sup>a</sup>P < 0.05 vs SGT group; <sup>d</sup>P < 0.01 vs normal group.

species and numbers in colorectal contents were analyzed. As shown in Table 2, compared to the normal group (7.98 ± 0.92, 8.90 ± 1.44) and SGT group (8.51 ± 0.46, 9.43 ± 0.69), the numbers of *Enterococcus* and *Enterobacteria* in the AGT group (8.76 ± 1.93, 10.18 ± 1.64) were significantly

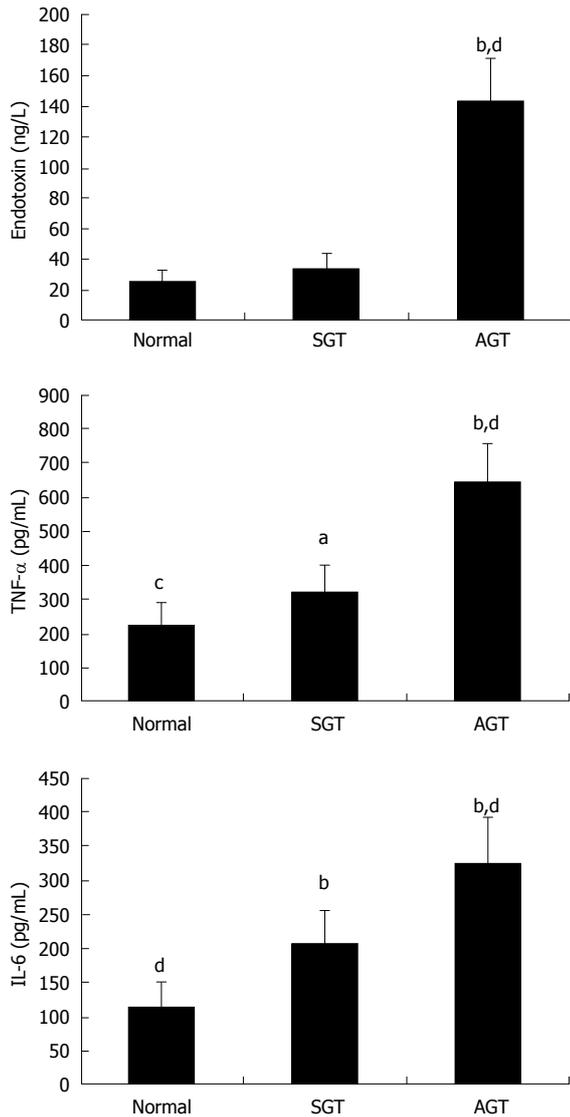
increased (both <sup>a</sup>P < 0.01, <sup>b</sup>P < 0.05, respectively). Meanwhile, compared to the normal group (9.62 ± 1.60, 9.93 ± 1.10) and SGT group (8.95 ± 0.04, 9.02 ± 1.14), the numbers of *Bifidobacterium* and *Lactobacillus* in the AGT group (7.83 ± 0.72, 8.87 ± 0.13) were remarkably reduced (both <sup>a</sup>P < 0.01, <sup>b</sup>P < 0.05, respectively). There were no statistical differences in bacterial species and counts between the normal group and the SGT group.

### UPLC-MS analysis and PLS-DA

In order to analyze the interactions between metabolic profile and CGD, caudal vein plasma was collected to perform metabonomics analysis by the method of UPLC-MS and PLS-DA. As shown in Figures 4-6, the metabolic profiles of plasma in rats from the SGT and AGT groups were deviated from those of the normal group gradually. We also found that metabolic alterations were more severely deviated in the AGT group vs the normal group, which suggested that the degree of metabolic change was positively associated with the severity of CGD in recipients following LT.

## DISCUSSION

Organ transplantation has become the radical method for treatment of many end-stage organic diseases<sup>[17,18]</sup>. Since the early experiences of the 1960s, liver transplant surgery has evolved over the decades and is now the standard of care in patients with end-stage liver diseases<sup>[19-21]</sup>. Although there has been consistent improvement in the overall survival rates for transplant recipients, organ shortages and CGD are still two major problems which hinder the development of organ transplantation.



**Figure 3** Plasma levels of endotoxin, tumor necrosis factor- $\alpha$ , and interleukin-6 in the different groups on the 21th day after liver transplantation. Normal group ( $n = 8$ ); SGT group ( $n = 8$ ); AGT group ( $n = 8$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs Normal group, respectively; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs SGT group, respectively;  $P$  not significant in the remaining parameters. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-6: Interleukin-6.

In general, chronic hepatic allograft dysfunction is defined as the declining of hepatic graft function irreversibly and gradually, expressed by increasing or persistent elevations in serum levels of alanine aminotransferase, alkaline phosphatase, or bilirubin (greater than two times the upper limit of normal)<sup>[5]</sup>. Chronic hepatic allograft dysfunction may result from a variety of causes including rejection<sup>[8]</sup>, vascular stenosis/thrombosis, *de novo* or recurrent infection, biliary complications<sup>[19]</sup> including stricture or stenosis, recurrent disease related to autoimmune mechanisms such as that seen in primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune chronic active hepatitis<sup>[5]</sup>. In addition, drug hepatotoxicity and the development of neoplasms such as a posttransplant lymphoproliferative disorder or the recurrences of hepatocellular carcinoma are important considerations<sup>[3]</sup>.

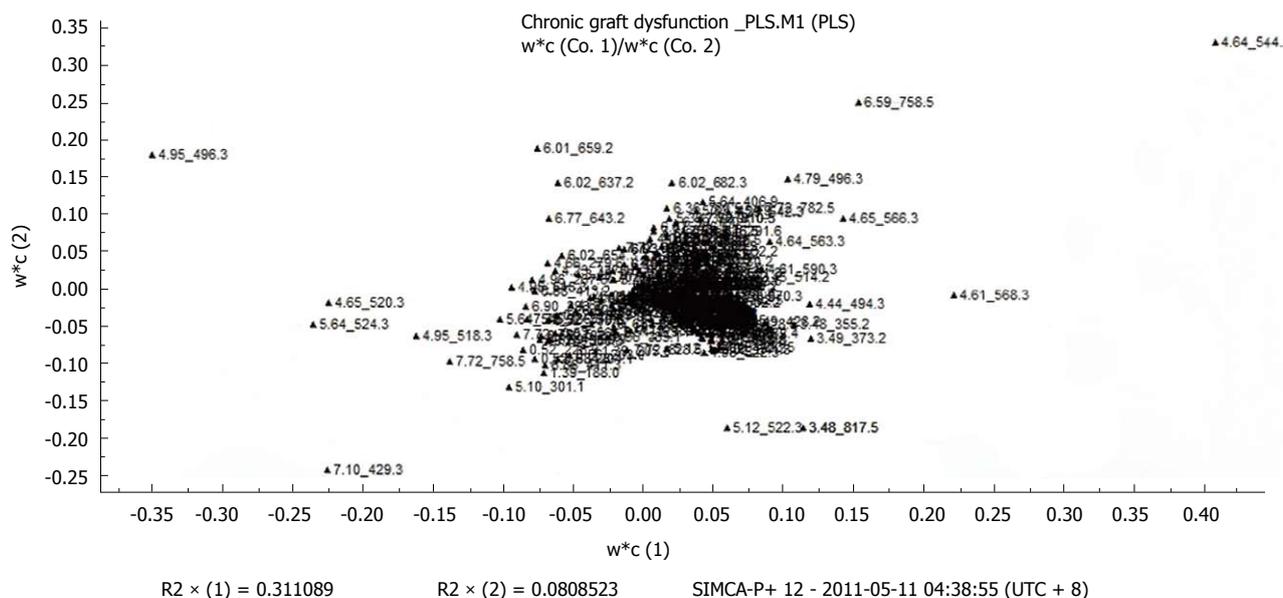
With the efforts in recent decades, survival after LT is 90% at 1 year and approximately 75% at 5 years<sup>[5]</sup>. However, biliary tract complications are still the “Achilles heel” of LT<sup>[22,23]</sup>. Despite great improvements in the surgical techniques and standardization of the method of biliary reconstruction, the biliary tract is still the most common site for postoperative complications. So far little is known about alterations of biliary tract when recipients suffer from CGD following LT. Thus, it has been indispensable to explore changes of biliary tract and relevant mechanisms of CGD to improve survival conditions of recipients following LT for a long time.

In our study, no rats died within 2 wk after LT since applications of immunosuppressants and sterile surgery in SPF-class laboratory. Life span of human is usually 60-90 years, while life expectancy of rats typically is 2-3 years, so we can define CGD of rats as loss of graft function after about one month post-operation. During the whole process of observation, a total of 8 rats in the LT groups died of hepatic graft diseases, such as partial hepatic necrosis and chronic bile duct hyperplasia, and the other 2 rats died of abdominal infection. Notably, 5 rats in these 8 rats that died of hepatic graft diseases died of chronic bile duct hyperplasia, which accounted for 5/8 (62.5%). We concluded that chronic bile duct hyperplasia was a pathological type of CGD and its occurrence frequency was closely associated with the causes of CGD following LT. This finding is consistent with the reports<sup>[24]</sup> that bile duct hyperplasia extending progressively is a pathology finding in a profile of auxiliary LT with portal vein arterialization in pigs.

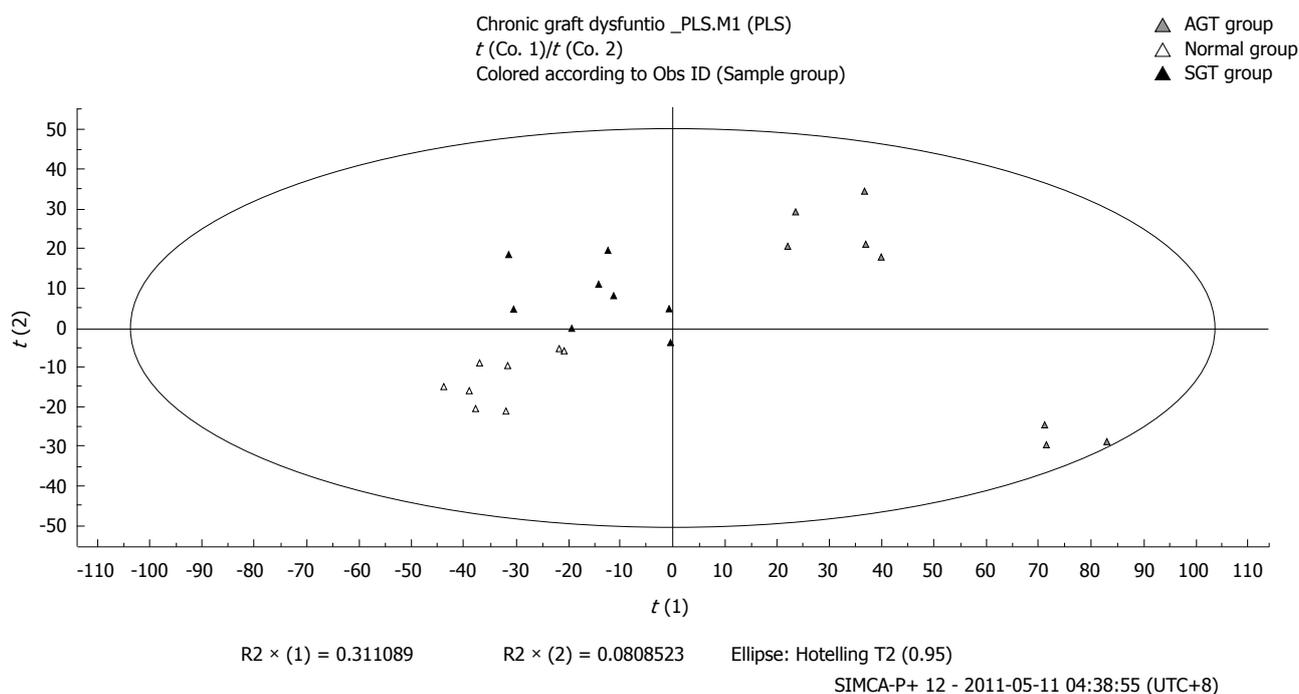
In order to explore influential factors and possible mechanisms of chronic bile duct hyperplasia, as a special type of CGD following LT, we further investigated alterations of serum endotoxin and cytokines, bacterial translocation, intestinal microflora, and metabolic profile in the different groups.

Endotoxin, which mainly originated from non-viable intestinal gram-negative bacteria, is a crucial medium to aggravate hepatic graft injury<sup>[25]</sup>. Plasma level of endotoxin not only can reflect intestinal barrier function<sup>[26]</sup>, but also can predict the defensive ability of body and the injury degree of hepatic graft. Our research found that plasma level of endotoxin in the AGT group remarkably increased compared to the other two groups, which suggested that intestinal barrier function was destroyed when recipients suffered from CGD following LT. Meanwhile, we also speculated that the elevation of plasma endotoxin was closely associated with the occurrence of CGD after LT in rats.

Inflammation plays a vital role in the progression of liver injury<sup>[27]</sup>. TNF- $\alpha$  and IL-6 are important proinflammatory mediators and can directly, or by inducing inflammatory cascades and enhancing the microvascular dysfunction of liver and intestine, aggravate the injury of hepatic graft<sup>[28]</sup>. Inflammatory mediators such as TNF- $\alpha$ , interferon- $\gamma$  (INF- $\gamma$ ) and interleukin (IL) have cytotoxicity, thus being considered as effectors of liver injury. Our results revealed that plasma levels of cytokines gradually



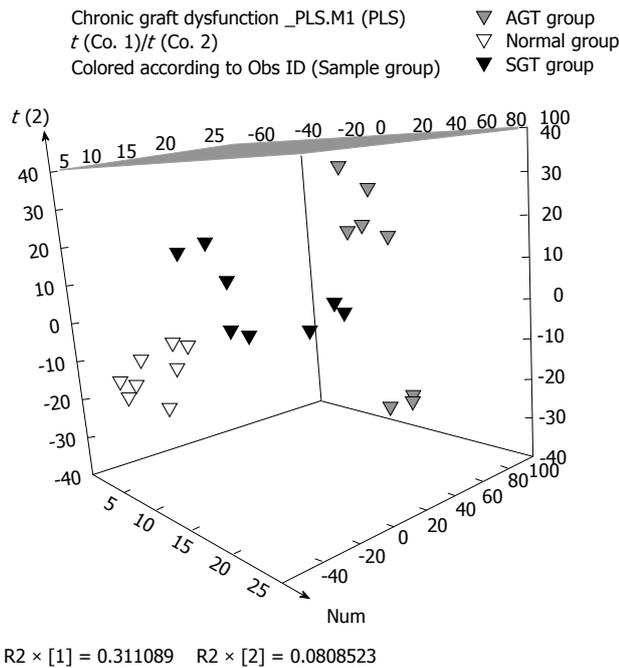
**Figure 4** Loading plot of all the different plasma samples. The marked numbers beside the points were the corresponding retention time and the mass-to-charge ratio (m/z) of each possible biomarkers.



**Figure 5** The partial least squares-discriminate analysis scores plot of the different groups. According to this plot, the metabolic profiles of the SGT and AGT groups deviated from those of the normal group gradually.

increased from the rats in the normal group to those in the syngeneic transplant group, and even to those in the allogeneic transplant group, suggesting that inflammatory reaction was positively associated with the severity of CGD in recipients. Thus, our findings support the concept that inflammation may be a component of the pathogenesis of CGD, which is in line with the report on inflammation and graft deterioration by Dahle *et al*<sup>[29]</sup>. However, the exact pathogenetic role of inflammatory cytokines in graft failure is still elusive.

Under certain conditions, the original bacteria in the intestine would cross a relatively complete intestinal epithelium to reach the sites of MLN, abdominal internal and external organs (such as liver, spleen and lung) as well as blood, and may cause the occurrence of infection, which is called “bacterial translocation”<sup>[30]</sup>. Normally, a small amount of bacteria and endotoxin can go through the intestinal wall, which may be associated with the maintenance of normal intestinal immune response and the activity of reticuloendothelial system<sup>[31,32]</sup>. In gen-



**Figure 6** The three-dimensional partial least squares-discriminate analysis scores plot of the different groups. According to this three-dimensional plot, the metabolic profiles of the AGT groups deviated more than those of the SGT group from those of the normal group. PLS: Partial least squares.

eral, three factors mainly attributed to the occurrence of BT: the destruction of intestinal barrier, the imbalance of intestinal microflora, and the reduction of immune defense<sup>[33]</sup>. There was rare bacterial growth in culture medium of biliary duct in the normal group, while bacterial counts significantly elevated in the AGT group. Bacterial identification mainly designated aerobic bacteria, including *Escherichia coli*, *Enterococcus*, *Protens vulgaris*, *Streptococcus agalactiae* and *Protens mirabilis*. This result suggested bacterial translocation occurred in recipients after LT under immunosuppression, which might aggravate the severity of CGD in some extent.

The commensal bacteria living in the human intestine play a pivotal role in the maintenance of intestinal homeostasis in their host<sup>[34]</sup>. The normal formation of intestinal microflora contributes not only to the prevention of enteritis caused by pathogens but also to immunological development and preservation<sup>[35]</sup>. Under physiological circumstances, intestinal bacteria keep an ecological balance of intestinal microflora. Many researches have indicated that some extrinsic factors, such as administration of abdominal surgery<sup>[36]</sup>, hepatic I/R injury<sup>[14]</sup>, gastrointestinal disorders<sup>[37]</sup> and LT<sup>[25]</sup>, can cause the imbalance of intestinal microflora. Our results also indicated that the imbalance of intestinal microflora was expressed by an increase in *Enterococcus* and *Enterobacteria*, and a reduction in *Bifidobacterium* and *Lactobacillus*, when recipients suffered from severe CGD in allogeneic transplant group. Thereafter we may speculate that the occurrence of CGD has some correlations with intestinal microflora.

In addition, we performed metabolomics analysis of

plasma in recipients following LT. As a system analysis approach, metabolomics can provide comprehensive information on the dynamic process of postoperative physiopathological development<sup>[15]</sup>. The systemic detection of chronic diseases can be obtained with metabolomics at an earlier stage compared to the clinical chemistry and histopathological assessment<sup>[15]</sup>. Our results on metabolomics analysis revealed that the metabolic profile of plasma gradually deviated from the normal parameters, from the rats in the syngeneic transplant group to those in the allogeneic transplant group. To some extent, this finding suggested that the mechanism of CGD might be explained by the alterations of plasma metabolic profile in recipients following LT. However, further exploration need to be taken for the accurate relationship between metabolic changes and CGD of recipients following LT.

In conclusion, according to the observation of one year for recipients following LT in rats, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function, intestinal microflora, and plasma metabolic profile, which will be the possible therapeutic targets for LT.

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## COMMENTS

### Background

Although early mortality rates after liver transplantation (LT) have fallen dramatically, the paradox is that long-term graft survival has barely improved over the last two decades. Chronic graft dysfunction (CGD) has become the biggest obstacle for long-term function of allograft and better life quality of patients. The biliary tract is still the most common site for postoperative complications. These complications not only affect allograft survival, but also have a major impact on the life quality for a hepatic allograft recipient.

### Research frontiers

So far little is known on biliary tract variation of CGD and its influential factors in recipients following LT. In our experimental study, according to the observation of one year for recipients following LT, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function, intestinal microflora and plasma metabolic profile.

### Innovations and breakthroughs

Chronic hepatic allograft dysfunction may result from a variety of causes including rejection, vascular stenosis/thrombosis, *de novo* or recurrent infection, recurrent disease related to autoimmune mechanisms such as that seen in primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune chronic active hepatitis. However, So far little is known about alterations of biliary tract when recipients suffer from CGD following LT. Through one-year observation of recipients following LT, we found that chronic bile duct hyperplasia is a pathological type of CGD following LT. Meanwhile, we elaborated the influential factors of this kind of CGD mainly including inflammatory response, intestinal

barrier function, intestinal microflora, and plasma metabolic profile. Furthermore, through the technique of ultra performance liquid chromatography-mass spectrometry analysis and partial least squares-discriminate analysis (PLS-DA), we explored the plasma metabolomics alterations during the period of CGD after LT, and indicated the relevance between plasma metabolomics and CGD after LT in rats.

### Applications

This study provides the experimental data for the research of CGD after organ transplantation in rats, and indicated that chronic bile duct hyperplasia is a kind of CGD following LT in rats. The relative influence factors will be the possible therapeutic targets to prevent or alleviate CGD after LT.

### Terminology

Chronic hepatic allograft dysfunction is defined as the declining of hepatic graft function irreversibly and gradually, expressed by increasing or persistent elevations in serum levels of alanine aminotransferase, alkaline phosphatase, or bilirubin (greater than two times the upper limit of normal). PLS-DA is a statistical method that can grasp the principal contradiction from the complexity, and thereby simplify something complexity, which can generate models that are tightly focused on the effects of interest.

### Peer review

This is a well designed experimental report on chronic allograft dysfunction after LT. The topic is of some interest due to the prolonged survival now being constantly achieved after LT. The authors described chronic bile duct hyperplasia as a type of chronic allograft dysfunction associated to inflammation of the intestinal mucosa

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## Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis

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### Abstract

**AIM:** To compare the influence of different transplant sites in bone marrow mesenchymal stem cell (MSC)-based therapy for liver fibrosis.

**METHODS:** MSCs isolated from Sprague Dawley (SD) rats were induced into hepatocyte-like cells. Liver fibrosis in SD rats was induced with carbon tetrachloride. Following hepatocyte induction *in vitro*, 4',6-diamidino-2-phenylindole (DAPI)-labeled MSCs were transplanted by intravenous, intrahepatic, and intraperitoneal injection. Histopathological staining, immunohistochemistry, and biochemical analysis were used to compare the morphological and functional liver regeneration among different MSC injection modalities. The expression differences of interleukins, growth factor, extracellular matrix, matrix metalloproteinases, and tissue inhibitor of metalloproteinase were examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) and

enzyme linked immunosorbent assay (ELISA).

**RESULTS:** Four days after exposure to hepatocyte differentiation medium, MSCs that did not express hepatocyte markers could express  $\alpha$ -fetoprotein, albumin, and cytokeratin 18. The results of histopathological staining, immunohistochemistry, and biochemical analysis indicated that intravenous injection is more effective at rescuing liver failure than other injection modalities. DAPI-labeled cells were found around liver lobules in all three injection site groups, but the intravenous group had the highest number of cells. PCR and ELISA analysis indicated that interleukin-10 (IL-10) was highest in the intravenous group, whereas *il1 $\beta$* , *il6*, *tnf $\alpha$*  and *tgf $\beta$* , which can be regulated by IL10 and are promoters of liver fibrosis, were significantly lower than in the other groups.

**CONCLUSION:** MSC administration is able to protect against liver fibrosis. Intravenous injection is the most favorable treatment modality through promotion of IL10 expression.

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**Key words:** Mesenchymal stem cells; Hepatocyte differentiation; Intravenous injection; Liver fibrosis; Interleukin-10

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## INTRODUCTION

Liver injury caused by chemical damage or viral infection often leads to liver fibrosis, which can lead to an impairment of liver function that requires medical intervention<sup>[1,2]</sup>. Although liver transplantation is by far the most effective treatment for liver cirrhosis, extensive clinical application of the technique is limited by the lack of donor organ availability<sup>[3]</sup>. Thus, it is very important to investigate different treatments and therapies for cirrhosis. Cell-based hepatocyte transplantation, a potential interventional procedure, provides an effective strategy and holds great promise for the treatment of impaired livers<sup>[4]</sup>. When compared to orthotopic liver transplantation, cell transplantation has the advantages of lower cost, lower risk, and a simpler procedure<sup>[5]</sup>. A single donor could serve multiple recipients, and excess cells could be cryopreserved for future use. However, the type of hepatocyte necessary for transplantation is less common in mature liver cells because of the paucity of cadaveric livers. Furthermore, with hepatocytes cultured *in vitro*, it is difficult to obtain mature and intact hepatocytes<sup>[6]</sup>. Thus, it is very important to investigate alternative, appropriate therapies for liver cirrhosis.

Mesenchymal stem cells (MSCs) are adherent, fibroblast-like, pluripotent and non-hematopoietic progenitor cells. They have been found to reside in most organs and tissues investigated to date, including bone marrow, adipose, dermis, muscular tissue, hair follicles, the periodontal ligament and the placenta. Previous studies<sup>[7,8]</sup> have demonstrated that MSCs can be differentiated into osteogenic, chondrogenic, adipogenic, myogenic, cardiomyogenic, and hematopoietic potential stromal cells. Moreover, apart from their differentiative abilities, MSCs play a supportive role in organ regeneration processes. In addition, several studies<sup>[9]</sup> have suggested that the use of MSCs *in vivo* should be safer than that of embryonic stem cells due to their higher chromosomal stability and lower tendency to form neoplasms in the recipient host. These reports indicate that MSCs are an attractive cell source for regenerative medicine.

A large number of *in vitro* studies indicate that bone marrow-derived MSCs can express the liver-specific marker  $\alpha$ -fetoprotein (AFP), cytokeratin 18 (CK18), and albumin. In addition, they can be involved in urea production, show liver-specific functions of cytochrome P450 activity, and store glycogen when co-cultured with adult liver cells or cultured in the presence of cytokines, such as fibroblast growth factor and hepatocyte growth factor<sup>[10,11]</sup>. The functional hepatocyte-like cells derived from MSCs might serve as cell sources for liver-targeted cell therapy<sup>[12]</sup>.

Hematopoietic stem cells (HSCs), another type of bone marrow-derived stem cell, also have multi-potent differentiation capabilities. The transplantation of HSCs can act as a substitute for hepatocyte transplantation in a murine model of tyrosinemia, and HSC transplantation can correct this metabolic liver disease<sup>[13,14]</sup>. However, the fusion process of hematopoietic stem cells with hepatocytes and the difficulty in maintaining hematopoi-

etic stem cells hamper their wide application to human disease treatment<sup>[15,16]</sup>. Sato *et al.*<sup>[17]</sup> examined the ability of fractionated human bone marrow-component MSCs to differentiate into hepatocytes *in vivo* by directly inoculating the cells into rat livers that had sustained chronic damage from alcohol treatment. Their results indicated that MSCs had a great ability to differentiate into hepatocytes without any evidence of fusion.

Besides treating acutely damaged tissue, MSCs also have the potential to reduce chronic fibrogenesis through the modulation of inflammation, collagen deposition, and remodeling. Although numerous studies have reported that bone marrow (BM)-derived MSCs can reduce carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis in mice, the mechanism by which MSCs repair the fibrosis is unclear, and the results are controversial<sup>[18-23]</sup>. For therapeutic applications, it will be important to understand the potency and possible repair mechanisms of MSCs. In the present study, we aimed to find and compare the best therapeutic effects among three different protocols of MSC engraftment (intraperitoneal, intravenous and intrahepatic transplantation) to treat CCl<sub>4</sub>-induced liver injury, as well as to elucidate the mechanisms that explain the differences between the effects of the cell transplant site.

## MATERIALS AND METHODS

### Isolation and culture of MSCs

MSCs were prepared from rat bone marrow as described previously. In brief, whole BM was flushed from the tibia and femur of Sprague Dawley (SD) rats (six-week-old males); cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 2 mmol/L L-glutamine; and purified for up to five passages. MSCs were grown to confluency before being detached by trypsin/ethylenediaminetetraacetic acid treatment. After detachment, cells were incubated with four phycoerythrin-conjugated antibodies: CD34, a hematopoietic progenitor marker; CD45, a leukocyte marker; CD90, which is also known as Thy-1; and/or CD29. Fluorescence-activated cell sorting was performed on at least 10 000 cells/sample using Cell Quest software (Beckman Coulter).

### Hepatocyte differentiation

Hepatic transplantation was performed as previously described<sup>[12]</sup>. Briefly, the cultured cells were harvested from the culture bottles with 0.25 g/L trypsin. Cultured cells at passage 3 were seeded in six-well cell culture plates. When the cells grew to 70% confluence, the control group was continuously cultured in DMEM supplemented with 10 mL/L fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. The hepatocyte differentiation group was cultured in  $\alpha$ -MEM supplemented with 10 mL/L FBS, 20 ng/mL hepatocyte growth factor (HGF), 20 ng/mL fibroblast growth factor (FGF)-4, 20 ng/mL epidermal growth factor (EGF), 100 U/mL penicillin and 100 U/mL streptomycin. In

Table 1 Gene primers used for detection

Gene	Sense	Anti-sense
<i>afp</i>	AACAGCAGAGTGCTGC- AAAC	AGTTTCGTCCCTCAGAAAG
<i>albumin</i>	ATACACCCAGAAAGCA- CCTC	CACGAATTGTGCGAATG
<i>ck18</i>	GGACCTCAGCAAGATC- ATGGC	CCACGATCTACGGGTAGT- TG
<i>mmp2</i>	CTATTCIGTCAGCACTT- TGG	CAGACTTTGGTCTCCAACCT
<i>mmp9</i>	AAATGTGGGTGTACAC- AGGC	TTCACCCGGTTGTGGAAACT
<i>timp1</i>	ATATICTGTCTGGATCG- GC	GCTTCGCACTCCTGTTT
<i>hgf</i>	TGGTGTTCACAAGCAA- TCCAGA	CCGTTCAGGTCATGCATTC
<i>pdgf</i>	GGCCTTCTTAAAGATTG- GTTCT	GCCTCATAGACCGCACCAAC
<i>il1β</i>	GCTGTGGCAGTACTCTA- TGICTTG	AGGTCGTCATCATCCACG- AG
<i>il2</i>	GACCGTGGAAATTCAT- CAGCA	GTCATCATCGAATGGCAC- TC
<i>il6</i>	CCACTTCACAAGTCGGA- GGCTTA	GTGCATCATCGCTTCATA- CAATC
<i>il10</i>	CAGACCCACATGCTCCG- AGA	CAAGGCTTGGAACCCAAG- TA
<i>il13</i>	AGGAGCTTATTGAGGAG- CTGAAGCA	TGGAGATGTTGGTCAGGGA- ATCCA
<i>infγ</i>	AGGCCATCAGCAACAA- CATAAGTG	GACAGCTTTGTGCTGGATCT- GTG
<i>tnfα</i>	AACTCGAGTGACAAGC- CCGTAG	GTACCACCACTGGTTGTCT- TTGA
<i>tgfb1</i>	TGCGCCTGCAGAGATT- AAG	AGGTAACGCCAGGAATTGT- TGCTA

each well, 2 mL of medium was added and changed every 4 d. The medium was stored at -20 °C until the albumin, AFP and urea assays were conducted. To determine the cell phenotype, the cultured cells were stained by anti-AFP and albumin (ALB) protein monoclonal antibodies according to the manufacturer's protocol<sup>[17]</sup>.

### CCl<sub>4</sub>-induced rat liver injury model

To establish the liver-damaged rat model, 0.5 mL/kg CCl<sub>4</sub> was injected subcutaneously into adult male SD rats (320 ± 20 g) twice a week for 4 wk. Control ("normal") animals were injected with the same volume of normal saline. The extent of hepatic damage was evaluated by biochemical analysis of blood samples and histopathological examination of liver tissue samples taken from sacrificed rats.

### Cell transplantation

For *in vivo* tracking of transplanted cells, MSCs from the SD rats were labeled with 4,6-diamidino-2-phenylindole (DAPI). Forty-five rats that suffered liver injury induced by CCl<sub>4</sub> were classified into three groups: intraperitoneal transplantation, intravenous transplantation, and intrahepatic transplantation. Each group's hepatocyte differentiated-MSCs were resuspended in 0.1 mol/L phosphate buffer solution (PBS) at a concentration of 10<sup>7</sup> cells/mL, and every rat was injected with 300 μL. Rats were sacrificed

at 28 d post-implantation. At that time, liver tissues and blood were obtained for analysis.

### Reverse transcription polymerase chain reaction and real-time polymerase chain reaction analysis

Total RNA was prepared using the RNeasy total RNA isolation kit (Invitrogen, United States). For cDNA synthesis, random hexamer primers (Invitrogen, United States) were used to prime reverse transcriptase reactions. The cDNA synthesis was carried out using Moloney-murine leukemia virus Superscript II reverse transcriptase (TaKaRa, Japan) following the manufacturer's instructions. For the semiquantitative polymerase chain reaction (PCR) detection of AFP, ALB, and CK18, 5 μL of cDNA-template was mixed with 2.5 μL of 10× PCR buffer, 0.5 μL of 10 mmol/L dNTPs, 0.5 μL of each primer (50 ng/μL), and 0.5 μL of polymerase (TaKaRa, Japan) in a total volume of 25 μL for each probe. PCR was performed in a programmable Biometra Uno-Thermobloc (Biometra, Germany). All samples were analyzed on 1% agarose gels. The size of the PCR fragments was estimated using a 100-base-pair ladder.

At three days post-MSC transplantation, quantitative PCR was carried out using standard protocols with the Quantitec SYBR Green PCR Kit (TaKaRa, Japan). The PCR mix contained SYBR Green Mix, 0.5 μmol/L primers (Table 1), 1 ng of DNA template and nuclease-free water to a final volume of 25 μL. PCR was performed in an ABI Prism 7000 Detection System (Applied Biosystems, United States). The percentage of gene expression was normalized as a function of *GAPDH* gene expression. Oligonucleotide primers for real-time PCR were obtained from TaKaRa (Japan), including matrix metalloproteinases (*mmp*2, *mmp*9), tissue inhibitor of metalloproteinase (*timp*1), *hgf*, *pdgf*, *il1β*, *il2*, *il6*, *il10*, *il13*, *infγ*, *tnfα* and *tgfb1*. Primer sequences are shown in Table 1.

### Biochemical analysis

Biochemical parameters including albumin, total bilirubin in serum (TBIL), and alanine aminotransferase (ALT) were analyzed with a biochemical analyzer (Roche Integra 800, Holliston, United States).

### Histopathological analysis and immunohistochemistry

At the time of sacrifice, liver tissue samples were collected and fixed in 3.7% formaldehyde for two days. Tissues were then dehydrated, cleared, and infiltrated with a histoprocessor for 16 h. Serial 3-μm sections were hematoxylin and eosin (HE) and Van Gieson's (VG) stained for histopathological analysis. For HE analysis, sectioned samples were stained with hematoxylin solution (Sigma-Aldrich, Germany) for 5 min followed by eosin for 5 min. For the VG stain, the sectioned samples were placed in hematoxylin solution for 5 min following a 10-min water wash, stained in VG (Biyuntian, China) solution for 3 min, and dehydrated in succession with 85%, 95% and 100% ethanol for 3 min.

Immunohistochemical staining was performed according to a previously reported method to evaluate

certain markers including alpha-smooth muscle actin ( $\alpha$ -SMA). After paraffin removal and microwave-based antigen retrieval, the sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS to quench endogenous peroxidase activity and then incubated with 5% goat serum to block the non-specific sites.  $\alpha$ -SMA (1:100) mAb was applied and incubated at 4 °C overnight (PBS was used as the negative control), followed by incubation with peroxidase-conjugated AffiniPure goat anti-mouse secondary antibody (Zhongshan Goldbridge, China) at 37 °C for 30 min. The specimens were then incubated with diaminobenzidine peroxidase substrate to obtain a brown stain and then subsequently counterstained with hematoxylin.

### Enzyme linked immunosorbent assay

Blood of the MSC- and CCl<sub>4</sub>-treated rats from the three different injection groups was collected at days 3, 7 and 14 post-transplantation. Serum samples were assayed for IL10 production with an IL10 enzyme linked immunosorbent assay (ELISA) Quantitation kit (Invitrogen, United States) according to the manufacturer's recommendation.

### Data analysis

Data are shown as the means and standard deviations. The statistical differences were analyzed using Student's *t* test for normally distributed values and by the *t* test for non-normally distributed values. Values of *P* < 0.05 were considered significant. The data represent mean  $\pm$  SD from at least three independent experiments.

## RESULTS

### Characterization of stem cell cultures

The cells were isolated by gradient density centrifugation. After approximately 3 d in culture, cells from the stromal fraction appeared as a monolayer of broad, flat cells (Figure 1A). As the cells approached 80% confluence, they differentiated into a more spindle-shaped, fibroblastic morphology (Figure 2B). More than 98% of cells expressed the MSC markers CD29 and CD90 (Figure 1C and D), and less than 5% of hematopoietic stem cells expressed leukocyte markers CD34 and CD45 (Figure 1E and F). They were easily expanded for up to 10 passages, maintaining the spindle-shaped, fibroblastic morphology and MSC markers of the undifferentiated state. Adipose-derived stem cells (ADSCs) did not spontaneously differentiate during *in vitro* culture.

### Differentiation to hepatocytes

When the cultured cells reached 70% confluence, they were treated with hepatocyte differentiation medium containing 20 ng/mL HGF, 20 ng/mL FGF-4, and 20 ng/mL EGF. After 4 d of hepatocyte induction, ADSCs displayed changes in cellular morphology including shrinkage of the cytoplasm and diameter, as well as the formation of round cells. The fibroblasts, as control cells, did not exhibit any change in morphology after being treated with differentiation medium. Before induction, the MSCs did not express hepatocyte markers. Four

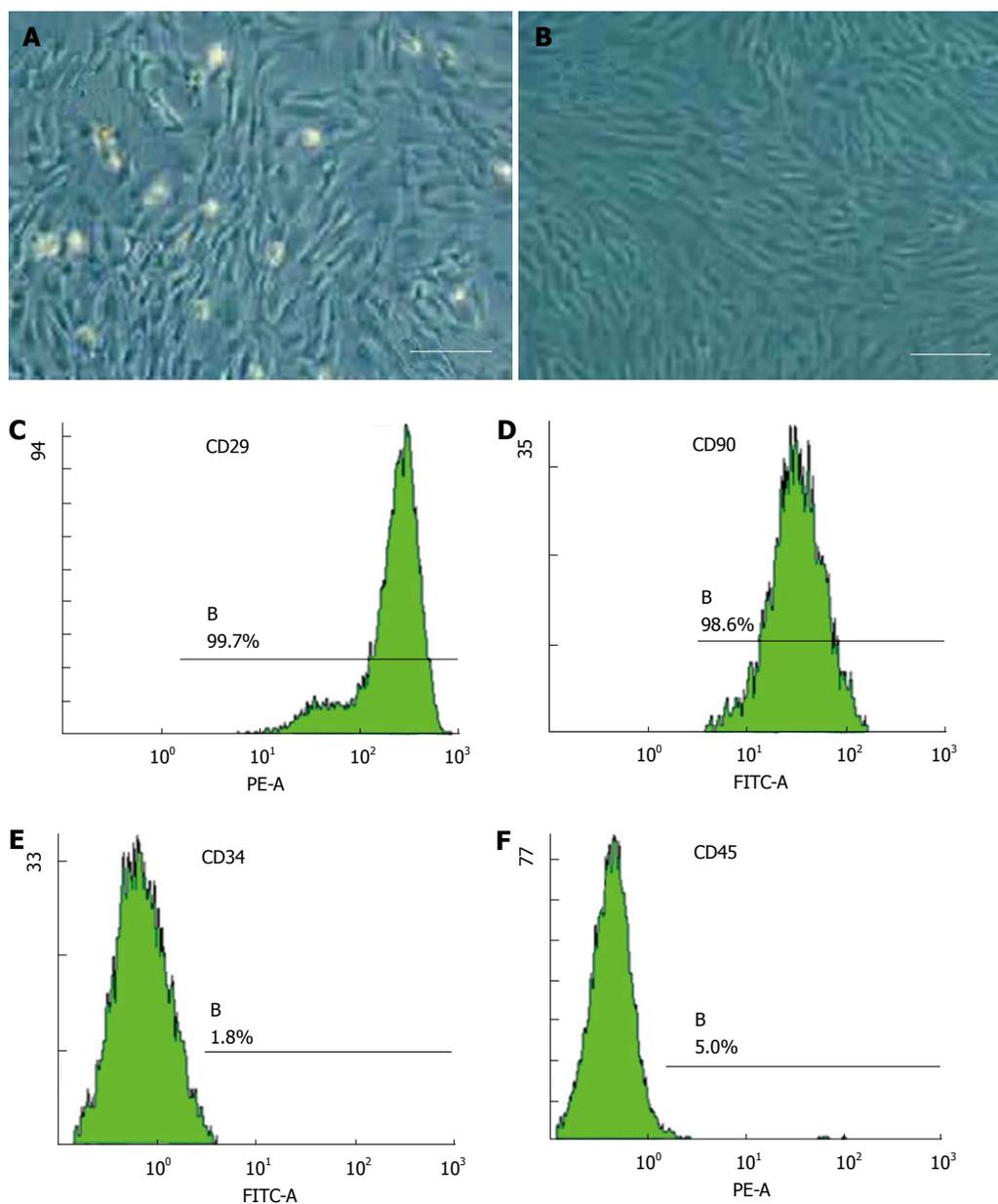
days after exposure to differentiation medium, the MSCs stained positive for the AFP and ALB hepatocyte markers (Figure 2A and B). Hepatocyte marker expression after hepatocyte induction was further confirmed by RT-PCR. In addition, *AFP*, *ALB* and *CK18* gene expression levels increased with prolonged exposure time in differentiation medium (Figure 2C). From these results, we can conclude that the MSCs differentiated into hepatocytes, and the proportion of differentiated cells account for approximately 70% of all induced cells.

### Intravenous injection of MSCs provides better treatment than other injection modalities

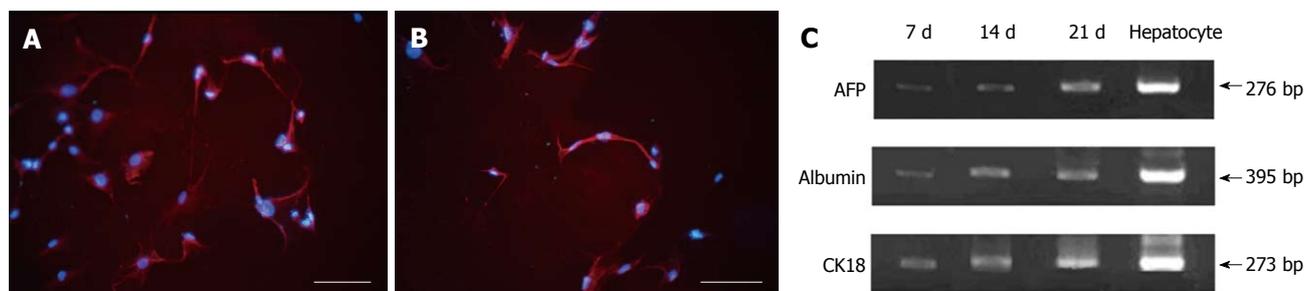
To explore cell homing, MSCs were labeled with DAPI before transplantation. Four days after transplantation, the number of surviving homing MSCs was highest in animals that received an intravenous injection. Surviving homing MSCs in animals that received an intrahepatic injection were more numerous than those observed in animals that received an intraperitoneal injection (Figure 3A-D). In addition, histological staining indicated that sham-injected, CCl<sub>4</sub>-treated rats suffered serious inflammation with non-normal liver lobules dispersed throughout the liver. The sham group also had large areas of collagen that were distributed along the edge of the liver lobules (Figure 3H and L). In the intraperitoneal and intrahepatic injection groups, the morphology of liver lobules was unclear, and collagen deposition was evident in the liver (Figure 3F, G, J and K). In the intravenous injection group, however, the liver lobules were normal (Figure 3E and I). Although there was mild inflammation in the portal or sinusoid areas, collagen accumulation was minimal in the liver. Immunohistochemistry of liver sections for  $\alpha$ -SMA expression revealed intense staining patterns in sham- and intraperitoneal-injected mice (Figure 3O and P). However,  $\alpha$ -SMA staining levels in intravenous- and intrahepatic-injected mice were significantly lower (Figure 3M and N). In addition, there were lower levels of  $\alpha$ -SMA staining in intravenous-injected mice than in intrahepatic-injected mice. To further compare the functional restoration of MSCs following intraperitoneal, intravenous, and intrahepatic injection, we quantitatively analyzed the levels of AFP, albumin, and TBIL production. The serum from rats given CCl<sub>4</sub> but not injected with MSCs, and from rats given neither MSCs nor CCl<sub>4</sub>, was used as control samples. Twenty-eight days post-injection, intravenously-injected MSCs were close to the level observed in normal rats. However, intraperitoneally-injected MSCs had not enough effective treatment when compared with MSCs intravenous injection group. The observed levels in the intrahepatic injection group fell between the other MSC-treated groups, but the levels were still closer to the levels observed in the intravenous injection group (Figure 4).

### Comparison of cytokine expression following intraperitoneal, intravenous and intrahepatic MSC treatment

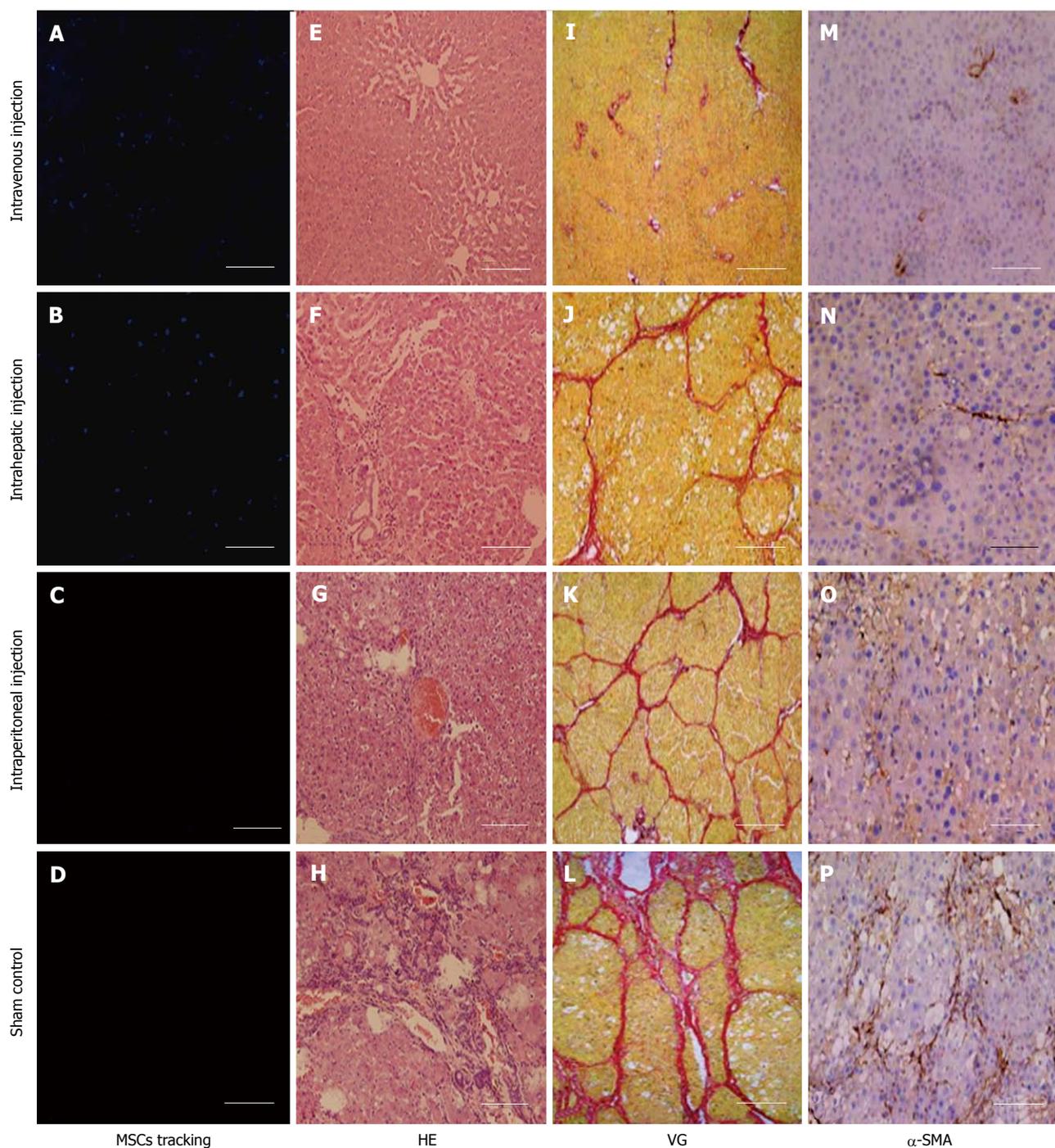
To further explore why intravenously-injected MSC treatment was more effective than either intraperitoneal or



**Figure 1 Mesenchymal stem cell culture and identification.** A: The morphology of mesenchymal stem cell (MSC) culture at 3 d (10 × 10 magnification); B: The morphology of MSCs approached 80% confluence (10 × 10 magnification); C: Fluorescence-activated cell sorting (FACS) analysis for MSC positive cell marker CD29; D: FACS analysis for MSC positive cell marker CD90; E: FACS analysis for MSC negative cell marker CD34; F: FACS analysis for MSC negative cell marker CD 45. Scale bars represent 100 μm. FITC: Fluorescein isothiocyanate; PE: Phycoerythrin.



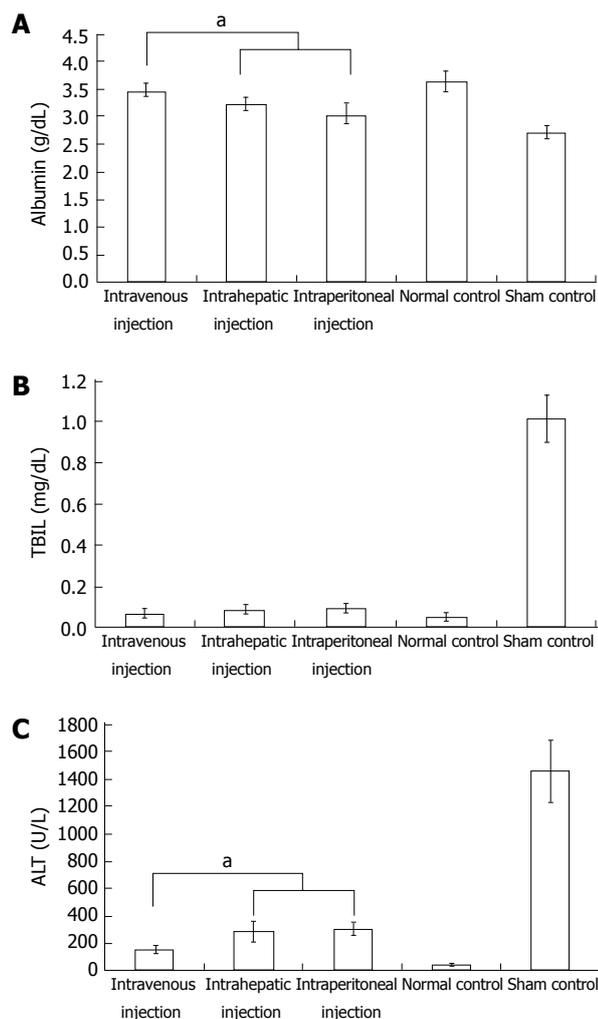
**Figure 2 Identification of mesenchymal stem cell transdifferentiation into hepatocytes.** Hepatocytes were used as positive control. A: Fluorescence immunostaining detection of α-fetoprotein (AFP) expression after 21 d induction in mesenchymal stem cells (MSCs) (10 × 40 magnification); B: Fluorescence immunostaining detection of albumin expression after 21 d induction in MSCs (10 × 40 magnification); C: Reverse transcription polymerase chain reaction detection of hepatocyte specific protein AFP, albumin and cytokeratin 18 (CK18) mRNA expression after 7, 14 and 21 d induction in MSCs. Scale bars represents 100 μm.



**Figure 3 Comparison of liver treatment efficacy in different mesenchymal stem cell transplanted modalities.** All figures are 10 × 10 magnification. Scale bars represent 100 μm. A, B, C and D: Fluorescence tracked 4',6-diamidino-2-phenylindole (DAPI)-labeled mesenchymal stem cells (MSCs) in liver. Blue dots are DAPI-labeled MSCs; A: MSC intravenous injection, and many DAPI-labeled cells can also be seen distributed in liver; B: MSC intrahepatic injection, and many DAPI-labeled cells can also be seen distributed in liver; C: MSC intraperitoneal injection, and few labeled cells can be seen in liver; D: MSC non-treated liver fibrosis; E, F, G and H: Hematoxylin and eosin (HE) analysis for detecting liver extracellular matrix (ECM) arrangement in liver fibrosis; E: MSC intravenous injection, and liver ECM arrangement was similar to normal liver; F and G: MSC intrahepatic and intraperitoneal injection, and ECM arrangement was disordered in both groups; H: MSC non-treated liver fibrosis; I, J, K and L: Van Gieson's (VG) staining analysis for detecting collagen in liver fibrosis; I: MSC intravenous injection, and little positive staining was detected; J and K: MSC intrahepatic and intraperitoneal injection, and a large number of collagen was deposited in liver lobules; L: MSC non-treated liver fibrosis; M, N, O and P: Immunohistochemical analysis for detecting the expression of the marker of myofibroblasts, alpha-smooth muscle actin ( $\alpha$ -SMA); M: MSC intravenous injection, and there was no immunoreactivity in this group; N and O: MSC intrahepatic and intraperitoneal injection, and a large amount of positive staining can be seen in both groups; P: MSC non-treated liver fibrosis.

intrahepatic injection, real-time PCR was used to quantitatively analyze the expression difference among the 3 transplant methods. As seen in Figure 5A and B, *mmp2*

and *mmp9* were expressed at their highest levels in the intravenous injection group, whereas *timp1* expression was the lowest in this group. The intraperitoneal injection



**Figure 4** Quantitative analysis for hepatocyte specific protein expression by biochemical analysis to compare the liver fibrosis treatment efficacy of intravenous, intrahepatic and intraperitoneal mesenchymal stem cell injection. Non-treated carbon tetrachloride (CCl<sub>4</sub>) group acted as normal control. Rats not injected with mesenchymal stem cells (MSCs) after CCl<sub>4</sub> processing were used as sham control. Data represent the mean ± SE, and <sup>a</sup>P < 0.05. A: Analysis for albumin expression; B: Analysis for total bilirubin in serum (TBIL) expression; C: Analysis for alanine aminotransferase (ALT) expression.

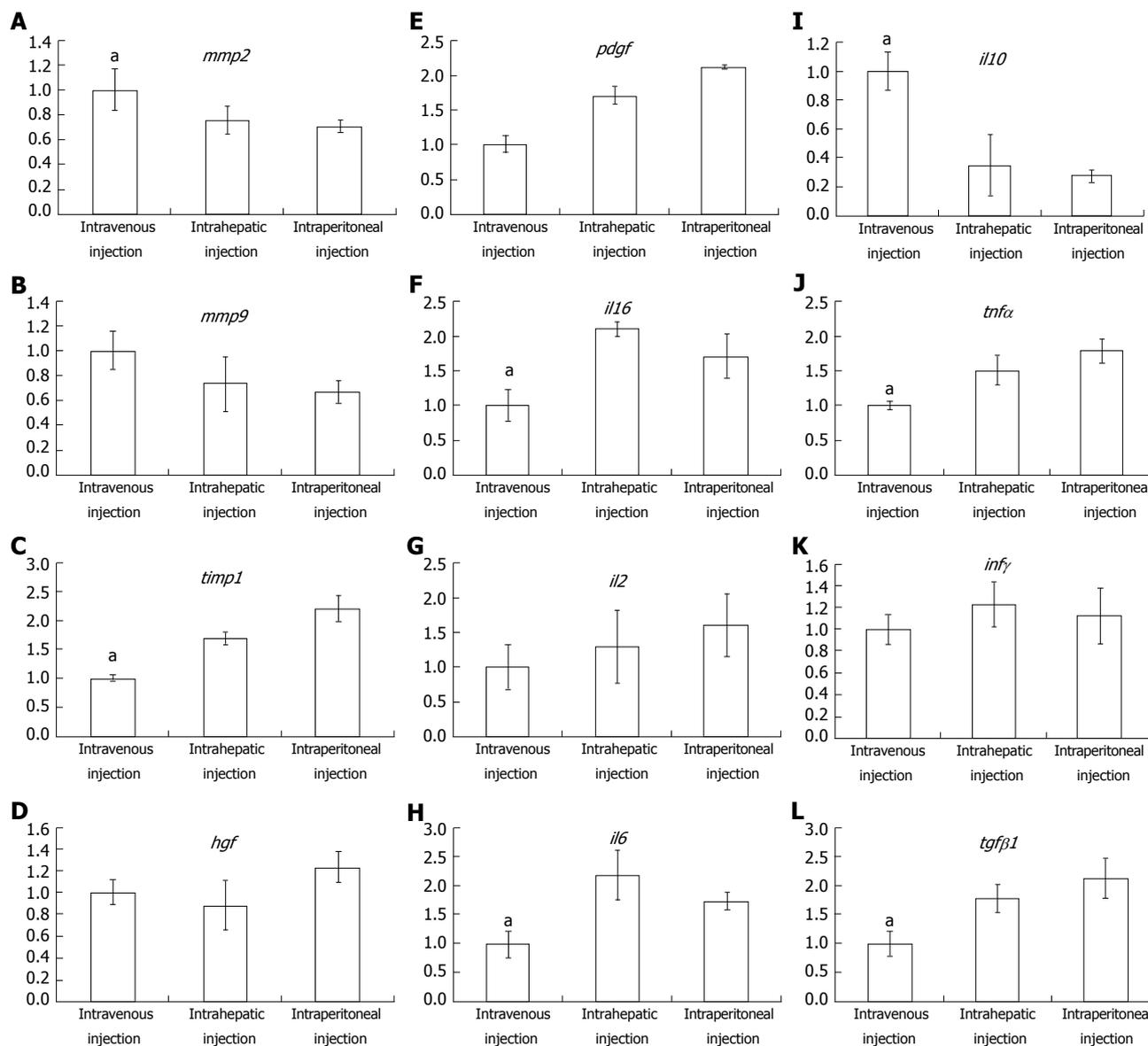
tion group, in contrast to the intravenous injection group, had the lowest *mmp2* and *mmp9* expression and the highest *timp1* expression. These results were in accordance with the observation that the fibrosis observed in the intravenous injection group was low relative to the two other groups. Besides assaying *mmp* and *timp* expression, cytokine expression was also assayed to detect whether the immune response played a pivotal role in the differential treatment response among the three experimental groups. The expression of *hgf* was slightly higher in the intravenous injection group than in the other two groups (Figure 5D). platelet-derived growth factor (PDGF), an important fibrosis enhancer, was not significantly different among the three groups. Similarly, expression levels of *pdgf*, *infγ* and *il2* were not significantly different among the three groups (Figure 5E, K and G). In the intravenous injection group, however, the expression levels of

*il1β*, *il6*, *tnfα* and *tgfβ* were significantly lower than in other groups, and the intraperitoneal injection group had the highest expression of these genes (Figure 5F, H, J and L). Interestingly, in the intravenous injection group, *il10*, a key factor in the regulation of the Th1-mediated immune response and homeostasis between matrix metalloproteinase (MMP) and the tissue inhibitors of the MMP (TIMP), was significantly higher when compared to the intrahepatic and intraperitoneal injection groups. Furthermore, the blood ELISA assay also indicated that IL10 production in the intravenous injection group was higher than in the other two groups (Figure 6).

## DISCUSSION

Liver fibrosis results from chronic injury to the liver in conjunction with excessive deposition of collagen and other components of the extracellular matrix (ECM), which is a characteristic of most types of chronic liver diseases<sup>[1]</sup>. If not effectively treated in time, liver fibrosis may transform into liver cirrhosis. Cirrhosis causes a number of complicating diseases, such as portal hypertension, hepatic encephalopathy, and gastrointestinal bleeding<sup>[2]</sup>. Thus, the discovery of a new approach to treating this disease would provide great clinical value. CCl<sub>4</sub>-induced hepatocyte necrosis has the ability to imitate human liver fibrosis disease in animals and has so far been a main method used in the development of animal treatment models<sup>[2,3]</sup>. In this study, CCl<sub>4</sub> was used to induce liver fibrosis in SD rats according to previous protocols. Histological staining indicated collagen deposition in the liver, an indicator of fibrosis. Further liver function testing revealed that ALB, TBIL and ALT levels changed from their normal, baseline levels of 3.65 ± 0.18, 0.049 ± 0.019, and 48.70 ± 9.86 to 3.02 ± 0.22, 1.018 ± 0.114, and 1458.78 ± 230.07, respectively. These findings, in accordance with previous reports, all indicate that liver fibrosis was induced by CCl<sub>4</sub>.

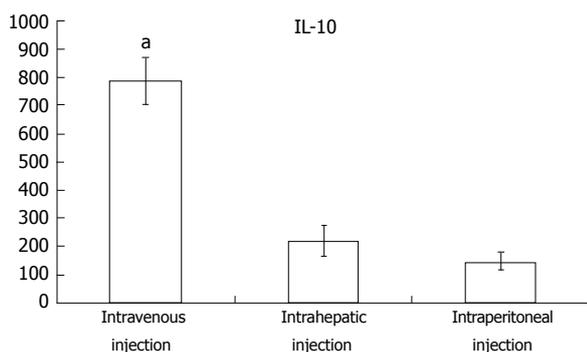
Liver transplantation is currently considered the best treatment for liver cirrhosis caused by fibrosis. However, there are some disadvantages that limit the expansive use of the procedure, such as liver shortages, high risks associated with surgery, and the risk of further post-surgery complications<sup>[3]</sup>. Although hepatocyte therapy has been proven to improve liver function, immune rejection and hepatocyte disorder in *in vitro* cultures have provided obstacles to the expansive use of this type of therapy. MSCs, in recent years, have shown potential as a post-transplantation fibrosis cure<sup>[24,25]</sup>. MSCs derived from bone marrow also contain HSCs and multipotent adult progenitor cells. Thus, fluorescence-activated cell sorting in this study was performed to identify the type of cultured cells obtained. More than 98% of cells stained positive for CD29 and CD90, which is in accordance with the diagnostic characteristics of MSCs. The cells stained negative for CD34 and CD45, which are indicative of hematopoietic cell lines. These results demonstrate that the cultured cells derived from the rat



**Figure 5** Real-time polymerase chain reaction analysis for detecting the expression difference of cytokines, growth factors and interleukin, which are involved in liver fibrosis progression, among different mesenchymal stem cell transplanted modalities. The histogram shows the relative expression level for intravenous, intrahepatic and intraperitoneal injections. Data represent the mean  $\pm$  SE, <sup>a</sup>*P* < 0.05. A and I: The expression of *mmp2* and *il10* in intravenous injection was significantly higher than other injection modalities; F, J, C, H and L: The expression of *il1β*, *tnfα*, *timp1*, *il6* and *tgfβ1* was significantly lower in intravenous injection than others; E, B, G, K and D: The expression of *pdgf*, *mmp9*, *il2*, *hgf*, *infγ* and *hgf* was not significantly different among the three mesenchymal stem cell injection modalities.

bone marrow consisted of more than 98% MSCs. HSCs that produce all of the blood lineages and liver epithelium have been previously shown to be capable of acting as a substitute for hepatocytes in transplantation in a murine model of tyrosinemia<sup>[26,27]</sup>. The benefits to the regenerating liver in the transplant recipients are derived from donor HSCs that fuse with host hepatocytes, rather than from transdifferentiating hematopoietic stem cells or hepatic stem cells present in bone marrow<sup>[15,16]</sup>. In a study by Sato *et al.*<sup>[17]</sup>, unlike HSCs, MSCs grafted directly to livers that had sustained chronic damage from allyl alcohol treatment were more successful than MSC+ and non-MSC/CD34- cells in differentiating into hepatocytes without any evidence of fusion. *In vitro*, multiple studies, including those of our group, indicate that MSCs can also

differentiate into hepatocytes when induced by cytokines, such as HGF, FGF-4 and EGF<sup>[28]</sup>. To assay the degree of hepatocyte differentiation of MSCs, real-time PCR and immunofluorescence staining were performed. High expression levels of AFP, ALB and CK18 were noted in induced MSCs when compared to non-induced MSCs. While some studies have examined only one marker, or a small number of them, most published data refer to multiple markers, of which expression is assessed at both the protein and mRNA levels. One of the most widely used markers is albumin secretion, together with the evaluation of AFP, metabolic enzymes, and cytoskeletal proteins<sup>[19]</sup>. AFP in the liver is a marker of immature liver cells or oval cells in adult livers. Albumin is a typical marker of mature hepatocytes. CK18 is expressed by several liver



**Figure 6** Enzyme linked immunosorbent assay analysis for detecting interleukin-10 protein expression in peripheral blood after intravenous, intrahepatic and intraperitoneal mesenchymal stem cell injection. Values shown are mean  $\pm$  SE,  $^aP < 0.05$ . IL-10: Interleukin-10.

cell types, including biliary epithelial cells and hepatic oval cells. In particular, a “cytokeratin switch” can be observed as a later process in the maturation of hepatocytes from bipotential progenitors. In fact, bipotential hepatoblasts express both CK-18 and CK-19, while mature hepatocytes express CK-18 alone, and CK-19 specifically identifies cholangiocyte populations<sup>[17]</sup>.

While MSCs are not the only reported adult stem cells that can treat liver fibrosis, they have advantages that make them more favorable for their use in clinical treatment, such as easy accessibility, minimal invasiveness, and fast proliferation<sup>[12]</sup>. In this study, we injected MSCs isolated from bone marrow into SD rats at three different body regions. We found that the liver function of the three groups was elevated compared to the control group. These observations were made by the biochemical analysis of ALB, TBIL and ALT and histological staining of collagen deposition. Although there was an elevation of liver function in all of the experimental groups, there were also differences among the groups. The expression of  $\alpha$ -SMA, which plays a pivotal role in liver fibrosis, was also significantly reduced after MSC transplantation. These results are similar to those seen in many previous reports and suggest that, apart from expressing specific markers, differentiated cells are capable of carrying out the functional activities of mature hepatocytes, which are involved in the supportive functions needed for regenerative medicine applications. These enzymatic functions should also be considered as more reliable “markers” of the successful differentiation of MSCs.

So far, there are two primary methods, non-induced and hepatocyte-induced MSC injection, for the treatment of livers with MSCs. Although these two methods have a potent ability to reverse liver fibrosis, the mechanism of treatment for non-induced MSCs is still unknown. There may be two factors by which MSCs protect against liver fibrosis. One is that MSCs which differentiate into hepatocytes, because of the *in vivo* niche, secrete numerous growth factors that promote liver regeneration<sup>[29,30]</sup>. Another possibility is that MSCs suppress hepatic stellate cell activity and secrete MMP, thereby eliminating ECM deposition<sup>[31]</sup>. In contrast to the primary rationale for us-

ing non-induced MSCs to treat liver fibrosis, some studies have demonstrated that MSCs injected into rats with cirrhotic livers differentiate mainly into myofibroblasts and hepatic stellate cells, both of which are promoters of liver fibrosis<sup>[32,33]</sup>. In the present study, to avoid liver fibrosis aggravation, transplanted MSCs were induced to differentiate into hepatocytes. The results of this method appear to promote improved liver regeneration, rather than liver cirrhosis aggravation, in CCl<sub>4</sub>-induced fibrotic rats.

In previous reports, Kuo *et al.*<sup>[12]</sup> demonstrated that intravenous MSC-derived hepatocyte transplantation was more effective in rescuing liver failure than intrasplenic transplantation. In this paper, we extended the effective comparison of MSC-engrafted sites, including intravenous, intraperitoneal, and intrahepatic transplantation. In agreement with the results of Kuo *et al.*<sup>[12]</sup>, intravenous transplantation in our experiments was better at rescuing liver fibrosis than transplantation *via* the other two methods. The effectiveness of intraperitoneal transplantation was better in the non-MSC-treated groups than in the other treated groups. The difference among the three groups was stratified by not only liver biochemical functional differences and collagen deposition but also by the distribution of MSC-derived hepatocytes in the recipient liver. Although the results of the present study and those of the Kuo group study are valuable for guiding MSC therapy for liver disease, they do not fully elucidate the causes of these transplant-site effectiveness differences.

Thus, we assayed for protein and mRNA expression differences of cytokines and interleukins that play an important role in fibrosis progression. HGF, a powerful mitotic promoter for hepatocytes, also has the ability to restrain collagen and transforming growth factor (TGF) $\beta$ 1 expression, thereby suppressing hepatic stellate cell activity<sup>[34,35]</sup>. PDGF can enhance liver fibrosis *via* stimulation of hepatic stellate cells expressing collagen and TIMP<sup>[36]</sup>. In this study, the mRNA expression of these two factors, however, was not significantly different among the three groups. Thus, they are not the factors leading to the observed difference caused by injection in different MSC transplant sites. Similarly, elevations of *hgf*, *pdgf*, *infy* and *il2* were also detected, but the differences did not reach statistical significance. *il1 $\beta$* , *il6*, *tnf $\alpha$* , and *tgf $\beta$*  were expressed at significantly lower levels after intravenous injection than after intraperitoneal or intrahepatic injection. The expression of *il10*, however, was highest in the intravenous injection group. These results demonstrate that IL10 expression may play a central role in mediating the superior effects of intravenously injected MSCs in ameliorating liver fibrosis. IL10 is an inhibitor of many cytokines that stimulate tissue fibrosis, such as IL6, tumor necrosis factor- $\alpha$  and TGF $\beta$ . In addition, IL10 can suppress TIMP-1 expression and thereby relieve MMP-1 to degrade liver collagen deposits<sup>[37,38]</sup>. The increased expression of *timp1* following intravenous injection is in contrast to the expression following other injection modalities, which supports such a viewpoint (Figure 5C). According to previous reports, an intravenous injection of MSCs can beneficially modulate

the host immune response by increasing the release of prostaglandin E2 from the BM-derived MSCs acting on the EP2 and EP4 receptors of the macrophages and by stimulating the production and release of IL10<sup>[39]</sup>. Blood ELISA assays indicated that IL10 expression in the intravenous injection group was higher than that in other groups. These results support the viewpoint that the main advantage provided by MSCs transplanted *via* injection into the venous blood is the stimulation of IL10 release, thereby supporting this method as an effective treatment of liver fibrosis.

In summary, MSC-derived hepatocytes are able to protect against liver fibrosis induced by CCl<sub>4</sub>. Intravenous injection, in contrast to the intraperitoneal and intrahepatic injection of MSCs, provides the most effective treatment to prevent fibrosis. These results are due to the fact that MSCs in the venous blood can stimulate IL10 release, which, in turn, can modulate the host immune response and homeostasis between TIMP and MMP.

## COMMENTS

### Background

Liver fibrosis causes many deadly diseases. Previous reports indicate that mesenchymal stem cells (MSCs) can facilitate recovery from chemically-induced liver damage and decrease liver fibrosis.

### Research frontiers

In recent years, MSCs have been reported in many studies to promote liver fibrosis regeneration at many different transplanted sites. However, there are no critical comparisons of how different transplant sites influence bone marrow MSC-based therapy for liver fibrosis, and the molecular mechanisms that influence the treatment differences are unknown.

### Innovations and breakthroughs

This study compares the treatment effectiveness of MSCs for intravenous, intrahepatic and intraperitoneal injection. The results suggest that intravenous injection, in contrast to intraperitoneal and intrahepatic MSC injection, provides the most effective treatment to prevent fibrosis. These results are due to the fact that MSCs in the venous blood can stimulate interleukin-10 (IL-10) release, which, in turn, can modulate the host immune response and homeostasis between tissue inhibitor of metalloproteinase and matrix metalloproteinases.

### Applications

This comparative study of treatment effectiveness influenced by transplanted sites supports the therapeutic principle of future MSC treatment.

### Terminology

Liver injury caused by chemical damage or viral infection often leads to liver fibrosis, which can lead to an impairment of liver function that requires medical intervention. MSCs that have been found to reside in most organs and tissues can be differentiated into hepatocyte-like cells *in vitro*, and rescue liver fibrosis. The authors found MSCs intravenously injected into the body impeded liver fibrosis caused by carbon tetrachloride more than intraperitoneal and intrahepatic injection. IL-10, which is a regulator of host immune response and metabolism homeostasis, may play an important role in mediating this phenomenon.

### Peer review

The authors compared the liver fibrosis treatment effectiveness among different MSCs injection modalities. The study revealed that MSCs intravenously transplanted evidently improved the functional protein expression and reduced the collagen deposition in injured liver more than other injection modalities. And further mechanism detection suggested intravenous injection of MSCs releases more IL10 that can ameliorate liver fibrosis by down-regulation of *il1β*, *il6*, *tnfα*, and *tgfb* expression. The results may represent a molecular mechanism of MSC therapy in liver injury.

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## Rebamipide suppresses diclofenac-induced intestinal permeability *via* mitochondrial protection in mice

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### Abstract

**AIM:** To investigate the protective effect and mechanism of rebamipide on small intestinal permeability induced by diclofenac in mice.

**METHODS:** Diclofenac (2.5 mg/kg) was administered once daily for 3 d orally. A control group received the vehicle by gavage. Rebamipide (100 mg/kg, 200 mg/kg, 400 mg/kg) was administered intragastrically once a day for 3 d 4 h after diclofenac administration. Intestinal permeability was evaluated by Evans blue and the FITC-dextran method. The ultrastructure of the mucosal barrier was evaluated by transmission electron microscopy (TEM). Mitochondrial function including mitochondrial swelling, mitochondrial membrane potential, mitochondrial nicotinamide adenine dinucleotide-reduced (NADH) levels, succinate dehydrogenase (SDH) and ATPase activities were measured. Small intestinal mucosa was collected for assessment of malondialdehyde (MDA)

content and myeloperoxidase (MPO) activity.

**RESULTS:** Compared with the control group, intestinal permeability was significantly increased in the diclofenac group, which was accompanied by broken tight junctions, and significant increases in MDA content and MPO activity. Rebamipide significantly reduced intestinal permeability, improved inter-cellular tight junctions, and was associated with decreases in intestinal MDA content and MPO activity. At the mitochondrial level, rebamipide increased SDH and ATPase activities, NADH level and decreased mitochondrial swelling.

**CONCLUSION:** Increased intestinal permeability induced by diclofenac can be attenuated by rebamipide, which partially contributed to the protection of mitochondrial function.

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**Key words:** Intestinal mucosal permeability; Mitochondria; Non-steroid anti-inflammatory drugs; Oxidative damage; Rebamipide; Tight junction

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## INTRODUCTION

It is well-known that traditional non-steroid anti-inflammatory drugs (NSAIDs) induce mucosal injuries in the lower gastrointestinal (GI) resulting in serious damage<sup>[1-5]</sup>. However, the mechanism of NSAIDs-related small intestinal mucosal injury is not yet clear. Bjarnason *et al*<sup>[6]</sup> suggested a hypothesis for the mechanism involved in the pathogenesis of NSAIDs-related enteropathy which was thought to be a multi-stage process, and included mitochondrial damage, increased intestinal permeability, decreased blood flow, and a reduction in prostaglandins. Several basic studies have supported the idea of increased permeability of the small intestinal mucosa as a central mechanism. Intestinal permeability regulates penetration of substances such as macromolecules, bile acids, bacteria and other intra-lumen toxins through the intestinal epithelial barrier. This may lead to low-grade intestinal inflammation by exposing the mucosa to luminal factors and might be the driving force in converting inflammation into ulcer<sup>[7]</sup>. Therefore, increased intestinal permeability was usually seen as the early stage of intestinal mucosal damage induced by NSAIDs. Many gastrointestinal inflammatory diseases, such as inflammatory bowel disease, can be detected through intestinal permeability tests, which can be used as an early predictor of relapse<sup>[8-11]</sup>. However, there are no effective drugs for the treatment of increased intestinal permeability induced by NSAIDs. Although proton pump inhibitors and prostaglandin analogues have a preventive effect in patients with traditional NSAIDs-induced upper GI adverse events, investigations into the preventive effects of drugs on NSAIDs-induced lower GI mucosal injury are inadequate.

Rebamipide is a mucosal protective agent which exerts a protective effect on NSAIDs-induced gastric injury through its antioxidant properties<sup>[12]</sup>. It was demonstrated that *Helicobacter pylori*-related macromolecular transepithelial transportation was reduced by the administration of rebamipide<sup>[13-14]</sup>, which was attributed to barrier integrity reinforcement. Kishimoto *et al*<sup>[15]</sup> reported that rebamipide prevented dextran sulfate sodium-induced ulcerative colitis in rats, which was related to mucosal barrier repair. In addition, rebamipide was shown to have a healing effect in a patient with corticosteroid-resistant ulcerative colitis<sup>[16]</sup>. However, few studies have evaluated the mechanism of rebamipide on intestinal permeability in NSAIDs enteropathy.

The aim of this research was to investigate the preventive effect and mechanism of rebamipide on intestinal permeability in a diclofenac-induced enteropathy model.

## MATERIALS AND METHODS

### Animals and reagents

Kunming mice, 6-8 wk old weighing  $20 \pm 2$  g were provided by the Laboratory Animal Center of Anhui Medical University. The mice were housed in animal facilities with 50% humidity and a 12:12-h light-dark cycle and

fed a standard pellet diet and tap water ad libitum. All experiments were performed in accordance with the institutional and national guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Anhui Medical University. Rebamipide was purchased from Zhejiang Otsuka Pharmaceutical Corporation, Zhejiang, China. Diclofenac sodium salt, Evans blue, rhodamine 123 and fluorescein isothiocyanate dextran (FITC-D) was purchased from Sigma Co. Acetylcysteine was obtained from Beijing Solarbio Science and Technology Co., Ltd, Beijing, China. Malondialdehyde (MDA), myeloperoxidase (MPO), ATPase and succinate dehydrogenase (SDH) detection kits were bought from Nanjing Jiancheng Institute of Biotechnology, Nanjing, China.

### Experimental protocol

Mice were randomly divided into the following five groups; control, diclofenac, rebamipide 100 mg/kg, 200 mg/kg, and 400 mg/kg<sup>[15]</sup>. Mice were administered diclofenac (2.5 mg/kg)<sup>[17]</sup> dissolved in 0.2% methylcellulose daily by oral gavage for 3 d, except the control group which received the vehicle. Rebamipide suspended in 0.2% methylcellulose dissolved in saline was dosed intragastrically once a day for 3 d 4 h after diclofenac administration. In the control and diclofenac groups, saline was given orally instead of rebamipide. At the end of the experiment, mice were sacrificed by decapitation, and the small intestine was quickly removed.

### Intestinal permeability

Small intestinal permeability was evaluated as previously described<sup>[18]</sup>. Briefly, mice were anesthetized with ether, the abdomen was opened and a 3 cm proximal portion of the ileum from the ileocecal junction was ligated by silk suture with care to prevent injury to the superior mesenteric vessels. The ileal luminal contents were washed out gently with 4-5 mL of phosphate buffered saline (PBS). The ileocecal end was ligated to prepare the ileal loop (3 cm), and then 0.2 mL of 1.5% (w/v) Evans blue in PBS was injected into the loop. Mice were warmed with an incandescent lamp and left undisturbed for 60 min without any signs of pain. Sixty min later, mice were sacrificed by decapitation. The ileal loop was rapidly dissected out, opened, rinsed with 6 mmol/L acetylcysteine, dried on filter paper at 37 °C for 24 h, and then weighed and incubated with 3 mL of formamide at 50 °C for 24 h. The amount of dye eluted was estimated using a spectrophotometer at a wavelength of 612 nm<sup>[19]</sup>. The amount of Evans blue permeating into the intestinal wall was calculated based on the standard curve of Evans blue in formamide.

Intestinal permeability to FITC-D with a molecular mass of 4000 Da was determined using a method previously described by Chen *et al*<sup>[20]</sup> with a minor modification. Mice were anesthetized and a 5 cm segment of the ileal loop was prepared by ligating the ileum at the 3 cm and 8 cm proximal portions from the ileocecal junction

with care to prevent injury to the superior mesenteric vessels. Then 0.2 mL of PBS (pH 7.4) containing 25 mg/kg FITC-D was injected into the loop. After 30 min, a blood sample (100  $\mu$ L) was taken by puncture of the portal vein under ether anesthesia and immediately diluted with 1.9 mL of 50 mmol/L Tris (pH 10.3) containing 150 mmol/L sodium chloride. The diluted plasma was centrifuged at 3000 g for 7 min and plasma FITC-D concentrations were determined by a fluorescence spectrophotometer at an excitation wavelength of 485 nm and an emission wavelength of 515 nm<sup>[21-22]</sup>.

#### Assessment of transmission electron microscopy

For transmission electron microscopy (TEM) assessment, an ileal specimen of about 1 cm in length from the ileocecal junction to the proximal portion was excised with a sharp scalpel and fixed in 2.5% glutaraldehyde for 4 h at 4°C, followed by fixation in osmic acid and embedding in Epon. Ultrathin sections were examined by a Hitachi TEM to detect ultrastructural injuries.

#### Intestinal MDA content and MPO activity

The dissected intestine was removed without any fat and mesenteries attached, and subsequently homogenized in physiologic saline for the detection of MDA content and MPO activity using commercial kits following the instruction procedures.

#### Preparation of liver mitochondrial and determination of mitochondrial functions

Mouse liver mitochondria were isolated as described by Johnson and Lardy<sup>[23]</sup>, as it was exceedingly difficult to obtain a high yield of mitochondria from intestinal tissue<sup>[24]</sup>. Briefly, the liver was rapidly removed and placed in medium containing 250 mmol/L sucrose, 10 mmol/L Tris and 1 mmol/L EGTA (pH 7.8) at 4 °C. The tissue was scissor minced and homogenized on ice. The homogenates was centrifuged at 600 g for 10 min and the resulting supernatant was centrifuged at 15 000 g for 5 min. The resulting mitochondrial pellet was then washed with the same medium without EGTA, and then centrifuged at 15 000 g for 5 min. The final mitochondrial suspension contained 5 mg/mL protein determined by Lowry's method.

#### Determination of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was evaluated from the uptake of rhodamine 123, which accumulates electrophoretically into energized mitochondrial in response to their negative-inside membrane potential<sup>[25]</sup>. Briefly, 1800  $\mu$ L of the phosphate buffer (pH 7.2) containing 250 mmol/L sucrose, 5 mmol/L  $\text{KH}_2\text{PO}_4$ , 3 mmol/L succinate and 0.3  $\mu$ mol/L rhodamine 123 was added to the cuvette, and the fluorescence was monitored by fluorescence spectrometry with excitation and emission wavelengths of 503 nm and 527 nm, respectively. After 30 s, the mitochondrial suspension (final concentration of 0.5 mg/mL protein) was added, and the fluores-

cence intensity was recorded continuously at 25 °C for 5 min. MMP was expressed by the relative value compared to the baseline intensity.

#### Measurement of mitochondrial swelling

Mitochondrial swelling was assessed by measuring the changes in absorbance of the suspension at 520 nm ( $\Delta$ ) by spectrophotometry according to Halestrap *et al.*<sup>[26]</sup>. The standard incubation medium for the swelling assay contained 250 mmol/L sucrose, 0.3 mmol/L  $\text{CaCl}_2$  and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 mL of phosphate buffer. A quantity of 1.8 mL of this suspension was added to both sample and reference cuvettes and 6 mmol/L succinate was added to the sample cuvette only, and the  $A_{520\text{ nm}}$  scanning was started and recorded continuously at 25 °C for 10 min. Swelling of mitochondrial was evaluated according to decreased values in absorption at 520 nm.

#### Determination of mitochondrial nicotinamide adenine dinucleotide-reduced level

The mitochondrial pyridine nucleotide, nicotinamide adenine dinucleotide-reduced (NADH), was monitored by measuring its autofluorescence with excitation and emission wavelengths of 360 nm and 450 nm, respectively, using a fluorescence spectrometer according to Minezaki *et al.*<sup>[27]</sup>. Mitochondria (2 mg protein) were added to 1.8 mL of phosphate buffer containing 6 mmol/L succinate and the autofluorescence of NADH was determined.

#### Determination of mitochondrial SDH and ATPase activity

The activities of mitochondrial SDH and ATPase were detected using kits as described in the instruction manuals. The quantity of Pi production represented the activity of ATPase and was measured by the active unit mmol Pi h mg protein. SDH activity was expressed as units/mg.protein.

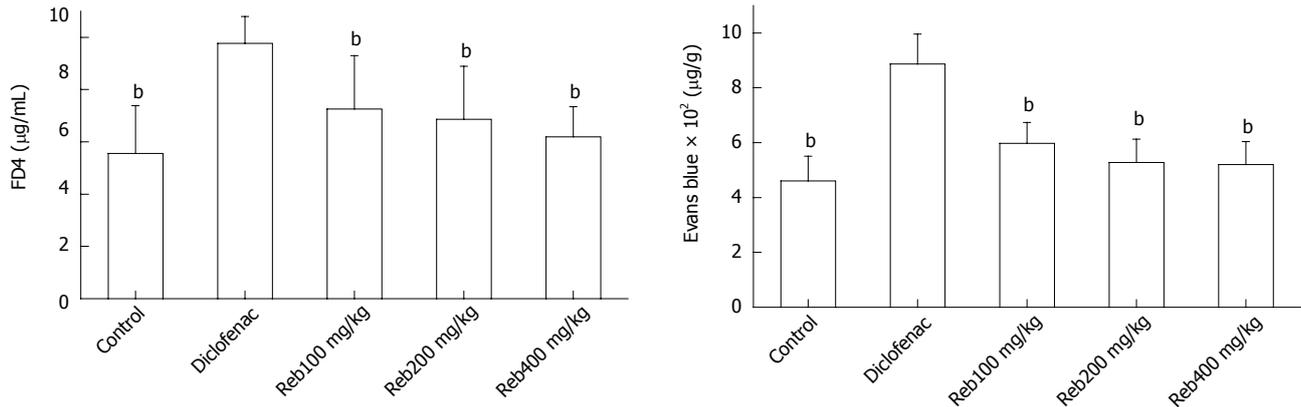
#### Statistical analysis

All results are expressed as mean  $\pm$  SEM. Statistical comparisons were made using the one-way analysis of variance (ANOVA) test. The level of significance was set to a *P*-value of 0.05. All tests were two-sided.

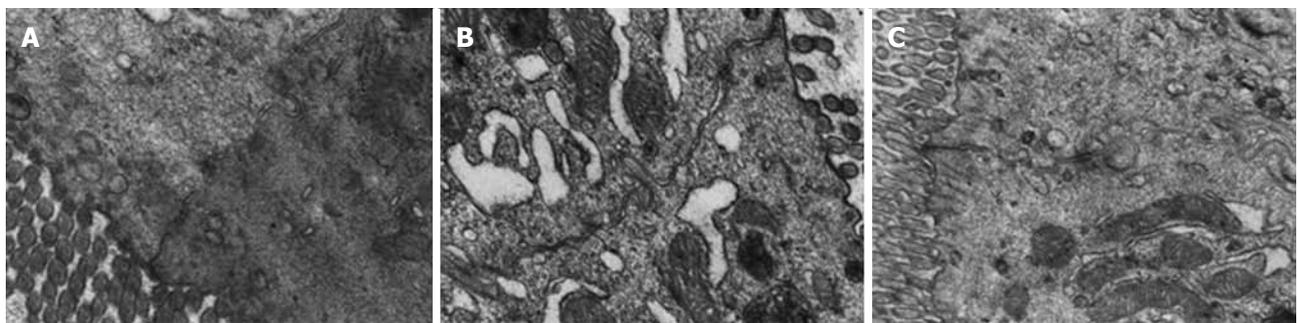
## RESULTS

### Effect of rebamipide on diclofenac-induced small intestinal permeability in mice

Non-absorbed macromolecules, such as EB and FITC-D, are often used as probes in intestinal permeability tests. The amount of Evans Blue which had permeated into the intestinal wall and the plasma FITC-D concentrations in the diclofenac group were significantly higher than those in the control group (*P* < 0.01, Figure 1). These results indicated that diclofenac damaged the small intestinal mucosal barrier, which resulted in an increase in intestinal permeability. Rebamipide significantly reduced Evans Blue and FITC-D permeation.



**Figure 1** Effects of rebamipide on diclofenac-induced small intestinal permeability in mice. Values are mean  $\pm$  SEM of data obtained from 8 mice in each group. <sup>b</sup> $P < 0.01$  compared with the diclofenac group.



**Figure 2** Transmission electron microscopic appearances of diclofenac-induced small intestinal injuries in mice (original magnification  $\times 20\,000$ ). A: Control group; B: Diclofenac group; C: Rebamipide group (400 mg/kg). In the diclofenac group, partial deformation of intestinal epithelial cells, intestinal microvillus reduction, disarrangement of the epithelial surface and broader junctional complexes, tight junction opening were seen. Rebamipide group showed regular and intensive microvillus, and ameliorated tight junction when compared with the diclofenac group.

### Effects of rebamipide on diclofenac-induced ultrastructure of the intestinal barrier in mice

TEM observations showed that the intestinal mucosa in the diclofenac group (Figure 2B) demonstrated visible injury and a portion of the intestinal epithelial cells were deformed, in addition, a significant reduction in intestinal microvilli, disarrangement of the epithelial surface, broader junctional complexes, and open tight junctions were observed when compared with the control group (Figure 2A). In contrast, the rebamipide group displayed nearly normal intestinal epithelial cells, regular and numerous microvilli, and clearly heightened tightness in the tight junctions (Figure 2C).

### Effects of rebamipide on small intestinal MDA content and MPO activity

Compared with the control group, the small intestinal MDA content and MPO activity were significantly increased in the diclofenac group ( $1.65 \pm 0.32$  vs  $0.97 \pm 0.28$  nmol/mg protein, and  $0.236 \pm 0.027$  vs  $0.159 \pm 0.025$  U/g, respectively, both  $P < 0.01$ ), indicating that diclofenac caused oxidative damage and inflammation in small intestinal mucosa. Rebamipide significantly reduced the MDA content and MPO activity demonstrating that the anti-oxidative and anti-inflammatory effects of rebamipide

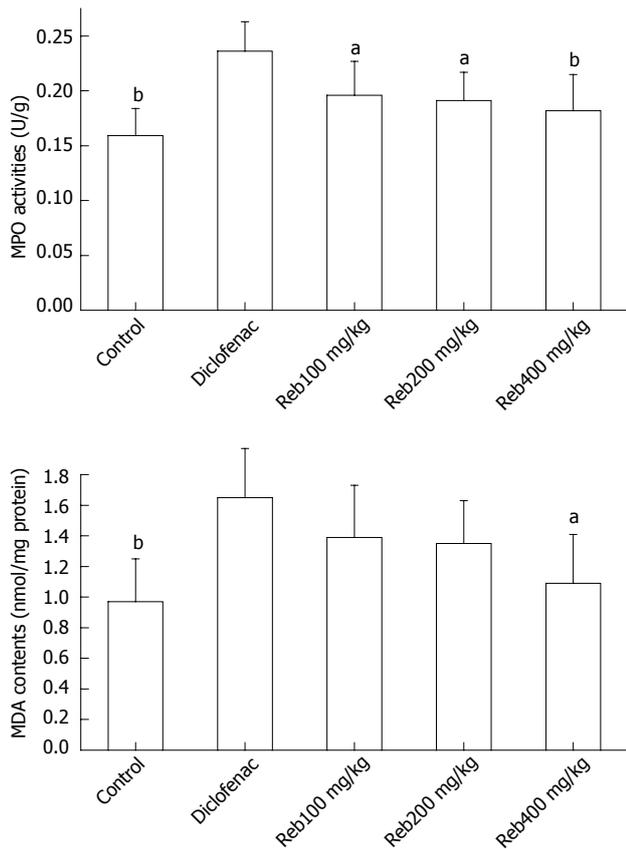
may be related to the reduction in small intestinal injury (Figure 3).

### Effects of rebamipide on diclofenac-induced impairment of liver mitochondrial functions, mitochondrial membrane potential

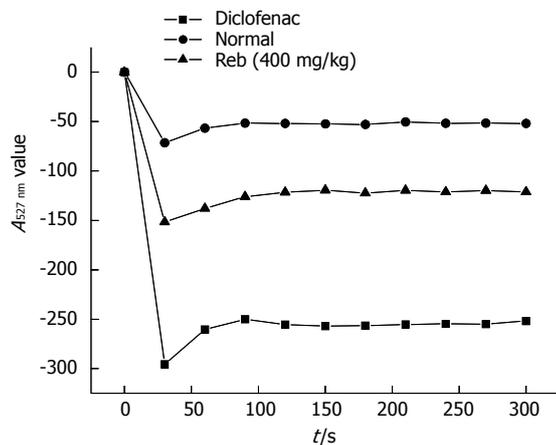
In the control group, the fluorescence intensity was rapidly reduced within 30 s after the mixture of mitochondria with rhodamine 123, it then began to rise and reached a steady-state within 90 s (Figure 4). Compared with the control group, the reduction in fluorescence intensity in the diclofenac group decreased slightly, indicating that the uptake of rhodamine 123 in mitochondria was smaller in the diclofenac group than in the control group. The reduction in mitochondrial rhodamine 123 uptake was reversed in the rebamipide group indicating that rebamipide significantly improved the impairment of MMP.

### Mitochondrial swelling

It has been shown that pH change and high calcium can cause mitochondrial swelling, which is detected by a reduction in mitochondrial absorbance at certain wavelengths. After adding the reaction buffer (at pH 7.2) or 0.3 mmol/L of  $\text{CaCl}_2$ , the mitochondrial absorbance at

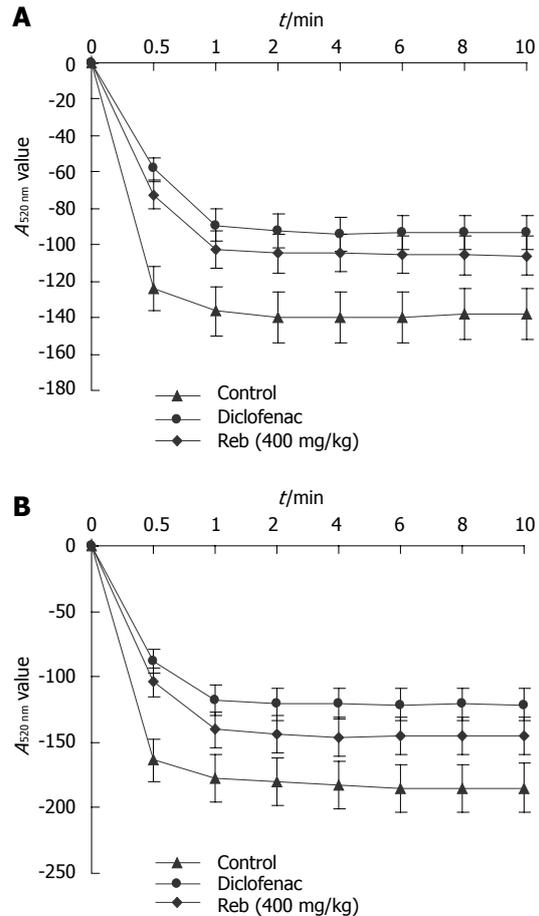


**Figure 3** Effects of rebamipide on small intestinal malondialdehyde content and myeloperoxidase activity. Values are mean  $\pm$  SEM of data obtained from 8 mice in each group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  compared with the diclofenac group. MDA: Malondialdehyde; MPO: Myeloperoxidase.



**Figure 4** Effects of rebamipide on diclofenac-induced liver mitochondrial membrane potential. In the control group, the fluorescence intensity was reduced within 30 s, and then began to rise and reached a steady state within 90 s. The fluorescence intensity reduction in the diclofenac group was smaller than that in the control group, indicating that the liver mitochondrial membrane potential was decreased by diclofenac administration. The reduction in the fluorescence intensity in the rebamipide group was greater than that in the diclofenac group indicating that rebamipide improved the impairment in mitochondrial function induced by diclofenac.

520 nm declined, indicating mitochondrial swelling due to abnormal osmotic pressure. The extent of absorbance

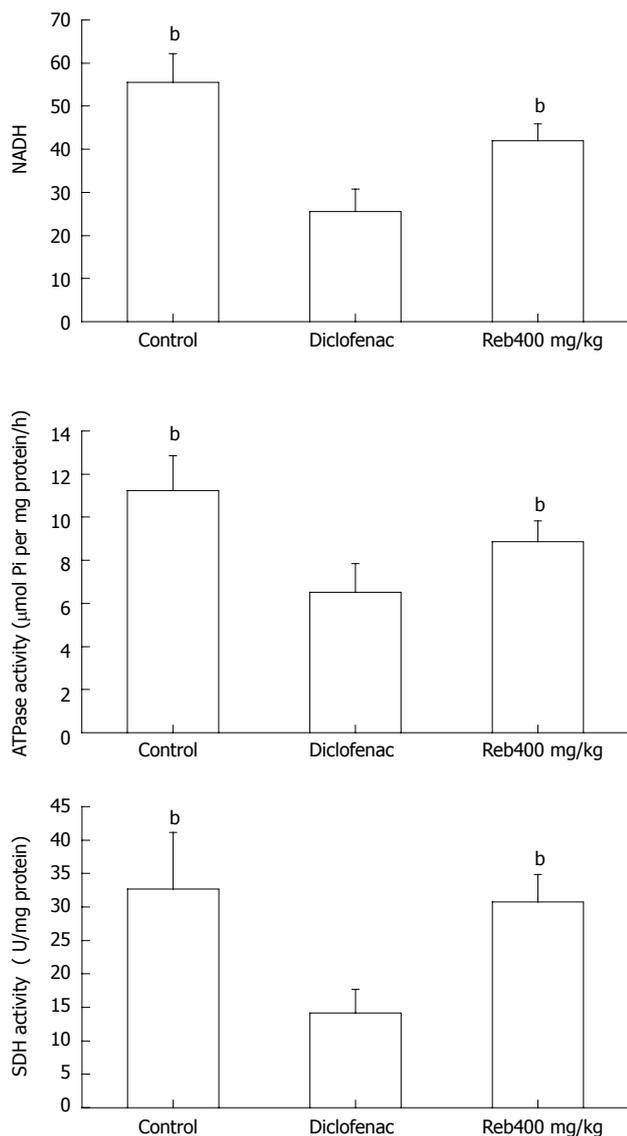


**Figure 5** Effects of rebamipide on diclofenac-induced liver mitochondrial swelling in mice. A: After adding the reaction buffer, the absorbance at 520 nm in the control mitochondria declined rapidly. The decrease was smaller in the presence of diclofenac compared with that in the control, demonstrating that liver mitochondrial dysfunction was induced by diclofenac administration. This reduction in absorbance was significantly increased in the presence of rebamipide, indicating that rebamipide improved impaired mitochondrial function; B: After adding 0.3 mmol/L CaCl<sub>2</sub> reaction buffer, the absorbance at 520 nm in the control mitochondria declined rapidly, suggesting significant swelling of mitochondria. The decrease was smaller in the presence of diclofenac compared with that in the control, demonstrating that liver mitochondrial dysfunction was induced by diclofenac administration. This reduction was significantly increased in the presence of rebamipide, indicating that rebamipide improved impaired mitochondrial function.

decrease in the diclofenac group was smaller than that in the control group (Figure 5A and B), demonstrating that liver mitochondrial dysfunction was induced by diclofenac administration. Compared with the diclofenac group, the absorbance of rebamipide was significantly increased, indicating that rebamipide improved impaired liver mitochondrial functions.

**Mitochondrial NADH levels, SDH and ATPase activities**

Compared with the control group, significant decreases in NADH levels, ATPase and SDH activities in liver mitochondrial were seen in the diclofenac group (Figure 6). Rebamipide significantly increased these levels and activities, demonstrating that rebamipide enhanced cellular stress response capacity and maintenance of mitochon-



**Figure 6** Effects of rebamipide on diclofenac-induced decreases in liver mitochondrial nicotinamide adenine dinucleotide-reduced levels, succinate dehydrogenase and ATPase activities in mice. Values are mean  $\pm$  SEM of data obtained from 8 mice in each group. <sup>b</sup> $P < 0.01$  compared with the diclofenac group. SDH: Succinate dehydrogenase; NADH: Nicotinamide adenine dinucleotide-reduced.

drial energy metabolism.

## DISCUSSION

Recently, several investigators have shown that the administration of NSAIDs induces small intestinal damage. Bjarnason *et al*<sup>[28]</sup> demonstrated that in 97 patients who took NSAIDs for more than 2 mo these agents caused enteropathy in 66% of these patients. Sigthorsson *et al*<sup>[29,30]</sup> demonstrated that intestinal adverse events, defined as hospitalization for intestinal perforation or hemorrhage, occurred in 72% of 286 patients who took 12 different NSAIDs. These trials showed that chronic NSAIDs use not only causes upper GI injury, but also lower GI injury. To date, there are no effective drugs to

prevent NSAID-induced lower GI complications.

Bjarnason *et al*<sup>[31]</sup> advocated that an increase in the permeability of small intestinal mucosa was an important factor in the mechanism of NSAID-induced small intestinal injury. Increased intestinal permeability leads to large molecules contained in food substances, bile acids, pancreatic juice, bacteria and other toxins within the lumen passing through the intestinal epithelial barrier, causing intestinal inflammation and injury. Therefore, the change in small intestinal permeability could directly reflect small intestinal mucosal integrity and barrier function, and help to identify early damage to intestinal mucosa.

In the present study, we investigated intestinal permeability induced by diclofenac and the protective effects of rebamipide. Because non-absorbed macromolecules, such as EB and FITC-D, are often used as probes in intestinal permeability tests, we investigated the amount of Evans Blue permeating into the intestinal wall ( $460.6 \pm 89.7$  *vs*  $887.1 \pm 108.3$ ) and the significant elevation in plasma FITC-D concentrations ( $5.56 \pm 1.82$  *vs*  $9.77 \pm 1.03$ ) (Figure 1). By using TEM technology, visible injury, deformed intestinal epithelial cells, a significant reduction in intestinal microvilli, disarrangement of the epithelial surface, broader junctional complexes, and opened tight junctions were observed in the diclofenac group (Figure 2B). Administration of rebamipide significantly improved small intestinal mucosal permeability and the ultrastructure changes showed more regular and numerous microvilli, and ameliorated tight junctions (Figure 1 and Figure 2C).

On the other hand, our results demonstrated that small intestinal injury induced by diclofenac was associated with increased permeability, ulcers and edema macroscopically, and small intestinal villi damage, partial epithelial denudation, and infiltration of inflammatory cells microscopically. Rebamipide reduced these small intestinal injuries both macroscopically and microscopically. Administration of rebamipide prevented small intestinal injury and histopathologic changes induced by diclofenac (data not shown).

Matysiak *et al*<sup>[13]</sup>, using HT29-19A intestinal epithelial cells, reported that rebamipide may exert its protective effect on gastric mucosa by reinforcing the epithelial barrier. Banan *et al*<sup>[32]</sup>, using human intestinal (Caco-2) cell monolayers, reported that rebamipide prevented oxidation of actin and led to the protection of actin cytoskeleton and intestinal barrier integrity against oxidant insult. These two reports show that rebamipide acts in the management of tight junctions in small intestinal mucosa. It is suggested that rebamipide improved intestinal mucosa integrity by reducing intestinal permeability.

Rebamipide removes oxygen free radicals in epithelial cells. It has been reported to promote healing and prevent relapse of gastric ulcers, which is attributed to inflammatory cell migration into the gastric mucosa<sup>[33]</sup>. Kim *et al*<sup>[34]</sup> reported that rebamipide significantly inhibited upper GI mucosal damage induced by NSAIDs in a randomized controlled trial carried out in healthy volun-

teers. In addition, rebamipide was protective in NSAID-induced lower GI injuries. Niwa *et al.*<sup>[35]</sup> reported that taking rebamipide plus diclofenac significantly prevented small intestinal injury compared with placebo plus diclofenac. However, the protective mechanism of rebamipide on intestinal mucosal injury remains unclear.

Many researchers have demonstrated that mitochondrial injury is part of the mechanism of intestinal damage induced by NSAIDs, which also includes dissipating the mitochondrial transmembrane potential, and inducing the mitochondrial permeability transition pore, which liberates cytochrome c, resulting in the caspase cascade and cellular lipid peroxidation, inducing cellular apoptosis. The fluorescent molecular probe, rhodamine 123, can enter the mitochondrial matrix through mitochondrial membrane potential dependent-substrate, and can reflect mitochondrial membrane potential by measuring the changes in optical density<sup>[25]</sup>. Research has shown that rebamipide exerted a protective effect on mitochondrial membrane stability in gastric epithelial cells<sup>[36]</sup>, however, the mechanism remains unknown. In our study, as it was exceedingly difficult to obtain a high yield of mitochondria from intestinal tissue<sup>[24]</sup>, mouse liver mitochondria were isolated to examine mitochondrial function instead of small intestinal mitochondria according to the method of Somasundaram. We found that rebamipide had a protective role in mitochondrial damage induced by diclofenac, which was related to the maintenance of mitochondrial membrane potential, an improvement in mitochondrial function, enhanced enzyme activities in the mitochondrial respiratory chain and the maintenance of energy metabolism, which indicated that the protective effect of rebamipide may be related to mitochondrial protection.

In conclusion, the administration of diclofenac in mice induced small intestinal mucosal damage and was associated with small intestinal hyperpermeation. Rebamipide showed a preventive effect on hyperpermeation, which was related to mitochondrial protection.

## COMMENTS

### Background

It is well-known that traditional non-steroid anti-inflammatory drugs (NSAIDs) induce mucosal injury in the lower gastrointestinal resulting in serious damage. However, the mechanism of NSAIDs-related small intestinal mucosal injury has not yet been clearly defined and the available protective drugs for NSAIDs enteropathy are inadequate.

### Research frontiers

Many researchers have demonstrated that mitochondrial injury is part of the mechanism of intestinal damage induced by NSAIDs, which also includes dissipating the mitochondrial transmembrane potential, and inducing the mitochondrial permeability transition pore, which liberates cytochrome c, resulting in the caspase cascade and cellular lipid peroxidation, inducing cellular apoptosis.

### Innovations and breakthroughs

The administration of diclofenac in mice induced small intestinal mucosal damage and was associated with small intestinal hyperpermeation. Rebamipide showed a preventive effect on the permeability induced by diclofenac, which was related to mitochondrial protection.

### Applications

These findings may provide experimental evidence for the clinical application of

rebamipide in the treatment of NSAIDs enteropathy.

### Terminology

NSAIDs enteropathy, mitochondria, rebamipide, intestinal mucosal permeability.

### Peer review

This is an interesting paper investigating the effects of NSAIDs on intestinal permeability and the protective effect of rebamipide on diclofenac-induced injury. However, for this kind of pharmacological study, a clear dose-dependency has not been found.

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## Differential diagnosis in patients with suspected bile acid synthesis defects

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### Abstract

**AIM:** To investigate the clinical presentations associated

with bile acid synthesis defects and to describe identification of individual disorders and diagnostic pitfalls.

**METHODS:** Authors describe semiquantitative determination of 16 urinary bile acid metabolites by electrospray ionization-tandem mass spectrometry. Sample preparation was performed by solid-phase extraction. The total analysis time was 2 min per sample. Authors determined bile acid metabolites in 363 patients with suspected defects in bile acid metabolism.

**RESULTS:** Abnormal bile acid metabolites were found in 36 patients. Two patients had bile acid synthesis defects but presented with atypical presentations. In 2 other patients who were later shown to be affected by biliary atresia and cystic fibrosis the profile of bile acid metabolites was initially suggestive of a bile acid synthesis defect. Three adult patients suffered from cerebrotendinous xanthomatosis. Nineteen patients had peroxisomal disorders, and 10 patients had cholestatic hepatopathy of other cause.

**CONCLUSION:** Screening for urinary cholanooids should be done in every infant with cholestatic hepatopathy as well as in children with progressive neurological disease to provide specific therapy.

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**Key words:** Cholestatic liver disease; Bile acid synthesis defects; Biliary atresia; Electrospray-ionization tandem-mass-spectrometry

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## INTRODUCTION

Inborn errors of bile acid synthesis can result in potentially treatable progressive cholestatic liver disease and neurological disease. Liver disease often presents in early childhood with jaundice, deficiency of fat-soluble vitamins, acholic stools and hepatomegaly. Serum aminotransferases and conjugated bilirubin are usually elevated and typically, gamma glutamyl transpeptidase activity is normal. Progressive neurological disease usually occurs later in childhood or early adulthood with pyramidal tract dysfunction, cerebellar signs, sensory-motor neuropathy and cognitive decline.

The synthesis of bile acids in the liver is the main pathway of cholesterol degradation. Many enzymes localized in different subcellular compartments of hepatocytes interact in the conversion of cholesterol to bile acids. Thus, defective functioning of one or more of these enzymes results in a deficiency of the final bile acids chenodeoxycholic acid and cholic acid (Figure 1) as well as the formation of atypical sterol intermediates (Table 1) including unusual bile acids and bile alcohols, which can be hepatotoxic or interfere with biliary secretion leading to cholestasis and malabsorption of fat-soluble vitamins. Severe liver disease in turn gives rise to serum aminotransferases and conjugated bilirubin levels while serum gamma glutamyl transpeptidase activity in patients with bile acid synthesis defects remains normal despite severe cholestasis<sup>[1]</sup>. This contributes to the fact that glutamyl transpeptidase is localized mainly at the luminal plasma membrane of bile duct epithelium cells. It increases if bile acids dissolve the glutamyl transpeptidase from this membrane. In patients with bile acid synthesis defects (and in those with a bile acid transporter defect) these bile acids are not produced. Therefore no increase in glutamyl transpeptidase is seen despite severe cholestasis<sup>[2]</sup>.

At the genetic level, 9 inborn errors in the bile acid biosynthetic pathways have been described to date: cerebrotendinous xanthomatosis, 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase/isomerase deficiency<sup>[3]</sup>,  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase (5 $\beta$ -reductase) deficiency<sup>[4]</sup>, oxysterol 7 $\alpha$ -hydroxylase deficiency<sup>[5]</sup>, cholesterol 7 $\alpha$ -hydroxylase deficiency<sup>[6]</sup>, as well as the peroxisomally located defects 2-methylacyl-CoA racemase (AMCAR) deficiency<sup>[7,8]</sup>, D-bifunctional protein deficiency<sup>[9,10]</sup>, sterol carrier protein X (SCPx) deficiency<sup>[11]</sup>. The clinically most important errors are depicted in Table 1.

Several analytical techniques are available for the de-

termination of bile acid profiles in urine and plasma<sup>[12-15]</sup>, but the most useful screening test is analysis of urinary cholanooids (bile acids and bile alcohols) by electrospray ionization-tandem mass spectrometry (ESI-MS/MS)<sup>[2]</sup>.

However, patients with severely compromised liver function of different origin may show patterns of bile acid metabolites resembling those of bile acid synthesis defects and patients with disorders of bile acid synthesis may present with atypical clinical symptoms making a straightforward diagnosis impossible. In this study urinary bile acids of 363 patients with suspected defects in bile acid metabolism were determined and in 36 patients an abnormal bile acid pattern was recognized. This paper focuses on 2 groups of patients: those with proven defects of bile acid synthesis, who present with atypical clinical symptoms and those with cholanooid profiles pointing to a defect of bile acid synthesis who were later shown to have a different disorder. We describe clinical presentations, present differential diagnoses and analyze the diagnostic pitfalls of the ESI-MS/MS method in these patients.

## MATERIALS AND METHODS

### Ethics

This study has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Sample collection for this study was approved by the ethics committee of the medical faculty, University of Heidelberg, Germany (No. 071/2005).

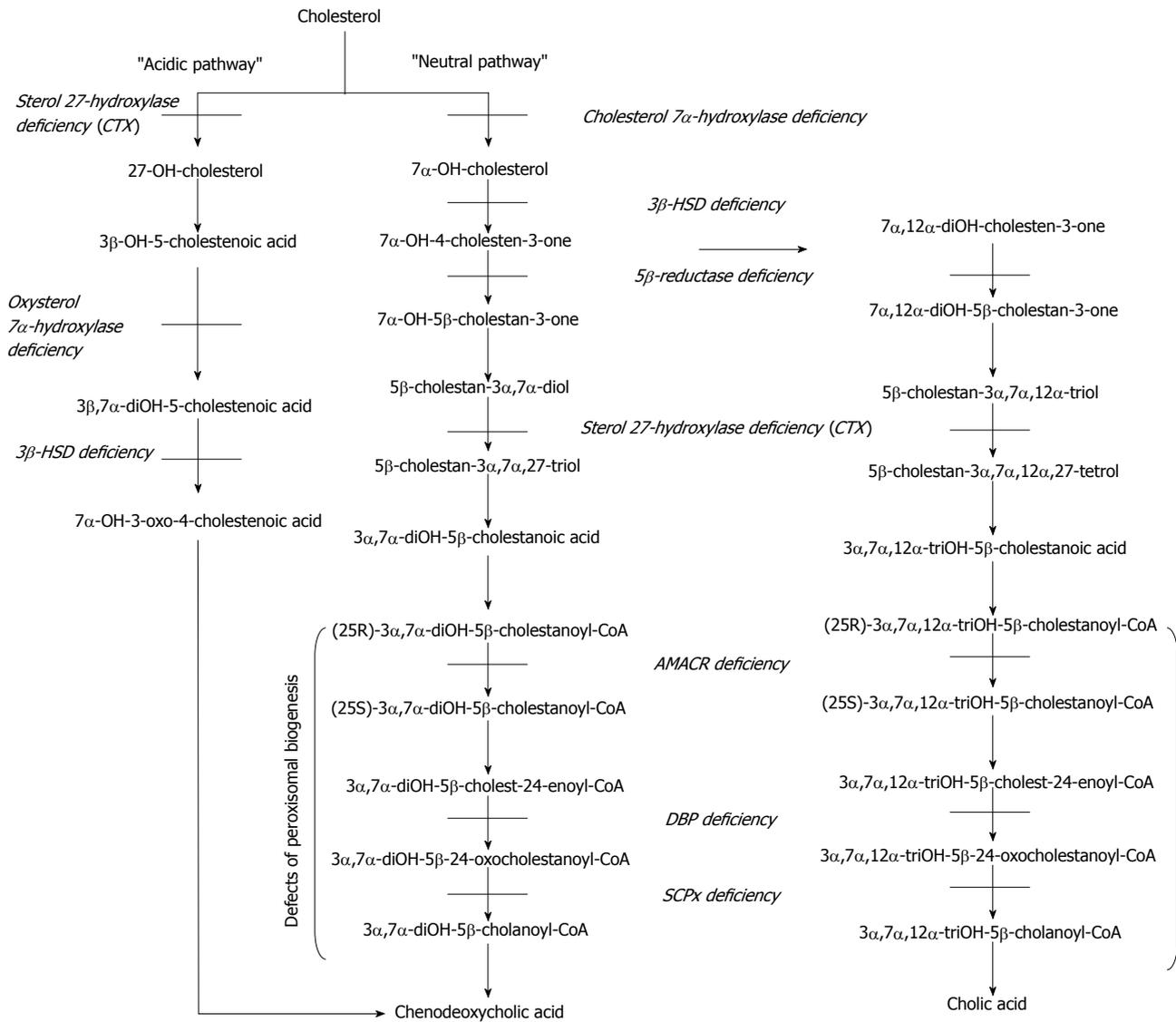
### Patients

The control population consisted of 100 healthy and non-symptomatic children, adolescents and young adults ranging in age from 10 d to 20 years. Urine specimens were collected in our hospital and used to establish the normal ranges of the 16 metabolites assayed. As positive controls, we additionally investigated patient samples collected previously at the UCL Institute of Child Health, London; these samples were obtained from patients with the following confirmed defects: 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase (3 $\beta$ -HSD) ( $n = 1$ ), 5 $\beta$ -reductase deficiency ( $n = 1$ ), peroxisomal biosynthesis disorders ( $n = 3$ ), and cerebrotendinous xanthomatosis ( $n = 3$ ).

After establishing reference ranges urine samples of patients with suspected bile acid synthesis defects were investigated in the clinical routine. To date samples of 363 patients have been analyzed. Thirty-six of those showed abnormal results and are further discussed in this study. The remaining 327 patients had normal or non-specific bile acid profiles. In the following, 3 clinically especially instructive histories from patients are briefly summarized.

### Patient MD

This girl, the second child of healthy Turkish parents who are first cousins, was born after 35 wk of an otherwise uneventful pregnancy. Shortly after birth a persistent



**Figure 1** The metabolic pathway of bile acid biosynthesis. The classical "neutral pathway" with chenodeoxycholic acid and cholic acid as end products is the main pathway in adults. In children, the alternative "acidic pathway" with chenodeoxycholic acid as major product is more active. The end products are exported as glycine or taurine conjugates into the bile canaliculi. Known metabolic blocks are displayed as bars and the specific defects in italic script. CTX: Cerebrotendinous xanthomatosis.

metabolic acidosis was observed, which was treated with bicarbonate supplementation. She also had nephrocalcinosis and failure to thrive. Because phosphate excretion was also elevated DeToni-Debré-Fanconi syndrome was suspected. At age 6 years she developed gait ataxia, muscle weakness, and external ophtalmoplegia. A severe deficiency of fat-soluble vitamins became evident (vitamin A: 0.89 μmol/L, reference range 1.09-3.07, vitamin E: 19.89 μmol/L, reference range 25-42). Aspartate aminotransferase (AST) was 66 U/L (reference range < 39), alanine aminotransferase (ALT) 46 U/L (reference range < 34), gamma glutamyltransferase (GGT) 22 U/L (reference range < 38), total bilirubin 24 μmol/L (reference range < 17), and conjugated bilirubin 12 μmol/L (reference range < 5). Abdominal ultrasound showed normal hepatic echotexture. Because multiple organ systems were involved a defect of mitochondrial energy metabolism

was suspected but lactate concentrations in blood and CSF (cerebrospinal fluid), determination of respiratory chain and pyruvate dehydrogenase complexes in frozen muscle as well as mutation analysis for pediatric mitochondrial disorders such as MERRF (myoclonic epilepsy with "ragged red fibres"), MELAS (encephalomyopathy, lactic acidosis, stroke-like episodes) and NARP (neuropathy, ataxia, retinitis pigmentosa) were all normal. After the diagnosis of the bile acid synthesis disorder 3β-hydroxy-Δ<sup>5</sup>-C<sub>27</sub>-steroid dehydrogenase deficiency was established by analysis of urinary bile acid metabolites, supplementation with vitamins A, E and K normalized her vitamin A levels but those of vitamin E remained below the normal range. After adding ursodeoxycholic acid and chenodeoxycholic acid, vitamin concentrations normalized quickly and remained so even after discontinuation of vitamin supplementation. Her gait improved significantly

**Table 1** Reference ranges of bile acid metabolites which accumulate in different bile acid biosynthesis defects

Defect	Metabolite	<i>m/z</i>	Median	Range
3 $\beta$ -hydroxy- $\Delta^5$ -C27-steroid dehydrogenase deficiency <i>HSD3B7</i> 16p11.2-12 OMIM: 23110	Glyco-dihydroxy-5-cholenoic acid	526	0.05	0.0-0.9
	Glyco-trihydroxy-5-cholenoic acid	542	0.07	0.0-0.4
	Dihydroxy-5-cholenoic acid sulphate	469	0.71	0.15-8.1
	Trihydroxy-5-cholenoic acid sulphate	485	0.08	0.0-2.0
$\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency <i>AKR1D1</i> 7q31 OMIM: 235555	Glyco-hydroxy-oxo-cholenoic acid	444	0.03	0.0-0.4
	Glyco-dihydroxy-oxo-cholenoic acid	460	0.04	0.0-2.4
	Tauro-hydroxy-oxo-cholenoic acid	494	0.02	0.0-0.5
	Tauro-dihydroxy-oxo-cholenoic acid	510	0.04	0.0-5.0
Defects of peroxisomal biogenesis Multiple OMIM: 214100	Tauro-tri-hydroxycholestanoic acid	556	0.02	0.0-0.3
	Tauro-tetra-hydroxycholestanoic acid	572	0.02	0.0-0.2
Sterol 27-hydroxylase deficiency (cerebrotendinous xanthomatosis) <i>CYP27A1</i> 2q33-qter OMIM: 213700	Glucuronide-5 $\beta$ -cholestane-tetrol	611	0.24	0.0-1.6
	Glucuronide-5 $\beta$ -cholestane-pentol	627	0.37	0.1-4.3
Cholestatic liver disease Multiple	Glyco-chenodeoxy-cholic acid	448	0.49	0.0-5.2
	Glyco-cholic acid	464	0.64	0.1-14.6
	Tauro-chenodeoxy-cholic acid	498	0.05	0.0-0.8
	Tauro-cholic acid	514	0.08	0.0-8.4

The disorders are listed with their Online Mendelian Inheritance in Man™ (OMIM) and their genetic defect, respectively. Reference ranges for bile acid metabolites which are increased in different bile acid synthesis defects were established in 100 healthy children, adolescents and young adults of age 10 d to 20 years. The metabolites are arranged to their appearance in different pathological conditions. Data are peak area ratios obtained for conjugates *versus* internal standard normalized to mol creatinine. The characteristic fragment ions used for detection of the metabolites are given as the mass-to-charge ratio (*m/z*).

and after a short period of treatment her muscle strength returned to normal.

### Patient AS

This baby girl was the first child of healthy non-consanguineous Tamil parents. At age 4 wk scleral icterus was observed. Additionally, she developed dark urine and pale stools, but her weight gain was within the normal range. Laboratory investigations at age 13 wk showed elevated AST (681 U/L, reference range < 74), ALT (344 U/L, reference range < 60) and normal GGT (99 U/L, reference range < 160). Total bilirubin was 268  $\mu$ mol/L (reference range < 21), conjugated bilirubin was 185  $\mu$ mol/L (reference range < 5). The gall bladder was not detectable in abdominal ultrasound, therefore biliary atresia was suspected. Explorative laparotomy showed a completely cirrhotic liver; there was no excretion of marker dye into the bile ducts. Due to the severe cirrhosis a Kasai procedure was not attempted. A primary liver transplantation was planned. Histological examination of a liver biopsy showed proliferating bile ducts and no giant cells consistent with biliary atresia; however, analysis of bile acids was suggestive of  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase deficiency. Subsequent DNA sequencing of the exons of the patient's *AKR1D1* gene failed to reveal mutations, thereby excluding the diagnosis of primary 5 $\beta$ -reductase deficiency. She was treated with cholic acid and ursodeoxycholic acid but her condition deteriorated with progressive ascites, which led to refer-

ral for liver transplantation. Segments 2 and 3 of her mother's liver were transplanted successfully at age 4.5 mo. Examination of the explanted liver and biliary tree confirmed the diagnosis of biliary atresia. Several days after transplantation she developed rejection, which was successfully treated with immunosuppression. At age 10 mo the patient was doing well.

### Patient KS

This boy was the first child of non-consanguineous German parents. His mother suffered from a stroke at the age of 20 years. Follow-up investigations were not performed. At the age of 6 years attention deficit hyperactivity disorder (ADHD) was diagnosed in patient KS and treatment with methylphenidate was initiated. After primary school he attended a secondary school for children with learning difficulties. His parents noted recurrent diarrhea, especially after consuming sweets. At the age of 14 years bilateral cataracts were diagnosed. After surgery he was referred for metabolic workup. Galactose metabolites, homocysteine and urinary oligosaccharides were all normal. Cranial magnetic resonance imaging (MRI) showed increased signal intensity in cerebellar white matter in T2. Analysis of urinary bile acid metabolites was suggestive of cerebrotendinous xanthomatosis. Sterol analysis of plasma showed a clearly elevated concentration of cholestanol (0.24  $\mu$ mol/L, reference range < 0.01), together with positive results of mutation analysis confirming the diagnosis. Treatment with chenodeoxycholic

**Table 2** Electrospray ionization-tandem mass spectrometry operating parameters for bile acid intermediates

	Glycine conjugates (IS included)	Taurine conjugates	Sulfatides	Glucuronides
Nebulizer gas (au)	11	11	11	11
Curtain gas (au)	9	9	9	9
Collision gas (au)	5	5	5	5
Ion spray voltage (V)	-4100	-4100	-4100	-4100
Temperature (°C)	200	200	200	200
Declustering potential (V)	-35	-35	-35	-35
Focusing potential (V)	-31	-31	-31	-31
Entrance potential (V)	-10	-10	-10	-10
Prefilter (V)	23	23	23	23
Collision energy (V)	-55	-129	-60	-75
Collision cell exit potential (V)	-9	-9	-9	-9
Daughter ion ( <i>m/z</i> )	74	80	97	85
Scan range of precursor ions ( <i>m/z</i> )	430 to 550	490 to 590	460 to 490	600 to 630

The mass spectrometer was operated in a negative precursor ion scan mode with a step-size of 0.1 amu. Nitrogen was used as nebulizer, curtain and collision gases. au: Arbitrary units; IS: D<sub>4</sub>-glycocholic acid.

acid normalized bowel movements and the sterol profile. The patient has not developed ataxia or xanthomata. At the age of 19 years he is an apprentice landscaper.

### Analysis of bile acid conjugates

Urine samples were stored at -20 °C until analysis. Sample preparation was performed by solid-phase extraction (SPE) using a Oasis™ HLB cartridge as described previously<sup>[16]</sup>. In brief, the cartridge was preconditioned with 2 × 1 mL dichloromethane/methanol (2:1, v/v) and 1 mL purified water. To a fixed volume of 300 µL urine 10 µL internal standard solution (100 µmol/L D<sub>4</sub>-glycocholic acid) was added. The urine sample was then applied onto the preconditioned cartridge and washed with 2 mL each of purified water and n-hexane. The bile acid conjugates were eluted with 300 µL of 700 mL/L aqueous methanol (v/v). The eluate was directly used for injection to tandem mass spectrometer. The sample preparation time for a series of 20 samples takes about 1.5 h.

ESI-MS/MS measurement was carried out using a triple quadrupole IONICS EP 10+ upgraded Perkin Elmer Sciex API 365 mass spectrometer equipped with a turbo spray ion source. The mobile phase contained acetonitrile/purified water (1:1, v/v). 10 µL eluate of the SPE cartridge was directly injected. The flow rate was 80 µL/min over a total run time of 2 min.

The 16 urinary bile acid metabolites (glycine and taurine conjugates, sulfates and glucuronides) were identified with negative electrospray ionisation. Characteristic fragment ions used to determine the bile acid metabolites according to<sup>[17-22]</sup> are given in Table 1 as their mass-to-charge ratio (*m/z*). ESI-MS/MS operating parameters for

the four precursor ion scans are given in Table 2.

Data acquisition was carried out by Analysis 1.4.1 software. The determination of the 16 relevant analytes in urine was semi-quantitative since only one internal standard, D<sub>4</sub>-glycocholic acid, was used as a reference quantity. Therefore the resulting values were given as analyte/internal standard peak-area ratios normalized to mol creatinine. Creatinine determination in urine was performed on an Olympus AU 400 analyzer using the creatinine kit (Beckman Coulter, Krefeld, Germany).

### Determination of 3β-hydroxy-Δ<sup>5</sup>-C<sub>27</sub>-steroid dehydrogenase activity in fibroblasts

Patient and control fibroblasts were cultured in Dulbecco's modified eagle medium with 10% bovine serum, and antibiotic supplements. After 5-10 passages cells were trypsinized, washed with PBS and stored as pellets at -70 °C until required.

The activity of 3β-hydroxy-Δ<sup>5</sup>-C<sub>27</sub>-steroid dehydrogenase was determined as described previously<sup>[17,18]</sup>. Briefly, fibroblasts were thawed on ice, resuspended in potassium phosphate buffer (0.1 mol/L, pH 7.5) and sonicated. Cell suspensions containing 1 mg protein were preincubated with <sup>14</sup>C-labelled 7α-hydroxycholesterol (0.05 mmol/L in 5 µL acetone) and buffer for 5 min in a 37 °C shaking water bath. The reaction was initiated with the addition of 50 µL of nicotinamide adenine nucleotide (NAD) (1.5 mmol/L), with a final reaction volume of 0.5 mL. After 1 hour the reaction was stopped with the addition of 2 mL of ethanol, followed by 3 mL of water. Extractions were performed twice with *n*-hexane/ethyl acetate (1:1 v/v). Negative controls included a reaction with extract from control fibroblasts without the addition of NAD, and an assay with no cellular extract added.

The extracts were dried under argon, re-dissolved in toluene/ethyl acetate (1:1 v/v) and spotted onto a thin layer chromatography (TLC) plate, which was developed using a mobile phase of toluene/ethyl acetate (1:1 v/v).

Visualization of sterol products was performed using a phosphoimager according to the manufacturer's recommendations (Fuji Medical Systems, Stamford, United States).

### Mutation analysis of HSD3B7 and AKR1D1 loci

Genomic DNA was extracted from cultured fibroblasts or whole blood collected into EDTA-containing tubes by standard methods. Individual exons together with an average of 25 base pairs of 5'- and 3'-flanking intronic DNA from the 3β-hydroxy-Δ<sup>5</sup>-C<sub>27</sub>-steroid dehydrogenase gene (*HSD3B7*) and Δ<sup>4</sup>-3-oxosteroid 5β-reductase gene (*AKR1D1*) were amplified and subjected to DNA sequence analysis as described previously<sup>[18,19]</sup>.

## RESULTS

### Reference intervals and control ranges

Bile acid conjugates were extracted from urine and 16 metabolites (6 glycine conjugates, 6 taurine conjugates, 2 sulfates and 2 glucuronides) were determined semi-quantita-

**Table 3** Peak area ratios of specific glycine and taurine conjugates in controls, patients with cholestatic liver disease (cholestasis) and patients AS and MK with suspected 5 $\beta$ -reductase deficiency

Metabolite	<i>m/z</i>	Controls ( <i>n</i> = 100)	Cholestasis ( <i>n</i> = 10)	AS	MK	MK <sup>1</sup>
Glyco-hydroxy-oxo-cholenoic acid	444	0-0.4	1-210	34	104	14
Glyco-dihydroxy-oxo-cholenoic acid	460	0-2.4	8-251	15	1331	1
Tauro-hydroxy-oxo-cholenoic acid	494	0-0.5	1-26	39	6	17
Tauro-dihydroxy-oxo-cholenoic acid	510	0-5	8-133	19	779	0
Glyco-chenodeoxycholic acid	448	0-5.2	2-10 754	4	9	25
Glyco-cholic acid	464	0.1-14.6	36-2814	17	49	47
Tauro-chenodeoxycholic acid	498	0-0.8	1-718	9	45	10
Tauro-cholic acid	514	0-8.4	6-749	9	10	1

Elevation of glycine and taurine conjugates of dihydroxy-oxo-cholenoic acid (*m/z* 460 and 510) and hydroxy-oxo-cholenoic acid (*m/z* 444 and 494) point to 3 $\beta$ -HSD. Elevation of glycine and taurine conjugates of cholic acid (*m/z* 448 and 464) and chenodeoxycholic acid (*m/z* 498 and 514) point to cholestatic liver disease. In patient AS, 5 $\beta$ -reductase and 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency were excluded by mutation analysis. The cholanoic profiles of patient MK with cystic fibrosis are in a decompensated state with severe cholestatic liver disease and after treatment with ursodeoxycholic acid, pancreatic enzymes and fat-soluble vitamins.

tively by ESI-MS/MS. We established reference ranges for these 16 metabolites studied here in 100 subjects ranging in age from 10 d to 20 years (Table 1); no age-dependency for any of these bile acid conjugates was found (data not shown).

### Bile acid metabolites in patient samples

**Cholestatic liver disease and cholanoic profiles mimicking 5 $\beta$ -reductase deficiency:** In patients with cholestatic liver disease analysis of urinary bile acid metabolites showed clearly increased excretion of glycine and taurine conjugates of cholic acid and chenodeoxycholic acid (*m/z* 448, 464, 498 and 514). In a subgroup of patients with non-specified cholestatic liver disease (*n* = 10) we additionally detected elevated excretion of glycine and taurine conjugates of dihydroxy-oxo-cholenoic acid (*m/z* 460, 510) and hydroxy-oxo-cholenoic acid (*m/z* 444, 494), however the elevation of cholic and chenodeoxycholic conjugates always exceeded the excretion of (di)-hydroxy-oxo-cholenoic acid conjugates (Table 3).

In patient AS with suspected biliary atresia excretion of glyco-(di)-hydroxy-oxo-cholenoic acid conjugates was elevated (Table 3). However, conjugates of cholic acid and chenodeoxycholic acid were very low for an infant with cholestasis. As some aspects of biliary atresia such

as absent gall bladder upon ultrasound and missing excretion of bile in the gut have been described previously in patients with 5 $\beta$ -reductase deficiency, we performed mutation analysis of the *AKR1D1* gene, which was normal, excluding 5 $\beta$ -reductase deficiency. Similarly, no mutations were detected in the 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase gene (*HSD3B7*).

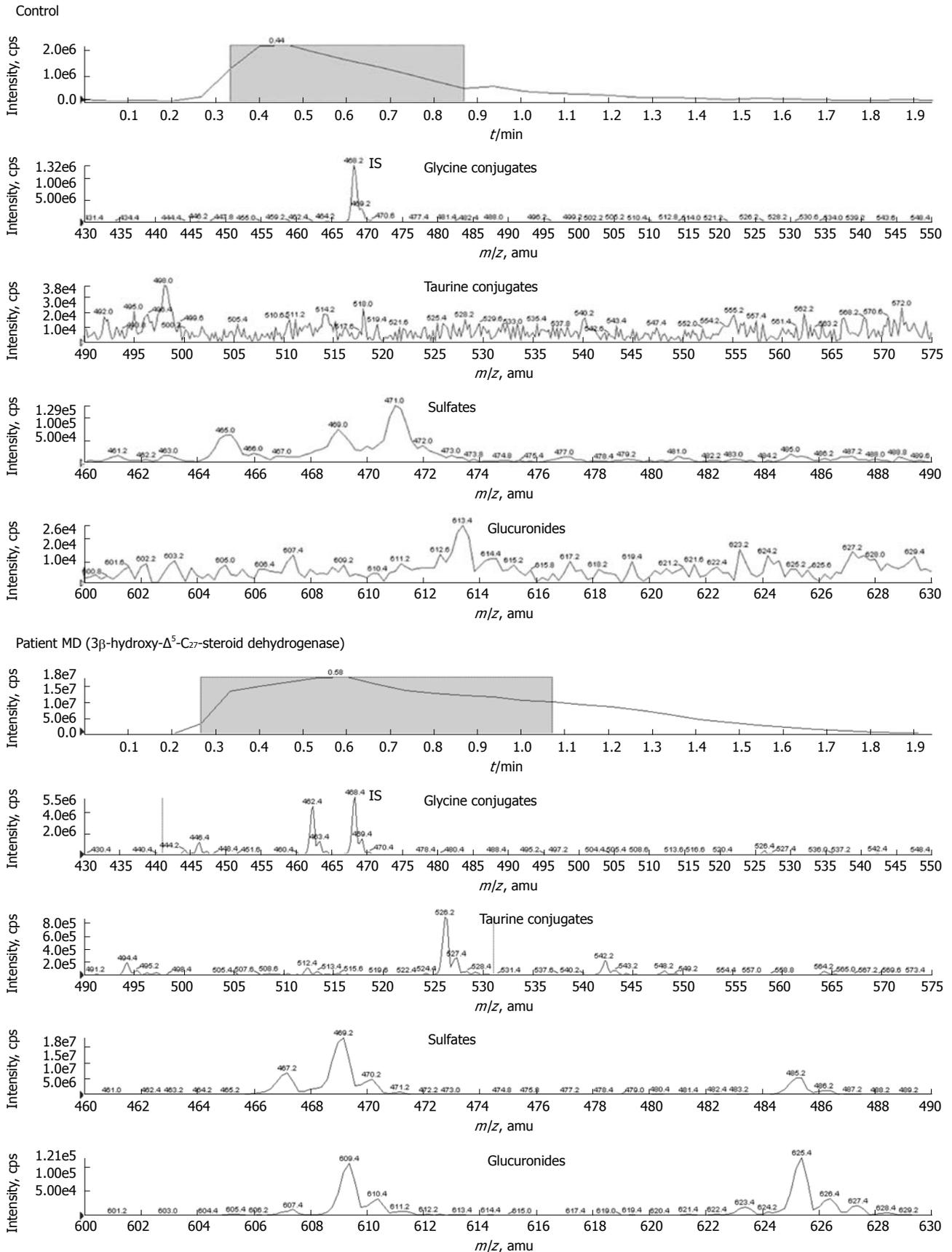
A quite similar cholanoic profile with low amounts of cholic and chenodeoxycholic acid conjugates was found in another patient, MK, a 7 mo old patient with failure to thrive and severe cholestatic liver disease (Table 3). In this patient cystic fibrosis was confirmed by an abnormal sweat test, abnormal ion transport in rectal epithelial cells and heterozygosity for the mutation  $\Delta$ F508 in the *CFTR* gene. Supplementation with ursodeoxycholic acid, pancreatic enzymes and fat-soluble vitamins completely normalized the cholanoic profile.

**3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency-patient MD:** Analysis of urinary bile acid metabolites in patient MD revealed large quantities of sulphate conjugates of di- and trihydroxy-5-cholenoic acid metabolites characterized by *m/z* 469 and 485, respectively. Glycine conjugates of di- and trihydroxy-5-cholenoic acid (*m/z* 526 and 542) were less pronounced, but clearly increased compared to controls (Figure 2). Peaks attributable to the glycine and taurine conjugates of chenodeoxycholic acid and cholic acid (*m/z* 448, 464, 498 and 514, respectively) were small or present at near baseline levels. This constellation of metabolites is characteristic of 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency.

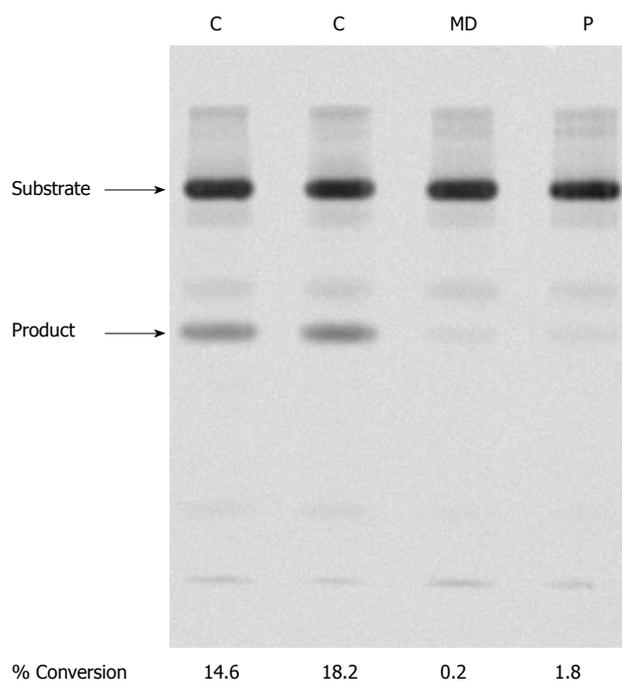
Mutation analysis of the *HSD3B7* gene showed homozygosity for a single base pair mutation (c.605G > C, GenBank Accession #AF277719) in exon 5 resulting in an amino acid substitution (p.G196A). This mutation has not been described before but has not been found in > 50 control DNAs.

To examine if this mutation affected enzyme activity we determined 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase activity in the patient's fibroblasts (Figure 3) as described in materials and methods. In control fibroblasts (C) two clear sterols were visualised after TLC developed under standard conditions. These sterols were identified by gas chromatography mass spectrometry as the substrate, 7 $\alpha$ -hydroxycholesterol and the product, 7 $\alpha$ -hydroxy-4-cholesten-3-one of the 3 $\beta$ -HSD enzyme. In fibroblasts of patient MD, little or no product was formed, indicative of severely reduced 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase activity. As a negative control, unaffected fibroblasts were incubated with substrate in the absence of NAD and the reaction subjected to TLC. In this case no product was observed on the silica plate (results not shown).

**Cerebrotendinous xanthomatosis-patient KS and others:** In patient KS analysis of urinary bile acid metabolites showed increased excretion of glucuronide-5 $\beta$ -cholestane-pentole (*m/z* 627) and less pronounced excretion of glucuronide-5 $\beta$ -cholestane-tetrole (*m/z* 611). Mutation



**Figure 2** Precursor ion spectra of urinary bile acid metabolites from patient MD with 3β-hydroxy-Δ<sup>5</sup>-C<sub>27</sub>-steroid dehydrogenase deficiency and control. The strongest signals were identified as sulphate conjugates of di- and trihydroxy-5-cholenoic acid scanning with *m/z* 469 and 485. Glycine conjugates of di- and trihydroxy-5-cholenoic acid (*m/z* 526 and 542) were less pronounced, but also increased.



**Figure 3** 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase activity in patient fibroblasts. In control cells (C) two sterols were observed after thin layer chromatography. These compounds were identified by gas chromatography mass spectrometry as the substrate, 7 $\alpha$ -hydroxycholesterol and product, 7 $\alpha$ -hydroxy-4-cholesten-3-one, of 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase. In fibroblasts of patient MD and another patient with known 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency (P), little or no 7 $\alpha$ -hydroxy-4-cholesten-3-one was formed.

analysis of the *CYP27A1* gene revealed compound heterozygosity for c.IVS6-1A and c.1213C > T (p.R405W) (University Medical Center Nijmegen, Department of Human Genetics, DNA-Diagnostics). These are known pathogenic mutations for cerebrotendinous xanthomatosis<sup>[20]</sup>.

Additionally we found elevated concentrations of 5 $\beta$ -cholestane-tetrol glucuronides (mean excretion 4, normal < 1.6) and -pentol glucuronides (mean excretion 28, normal < 4.3) in three adults unrelated to KS and to each other with previously confirmed cerebrotendinous xanthomatosis.

### Peroxisomal disorders

We analyzed urinary bile acid metabolites in 21 infants with suspected peroxisome biogenesis defects. All patients had profound neurological abnormalities and clearly elevated concentrations of hexacosanoic acid (C26:0). In 16 patients, tauro-tetra-hydroxycholestanic ( $m/z$  572) was elevated, consistent with a peroxisome biogenesis defect. Of these patients 12 additionally showed an elevation of tauro-tri-hydroxycholestanic acid ( $m/z$  556), but this increase was always lower than that measured for tauro-tetra-hydroxycholestanic acid. In two patients there was no elevation of tauro-tetra-hydroxycholestanic acid but increases of tauro-tetra-hydroxy-24-ene-cholestanic acid ( $m/z$  570) and tauro-penta-hydroxy-24-ene-cholestanic acid ( $m/z$  586) were detected, pointing to D-bifunctional protein deficiency (*HSD17B4* gene).

In a previously described 45-year old patient (HPH)<sup>[11]</sup> with dystonic head tremor, spasmodic torticollis and leucoencephalopathy in MR imaging we found increased excretion of 27-nor-5 $\beta$ -cholestanepentol-glucuronide ( $m/z$  613) and 27-nor-5 $\beta$ -cholestanhexol-glucuronide ( $m/z$  629). This patient was found to suffer from a defect of peroxisomal sterol carrier protein X (SCPx)<sup>[11]</sup>.

### DISCUSSION

Defects of bile acid synthesis can present with a wide variety of clinical symptoms from early infancy to adulthood. Cholestatic liver disease, which is often described as the leading symptom, may not be evident as in patient MD in the present cohort. Instead impaired absorption of fat-soluble vitamins may result in a multisystem disorder with predominantly neurological symptoms in early childhood. It is likely that vitamin E deficiency contributed to the ataxia seen in MD; spinocerebellar ataxia is a common feature of vitamin E deficiency (as seen in untreated abetalipoproteinemia). Myopathy has also been reported in vitamin E deficiency in both man and experimental animals.

Cataracts are often the first sign of cerebrotendinous xanthomatosis in school children and adolescents, with xanthomas and ataxia usually occurring later in life. Treatment with chenodeoxycholic acid positively influences neurological disease and avoids development of xanthomata and arteriosclerosis<sup>[21]</sup>, therefore determination of bile acid metabolites and sterols should be included in the diagnostic workup of cataract in this age group.

In patients with peroxisomal disorders determination of bile acid metabolites is helpful in distinguishing between defects of peroxisome biogenesis versus single enzyme deficiencies. An elevated excretion of tauro-tetrahydroxy-cholestanic acid is highly suggestive of a defect in peroxisome biogenesis whereas increased excretion of tauro-tetra-hydroxy-24-ene-cholestanic acid and tauro-penta-hydroxy-24-ene-cholestanic acid point to a D-bifunctional protein defect. It is important to note that a normal bile acid profile does not exclude peroxisome biogenesis disorders as some patients may present without urinary bile acid abnormalities. Importantly bile acid analysis may elucidate peroxisomal defects with predominantly neurological presentations as in peroxisomal SCPx or  $\alpha$ -methyl-acyl-CoA racemase deficiencies, in which analysis of very long-chain fatty acids is not of diagnostic value<sup>[11]</sup>.

Earlier studies<sup>[22]</sup> have indicated that non-specific cholestatic liver disease may result in elevated excretion of hydroxy- and dihydroxy-oxo-choleonic acids mimicking 5 $\beta$ -reductase deficiency. However, non-specific cholestatic liver disease can usually be distinguished from 5 $\beta$ -reductase deficiency by the finding of elevated urinary concentrations of the primary bile acids cholic acid and chenodeoxycholic acid. From our study cohort 2 examples of patients with elevated hydroxy- and dihydroxy-oxo-choleonic acids and low primary bile acids are de-

scribed, one presenting with biliary atresia (patient AS) and the other (patient MK) with cystic fibrosis, cholestasis and severe malabsorption.

Since one patient with 5 $\beta$ -reductase deficiency had a clinical presentation resembling biliary atresia 5 $\beta$ -reductase deficiency should be considered in the differential diagnosis of biliary atresia<sup>[19]</sup>. Although liver transplantation is curative for both biliary atresia and 5 $\beta$ -reductase deficiency, it is crucial to define the molecular basis of the diseases in these individuals prior to counselling of the parents regarding recurrence risk in further pregnancies. In our patient AS, mutation analysis of the 5 $\beta$ -reductase gene (*AKR1D1*) excluded a deficiency in bile acid synthesis leaving biliary atresia as the most likely diagnosis, so that she subsequently underwent successful liver transplantation.

In patient MK, the common  $\Delta$ F508 mutation was detected in the *CFTR* gene, and this individual experienced a normalization of her urinary cholanoic profile after supplementation with pancreatic enzymes, vitamins and ursodesoxycholic acid.

The mechanism underlying the aberrant cholanoic profiles in patients AS and MK is not clear. There may be secondary inhibition of 5 $\beta$ -reductase by an as yet undefined substance occurring in both conditions.

Because of the clinical variability associated with atypical presentations, as in the patients described in this article, the availability of a quick and reliable method for determination of bile acid metabolites is essential in the diagnostic work-up. The ESI-MS/MS method described is fast and reliable. However, in some patients cholanoic profiles suggesting 5 $\beta$ -reductase deficiency are obtained. In these cases mutation analysis of the *AKR1D1* gene is necessary. If 5 $\beta$ -reductase deficiency is excluded cystic fibrosis or biliary atresia should be considered in the differential diagnosis.

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## COMMENTS

### Background

Bile acid synthesis defects can result in progressive cholestatic liver disease and neurological disease. They can be treated effectively with bile acid supplementation when determined early but result in permanent liver failure requiring liver transplantation or irreversible neurological damage when diagnosed at a late stage. Therefore knowledge of clinical symptoms and early diagnosis are important.

### Research frontiers

Analysis of urinary bile acids by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) is the method of choice to screen for bile acid synthesis defects. However, patients with liver disease of other origin may show similar patterns of bile acid metabolites. Moreover patients with disorders of bile acid synthesis may present with atypical clinical symptoms.

## Innovations and breakthroughs

In the present study authors analyzed urinary bile acid metabolites in a large number of patients, in whom bile acid synthesis defects were suspected. In patients with abnormal bile acid profiles authors performed complete biochemical and molecular diagnostic workup. The pitfalls of the ESI-MS/MS method were evaluated and differential diagnoses discussed.

## Applications

To expand the knowledge about clinical presentation of bile acid synthesis defects and the differential diagnoses. To be aware of pitfalls of the ESI-MS/MS method and further diagnostic procedures to confirm or exclude defects of bile acid synthesis.

## Peer review

This work is well written. Although the group of patients with bile acid synthesis defects is small, it is important to make the clinical diagnosis. This paper will be particularly interesting for pediatricians.

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## Evaluation of acoustic radiation force impulse imaging for determination of liver stiffness using transient elastography as a reference

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### Abstract

**AIM:** To evaluate cut-off values and performance of acoustic radiation force impulse imaging (ARFI) using transient elastography [FibroScan® (FS)] as a reference.

**METHODS:** Six hundred and six patients were enrolled in this study. All patients underwent liver stiffness measurement with FS (FS-LS) and ARFI (with shear wave velocity quantification; ARFI-SWV) and the performance of ARFI in comparison to FS was determined. Sixty-eight patients underwent liver biopsy.

**RESULTS:** Significantly higher success rates for the determination of liver stiffness were found using ARFI

as compared to FS [604/606 (99.7%) vs 482/606 (79.5%),  $P < 0.001$ ]. ARFI-SWV correlated significantly with FS-LS ( $r = 0.920$ ,  $P < 0.001$ ). ARFI-SWV increased significantly with the stage of fibrosis ( $1.09 \pm 0.13$  m/s for patients with no significant fibrosis (FS-LS  $< 7.6$  kPa);  $1.46 \pm 0.27$  m/s for patients with significant liver fibrosis ( $7.6 < \text{FS-LS} \leq 13.0$  kPa); and  $2.55 \pm 0.77$  m/s for patients with liver cirrhosis (FS-LS  $> 13.0$  kPa)). ARFI-SWV cut-off values were identified for no significant fibrosis (1.29 m/s; sensitivity 91.4% and specificity 92.6%) and for liver cirrhosis (1.60 m/s; sensitivity 92.3% and specificity 96.5%). The optimal cut-off value for predicting liver fibrosis ( $F \geq 2$ ) was 1.32 m/s (sensitivity 87.0% and specificity 80.0%) and for liver cirrhosis (F4) 1.62 m/s (sensitivity 100% and specificity 85.7%), for patients who underwent liver biopsy. An excellent inter- and intraobserver reproducibility was observed for ARFI-SWV determinations.

**CONCLUSION:** An ARFI-SWV cut-off value of 1.29 m/s seems to be optimal for patients with no significant liver fibrosis and 1.60 m/s for patients with liver cirrhosis.

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**Key words:** Acoustic radiation force impulse imaging; Elastography; Fibroscan; Liver

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## INTRODUCTION

Liver biopsy is currently considered the gold standard for assessing hepatic fibrosis or cirrhosis<sup>[1]</sup>. However, it is an invasive procedure with rare but potentially life threatening complications. In addition, the accuracy of liver biopsy for assessment of fibrosis may suffer from sampling errors and interobserver variability<sup>[2-6]</sup>. Therefore, research has been focused on the evaluation of non-invasive methods for assessment of liver fibrosis, such as routine biological and hematologic tests, surrogate serum fibrosis markers and measurement of liver elasticity<sup>[7-11]</sup>. Considerable experience exists for transient elastography<sup>[12]</sup>. Transient elastography [FibroScan<sup>®</sup> (FS)] is a rapid, non-invasive, and reproducible method for measuring liver stiffness (FS-LS). A strong association between FS-LS and the degree of liver fibrosis was demonstrated in patients with chronic hepatitis<sup>[13-15]</sup>. A cut-off value of 13 kPa has been established for the discrimination between liver fibrosis and cirrhosis<sup>[13]</sup>. For discrimination of fibrosis from no significant fibrosis a cut-off value of 7.6 kPa was suggested<sup>[16]</sup>. However, FS is limited in patients with ascites or a body mass index above 28 kg/m<sup>2</sup><sup>[17]</sup>. Further limitations of FS have been described in several studies<sup>[18-21]</sup>.

Another noninvasive tool for the detection of liver fibrosis is the acoustic radiation force impulse (ARFI) imaging technology<sup>[22-24]</sup>. ARFI imaging has been incorporated into a conventional ultrasonographic (US) device (Acuson S2000; Siemens Medical Solutions, Mountain View, CA, United States). ARFI imaging technology involves the mechanical excitation of tissue using short-duration acoustic pulses in a region of interest, producing shear waves that spread away from the region of interest<sup>[25-27]</sup>. By recording the shear wave-front and correlating these measurements with the elapsed time, the shear wave velocity-SWV (m/s) can be quantified (ARFI-SWV). The SWV increases with stiffness. Thus, the measured SWV is an intrinsic and reproducible property of the tissue<sup>[28-30]</sup>. A significant correlation between ARFI imaging, serum fibrosis marker tests, and the histologic fibrosis stage was reported in a few pilot studies<sup>[31-33]</sup>.

In this study, we compared FS-LS with ARFI-SWV. Using the known cut-off values of 7.6 kPa and 13 kPa for FS we established cut-off values for ARFI-SWV for discriminating no significant liver fibrosis from significant liver fibrosis and significant liver fibrosis from liver cirrhosis, respectively. Furthermore, inter- and intraobserver reproducibility was studied for ARFI.

## MATERIALS AND METHODS

### Patients

This study was approved by the ethics committee of Heinrich Heine University. A total of 606 patients who had consulted the Hepatology Unit of the University Hospital Düsseldorf, Germany were included in this study. Aetiology of the liver disease was determined according to standard diagnostic criteria. Patients' characteristics and aetiologies of liver diseases are shown in Table 1.

**Table 1** Characteristics of patients at the time of liver stiffness measurement (*n* = 606)

Patients, <i>n</i>	606
Male, <i>n</i> (%)	363 (59.9%)
Age (yr)	53 ± 17
ALT (IU/L)	59 ± 159
AST (IU/L)	53 ± 142
GGT (IU/L)	104 ± 218
AP (IU/L)	97 ± 84
Total bilirubin (mg/dL)	1.1 ± 0.4
Prothrombin time (% of normal)	96 ± 22
Chronic liver diseases, <i>n</i> (%)	
Non-alcoholic steatohepatitis	236 (38.9%)
Chronic hepatitis B	48 (7.9%)
Chronic hepatitis C	97 (16.0%)
Alcoholic liver disease	52 (8.6%)
Autoimmune hepatitis	18 (3.0%)
PBC/PSC	12 (2.0%)
Others	14 (2.3%)
Healthy controls	129 (21.3%)
Liver biopsy available, <i>n</i> (%)	68 (11.2%)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT:  $\gamma$ -glutamyl-transpeptidase; AP: Alkaline phosphatases; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

### Liver stiffness measured by FibroScan

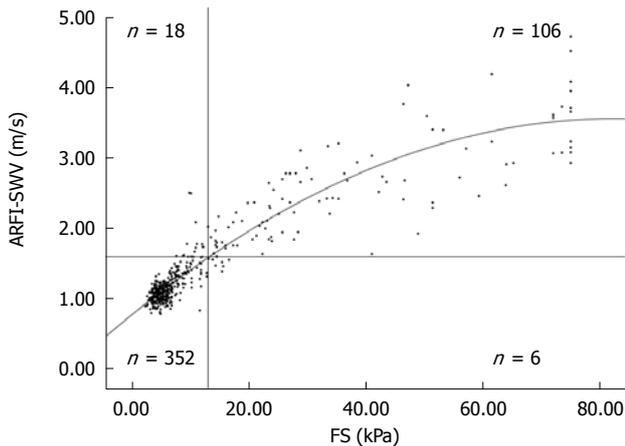
Details of the technical background and examination procedure have been described previously<sup>[12]</sup>. The tip of the probe transducer was placed on the skin between the ribs over the right liver lobe. The measurement depth was between 25 mm and 65 mm below the skin surface. Ten measurements were obtained in each patient. Determination of liver stiffness was considered valid, when a success rate of at least 60% was obtained. The results were expressed in kPa. The median value was taken as representative.

### Acoustic radiation force impulse-shear wave velocity determination

In all patients, ARFI imaging (Acuson S2000, Virtual Touch Tissue Quantification mode) and transient elastography (TE; FibroScan; Echosens, Paris, France) were performed on the same day. The examination was performed in the right lobe of the liver, through the intercostal space, at the same site as the transient elastography measurement. A measurement depth of 2 cm below the liver capsule was chosen to standardize the examination for ARFI-SWV. The mean value of ten measurements was taken as representative.

### Liver histology and quantification of liver fibrosis

A subgroup of 68 patients underwent liver biopsy in the previous six months. Patients with histological proven liver cirrhosis in the previous two years were also included. Liver biopsy specimens were fixed in formalin and embedded in paraffin. Liver fibrosis was evaluated semiquantitatively according to the METAVIR scoring system. Fibrosis was staged on a 0-4 scale as follows: F0: No fibrosis; F1: Portal fibrosis without portal septa; F2: Portal fibrosis with few septa; F3: Numerous septa with-



**Figure 1 Correlation of acoustic radiation force impulse with FibroScan ( $r = 0.920$ ;  $P < 0.001$ ).** The vertical line represents the cut-off value of 13 kPa for FS and the horizontal line represents the cut-off value of 1.60 m/s for acoustic radiation force impulse ( $n = 482$ ); using the cut-off value of 1.60 for the discrimination of liver fibrosis from liver cirrhosis for acoustic radiation force impulse imaging shear wave velocity, 458 of the 482 patients (95.1%) were classified correctly. Six (1.2%) patients were classified false-negative (FS-LS  $\geq 13$  kPa and acoustic radiation force impulse imaging shear wave velocity  $< 1.60$  m/s), and 18 (3.7%) were classified false-positive (FS-LS  $< 13$  kPa and ARFI-SWV  $\geq 1.60$  m/s. FS: FibroScan®.

out cirrhosis; and F4: Cirrhosis.

### Statistical analysis

Data were entered in SPSS (version 19.0, Inc., Munich, Germany). A  $\chi^2$  or Fisher's exact test ( $F$ -test) was used to compare categorical variables, and a Mann-Whitney test was used for the comparison of continuous variables. The significance level was set at 0.05, and all  $P$  values were two-tailed. A Pearson's test was performed to study the correlation between FS-LS and ARFI-SWV.

For no significant fibrosis (FS  $\leq 7.6$  kPa) and liver cirrhosis (FS  $> 13.0$  kPa), the diagnostic performance of ARFI was assessed using receiver operating characteristic (ROC) curves. 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 area under the ROC curve (AUROC). AUROC-values close to 1.0 indicated high diagnostic accuracy. ROC curves were generated for patients with FS  $\leq 7.6$  kPa, and patients with FS  $> 13$  kPa. Optimal cut-off values for ARFI were chosen to maximize the sum of sensitivity and specificity, positive and negative predictive values were computed for these cut-off values. Using this analysis SWV cut-off values were identified for patients with no significant fibrosis (FS  $\leq 7.6$  kPa) and patients with liver cirrhosis (FS  $> 13.0$  kPa).

Intraobserver and interobserver agreement was analysed using the intraclass correlation coefficient (ICC)<sup>[34]</sup>. ICC values range from +1 (100% agreement; all the variability being due to patient characteristics) to -1 (100% disagreement; all the variability being due to the rater's performance). Interobserver agreement was calculated as the agreement between the first liver ARFI measurements of the two observers. Intraobserver agreement was calcu-

lated as the agreement between the first and the second ARFI evaluation. The agreement of liver stiffness between the right liver lobe and left liver lobe was calculated using the ICC. Agreement was classified as poor (ICC = 0.00 to 0.20), fair to good (ICC = 0.40 to 0.75) or excellent (ICC = 0.75).

## RESULTS

A total of 606 patients were enrolled in this study. Their characteristics at the time of the FibroScan/ARFI examination are summarised in Table 1 (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1856085/table/tbl1/>). Aetiologies of chronic liver diseases were non-alcoholic steatohepatitis ( $n = 236$ ), hepatitis C virus ( $n = 97$ ) or hepatitis B virus infection ( $n = 48$ ), alcoholic liver disease ( $n = 52$ ), primary biliary cirrhosis/primary sclerosing cholangitis ( $n = 12$ ), autoimmune hepatitis ( $n = 18$ ), and others ( $n = 14$ ). In addition, another 129 patients without any liver diseases were included in the study.

### Comparison of success rates for acoustic radiation force impulse and FibroScan

FS-LS ranged from 2.3 kPa to 75.0 kPa (median 6.0 kPa) and ARFI-SWV ranged from 0.77 m/s to 4.72 m/s (mean  $1.5 \pm 0.77$  m/s). Mean depth of the area where ARFI-SWV measurement was performed was  $4.51 \pm 0.56$  cm. The overall success rate was  $77.8\% \pm 28.5\%$  for FS compared to  $93.3\% \pm 9.87\%$  for ARFI ( $P < 0.001$ ). A liver stiffness measurement success rate of 100% was observed in 262 (43.2%) patients by FS compared to 373 patients (61.6%,  $P < 0.001$ ) by ARFI.

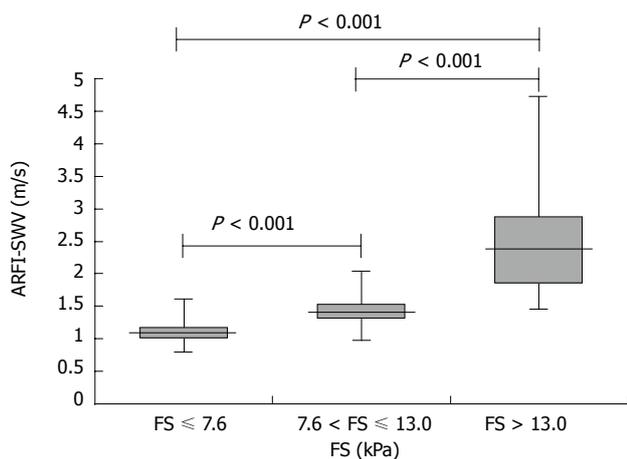
A valid liver stiffness determination (success rate of at least 60%) was observed in 482/606 (79.5%) by FS compared to 604/606 (99.7%) by ARFI ( $P < 0.001$ ). This difference was mostly due to a large distance between the skin surface and the liver capsule, which is associated with overweight. The success rate of FS was significantly dependent upon the distance between the skin surface and liver capsule (success rate 0%:  $3.27 \pm 0.34$  cm; success rate between 1% and 59%:  $2.81 \pm 0.56$  cm; and success rate  $\geq 60\%$ :  $2.41 \pm 0.52$  cm;  $P < 0.001$  for all differences).

After exclusion of all patients with an invalid liver stiffness determination (success rates below 60%) in one of the techniques, 482 patients remained for the following analysis.

### Correlation of acoustic radiation force impulse with FibroScan

To analyse the correlation between FS-LS and ARFI-SWV, a Pearson test was performed. There was a significant correlation between these two methods ( $P < 0.001$ ;  $r = 0.920$ ; Figure 1)

In consideration of the cut-off values for the different stages of liver fibrosis for FS, the following frequencies were observed: 297 (61.6 %) no significant fibrosis (FS-LS  $\leq 7.6$  kPa), 73 (15.2%) significant fibrosis ( $7.6$  kPa  $<$  FS-LS  $\leq 13.0$  kPa), and 112 (23.2%) cirrhosis (FS-LS  $> 13.0$  kPa). Mean ARFI-SWV was  $1.09 \pm 0.13$  m/s



**Figure 2** Acoustic radiation force impulse imaging shear wave velocity for the different stages of fibrosis. Box plots show median values with 25th and 75th percentiles of shear wave velocity determined by acoustic radiation force impulse. (FS ≤ 7.6 kPa: No significant fibrosis; 7.6 kPa < FS ≤ 13.0 kPa: Significant fibrosis; FS > 13.0 kPa: Liver cirrhosis).

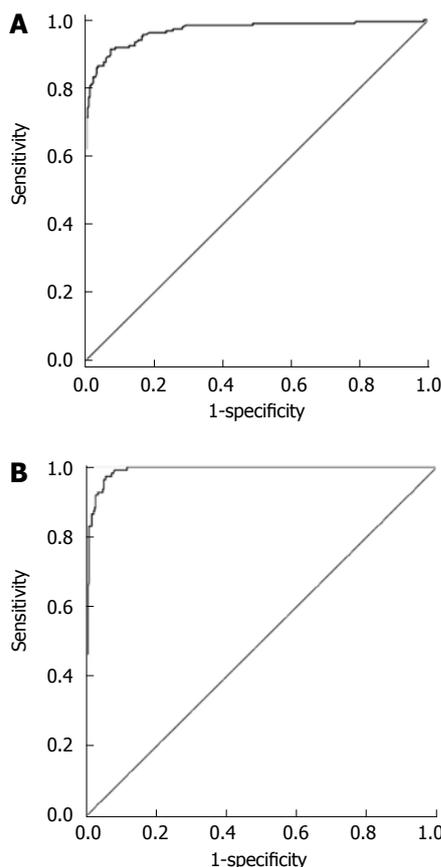
(range 0.80-1.61 m/s) for patients with no significant fibrosis (FS-LS < 7.6 kPa), compared to 1.44 ± 0.26 m/s (range 0.98-2.03 m/s) for patients with significant liver fibrosis, and 2.55 ± 0.77 m/s (range 1.47-4.72 m/s) for patients with liver cirrhosis. ARFI-SWV was significantly different between patients according to their fibrosis stage ( $P < 0.001$ ). Figure 2 shows box plots of ARFI-SWV for the three groups.

**Receiver operating characteristic analysis of acoustic radiation force impulse**

The diagnostic value (ROC curves) of liver stiffness measurement for patients with FS-LS < 7.6 kPa and patients with FS-LS > 13.0 kPa is shown in Figure 3. Corresponding AUROC values and 95% confidence intervals were 0.969 (95% CI: 0.952-0.985) for FS-LS < 7.6 kPa and 0.991 (95% CI: 0.985-0.997) for FS-LS > 13 kPa. Based on the ROC curves the optimal cut-off values for ARFI were chosen to maximize the sum of sensitivity and specificity. These cut-off levels were 1.29 m/s (sensitivity 91.4% and specificity 92.6% for FS-LS < 7.6 kPa) and 1.60 m/s (sensitivity 92.3% and specificity 96.5% for FS-LS > 13 kPa). The corresponding positive predictive value was 0.93 and the negative predictive value was 0.90 for FS-LS ≤ 7.6 kPa. When 1.60 m/s was chosen as the cut-off value for liver cirrhosis, the positive and negative predictive values were 0.85 and 0.98, respectively (Table 2).

Using these cut-off values for ARFI-SWV, 458 of the 482 patients (95.1%) were classified correctly. Six (1.2%) patients were classified false-negative (FS-LS ≥ 13 kPa and ARFI-SWV < 1.60 m/s), and 18 (3.7%) were classified false-positive (FS-LS < 13 kPa and ARFI-SWV ≥ 1.60 m/s; Figure 1).

After dividing the patients according to the aetiologies of chronic liver diseases [non-alcoholic steatohepatitis ( $n = 157$ ) vs others ( $n = 325$ )], the following cut-off values for ARFI were chosen (FS-LS > 13kPa): 1.52 m/s



**Figure 3** Receiver operator characteristic curve for acoustic radiation force impulse for the prediction of no significant fibrosis and liver cirrhosis ( $n = 482$ ). A: Receiver operator characteristic curve for acoustic radiation force impulse for the prediction of no significant fibrosis (FS < 7.6 kPa); B: Receiver operator characteristic curve for acoustic radiation force impulse for the prediction of liver cirrhosis (FS > 13.0 kPa).

for patients with non-alcoholic steatohepatitis (sensitivity 100% and specificity 96.6%; AUROC 0.990; 95% CI: 0.977-1.000) compared to 1.64 m/s for patients with liver diseases other than non-alcoholic steatohepatitis (sensitivity 94.2% and specificity 96.2%; AUROC 0.988; 95% CI: 0.980-0.996).

**Relationship between liver stiffness and liver histology**

68 patients underwent liver biopsy. A valid liver stiffness determination (success rate of at least 60%) was observed in 59/68 (86.8%) by FS compared to 68/68 (100%) by ARFI ( $P = 0.003$ ).

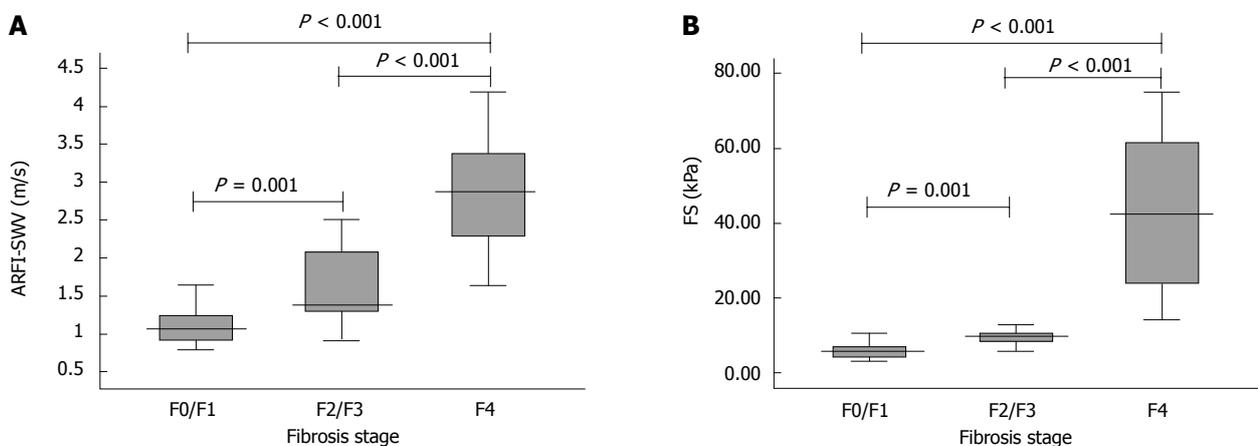
Liver stiffness measurements by ARFI ranged from 0.79 m/s to 4.17m/s. For patients without significant fibrosis ( $F \leq F1$ ,  $n = 23$ ), mean ARFI-SWV was 1.11 ± 0.24 m/s, 1.78 ± 0.88 m/s for patients with moderate fibrosis (F2 and F3;  $n = 17$ ), and 2.87 ± 0.76 m/s for liver cirrhosis (F4;  $n = 28$ ). Liver stiffness measured by ARFI was significantly different between patients according to their fibrosis stage ( $P = 0.001$  for  $F \leq F1$  vs F2/F3;  $P < 0.001$  for F2/F3 vs F4; and  $P < 0.001$  for  $F \leq F1$  vs F4; Figure 4A).

AUROC values and 95% confidence intervals were 0.934 (95% CI: 0.870-0.998) for liver cirrhosis (F4) and 0.929 (95% CI: 0.870-0.987) for  $F \leq F1$ . Based on the

**Table 2** Diagnostic accuracy of acoustic radiation force impulse imaging shear wave velocity and liver stiffness measured by transient elastography

	AUROC	Cut-off	PPV	NPV	Sensitivity (%)	Specificity (%)
Comparison to all patients [ARFI-SWV ( <i>n</i> = 482)]						
FS < 7.6 kPa	0.969	1.29 m/s	0.93	0.90	91.4	92.6
FS > 13.0 kPa	0.991	1.60 m/s	0.85	0.98	92.3	96.5
Comparison with liver biopsy [ARFI-SWV ( <i>n</i> = 68) and FS-LS ( <i>n</i> = 59)]						
Non significant liver fibrosis						
ARFI-SWV ( <i>n</i> = 23)	0.929	1.32 m/s	0.83	0.91	87.0	80.0
FS-LS ( <i>n</i> = 20)	0.920	7.6 kPa	0.85	0.95	94.9	85.0
Liver cirrhosis						
ARFI-SWV ( <i>n</i> = 28)	0.934	1.62 m/s	1.0	0.85	100	85.7
FS-LS ( <i>n</i> = 24)	0.958	13.0 kPa	1.0	0.91	100	91.4

AUROC: Area under the receiver operating characteristic curves; CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value; ARFI-SWV: Acoustic radiation force impulse imaging shear wave velocity; FS-LS: Liver stiffness measured by transient elastography.



**Figure 4** Acoustic radiation force impulse imaging shear wave velocity and liver stiffness measured by transient elastography for the different fibrosis stages in patients who underwent liver biopsy (F0/F1, F2/F3, and F4). A: ARFI-SWV for the different fibrosis stages in patients who underwent liver biopsy. Box plots show median values with 25th and 75th percentiles of shear wave velocity determined by ARFI (*n* = 68); B: FS-LS for the different fibrosis stages in patients who underwent liver biopsy (F0/F1, F2/F3, and F4). Box plots show median values with 25th and 75th percentiles of FS-LS (*n* = 59). ARFI-SWV: Acoustic radiation force impulse imaging shear wave velocity; FS-LS: Liver stiffness measured by transient elastography.

ROC curves, the discriminating cut-off values for ARFI were chosen to maximize the sum of sensitivity and specificity. These cut-off levels were 1.32 m/s for  $F \leq F1$  (sensitivity 87.0% and specificity 80.0%) and 1.62 m/s for liver cirrhosis (F4) (sensitivity 100% and specificity 85.7%). The corresponding positive predictive value was 0.83 and the negative predictive value was 0.91 for non-significant fibrosis and 1.0 and 0.85 for liver cirrhosis (Table 2).

Liver stiffness measurements by FS ranged from 2.9 kPa to 75 kPa. For patients without significant fibrosis ( $F \leq F1$ , *n* = 20), mean FS-LS was  $7.6 \pm 8.1$  kPa,  $13.8 \pm 17.1$  kPa for patients with moderate fibrosis (F2 and F3; *n* = 15), and  $42.6 \pm 20.5$  kPa for liver cirrhosis (F4; *n* = 24). Liver stiffness measured by FS was significantly different between patients according to their fibrosis stage ( $P = 0.001$  for  $F \leq F1$  vs F2/F3;  $P < 0.001$  for F2/F3 vs F4; and  $P < 0.001$  for  $F \leq F1$  vs F4; Figure 4B).

AUROC value for non-significant fibrosis (F0/F1) was 0.920 (95% CI: 0.841-0.998) with a sensitivity of 94.9% and specificity of 85.0% when 7.6 kPa was chosen

as the cut-off value. AUROC value and 95% confidence intervals were 0.958 (95% CI: 0.900-1.000) for liver cirrhosis (F4) with a sensitivity of 100% and specificity of 91.4% when 13.0 kPa was chosen as the cut-off value. The corresponding positive predictive value was 0.85 and the negative predictive value was 0.95 for non-significant liver fibrosis (F0/F1) and 1.0 and 0.91 for liver cirrhosis (F4; Table 2).

#### Inter- and intraobserver reproducibility; differences between the right and left liver lobes

In order to evaluate the reproducibility of ARFI-SWV measurements, 18 patients were examined repeatedly. For interobserver reproducibility, the patients were examined by two observers consecutively. An excellent agreement between the observers was found (ICC = 0.945; 95% CI: 0.844-0.981). For intraobserver reproducibility, one observer examined the patients twice directly in series. The intraobserver reproducibility was also excellent (ICC = 0.975; 95% CI: 0.906-0.993).

In order to study if there was a difference when ARFI

was performed in the right liver lobe or left liver lobe, 18 patients underwent measurement of ARFI-SWV in both liver lobes. The mean distance between skin surface and the liver capsule for the right and left liver lobes were  $2.24 \pm 0.52$  cm and  $2.54 \pm 0.62$  cm, respectively ( $P = 0.143$ ). ARFI-SWV did not differ significantly between the two liver lobes ( $1.36 \pm 0.41$  m/s *vs*  $1.51 \pm 0.53$  m/s,  $P = 0.143$ ). The agreement between both liver lobes, however, was moderate (ICC = 0.589; 95% CI: 0.135-0.851).

**Dynamics measured by FibroScan and acoustic radiation force impulse**

Fifty patients underwent liver stiffness measurement at least twice (mean  $\pm$  SD,  $2.3 \pm 1.2$ ). The mean interval between the two measurements was  $73.6 \pm 56.6$  d. FS-LS did not change in 24 patients, increased in 9 and decreased in 17 patients. ARFI-SWV changed in parallel to FS-LS. The behaviour of ARFI-SWV over time was assessed in 24 patients, in which FS-LS remained constant, increased or decreased over time (8 patients in each group) (Figure 5).

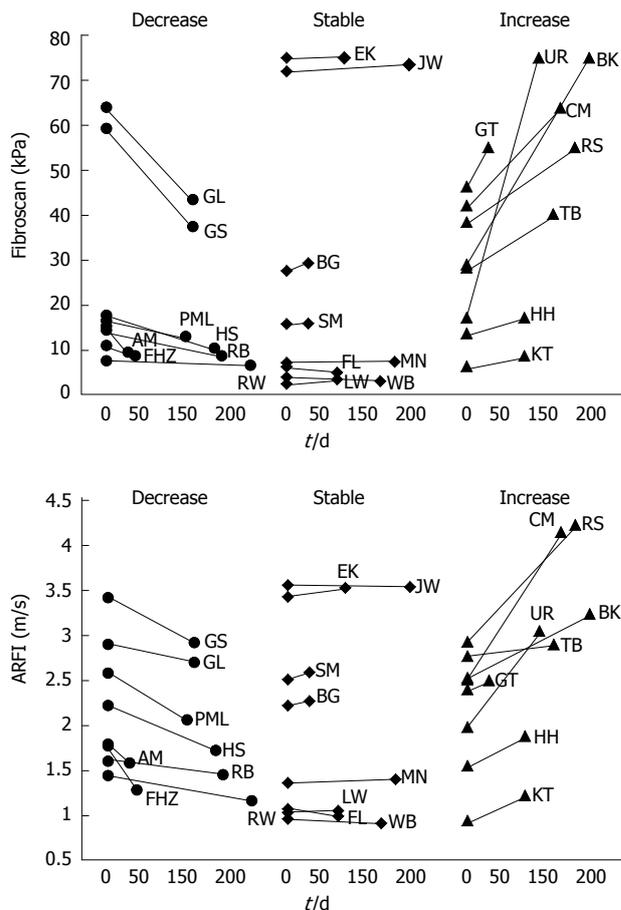
**DISCUSSION**

Liver biopsy is currently considered to be the gold standard for detection of liver fibrosis/cirrhosis, but is associated with serious complications<sup>[1]</sup>. On the other hand, cut-off values for FS-LS are available, which were evaluated in a meta-analysis including more than 8000 patients<sup>[16]</sup>. All these patients underwent liver biopsy, and these cut-off values were compared with the gold standard. Therefore, we used TE as the standard to evaluate ARFI technology. This allowed us to avoid any liver biopsy-associated complications.

The data in this study suggest that non-invasive fibrosis/cirrhosis assessed by measuring ARFI-SWV shows an excellent agreement with the established FS-LS measurements and can be successfully employed in patients, where FS-LS measurements were unsuccessful. Furthermore, as already reported for FS-LS determinations<sup>[35]</sup>, both intra- and interobserver variability were excellent for ARFI-SWV measurements.

A significantly higher success rate was observed for ARFI compared to FS ( $78.6\%$  *vs*  $99.8\%$ ,  $P < 0.001$ ). A significant inverse relationship between the success rate by FS and the distance between the skin surface and liver capsule was observed. ARFI is less dependent on this factor. Another advantage is that ARFI is performed under the control of conventional B-mode sonography. The observer can select and place the region of interest under visual control.

Mean ARFI-SWV increased significantly with the stage of fibrosis [ $1.09 \pm 0.13$  m/s (range 0.80-1.61 m/s)] for patients with no significant fibrosis (FS-LS  $< 7.6$  kPa);  $1.44 \pm 0.26$  m/s (range 0.98-2.03 m/s) for patients with significant liver fibrosis ( $7.6 < \text{FS-LS} \leq 13.0$  kPa); and  $2.55 \pm 0.77$  m/s (range 1.47-4.72 m/s) for patients with liver cirrhosis ( $13.0 < \text{FS-LS}$ ).



**Figure 5** Behaviour of acoustic radiation force impulse imaging shear wave velocity over time in 24 patients, in which FS-LS remained constant, increased or decreased over time (8 patients in each group). FS-LS: Liver stiffness measured by transient elastography.

We defined cut-off values for patients with no significant fibrosis and patients with liver cirrhosis. They were chosen so that the sum of sensitivity and specificity was maximal. A cut-off value for ARFI-SWV of 1.29 m/s was associated with a sensitivity of 91.4% and specificity of 92.6% for patients with FS-LS  $< 7.6$  kPa and a cut-off value of 1.60 m/s for patients with FS-LS  $> 13.0$  kPa with a sensitivity of 92.3% and specificity of 96.5%. Both cut-off values indicated high diagnostic accuracy for no significant fibrosis or liver cirrhosis, respectively. These cut-off values were confirmed by the subgroup-analyses. The cut-off values identified in patients who underwent liver biopsy did not differ significantly from the cut-off values taken from the correlation between both liver stiffness measurements ( $1.29$  m/s *vs*  $1.32$  m/s for patients without significant liver fibrosis;  $1.60$  m/s *vs*  $1.62$  m/s for patients with liver cirrhosis).

Experience with ARFI is limited and there are only a few published studies on small numbers of patients<sup>[31-33,36-40]</sup>. The largest study published by Palmeri *et al*<sup>[36]</sup> included 172 patients with non-alcoholic fatty liver disease. The present study included patients with different liver diseases and used FS-LS cut-off values from a meta-analysis of studies which included patients with different liver dis-

eases<sup>[16]</sup>. In the above-mentioned studies, ARFI-SWV cut-off values between 1.30 m/s and 1.37 m/s were reported for no significant liver fibrosis and 1.75–2.00 m/s for liver cirrhosis<sup>[31,32,38,40]</sup>. These cut-off values differ from those determined in our study, and may be due to differences in the liver diseases studied, sample size and the sensitivities and specificities chosen. In our substudy, which included patients with non-alcoholic steatohepatitis, the cut-off value was lower than for other patients, which may be due to the softening effect of steatosis. This effect has been described previously<sup>[41]</sup>. While liver stiffness measurement by FS is possible only in the right liver lobe, measurement of liver stiffness by ARFI is practicable in both liver lobes. Agreement of the measured liver stiffness between both liver lobes was moderate, but should be investigated in a larger study population. Thus, at present ARFI should be performed in the right liver lobe. Dynamics in the liver stiffness measured by FS have been described previously<sup>[18,19,21]</sup>. A congruent behaviour of ARFI-SWV and FS-LS dynamics over time was observed.

In conclusion, ARFI-SWV correlated significantly with FS-LS. ARFI can be performed in a significantly higher proportion of patients compared to FS. The most important advantage of ARFI over FS is the visual control by B-mode sonography and the variable depth of the measurement. A cut-off value of 1.29 m/s seems to be optimal for no significant fibrosis and 1.60 m/s for liver cirrhosis. ARFI-SWV did not depend on the observer. The sensitivity and specificity for the detection of liver cirrhosis seems to be comparable for both methods when liver biopsy is taken as the reference.

## COMMENTS

### Background

Liver biopsy is currently considered the gold standard for assessing hepatic fibrosis or cirrhosis, but is associated with complications. Thus, research has been focused on the evaluation of methods for the assessment of liver fibrosis. Transient elastography [FibroScan® (FS)] and acoustic radiation force impulse imaging (ARFI) are two methods used to detect liver fibrosis/cirrhosis.

### Research frontiers

A strong association between FS-LS and the degree of liver fibrosis was demonstrated in patients with chronic hepatitis. The experience with ARFI is limited. In this study the authors found a strong correlation between FS and ARFI. Using FS as a reference they evaluated cut-off values for ARFI. To evaluate the reproducibility of ARFI, an intra- and interobserver study was performed, without any significant results. It is known that liver stiffness shows a dynamic development, this point was also observed using ARFI.

### Innovations and breakthroughs

Non-invasive methods for the assessment of liver fibrosis are of great interest. FS and ARFI are two methods used to detect liver fibrosis/cirrhosis. The experience with ARFI is very limited. Significantly higher success rates for the determination of liver stiffness were found using ARFI as compared to FS. A strong correlation between liver stiffness measured by FS and ARFI was shown. An ARFI-SWV cut-off value of 1.29 m/s seems to be optimal for patients with no significant liver fibrosis and 1.60 m/s for patients with liver cirrhosis.

### Applications

ARFI is an additional non-invasive tool to detect liver fibrosis/cirrhosis. An ARFI-SWV cut-off value of 1.29 m/s seems to be optimal for patients with no significant liver fibrosis and 1.60 m/s for patients with liver cirrhosis.

### Terminology

FS and ARFI are two non-invasive methods to detect liver fibrosis/cirrhosis by

measuring liver stiffness. Both methods show a strong correlation between liver stiffness and the stage of liver fibrosis. These methods did not show any dependence on the observer. These tools can reduce the number of liver biopsies which is associated with complications.

### Peer review

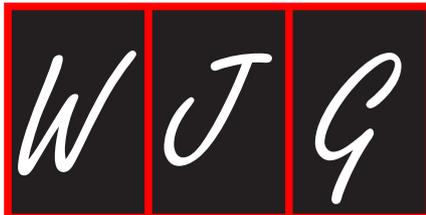
The authors reported the efficacy of acoustic radiation force impulse for determination of liver stiffness. The report is well written.

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## Framework for assessing quality of care for inflammatory bowel disease in Sweden

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### Abstract

**AIM:** To create and apply a framework for quality assessment and improvement in care for inflammatory bowel disease (IBD) patients.

**METHODS** A framework for quality assessment and improvement was created for IBD based on two generally acknowledged quality models. The model of Donabedian (Df) offers a logistical and productive perspective and the Clinical Value Compass (CVC) model adds a management and service perspective. The framework creates a pedagogical tool to understand the

balance between the dimensions of clinical care (CVC) and the components of clinical outcome (Df). The merged models create a framework of the care process dimensions as a whole, reflecting important parts of the IBD care delivery system in a local setting. Clinical and organizational quality measures were adopted from clinical experience and the literature and were integrated into the framework. Data were collected at the yearly check-up for 481 IBD patients during 2008. The application of the quality assessment framework was tested and evaluated in a local clinical IBD care setting in Jönköping County, Sweden.

**RESULTS:** The main outcome was the presentation of how locally-selected clinical quality measures, integrated into two complementary models to develop a framework, could be instrumental in assessing the quality of care delivered to patients with IBD. The selected quality measures of the framework noted less anemia in the population than previously reported, provided information about hospitalization rates and the few surgical procedures reported, and noted good access to the clinic.

**CONCLUSION:** The applied local quality framework was feasible and useful for assessing the quality of care delivered to IBD patients in a local setting.

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**Key words:** Quality measures; Inflammatory bowel disease; Value compass; Donabedian; Quality improvement

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## INTRODUCTION

In modern healthcare, there is often a gap between the expected level of healthcare delivery and the actual healthcare provided, as shown by McGlynn *et al*<sup>[1]</sup>. This is also true for the care of inflammatory bowel disease (IBD), as highlighted recently in an editorial by Siegel<sup>[2]</sup> and previously by Reddy *et al*<sup>[3]</sup> as well as by the American Gastroenterology Association<sup>[4]</sup> several years ago. There is still no framework or general quality measures for IBD as noted by Kappelman<sup>[5]</sup>, who called for action and challenged the gastroenterology community to correct this.

IBD is a chronic disease with two primary subtypes; Crohn's disease (CD), and ulcerative colitis (UC)<sup>[6]</sup>. The incidence of CD and UC in Sweden is approximately 6 and 15 per 100 000 inhabitants, respectively, and the prevalence is approximately 150 and 300 per 100 000, respectively<sup>[7]</sup>. Because of the early age at onset and the absence of curative treatment, the vast majority of patients require lifelong medical care, which periodically leads to intensive outpatient contact, hospitalizations, and occasionally surgery. Improved quality of care aims to minimize the symptoms of the disease, improve quality of life, and meet the goal of delivering the best possible value of care to the patient<sup>[8]</sup>. These targets are well captured in the Institute of Medicine's mnemonic, stressing the need for safe, timely, efficient, evidence-based, effective, and patient-centered care (STEEEP)<sup>[9]</sup>.

During the first years of the new millennium, the structure of care for IBD patients within the Gastroenterology Unit at the Department of Internal Medicine, Highland Hospital, Eksjö, Sweden was significantly redesigned as previously reported<sup>[10,11]</sup>. Along with the redesign, the need to be able to monitor the changes and the quality of care became obvious. Obvious also was the absence of any known framework and quality measures for the assessment of quality of care for IBD. To bridge this gap, a selection of clinical and organizational parameters were integrated into two generally acknowledged quality models adopted from Donabedian (Df)<sup>[12]</sup> and the Clinical Value Compass (CVC)<sup>[13]</sup>, and were merged to form a quality framework. The collection of quality measures was accomplished as a part of the ordinary yearly check performed by a specialist nurse or by a gastroenterologist. The selected measures were integrated and applied to the quality framework as a means to assess the quality of IBD care in the local setting.

A quality assessment tool may be developed in several ways, and there are several critical steps when creating a quality framework; these include design, implementation,

and utilization. Each of these factors must be addressed before the framework can be used. Furthermore, before the process of introducing a framework begins, insight into the complexity of care, an understanding of the systems used, and sound professional knowledge, all coupled with both enthusiasm and leadership, are required<sup>[8,14,15]</sup>.

In this study, two generally acknowledged quality models were used. The first, according to Df<sup>[12]</sup>, has been discussed previously by Kappelman *et al*<sup>[5]</sup> and testing on IBD care was suggested. Donabedian advises that the following questions are to be asked before using a quality framework<sup>[12]</sup>: "who and what activities are to be assessed"; "how are these activities supposed to be conducted"; and "what are they to accomplish?" These are all important questions to raise and are possible to apply to health care institutions. The model according to Donabedian derives the quality of care from the components of structure, process, and outcome. Structure denotes the attributes of the setting and includes the facilities, equipment, human resources, and organizational structure. Processes are defined by what is actually done in delivering and receiving care. Furthermore, outcome denotes the effects of care on the health status of patients and populations, conveys a production management perspective, and frames a delivery-focused approach by the organization.

The second model is the CVC<sup>[13]</sup>. It was derived from a management customer area, and offers a flexible framework where the outcomes of health care are perceived in four dimensions as follows: (1) functional; (2) **economic**; (3) satisfaction with health care; and (4) **clinical outcome**. The use of already existing measures is favored to avoid add-on routines, making it possible to fulfill the intertwined assignment to both manage the patient and improve care by measuring outcomes<sup>[16]</sup>.

The Df offers a logistical, productive perspective to the studied case, and the CVC adds a management and service perspective. The framework creates a pedagogical tool to understand the balance between the dimensions of clinical care (CVC) and the components of clinical outcome (Df). Together they create a framework of the care process dimensions as a whole, reflecting important parts of the IBD care delivery system in a local setting.

Quality measures are valuable means of improving clinical practice. The use of quality measures may be defined as the process of collecting, computing, and presenting quantified constructs for the managerial purposes of following up, monitoring, and improving organizational performance<sup>[17]</sup>. The basis of this argument is that they play a significant role in the coordination of organizational activity<sup>[18]</sup>, decision-making, prioritization<sup>[19]</sup>, comparisons, and initiation of improvement processes<sup>[20]</sup>. In every effort to measure the performance, it is important to consider the desired application of the information obtained. The application of the information may be to control, budget, motivate, or improve the care<sup>[21]</sup>. As part of the explorative case study, well established measures such as hemoglobin, quality of life, medication, and

access to care, which were practical to perform and used in daily clinical life, were chosen after a review of relevant literature and from clinical experience<sup>[22-24]</sup>.

The aim of this study was two fold; firstly, to apply a generally acknowledged quality framework to the assessment and improvement of care for IBD, and secondly, to study and evaluate its application in a local clinical IBD care setting in Jönköping County, Sweden.

## MATERIALS AND METHODS

The measures in this study originate from the Gastroenterological Unit responsible for all IBD care in the area, which is a part of the Department of Internal Medicine at the Highland Hospital in Eksjö, Jönköping County, Sweden. The unit includes an outpatient clinic, an inpatient ward with 15 beds, and an affiliated unit for endoscopic examinations<sup>[11]</sup>. The Highland health care system consists of eight health care centers for primary care, and the 280-bed Highland Hospital responsible for secondary and acute care, in all serving 110 000 inhabitants. The health care delivered is tax financed, and the county council functions both as insurer and provider of the care.

To date, no quality measures for IBD care have been generally approved. Feasible and practical quality measures were selected in order to evaluate the quality of care delivered within the local setting. The first act was to organize a registry with information, including patient addresses, diagnosis, disease duration, smoking habits, weight, and sex. Further information about the current prescribed medication and whether any surgical intervention had been performed was added to the files. Hemoglobin was chosen as the clinical marker to find anemia in the population, which may go undetected in many patients<sup>[25]</sup>. Further quality measures assessing the access to care<sup>[10]</sup> and quality of life (QoL) were chosen and integrated into the framework. Access was measured as the number of days from the referral being sent from the primary care physician until the patient received a scheduled consultation at the outpatient clinic, as well as the clinic's ability to offer an acute visit within two days after contact by a known patient. QoL was measured by using the short health scale (SHS)<sup>[26,27]</sup>. SHS is a questionnaire consisting of four questions about symptoms, function, worry and general health associated with the disease, reported on a 6-point graded likert scale. Patients were diagnosed according to clinical, endoscopic, and microscopic findings, and were sub-typed as having UC or CD. A senior gastroenterologist confirmed the diagnosis and registration of each patient. The status of the disease, i.e., subjectively experienced activity, was reported by the patients on the day of the annual check-up. Tumor surveillance colonoscopy was offered and performed according to guidelines for more than 95% of relevant patients. At the end of 2008, 481 patients were included in the local registry.

During the year, all patients were offered an annual

check-up, which was preceded by a letter including a quality of life questionnaire and instructions for laboratory testing (hemoglobin) that could be performed at any of the primary care centers. An important part of the annual check-up was to remind the patient to contact the nurse by telephone with any questions or worries raised during the remainder of the year. Reinforcing this opportunity for telephone access was aimed toward avoiding misdirected care for IBD to other care settings such as the Emergency Department. In the redesigned clinical model, there was also a guarantee that access to an unscheduled visit for acute symptoms would be available within two days at all times. Data was collected by the specialist nurse or gastroenterologist at the time of the check-up, and computed every quarter but presented once a year.

In Table 1 an overview of the definitions, quality dimensions and components, purposes behind the measures, operational definitions, and data sources of the quality measures are integrated into the two quality models together creating the framework.

### Ethical considerations

The ethical committee at the University of Linköping, Sweden, approved this study.

## RESULTS

The first main finding is the presentation regarding how locally-selected clinical quality measures, integrated into two complementary models to create a framework, could be instrumental in assessing the quality of care delivered to patients with IBD. Further, the second main finding is the results presented in Table 2 for the local IBD population using the framework. The data describe the epidemiology of a patient population in the local care setting for IBD. To be stressed is the fact that more than 95% of the patients with IBD in the area are cared for by our care unit. The incidence of IBD was slightly below the expected level according to Swedish data<sup>[7]</sup>. This is probably explained by the older age distribution in the studied rural area. The prevalence of anemia is less than previously reported. Medication is presented for Crohn's disease and ulcerative colitis. Immunosuppressive medication, cortisone and anti-TNF-alpha are prescribed more for Crohn's disease compared to ulcerative colitis. Further, 5-aminosalicylic acid is prescribed more for ulcerative colitis compared to Crohn's disease. Table 2 show good access to care. Few surgical interventions were performed over the year. Three patients with ulcerative colitis underwent colectomy and three patients with Crohn's disease underwent incisions due to fistulas or strictures. No tumor was found in the population. Data was not processed statistically for differences between groups.

In the years before 2008, an average of 75% of the registered patients had a complete annual check-up documented, i.e., a telephone call or a visit in combination with QoL and/or laboratory tests. In 2008, patients without

**Table 1** Overview of the quality framework presenting definitions, purposes, data sources and operational definitions for the adopted quality measures as well as properties of the applied models

	Characteristics of measures included in the framework					Properties of the models included in the framework	
	Quality measure	Definition of measure	Data source and data collection	Operational definition of measure	Purposes for the measure adapted from Behn	Quality dimension according to the clinical value compass	Quality components as part of the quality model of Donabedian
Patient data	Diagnosis	Inflammatory bowel disease	Local gastro registry	Crohn's disease and ulcerative colitis	Control, evaluation	Clinical dimension	Outcome
	Gender	Sex	Local gastro registry	female:male	Control, learning	Clinical dimension	Structure
	Age		Local gastro registry	Age [mean (SD)] range	Control, learning	Clinical dimension	Structure
	Disease duration	Début year	Local gastro registry	Years since time of diagnosis [mean (SD)] range	Control, evaluation	Clinical dimension	Outcome
Laboratory measures	Hemoglobin	Blood sample enabling detection of anemia associated with chronic disease, blood loss, or iron deficiency	Local gastro registry Tests were performed at the nearest primary care center and reported electronically	Cut-off points were defined as: mean (SD) normal $\geq 120$ g/L, anemia 100-119 g/L severe anemia $< 100$ g/L missing	Control, evaluation	Clinical dimension	Outcome
Medication	Prescribed medicine	Currently prescribed preventive medication	Local gastro registry	Prescribed medication: 5-ASA cortisone immunosuppressive anti-TNF- $\alpha$ no medication	Control, evaluation	Clinical dimension	Process
Surgical interventions	Incidence of surgery	Surgical interventions associated with IBD	ERS, searched for ICD codes for surgical interventions and IBD once a year	Type and numbers of surgical interventions: colectomy hemicolectomy loop ileostomy perianal/ fistula/ stricture incision revision abdominal scar	Evaluation	Clinical and cost dimension	Process
	Tumor incidence	Incidence of gastrointestinal tumors associated with IBD	Data from the national tumor registry retrieved once a year	Number and type of intestinal tumors associated with IBD according to diagnosis in records as ICD code	Evaluation	Clinical and cost dimension	Outcome
Quality of life	The Short Health Scale, SHS	SHS is a health related quality of life questionnaire consisting of four questions graded on a 6 point Likert scale.	Local gastro registry	Percent scoring 1 to 3 representing that the goal of the care was reached symptoms functioning worry wellbeing	Evaluation	Functional dimension	Outcome
Access to care	Waiting time	Referral from primary to secondary care	Local administrative data base	Number of days from the referral being sent from the primary care physician until the patient received a scheduled consultation at the outpatient clinic	Motivation, budget, learning, evaluation, promotion	A proxy for the satisfaction dimension	Process and outcome
	Waiting time for known patients	An acute visit is used for an urgent need of assessment due to deteriorating disease	Local administrative data base	The clinic's ability to offer an acute visit within two days after contact for known IBD patients	Motivation, budget, learning, evaluation, promote Learning	A proxy for the satisfaction dimension	Process
	Contact route (before being admitted to hospital)	The place for the decision to admit the patient for inpatient care, i.e. either at the ER or the outpatient clinic	ERS Contact route was decided after finding indicators such as: where the note was written, if the note was written by an on call colleague or a gastroenterologist	The ERS was searched to find out where the decision was either at the ER or from the outpatient clinic		Cost and a proxy for the satisfaction dimension	Process

Hospitalization	Hospitalization	Individual and total numbers of admittances for IBD patients	ERS was searched for ICD codes and national data was retrieved from the National Board of Health and Welfare	ERS documented ICD code for IBD and hospitalisation	Motivation, budget, evaluation	Cost dimension	Process and outcome
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IBD: Inflammatory bowel disease; ICD: International classification of diseases; ERS: Electronic record system.

**Table 2** Quality framework applied to the inflammatory bowel disease care setting at the Department of Internal Medicine in Highland Hospital, Eksjö, Jönköping County, Sweden

Quality measures from 2008		Crohn's disease	Ulcerative colitis
Patient data	Diagnosis	194	261
	Gender		
	Female:male	44%:56%	42%:58%
	Age (yr)		
	Mean (SD)	53 (± 15)	51 (± 15)
	Range	18-90	20-91
	Disease duration		
	Years since time of diagnosis		
	Mean (SD)	20 (± 13)	14 (± 10))
	Range	0-58	0-53
Laboratory measures	Hemoglobin		
	Mean (SD)	140 (± 12)	143 (± 13)
	Normal ≥ 120 g/L	95%	96%
	Anemia 100-119 g/L	4%	4%
	Severe anemia < 100 g/L	< 1%	0
	Missing	16%	17%
Medication	Prescribed medicine		
	5-ASA	43%	56%
	Cortisone	16%	4%
	Immunosuppressant	34%	12%
	Anti-TNF-α	8%	2%
	No medication	31%	40%
Surgical interventions	Incidence of surgery		
	Type and numbers of surgical interventions:		
	Colonectomy		3
	Hemicolectomy	1	1
	Loop ileostomy		1
	Perianal/fistula/stricture incision	3	1
	Revision abdominal scar	1	
	Tumor incidence		
	Number and type of intestinal tumors associated with IBD according to diagnosis in records as ICD code	0	0
Quality of life	The Short Health Scale, SHS		
	Percent scoring 1 to 3 representing that the goal of the care was reached		
	symptoms	95%	98%
	functioning	88%	95%
	worry	91%	94%
	wellbeing	97%	96%
Access to care	Waiting time		
	Number of days from the referral being sent from the primary care physician until the patient received a scheduled consultation at the outpatient clinic	< 3 wk	< 3 wk
	Waiting time for known patients		
	The clinic's ability to offer an acute visit within two days after contact for known IBD patients	< 2 d	< 2 d
	Contact route (before being admitted to hospital)		
	The ERS was searched to find out where the decision was either at the ER or from the outpatient clinic	50%/50%	50%/50%
Hospitalization	Hospitalization		
	ERS documented ICD code for IBD and hospitalisation	29	17

Data from the annual check-up 2008. IBD: Inflammatory bowel disease; ICD: International classification of diseases; 5-ASA: 5-aminosalicylic acid; ERS: Electronic record system; SHS: Short health scale.

complete annual check-ups were offered a new visit or telephone call at the end of the year. Using this approach,

98% (471/481) of the IBD population had a documented annual check-up during 2008. Of nine patients not receiving a check-up, four refrained from participating in the study and five were missing. One patient (with CD) was excluded from the study because of particularly severe disease demanding various levels of hospitalization on a more or less continuous basis.

## DISCUSSION

Quality improvement (QI) forms a link between the study of disease (science) and clinical care (management)<sup>[28]</sup> and provides better management of the planning, delivery, and assessment of care. The need for a general assessment tool for IBD care has been emphasized several times over a number of years<sup>[3,5]</sup>. This study is, to the best of our knowledge, one of the first to present how two generally acknowledged quality models<sup>[12,13]</sup> with integrated clinical quality measures can be applied as a quality framework and tested in clinical practice at a single center in an IBD population. The intent was to evaluate the quality of care delivered to a population of patients with IBD in the Highland health care area, Jönköping County, Sweden. Because there are few other frameworks currently available, there are problems with comparing results and usage, which needs to be done when future research is available.

The framework offers a map of the epidemiology of all patients affected by IBD in a local setting. This is a prerequisite and a foundation for any further analysis and improvement effort. Interesting results were found in the population as presented in the framework. Anemia is a well-known complication of IBD, caused by a combination of bone marrow suppression secondary to chronic inflammation and blood loss from intestinal bleeding. The reported prevalence of anemia from different IBD care settings and patient populations ranges from 9% to 74%<sup>[29]</sup>. In this study, anemia was detected in 4% of UC patients and 5% of CD patients, as shown in Table 1. Less than 1% had severe anemia. However, the mean hemoglobin for all study groups was comparable to that of a healthy control population. The detected prevalence of anemia has even improved compared to previous findings in the same population<sup>[10]</sup>. The clinic has used the findings of incipient anemia to offer extra visits to the outpatient clinic, and/or more thorough laboratory investigations to identify the reasons behind these findings. The analysis of hemoglobin is inexpensive, valid, and simple to perform. Treatment of anemia on an individual level is well established. Altogether, it is a feasible and useful finding to apply as a quality measure within a population.

Knowledge of how well guidelines for medication are implemented in an IBD patient population is sparse. The prescription pattern presented is in line with reports from centers in Norway<sup>[30,31]</sup> and Canada<sup>[32]</sup>. It provides an example of how quality measures can be directly related to guidelines and thus provides important information about the quality of care delivered<sup>[33]</sup>. The incidence and type of surgery is presented in Table

1. Surgical intervention rates were low in our study, and should be interpreted cautiously. The figures of access and number of hospital admittances for IBD could be used in future work as a benchmark for other clinics and as comparisons to national trends in Sweden or in America<sup>[34,35]</sup>.

The future use of the framework is associated with the way in which data retrieval could be improved. This could be done in several ways. One way would be to retrieve the data directly from the electronic medical record (EMR), and a second way would be to provide opportunities for the patient to deliver self-reported outcome measures directly into the EMR. In order to achieve this for additional quality measures, several steps are required. First, the suggested framework and measures need to be tested, discussed, and refined in a broader setting. Secondly, the measures need to be presented and followed as “real time” data on both an individual and a group/subgroup level in order to allow benchmarking. Thirdly, it should be possible to correlate quality measures with prescriptions, days off from work, and further changes in medication and/or treatment. An example of a “feed-forward” quality register<sup>[36]</sup> is already in place for patients with rheumatoid arthritis within the Swedish Rheumatoid Arthritis Registry (SRAR)<sup>[37,38]</sup>. In the SRAR register, it is possible to track individual patients as well as patient populations both locally and nationally and use this information to, for example, correlate their clinical status with the timing of newly prescribed biological drugs and days off from work<sup>[39]</sup>. The SRAR is regarded as one of the best quality registries in Sweden, and can serve as a model for future IBD registry work.

This study presents how locally-selected clinical quality measures, integrated into two complementary models to develop a framework, could be instrumental in assessing the quality of care delivered to patients with IBD. The selected quality measures noted less anemia in the population than previously reported, provided information about hospitalization rates and the few surgical procedures reported, and noted good access to the clinic. We believe that this approach of organizing and regularly utilizing data within our system is sustainable, and will enable future improvement in the quality and value of care for our IBD patients. We propose that the suggested framework and quality measures should be further tested, evaluated, and refined within the gastroenterological community.

## COMMENTS

### Background

In modern healthcare, there is often a gap between the expected level of healthcare delivery and the actual healthcare provided. This is also true for the care of inflammatory bowel disease (IBD) as highlighted by the American Gastroenterology Association. These stakeholders have called for action and challenged the gastroenterology community to find systems for quality assessment and improvement in IBD.

### Research frontiers

Since the publication “Crossing the Quality Chasm” by the Institute of Medicine in America on the brink of the new millennium, the urge to improve quality of care

has been one of the main focuses in health care research. Unfortunately, few publications connecting this area to IBD have been published since that time.

### Innovations and breakthroughs

The main outcome was the presentation of how locally-selected clinical quality measures, integrated into two complementary models to develop a framework, could be instrumental in assessing the quality of care delivered to patients with IBD. The selected quality measures of the framework noted less anemia in the population than previously reported, provided information about hospitalization rates and the few surgical procedures reported, and also noted good access to the clinic.

### Applications

The framework offers a map of the epidemiology of all patients affected by IBD in a local setting. This is a prerequisite and a foundation for any further analysis and improvement effort.

### Peer review

In this study, the authors created and applied a framework for quality assessment and improvement in IBD. They showed that the locally selected clinical quality measures, integrated into two complementary models to create a framework, could be instrumental in assessing the quality of care delivered to patients with IBD.

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## Autoimmune thyroid diseases and *Helicobacter pylori*: The correlation is present only in Graves's disease

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### Abstract

**AIM:** To investigate the correlation between autoimmune thyroid diseases (ATDs) and the prevalence of Cag-A positive strains of *Helicobacter pylori* (*H. pylori*) in stool samples.

**METHODS:** Authors investigated 112 consecutive Caucasian patients (48 females and 4 males with Graves' disease and 54 females and 6 males with Hashimoto's thyroiditis HT), at their first diagnosis of ATDs. Authors tested for *H. pylori* in stool samples using an amplified enzyme immunoassay and Cag-A in serum samples using an enzyme-linked immunoassay method (ELISA). The results were analyzed using the two-sided Fisher's exact test and the respective odds ratio (OR) was calculated.

**RESULTS:** A marked correlation was found between the presence of *H. pylori* ( $P \leq 0.0001$ , OR 6.3) and, in particular, Cag-A positive strains ( $P \leq 0.005$ , OR 5.3) in Graves' disease, but not in Hashimoto's thyroiditis,

where authors found only a correlation with Cag-A strains ( $P \leq 0.005$ , OR 8.73) but not when *H. pylori* was present.

**CONCLUSION:** The marked correlation between *H. pylori* and Cag-A, found in ATDs, could be dependent on the different expression of adhesion molecules in the gastric mucosa.

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**Key words:** Autoimmunity; Cag-A; Graves' disease; Hashimoto's thyroiditis; *Helicobacter pylori*; Hyperthyroidism; Hypothyroidism

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### INTRODUCTION

Autoimmune thyroid diseases (ATDs) are represented, essentially, by Hashimoto's thyroiditis (HT) and its variants (postpartum and sporadic thyroiditis), Graves' disease (GD) and atrophic thyroiditis<sup>[1]</sup>. A typical marker of HT and GD is the presence of autoantibodies against thyroglobulin (TgAbs), thyroperoxidase (TPOAbs) and thyrotropin receptor (TRAbs)<sup>[2]</sup>. Both genetic and environmental factors are involved in the pathogenesis of ATDs. Some bacteria and viruses are suspected of being able to mimic the antigenic profile on the thyroid

cell membrane, and play an important role in the onset of autoimmune diseases<sup>[3-6]</sup>. *Helicobacter pylori* (*H. pylori*) infection is found worldwide and has an incidence of up to 50% in the population of developed countries<sup>[7]</sup>. A cohort effect has been demonstrated for such infection, and a higher prevalence rate is found in the elderly and in males<sup>[8]</sup>. *H. pylori* is a gram-negative, motile bacterium, which typically colonizes and infects the gastric mucosa; the most virulent strains can usually be identified by the presence of the cytotoxin-associated gene A (Cag-A) antigen<sup>[9]</sup>. Therefore, the microorganism is responsible for gastric diseases such as gastritis, gastric/duodenal ulcers and carcinomas.

Several studies<sup>[10-12]</sup> have shown a positive correlation between the presence of *H. pylori* and HT, although others did not find such an association<sup>[13,14]</sup>. Moreover, we recently demonstrated a noteworthy correlation between *H. pylori* infection and GD, independent of hormonal status<sup>[15]</sup>.

The aim of this study was to investigate the prevalence of *H. pylori* in ATDs and, in particular, HT, to help clarify the controversial results observed in previous studies. We detected the presence of *H. pylori* in fresh stool samples from our patients using an enzyme immunoassay method, and Cag-A positivity using a serological test.

## MATERIALS AND METHODS

### ATDs patients

We studied 112 consecutive Caucasian patients (48 females and 4 males with GD and 54 females and 6 males with HT), at their first diagnosis of ATDs, enrolled over a period of 18 mo (from October 2008 to March 2010). The mean age ( $\pm$  SD) of the ATDs patients was  $49.7 \pm 6.6$  years ( $48.8 \pm 3.9$  years for GD patients and  $50.2 \pm 9.7$  years for HT patients). The study inclusion criteria were previously reported<sup>[15]</sup>. Briefly, these criteria included the absence of other diseases, a negative anamnesis for antimicrobial drugs use for at least three months and the absence of dyspeptic symptoms (epigastric pain, nausea, heartburn) or gastric diseases. The study was approved by the ethical committee of our institution and informed consent was obtained from each patient.

GD diagnosis was defined by hormonal hyperthyroidism [suppressed thyrotrophin (TSH), elevated FT3 and FT4], diffuse and high iodine capture on thyroid scintigraphy, and positive titers of TPOAbs, TgAbs and TRAbs. To eliminate possible bias between subclinical and frank primary hypothyroidism, HT diagnosis was defined by a cut-off value higher than 35 mU/mL TSH, low FT3 and FT4 values, positive titers of TPOAbs and TgAbs and hypoechogenicity pattern on echography (Table 1).

### Controls

The control population was composed of 100 body mass index-, socio-economic- and inclusion criteria class-matched individuals (90 females and 10 males, mean age  $49.0 \pm 4.5$  years, Table 1). All of these subjects showed

Table 1 Clinical characteristics of the investigated groups

Group	n	Sex female/male	Age (yr) (mean $\pm$ SD)	Smokers yes/no	Ophthalmopathy yes/no
Control	100	90/10	49.0 $\pm$ 4.5	44/56	-
GD	52	48/4	48.8 $\pm$ 3.9	21/31	34/18
HT	60	54/6	50.2 $\pm$ 9.7	26/34	0/60
ATDs	112	102/10	49.2 $\pm$ 6.9	47/65	34/112

No significant statistical differences in sex and age were present among the different groups. ATDs: Autoimmune thyroid diseases; HT: Hashimoto's thyroiditis; GD: Graves' disease.

normal TSH, FT3 and FT4 values with absent titers of TPOAbs, TgAbs and TRAbs.

### Study of the presence of *H. pylori* in stool samples

The tests were performed by laboratory technicians blinded to the subject's diagnosis. Fresh stool samples were obtained and tested using an amplified enzyme immunoassay for the detection of *H. pylori* antigens (Amplified IDEIA *H. pylori* StAR, Oxoid, United Kingdom). This test is highly specific for *H. pylori* antigens (sensitivity 95%, specificity 95%), with no cross-reactivity with other microorganisms. An absorbance value  $> 0.150$  using a dual wavelength (450/620 to 650 nanometers) was considered positive for the presence of *H. pylori*.

### Detection of Cag-A antibodies

Fresh serum samples were tested with the enzyme-linked immunoassay method (ELISA, Radim, Pomezia, Italy, sensitivity 93.7%, specificity 100%). Anti-Cag-A immunoglobulin-G values greater than 15 units/mL were regarded as Cag-A positive.

### Statistical analysis

The relationship between the different studied groups, in terms of *H. pylori* and Cag-A positivity, was investigated with the two-sided Fisher's exact test and calculation of the respective odds ratio (OR, with 95% confidence interval, using the approximation of Woolf, Instat 3.06, Graphstat Software Inc., San Diego, CA, United States).  $P \leq 0.05$  was considered significant.

## RESULTS

### Detection of *H. pylori* in fresh stool samples

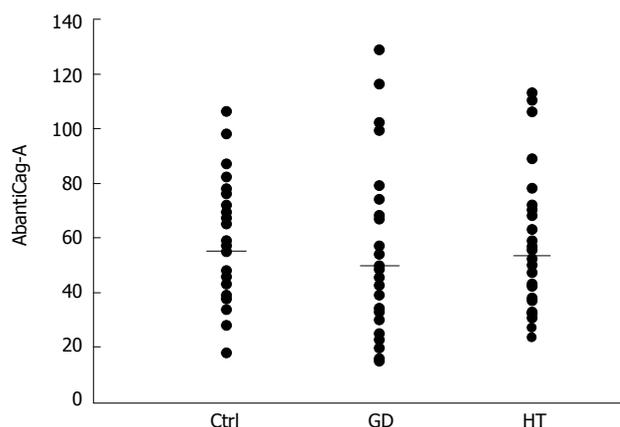
Of 112 ATDs patients, 43/52 (82%) in the GD group and 28/60 (46%) in the HT group were positive for *H. pylori* infection, *vs* 43 [43.0%,  $P \leq 0.0001$ , OR 6.3 (2.7-14.3)] *vs* the GD group, not significant *vs* the HT group] of 100 controls (Table 2).

Immunoassay testing on the stool samples confirmed that the observed *H. pylori* positivity was dependent on ongoing *H. pylori* presence in the gastric mucosa and not on past infection. Furthermore, no correlation was found between the presence of *H. pylori* and smoking habit in the two groups of ATDs patients (data not shown).

**Table 2** The HP- and CagA-positivity in patients in the different study groups

Group	n	HP-	HP+	CagA+	CagA-	Overall CagA+
Control	100	57	43	21	22	21/100
GD total	52	9	43 <sup>c</sup>	36 <sup>b</sup>	7	36 <sup>c</sup> /52
HT total	60	32	28 <sup>1</sup>	25 <sup>b</sup>	3	25 <sup>b</sup> /60
ATDs total	112	41	71 <sup>2</sup>	61 <sup>c</sup>	10	61 <sup>c</sup> /112

<sup>a</sup> $P \leq 0.05$  vs control, <sup>b</sup> $P \leq 0.005$ , <sup>c</sup> $P \leq 0.0001$ . <sup>1</sup>Not significant; <sup>2</sup>Not calculated.



**Figure 1** The AbantiCag-A levels are shown for the investigated groups. The bars show the different means. No significant difference was found among the different groups. GD: Graves' disease; HT: Hashimoto's thyroiditis.

### Cag-A positivity in the serum of *H. pylori*-positive patients

Thirty-six (83.7%) of 43 *H. pylori*-positive GD patients and 25/28 (89.2%) *H. pylori*-positive HT patients were positive for Cag-A antigens vs 21/43 [48.8%,  $P \leq 0.005$ , OR 5.3 (1.9-14.7) vs the GD group and  $P \leq 0.005$ , OR 8.73 (2.7-33.0) vs the HT group] of infected controls. Again, considering the overall prevalence of infection by Cag-A-positive *H. pylori* in the studied groups of ATDs patients, the results were statistically significant, [61 of 112 or 54.4% vs the controls, 21 of 100 subjects or 21%,  $P \leq 0.0001$  OR 4.5 (2.4-8.2), Table 2].

Cag-A antibody levels, expressed in mU/mL [control  $56 \pm 24.3$  (mean  $\pm$  SD), GD  $50.3 \pm 28.6$ , HT  $54.1 \pm 22.6$ ], were similar among the three groups of investigated subjects (Figure 1) and did not correlate with the respective titers of TgAbs, TPOAbs or TRAbs (data not shown).

## DISCUSSION

*H. pylori* infection is found world wide and affects up to 50% of the population of developed countries, such as Italy, and the most virulent strains are identified by the presence of Cag-A antigens<sup>[9]</sup>. Recently, a significant correlation was shown between the Cag-A carrier *H. pylori* strains and GD, independent of the hormonal status of the investigated patients<sup>[13]</sup>. Moreover, other studies have

investigated the association between such microorganisms and HT, however, the results are controversial. Some investigations point to a noteworthy correlation<sup>[10,11,12]</sup>, others do not<sup>[13,14]</sup>. The use of different techniques to assess *H. pylori* infection could explain these conflicting conclusions. For instance, serological detection of *H. pylori* antibodies can not discriminate between past and ongoing infection. Conversely, the <sup>13</sup>C-urea breath test and immunoassay test on fresh stool samples can only detect ongoing *H. pylori* infection, therefore these tests are currently considered the preferred non-invasive methods of investigation<sup>[7]</sup>. Moreover, the presence of similar antigenic sites for Cag-A and TPO could cause false positive results in the Abs titers against *H. pylori*, leading to a bias in group selection of the enrolled patients<sup>[16]</sup>. In addition, the different grade of thyroid function in HT patients, such as subclinical or frank hypothyroidism, could be a misleading factor.

Our results, using a stool antigen test, confirmed that a correlation was present between *H. pylori* and hyperthyroid GD patients, but this correlation was not seen in hypothyroid HT patients.

In accordance with the guidelines<sup>[7]</sup>, we did not perform further invasive exams, such as gastroscopy, in consideration of the age (usually under 45 years old in the investigated patients) and the absence of digestive symptoms in *H. pylori*-positive patients.

Several factors could be considered to explain the different results regarding *H. pylori* prevalence in GD and HT. Usually, the onset and/or progression of ATDs are dependent on different autoimmune mechanisms. Cellular autoimmunity with the TH1 profile of CD4 + T helper precursor cells is predominant in HT, whereas humoral autoimmunity (production of TRAbs or TSH-receptor blocking antibodies) with the TH2 profile is prevalent in GD and atrophic thyroiditis<sup>[17]</sup>. These different activated profiles in ATDs induce the expression of different panels of cytokines, such as interleukin (IL)-4, IL-5, IL-6 and IL-10 in GD and IL-2, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in HT<sup>[17]</sup>. Also, the opposite thyroid function, i.e., hyperthyroidism in GD vs hypothyroidism in HT, could be another factor leading to the controversial results on *H. pylori* prevalence in GD and HT patients.

In our study, both GD and HT show a comparable elevated prevalence of Cag-A positive strains in *H. pylori*-positive patients, in agreement with previous observations in TH patients<sup>[11]</sup>.

The involved factors could operate through a common pathway, such as the glycoconjugates-mediated adhesion of *H. pylori* to the gastric mucosa, which represents a crucial step in the establishment of successful infection. *H. pylori* glycan receptors include fucosylated ABO blood group antigens<sup>[18,19]</sup> and glycans with charged groups, such as sialic acid<sup>[20]</sup> or sulfate<sup>[21]</sup>, and neolacto core chains<sup>[22]</sup>. Two different *H. pylori* adhesins have been characterized on the basis of their interactions with the receptors: the

blood group antigen-binding adhesin (BabA) is specific for H type-1 and Lewis b antigens, admitting terminal blood groups A and B glycan determinants, whereas the sialic acid binding adhesin (SabA) recognizes the Sialyl-Lewis a and Sialyl-Lewis x antigens<sup>[20,23]</sup>.

In particular, the potential effect of the suggested factors, such as hyperthyroidism or the production of cytokines induced by humoral immunity, could modify the profile of the adhesion molecules expressed on the gastric mucosa, increasing *H. pylori* binding in GD and selecting the Cag-A positive strains in ATDs.

Regarding the pathogenetic role of HP in the onset of ATDs, it has been postulated that viral and bacterial infections could play a noteworthy role. Usually, elevated levels of antibodies against some bacteria have been found in GD patients<sup>[4,6]</sup> and, conversely, an antigen structure, such as TSH-binding protein, is described in many gram-positive and gram-negative bacteria<sup>[24]</sup>. Moreover, Cag-A positive *H. pylori* strains show some nucleotide sequence similarity to TPO sequence<sup>[25]</sup>. A positive linear regression between *H. pylori*-Abs titers and microsomal autoantibodies<sup>[9]</sup> and a significant reduction in these antibodies after *H. pylori* eradication have been demonstrated<sup>[26]</sup>. Therefore, cross-reactivity of the antibodies produced against thyroid antigen structures during *H. pylori* infections could potentially induce a biological effect<sup>[27]</sup>, in a similar way to that of *H. pylori* which triggers the onset of autoantibodies against the H<sup>+</sup>K<sup>+</sup>-ATPase in the gastric autoimmunity<sup>[28,29]</sup>. Moreover, the increased prevalence of *H. pylori* in GD, on first diagnosis, and the observation that, usually, *H. pylori* infection starts during childhood<sup>[30]</sup>, suggest that the bacterium could be present before the onset of the autoimmune disease. Larizza *et al*<sup>[31]</sup> proposed that *H. pylori* infection can induce and/or worsen the course of GD in susceptible young patients, carrying the human leukocyte DRB1\*0301 antigen. The authors also suggested that *H. pylori* eradication could prevent GD in these "at high risk" children.

Conversely, hyperthyroid GD patients could just be more susceptible to *H. pylori* infection, and the presence of the microorganism could represent an epiphenomenon, not involved in the onset of the autoimmune disease.

In conclusion, we report an increased *H. pylori* prevalence only in hyperthyroid GD patients, but not in hypothyroid HT patients, although the strains involved in both GD and HT are, prevalently, carriers of Cag-A antigens. These results suggest the execution of screening for *H. pylori* in ATDs patients, taking into account either the presence of virulent strains in autoimmune diseases and the increased *H. pylori* prevalence in GD. Therefore, a possible role of *H. pylori* infection might be postulated for GD, but further studies are needed to confirm such a hypothesis.

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## COMMENTS

### Background

Autoimmune thyroid diseases (ATDs) are represented, essentially, by Hashimoto's thyroiditis (HT) and its variants (postpartum and sporadic thyroiditis) and Graves' disease (GD). *Helicobacter pylori* (*H. pylori*) infection is found worldwide with an incidence of up to 50% in the population of developed countries and a possible correlation has been suggested between the bacterium and ATDs.

### Research frontiers

A wide range of diseases are correlated with the presence of *H. pylori* and a possible pathogenetic role is suspected.

### Innovations and breakthroughs

A noteworthy correlation between *H. pylori* and GD, but not with HT, has been demonstrated. In contrast, the prevalence of Cag-A expression was increased in both ATDs.

### Applications

Screening for *H. pylori* in ATDs patients is suggested, taking into account either the presence of virulent strains in autoimmune diseases and the increased *H. pylori* prevalence in GD.

### Peer review

The manuscript is well written and the methods are adequate. The results justify the conclusions drawn.

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## Predictability of outcome of caustic ingestion by esophagogastroduodenoscopy in children

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### Abstract

**AIM:** To assess the necessity of esophagogastroduodenoscopy (EGD) to predict the outcome of caustic ingestion in children.

**METHODS:** The study included 206 children who underwent EGD because of ingestion of caustic substances between January 2005 and August 2010. Retrospective analysis of data of the patients was performed.

**RESULTS:** The male/female ratio was 1.6 and mean age was  $38.1 \pm 28.8$  mo. The caustic substances were acidic in 72 (34.9%) cases, alkaline in 56 (27.2%), liquid household bleach in 62 (30.1%), and unknown in 16 (7.8%). Fifty-seven (27.7%) patients were symptom-free. Significant clinical findings were observed in 149 (72.3%) patients. Upper gastrointestinal endoscopy findings of esophageal injury were grade 0 in 86 (41.7%) patients, grade 1 in 49 (23.8%), grade 2a in 42 (20.4%), grade 2b in 28 (13.6%), and grade 3a in 1 (0.5%) patient. 35 patients with grade 2a, 2b, and 3a injuries underwent esophageal dilation at second

week of ingestion. Esophageal stricture, which necessitated a regular dilation program developed in 13 of the aforementioned 35 patients. There is no statistically significant difference in the rate of development of esophageal stricture between the patients who ingested acidic (15.3%) and alkaline (8.9%) substances ( $P = 0.32$ ). Severe gastric injury was detected in 38 (18.5%) patients. The rate of development of gastric injury was significantly higher in the acidic group (14%) than in the alkaline group (2.9%) ( $P = 0.001$ ). Out of 149 patients with clinical findings, 49 (32.9%) patients had no esophageal injury and 117 (78.5%) patients had no gastric lesion. Esophageal and severe gastric injuries were detected in 20 (35.1%) and 8 (14%) of patients with no clinical findings respectively. Pyloric stenosis developed in 6 patients. Pyloric obstruction improved with balloon dilation in 2 patients. Mean hospitalization time were  $1.2 \pm 0.5$  d for grade 0 and  $2.3 \pm 5$  d for grade 1 and  $6.3 \pm 6.2$  d for grade 2a and  $15.8 \pm 18.6$  d for grade 2b. It was significantly longer for patients with grade 2a and 2b injuries ( $P = 0.000$ ).

**CONCLUSION:** Endoscopy is an effective technique for determining the presence of esophageal and gastric damage and to avoid unnecessary treatment in patients with no or mild injury.

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**Key words:** Endoscopy; Caustic; Injury; Esophagus; Stomach

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## INTRODUCTION

Corrosive ingestion is an important social and medical problem due to associated early and long-term complications, including bleeding, perforation, systemic complications (renal insufficiency, hepatic dysfunction, and diffuse intravascular coagulation), esophageal stricture, fistula, gastric outlet obstruction, and cancer<sup>[1-4]</sup>. Corrosive injury may also lead to economic hardship due to medical costs and psychosocial problems in affected children, including behavioral and educational, as well as domestic problems<sup>[5]</sup>. Although the use of child proof packages or containers is increasing, caustic ingestion is still an important problem in children because of uncontrolled and cheaper cleaners which have been introduced through common uncontrolled markets in developing countries<sup>[6,7]</sup>.

Several studies<sup>[8,9]</sup> indicate that clinical signs are not always helpful in predicting the degree of injury and subsequent stricture formation. esophagogastroduodenoscopy (EGD) is the most effective method for establishing the severity of injury and treatment planning. However the reported studies<sup>[8-10]</sup> that investigated the role of endoscopy in caustic ingestion focused especially on esophageal injury. There are several studies with limited number of patients which emphasizes the gastric findings with detailed findings and results of caustic injury. The aim of the present study is to determine significance and necessity of the EGD to predict the esophageal and gastric outcome of caustic ingestion and its effect on planning the treatment strategies in children.

## MATERIALS AND METHODS

The study included 206 children that underwent EGD in a single institution because of accidental caustic substance ingestion between January 2005 and August 2010. We didn't perform EGD in patients with questionable history of ingestion if they were asymptomatic and had no oropharyngeal finding. Age and gender of the patients, chemical properties of the caustic substances, clinical findings, endoscopic findings, treatment modalities, feeding methods, and long-term complications were analyzed retrospectively. Hematemesis, oropharyngeal fibrinous lesions, severe mucosal edema, vomiting, drooling, oropharyngeal hyperemia and respiratory distress were considered positive clinical findings.

Ampicillin with sulbactam and ranitidine were routinely administered to all patients before EGD. EGD was performed in all patients under general anesthesia by a fiberoptic Pentax LH-150PC (Japan) endoscope. Endoscopy was performed within 48 h of initial injury. Endoscopic findings were graded by using a modification of the method of Di Costanza which was used by

Poley *et al*<sup>[4]</sup> as grade 0: normal; grade 1: mucosal edema and hyperemia; grade 2a: hemorrhagic, bullous mucosa, exudates, fibrinous membranes, or superficial ulceration; grade 2b: circumferential ulceration in addition to grade 2a; grade 3: scattered small necrotic areas, and black or brown mucosa. Grade 2a, 2b, and 3 were defined as severe lesions. Gastric injury was classified as normal (normal mucosal appearance, edematous or hyperemic mucosa) or severe (exudates, fibrinous membrane, superficial ulceration, scattered small necrotic area and hemorrhagic, black or brown mucosa).

Intravenous antibiotics and H<sub>2</sub> (histamin-2) receptor blocker were discontinued in patients with grade-0 and grade-1 injuries. Patients with grade-0 and grade-1 esophageal injury without severe gastric injury were fed orally and discharged after endoscopy. Gastric decompression and medical treatment which included steroid, intravenous antibiotics (ampicillin-sulbactam, netilmicin, and metronidazol) and H<sub>2</sub> receptor blocker were given in patients with grade 2 and 3 injuries or severe gastric injury. All patients who had grade-2 or 3 esophageal injury without severe gastric injury were fed *via* nasogastric tube after endoscopy. Enteral nutrition was not started and total parenteral nutrition (TPN) was given in patients who had severe gastric injury which was characterized with mucosal necrosis. A repeat endoscopy was performed for the reevaluation of the esophageal and gastric injury, and to start dilation within 7-13 d after caustic ingestion in patients with high suspicion about development of esophageal stricture and grade 2 or higher injury. Oral nutrition was initiated after detection of esophageal and gastric amelioration during the repeat endoscopy. Barium meal studies were performed in all patients with severe esophageal or gastric injuries at the end of the third week of ingestion to determine if esophageal or pyloric stricture was present. Dilation management was started in patients with esophageal or pyloric stricture.

### Statistical analysis

Statistical analysis was performed using SPSS v.11.5 software. Data were analyzed using descriptive statistical methods. Differences between groups were analyzed using the chi square test for categorical variables and Mann Whitney-U for continuous variables. *P* < 0.05 was considered statistically significant.

## RESULTS

The male/female ratio was 129/77 and mean age was 38.1 ± 28.8 mo (range: 4 mo-15 years). The caustic substances were acidic in 72 (34.9%) patients, alkaline in 56 (27.2%), liquid household bleach in 62 (30.1%), and unknown substance in 16 (7.8%) patients. Fifty-seven patients (27.7%) were symptom-free on admission. Positive clinical findings were observed in 149 (72.3%) patients. The median time of presentation was 1 h (range 10 min-7 d) and mean time of endoscopy was 1.56 ± 1.39 d (range: 1-10 d). Endoscopy was performed within 48 h of injury

**Table 1** Endoscopic findings of the patients number based on ingested caustic substance *n* (%)

Esophageal grade	Acidic	Alkaline	Liquid household bleach	Unknown content	Total
Grade 0	14	18	44	10	86 (41.7)
Grade 1	11	20	15	3	49 (23.8)
Grade 2a	25	12	2	3	42 (20.4)
Grade 2b	21	6	1	0	28 (13.6)
Grade 3a	1	0	0	0	1 (0.5)
	72 (34.9)	56 (27.2)	62 (30.1)	16 (7.8)	206 (100)

**Table 2** Esophageal stricture in relation to esophageal injury *n* (%)

Grade of esophageal injury	Stricture rate
Grade-0	0 (0)
Grade-1	0 (0)
Grade 2a	6 (37.5)
Grade 2b	9 (56.25)
Grade 3	1 (6.25)
Total	16 (100)

in 185 (89.8%) patients and was performed after 48 h in 21 (10.2%) patients because of late presentation. Esophageal findings according to the type of ingested substance are summarized in Table 1. One hundred and thirty-three patients with no or grade 1 esophageal injury were fed and discharged after endoscopy. Two patients who had grade 1 esophageal injury were hospitalized for 21 d and 30 d respectively because of severe gastric injury.

A repeat endoscopy and bouginage were performed within 7 d to 13 d after initial procedure in 35 patients with grade 2a and grade 2b esophageal injury and high suspicion about development of esophageal stricture. Esophageal stricture was detected in 16 patients with barium meal study. Thirteen of them needed more than 1 dilation. The correlation of degree of esophageal injury and stricture formation. The stricture rate was 15.3% (11 patients) and 8.9% (5 patients) among the patients with acidic and alkaline injuries, respectively (Table 2). No esophageal strictures developed in patients that ingested liquid household bleach or unknown caustic substances. Although 63.6% of patients who developed esophageal stricture ingested acidic substances, there is no statistically significant difference in the rate of development of esophageal stricture between the patients who ingested acidic and alkaline substances (*P* = 0.32).

Severe gastric injury was detected in 38 (18.4%) patients (Table 3). Gastroscopy could not be performed in 2 patients that ingested acidic substances because of severe esophageal edema. Gastric injury was more severe than esophageal injury in 7 (3.4%) patients. The rate of development of gastric injury was significantly higher in the acidic group than in the alkaline group (*P* = 0.001). Septicemia developed in four patients (1.9%) after oral feeding which was started after revealing the

normal esophageal appearance with partially improved initial severe gastric mucosal injury by control endoscopy. Pyloric stenosis developed in 6 patients. Five patients ingested acidic substances while one patient ingested liquid household bleach. Three of them had no clinical signs after ingestion. Endoscopy revealed grade 2a and grade 2b esophageal injuries in 2 and 4 of them, respectively. Severe gastric injuries, especially of the antral and pyloric areas, were observed in all 6 patients. Endoscopic balloon dilation of the pylorus was attempted in 4 of these patients and pyloric obstruction improved with dilation in 2 of them. One month following ingestion gastrojejunostomy and Heineke-Miculicz pyloroplasty were required in three and one remaining patients respectively.

Esophageal and gastric endoscopic findings according to clinical findings are summarized in Tables 4 and 5. The sensitivity and specificity of all clinical findings regarding severe esophageal injury were calculated as 80.6% and 32.8%, respectively. However sensitivity and specificity of all clinical findings regarding severe gastric injury were calculated as 75.7% and 29%, respectively. Tracheal injury was observed only in 1 patient. Duodenal injury was not detected in any of the patients. No complications related to endoscopy were observed in any of the patients.

TPN was required in 15 (7.3%) patients, of which 14 had severe gastric, antral or pyloric injury; six patients developed pyloric stenosis. One patient with esophageal injury without gastric injury required TPN because of enteral nutrition intolerance. Mean hospitalization time were 1.2 ± 0.5 d for grade 0 and 2.3 ± 5 d for grade 1 and 6.3 ± 6.2 d for grade 2a and 15.8 ± 18.6 d for grade 2b. It was significantly longer for patients with grade 2a and 2b injuries (*P* = 0.000).

**DISCUSSION**

Extent of injury following caustic ingestion depends on amount, concentration and pH of substance and tissue contact time<sup>[8,11]</sup>. Alkaline injury caused liquefaction necrosis which results in deep penetration of tissue<sup>[11]</sup>. Alkaline injury appears mostly in esophagus. However acidic injury causes coagulation necrosis which limits deep penetration. Acidic substance rapidly transit to the stomach because of their low viscosity and specific gravity. This condition results gastric injury more than esophageal injury<sup>[11]</sup>.

The late complications of caustic ingestion are closely related to the depth and extent of the esophageal or gastric injuries. Several clinical approaches and treatment modalities were recommended in injured children<sup>[6,12-15]</sup>. However, to estimate the risk of stricture formation, to begin early and appropriate treatment, and to prevent unnecessary malnutrition and medication use, the presence of esophageal and gastric damage should be documented. Several diagnostic trials were conducted for this purpose and included radiocontrast esophagography, scintigraphy, and esophageal ultrasound; however, the usefulness and prognostic value of these methods remain

**Table 3 Gastroscopic findings according to caustic substance n (%)**

	Substance				Total (n = 206)
	Acidic (n = 72 )	Alkaline (n = 56)	Liquid household bleach (n = 62 )	Unknown content (n = 16)	
Severe gastric injury	29 (14)	6 (2.9)	2 (1)	1 (0.5)	38 (18.4)

**Table 4 Severity of esophageal lesions in relation to clinical findings n (%)**

Clinical findings	Grade of esophageal injury				
	0	1	2a	2b	3
Normal (n = 57) (27.7%)	37 (17.9)	9 (4.4)	9 (4.4)	2 (1)	0
Positive clinical findings (n = 149) (72.3%)	49 (23.8)	40 (19.4)	33 (16)	26 (12.6)	1 (0.5)

**Table 5 Severity of gastric lesions in relation to clinical findings n (%)**

Clinical findings	Gastric injury	
	Normal	Severe
Normal (n = 57) (27.7%)	49 (23.7)	8 (3.9)
Positive clinical findings (n = 149) (72.3%)	117 (56.8)	30 (14.6)

Gastroscopy could not be performed in 2 (1%) patients with positive clinical findings because of severe esophageal edema.

controversial<sup>[16,17]</sup>. The predictability of esophageal injury based on signs and symptoms, and the necessity of upper gastrointestinal endoscopy has been addressed in previous reports<sup>[1,2,4,8,9]</sup>. Although Gaudreault *et al*<sup>[9]</sup> considered vomiting, dysphagia, excessive salivation, abdominal pain, refusal to drink, and oropharyngeal burn specific clinical signs and symptoms of caustic injury, they observed severe esophageal burns in only 18%-33% of patients with these findings and concluded that clinical signs or symptoms cannot predict esophageal injury. Nonetheless, endoscopic evaluation was suggested as a mandatory intervention in symptomatic corrosive-injured patients, but it was not necessary for asymptomatic patients, especially those with a questionable history<sup>[3,18]</sup>. Lamireau *et al*<sup>[8]</sup> reported that vomiting, drooling, and oropharyngeal lesions were not predictors of esophageal injury; however, respiratory symptoms, hematemesis, or the presence of at least 3 symptoms were highly predictive of severe gastrointestinal injury, even though their sensitivity was low. However none of the authors stated the gastric injury in detail and its relation with symptoms and outcome of caustic injury.

We observed severe esophageal injury in 19.3% of our patients that did not have symptoms on the contrary 59.7% of the patients with positive clinical symptoms have no or grade 1 esophageal injury. Fifty percent of patients who developed pyloric stenosis did not have any clinical findings. We want to emphasize that clinical findings are not predictors of esophageal or gastric injury. Although sensitivity of clinical findings was relatively high; specificity of findings was low. Therefore these results support that the absence of any oropharyngeal lesion

does not rule out the severe esophageal or gastric injury.

Endoscopic evaluation is the most effective and widely used technique for establishing the severity of injury. The burned esophagus is weakest between the 7th and 21st d of injury<sup>[5]</sup>; the frequency of endoscopic complications such as fistulas, perforation, and bleeding usually increase in patients with high-grade injury during this period<sup>[5,5]</sup>. Early endoscopy is recommended, especially in the first 24-48 h<sup>[11,3,18]</sup>.

It is usually recommended to stop endoscopy at the first circumferential esophageal burn because of the risk of perforation beyond this point<sup>[19]</sup>; however; we think that this approach might cause a more severely burned esophagus or stomach to be missed. We observed severe gastric injury in 18.4% patients; gastric injury was more severe than esophageal injury in 3.4% of patients. We performed endoscopy after 48 h of injury in 10.2% of patients because of late presentation to the hospital. In contrary to the ordinary knowledge, we performed complete upper gastrointestinal endoscopy to reveal gastric injury even in patients in which severe esophageal injury was detected through endoscopy in all patients except two. However, there were no complications due to late or complete EGD.

The most frequent complication of corrosive substance ingestion is esophageal stricture. The rate of stricture formation is reported to be between 2% and 63%<sup>[5,6,9,13,20]</sup>. Baskin *et al*<sup>[20]</sup> reported that 4.7% of patients with grade 2a injury and 26% of those with grade 2b injury developed esophageal stricture. Huang *et al*<sup>[6]</sup> reported that all patients with grade 2 and 3 injury developed esophageal stricture. Overall, incidence of esophageal stricture was 7.8% in our study. Fourteen point three percent of our patients with grade 2a esophageal injury developed esophageal stricture. This rate was 32.1% and 100%, respectively, in patients with grade 2b and grade 3 esophageal injuries. We think that the partially low overall rate of esophageal stricture was related to the treatment strategies which were directed through the findings of EGD. Because we started early dilation within 7-13 d after initial procedure in patients who had grade 2 and 3 esophageal injury<sup>[3]</sup>. Therefore early dilation is suggested

to improve the outcome of esophageal injury and reduce the number of patients that develop esophageal stricture which was detected at the third week of ingestion.

We did not observe a significant statistical difference in the rate of development of esophageal injury between the acidic and alkaline ingestion groups in contrary to the literature, however esophageal and gastric injury occurred less frequently in the liquid household bleach and unknown content groups than in the acid and alkaline groups. Although 68.7% of the patients who developed esophageal stricture ingested acidic substances, there was no difference in the rate of stricture formation between the acidic and alkaline ingestion groups.

In the present study gastric injuries occurred with greater frequency in the acidic group than in the alkaline group [acidic (40.2%) *vs* alkaline (10.7%)]. Several studies<sup>[21-24]</sup> report that the overall incidence of gastric outlet obstruction is 5%-10% and that surgical correction is the preferred treatment modality in these patients. We observed gastric injury in 8.4% of our patients and gastric outlet obstruction occurred in 15.7% of them. Another advantage of EGD in patients with gastric injury is attempt of balloon dilation for pyloric stenosis.

We think that overlooking gastric injury distal to the upper circumferential esophageal injury is prevented by the gastroscopy which is completed even severe circumferential esophageal injury is detected. Also the widespread use of endoscopy and endoscopic dilation reduce the necessity for surgical procedures to treat corrosive-induced gastric outlet obstruction.

In conclusion, we think that endoscopy which is a mandatory and effective technique should be performed to prevent unnecessary hospitalization and medication use, to plan initial treatment and to predict the patients who are under the risk of developing esophageal stricture and/or gastric outlet obstruction. EGD can be performed without complications in experienced hands. Additionally EGD has advantages such as to establish treatment and follow-up strategies and improving the clinical outcome of the children with caustic ingestion.

## COMMENTS

### Background

Corrosive ingestion is still an important problem causing serious esophageal and gastric injuries which end up with esophageal strictures and pyloric obstruction in children. There are several studies with limited number of patients which emphasizes the gastric findings and results of caustic injury. Endoscopy is the most effective method for establishing the severity of injury.

### Research frontiers

There have been several studies which indicate that clinical signs are not always helpful in predicting the degree of injury and subsequent stricture formation of esophagus and pylorus. Endoscopy provides detailed information about the grade of corrosive injury thus helps to predict esophageal and gastric outcomes.

### Innovations and breakthroughs

The present retrospective study investigated the necessity of endoscopy to predict the outcome of caustic ingestion in children. This study suggests that endoscopy should be performed in all patients who experienced caustic ingestion except the patients with a questionable history and had no symptoms. Also severe gastric injury was observed in 18.4% patients and more severe than

esophageal injury in 3.4% of patients in this study. Therefore complete upper gastrointestinal endoscopy should be performed to prevent misdiagnosis.

### Applications

This article provides important data about significance and necessity of the endoscopy in patients with caustic ingestion. It is important to establish the severity, penetration and extent of injury to plan the treatment strategies in children with caustic ingestion.

### Terminology

Upper gastrointestinal endoscopy and esophagogastroduodenoscopy (EGD) are direct visual examination of mouth, esophagus, stomach and duodenum through an endoscope. Stricture is narrowing of the lumen. Total parenteral nutrition is defined as feeding of patient intravenously by bypassing of digestive system.

### Peer review

The manuscript is a reasonable retrospective review of 206 children who underwent EGD because of caustic ingestion. Severe gastric injury was noted in 18.5% and endoscopies proved safe even in the setting of severe esophageal injury.

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## Bevacizumab as a second- or later-line of treatment for metastatic colorectal cancer

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### Abstract

**AIM:** To determine the efficacy of bevacizumab in patients with metastatic colorectal cancer (MCRC) who have failed prior chemotherapy without bevacizumab.

**METHODS:** Between March 2002 and June 2010, 40 patients in South Korea with MCRC who were treated with bevacizumab plus chemotherapy as a second or later-line treatment were analyzed retrospectively for their overall response rate (ORR), overall survival (OS), and progression-free survival (PFS). The tumor re-

sponses were assessed using the Response Evaluation Criteria in Solid Tumors guidelines.

**RESULTS:** All of the patients had progressed under prior chemotherapy without bevacizumab. Three patients (7.5%) exhibited an ORR, twenty one patients (52.5%) exhibited stable disease (SD), and fifteen patients (37.5%) exhibited disease progression. The median duration of the OS and PFS were 14.0 mo and 6.13 mo respectively. The median OSs were 16.60, 14.07 and 13.00 mo for second-line, third-line and fourth- or later-line treatments, respectively. The median PFSs were 7.23, 7.30 and 3.87 mo for the second-line, third-line and fourth- or later-line treatments, respectively.

**CONCLUSION:** In patients with MCRC, bevacizumab combined chemotherapy may be beneficial during second- or later-line treatment.

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**Key words:** Colorectal cancer; Metastasis; Bevacizumab; Efficacy; Second- or later-line

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Park LC, Lee HS, Shin SH, Park SJ, Park MI, Oh SY, Kwon HC, Baek JH, Choi YJ, Kang MJ, Kim YS. Bevacizumab as a second- or later-line of treatment for metastatic colorectal cancer. *World J Gastroenterol* 2012; 18(10): 1104-1109 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i10/1104.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i10.1104>

### INTRODUCTION

Metastatic colorectal cancer (MCRC) is a common can-

cer, and significant advances have been made in the treatment of this disease. The integration of oxaliplatin or irinotecan chemotherapies in combination with 5-fluorouracil (5-FU) and leucovorin (LV) as a front-line therapy for MCRC is associated with significant improvements in the time-to-progression and overall survival (OS)<sup>[1-3]</sup>.

The recent integration of bevacizumab in front-line therapy for MCRC has resulted in an additional positive impact on the outcome for colorectal cancer patients. Vascular endothelial growth factor (VEGF) is a diffusible, homodimeric glycoprotein this is produced by healthy and neoplastic cells and is a key promoter of angiogenesis under physiologic and pathologic conditions, including tumor progression<sup>[4]</sup>. The VEGF family includes six members [VEGF-A-E and placental growth factor (PIGF)]. The major mediator of tumor angiogenesis is VEGF-A<sup>[5]</sup>. Bevacizumab is a recombinant, humanized, monoclonal antibody directed against the VEGF ligand (VEGF-A); bevacizumab binds all isoforms of VEGF-A with a high affinity<sup>[6]</sup>. The inhibition of VEGF with bevacizumab has been shown to result in tumor reduction of colon cancer in xenograft models and acts in synergy with chemotherapy<sup>[7]</sup>. The addition of bevacizumab to 5-FU-based combination chemotherapy has been shown to result in a statistically significant and clinically meaningful improvement in survival among patients with MCRC<sup>[8-11]</sup>. The addition of bevacizumab to the FOLFOX chemotherapy regimen, consisting of 5-FU, LV, and oxaliplatin, provides a statistically significant and clinically meaningful improvement in OS, compared to FOLFOX alone, in patients with advanced or metastatic disease in whom the disease has progressed after adjuvant chemotherapy with FOLFIRI (5-FU, LV and irinotecan)<sup>[12,13]</sup>. However, based on the inconclusive results from multicenter studies, the role of bevacizumab in the treatment of patients with a disease that is refractory to 5-FU, irinotecan, and oxaliplatin is not yet known<sup>[14-16]</sup>.

The present retrospective study was designed to determine the efficacy and safety of bevacizumab combined with chemotherapy in patients with MCRC who have failed prior chemotherapy without bevacizumab.

## MATERIALS AND METHODS

### Patients

Between April 2005 and June 2010, 40 patients with MCRC were treated with bevacizumab plus chemotherapy as a second- or later-line treatment in Busan, South Korea. Patients who were eligible for the present study suffered from histologically-confirmed MCRC. Other inclusion criteria included an age of at least 20 years, and a life expectancy of > 3 mo. There were no limitations on the number of prior therapies or on the Eastern Cooperative Oncology Group (ECOG) performance status. However, adequate hematologic (an absolute neutrophil count > 1500/ $\mu$ L, hemoglobin > 9.0 g/dL, and a platelet count > 75 000/ $\mu$ L), hepatic (bilirubin < 2.0 mg/dL and transaminase levels < 3 times the upper normal limit), and renal

functions (creatinine < 1.5 mg/dL and urinary excretion  $\leq$  500 mg of protein per day) were required.

The exclusion criteria included the presence of clinically significant cardiovascular disease, uncontrolled hypertension, central nervous system metastasis, major surgery within 6 wk, pregnancy or lactation, non-healing wounds, bleeding diatheses, the regular use of aspirin (> 325 mg/d) or other non-steroidal anti-inflammatory agents, pre-existing bleeding diatheses or coagulopathies, the need for full-dose anticoagulation or prior bevacizumab therapy.

### Treatment schedule and dosing

Among the 40 patients in the present study, 12 patients received bevacizumab plus oxaliplatin-containing chemotherapy, 19 patients received bevacizumab plus irinotecan-containing chemotherapy, 8 patients received bevacizumab plus 5-FU and LV (FL), and 1 patient received bevacizumab alone. Bevacizumab plus FOLFOX or FOLFIRI chemotherapy was administered every 2 wk and consisted of the following: intravenous (IV) oxaliplatin (85 mg/m<sup>2</sup>) or irinotecan (150 mg/m<sup>2</sup>) over 2 h; IV LV (200 mg/m<sup>2</sup>) over 2 h, followed by a bolus of 5-FU (400 mg/m<sup>2</sup>); and infusional 5-FU (600 mg/m<sup>2</sup>) over 22 h, with FL repeated on day 1. The FL chemotherapy was administered every 4 wk [infusional 5-FU (375 mg/m<sup>2</sup>) over 24 h, and the IV LV (20 mg/m<sup>2</sup>)] was administered over 2 h for 5 d. Bevacizumab was administered intravenously at 5 mg/kg over 30 min to 90 min every 2 wk, prior to FOLFOX, FOLFIRI, or FL.

### Assessment

The objective of the present study was to evaluate the overall response rate (ORR), OS, progression-free survival (PFS), and toxicity of bevacizumab in patients who failed prior treatment. The tumor responses were assessed using Response Evaluation Criteria in Solid Tumors guidelines<sup>[17]</sup>. Progression was defined as a 20% increase at the time of disease progression.

The toxicities were graded using the NCI Common Terminology Criteria for Adverse Events (version 3.0)<sup>[18]</sup>. Radiographic assessments were performed at baseline (within 4 wk before starting chemotherapy) and every 6 wk to 8 wk. Radiologic evaluation consisted of a chest X-ray, bone scan, chest computed tomography (CT) scan, and abdominopelvic CT scan.

### Statistical analysis

All of the patients who received at least three dose of therapy were included in the PFS and OS analyses. The OS was defined as the time elapsed between the initiation of the study therapy to the date of death from any cause. The PFS was defined as the time elapsed between the initiation of the study therapy to the date of the progressive disease (PD). Patients who died without a documented PD were considered to have had a PD at the time of death. Patients who were removed from therapy for

Table 1 Patient baseline characteristics <i>n</i> (%)	
Characteristics	Value
<i>n</i>	40
Age (yr), median (range)	55.50 (26-76)
Gender	
Male	22 (55.0)
Female	18 (45.0)
Primary tumor location	
Colon cancer	10 (25.0)
Rectal cancer	30 (75.0)
ECOG	
0-1	31 (77.5)
≥ 2	9 (22.5)
Metastatic sites	
Liver only	6 (15.0)
Lung only	4 (10.0)
Liver and lung only	8 (20.0)
2 sites, including liver or lung	6 (15.0)
2 sites, excluding liver and lung	2 (5.0)
≥ 3 sites	9 (22.5)
1 site, excluding liver and lung	5 (12.5)
Number of metastatic sites	
1 site	15 (37.5)
≥ 2 sites	25 (62.5)
Histologic type	
Well	9 (22.5)
Moderate	9 (22.5)
Poor	1 (2.5)
Unknown	21 (52.5)
Previous chemotherapy	
Fluoropyrimidine + oxaliplatin	31
Fluoropyrimidine + irinotecan	25
Capecitabine combined	15
Fluoropyrimidine combined	6
Line number of bevacizumab	
2nd line	17 (42.5)
3rd line	13 (32.5)
4th or later-line	10 (25.0)
Chemotherapy associated with bevacizumab	
Oxaliplatin-combined	12 (30.0)
Irinotecan-combined	19 (47.5)
5-fluorouracil-combined	8 (20.0)
Bevacizumab alone	1 (2.5)

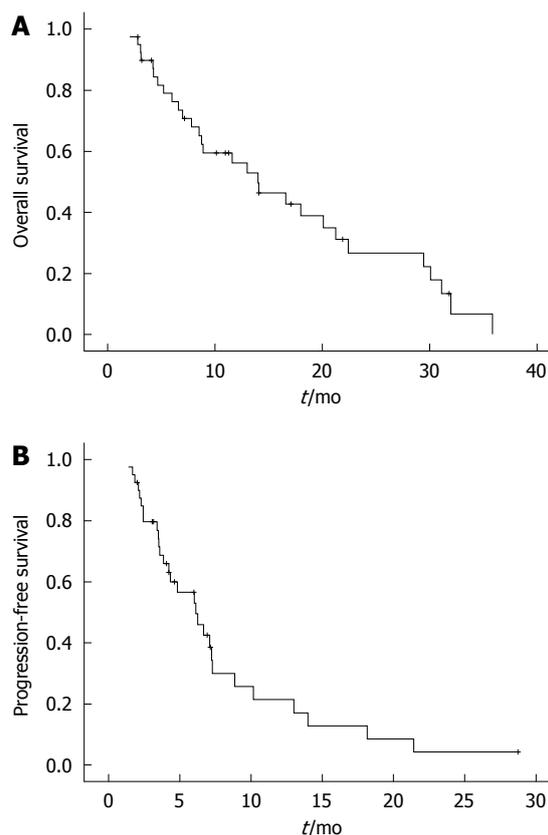
ECOG: Performance status score.

toxicity without clinical or radiographic evidence of PD were censored. Patients who were lost to follow-up were censored at the last contact date. Survival curves were estimated using the Kaplan-Meier method.

## RESULTS

### Patient characteristics

The median age of the patients in the present study was 55.5 years. Forty patients received FOLFOX, FOLFIRI or FL, plus bevacizumab or bevacizumab alone, as a therapy. The metastatic sites were primarily located in the liver and/or lung (45.0%). Thirty one patients had previously undergone treatment with the FOLFOX regimen, twenty-five had undergone treatment with FOLFIRI, fifteen had undergone treatment with capecitabine alone and six had undergone treatment with FL. Seventeen patients (42.5%) were treated with bevacizumab combined



**Figure 1** Curves for the overall survival, and progression-free survival in patients with metastatic colorectal cancer after bevacizumab combined chemotherapy as a second- or later-line treatment. A: Curves for the overall survival in patients with metastatic colorectal cancer after bevacizumab combined chemotherapy as a second- or later-line treatment; B: Curves for progression-free survival in patients with metastatic colorectal cancer after bevacizumab combined chemotherapy as a second- or later-line treatment.

chemotherapy as a second-line treatment, thirteen (32.5%) were treated as a third-line treatment, and ten (25.0%) were treated as a fourth- or later-line treatment. The majority of the patients were treated with Bevacizumab combined chemotherapy, including oxaliplatin (30.0%), irinotecan (47.5%), and fluoropyrimidine (20.0%). One patient (2.5%) was treated using bevacizumab alone, without the addition of other chemotherapy regimens. Additional patient demographics are summarized in Table 1.

### Efficacy

Three patients had partial responses, resulting in an ORR of 7.5%. Twenty-one patients exhibited a stable disease (SD), and fifteen patients exhibited a PD. The response rates of second-line, third-line, and fourth- or later-line treatments were noted in Table 2.

The median duration of the OS and PFS was 14.0 mo and 6.13 mo, respectively (Table 3 and Figure 1). The median OSs were 16.60 mo, 14.07 mo and 13.00 mo for the second-line, third-line and fourth- or later-line treatments, respectively. The median PFSs were 7.23, 7.30, and 3.87 mo for the second-line, third-line and fourth- or later-line treatments, respectively.

Table 2 Response to treatment *n* (%)

Response	Patients	CR	PR	SD	PD	Unknown
Overall	40	0 (0.0)	3 (7.5)	21 (52.5)	15 (37.5)	1 (2.5)
2nd line	17	0 (0.0)	2 (13.3)	11 (64.7)	4 (23.5)	0 (0.0)
3rd line	13	0 (0.0)	1 (7.7)	6 (46.2)	5 (38.5)	1 (7.7)
4th or later-line	10	0 (0.0)	0 (0.0)	4 (40.0)	6 (60.0)	0 (0.0)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 3 Analysis of survival

End point	Median follow-up duration (range) (mo)	mOS (95% CI) (mo)	mPFS (95% CI) (mo)
Overall	23.87 (6.13-77.83)	14.00 (7.77-20.23)	6.13 (3.94-8.33)
2nd line	22.37 (6.13-56.10)	16.60 (3.22-29.98)	7.23 (6.45-8.02)
3rd line	20.03 (13.87-57.77)	14.07 (6.89-21.25)	7.30 (3.84-10.77)
4th and later-line	51.12 (17.87-77.83)	13.00 (4.48-21.52)	3.87 (2.78-4.95)

mOS: Median overall survival; mPFS: Median progression-free survival; median follow-up duration: From diagnosis to last follow-up date or death date. CI: Confidence interval.

Table 4 Summary of the data after bevacizumab-combined chemotherapy as second- and later-line treatment in patients with metastatic colorectal cancer after failure of irinotecan, oxaliplatin and 5-fluorouracil

Ref.	Treatment line	Treatment regimen	<i>n</i>	ORR (%)	Median PFS (mo)	Median OS (mo)
Giantonio <i>et al</i> <sup>[12]</sup>	Second	BV + FOLFOX4	287	22.7	7.3	12.9
		FOLFOX4	285	8.6	4.7	10.8
		BV	234	3.3	2.7	10.2
Yildiz <i>et al</i> <sup>[20]</sup>	Second	BV + Irinotecan-based therapy	40	20.0	6.0	14.0
Chen <i>et al</i> <sup>[14]</sup>	Third	BV + FU/LV	100	4.0	3.7	9.1
Kwon <i>et al</i> <sup>[21]</sup>	Third	BV + FOLFIRI	14	28.5	3.9	10.9
Lièvre <i>et al</i> <sup>[22]</sup>	Second or later-line	BV + FOLFIRI or FOLFOX	31	32.2	9.7	18.4
Kang <i>et al</i> <sup>[16]</sup>	Third or later-line	BV + FOLFIRI or FOLFOX	42	9.5	5.3	9.5
Park <i>et al</i>	Second or later-line	BV + FOLFIRI or FOLFOX	40	7.5	6.13	14.0

BV: Bevacizumab; FOLFOX4: Fluoropyrimidine + oxaliplatin; FOLFIRI: Fluoropyrimidine + irinotecan; FU/LV: Fluoropyrimidine + leucovorin; OS: Overall survival; PFS: Progression-free survival; ORR: Overall response rate.

## DISCUSSION

In colorectal cancer, the use of bevacizumab has been shown to result in an improvement in survival rates and response rates. Bevacizumab was investigated after a randomized phase II study in combination with FL as part of the first-line of treatment of MCRC and resulted in a considerable improvement in efficacy when compared with the FL control<sup>[9,10,14]</sup>. Based on these data, phase III studies were conducted. Compared with irinotecan plus 5-FU/LV (IFL) alone, IFL plus bevacizumab improved the PFS, the ORR and the OS<sup>[8]</sup>. In the TREE-2 trial, previously untreated patients with MCRC were randomly assigned to bevacizumab and one of the three oxaliplatin- and 5-FU-containing regimens used in the TREE 1 trial (FOLFOX, oxaliplatin plus bolus 5-FU/LV, or capecitabine plus oxaliplatin). The bevacizumab-containing arms resulted in an improvement in the OS compared with the non-bevacizumab-containing groups in the TREE-1 study<sup>[19]</sup>. The administration of bevacizumab resulted in a superior response rate, PFS and OS in the

treatment of MCRC in the second-line setting. Supporting evidence was presented by the ECOG 3200, a phase III study randomizing patients who progressed after first-line IFL to FOLFOX plus bevacizumab *vs* FOLFOX alone<sup>[12]</sup>. In the second-line setting, another study showed that bevacizumab plus irinotecan was an active and safe treatment option for patients failing oxaliplatin-based therapy<sup>[20]</sup>. The role of bevacizumab in combination with FL as a third-line treatment was studied in a phase II trial of patients who failed irinotecan- and oxaliplatin-based chemotherapy regimens. Based on previous study, the use of third-line FL plus bevacizumab in chemoresistant patients is considered an ineffective treatment<sup>[14]</sup>. However, additional reports presented different results than this previous report after bevacizumab combined chemotherapy as a third-line treatment. Bevacizumab with FOLFIRI was reported to be well tolerated and to be a feasible treatment in patients with heavily treated advanced MCRC<sup>[21]</sup>. Two studies evaluated the efficacy and safety of bevacizumab plus FOLFIRI or FOLFOX in MCRC after failure with FOLFIRI and FOLFOX using a

retrospective analysis. These studies concluded that bevacizumab plus FOLFIRI or FOLFOX as third-line or later treatment in patients with MCRC resulted in a modest activity and was relatively tolerable<sup>[16,22]</sup>. A summary of the data during bevacizumab-combined chemotherapy as a second- or later-line treatment in patients with MCRC is shown in Table 4.

In the present study, the ORR was 7.5% for all of the patients, and the median duration of the OS and PFS was 14.0 mo and 6.13 mo, respectively. We suggest that bevacizumab combined chemotherapy as a second- or later-line treatment is an active and tolerable treatment in patients with MCRC after failure to response to previous chemotherapy.

## COMMENTS

### Background

The addition of bevacizumab to 5-fluorouracil (5-FU)-based combination chemotherapy as the first-line treatment results in a clinically meaningful improvement in the survival of patients with metastatic colorectal cancer (MCRC).

### Research frontiers

Based on the inconclusive results from multicenter studies, the role of bevacizumab in the treatment of patients with disease that is refractory to 5-FU, irinotecan, and oxaliplatin is unclear.

### Innovations and breakthroughs

In previous studies, bevacizumab combined chemotherapy as a second- or later-line treatment was shown to have controversial results. In the present study, three patients exhibited partial responses, resulting in an overall response rate of 7.5%. The median duration of the overall survival (OS) and progression-free survival (PFS) was 14.0 mo and 6.13 mo, respectively. The median OSs were 16.60, 14.07 and 13.00 mo for the second-line, third-line and fourth- or later-line treatments, respectively. The median PFSs were 7.23 mo, 7.30 mo, and 3.87 mo the second-line, third-line and fourth- or later-line treatments, respectively.

### Applications

The results of the present study suggest that bevacizumab combined chemotherapy as a second- or later-line treatment is an active and tolerable treatment option for patients with MCRC after failure to previous chemotherapy.

### Peer review

The present study is a good descriptive retrospective study that evaluated the efficacy of bevacizumab plus chemotherapy in patients with MCRC who have failed prior chemotherapy without bevacizumab.

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## Is hepatic neoplasm-related pyogenic liver abscess a distinct clinical entity?

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### Abstract

**AIM:** To compare the clinical characteristics of pyogenic liver abscess (PLA) in patients with and without hepatic neoplasm (HN).

**METHODS:** Authors performed a retrospective analysis involving patients with PLA. The demographic, clinical features, laboratory and imaging findings, management and outcome of patients with and without HN were studied.

**RESULTS:** From January 2000 to December 2009 inclusive, 318 patients (35 with HN) had PLA, and mean age and comorbidity were comparable between the two groups. More patients with HN experienced right upper quadrant pain (68.6% vs 52.7%,  $P < 0.04$ ), developed jaundice (14.3% vs 5.7%,  $P < 0.03$ ) and hepatomegaly (17.1% vs 3.9%,  $P < 0.01$ ), and had higher serum total bilirubin level (43.3  $\mu\text{mol/L}$  vs 30.0  $\mu\text{mol/L}$ ,  $P = 0.05$ ). Most patients in both groups had PLAs in the right hepatic lobe, and biliary tract disorder was the most common underlying cause (71.4% and 61.8%). However, more PLAs in the HN group were associated with thicker abscess wall (37.1% vs 19.4%,  $P < 0.01$ ), septal

lobulation (77.1% vs 58%,  $P < 0.02$ ), gaseous cavitation (17% vs 7.8%,  $P = 0.03$ ), portal thrombophlebitis (11.4% vs 1.8%,  $P < 0.01$ ) and aerobilia (25.9% vs 5.5%,  $P < 0.01$ ). Mixed bacterial growth (40% vs 15.2%,  $P < 0.01$ ) and Gram-negative bacilli (22.8% vs 60.4%,  $P < 0.01$ ) were dominant isolates in PLAs with and without HN, respectively. Although incidence of the complications was comparable between the two groups, patients with HN had a higher mortality rate than those without (71.4% vs 8.8%,  $P < 0.01$ ). Multivariate logistic regression analysis revealed underlying active malignancy [odds ratio (OR): 40.45, 95% CI: 14.76-111.65], hypoalbuminemia (OR: 1.22, 95% CI: 1.14-1.38), disseminated intravascular coagulation (OR: 3.32, 95% CI: 1.19-9.69) and acute coronary syndrome (OR: 4.48, 95% CI: 1.08-17.8) were independent risk factors associated with mortality. However, several HN cases, presented concurrently with PLAs, were found to have curative resectable tumors and had good prognosis after surgery.

**CONCLUSION:** PLA associated with HN tends to form a distinct clinical syndrome with a different extent of clinical manifestations, radiological and microbiological features and complications.

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**Key words:** Pyogenic liver abscess; Hepatic neoplasms; Hepatic malignancy; Liver abscess

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Law ST, Li KK. Is hepatic neoplasm-related pyogenic liver abscess a distinct clinical entity? *World J Gastroenterol* 2012; 18(10): 1110-1116 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i10/1110.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i10.1110>

## INTRODUCTION

Pyogenic liver abscess (PLA) has been recognized since the time of Hippocrates<sup>[1-4]</sup>. It is the most common type of visceral abscess; in a report of 540 cases of intra-abdominal abscesses, PLA accounted for 48% of visceral abscesses. This condition is potentially life threatening, with a mortality rate ranging from 10% to 40%<sup>[5,6]</sup>. The previous reports have indicated that hepatic malignancy is the major risk and poor prognostic factor for PLA<sup>[7,8]</sup>. Clinically, it is mandatory for clinicians to rule in/out any coexisting tumor component in the PLA, so that appropriate neoadjuvant therapy or even surgical resection if localized can be offered promptly after treatment of the infection. However, to the best of our knowledge, the issue of the prediction of the presence of hepatic malignancy in PLA has never been investigated.

Thus, the aim of this study was to review the characteristics of patients with and without hepatic neoplasm (HN)-associated PLA in the following areas: risk factors, clinical features, characteristics features of liver abscess, treatment and outcome.

## MATERIALS AND METHODS

The records of patients discharged from Tuen Mun Hospital in Hong Kong with a diagnosis of PLA (International Classification of Diseases code 572.0) between January 2000 and December 2009 were reviewed. Cases were identified through searching the hospital database (Clinical Management System of Hospital Authority of Hong Kong). Underestimation of actual caseload is possible because coding might not have included patients suffering from an underlying disease.

The case definition of PLA required patients to have one or more filling defects on liver imaging [either ultrasound or computed tomography (CT)], together with either (1) complete resolution of radiological abnormalities following antimicrobial therapy with/without positive blood/pus culture, or (2) histological proof of microbiological infection without underlying neoplasm.

The diagnosis of hepatic tumor abscess was based on the presence of hepatic tumor of primary or secondary origin plus one or both of the following: (1) positive pus culture; and (2) partial resolution of radiological abnormalities following antimicrobial therapy.

The clinical records of these patients were retrospectively reviewed to obtain the demographic characteristics, clinical features, laboratory, imaging findings, treatment methods and final outcomes. Recurrence was defined as the development of new clinical and radiological changes subsequent to clinical and/or radiological resolution.

### Statistical analysis

The data were compiled and analyzed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, United States). All continuous variables were expressed as mean  $\pm$  SD. Categorical variables were reported as percentages. Student's *t* test,  $\chi^2$  test, Fisher's exact test and Mann-

**Table 1** Demographic and clinical characteristics of patients with/without hepatic neoplasm-associated pyogenic liver abscess (mean  $\pm$  SD) *n* (%)

Variable	HN group ( <i>n</i> = 35)	Non-HN group ( <i>n</i> = 283)	<i>P</i> value
Age (yr)	67.7 $\pm$ 16.2	65.4 $\pm$ 15.13	0.40
Sex (male: female)	18:17	163:120	
Comorbidity			
Diabetes mellitus	9 (25.7)	81 (28.6)	0.36
Hypertension	12 (34.3)	78 (27.6)	0.20
Ischemic heart disease	1 (2.9)	23 (8.1)	0.14
Stroke	2 (5.7)	31 (11.0)	0.17
Duration of symptoms before presentation (d)	3.7 $\pm$ 2.8	4.9 $\pm$ 4.9	0.18
Symptoms and signs			
Fever and chill	34 (97.1)	268 (94.7)	0.27
Right upper quadrant pain	24 (68.6)	149 (52.7)	< 0.04
Diarrhea	1 (2.9)	21 (7.5)	0.16
Cough and sputum	6 (17.1)	59 (20.8)	0.41
Jaundice	5 (14.3)	16 (5.7)	< 0.03
Hepatomegaly	6 (17.1)	11 (3.9)	< 0.01
Right pleural effusion/ consolidation (on admission)	3 (8.6)	39 (13.8)	0.20

HN: Hepatic neoplasm.

Whitney *U* test were used when appropriate.  $P \leq 0.05$  was considered statistically significant.

## RESULTS

From January 2000 to December 2009 inclusive, a total of 318 patients were diagnosed with PLA, 35 (11%) of which were associated with HN. There were 29 cases of hepatopancreatobiliary (HPB) malignant disease and causes identified for the other patients were as follows: colonic cancers in three patients, and hepatocellular, gastric and breast cancer in one patient each. Among 29 cases of HPB neoplasms, there were 20 cases of cholangiocarcinoma (13 intrahepatic and seven extrahepatic cholangiocarcinoma), six cases of gallbladder cancer, and three of pancreatic cancer. In the HN group, 10 patients had neoplasms that presented concomitantly with the development of PLA, and they included four gallbladder cancers, two intrahepatic cholangiocarcinomas, two cancers of the head of the pancreas, and one each of breast and cecal cancer. Although they presented concurrently, most of them were found to be widely metastatic by imaging, and unable to undergo curative resection. Only four cases had localized tumors on presentation: two cases each of intrahepatic cholangiocarcinoma and cancer of the head of the pancreas. They remained stable after the resection of the tumors. For the patients whose HNs presented prior to PLAs, the median period of time was 5 mo (range: 2-17 mo).

### Demographic characteristics

The demographic characteristics of these patients are shown in Table 1. There was no sex dominance in the HN group (male to female ratio: 1.06). The mean age

**Table 2** Laboratory findings and radiological features of patients with/without hepatic neoplasm-associated pyogenic liver abscess *n* (%)

Laboratory parameters	HN group ( <i>n</i> = 35)	Non-HN group ( <i>n</i> = 283)	<i>P</i> value
Hemoglobin (mean, g/dL)	10.0	11.3	0.22
White cell count (mean, 10 <sup>9</sup> /L)	18.9	16.8	0.13
ESR (mean, mm/h)	82.0	78.9	0.84
CRP (mean, mg/L)	120.0	141.8	0.56
Albumin (mean, g/L)	27.1	29.6	0.56
Total bilirubin (mean, mol/L)	43.3	30.0	0.05
Alanine aminotransferase (mean, U/L)	64.1	84.1	0.16
Hb <sub>a1c</sub> (mean, %)	10.3	9.1	0.41
Bacteremia	13 (37.1)	120 (42.4)	0.27
Radiological features			
Size (cm, mean ± SD)	6.8 ± 3.1	6.1 ± 3.0	0.19
Site			
Right lobe	22 (62.9)	156 (55.1)	0.19
Solitary/multiple	15 (43)/ 7 (20)	133 (47)/ 23 (8.1)	
Left lobe	7 (20)	82 (29)	0.13
Solitary/multiple	5 (14.3)/ 2 (5.7)	73 (25.8)/ 9 (3.2)	
Bilobar	3 (8.6)	37 (13.1)	0.22
Echogenicity			
Hypoechoic	31 (88.6)	256 (90.5)	0.36
Hyperchoic/heterogenous	4 (11.4)	27 (9.5)	0.37
Thicken wall	13 (37.1)	55 (19.4)	< 0.01
Rim enhancement	26 (74.3)	176 (62.2)	0.08
Septal lobulation	27 (77.1)	164 (58.0)	< 0.02
Aerobilia	7 (25.9)	16 (5.5)	< 0.01
Fluid/gaseous cavitation	33 (94.3)/ 6 (17.1)	243 (85.9)/ 21 (7.8)	0.08/ 0.03
Portal thrombophlebitis	4 (11.4)	5 (1.8)	< 0.01
Subcapsular rupture of the abscess	2 (5.7)	16 (5.6)	0.49

HN: Hepatic neoplasm; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

was 67.7 years (range: 24-91 years; median: 72 years) in the HN group and 65 years (range: 24-97 years; median: 65 years) in the non-HN group (data not shown). There was no significant difference in age and sex between the two groups (*P* = 0.40). Major comorbidities of the two groups were similar and included hypertension, ischemic heart disease, diabetes mellitus and stroke.

### Clinical features

The clinical features of PLA in patients with and without HN are shown in Table 1. Patients with HN tended to have more acute onset of symptoms than did patients without HN. As shown in Table 1, the most common presenting features were fever, chills and right upper quadrant pain. The HN group experienced right upper quadrant pain (68.6% *vs* 52.7%, *P* < 0.04), jaundice (14.3% *vs* 5.7%, *P* < 0.03) and hepatomegaly (17.1% *vs* 3.9%, *P* < 0.01) more than did the non-HN group, with a significant difference between them. About 20% of patients in both groups experienced respiratory symptoms but only half of these patients had abnormalities in chest radiography.

**Table 3** Bacteriology, pathogenesis and confirmatory investigations of hepatic neoplasm- and non-hepatic neoplasm-associated pyogenic liver abscess *n* (%)

Bacteriology	HN group ( <i>n</i> = 35)	Non-HN group ( <i>n</i> = 283)	<i>P</i> value
Gram-positive organism	6 (17.2)	23 (8.1)	0.04
<i>Streptococcus milleri</i>	3 (8.6)	18 (6.4)	
Others	3 (8.6)	5 (1.7)	
Gram-negative organism	8 (22.8)	171 (60.4)	< 0.01
<i>Escherichia coli</i>	2 (5.7)	27 (9.5)	
<i>Klebsiella</i> spp	5 (14.2)	135 (47.7)	
Others	1 (2.9) <sup>1</sup>	9 (3.2) <sup>2</sup>	
Mixed growth	14 (40.0)	43 (15.2)	< 0.01
Unknown	7 (20.0)	46 (16.3)	< 0.05
Antibiotic resistance	10 (28.6)	49 (17.3)	0.05
Pathogenesis			
Biliary tract disorder	25 (71.4)	175 (61.8)	0.14
Portal pyemia	3 (8.6)	11 (3.9)	0.10
Direct spread	5 (14.3)	0 (0)	< 0.01
Hematogenous	2 (5.7)	5 (1.8)	0.07
Cryptogenic	0 (0)	15 (5.3)	0.08
Not investigated	0 (0)	77 (27.2)	< 0.01

<sup>1</sup>Bacteriodes (*n* = 1); <sup>2</sup>Enterobacter (*n* = 4), Citrobacter (*n* = 2), Fusobacterium (*n* = 2), Proteus (*n* = 1). HN: Hepatic neoplasm.

### Laboratory and radiological features

The laboratory findings are summarized in Table 2. Anemia, leukocytosis, high erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), hypoalbuminemia, and elevated total bilirubin and alanine aminotransferase were common in the two groups. The HN group tended to have a higher serum bilirubin level (43.3 μmol/L *vs* 30.0 μmol/L, *P* = 0.05). About 40% of the patients had positive bacterial growth in blood culture on admission. Ultrasonography (USG) and CT of the abdomen were performed in two (5.7%) and nine (25.7%) patients with HN, and 81 (28.6%) and 20 (7.1%) patients without HN, respectively. Combined imaging was performed in 24 (68.6%) and 182 (64.3%) patients with and without HN, respectively. The characteristics of PLA found by radiological imaging are shown in Table 2. The majority of the patients in both groups had right lobe PLA of comparable size. Most PLAs in the two groups appeared as hypoechoic nodules on USG imaging. Both groups had the following common features: rim enhancement, septal lobulation and fluid cavitation in CT imaging, with greater frequency in the HN group. In contrast, more patients in the HN group had thicker (37.1% *vs* 19.4%, *P* < 0.01) abscess walls (i.e., thickened abscess wall is defined as wall thickness > 1 cm<sup>[9]</sup>) than those in the non-HN group had, but both carried a similar risk of rupture. Moreover, aerobilia, gaseous cavitation and portal thrombophlebitis were experienced more in the HN group, with a significant difference between the two groups (*P* < 0.05).

### Underlying etiology

The etiologies of the two groups are summarized in Table 3. The pattern of the causative organisms was entirely different between the two groups. Most of the

**Table 4** Management and outcomes of pyogenic liver abscess with/without hepatic neoplasm *n* (%)

	HN group ( <i>n</i> = 35)	Non-HN group ( <i>n</i> = 283)	<i>P</i> value
Intervention			
Imaging-guided aspiration ± drainage	27 (77.1)/ 6 (17.1)	233 (82.3)/ 206 (72.8)	0.23/ < 0.01
Surgical drainage	1 (2.9)	18 (6.4)	0.20
Complications and outcomes			
Metastatic infection	0 (0)	17 (5.7)	0.07
Septic shock	14 (40)	77 (27.2)	0.06
DIC <sup>1</sup>	6 (17.1)	44 (15.5)	0.39
Acute coronary syndrome	2 (5.7)	14 (4.9)	0.42
Respiratory/renal failure	1 (2.9)	14 (4.9)	0.29
ICU care <sup>2</sup>	1 (2.9)	31 (11)	0.07
Recurrence	1 (2.9)	16 (5.7)	0.25
Death	25 (71.4)	25 (8.8)	< 0.01

<sup>1</sup>For disseminated intravascular coagulation; <sup>2</sup>For intensive care unit. HN: Hepatic neoplasm.

microorganisms isolated from the HN group were mixed growth (40%), followed by Gram-negative and Gram-positive isolates, whereas Gram-negative isolates were dominant in the non-HN group, followed by polymicrobial isolates and Gram-positive cocci. *Klebsiella* spp. were the dominant Gram-negative organisms in both groups.

Biliary tract disorder was the most common cause of disease in the two groups. In 14 (4.4%) patients PLA was due to portal pyemia: three patients were from the HN group and all had advanced colon cancer with multiple hepatic metastasis. The remaining 11 were from the non-HN group: seven with colonic diverticulitis shown by abdominal CT scan; two with active inflammatory bowel disease; and two with a recent history of acute appendicitis. Five patients from the HN group had PLAs caused by the direct spread of infection. One patient developed PLA after transarterial chemoembolization for hepatocellular carcinoma and the organism involved was *Staphylococcus aureus*, thus the infection was probably due to catheter delivery of the skin contaminant. The other four cases had advanced carcinoma of the gallbladder with local invasion into the liver parenchyma. Seven cases (two from the HN group) were caused by hematogenous spread: In the HN group, these were cases of advanced gastric and breast cancer and both had multiple liver metastases. Causes identified in the other group were as follows: urinary tract infection in two patients, and one each with pneumonia, right psoas abscess and continuous ambulatory peritoneal dialysis peritonitis. Lastly, > 20% of patients in the non-HN group had not been investigated for the underlying causes of PLA.

### Management and treatment outcomes

The management and outcomes of the two groups are summarized in Table 4. All patients received intravenous broad-spectrum antibiotics after assessment for sepsis. Antibiotic resistance was detected in 10 (28.6%) isolates from the HN group, but in only 49 (17.3%) from patients without HN, with a significant difference between the

two groups ( $P = 0.05$ ). Antibiotic therapy was the only treatment for seven and 32 patients in the HN and non-HN groups, respectively, and the reason for not performing image-guided aspiration and drainage was the small size of the lesion (< 3 cm in diameter). Among the HN group, USG-guided needle aspiration was performed in 27 (77.1%) patients and continuous catheter drainage in six (17.1%). In the non-HN group, 233 (82.3%) patients had USG-guided needle aspiration and among these, 206 (72.8%) patients had continuous catheter drainage, with a significant difference between the two groups. A pigtail catheter was not inserted in these 48 patients after percutaneous aspiration because the abscess was too solid or it was completely collapsed after aspiration, thus, further catheter drainage was not considered useful. Surgical drainage was required in one (2.9%) and 18 (6.4%) patients with and without HN, respectively. The main reason for laparotomy among these patients was the presentation of acute abdomen. There were no patients who failed to respond to antibiotics with/without percutaneous drainage.

No patients with HN had detectable metastatic infections, whereas 16 (5.7%) patients without HN experienced distant spread of the infection: urinary tract infection in eight, endophthalmitis in three, pneumonia in two, and right empyema, right psoas abscess and simultaneous endophthalmitis and urinary tract infection in one patient each. Septic shock developed in 40% of patients with HN but in only 25% of those without HN, with no significant difference between the two groups. Other complications, such as disseminated intravascular coagulation (DIC), acute coronary syndrome and respiratory/renal failure had comparable incidence between the two groups. The intensive care unit was offered to 31 (11%) patients without HN but only one (2.9%) patient with HN, with no significant difference between the two groups. Recurrence rate of PLA was low in both groups. Twenty-five (71.4%) patients with HN died during the management, all of which was attributed to the underlying HNs, in which all except one had the septic process under control. Only one case died due to uncontrolled *Clostridium perfringens* septicemia on day 7 of hospitalization. The non-HN group had only 25 (8.8%) deaths and causes were as follows: 17 with liver abscesses, six with hospital-acquired pneumonia, two with cerebrovascular accident, and one with uncontrolled bleeding duodenal ulcer, with a significant difference between the two groups (log rank test < 0.01) (Figure 1). By using multivariate logistic regression analysis, underlying active malignancy, hypoalbuminemia, DIC and acute coronary syndrome were independent risk factors associated with mortality for these two groups (Table 5). These combined risk factors accounted for 49% (Nagelkerke  $r^2$ : 0.49) of the mortality risk and HN was the most significant clinical variable in this model.

### DISCUSSION

PLA has been recognized since the time of Hippocrates. During recent decades, despite advances in microbiology,

Table 5 Summary of multivariate logistic regression analysis of mortality of pyogenic liver abscess

Variable <sup>1</sup>	Wald estimate <sup>2</sup>	$\beta$ coefficient (B)	OR <sup>3</sup> (95% CI)	P value
Hepatic malignancy	51.5	3.7	40.45 (14.76-111.65)	0.00
Hypoalbuminemia	15.2	0.2	1.22 (1.14-1.38)	0.00
DIC	5.2	1.2	3.32 (1.19-9.69)	< 0.05
Acute coronary syndrome	4.3	1.5	4.48 (1.08-17.80)	0.02
Impaired renal function	0.1	0	1.00 (0.82-1.12)	0.76
Total bilirubin	0.3	0	1.00 (0.71-1.51)	0.48
Septic shock	0.9	0.03	1.03 (0.82-2.67)	0.39
Surgical intervention	0.11	0.13	1.14 (0.71-3.90)	0.74

<sup>1</sup>Variables were entered into the model by stepwise forwards method; only those clinically interested were listed; <sup>2</sup>Wald estimate corresponds to the importance of the variable in the model; <sup>3</sup>Odds ratio (OR): calculated by  $\text{Exp}(B)$ . DIC: Disseminated intravascular coagulation.

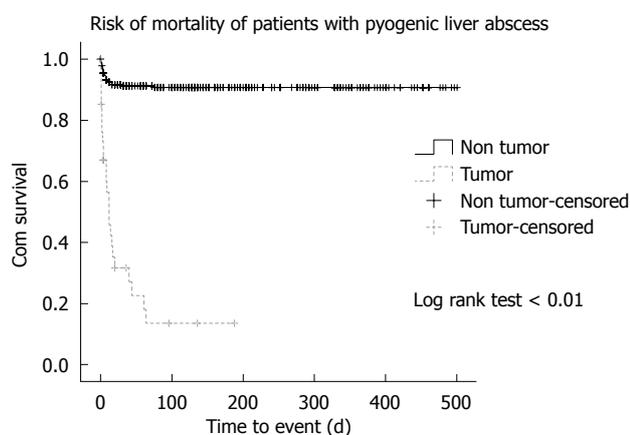


Figure 1 Kaplan-Meier estimates of overall risk of mortality of pyogenic liver abscess in patients with and without hepatic neoplasm.

imaging-guided intervention and antibiotic therapy, PLA is still a potential life-threatening condition with a mortality rate ranging from 10% to 40%, and one of the reasons is the increasing number of patients with primary or secondary HNs<sup>[5-8]</sup>. In general, only 5% of malignant liver tumors are of primary origin, whereas the rest are assumed to be metastases<sup>[10]</sup>. In fact, the liver is the second most common site of metastasis next to lymph nodes. Our study revealed two important findings. First, the primary HNs, instead of liver metastases, were dominant in those PLAs associated with HN. The liver is resistant to infection because it is one of the reticuloendothelial systems in the body and harbors numerous lymphoid cells. Second, the primary site of the HN is also crucial for the development of PLAs because HBP malignant disease was dominant within the HN group.

In the present study, the mean age of the patients and the prevalence of their comorbidity were comparable with previous studies. Men and women were equally affected in patients with HN, whereas there was male dominance in patients without HN, as in most previous studies<sup>[11-17]</sup>. The duration of symptoms before admission was shorter in the HN group than the non-HN group. This is because a higher proportion of the cases in HN groups were due to the biliary tract pathology. Seeto *et al.*<sup>[18]</sup> have found that

PLA presents most acutely (3 d) if it is due to biliary tract disorder.

The patients in both groups presented with similar clinical features: fever, chill and right upper quadrant pain were frequent symptoms. Because of the presence of hepatic tumors of either primary or secondary, there was a higher incidence of right upper quadrant pain, jaundice and hepatomegaly on physical examination in patients with HN.

Abnormalities in laboratory parameters were similar in both groups, mainly anemia, leukocytosis, high ESR and CRP, deranged liver function test, with elevated serum bilirubin and alkaline phosphatase. However, the change in serum alanine aminotransferase was less pronounced in patients with HN, which may relate to the dominance of cancer of the biliary system among the HNs, thus reflecting the probable secondary biliary cirrhotic change in liver parenchyma. Right lobe involvement was dominant in both groups of patients, which is consistent with prior reports<sup>[19]</sup>. The right lobe dominance is due to its size and propensity to receive most of the portal blood flow. Bilobar involvement was only seen in 8.6% of the HN group. This is because bilobar tumors are more common in hepatic metastases of extra-HPB origin, which only contributed five (14.3%) cases in our series.

PLAs with and without HN had several similar radiological features in our study. USG appearance of both groups might range from hyperechoic to hypoechoic and this variation has a close relationship to the pathological stage of PLA. During the very early stage of abscess formation, the hepatocytes/tumor cells undergo acute inflammation and thus the abscess might appear solid, i.e., hyperechoic. When these cells start to become necrotic, the abscess liquefies with increasing fluid content and surrounding edema. This is also the stage most patients with PLA present clinically, and thus most PLAs, with or without HN, are hypoechoic by USG and are fluid-containing lesions with rim enhancement in CT contrast studies. In contrast, our study demonstrated that PLAs with and without HNs had several contrast features in imaging. First, within the hepatic tumor tissue, the tumor cells will secrete cytokine to stimulate the adjacent fibrous stroma tissue to grow, therefore, thickened abscess wall

and septation were more commonly seen in PLAs with HN<sup>[20]</sup>. Second, there was a higher incidence of aerobilia and portal thrombophlebitis in patients with HN that might be related to the high incidence of HPB malignancies in the HN group. Third, the gaseous cavitation was more commonly seen in PLAs associated with HN. This might be explained by the fact that the tumor cells have high energy demand for rapid duplication, and this leads to accumulation of lactic acid through their glycolytic pathway. Subsequently, the acidic environment stimulates the formic hydrogen lyase of the bacteria to produce carbon dioxide and hydrogen through mixed acid fermentation<sup>[21]</sup>. The comparable risk of rupture of PLA associated with/without HN might be explained by the combined counteracting effects of the wall thickness and the gaseous cavitation inside the abscess.

The bacteriological pattern in PLA was entirely different between the two groups in which there was a higher incidence of polymicrobial isolates in the abscesses of the HN group. This may be related to the mixture of microorganisms in the normal flora in the gastrointestinal and biliary tract. There are three possible mechanisms for the bacterial flora to develop PLA in the presence of HN: malignant biliary obstruction caused by HPB malignancies, portal venous suppuration, which usually happens in colorectal tumor, and bacterial seeding *via* hepatic artery following systemic chemotherapy<sup>[22]</sup>. Although only some patients were investigated by colonoscopy, endoscopic retrograde cholangiopancreatography and endoscopic ultrasound were performed, the biliary disease was still the most common identifiable cause of PLA in patients without HN as in other recent studies<sup>[18,23,24]</sup>.

In our study, most of the patients were managed by combination of antibiotic and imaging-guided aspiration with/without drainage. There was no difference in the complications between the two groups during the imaging-guided intervention. No tumor seedlings were reported in the HN group, and this might have been due to only minute quantities of tumor cells in the central region of the tumor mass. Thus, imaging-guided aspiration of the hepatic tumor abscess is regarded as a relatively safe procedure. Although both groups of patients responded similarly in the initial phase of management, the patients with HN tended to have a more fulminant clinical course with a higher incidence of septic shock, DIC and acute coronary syndrome. There was no detected metastatic infection in patients with HN and this might be explained by the difficulty for the bacteria to disseminate within the tumor tissue. Surprisingly, underlying active hepatic malignancy was less likely to be associated with the recurrence of PLA, because a considerable proportion of the patients with HN died. This would have made an apparently negative association between malignancy and recurrence.

In the present study, the overall mortality rate of PLA was 16%, which is comparable to other studies<sup>[25]</sup>. The higher mortality rate of patients with HN was due to underlying active malignancy and higher incidence of the complications mentioned previously. Half of the deaths

in the HN group happened within the first 12 wk. Although there were two cases each of intrahepatic cholangiocarcinoma and cancer of the head of the pancreas that presented concurrently with PLAs, these four cases were found to have localized tumors and thus curative resection could be performed after treatment of PLA. Thus, it is justifiable to have early follow-up imaging with/without biopsy for those PLAs that have a suspicion of malignancy. Despite the advanced stage of the disease, early diagnosis of the neoplastic component is warranted because neoadjuvant target therapy might modify its natural course. In our study, the most important determinants of mortality rate were underlying active hepatic malignancy, hypoalbuminemia, DIC and acute coronary syndrome. The prognostic value of underlying malignancy has been recognized in other studies<sup>[24,26]</sup>. Hypoalbuminemia reflects the duration of the growth of the liver abscess and the degree of impairment of hepatic synthetic function. DIC and acute coronary syndrome may relate to the severity of the septic process.

PLA associated with HN tends to have a distinct clinical syndrome with a different extent of clinical manifestations, radiological and microbiological features, and complications. The initial antibiotic of choice should be broad-spectrum with coverage of both Gram-positive and -negative microorganisms. These PLAs can be safely treated with image-guided aspiration with/without drainage, with negligible risk of tumor seedlings. Early follow-up imaging should be considered for those PLAs with potential risk of malignancy.

## COMMENTS

### Background

Hepatic neoplasm (HN) is a recognized cause of pyogenic liver abscess (PLA). It is well-known that HN can mimic PLA on presentation and it is difficult to identify the coexisting hepatic tumor component in PLA, thus, PLA associated with HN is a diagnostic challenge to clinicians. There have been few studies focused on this subject.

### Research frontiers

Due to the better understanding of carcinogenesis and the advancement of molecular medicine, treatments based on molecular targets are being developed and these might have the potential to modify the clinical course of these neoplasms.

### Innovations and breakthroughs

PLA associated with HN tends to have a distinct clinical syndrome with a different extent of clinical manifestations, radiological and microbiological features, and complications. Several cases, presented concurrently with PLAs, were found to have curative resectable tumors and had good prognosis after surgery.

### Applications

The initial antibiotic of choice should be broad-spectrum with coverage of both Gram-positive and -negative microorganisms. These PLAs can be safely treated with image-guided aspiration with/without drainage, with negligible risk of tumor seedlings. Early follow-up imaging should be considered for those PLAs with potential risk of malignancy.

### Peer review

This is a unique manuscript documenting the clinical course of liver abscess associated with/without malignancy. The authors report differences between two groups (liver abscess with and without malignancy) and liver abscess with malignancy was associated with unfavorable prognosis. The viewpoint of this manuscript is interesting, but the clinical impact of this study is not fully discussed.

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## Association between gastric cancer and -1993 polymorphism of *TBX21* gene

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### Abstract

**AIM:** To investigate the association between the polymorphism of *TBX21* gene and the risk of gastric cancer in a Chinese population.

**METHODS:** The -1993 polymorphism located in *TBX21* gene promoter region was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The risk between *TBX21* gene genotype and gastric cancer was determined by multivariate logistic regression analysis in 220 gastric cancer patients and 262 cancer-free controls matched by age, sex and ethnicity.

**RESULTS:** Compared with the *TBX21* -1993TT geno-

type, the -1993CC genotype exhibited a significantly elevated risk for gastric cancer [Odds ratio (OR) = 3.42, 95% confidence interval (CI): 1.41-8.31]. The relationship between the -1993 polymorphic genotype and the invasive status such as lymph node and distant metastasis was found among the gastric cancer patients (OR = 4.02, 95% CI: 1.87-8.66; OR = 7.02, 95% CI: 3.44-14.34, respectively).

**CONCLUSION:** *TBX21* -1993 polymorphism might contribute to the risk of gastric cancer, especially to the distant metastasis.

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**Key words:** *TBX21* gene; Gastric cancer; Polymorphism; Genetic susceptibility; Association analysis

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Zhang LH, Li Q, Li P, Zhu ST, Wang J, Yang HL, Xu CQ, Guo XH. Association between gastric cancer and -1993 polymorphism of *TBX21* gene. *World J Gastroenterol* 2012; 18(10): 1117-1122 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i10/1117.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i10.1117>

### INTRODUCTION

Gastric cancer is the fourth most common cancer, which approximately accounts for 12% of all cancer deaths and currently ranks second in global cancer mortality only behind the lung cancer<sup>[1]</sup>. Until recently, although the global incidence of gastric cancer has been dramatically

decreased in different regions, it still represents a leading cause of death worldwide, especially in China and other Asian countries<sup>[2]</sup>. It is estimated that more than 930 000 new cases of gastric cancer would be diagnosed each year in the world. Although the different management approaches were performed, a minimum of 700 000 patients would die from this malignant disease<sup>[3]</sup>.

In the previous study, the dietary factors, such as nitrates, smoked fish and salted meats, moldy foods containing aflatoxin, and the infection with *Helicobacter pylori* (*H. pylori*), were thought to be involved in the development of gastric cancer<sup>[4]</sup>, but the detailed mechanisms of gastric carcinogenesis are still unknown. In spite the new insight of the immune surveillance, the molecular elimination mechanism and host susceptibility to malignant gastric cell still remained largely unsolved. The susceptibility of the host genetic factor to malignant disease is a dynamic interactive process, which is thought to be involved in the balance between the host immune response and the malignant cell apoptosis. Moreover, multitudinous cytokines played crucial roles against the malignant transformation of normal cells in this process<sup>[5]</sup>.

Although the genetic basis of host susceptibility to the development of gastric cancer had not been clearly discovered, our previous study reported that the -1661A/G polymorphism located at *cytotoxic T lymphocyte-associated antigen-4* (*CTLA-4*) gene promoter might significantly increase the risk of gastric cancer development<sup>[6]</sup>. Similarly, in the immune-regulated area, researches demonstrated that the *TBX21* gene (NCBI OMIM nos.\*604895), which encoded the transcription factor T-bet (T-box expressed in T cells), was a key transcriptional activator to induce the differentiation from helper T cells to Th1 cells<sup>[7]</sup>. In the previous experiments, the expression of *TBX21* gene was proved to be associated with breast cancer and the T-bet protein played a key role in natural killer (NK) cell-mediated melanoma metastatic disease<sup>[2,8]</sup>. These evidences indicated that the expression of *TBX21* gene was closely associated with the development of cancer through the Th1 and NK cells pathway<sup>[9]</sup>. Moreover, the -1993 polymorphism located in *TBX21* gene promoter region had been verified to alter the transcription factor binding and regulating the *TBX21* gene expression<sup>[10]</sup>. Hence, in this study, we systematically scrutinized the -1993 genetic polymorphism and assessed the potent susceptibility of this *TBX21* gene (SNP) to gastric cancer in the Chinese population.

## MATERIALS AND METHODS

### Patients

This population-based case-control study was carried out in two hospitals: Beijing Friendship Hospital, Capital Medical University and Qianfoshan Hospital, Shandong University, China. Followed an identical protocol, each hospital was responsible to recruit the gastric cancer patients consecutively from 2005 through 2010. All patients were histopathologically confirmed by endoscopic biopsy

or surgical specimen. At the same time, a comparable group of normal controls without gastric cancer or other disease was included in this study from the two hospitals. Two hundred and twenty eligible patients with gastric cancer (124 males and 96 females) were enrolled in this study. The individuals who possessed secondary, recurrent malignancies, and accepted blood transfusion were excluded. There were eight cases of gastric cancers located in cardia, 117 in non-cardia, six in the upper third, 49 in the middle third, and 40 in the lower third. Two hundred and sixty two controls (143 males and 119 females) proved to be free from any malignant diseases by the physical examination were randomly and simultaneously recruited from the two hospitals. Except for matching the gastric cancer patients by gender and age (within 5 years), control subjects with severe clinical symptoms, previous diagnosis of cancer, and genetic disease were excluded. The mean age of the gastric cancer patients was  $41.14 \pm 7.10$  years (mean  $\pm$  SD) and the control group was  $41.69 \pm 8.83$  years. There were no statistically significant differences in current smoking or drinking status, age, sex, and race between the two groups. All participants were of Han nationality from Northern China. The minor distinct ethnic groups and migrants from other countries were not included. This study protocol was approved by the Institutional Review Boards of participating institutions, and each participant should provide a signed informed consent before the two mL peripheral blood sample was collected.

### DNA extraction and genotyping method

According to the standard protocol as described in a previous study<sup>[11]</sup>, genomic DNA was extracted from peripheral blood leukocytes using sodium dodecyl sulphate lysis and proteinase K digestion followed by the standard phenol-chloroform extraction. DNA samples were quantified and subjected to specific polymerase chain reaction (PCR) amplification as described below. The -1993 polymorphism within *TBX-21* gene promoter region was identified by the reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers which were used in PCR amplification for the promoter specific fragments were described previously<sup>[10]</sup>. The PCR was performed in a 20  $\mu$ L total reaction volume containing 2  $\mu$ L 10  $\times$  PCR buffer (Qiagen Inc., Hilden, Germany), 1.5 mmol/L MgCl<sub>2</sub>, 0.5  $\mu$ mol/L each primer, 0.2 mmol/L dNTP, 1.2 U Taq polymerase (Qiagen Inc., Hilden, Germany) and 200 ng of genomic DNA. After an initial denaturation at 95  $^{\circ}$ C for 5 min, the DNA was amplified by 35 cycles of 94  $^{\circ}$ C for 30 s, 62.0  $^{\circ}$ C for 40 s, and 72  $^{\circ}$ C for 45 s, with a final elongation at 72  $^{\circ}$ C for 10 min on the Gene-Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA, United States). PCR products were purified using a Multi-Screen-PCR purifying plate (Millipore Company, Billerica, MA, United States). Under the condition recommended by the manufacturer's instruction, the purified PCR products were digested by the restrictive enzyme *Hha* I (New England Biolabs Beverly, MA, United States). The digest-

Table 1 Distribution of *TBX21* -1993T/C allelic frequencies in cases and controls *n* (%)

Variables	Case	Control	Adjusted OR (95% CI)	<i>P</i> trend
Age (yr) (mean ± SD)	41.14 ± 7.10	41.69 ± 8.83		0.884 <sup>1</sup>
Gender				
Male	124 (56.36)	143 (54.58)		0.695 <sup>2</sup>
Female	96 (43.64)	119 (45.42)		
Smoking status				
Never	160 (72.73)	201 (76.72)		0.314 <sup>2</sup>
Always	60 (27.27)	61 (23.28)		
Drinking status				
Never	149 (67.73)	188 (71.76)		0.337 <sup>2</sup>
Always	71 (32.27)	74 (28.24)		
Family history of cancer				
No	181 (82.27)	240 (91.60)		0.002 <sup>2</sup>
Yes	39 (17.73)	22 (8.40)		
<i>TBX21</i> -1993				
T	356 (80.91)	473 (90.27)	1.00	-
C	84 (19.09)	51 (9.73)	2.06 (1.44-2.96)	< 0.001 <sup>2</sup>
T/T	154 (70.00)	219 (83.59)	1.00	-
T/C	48 (21.82)	35 (13.36)	1.84 (1.13-3.01)	0.015 <sup>3</sup>
C/C	18 (8.18)	8 (3.05)	3.42 (1.41-8.31)	0.007 <sup>3</sup>

<sup>1</sup>Mann-Whitney *U* test; <sup>2</sup> $\chi^2$  test; <sup>3</sup>Multivariate unconditional logistic regression model. SD: Standard deviation; CI: Confidence interval; OR: Odds ratio.

ed PCR products were fractionated on 4% agarose Tris-borate-EDTA gel (Agarose 1000; Gibco BRL, Rockville, MD, United States) and stained with ethidium bromide. All assays were conducted blindly by two researchers without the knowledge of case or control status. Additionally, about 10% of the samples were randomly selected and retested, and the results were 100% concordant.

### Statistical analysis

The frequency distributions of demographic variables and putative risk factors to gastric cancer, including age, gender, smoking or drinking status, and family history of cancer were examined in case and control groups by Mann-Whitney *U* test or Chi-square test. Multivariate unconditional logistic regression was used to determine the statistical significance of interaction between the certain variables and gastric cancer. Odds ratios (ORs) were adjusted by matching the variables. The Chi-square was used to compare the observed genotype distributions with the expected genotype by the Hardy-Weinberg equilibrium. All calculations were performed using the SPSS/Win statistical program (version 11.5.1 for Windows; SPSS Inc, Chicago, IL, United States). The statistical tests were two-sided, and the values of *P* < 0.05 were considered statistically significant.

## RESULTS

### Study population characteristics

In our experiment, *TBX21* gene -1993 loci within its promoter region were found in 482 Chinese individuals consisting of 220 gastric cancer patients and 262 controls, and the substitution of nucleotide from T to C was detected by PCR-RFLP method. The observed -1993 T/C genotypes of *TBX21* gene were in Hardy-Weinberg equilibrium (*P* > 0.05). There were no statistically significant

differences in age (*P* = 0.884) or gender distribution (*P* = 0.695) between patients and controls by the Mann-Whitney *U* test or Chi-square test (Table 1). The family cancer history in case group showed statistically significant difference from the control group (*P* = 0.002). The amounts of alcohol intake and tobacco smoking were similar between patients and controls.

### Association between *TBX21* -1993 polymorphism and gastric cancer

Under the subgroup analysis stratified by the confounding factors or as a whole group analysis in the gastric cancer cases, the association between the genotype frequencies of *TBX21* gene -1993T/C polymorphism and gastric cancer is shown in Tables 1 and 2. The distribution of the T allele at the -1993 loci was significantly different between the control and case groups (OR = 2.06, 95% CI: 1.44-2.96, Table 1). The -1993TC and -1993CC genotypes were associated with the risk of gastric cancer (OR = 1.84, 95% CI: 1.13-3.01; OR = 3.42, 95% CI: 1.41-8.31; respectively). When the data were stratified by gender, age, smoking or drinking status and the family history of cancer, the stronger association was revealed in the male patients aged less than 65 years with frequent smoking, especially in the patients who had family histories of cancer (OR = 3.99, 95% CI: 1.06-15.03, Table 2).

### Clinicopathological association between patients and *TBX21* -1993 polymorphism

The association between the clinicopathological characteristics of the gastric cancer and the genotype distributions is shown in Table 3. In the multivariate unconditional logistic regression analysis, the clinicopathological parameters did not show any significant relations to -1993TC genotype in age (*P* = 0.218), gender (*P* = 0.388), tumor size (*P* = 0.369), degree of differentiation (*P* =

Table 2 Multivariate logistic regression analysis of *TBX21* -1993T/C stratified by the selected variables

Variables	Cases/controls		Adjusted OR (95% CI)	P
	T/T	T/C + C/C		
Gender				
Male	82/118	42/25	2.37 (1.31-4.31)	0.005
Female	72/101	24/18	1.89 (0.95-3.76)	0.071
Age (yr)				
< 55	94/114	50/27	2.79 (1.61-4.81)	< 0.001
55-65	56/59	15/11	2.13 (1.36-3.31)	0.001
> 65	4/16	1/5	0.58 (0.04-7.92)	0.683
Smoking status				
Never	118/167	42/34	1.77 (1.06-2.97)	0.029
Always	36/52	24/9	3.38 (1.31-8.71)	0.012
Drinking status				
Never	105/155	44/33	2.00 (1.18-3.89)	0.010
Always	49/64	22/10	2.48 (1.04-5.90)	0.040
Family history of cancer				
No	131/201	50/39	1.99 (1.23-3.21)	0.005
Yes	23/18	16/4	3.99 (1.06-15.03)	0.041

CI: Confidence interval; OR: Odds ratio.

Table 3 Clinicopathological characteristics analysis of 220 gastric cancer patients and *TBX21* -1993T/C polymorphism *n* (%)

Variables	All	T/T	T/C	C/C	Adjusted OR (95% CI)	P
Age (yr)						
≥ 60	26 (11.82)	21 (80.77)	3 (11.54)	2 (7.69)	1.93 (0.68-5.47)	0.218
< 60	194 (88.18)	133 (68.55)	45 (23.20)	16 (8.25)		
Gender						
Male	124 (56.36)	82 (66.13)	29 (23.39)	13 (10.48)	0.70 (0.32-1.56)	0.388
Female	96 (43.64)	72 (75.00)	19 (19.79)	5 (5.21)		
Tumor size						
≥ 5 cm	123 (55.91)	84 (68.29)	30 (24.39)	9 (7.32)	0.75 (0.40-1.41)	0.369
< 5 cm	97 (44.09)	70 (72.16)	18 (18.56)	9 (9.28)		
Degree of differentiation						
G1-2	107 (48.64)	70 (65.42)	23 (21.50)	14 (13.08)	1.64 (0.87-3.07)	0.124
G3-4	113 (51.36)	84 (74.34)	25 (22.12)	4 (3.54)		
TNM stage						
Stage I / II	67 (30.45)	47 (70.15)	13 (19.40)	7 (10.45)	0.73 (0.36-1.46)	0.370
Stage III / IV	153 (69.55)	107 (69.93)	35 (22.88)	11 (7.19)		
Lymph node status						
N <sub>0</sub>	78 (35.45)	66 (84.62)	6 (7.69)	6 (7.69)	4.02 (1.87-8.66)	< 0.001
N <sub>1-3</sub>	142 (64.55)	88 (61.97)	42 (29.58)	12 (8.45)		
Distant metastasis						
M0	148 (67.27)	121 (81.76)	18 (12.16)	9 (6.08)	7.02 (3.44-14.34)	< 0.001
M1	72 (32.73)	33 (45.83)	30 (41.67)	9 (12.50)		

CI: Confidence interval; OR: Odds ratio.

0.124), and the TNM stage ( $P = 0.370$ ). In contrast, the patients were dichotomized in TC + CC and TT genotypes, a significantly higher rate of pN<sub>1-3</sub>-category was detected in TC + CC allele carriers (38.03%). It showed that the -1993 SNP was significantly associated with the lymph node metastasis ( $P < 0.001$ , OR = 4.02, 95% CI: 1.87-8.66; Table 3). Moreover, the eventual statistical results revealed that the TC+CC genotype in gastric cancer patients was more closely associated with the tumor distant metastasis with respect to the frequency of wild genotype TT ( $P < 0.001$ , OR = 7.02, 95% CI: 3.44-14.34).

## DISCUSSION

In antitumor immunity, the local progression or distant

metastasis of primary tumors was extrinsically controlled by type 1 immune responses, particularly *via* the cytokine interferon (IFN)- $\gamma$  pathway<sup>[12-14]</sup>. The secretion of IFN- $\gamma$  was highly dependent on helper T cells, and then the helper T cell-dependent type 1 (IFN- $\gamma$ )-related responses promoted the tumor immunogenicity and inhibited the tumor cell progression. The transcription factor T-bet (*TBX21*; GenBank database accession number: AC003665) gene is a member of the T-box family, which is located on chromosome17q21.32, and plays a critical role in the development of type 1 helper T cells<sup>[15]</sup>. In the *T-bet* gene knockout mice, it exhibited a dramatic difference from the wild-type mice in the expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-12, and IL-13<sup>[16,17]</sup>. The levels of these cytokines significantly decreased in T-bet knockout mice

compared with the wild-type mice. Because the infection of *H. pylori* is one of the major risk factors for gastric cancer, T-bet seemed to be important for *H. pylori*-induced gastric cancer in mice<sup>[18]</sup>. In mouse T-bet transgenic adenocarcinoma model, another study demonstrated that T-bet could affect the primary tumor incidence and lead to a moderate decrease in the rate of tumor progression. Meanwhile, T-bet not only possessed a moderate effect on the primary cancers but primarily exerted a significant suppressor capability in the metastasis of cancer<sup>[19]</sup>. These findings strongly supported that the T-bet regulation role in immune response was related to cancer risk and could account for a portion of this risk in cancer metastasis<sup>[20]</sup>.

This is the first epidemiologic study to evaluate the association between -1993 variant of *TBX21* gene and gastric cancer. A positive association was observed between -1993T/C and gastric cancer. When analyzed by genotypes, the CC genotype was strongly associated with the gastric cancer development after adjusting for potential confounders (OR = 3.42). When stratified by gender, age, smoking or drinking status, and the family history of cancer, the adjusted OR was 3.38 (95% CI: 1.31-8.71) for smokers and the adjusted OR was 3.99 (95% CI: 1.06-15.03) for the individuals with cancer family histories.

In our present experiment, when stratified by age and gender among gastric cancer patients, no positive association was found between the -1993 polymorphism and gastric cancer. Similarly, no clear difference was noticed between this polymorphic site and clinicopathological characteristics such as tumor size, degree of differentiation, and TNM stage status. Although no association was observed between -1993TC and the progression of gastric cancer, the invasive status of lymph node was related to the distant metastasis rate of tumor (OR = 4.02 and 7.02). The results revealed that the *TBX21* -1993T/C polymorphism played a crucial role in the process of gastric cancer metastasis. To our best knowledge, *TBX21* -1993T/C polymorphism has not been investigated in gastric cancer. However, the SNPs have often been observed to be amplified in virus infection<sup>[21-22]</sup>, systemic sclerosis<sup>[23]</sup>, asthma<sup>[24]</sup>, rheumatoid arthritis<sup>[25]</sup>, and systemic lupus erythematosus<sup>[26]</sup>, suggesting that our results are not by chance.

In summary, this population-based case-control study indicated that the host *TBX21* -1993T/C polymorphism was associated with an increased risk of gastric cancer, especially with the distant metastasis. The findings suggested that host *TBX21* -1993 polymorphism might be a potential marker to identify the individuals who were in the risk of gastric cancer, especially in China. Because of the limited sample size and single race for these genotypes, further replicated studies or pooled analyses are needed to confirm our results and the interaction study between the *H. pylori* infection and the genetic factors should be conducted as well.

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## COMMENTS

### Background

*TBX21*, which played a critical role in modulating the development of naive T lymphocyte, has been thought to be a susceptibility gene in the progress of cancer. In this study, the authors investigated the association between the potentially functional polymorphism of *TBX21* gene and the risk of gastric cancer in the Chinese population.

### Research frontiers

Gastric cancer is the one of the most common cancers in the Chinese population, many studies have reported that the expression of *TBX21* gene is closely associated with the development of cancer through the Th1 and NK cells pathway. In this study the authors reported that the -1993 genetic polymorphism located in *TBX21* gene promoter region and assessed the potent susceptibility of this *TBX21* gene to gastric cancer in the Chinese population.

### Innovations and breakthroughs

This is one of the first studies of its kinds to investigate whether the host *TBX21* -1993T/C polymorphism was associated with an increased risk of gastric cancer, especially the distant metastasis. The findings suggested that host *TBX21* -1993 polymorphism might be a potential marker to identify the individuals who were at the risk of gastric cancer.

### Applications

This study provides the evidence of the association between gastric cancer and the -1993 polymorphism of *TBX21* gene. Because of the limited sample size and single race for these genotypes, further studies are needed to confirm the findings.

### Terminology

The *TBX21* gene (NCBI OMIM nos.\*604895), which encoded the transcription factor T-bet (T-box expressed in T cells), is a key transcriptional activator to induce the differentiation from helper T cells to Th1 cells. The gene polymorphism represents the different base substitution or replacement in the DNA sequence.

### Peer review

In the present study, the *TBX21*-1993 polymorphism was suggested to be associated with the risk of gastric cancer, in particular, the distant metastasis. The finding overall is interesting.

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## Interleukin-8, a promising predictor for prognosis of pancreatic cancer

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### Abstract

**AIM:** To investigate the value of interleukin-8 (IL-8), a pro-inflammatory chemokine, in predicting the prognosis of pancreatic cancer.

**METHODS:** Expression of IL-8 and its receptor CXCR1 was assessed by immunohistochemistry in pancreatic cancer and chronic pancreatitis samples. Enzyme-linked immunosorbent assay was used to detect the serum IL-8 levels in pancreatic cancer patients. Human pancreatic cancer tissues were heterotopically transplanted to the immune-deficiency mice to evaluate the effect of serum IL-8 on the tumorigenesis of the cancer samples.

**RESULTS:** IL-8 and CXCR1 proteins were both over-expressed in pancreatic adenocarcinoma samples (55.6% and 65.4%, respectively) compared with the matched para-cancer tissues (25.9% and 12.3%,  $P < 0.01$ ), or chronic pancreatitis (0% and 25%,  $P < 0.05$ ). Serum IL-8 levels in pancreatic cancer patients ( $271.1 \pm 187.7$  ng/mL) were higher than in other digestive system tumors, such as gastric cancer ( $41.77 \pm 9.11$  ng/mL,  $P = 0.025$ ), colorectal carcinoma ( $78.72 \pm 80.60$  ng/mL,  $P = 0.032$ ) and hepatocellular carcinoma ( $59.60 \pm 19.80$  ng/mL,  $P = 0.016$ ). *In vivo* tumorigenesis analysis further proved that tumor tissues from patients with higher serum IL-8 levels grew faster than those with lower IL-8 levels.

**CONCLUSION:** IL-8 can be a fine serum marker for predicting the prognosis pancreatic cancer.

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**Key words:** Pancreatic cancer; Interleukin-8; CXCR1; Immunohistochemistry; Tumor implantation; Enzyme-linked immunosorbent assay

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### INTRODUCTION

Interleukin-8 (IL-8) is a pro-inflammatory factor, belonging to CXC chemokine family. It was initially named neutrophil-activating peptide-1 for its potent chemotactic activity on granulocytes in inflammatory and immune

diseases<sup>[1,2]</sup>. Recently, it has been shown that IL-8 plays a critical role in cancer invasion, angiogenesis and metastasis<sup>[3-6]</sup> and is considered as an important component of tumor microenvironment<sup>[7,8]</sup>. The significance of tumor microenvironment, which can be described as the “soil” of cancer cells, has been emphasized, especially the cancer-stroma interaction, which has become critical determinants of cancer behavior<sup>[9]</sup>. Stromal cells can produce IL-8 to influence the ability of invasion or metastasis of cancer cells, and the cancer cells themselves can also secrete IL-8 in an autocrine or paracrine manner, such as in breast cancer<sup>[10]</sup>, gastric cancer<sup>[4]</sup>, colon cancer<sup>[11]</sup>, cervical cancer<sup>[12]</sup>, pancreatic cancer<sup>[8,13]</sup> and leukemia<sup>[14,15]</sup>.

Pancreatic cancer is one of the most aggressive human malignancies and often with a dismal prognosis. The overall 5-year survival rate is only 5% or even less. Patients with pancreatic cancer have already been at advanced stage in their first visit to hospital. Around 15%-20% of the patients have the chance for tumor resection and the rest can only receive adjuvant therapies<sup>[16]</sup>. Although new drugs and techniques have been developed in treating pancreatic cancer, their therapeutic effects varied. Even the first-line anticancer drug, gemcitabine, still has not achieved satisfactory results in improving patients' outcome. The overall survival of pancreatic cancer patients is quite low, it is therefore, very important to predict the prognosis of the patients so as to enable more active treatment to prolong their lives.

Many studies<sup>[8]</sup> have revealed that pancreatic cancer highly produces IL-8, which can promote angiogenesis and invasion of tumors. Serum IL-8 levels were elevated in pancreatic cancer patients<sup>[17]</sup>, suggesting the feasibility of IL-8 to be a fine marker in predicting the outcome of the patients. The main aim of the present study is to investigate the prognostic value of IL-8 in pancreatic cancer patients. We examined the expression and secretion levels of IL-8 in both tumor tissue and human blood. Furthermore, we implanted the cancer tissues from patients with various serum IL-8 levels subcutaneously to the nude mice and observed the growth of each xenograft. Based on these *in vitro* and *in vivo* analyses, we aim to more precisely define the role of IL-8 in predicting the prognosis of pancreatic cancer patients.

## MATERIALS AND METHODS

### Sample collection

Eighty-one pancreatic ductal adenocarcinoma (PDC) specimens with matched para-cancerous pancreas were collected during resection in the surgically treated patients. Five cases of chronic pancreatitis (CP) served as controls. All patients with pancreatic cancer had been followed up for survival and outcome until October 2008. Except for four patients who were alive till the end of follow-up, the rest all died.

The clinical information of the patients is presented in Table 1. Besides the patients with PDC, patients with chronic and acute pancreatitis (AP) were also included.

**Table 1** Clinical data of patients subjected to enzyme-linked immunosorbent assay analysis for serum interleukin-8

Groups	Sex		Age (yr)	
	Male	Female	Range	Median
PDC	13	14	45-80	58
CP	13	2	9-58	40
AP	3	5	37-73	51
DA	3	5	47-65	58
GC	1	2	47-55	53
CRC	3	1	48-68	63
HCC	1	1	55-72	63.5

PDC: Pancreatic ductal carcinoma; CP: Chronic pancreatitis; AP: Acute pancreatitis; DA: Duodenal adenocarcinoma; GC: Gastric carcinoma; CRC: Colorectal carcinoma; HCC: Hepatocellular carcinoma.

**Table 2** Background information of cell lines for interleukin-8 detection

Cell lines	Cell origin	Number in cell line library
BxPC3	Primary adenocarcinoma	CRL-1687 (ATCC)
CFPAC-1	Liver metastasis from adenocarcinoma	CRL-1918 (ATCC)
SW1990	Spleen metastasis from adenocarcinoma	CRL-2172 (ATCC)
Patu 8988s	Liver metastasis from adenocarcinoma	ACC 204 (DSMZ)

ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures.

Those with other carcinomas from gastrointestinal system such as stomach, large bowel and liver were also investigated as controls. Diagnosis for all these patients was pathologically confirmed by HE staining. Supernatant from four pancreatic cancer cell lines (BxPC3, SW1990, CFPAC-1 and Patu 8988s) was collected after the cells were cultured for 48 h. Detailed information of these cell lines is shown in Table 2.

### Ethics

This study was approved by the Ethics Committee of the Second Military Medical University (Shanghai, China). Informed consent was obtained from each patient before tissue specimen and blood samples were collected.

### Immunohistochemical analysis

Unstained 3- $\mu$ m sections were cut from the paraffin blocks and deparaffinized by routine procedures. Envision solution (K4065, DAKO, Denmark) was added to detect the primary antibody and 3,3'-diaminobenzidine was applied as chemicon. Sections were counterstained with hematoxylin. Primary antibodies against IL-8 (AHC0881) and CXCR1 (AHR1522Z) were purchased from BioSource (California, United States). In immunohistochemical (IHC) analysis, all the focal cases were considered negative and cases showing diffused expression were considered positive. Only the positive signals in epithelial cells were taken into account.

### Enzyme-linked immunosorbent assay

The concentration of serum IL-8 in the patients was determined quantitatively with human IL-8 enzyme-linked immunosorbent assay (ELISA) detection kit provided by BioSource following the manufacturer's instructions. The up-limit of the test was 500 ng/mL.

### Animal models and heterotopic implantation of human tumor tissues

Eleven severe combined immunodeficiency (SCID) nude mice aged 6-7 wk were housed under specific pathogen-free conditions. Animals were inoculated with subcutaneously (sc) transplanted fresh tissue cubes (1 mL) from pancreatic cancer patients with a detected serum IL-8 level. Animals were monitored daily by general clinical observation throughout the study period. Tumor volumes were calculated based on the following formula: tumor volume = (length × width<sup>2</sup>)/2. Animals were euthanized when their tumors reached an appropriate size (0.3 cm<sup>3</sup>) and the latent period was recorded. All experimental manipulations were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Biomedical Ethics Committee of the Second Military Medical University (Shanghai, China).

### Histological analysis of implanted tumor tissues

After implanted with human tumor tissues heterotopically, animals with growing tumor were sacrificed by craniocervical dislocation. The mice were then weighed, and the sc tumor tissues were excised. Part of the tumor tissues underwent routine histological examination and the rest was stored in liquid nitrogen for future use. Histology of the corresponding human tumor tissues was also reviewed.

### Statistical analysis

The IHC results were analyzed by  $\chi^2$  test. And the ELISA assay results were expressed as arithmetic mean ± SD. The comparisons between groups of patients were made using analysis of variance, and Fisher's Least Significant Difference test was used for animal models. The difference was considered significant at  $P < 0.05$ . SPSS 10.0 was applied as a statistical tool. Survival rates were calculated by the Kaplan-Meier method.

## RESULTS

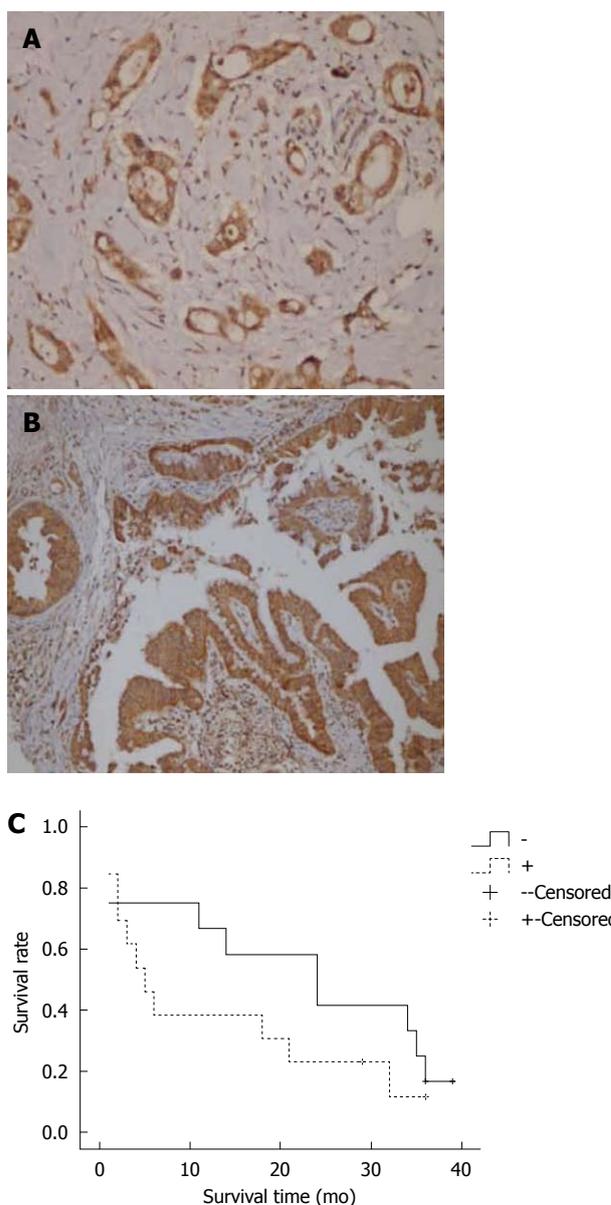
### IL-8 and CXCR1 expression in PDC

IL-8 was expressed in 55.6% (45/81) of pancreatic cancer specimens, whereas in 25.9% (21/81) of non-cancer tissues ( $P < 0.01$ ,  $\chi^2 = 14.727$ ) (Table 3). IL-8 immunoreactivity was absent in CP (0%, 0/5) ( $P = 0.016$ ,  $\chi^2 = 5.827$ ) (Table 3). The positive signal was localized in the cytoplasm of cancerous or normal ductal cells (Figure 1). Focal stromal cells could express both IL-8 and CXCR1. The relationship between the expression of IL-8 or CXCR1 and the histological grading of pancreatic cancer is shown in Table 3. The CXCR1 expression level was significantly higher in pancreatic cancer samples (65.4%,

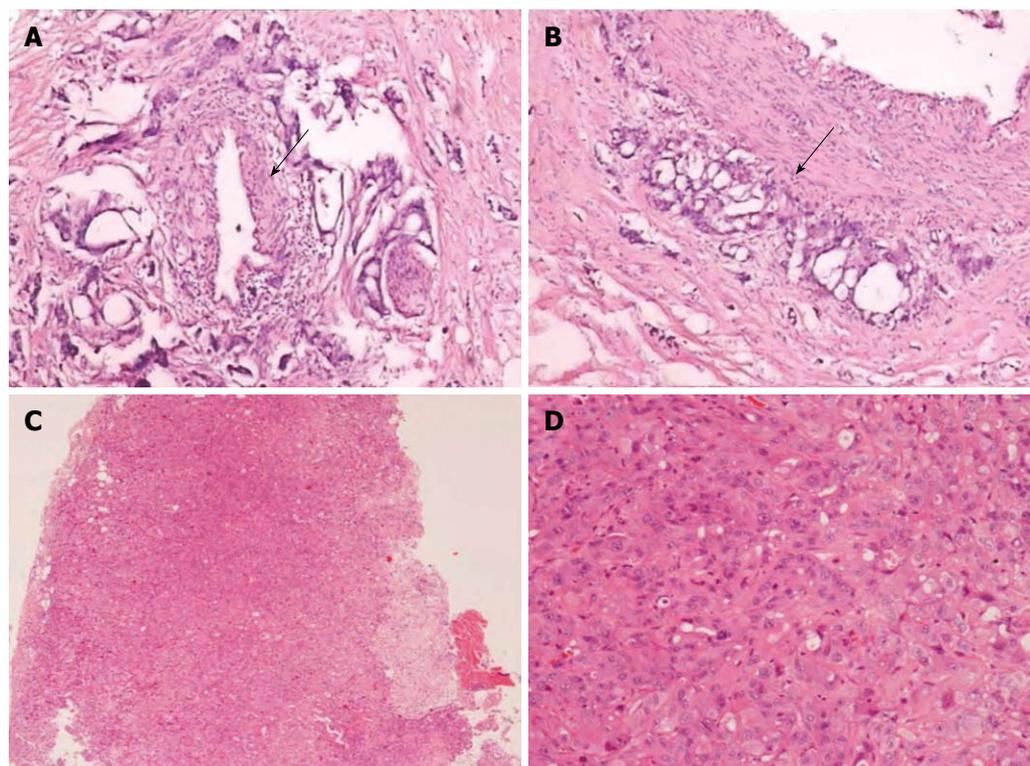
**Table 3** Interleukin-8 and CXCR1 expression in pancreatic cancer, matched para-cancer tissue and chronic pancreatitis

	Pancreatic cancer differentiation			Matched para-cancer tissue	Chronic pancreatitis
	Well	Moderate	Poor		
IL-8					
+	5	33	7	21	0
-	11	22	3	60	5
CXCR1					
+	10	35	8	10	1
-	6	20	2	71	4

IL-8: Interleukin-8.



**Figure 1** Interleukin-8 and CXCR1 expression in pancreatic cancer. A: Interleukin-8 (IL-8) expression in pancreatic cancer (× 200); B: CXCR1 expression in pancreatic cancer (× 200); The positive signals of IL-8 and CXCR1 were mainly located in the cytoplasm of cancer cells. For IL-8, focal positivity was found in the fibroblasts; C: Survival analysis of pancreatic cancer patients. "Censored" means patients were still alive till the end of follow-up study. The patients without IL-8 expression survived longer than those IL-8 positive.



**Figure 2** Histology of human pancreatic cancer tissue and transplanted tumor in SCID mice. A: Both vascular (arrow) and neural invasion (cross) were seen in the original human pancreatic ductal adenocarcinoma (PDC) ( $\times 100$ ); B: Cancer cells were infiltrating the medium-sized vessel in human PDC tissues ( $\times 100$ ) (arrow). Desmoplastic response was obvious in the human PDC tissues; C, D: Transplanted cancer tissue in SCID mice in low power (C,  $\times 40$ ) and in high power (D,  $\times 200$ ). Cancer cells were almost clustered and poorly differentiated. In some areas, primitive gland formation could be seen (arrow). Cells grew into sheet with very less stromal reaction than the original human tissues.

**Table 4** Interleukin-8 concentration in the supernatant of pancreatic cancer cell lines

Cell line	IL-8 concentration (ng/mL)
BxPC3	81.38
CFPAC-1	3.906
SW1990	> 500
Patu 8988s	498.9

SW1990 and Patu8988 originated from metastatic sites showed much higher levels of interleukin-8 (IL-8) than BxPC3 and CFPAC-1.

53/81) than in samples from the adjacent non-cancerous pancreas (12.3%, 10/81) ( $P < 0.001$ ,  $\chi^2 = 48.026$ ). No statistical difference was observed between histological grading and IL-8 or CXCR1 expression.

Overall survival analysis was performed in the patients. Most people died within 3 years after surgery and only four were alive till the end of follow-up. The median survival was 5 mo (range, 1-36 mo) in the IL-8 positive group and 24 mo (range, 1-39 mo) in the IL-8 negative group. Although IL-8 positive patients seemed to live shorter than IL-8 negative ones, no significant difference was observed by Kaplan-Meier method ( $P = 0.245$ ).

#### Serum IL-8 levels in pancreatic cancer patients and pancreatic cancer cell lines

Serum IL-8 level was measured in patients with PDC ( $n$

= 27), AP ( $n = 8$ ), CP ( $n = 15$ ) and other kinds of cancer from digestive system, such as duodenal adenocarcinoma (DA,  $n = 4$ ), gastric carcinoma (GC,  $n = 3$ ), rectal colonic carcinoma (CRC,  $n = 4$ ), and hepatocellular carcinoma (HCC,  $n = 2$ ). Blood was collected before surgery in the cancer patients. The serum IL-8 levels were significantly higher in PDC ( $271.1 \pm 187.7$  ng/mL) than in CP ( $97.02 \pm 130$  ng/mL,  $P = 0.002$ ), AP ( $133.6 \pm 162.9$  ng/mL,  $P = 0.041$ ), GC ( $41.77 \pm 9.11$  ng/mL,  $P = 0.025$ ), CRC ( $78.72 \pm 80.6$  ng/mL,  $P = 0.032$ ) or HCC ( $59.6 \pm 19.8$  ng/mL,  $P = 0.016$ ). No significant difference was observed between PDC and DA ( $168.7 \pm 212.4$  ng/mL,  $P = 0.247$ ). Two cancer cell lines originated from metastatic site (SW1990 and Patu 8988s) showed a much high level of IL-8 secretion (Table 4).

#### Histology of human tumor tissues implanted into SCID mice

Tumors subcutaneously implanted into the SCID mice were mostly composed of strands of cells, and glandular architectures were not very obvious (Figure 2). Intracellular vacuoles could be observed in some regions as immature or primary glands, indicating their adeno-epithelial origin. Unlike cancers growing in human pancreas, the desmoplastic reaction was not so remarkable and only several fibroblastoid spindle cells appeared in the implanted cancer tissues. Nerve and vessel invasion by can-

cer cells was easily observed in human cancer tissues, but none was detected in transplanted cancer tissues.

### ***In vivo* growth of heterotopically transplanted tumor tissues and its correlation with serum IL-8 levels**

Among all the 11 cases of human tumor tissues implanted into SCID mice, seven tumors grew subcutaneously in animals. We set the terminal goal of growth at 0.3 cm<sup>3</sup> and recorded the latent period. The slowest growing period was 93 d and the fastest was 44 d. Seven animals with tumors were divided into three groups based on the median latent period of 61 d for analysis: group 1, failed to form tumor,  $n = 4$ ; group 2, latent period < 61 d,  $n = 3$ ; and group 3, latent period  $\geq 61$  d,  $n = 4$ . Serum IL-8 level in group 2 ( $140.1 \pm 33.4$  ng/mL) was much higher than in group 1 ( $23.4 \pm 17.4$  ng/mL) and group 3 ( $23.2 \pm 16.1$  ng/mL) ( $P < 0.001$ ). There was no significant difference between group 1 and group 3 ( $P = 0.998$ ).

## **DISCUSSION**

In this study, the expression level of IL-8 and its receptor CXCR1 was elevated in pancreatic cancer and serum IL-8 level was significantly higher in patients with pancreatic cancer than in those with pancreatitis and also higher than in the patients with other kinds of tumors from digestive system. *In vivo* tumor tissues from pancreatic cancer patients with a higher serum IL-8 level grew faster and behaved more aggressively than those with low serum IL-8 levels. Follow-up data showed that patients with high serum IL-8 levels had a relatively lower survival than those with low serum IL-8 levels. Due to the insufficient number of patients involved in the follow-up analysis, no statistical difference was achieved in the two groups.

IL-8 is a member of the CXC chemokine family and is a chemotactic factor for T cells, neutrophils, and basophils. Besides its pro-inflammatory role, IL-8 has been evaluated as a pro-oncogenic effector in various types of human cancers, including leukemia, astrocytoma, melanoma, breast cancer, ovarian cancer, lung cancer, prostate cancer, colon cancer, renal cell carcinoma, gastric cancer and pancreatic cancer<sup>[18]</sup>. The most critical effect of IL-8 on cancer cells is its strong angiogenic potential and ability of promoting invasion and metastasis<sup>[19-21]</sup>. *In vitro* IL-8 can enhance the proliferation and survival of endothelial cells and through its receptor CXCR1 can upregulate the expression of matrix protein, MMP-2 and MMP-9<sup>[22]</sup>. IL-8 can mimic the role of vascular endothelial growth factor (VEGF), transactivate VEGFR2 and promote angiogenesis<sup>[23]</sup>. Various signals or pathways can induce IL-8 expression in cancers<sup>[24-26]</sup> and the whole IL-8-involved network is very complicated. It has been confirmed that nuclear factors, NF- $\kappa$ B and AP-1 are the upstream regulators of transcription of IL-8 mRNA, both of which may cooperate with each other in the production of IL-8. In our study, a higher expression level of IL-8 and CXCR1 was detected in pancreatic cancer tissues than in para-cancerous pancreas. Although IL-8 could be el-

evated in colon and gastric cancers, the serum IL-8 level in pancreatic cancer was remarkably higher than in colon and gastric cancers. Moreover, IL-8 level was even higher in acute pancreatitis, in which IL-8 is considered to be a reliable indicator in evaluating the severity of inflammation and necrosis<sup>[27]</sup>. These data suggest that pancreatic cancer cells have a higher capability to produce chemokines than inflammatory cells, and function in an autocrine manner, which fulfills the composition of tumor microenvironment.

We have no direct experimental information on which cells are responsible for the secretion of IL-8, but according to the IHC analysis, IL-8 expression occurred mostly inside cancer cells, indicating that they might be the main source of IL-8. Pancreatic cancer cell lines have been investigated for IL-8 secretion and its mRNA expression, and quite a number of these cell lines showed high levels of IL-8 in the supernatant and mRNA expression<sup>[28]</sup>. We also analyzed IL-8 levels in the cultured supernatant of four pancreatic cancer cell lines, and found that those cell lines originated from the primary tumor site produced less IL-8 than those selected from metastatic site. Nomura *et al.*<sup>[29]</sup> demonstrated similar results using two sublines of pancreatic cancer, both of which were sequentially selected from the parental cell line and possessed a high potential of organ metastasis, and the high IL-8 expression was closely correlated with the aggressive behavior of cancer cells. Besides cancer cells, inflammatory cells in the tumor stroma is also one of the important sources of IL-8 secretion. Neutrophils can not only promote tumor destruction<sup>[30]</sup>, but also increase the growth of tumor cells<sup>[31]</sup>. Neutrophil-dependent release of VEGF-A leads to subsequent recruitment of neutrophils, resulting in angiogenesis.

Our survival analysis showed that IL-8 positive pancreatic cancer patients had a lower survival than IL-8 negative ones, whereas no significant difference was observed between the two groups possibly due to the insufficient number of cases involved. *In vivo* preclinical experiments of animal models on IL-8 have been conducted by many labs using various tumor cell lines, such as prostate cancer, lung cancer, breast cancer and pancreatic cancer<sup>[18]</sup>. In these cell lines, a high level of IL-8 is directly correlated with tumor growth, angiogenesis and metastasis in nude mice. But in ovarian cancer, IL-8 was negatively involved during tumorigenesis in animal models. Lee *et al.*<sup>[30]</sup> demonstrated that the inverse regulation of IL-8 on xenograft growth was mainly mediated by its induction of neutrophil infiltration. In our animal model, we for the first time used the clinically resected pancreatic cancer tissues and transplanted them sc into SCID mice. The infiltration of inflammatory cells was not as evident as that observed by Lee *et al.*<sup>[30]</sup> in ovarian cancer. The growth rate of tumors was significantly correlated with the serum level of IL-8 of the corresponding patients. The higher serum IL-8 level in the patients, the faster the tumor grew, indicating the more aggressive phenotype of this tumor, the poorer prognosis of the patients. IL-8

level can be measured in the tumor tissues resected surgically and in the patients' sera, it can also be detected in other kinds of clinical samples, including pancreatic juice obtained from the duodenum. Moreover, IL-8 concentration in pancreatic juice could be used to discriminate between normal pancreas and pancreatic cancer<sup>[32]</sup>.

In summary, IL-8 was highly expressed in pancreatic cancer in both tumor tissues and blood samples. *In vivo* analysis showed that IL-8 would be a sensitive marker in predicting prognosis and monitoring disease progression of the pancreatic cancer patients. The patients with high serum levels of IL-8 should receive more active treatment due to the more aggressive biology of their cancer. Besides its prognostic value, IL-8 may represent a promising target for the development of adjuvant therapy for pancreatic cancer.

## COMMENTS

### Background

Many studies have revealed that pancreatic cancer highly produces IL-8 and IL-8 can promote angiogenesis and invasion of tumors. Serum IL-8 level was elevated in pancreatic cancer patients, suggesting the feasibility of IL-8 to be a fine marker in predicting outcomes of pancreatic cancer patients.

### Research frontiers

The present study is to confirm the prognostic value of IL-8 in pancreatic cancer patients. The authors examined the expression and secretion levels in tumor tissues and human blood, respectively. Cancer tissues from patients with various serum IL-8 levels were implanted subcutaneously into nude mice to observe the growth of each xenograft.

### Innovations and breakthroughs

ELISA was used to detect the serum IL-8 levels in pancreatic cancer patients. Human pancreatic cancer tissues were transplanted heterotopically into immune-deficiency mice to evaluate the effect of serum IL-8 on the tumorigenesis of cancer samples. Besides its prognostic value, IL-8 may represent a promising target for the development of adjuvant therapy for pancreatic cancer.

### Peer review

This issue is of great interest and is definitely offering the opportunity to more accurately predict prognosis of patients affected by pancreatic carcinoma and to monitor the disease progression.

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## Quantification of choline concentration following liver cell apoptosis using $^1\text{H}$ magnetic resonance spectroscopy

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### Abstract

**AIM:** To evaluate the feasibility of quantifying liver choline concentrations in both normal and apoptotic rabbit livers *in vivo*, using  $^1\text{H}$  magnetic resonance spectroscopy ( $^1\text{H}$ -MRS).

**METHODS:**  $^1\text{H}$ -MRS was performed in 18 rabbits using a 1.5T GE MR system with an eight-channel head/neck receiving coil. Fifteen rabbits were injected with sodium selenite at a dose of 10  $\mu\text{mol}/\text{kg}$  to induce the liver cell apoptosis. Point-resolved spectroscopy sequence-localized spectra were obtained from 10 livers once before and once 24 h after sodium selenite injection *in*

*vivo*. T1 and T2 relaxation time of water and choline was measured separately in the livers of three healthy rabbits and three selenite-treated rabbits. Hematoxylin and eosin and dUTP-biotin nick end labeling (TUNEL) staining was used to detect and confirm apoptosis. Choline peak areas were measured relative to unsuppressed water using LCModel. Relaxation attenuation was corrected using the average of T1 and T2 relaxation time. The choline concentration was quantified using a formula, which was tested by a phantom with a known concentration.

**RESULTS:** Apoptosis of hepatic cells was confirmed by TUNEL assay. In phantom experiment, the choline concentration (3.01 mmol/L), measured by  $^1\text{H}$ -MRS, was in good agreement with the actual concentration (3 mmol/L). The average T1 and T2 relaxation time of choline was  $612 \pm 15$  ms and  $74 \pm 4$  ms in the control group and  $670 \pm 27$  ms and  $78 \pm 5$  ms in apoptotic livers *in vivo*, respectively. Choline was quantified in 10 rabbits, once before and once after the injection with sodium selenite. The choline concentration decreased from  $14.5 \pm 7.57$  mmol/L before sodium selenite injection to  $10.8 \pm 6.58$  mmol/L (mean  $\pm$  SD,  $n = 10$ ) after treatment ( $Z = -2.395$ ,  $P < 0.05$ , two-sample paired Wilcoxon test).

**CONCLUSION:**  $^1\text{H}$ -MRS can be used to quantify liver choline *in vivo* using unsuppressed water as an internal reference. Decreased liver choline concentrations are found in sodium selenite-treated rabbits undergoing liver cell apoptosis.

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**Key words:** Cell apoptosis; Magnetic resonance spectroscopy; Quantification; Choline; *In vivo*

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## INTRODUCTION

Cell apoptosis is a form of programmed cell death. Compared with necrosis, cell apoptosis does not damage the neighboring cells. Primary tumors are thought to be a result of inadequate apoptosis<sup>[1]</sup>. Through detecting apoptosis, the progression of a tumor can be evaluated and drug effects can be determined so as to adopt appropriate therapies<sup>[2]</sup>. It is, therefore, essential to develop a non-invasive method that can be used to assess apoptosis of tissues *in vivo*.

Proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS) is a powerful tool which can be used for non-invasive measurement of compounds *in vivo*. Several previous studies<sup>[3-6]</sup>, evaluated the role of <sup>1</sup>H-MRS in characterization of apoptosis with lipid signals. The most important non-lipid signals are from the choline-containing compounds at 3.2 ppm. However, changes in these signals were not universal, at least in the early stages of apoptosis<sup>[7]</sup>. One of our previous studies, using *in vitro* 9.4T high resolution magnetic resonance spectroscopy, has shown decreased total choline compounds and free choline in the rat apoptotic liver tissues as compared with the healthy rats<sup>[8]</sup>.

The liver is a particularly suitable and interesting organ for metabolic studies using *in vivo* magnetic resonance spectroscopy due to its rich metabolic activities and location at the body surface<sup>[9]</sup>. However, difficulties in measurement may mostly come from respiratory movement, which leads to limited spectral resolution, especially in the clinical environment. Several previous *in vivo* studies<sup>[10,11]</sup> focused on choline-to-lipid ratios in liver tumors. These studies have shown increased choline-to-lipid ratios with the progression of hepatocarcinogenesis<sup>[10]</sup>, and decreased choline-to-lipid ratios after treatment<sup>[11]</sup>. However, quantitative results are more suitable for horizontal and vertical comparison. Two preliminary reports<sup>[12,13]</sup> have quantified the choline concentrations in human hepatic tumors using separate reference standards of an external phantom and internal water signal. Tissue water reference method has been used for quantification of the choline level in <sup>1</sup>H-MR spectra of brain<sup>[14]</sup>, breast, muscle<sup>[15]</sup>, and ovary<sup>[16]</sup>, and is considered as a simple and practical approach in the clinical environment. Fischbach *et al*<sup>[17]</sup> applied LCMoDel to quantify choline relative to water, although the relaxation attenuation was not corrected. Accurate quantification requires correction for the relaxation attenuation, especially for point-resolved spectroscopy sequence (PRESS) sequence<sup>[18,19]</sup>.

The aim of this study was to use an *in vivo* rabbit model (1) to evaluate the feasibility of quantifying choline

concentrations of healthy and apoptotic livers with *in vivo* <sup>1</sup>H-MRS using the unsuppressed water signal as the internal reference, and to correct the relaxation attenuation, and (2) to compare the observed changes of choline with our previous *in vitro* results.

## MATERIALS AND METHODS

### Ethics

This study was approved ethically by the Animal Ethics Committee of Shantou University Medical College.

### Phantom and animal

A standard spectroscopy phantom provided by GE Medical Systems (25-cm-diameter MRS HD sphere; General Electric Company) was used. The metabolites in the phantom are 3.0 mmol/L choline chloride, 10.0 mmol/L creatine hydrate, 12.5 mmol/L N-acetylaspartic acid, 7.5 mmol/L myo-inositol, 12.5 mmol/L L-Glutamic acid and 5 mmol/L lactate, containing 0.1% sodium azide, 0.1% Magnavis, 50 mmol/L potassium dihydrogen phosphate and 56 mmol/L sodium hydroxide. This phantom was used in previous studies in the brain<sup>[20]</sup> and liver<sup>[13]</sup> as the quantification standard.

New Zealand white rabbits ( $n = 18$ ) weighing  $1.9 \pm 0.3$  kg were used in this study. In the control group, rabbits ( $n = 3$ ) were injected with saline and were sacrificed for the liver histological analysis after the <sup>1</sup>H-MRS measurement. In the experimental group, the rabbits ( $n = 15$ ) were injected with sodium selenite at a dose of 10  $\mu$ mol/kg. Spectra were obtained once before and once 24 h after sodium selenite injection, and the rabbits were sacrificed after MRS. Prior to the MRS, rabbits were fasted for six hours and anesthetized with 1 mL/kg sodium pentobarbital through ear vein injection. Shen *et al*<sup>[21]</sup> observed that Se was able to induce apoptosis in *in vitro* HepG2 cells and found that selenite-induced apoptosis was both time- and dose-dependent<sup>[22]</sup>. The duration and dose of sodium selenite were determined according to their studies. All the rabbits were supplied from the Laboratory Animal Center of Shantou University Medical College.

### Magnetic resonance imaging and MRS

All studies were performed on a 1.5-T HDxt MR scanner (Signa Systems, GE Healthcare) using an eight-channel head/neck receiving coil. At first, the phantom was used for the regular system stability check and served as a test to confirm the quantification strategy *in vivo*. For the phantom measurement, magnetic resonance imaging (MRI) consisted of axial and coronal localizer sequences for positioning the single voxel (20 mm  $\times$  20 mm  $\times$  20 mm) within the phantom solution. For *in vivo* measurement, rabbits were placed in the prone position and the liver region was situated in the center of the coil. The abdomen was immobilized with cushions to reduce respiratory movement. MRI consisted of images followed by the Propeller fast spin echo T2-weight sequence (TE/TR: 110 ms/6000 ms; field of view: 20 cm  $\times$  20 cm; slice thickness: 4 mm; slice space: 0.5 mm) to define the

position of volume of interest (VOI).

Proton MRS was performed using a PRESS (TE/TR: 35 ms/1500 ms; total scan number: 128; VOI: 15 mm × 15 mm × 15 mm). Water suppression was obtained with chemical shift selective saturation. The volume saturation suppression (VSS) pulse was oblique and placed on the edge of the voxel for shimming and reducing motion artifact. T1 and T2 relaxation time of choline and water was separately measured in the phantom, three rabbits in the control group, and three selenite-injected rabbits. For T1 measurement, the TE was kept constant at 35 ms and the TR varied from 1130 ms to 3000 ms for five measurements. For T2 measurement, the TR was kept constant at 1500 ms and the TE varied from 30 ms to 135 ms for five measurements.

### Data analysis

The raw spectral data were input into a SAGE software package (GE Healthcare). The peak amplitudes of the unsuppressed water signal at 4.7 ppm and choline compound signals at 3.2 ppm were measured with SAGE. The T2 relaxation time of MR-visible water and choline was measured in SAGE using the macro T2 fit. T1 time was obtained by fitting the data to a mono-exponential model as a function of TR, and TE to a mono-exponential model with an in-house program. The average T1 and T2 values for Cho and water were used for correcting the relaxation attenuation with the following equation.

Choline was quantified using commercially available LCModel (version 6.2-2B) software, according to Dr. Provencher<sup>[17]</sup>, which is suitable for fitting the spectra collected by PRESS sequence (TE = 35 ms) in a GE 1.5T scanner. The mode of only-choline-2 was selected for phantom analysis. Using the prior knowledge incorporated into the spectral fitting, phase and baseline correction were automatically processed with LCModel. The control parameters were adjusted according to the LCModel manual.

Spectra were discarded if the reported signal-to-noise ratio (SNR) from LCModel was less than 15, or if the SD of choline was more than 20%. The ratio of the choline resonance area to the unsuppressed water resonance area was acquired. These ratios were converted to approximate mmol/L units by setting the concentration of water in the phantom as 55 556 mmol/L according to the manual. This ratio was measured *in vivo* using the analysis mode of liver-6. The water concentration was assumed to be 47 778 mmol/L for an 86% water content of the liver. The absolute concentration of choline was measured by the following formula modified from the reference<sup>[15]</sup>.

$$[Cho] = A_{Cho/H_2O} \times \frac{n_{H_2O}}{n_{Cho}} \times \frac{fT1(H_2O)}{fT1(Cho)} \times \frac{fT2(H_2O)}{fT2(Cho)} \times CF_{Lipid}$$

Where  $A_{Cho/H_2O}$  = the area ratio of choline to unsuppressed water,  $n_{H_2O} = 2$ ,  $n_{Cho} = 9$  (from three CH<sub>3</sub> groups),  $fT1 = 1 - \exp(-TR/T1)$ , and  $fT2 = \exp(-TE/T2)$ .  $CF_{Lipid}$  represents a correction factor that is equivalent to  $A_{H_2O}/(A_{H_2O} + A_{Lipid})$  measured from the non-suppressed water.

### Histopathology

All animals were sacrificed under anesthesia after the

MRS. Livers were removed and washed with physiological saline. Macroscopic examination followed by visual inspection was performed, and representative sections were taken. Formalin fixed samples were embedded in paraffin and 2-mm thin sections were cut. Samples were stained with hematoxylin and eosin and examined under light microscopy to identify apoptotic characteristics of vacuolated hepatocytes and the degree of lipid deposition in the parenchyma.

Shen *et al.*<sup>[21]</sup> found that selenite-induced apoptosis could be evaluated by dUTP nick end labeling (TUNEL) assay and *in vitro* HepG2 cell morphological changes. The apoptotic cells were observed and analyzed in this study according to the reported methods. After the homogenized liver tissue was filtered through a 40 μm cell strainer, the cells were washed with PBS and then were centrifuged at 1500 r/min for 5 min to remove cell debris. After this procedure was repeated three times, the cells were fixed with 4% paraformaldehyde for 30 min. Apoptotic cells were stained with transferase-mediated TUNEL using a One Step TUNEL Apoptosis Assay Kit (Beyotime, China). The TUNEL reaction took place with the addition of reaction mixture (containing nucleotides and TdT enzyme), which was incubated for 60 min at 37 °C in the dark. After wash with phosphate buffer solution (PBS), cells were finally resuspended in PBS for flow cytometry analysis (XL MCL, Beckman Coulter, United States). The data obtained from flow cytometry were analyzed using Expo32 ADC Analysis software for calculating the percentage of apoptotic cells in each group.

### Statistical analysis

All data were presented as a mean ± SD. Statistical significance was calculated using two-sample relative Wilcoxon test and was accepted at  $P \leq 0.05$ . The relationship between the percentage of apoptotic cells and choline concentration was investigated using the linear regression analysis. All analyses were performed using SPSS version 13.0 (SPSS Inc, United States).

## RESULTS

### Phantom studies

T1 and T2 relaxation time of choline was 1129 ms and 236 ms, respectively, while T1 and T2 relaxation time of water was 3172 ms and 206 ms. The fitted data was acceptable for the low SD of choline (9%). The calculated choline concentration was 3.01 mmol/L, which was in agreement with the known choline concentration in the phantom (3 mmol/L).

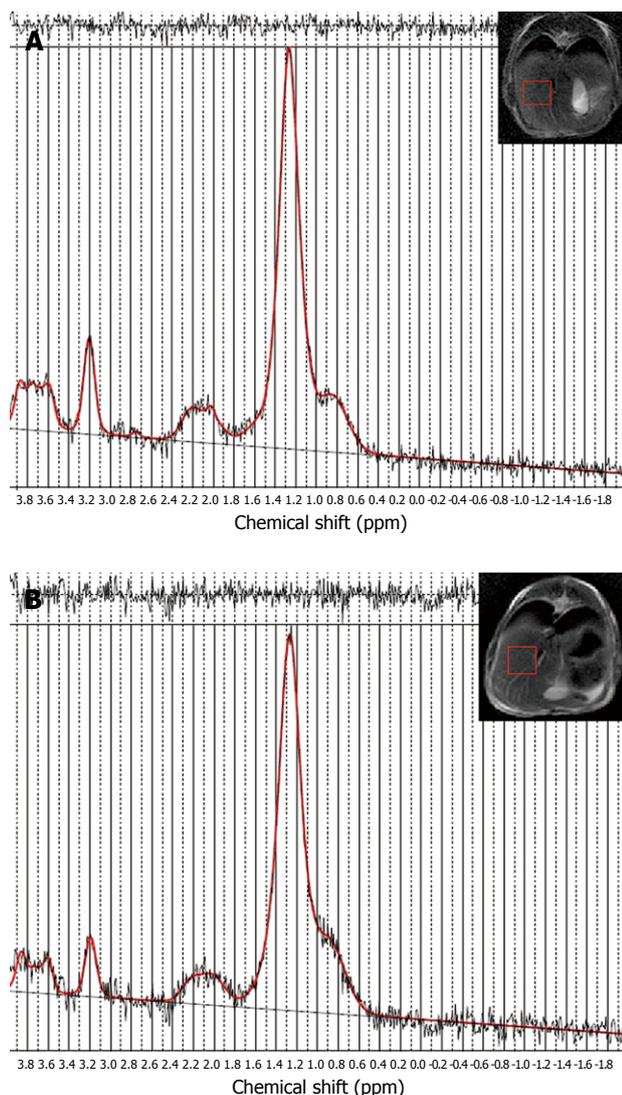
### In vivo studies

Choline spectra were obtained from all rabbits in the control group and 10/14 selenite-treated rabbits. Among the four failures in experimental group, one rabbit died during the model-making process, two failed in MRS after injection of sodium selenite due to prescan failure, and one spectrum was rejected after the injection of so-

**Table 1** Summary of data quality related parameters ( $n = 10$ , mean  $\pm$  SD)

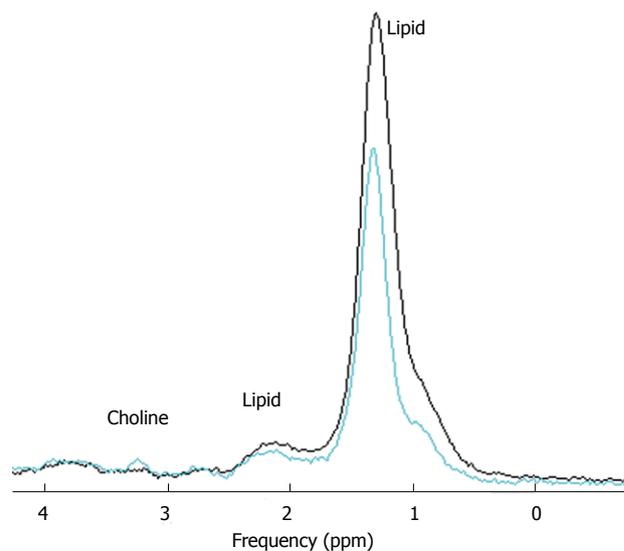
	SD of choline (%)	SNR of spectra	FWHM (Hz)	WS (%)
Before injection	8.5 $\pm$ 4.1	46.4 $\pm$ 19.4	12.6 $\pm$ 2.0	94.3 $\pm$ 2.8
After injection	10.5 $\pm$ 4.6	26.9 $\pm$ 14.3	11.8 $\pm$ 7.0	95.4 $\pm$ 2.5

Standard deviation (SD) of choline and signal-to-noise ratio (SNR) of spectra were produced by LCMoel. Full-width at half-maximum (FWHM) and water suppression (WS) was provided by the magnetic resonance machine.



**Figure 1** LCMoel output of point-resolved spectroscopy-localized single-voxel  $^1\text{H}$  magnetic resonance spectra from the liver of one rabbit before (A) and after injection of sodium selenite (B). The locations of the spectroscopic volume of interest are indicated by the squares in the Propeller FSE T2W image. The decreased choline-to-water area was observed.

dium selenite with a full-width at half-maximum (FWHM)  $> 20$  and there was no metabolite resonance peak within a range from 0 ppm to 4.0 ppm. One spectrum of liver cell apoptosis induced by sodium selenite was excluded due to a higher choline SD ( $> 20\%$ ). The spectra from ten rabbits before and after the injection with sodium selenite were finally adopted (Table 1).



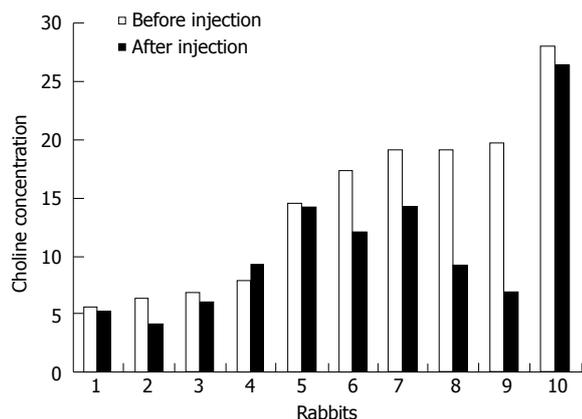
**Figure 2**  $^1\text{H}$  magnetic resonance spectra from a rabbit liver before (green line) and after injection of sodium selenite (black line). The decreased choline peak was observed at 3.2 ppm. However, the choline peak from the liver after injection of sodium selenite was too weak, with a choline SD  $> 20\%$ .

The average T1 and T2 relaxation time of choline was  $612 \pm 15$  ms and  $74 \pm 4$  ms in the control group ( $n = 3$ ) and  $670 \pm 27$  ms and  $78 \pm 5$  ms in apoptotic livers ( $n = 3$ ). The average T1 and T2 relaxation time of water was  $653 \pm 23$  ms and  $48 \pm 3$  ms in the control group ( $n = 3$ ), and  $719 \pm 37$  and  $50 \pm 5$  ms in apoptotic livers ( $n = 3$ ).

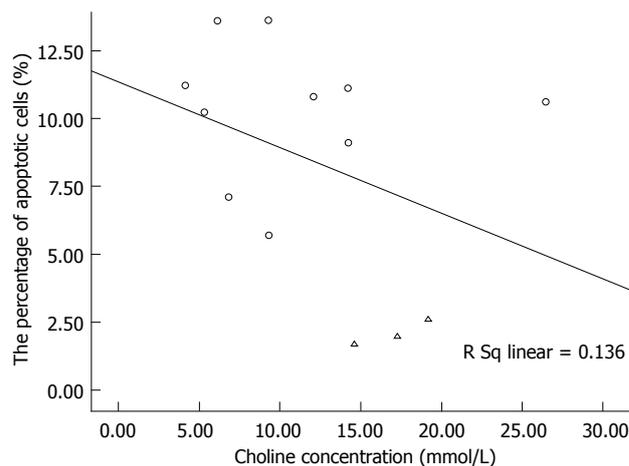
PRESS-localized single-voxel  $^1\text{H}$ -MR spectra from the liver of one rabbit before and after injection of sodium selenite can be well fitted using LCMoel (Figure 1). A decreased choline peak was observed at 3.2 ppm (Figure 2). The calculated average T1 and T2 relaxation time of choline and water in both normal and apoptotic livers were used to calculate the choline concentrations. Decreased choline concentrations ranged from  $14.5 \pm 7.6$  mmol/L ( $n = 10$ ) before injection of sodium selenite to  $10.8 \pm 6.6$  mmol/L ( $n = 10$ ) 24 h after that treatment ( $Z = -2.395$ ,  $P < 0.05$ , two-sample relative Wilcoxon test), although increased choline was also found in one rabbit (Figure 3). The calculated concentrations of choline varied from 5.5 mmol/L to 28.1 mmol/L in normal liver and 4.1 mmol/L to 26.4 mmol/L in apoptotic liver after injection of sodium selenite.

### Histopathology

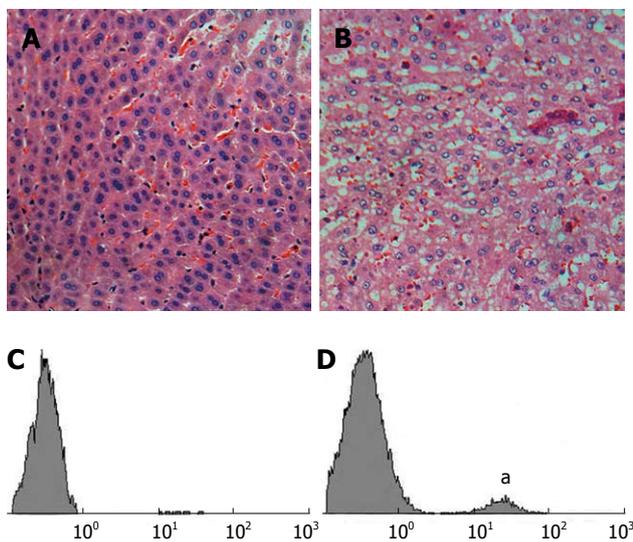
Histological differences between two groups were observed by hematoxylin and eosin staining. Normal liver had no observable effects on the microscopic distribution of vacuolated hepatocytes in liver tissues (Figure 4A and B). Apoptosis of hepatic cells in both groups was confirmed by TUNEL assay and the increased ratio of apoptotic to normal cells was detected by flow cytometry (Figure 4C and D). The average ratio was  $2.1\% \pm 0.5\%$  ( $n = 3$ ) *vs*  $10.3\% \pm 7.6\%$  ( $n = 10$ ), respectively. However, the linear regression analysis revealed no significant linear relationship between choline concentration and the



**Figure 3** Barplot shows the choline concentration of livers from 10 rabbits before and after injection of sodium selenite. The two-sample Wilcoxon test revealed significant differences between the groups ( $Z = -2.395, P < 0.05$ ).



**Figure 5** Scatter diagram shows the correlation between the percentage of apoptotic cells and choline concentration in the control group ( $\Delta$ ) and the experiment group after injection of sodium selenite ( $\circ$ ). The linear regression analysis revealed no significant linear relationship between variables ( $R = 0.369, F = 1.734, P > 0.05$ ).



**Figure 4** Light microscopy of representative hematoxylin and eosin stained histological sections of liver (A and B, x 200) and flow cytometry of liver apoptotic cells stained by dUTP-biotin nick end labeling assay (C and D). A, C: Normal rabbits; B, D: Rabbits injected with sodium. The elevated apoptotic cell peak labeled by A was observed.

percentage of apoptotic cells ( $R = 0.369, F = 1.734, P > 0.05$ ) (Figure 5).

## DISCUSSION

Noninvasive quantitative measurement of choline by proton MRS is important for the assessment of tumor characterization, grading, and post-treatment evaluation. The data in this study showed the feasibility of measuring choline concentration in rabbit liver *in vivo* using 1.5T MR by appropriate acquisition and quantification methods.

Choline spectra were obtained from three healthy rabbits and 10/14 selenite-treated rabbits. Phase and frequency shifts were found in some liver spectral frames. We deduced that this may be due to respiratory motion, which could also lead to inhomogeneous B0 and B1 fields and result in broadening of the spectral resonanc-

es. According to the data quality, the FWHM and water suppression of spectra in selenite-treated rabbits were better than those in normal rabbits due to decreased SNR and SD of choline. The possible reason is that rabbits injected with sodium selenite were quieter and therefore had reduced movement of the tissue. However, liver tissue necrosis may cause signal non-uniformity and worsen the effect of shimming, even leading to pleural effusion after injection of sodium selenite.

It is very important to make optimal choices about the spectroscopic sequence and parameters. The PRESS technique was used for its higher SNR and non-sensitivity to movement. The quality of MR spectra also relies on adequate technics, such as effective water suppression and prescan adjustment, such as the position of the VOI and adequate anesthetic dose. The VSS pulse was used to reduce the voxel misregistration leading to outer voxel contamination, and to improve the effect of shimming. Although it is a time-consuming process, it is of importance to obtain good quality spectra that allow for more accuracy of metabolite identification and quantification.

Absolute quantification remains a challenge due to the lack of relatively stable metabolites used as the standard. Therefore, metabolite concentrations were reported in terms of metabolite ratios such as choline/lipid under the assumption of constancy of the reference compound<sup>[23,24]</sup>. Unfortunately, variations in choline and lipid must be taken into account. The use of the lipid peak as an internal reference was not considered for this study, as the range of lipids is very variable<sup>[25]</sup>.

LCModel software is widely used in the field of quantitative analysis. Compared with other fitting programs, phase and baseline correction can be automatically processed and overlapping spectra can be better resolved. In this study, unsuppressed water was used as an internal reference to acquire the metabolite area ratio of choline to water. However, accurate choline quantification requires additional correction for some factors,

such as the concentration and relaxation attenuation of tissue water as well as the relaxation attenuation of choline. Otherwise, the measurement can only be presented as concentrations in arbitrary institutional units (a.u.).

T1 and T2 relaxation time of choline and water *in vivo* was measured in this study. A number of studies reported the relaxation time of human hepatic tumor metabolites. At 3T, T1 and T2 values of water in the phantom were measured by Li *et al.*<sup>[13]</sup> to be 3420 ms and 370 ms, respectively, and choline T2 relaxation time in HCC patients ranged from 88 ms to 161 ms. These values are similar to the T1 and T2 relaxation time of water (3172 and 206 ms) and T2 relaxation time of choline ( $78 \pm 5$  ms in apoptotic liver) in the present study. In the study by Goldberg *et al.*<sup>[26]</sup> T1 and T2 relaxation time of water in solid lesions was  $1004 \pm 234$  ms and  $80 \pm 18$  ms at 1.5T by MRI. These values are higher than our data ( $719 \pm 37$  ms and  $50 \pm 5$  ms in cell apoptotic livers). The differences of T1 and T2 relaxation time may be attributed to the magnetic field strength, subjects, and measurement methods. Enhanced field strength led to increase in T1 and minor decrease in T2. Moreover, this procedure was subject to failure caused by the movement. Due to individual differences, variable relaxation time should be taken into consideration.

The liver choline concentration *in vivo* seems not universal. In the report by Fischbach *et al.*<sup>[12]</sup>, it was  $7.7 \pm 7.3$  a.u. in normal liver parenchyma of volunteers and  $7.3 \pm 4.3$  a.u. in normal-appearing liver parenchyma of patients with hepatic tumors using LCMoel at 3T. However, Li *et al.*<sup>[13]</sup> reported that the choline concentrations in four patients ranged from 3.4 mmol/L to 14.0 mmol/L, and in healthy volunteers  $1.3 \pm 0.9$  mmol/L. It was  $1.0 \pm 0.7$  a.u. in the normal liver tissues of rats and  $5.6 \pm 1.5$  a.u. in tumor tissues reported by Chen *et al.*<sup>[27]</sup>. The choline concentration in our study ( $14.5 \pm 7.6$  mmol/L in normal and  $10.8 \pm 6.6$  mmol/L in apoptotic livers) is higher than the above values. In addition to field strength and different subjects as well as the quantification method, the assumed tissue water concentration may be the main reason leading to our higher values. The water concentration was assumed to be 47 778 mmol/L for an 86% water content of liver. This value may be slightly higher for including parts of MR-invisible water. However, the real water concentration of liver is hard to measure *in vivo* and might vary between individuals.

The basis set is another important issue in quantification with LCMoel. It included the metabolites of choline and lipid as well as glycogen in analysis mode of liver-6. Therefore, the above metabolites can be measured in our study. Nevertheless, a choline compound consists of glycerophosphorylcholine, phosphorylcholine and free choline, which can be resolved *in vitro* at 9.4T<sup>[8]</sup>. As for the much poorer spectral resolution and SNR of *in vivo* MRS, only choline is included in the basis set.

Se-induced apoptosis has been studied in a number of cancer cells *in vitro* and the results generally suggest the involvement of apoptosis in Se-induced cytotoxic and

anti-proliferative effects against cancer cells<sup>[21]</sup>. Choline is a nutrient essential for the normal function of cells and is usually considered as a marker of cell growth<sup>[28,29]</sup>. The data in our study support that choline concentrations are decreased with the elevated number of apoptotic liver cells. This result is consistent with the results of Blankenberg<sup>[30]</sup> and our results *in vitro*<sup>[8]</sup>. However, it is insufficient to use choline as a possible apoptosis biomarker in this study. High concentrations of selenite can induce oxidative stress and make tissue undergo necrosis, which also leads to a decreased choline concentration. Therefore, it is essential to confine the time window to early apoptosis in future studies. The increased choline after injection of sodium selenite may be caused by the experimental control. This phenomenon might be related to individual diversities.

In conclusion, this work demonstrates the feasibility of noninvasive measurement of liver choline concentrations using *in vivo* 1.5T clinical MR. Although there are limitations as outlined above, this method has the potential to characterize liver lesions and determine therapeutic responses. Moreover, it lays a foundation for future investigations of cell apoptosis *in vivo*<sup>[8,9,31]</sup>.

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## COMMENTS

### Background

Through detecting apoptosis, the progression of a tumor can be evaluated and drug effects can be determined. It is, therefore, essential to develop a non-invasive method that can be used to assess apoptosis of tissues *in vivo*. The liver is a particularly suitable and interesting organ for metabolic studies owing to its rich metabolic activities and location at the body surface. However, the difficulties in measurement *in vivo* may occur due to respiratory movement.

### Research frontiers

Changes in choline-containing compounds at 3.2 ppm seem not to be universal, at least in the early stages of apoptosis. In this study, the decreased choline concentrations were observed in the sodium selenite-induced rabbit liver cell apoptosis *in vivo*.

### Innovations and breakthroughs

Several previous *in vivo* studies focused on the choline-to-lipid ratios in liver tumors. This may be the first study to attempt to quantify the metabolite concentrations of both normal and apoptotic rabbit livers *in vivo* using single-voxel 1H-magnetic resonance spectroscopy (MRS) with unsuppressed water signal as the internal reference, and to correct the relaxation attenuation.

### Applications

This study demonstrates the feasibility of noninvasive measure of liver choline concentrations *in vivo* in a 1.5T clinical MR environment. Moreover, it lays a foundation for future investigations of cell apoptosis *in vivo*.

### Peer review

The authors have tried to evaluate the feasibility of quantifying liver choline concentrations in both normal and apoptotic rabbit livers *in vivo* using <sup>1</sup>H MRS. Based on the *in vitro* study using a phantom model and an *in vivo* study using 18 rabbits, the authors conclude that MRS can be used to quantify choline in rabbit liver *in vivo* using unsuppressed water as an internal reference. This is an interesting animal study and the authors needed to be lauded for their efforts.

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## Large-vessel thrombosis in intestinal Behçet's disease complicated with myelodysplastic syndrome and trisomy 8

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### Abstract

Behçet's disease is characterized by recurrent oral ulcers, genital ulcers, uveitis and skin lesions. Myelodysplastic syndrome (MDS) is characterized by problems due to ineffective hematopoiesis. Several studies have identified a relationship between MDS and Behçet's disease, especially intestinal Behçet's disease. Trisomy 8 seems to play an important role in these disorders as well. The present case was a 24-year-old woman who had a huge tonsil ulcer with initial symptoms of odynophagia and intermittent fever. We also noted folliculitis on her upper back. Five days later, she began to experience diarrhea and abdominal pain. Abdominal computed tomography and subsequent surgery revealed ileum perforation and enterocolitis with multiple ulcers. Later, she was admitted again for a vulvar suppurative ulcer and suspicious Bartholin's cyst infection. The patient's clinical presentations met the criteria for Behçet's disease. Six months after the bowel perforation event, we noted the development of pancytopenia in a routine laboratory examination. All the examinations led to the diagnosis of MDS

with trisomy 8. The most unusual finding was that multiple large vessel thrombi developed during follow-up. Previous studies have suggested that trisomy 8 in MDS leads to concurrent intestinal Behçet's disease. Moreover, the inflammatory and immune genes related to thrombus formation are overexpressed in cases of MDS with trisomy 8. Trisomy 8 must play a role in thrombosis. Further studies are needed to help clarify the pathophysiology and pathogenesis of these disorders.

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**Key words:** Behçet's disease; Myelodysplastic syndrome; Trisomy 8; Intestinal ulcers; Thrombosis

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### INTRODUCTION

Behçet's disease is a multisystem inflammatory disease characterized by recurrent oral ulcers, genital ulcers, uveitis, and skin lesions. Many other systems can be involved, such as the gastrointestinal tract, central nervous system and cardiovascular system; the disease can also cause arthritic joints.

Myelodysplastic syndrome (MDS) is a blood disease that easily converts to acute leukemia. It is characterized by stem cell disorders, multi-lineage dysplasia, and pancytopenia due to ineffective hematopoiesis.

Behçet's disease and MDS are two different disease entities. However, an association between the two diseases has been reported in an increasing number of cases. Most of the patients who suffer from the two diseases have intestinal ulcers. Some previous studies have also identified a statistically significant relationship between trisomy 8 and intestinal Behçet's disease with MDS.

We report the case of a patient with trisomy 8 who was diagnosed with intestinal Behçet's disease and MDS. We incidentally found multiple thrombi in the major veins.

## CASE REPORT

A 24-year-old woman was admitted to our hospital due to odynophagia and intermittent fever for 1 wk. The initial findings were a huge tonsil ulcer with a pus-like coating. We also noted several spots of folliculitis on her upper back. The laboratory examination revealed the following: white cell count  $12.5 \times 10^3/\text{mm}^3$ , red blood cell count  $3.80 \times 10^6/\text{mm}^3$ , hemoglobin 14.1 g/dL, platelet count  $150 \times 10^3/\text{mm}^3$ , alanine aminotransferase 24 IU/L, creatinine 0.9 mg/dL, Na 141 mEq/L and K 3.3 mEq/L. She was initially treated for acute suppurative tonsillitis. However, the symptoms persisted after the administration of antibiotics. Five days later, she began to experience diarrhea, abdominal pain and dyspnea. We arranged for abdominal computed tomography (CT), and the results showed ileus, edematous bowels, right-side colon dilation, ascites and free air. She then underwent an operation, and ileum perforation and enterocolitis with multiple ulcers were found (Figure 1). The pathology report identified multiple ulcers with transmural necrotizing inflammation in the colon and ileum.

About 2 mo later, the patient returned to our hospital due to increased vaginal discharge, itching and pain. Fever and chills followed these symptoms. She was admitted again for a vulvar suppurative ulcer and a suspicion of Bartholin's cyst infection. After antibiotic treatment, these symptoms improved, and she then received outpatient clinical follow-up care.

The patient's clinical presentations met the International Study Group's criteria for Behçet's disease; these include recurrent oral ulcers (> 3 times in a year), frequent genital ulcers (twice in the past 3 mo), folliculitis on the upper back and multiple bowel ulcers with perforation.

Three months after the second hospitalization, the patient was admitted again due to adhesion ileus. Unexpectedly, abdominal CT found thrombi in the patient's bilateral internal iliac vein, common iliac vein and inferior vena cava (Figure 2); the multiple thrombi were not noted in the previous CT scan.

Six months after the bowel perforation event, we noted the development of pancytopenia in a routine laboratory examination. The initial hemogram showed the following: white cell count  $1.0 \times 10^3/\text{mm}^3$ , red blood cell count  $1.45 \times 10^6/\text{mm}^3$ , hemoglobin 5.8 g/dL and platelet count



Figure 1 Ileum perforation and multiple transmural ulcers.



Figure 2 Abdominal computed tomography image shows a dilated bowel loop and a large thrombus (arrow) in the inferior vena cava.

$13 \times 10^3/\text{mm}^3$ . The differential count of the white blood cells was as follows: neutrophil-seg 18%, neutrophil-band 13%, lymphocytes 62%, monocytes 1%, eosinophils 2%, metamyelocytes 2% and atypical lymphocytes 62%. Bone marrow biopsy was performed for further evaluation and revealed profound hypocellularity of the marrow with a marked decrease of trilinear hematopoietic elements and focal aggregates of lymphoplasmacytic cells. Cytogenetic analysis demonstrated the presence of two cell lines: 48, XX, +8, +9 (11)/46, XX (9). Of the 20 cells examined, 11 showed an abnormal female karyotype with extra 8 and 9 chromosomes, and the remaining nine cells had normal, complete sets of chromosomes. These findings led to the diagnosis of MDS and refractory anemia. The overall picture was compatible intestinal Behçet's disease with MDS and chromosomal abnormality.

## DISCUSSION

Behçet's disease is characterized by recurrent oral ulcers, genital ulcers, uveitis, and skin lesions. Other systems can be involved, such as the gastrointestinal tract, central nervous system and cardiovascular system, and the disease can lead to arthritic joints. The exact etiology and pathogenesis of Behçet's disease are still being investigated. From previous studies, we know that genetic factors play key roles in its pathogenesis. MDS, however, is character-

ized by stem cell disorders, multi-lineage dysplasia and pancytopenia due to ineffective hematopoiesis.

Behçet's disease is generally considered to be an immunological disease. MDS is related to many immunological abnormalities, and presently, several types of immunomodulatory therapies, such as antithymocyte globulin and cyclosporin A<sup>[1]</sup>, have been used to treat MDS. These two diseases are currently thought to share some immunological characteristics. For example, extensive intramedullary cell death in an MDS patient is proposed to be strongly related to tumor necrosis factor (TNF)- $\alpha$ . In addition, the concentration of TNF and soluble TNF receptors is increased in the serum of patients with active Behçet's disease<sup>[2]</sup>. We conclude that some correlation exists in the pathogenesis of the two diseases.

The co-occurrence of Behçet's disease and MDS has been reported in an increasing number of cases<sup>[3]</sup>. Most of the patients who suffer from the two diseases have intestinal ulcers. In light of the previous literature, intestinal Behçet's disease is believed to be partly derived from MDS<sup>[4-7]</sup>, and most of these patients have trisomy 8. Kimura *et al*<sup>[6]</sup> have identified a statistically significant relationship between trisomy 8 and intestinal Behçet's disease with MDS.

Chromosomal abnormalities are observed in about 40% of patients with MDS, but trisomy 8 is found in only about 5% of the MDS population<sup>[8]</sup>. The high frequency of trisomy 8 in cases of intestinal Behçet's disease complicated with MDS suggests that trisomy 8 plays an important role in the pathogenesis of intestinal ulcers in the context of Behçet's disease.

Most cases of trisomy 8 with intestinal Behçet's disease complicated with MDS have been reported in Japan. A single case or a few cases have been reported in Italy, Korea, the United States, Germany, Spain, Israel and the United Kingdom<sup>[3]</sup>. We reported the case of a young woman who was diagnosed with intestinal Behçet's disease with MDS and trisomy 8. There are many compositions of trisomy 8; our patient was 48, XX, +8, +9, which is one of the karyotypes reported previously.

The most unusual finding in our reported case was that multiple large vessel thrombi developed during follow-up. Thrombosis related to Behçet's disease usually occurs in veins, and involvement of the arteries is less common<sup>[9]</sup>. Our patient developed thrombi in the bilateral internal iliac veins, common iliac veins and inferior vena cava. Thrombosis related to Behçet's disease is found more frequently in men<sup>[9]</sup>, although our patient was a young woman.

Kimura *et al*<sup>[6]</sup> have found that MDS patients with trisomy 8 tend to develop thrombosis and intestinal ulcers, but no definite cause for the vessel thrombus has been identified. Other researchers have noted neutrophil function abnormality and inflammatory cytokine overproduction in cases of MDS<sup>[10,11]</sup>. These immunological disorders, particularly cytokine overproduction, may lead to injury and inflammation of the endothelium.

Chen *et al*<sup>[12]</sup> have found that inflammatory and im-

mune genes, such as transforming growth factor (TGF)- $\beta$ , TGF- $\beta$  receptor, interleukin (IL)-10, IL-7 receptor and vascular cell adhesion molecule (VCAM)-1, are overexpressed in MDS patients with trisomy 8. The exact role of TGF- $\beta$  is controversial. Some researchers have suggested that it exacerbates neointima formation by inhibiting endothelial regeneration and promoting fibrosis, and some have shown that it protects against lipid lesion formation in atherosclerosis<sup>[13]</sup>. IL-10 and IL-7 are thought to contribute to the formation of atherosclerosis<sup>[14,15]</sup>. VCAM-1 has the ability to facilitate thrombus formation.

These previous studies have suggested that the presence of trisomy 8 in MDS leads to the patient having concurrent intestinal Behçet's disease. Moreover, the inflammatory and immune genes related to thrombus formation are overexpressed in cases of MDS with trisomy 8. Trisomy 8 must play a role in blood vessel thrombosis. We therefore hypothesize that trisomy 8 induces the activation of an abnormal inflammatory process and immune gene expression, eventually leading to or even aggravating blood vessel injury and thrombus formation. This may explain why our patient, with Behçet's disease complicated with MDS and trisomy 8, developed multiple vessel thrombi. In the near future, trisomy 8 may become a helpful predictor of Behçet's disease prognosis or outcome, especially in the case of intestinal ulcers or blood vessel thrombosis. Further studies are needed to help clarify the pathophysiology and pathogenesis of these disorders.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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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 Moorseelaar 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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- 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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- 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 dis-

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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

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## Genetically modified mouse models for the study of nonalcoholic fatty liver disease

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance, and type 2 diabetes. NAFLD represents a large spectrum of diseases ranging from (1) fatty liver (hepatic steatosis); (2) steatosis with inflammation and necrosis; to (3) cirrhosis. The animal models to study NAFLD/nonalcoholic steatohepatitis (NASH) are extremely useful, as there are still many events to be elucidated in the pathology of NASH. The study of the established animal models has provided many clues in the pathogenesis of steatosis and steatohepatitis, but these remain incompletely understood. The different mouse models can be classified in two large groups. The first one includes genetically modified (transgenic or knockout) mice that spontaneously develop liver disease, and the second one includes mice that acquire the disease after dietary or pharmacological manipulation. Although the molecular mechanism leading to the development of hepatic steatosis in the pathogenesis of NAFLD is complex, genetically modified animal models may be a key for

the treatment of NAFLD. Ideal animal models for NASH should closely resemble the pathological characteristics observed in humans. To date, no single animal model has encompassed the full spectrum of human disease progression, but they can imitate particular characteristics of human disease. Therefore, it is important that the researchers choose the appropriate animal model. This review discusses various genetically modified animal models developed and used in research on NAFLD.

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**Key words:** Nonalcoholic fatty liver disease; Steatosis; Steatohepatitis; Knockout; Animal models

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### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a histological spectrum of liver disease associated with obesity, diabetes and insulin resistance that extends from isolated steatosis to steatohepatitis and cirrhosis. Besides being a potential cause of progressive liver disease, steatosis has been shown to be an important cofactor in the pathogenesis of many other liver diseases. Mouse models have been developed and the different mouse models can be classified in two major groups. The first one includes genetically modified (transgenic or knockout) mice that spontaneously develop liver disease, and the second one

includes mice that acquire the disease after dietary or pharmacological manipulation. NAFLD and nonalcoholic steatohepatitis (NASH) are increasing due to the prevalence of the metabolic syndrome linked to visceral adiposity, insulin resistance, dyslipidemia and type two diabetes. In this context, research has been undertaken using animal models for human steatosis and NAFLD to NASH disease progression. Most of the animal models develop a fatty liver and many develop aspects of steatohepatitis. However, spontaneous development of fibrosis is very rare. Because it is highly unlikely that NAFLD in the human population is monogenic, study of animals with deletion or over-expression of a single gene may not mimic etiology of the human disease at the molecular level. Likewise, choice of experimental diet may not mimic the human diets associated with development of NAFLD in man. Although rodent models of hepatic steatosis and/or insulin resistance do not always perfectly reproduce the human pathology of NAFLD, the use of transgenic, knockout, and knockdown mouse models have helped over the past years to better our understanding of the molecular determinants of NAFLD. This literature review describes different genetically modified mouse models that exhibit histological evidence of hepatic steatosis or, more variably, steatohepatitis.

## GENETIC MODELS FOR NAFLD

### *ob/ob* mice

The *ob/ob* mice carry a spontaneous mutation in the leptin gene (leptin-deficient). These mice are hyperphagic, inactive, extremely obese and are severely diabetic, with marked hyperinsulinemia and hyperglycemia. *ob/ob* mice develop NASH spontaneously<sup>[11]</sup>, but unlike human NAFLD, *ob/ob* mice do not spontaneously progress from steatosis to steatohepatitis. *ob/ob* mice require a 'second hit' to be administered in order to trigger progression to steatohepatitis. This may be provided by exposure to small doses of lipopolysaccharide (LPS) endotoxin, ethanol exposure or hepatic ischemia-reperfusion challenge which all provoke a severe steatohepatitis and frequently acute mortality<sup>[2-5]</sup>. *ob/ob* mice require other stimuli such as a methionine choline deficient (MCD) diet or a high fat diet to trigger progression to steatohepatitis. The effects of leptin deficiency on several aspects of physiology increase the complexity of studies while using this strain<sup>[6]</sup>. Similarly, the limited fibrotic capacity of a leptin-deficient model means that it is best suited to studies investigating the mechanisms behind the development of steatosis and the transition to steatohepatitis. Recent work demonstrates that the apparent flaws in this model can be turned to advantage, providing new insights into stellate cell function and the progression to fibrosis.

### *db/db* mice

The *db/db* mice have a natural mutation in the leptin receptor (*Ob-Rb*) gene<sup>[7]</sup>. These mice are obese with insulin resistance, and are able to develop macrovesicular he-

patic steatosis. These mice readily develop symptoms of NASH upon induction with a second hit, such as feeding with an MCD diet<sup>[8]</sup>. These mice have normal or elevated levels of leptin but are resistant to its effects. Studies have shown that the *db* gene encodes the leptin receptor (OB-R) which is structurally similar to a class I cytokine receptor<sup>[9,10]</sup>. There are two isoforms; the short OB-Ra isoform has not been shown to have any signaling activity. In contrast, the OB-Rb isoform has a long intracytoplasmic region that contains signal transduction motifs which activate the JAK/STAT protein kinase signal transduction cascade<sup>[11]</sup>. *db/db* mice carry a sequence insertion at the 3' end of the mRNA transcript exactly where the OB-Ra and OB-Rb transcripts diverge. This insertion contains a stop codon that leads to the premature termination of the OB-Rb long intracellular signaling domain, loss of function and consequently leptin resistance<sup>[12]</sup>.

### *Yellow-obese agouti (Ay)* mice

KK-*A<sup>y</sup>* mice are a cross-strain of diabetic KK mice<sup>[13]</sup> and lethal yellow (*A<sup>y</sup>*) mice, which carry mutation of the agouti(a) gene on mouse chromosome 2<sup>[14]</sup>. KK-*A<sup>y</sup>* mice develop maturity-onset obesity, dyslipidemia, and insulin resistance, in part because of the antagonism of melanocortin receptor-4 by ectopic expression of the agouti protein<sup>[14]</sup>. Importantly, these mice present hyperleptinemia and leptin resistance without defects in the *ObR* gene, and the expression of adiponectin is conversely down-regulated<sup>[15,16]</sup>. The phenotype of KK-*A<sup>y</sup>* mice, including altered adipokine expression, quite resembles metabolic syndrome in humans indicating the potential usefulness of this strain as a model of metabolic syndrome NASH<sup>[17,18]</sup>. In fact, KK-*A<sup>y</sup>* mice are more susceptible to experimental steatohepatitis induced by MCD diet.

### *CD36<sup>-/-</sup>* mice

A valuable model for the study of the effects of alteration in fatty acid (FA) utilization on insulin responsiveness is the recently generated CD36-deficient mouse<sup>[19,20]</sup>. CD36, also known as fatty acid translocase (FAT)<sup>[21]</sup>, is a multispecific, integral membrane glycoprotein<sup>[22,23]</sup> that has been identified as a facilitator of FA uptake. Its function in binding and transport of FA was documented *in vitro* by affinity labeling with FA derivatives and by cell transfection studies<sup>[23,24]</sup>. The CD36-deficient mouse exhibits greater than 60% decrease of FA uptake and utilization by heart, skeletal muscle, and adipose tissues and thereby increases FA delivery to liver and exhibits increased plasma free fatty acid (FFA) and triglyceride (TG) levels<sup>[20]</sup>. The pathogenic role of FAT/CD36 in hepatic steatosis in rodents is well-defined<sup>[25]</sup>.

### *Phosphoenolpyruvate carboxykinase-sterol regulatory-element binding protein 1a-mice*

Sterol regulatory-element binding protein (SREBP) family members have been established as transcription factors regulating the transcription of genes involved in cholesterol and FA synthesis. *In vivo* studies have demonstrated that

SREBP-1 plays a crucial role in the dietary regulation of most hepatic lipogenic genes<sup>[26,27]</sup>. Physiological changes of SREBP-1 protein in normal mice by dietary manipulation such as placement on high carbohydrate diets, polyunsaturated FA-enriched diets, and fasting-refeeding regimens has been reported<sup>[28,29]</sup>. SREBP-1a transgenic mice, under the control of liver specific Phosphoenolpyruvate carboxykinase promoter (TgSREBP-1a), show a massively enlarged liver and atrophic peripheral white adipose tissue, and develop steatosis<sup>[30]</sup>.

#### **aP2-NSREBP-1c mice**

Leptin has similar effects in lipodystrophic rodents, most notably in aP2-nSREBP-1c transgenic mice. These animals express a truncated, constitutively active form of the SREBP-1c transcription factor under the control of the adipose tissue specific aP2 promoter and develop lipodystrophy with very low plasma leptin levels. These mice are also hyperphagic and have massive fat accumulation in peripheral tissues with hyperglycemia and hyperinsulinemia<sup>[31,32]</sup>. This mouse model has markedly reduced body fat and develops liver steatosis, profound insulin resistance, and increased levels of triglycerides<sup>[31,32]</sup>.

#### **aP2-diphtheria toxin mice**

The aP2/DTA mice have low serum leptin levels and are hyperphagic. These mice when fed a control diet are hyperlipidemic, hyperglycemic, and have hyperinsulinemia indicative of insulin-resistant diabetes. These mice are born normally and initially lack any distinguishing phenotypic features, but develop atrophy and necrosis of the adipose tissue at five to six months resulting in the complete absence of subcutaneous or intra-abdominal adipose tissue at eight to nine months of age<sup>[33]</sup>. This late onset of adipose tissue loss is associated with reduced leptin levels, increased food consumption, hyperlipidemia, hyperglycemia and insulin resistance. Monosodium glutamate-treated aP2/DTA mice develop gross hepatomegaly as a result of severe fatty changes in the liver<sup>[33]</sup>.

#### **A-ZIP/F-1 mice**

The A-ZIP/F-1 mice express a dominant negative version of the C/EBP $\alpha$  leucine zipper domain that potently interferes with adipocyte differentiation<sup>[34]</sup>. The A-ZIP/F-1 mouse (*A-ZIPTg/+*) is a model of severe lipotrophic diabetes and is insulin resistant, hypoleptinemic, hyperphagic, and shows severe hepatic steatosis. This mouse has essentially no white adipose tissue, reduced brown fat and severe metabolic phenotype with a reduced life span. These mice display massive hepatomegaly causing increased body weight, liver steatosis, severe diabetes (hyperglycemia, hyperinsulinemia, hyperphagic, polydipsia and polyuria), and are hypertensive<sup>[35]</sup>. They have increased triglycerides and FFA levels, alveolar foam cells and reduced leptin levels. These mice are unable to sustain glucose levels during fasting. The insulin resistance and much of the liver steatosis in the A-ZIP/F-1 mice can be reversed by transgenic over-expression

of leptin<sup>[36]</sup> or by transplanting normal adipose tissue<sup>[37]</sup>. By contrast, transplantation of adipose tissue from *ob/ob* mice did not reverse the phenotype of the A-ZIP/F-1 mice indicating that leptin deficiency strongly contributes to the metabolic complications in lipodystrophy<sup>[38]</sup>.

#### **Peroxisome proliferator-activated receptor alpha<sup>-/-</sup> mice**

Peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) is expressed in the liver and other metabolically active tissues including striated muscle, kidney and pancreas<sup>[39,40]</sup>. Many of the genes encoding enzymes involved in the mitochondrial and peroxisomal FA beta-oxidation pathways are regulated by PPAR $\alpha$ . In wild-type mice, peroxisome proliferators are compounds that induce lipid catabolism and an associated intracellular increase in peroxisome number and enzymatic activity. PPAR $\alpha$  mutated mice exhibit alterations of intracellular lipid processing, particularly in response to peroxisome proliferators. Mice deficient in PPAR $\alpha$  exhibit severe hepatic steatosis when subjected to fasting for 24-72 h, indicating that a defect in PPAR $\alpha$ -inducible FA oxidation accounts for severe FA overload in liver, causing steatosis, in contrast to the wild-type mice<sup>[41,42]</sup>.

#### **Galactin-3 knockout mice**

Galectin-3, a beta-galactoside-binding animal lectin, is a multifunctional protein. Galectin-3 plays a role in the regulation of hepatic stellate cell (HSC) activation *in vitro* and *in vivo*, thereby identifying galectin-3 as a potential therapeutic target in the treatment of liver fibrosis. This model plays a role in investigating liver carcinogenesis based on a natural history of NAFLD<sup>[43]</sup>. Previous studies have also suggested that galectin-3 may play an important role in inflammatory responses. The livers of *gal3(-/-)* male mice at six months of age displayed mild to severe fatty change. The liver weight per body weight ratio, serum alanine aminotransferase levels, liver triglyceride levels, and liver lipid peroxide in *gal3(-/-)* mice were significantly increased compared with those in *gal3(+/+)* mice. Furthermore, the hepatic protein levels of advanced glycation end-products (AGE), receptor for AGE, and PPAR $\gamma$  were increased in *gal3(-/-)* mice relative to *gal3(+/+)* mice<sup>[43,44]</sup>.

#### **Acetyl CoA oxidase<sup>-/-</sup> mice**

Acyl-coenzyme A oxidase (AOX) is the rate-limiting enzyme in peroxisomal FA  $\beta$ -oxidation for the preferential metabolism of very long-chain FAs. AOX null (*AOX(-/-)*) mice have defective peroxisomal  $\beta$ -oxidation and exhibit steatohepatitis. Microvesicular fatty change in hepatocytes is evident at 7 d. At 2 mo of age, livers show extensive steatosis and they have clusters of hepatocytes at periportal areas with abundant granular eosinophilic cytoplasm rich in peroxisomes. At 4-5 mo there is increased PPAR $\alpha$ , cytochrome P450, Cyp 4a10, and Cyp4a14 expression. By 6 to 7 mo, however, there is a compensatory increase in FA oxidation and reversal of hepatic steatosis resulting from hepatocellular regenera-

tion<sup>[45,46]</sup>. The *AOX*<sup>-/-</sup> mice proceed to develop adenomas and carcinomas by 15 mo of age<sup>[46,47]</sup>.

### **Aromatase (CYP 19)-deficient mice**

Aromatase P450 (CYP19) is an enzyme catalysing the conversion of androgens into estrogens<sup>[48]</sup>. These models present dyslipidemia, central obesity, hypercholesterolemia, hyperinsulinemia, hyperleptinemia, and hypertriglyceridemia<sup>[49]</sup>, and importantly the male mice have hepatic steatosis. Aromatase knockout (ArKO) mice have a similar phenotype to that of estrogen receptor null mice with increased gonadal fat pad weight<sup>[50]</sup>. Only ArKO males have elevated hepatic triglyceride levels leading to hepatic steatosis partly due to an increase in expression of enzymes involved in *de novo* lipogenesis and transporters involved in FA uptake<sup>[51-53]</sup>.

### **MTP<sup>-/-</sup> mice**

Mitochondrial  $\beta$ -oxidation of FAs is the major source of energy for skeletal muscle and the heart, and it plays an essential role in intermediary metabolism in the liver and impairment of mitochondrial  $\beta$ -oxidation in pathogenesis of NAFLD. The fetuses of *Mtpa*<sup>-/-</sup> mice accumulate long chain FA metabolites and have low birth weight compared with the *Mtpa*<sup>+/-</sup> and *Mtpa*<sup>+/+</sup> littermates. *Mtpa*<sup>-/-</sup> mice suffer neonatal hypoglycemia and sudden death 6-36 h after birth. Analysis of the histopathological changes in the *Mtpa*<sup>-/-</sup> pups revealed rapid development of hepatic steatosis after birth and, later, significant necrosis and acute degeneration of the cardiac and diaphragmatic myocytes. However, studies by Ibdah *et al*<sup>[54]</sup> indicated that aged but not young *MTPA*<sup>+/-</sup> mice developed hepatic steatosis with elevated alanine aminotransferase (ALT), basal hyperinsulinemia, and increased insulin compared with *MTPA*<sup>+/+</sup> littermates. Significant hepatic steatosis and insulin resistance developed concomitantly in the *MTPA*<sup>+/-</sup> mice at 9-10 mo of age. The cause resides in heterozygosity for  $\beta$ -oxidation defects that predisposes to NAFLD and insulin resistance in aging mice<sup>[55]</sup>.

### **Phosphatase and tensin homologue -/- mice**

Phosphatase and tensin homologue (PTEN) is a multifunctional phosphatase whose substrate is phosphatidylinositol-3,4,5-triphosphate and which acts as a tumor suppressor gene that downregulates phosphatidylinositol kinases<sup>[56,57]</sup>. Hepatocyte-specific PTEN-deficient mice spontaneously develop steatosis, steatohepatitis, and hepatocellular carcinoma<sup>[58,59]</sup>. By 10 wk of age, these mice have increased concentrations of triglyceride and cholesterol esters, and a histological analysis displays micro- and macrovesicular lipid vacuoles. At 40 wk of age, they have macrovesicular steatosis, Mallory bodies, ballooning degeneration, and sinusoidal fibrosis<sup>[59-60]</sup>. Mice that are homozygous for this allele are viable, fertile, and normal in size and do not display any gross physical or behavioral abnormalities. When crossed to a strain expressing Cre recombinase in liver, this mutant mouse

strain may be useful in studies of fatty liver and insulin signaling. Piguet *et al*<sup>[61]</sup> have investigated the effects of hypoxia in the PTEN-deficient mouse, a mouse model that develops NAFLD. The authors also showed that a short period (7 d) of exposure to hypoxia aggravates the NAFLD phenotype, causing changes in the liver that are in keeping with NASH, with increased lipogenesis and inflammation.

### **Methionine adenosyl transferase 1A -/- mice**

Mice deficient in methionine adenosyl transferase 1A (the enzyme responsible for SAM synthesis in the adult liver) have a decrease in hepatic SAM levels and spontaneously develop steatosis, NASH, and hepatocellular carcinoma (HCC)<sup>[62]</sup>. By three months of age, these mice have hepatomegaly with macrovesicular steatosis. These mice also have increased mRNA levels of CYP2E1 and UCP2, and levels of glutathione. Also, these mice have changes in the expression of genes involved in cell proliferation of lipid and carbohydrate metabolism<sup>[63]</sup>. These mice are predisposed to liver injury and have impaired liver regeneration after partial hepatectomy<sup>[64]</sup>.

### **Adiponectin null mice**

Adiponectin is an adipokine abundantly produced from adipocytes<sup>[65,66]</sup>. Adiponectin is an anti-inflammatory adipocyte-derived plasma protein known to alleviate steatosis and inflammation in NAFLD<sup>[65-67]</sup>. Two adiponectin receptors (adipoR1 and adipoR2) have been identified and found to be expressed in various tissues<sup>[68]</sup>. AdipoR1 is abundantly expressed in skeletal muscles, whereas adipoR2 is present predominantly in the liver, suggesting a role of adipoR2 in hepatic adiponectin signaling<sup>[68,69]</sup>. The physiological roles of adipoR1 and adipoR2 have recently been investigated by several laboratories in *adipoR1/2* knockout mice. Both *adipoR1* and *adipoR2* knockout mice exhibit mild insulin resistance<sup>[70]</sup>. In *adipoR1/R2* double knockout mice the binding and actions of adiponectin are abolished, resulting in increased tissue triglyceride content, inflammation oxidative stress<sup>[70-73]</sup> and mice exhibit impaired liver regeneration and increased hepatic steatosis.

### **Bid null mice**

The protein Bid is a participant in the pathway that leads to cell death (apoptosis), mediating the release of cytochrome from mitochondria in response to signals from "death" receptors known as tumor necrosis factor (TNF) receptor 1/Fas on the cell surface. Genetic inactivation of Bid, a key pro-apoptotic molecule that serves as a link between these two cell death pathways, significantly reduced caspase activation, adipocyte apoptosis, prevented adipose tissue macrophage infiltration, and protected against the development of systemic insulin resistance and hepatic steatosis independent of body weight<sup>[74,75]</sup>. These mice can be used in research based on adipocyte apoptosis which is a key initial event that contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis associated with obesity.

**Fas adipocyte-specific (AfasKO) null mice**

Fas (CD95), a member of the TNF receptor super family, is a major contributor to apoptosis in many cells. Fas activation may contribute to obesity-induced insulin resistance, since mice lacking Fas in adipocytes were partly protected from developing insulin resistance. In particular, Fas activation led to increased release of pro-inflammatory cytokines, and reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes<sup>[75]</sup>. Fas-deficient (Fas-def) mice show increased insulin-stimulated glucose incorporation when compared to wild type (WT) with higher expression levels of Akt<sup>[76,77]</sup>.

**Interleukin-6 KO mice**

Interleukin-6 (IL-6) is an adipocytokine associated with NAFLD and obesity that is secreted in larger amounts by visceral fat compared to subcutaneous fat in obese adults<sup>[78]</sup>. Increased systemic IL-6 is associated with increased inflammation and fibrosis in NAFLD patients<sup>[79]</sup>. Expression of IL-6, a major proinflammatory cytokine, is increased in animal models of NAFLD. Hepatic IL-6 production may also play an important role in NASH development, as well as in systemic insulin resistance and diabetes. IL-6 is elevated in the plasma and peripheral blood monocytes of patients with fatty diseases, including alcoholic liver disease and non-alcoholic steatohepatitis, and elevation of IL-6 correlates with the progression and severity of liver disease, suggesting that IL-6 may be involved in the pathogenesis of fatty liver disease<sup>[80,81]</sup>. Studies using *Il6*<sup>-/-</sup> mice show these animals display obesity, hepatosteatosis, liver inflammation and insulin resistance when compared with control mice on a standard chow diet<sup>[82]</sup>.

**TNF alpha KO mice**

TNF- $\alpha$  appears to play a central role in the development of hepatic steatosis. TNF- $\alpha$ , by mechanisms not completely defined, is over expressed in the liver of obese mice and is an important mediator of insulin resistance in both diet-induced and *ob/ob* models of obesity<sup>[83,84]</sup>. Data from animal and clinical studies indicate that TNF- $\alpha$  mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver damage<sup>[85,86]</sup>. Mice homozygous for the TNF targeted mutation are viable and fertile. Further, male mutant mice at 28 wk old display lower insulin, triglyceride, and leptin levels compared to wild type controls.

**NEMO<sup>LPC-KO</sup> mice**

The I $\kappa$ B kinase (IKK) subunit NEMO/IKK $\gamma$  is essential for activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B), which regulates cellular responses to inflammation. NEMO-mediated NF- $\kappa$ B activation in hepatocytes has an essential physiological function to prevent the spontaneous development of steatohepatitis and hepatocellular carcinoma. These mice were generated with liver parenchymal cell-specific knockout of these subunits (*NEMO*<sup>LPC-KO</sup>, *IKK2*<sup>LPC-KO</sup>) by crossing mice carrying loxP-

flanked *Nemo*<sup>[87]</sup> or *Ikk2*<sup>[88]</sup> alleles with Alfp-cre transgenic mice that mediate efficient Cre recombination in liver parenchymal cells, including hepatocytes and biliary epithelial cells, but not in endothelial or Kupffer cells<sup>[89]</sup>. *NEMO*<sup>LPC-KO</sup> mice were born and reached weaning age at the expected Mendelian frequency<sup>[90]</sup>. These mice showed efficient ablation of the respective proteins in whole-liver extracts and NF- $\kappa$ B activity in the liver was completely abolished<sup>[91,92]</sup>. Hepatocytes are LPS sensitive. When fed a high-fat diet, mice had reduced  $\beta$ -oxidation and upregulated PPAR- $\gamma$ , SREBP1 and FA synthase causing increased *de novo* lipid synthesis and macrovesicular steatosis with increased HCC occurrence<sup>[91,92]</sup>.

**Jun N-terminal kinase 1 null mice**

Jun N-terminal kinase (JNK) 1 null mice have less hepatic inflammation and fibrosis when fed a choline-deficient, l-amino acid-defined (CDAA) diet due to the absence of JNK1 in immune cells. As JNK is activated by oxidants and cytokines and regulates hepatocellular injury and insulin resistance, this kinase may mediate the development of steatohepatitis. JNK promotes the development of steatohepatitis as MCD diet-fed *JNK* null mice have significantly reduced levels of hepatic triglyceride accumulation, inflammation, lipid peroxidation, liver injury, and apoptosis compared with wild-type and *JNK2*<sup>-/-</sup> mice<sup>[93,94]</sup>. Hence JNK1 is responsible for JNK activation that promotes the development of steatohepatitis in the MCD diet model<sup>[93]</sup>. *JNK1* KO produces lean, male *JNK1* KO mice which have decreased body weights, fasting blood glucose levels, and fasting blood insulin levels compared to their wild-type controls<sup>[94]</sup>. This model can be used to study a combination of genetic and dietary challenges that constitute the disease etiology for NASH development and mimic more closely the pathogenesis of human NAFLD/NASH.

**Toll-like receptor 9 KO mice**

Development of NASH involves the innate immune system and is mediated by Kupffer cells and HSCs. Toll-like receptor 9 (TLR9) is a pattern recognition receptor that recognizes bacteria-derived cytosine phosphate guanine-containing DNA and activates innate immunity. Mice deficient in TLR9 have reduced steatohepatitis and fibrosis<sup>[95]</sup>. Hence this model can be used to study NAFLD involving innate immunity.

**LDLR KO and farnesoid X receptor KO mice**

Farnesoid X receptor (FXR) is essential for regulating bile-acid synthesis and transport. Mice with FXR deficiency have severe impairment of bile-acid homeostasis and manifest systemic abnormalities including altered lipid and cholesterol metabolism features known to be associated with the metabolic syndrome and NASH. Kong *et al*<sup>[96]</sup> studied LDL receptor knockout (*LDLR*<sup>-/-</sup>) mice fed with a high-fat diet for 5 mo, and checked whether FXR deficiency contributed to NASH development. Both high-fat diet and FXR deficiency increased

serum ALT activity, whereas only FXR deficiency increased bile-acid and ALP levels. FXR deficiency and high-fat feeding increased serum cholesterol and triglycerides. Although high-fat diet led to macrosteatosis and hepatocyte ballooning in livers of mice regardless of genotype, no inflammatory infiltrate was observed in the livers of *LDLR*<sup>-/-</sup> mice. In contrast, in the livers of *LDLR*<sup>-/-</sup>/*FXR*<sup>-/-</sup> mice, foci of inflammatory cells were observed when they were fed with control diet and were greatly increased when fed with the high-fat diet<sup>[96,97]</sup>. This model can be used to study a combination of genetic and dietary challenges that constitute the disease etiology for NASH development and mimic more closely the pathogenesis of human NAFLD/NASH.

### **Myd88 KO mice**

Chemokines, strongly induced by TLR stimulation, play an important role in the development of metabolic syndrome including NAFLD. TLR4- and MyD88-deficient mice, which are resistant to metabolic syndrome, show reduced chemokine production compared with WT mice<sup>[98,99]</sup>. MyD88 is a key molecule in the development of metabolic syndrome including NAFLD<sup>[98,99]</sup>. MyD88, an adaptor protein for all TLRs except for TLR3, is required for the expression of various inflammatory cytokines and chemokines<sup>[100]</sup>. MyD88-deficient mice are protected from metabolic syndrome as well as atherosclerosis<sup>[98,99]</sup> and from liver injury induced by bile duct ligation or carbon tetrachloride<sup>[101,102]</sup>. Miura *et al*<sup>[100]</sup> demonstrated that MyD88-deficient mice on a CDAA diet show less steatohepatitis with less insulin resistance compared with wild type mice. Inflammatory cytokines and fibrogenic factors are also significantly suppressed in MyD88-deficient mice compared with wild type mice fed a CDAA diet<sup>[100]</sup>.

### **Fatty liver dystrophy knockout mice**

Fatty liver dystrophy (*fld*) is a spontaneous point mutation in *Lpin1* which occurred on C3H/HeJ in 1994. An unstable gait and tremor at 3 wk of age was initially observed in these mice. The pups from these mice have a fatty liver before reaching weaning age. Mice carrying mutations in the *fld* gene have features of human lipodystrophy, a genetically heterogeneous group of disorders characterized by loss of body fat, fatty liver, and hypertriglyceridemia and insulin resistance<sup>[103]</sup>. Homozygous *fld* mice have an enlarged, fatty liver and hypertriglyceridemia that resolve to normal during the weaning transition. However, decreased overall size, decreased lipid in the fat pads and a peripheral neuropathy persist throughout the lifespan. This peripheral neuropathy manifests as a tremor and an unsteady gait shortly after 10 d of age and worsens with age. As with the original mutation of *fld*, homozygous females will breed and raise their litters but homozygous males do not breed.

### **Platelet endothelial cell adhesion molecule-1 null mice**

Platelet endothelial cell adhesion molecule-1 (PECAM-1)

is a 130-kDa transmembrane glycoprotein expressed on blood and vascular cells. Goel *et al*<sup>[104]</sup> demonstrated that genetic deficiency of PECAM-1 potentiates the development and progression of NASH. After 3 wk on an atherogenic diet, these mice developed mild microvesicular steatosis predominantly in hepatic parenchymal cells in the centrilobular region. At 9 and 18 wk on the atherogenic diet, more severe steatosis with lobular and sinusoidal inflammation developed in the livers, which are consistent with the typical histological features of steatohepatitis<sup>[104]</sup>.

### **ApolipoproteinB 38.9 mutant mice**

Fatty liver is prevalent in apolipoproteinB (apoB)-defective familial hypobetalipoproteinemia (FHBL). Similar to humans, mouse models of FHBL produced by gene targeting (*apoB*<sup>+/38.9</sup>) manifest low plasma cholesterol and increased hepatic TG even on a chow diet due to impaired hepatic VLDL-TG secretive capacity. These mice will be useful to study the genetic and molecular mechanism of apoB defects and lipid metabolism/liver fat accumulation, the relationship between hepatic steatosis and insulin resistance, and the progression of advanced NAFLD and atherosclerosis<sup>[105]</sup>.

### **Cystathionine-synthase deficient mice**

Cystathionine-synthase (CBS) deficiency causes severe hyperhomocysteinemia, which confers diverse clinical manifestations, notably liver disease. Robert *et al*<sup>[106]</sup> reported that CBS-deficient mice showed inflammation, fibrosis, and hepatic steatosis. These mice also had pathological resemblance to steatohepatitis and a pattern of perivenous and pericellular hepatic fibrosis around lipid-laden hepatocytes. CBS KO mice develop hepatic steatosis more tardily than inflammation and fibrosis at 8-32 wk old.

In addition to the above KO animals, Postic *et al*<sup>[107]</sup> has demonstrated a few animal models modulating enzymes in FA synthesis.

### **Acc2KO mice**

Acetyl-CoA carboxylase (ACC) catalyzes the synthesis of malonyl-CoA, the metabolic intermediate between lipogenesis<sup>[108]</sup> and  $\beta$ -oxidation<sup>[109]</sup>; this lipogenic enzyme has garnered significant attention over recent years. In mammals, two ACC isoforms exist, each with distinct tissue distribution and physiological roles: ACC1 is highly expressed in liver and adipose tissue, whereas ACC2 is predominantly expressed in heart and skeletal muscle and, to a lesser extent, in liver<sup>[110]</sup>. It is believed that only ACC1, but not ACC2, is committed to *de novo* lipogenesis in liver. Targeting ACC has beneficial effects on both hepatic steatosis and insulin resistance. ACC1-knockout mice (*Acc1*<sup>-/-</sup> mice and *ACC2*<sup>-/-</sup> mice) have been developed to study the effect.

### **SCD KO mice**

SCD1 has recently become a target of interest for the reversal of hepatic steatosis and insulin resistance<sup>[111]</sup>.

**Table 1** Potential candidate genes in fatty liver disease

Category of genes	Examples
Genes affecting insulin resistance	ADIPOQ, AKT2, ENPP1, IRS1, PPARG, HFE, resistin
Genes affecting hepatic lipid synthesis and uptake	DGAT2, SLC25A13, ACC, ELOVL6, SCD1, GPAT, SREBP1
Genes affecting hepatic lipid uptake	APOC3
Genes affecting hepatic triglyceride hydrolysis	PNPLA2, CGI-58, LIPA
Genes affecting hepatic lipid export	APOB, MTTP, PEMT
Genes affecting hepatic oxidative stress	GCLC, NOS2, SOD2, HFE, UCP2, MAT1A, GST, GSH-Px
Genes affecting immune regulation	ADIPOQ, ADIPOR1, ADIPOR2, STAT3, TNF $\alpha$ , IL10, IL6, CTLA-4, IL-4, IL-18
Genes influencing disease progression and fibrosis	TGF- $\beta$ 1, 3, PPAR $\alpha$ , DDX5, CPT1A, angiotensin II
Genes influencing response to endotoxin	CD14, TLR4, NOD2

ADIPOQ: Adiponectin; AKT: Beta serine/threonine-protein kinase; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; IRS-1: Insulin receptor substrate 1; PPARG: Peroxisome proliferator-activated receptor gamma; HFE: Hemochromatosis gene; DGAT2: Diacylglycerol acetyltransferase-2; SLC25A13: Solute carrier family 25 Member 13 (citrin); ACC gene: Acetyl-CoA carboxylase alpha; ELOVL6: Elongation of very long chain fatty acids; SCD1 gene: Stearoyl-CoA desaturase gene; GPAT: Glycerol-3-phosphate acyltransferase; SREBP1: Sterol regulatory element-binding transcription factor 1; APOC3: Apolipoprotein C-III; PNPLA2: Patatin-like phospholipase domain containing 2; CGI58: Comparative gene identification-58; LIPA: Lipase A; APOB: Apolipoprotein B; MTTP: Microsomal triglyceride transfer protein; PEMT: Phosphatidylethanolamine N-methyltransferase; GCLC: Glutamate-cysteine ligase, catalytic subunit; NOS2: Nitric oxide synthases2; SOD2: Superoxide dismutase-2; UCP2: Uncoupling protein 2; MAT1A: Methionine adenosyltransferase I alpha; GST: Glutathione S-transferase; ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase; CTGF: Connective tissue growth factor; CTLA-4: Cytotoxic T-cell associated antigen-4; GSH-Px: Glutathione peroxidase; STAT3: Signal transducer and activator of transcription 3; IL: Interleukin; PPAR: Peroxisomal proliferator activated receptor; SCD-1: Stearoyl CoA desaturase-1; TLR: Toll-like receptor; TNFR: TNF- $\alpha$  receptor; DDX5: DEAD box protein 5; CPT1A: Carnitine palmitoyltransferase 1A (liver); NOD2: Nucleotide-binding oligomerization domain containing 2.

SCD1 catalyzes the synthesis of monounsaturated FAs, particularly oleate (C18:1n-9) and palmitoleate (C16:1n-7), which are the major components of membrane phospholipids, TGs, and cholesterol esters. Mice with SCD1KO (*Scd1*<sup>-/-</sup> mice) show decreased lipogenic gene expression and increased  $\beta$ -oxidation and are protected from diet-induced obesity and insulin resistance when fed a HC/HF diet<sup>[112,113]</sup>. Inhibition of SCD1 using an ASO strategy (targeting SCD1 in both liver and adipose tissues) prevents many of the HF/HC-diet metabolic complications, including hepatic steatosis and postprandial hyperglycemia<sup>[114,115]</sup>.

### ELOVL6 KO mice

*Elovl6*<sup>-/-</sup> mice are protected against the development of hepatic insulin resistance when fed a HF/HC diet, despite the accumulation of palmitate concentrations. Improvement in insulin signaling (as evidenced by the restoration in insulin-mediated Akt phosphorylation)

occurred despite hepatic steatosis and marked obesity in *Elovl6*<sup>-/-</sup> mice<sup>[116]</sup>. While these results are somewhat surprising given the role of palmitate as a potent inducer of insulin resistance (at least in primary cultures of hepatocytes)<sup>[117]</sup>, they are also interesting since they indicate that the hepatic FA composition, and particularly the conversion of palmitate to stearate, is crucial for insulin sensitivity. It should be noted that the reduced SCD1 expression observed in livers of *Elovl6*<sup>-/-</sup> mice could have also contributed to the amelioration of insulin resistance in these mice<sup>[116]</sup>.

### ChREBP knockdown mice

ChREBP knockdown led to the expected inhibition of L-PK, ACC, FAS and SCD1 as well as GPAT. While a carbohydrate-response element was previously identified in the promoter region of the *GPAT* gene<sup>[118]</sup>, its expression was found to be unaffected in the liver of ChREBP-knockout mice upon refeeding<sup>[119]</sup>. It is possible that the nutritional regulation of GPAT may be more sensitive to insulin *via* SREBP-1c than to glucose *via* ChREBP. Nevertheless, following ChREBP knockdown, a resultant decrease in lipogenic rates was observed in shChREBP-RNA-treated *ob/ob* mice, leading to a 50% reduction in hepatic and circulating TG concentrations<sup>[120]</sup>. ChREBP knockdown not only affected the rate of *de novo* lipogenesis but also had consequences for  $\beta$ -oxidation. Therefore, similarly to the liver-specific knockout of SCD1 (LKO mice)<sup>[121]</sup>, the coordinated modulation in FA synthesis and oxidation in liver led to an overall improvement of lipid homeostasis in ChREBP-deficient mice. The decrease in lipogenic rates observed in LKO mice was at least partially attributed to a decrease in ChREBP nuclear protein content<sup>[122]</sup>. Clearly, ChREBP needs now to be considered as a key determinant of the molecular regulation of the lipogenic pathway.

## CLASSIFICATION OF SOME ANIMAL MODELS WITH DISRUPTION OF GENES INVOLVED IN NAFLD

Table 1 presents a number of candidate genes that are involved in the pathogenesis of NAFLD and a few are discussed below.

### Genes affecting lipid metabolism

**Pemt KO animals:** *Pemt*<sup>-/-</sup> mice have two selectively disrupted alleles of the *Pemt-2* gene at exon 2<sup>[123]</sup>, which encode PEMT, and do not express any PEMT activity in liver. Therefore these mice completely depend on dietary choline intake to meet daily choline requirements. When fed a diet deficient in choline and insufficient in methionine, *Pemt*<sup>-/-</sup> mice develop decreased PtdCho concentrations in hepatic membranes, leading to severe liver damage and death; a choline supplemented diet prevents this<sup>[124]</sup> and, if provided early enough, can reverse hepatic damage.

**DGAT2 mice:** DGAT2, an isoform of the enzyme acylCoA: diacylglycerol acyltransferase, catalyses the final stage of triglyceride synthesis in the liver<sup>[125]</sup>. Overexpression of DGAT2 in mice led to a 2.4-fold increase in hepatic triglyceride content, but no effect on production of VLDL triglyceride or apoB<sup>[126]</sup>. In addition, mice on a high-fat diet that overexpress DGAT develop fatty liver but not glucose or insulin intolerance<sup>[127]</sup>, showing that hepatic steatosis can occur independently of insulin resistance. Interestingly, antisense therapy reducing DGAT improves hepatic steatosis, but not insulin sensitivity<sup>[128]</sup>.

**Apolipoprotein C-III:** Apolipoprotein C-III (apoC-III) is the most abundant C apolipoprotein in human plasma, where it is present as an 8.8-kDa mature protein on chylomicrons, VLDL and HDL. ApoC-III is synthesized in the liver and in minor quantities by the intestine<sup>[129]</sup>. Several lines of evidence have implicated apoC-III as contributing to the development of hypertriglyceridemia in the human population. Investigation in *apoC3*<sup>-/-</sup> mice supports the concept that apoC-III is an effective inhibitor of VLDL TG hydrolysis and reveals a potential regulating role for apoC-III with respect to the selective uptake of cholesteryl esters<sup>[130]</sup>.

#### Genes affecting insulin resistance/sensitivity

**IRS 1:** Studies on mice with targeted disruption of the *Irs* genes lend some support to both situations. *Irs1* knockout (*Irs1*<sup>-/-</sup>) mice show significant embryonic and postnatal growth retardation, suggesting that IRS-1 plays a key role in relaying the growth-stimulating effects of insulin and insulin-like growth factor. IRS-1-deficient mice also have insulin resistance and mild glucose intolerance, but do not develop diabetes<sup>[131,132]</sup>.

**Ecto-nucleotide pyrophosphate phosphodiesterase:** Ecto-nucleotide pyrophosphate phosphodiesterase (ENPP1) has been shown to negatively modulate insulin receptor and to induce cellular insulin resistance when over-expressed in various cell types. Systemic insulin resistance has also been observed when ENPP1 is over-expressed in multiple tissues of transgenic models and is largely attributed to tissue insulin resistance induced in skeletal muscle and liver. In the presence of a high fat diet, ENPP1 over-expression in adipocytes induces fatty liver, hyperlipidemia and dysglycemia, thus recapitulating key manifestations of the metabolic syndrome<sup>[133]</sup>.

**Transcription factor 7-like 2:** Transcription factor 7-like 2 (TCF7L2) is a receptor for  $\beta$ -catenin and regulates the expression of a multitude of genes involved in cellular metabolism and growth. Various studies<sup>[134-136]</sup> have linked TCF7L2 variation with impaired insulin secretion and risk of diabetes, possibly mediated by altered  $\beta$ -cell glucose response. In addition, it regulates adipokine secretion and triglyceride metabolism through effects on PPAR- $\gamma$ , CCAAT/enhancer-binding protein, and lipoprotein lipase; TCF7L2 SNPs are associated with serum

triglyceride concentrations in familial hyperlipidemia<sup>[137]</sup>.

#### Genes affecting oxidative stress

**Glutamate-cysteine ligase:** Glutamate-cysteine ligase (GCLC) is the first and rate-limiting enzyme in the synthesis of glutathione, the major antioxidant in the liver. Liver-specific deletion of GCLC in mice rapidly leads to hepatic steatosis and progressive severe parenchymal damage<sup>[138]</sup>.

**Nitric oxide synthase:** Yoneda *et al.*<sup>[139]</sup>, who studied associations of PPAR $\gamma$ C1 $\alpha$ , also examined the influence of SNPs in the inducible nitric oxide synthase (*NOS2*) gene on their NAFLD cohort. iNOS is expressed as part of the inflammatory response and in the presence of superoxide radicals forms peroxynitrite, which can cause endoplasmic reticulum stress and cell death<sup>[140]</sup>. iNOS-deficient mice develop NASH with high fat diets<sup>[141]</sup>.

**Superoxide dismutase-2:** Elevated hepatic reactive oxygen species play an important role in pathogenesis of liver diseases, such as alcohol-induced liver injury, hepatitis C virus infection, and nonalcoholic steatohepatitis. Satoshi *et al.*<sup>[142]</sup> observed significant increases in lipid peroxidation and TG in the liver of *Sod1* KO and double KO mice but not in the liver of *Sod2* KO mice.

#### Genes affecting immune regulation

**Signal transducer and activator of transcription 3:** Signal transducer and activator of transcription 3 (STAT3) is an acute-phase transcription factor; after hepatic necrosis it activates pathways associated with liver regeneration and acute inflammation<sup>[143]</sup>. STAT3 is also implicated in nutrient metabolism and developing metabolic syndrome. Transgenic mice with hepatic deficiency of STAT3 develop insulin resistance and disturbed glucose homeostasis; whereas the constitutive liver specific expression of STAT3 in diabetic mice reduces blood glucose and plasma insulin concentrations and downregulates gluconeogenic gene expression<sup>[144]</sup>.

## CONCLUSION

Inbred strains of mice provide convenient tools to study the pathogenesis of NAFLD because they provide the opportunity to control genetic and environmental factors that might influence the natural history of NAFLD. Various genetic alterations or environmental stressors producing a similar phenotype prove that many different immunological, neuronal and hormonal factors are involved in the pathogenesis of NAFLD<sup>[145,146]</sup>. Transgenic mouse models also represent gene mediation to NAFLD. Therefore, any one of these animal models could be used to clarify how altered cross talk among immune cells, neurons and endocrine cells promote NAFLD. In contrast to human genetic studies, animal studies have found genes that consistently produce disease-like phenotypes, and the underlying genetic basis for the phenotypes in these models have often been

elucidated. Animal studies on NAFLD frequently reveal significant single-locus effects that can be reproduced across species and/or strains. Such “disease genes” in animal models can be found relatively easily using linkage mapping techniques in crossed inbred lines. Similarly, transgenic animals or genetically manipulated animals for NAFLD can reveal significant effects of candidate alleles in well-defined genetic backgrounds. This review has explored some of the advantages and disadvantages of a few genetically modified mouse models of NAFLD that would be useful in understanding the connections between lipid metabolism, host defences, environmental triggers, genetic variability, inflammatory recruitment, and fibrogenesis. These models will also serve as important platforms for assessing therapeutic strategies, which is an essential area of study.

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## Trends on gastrointestinal bleeding and mortality: Where are we standing?

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### Abstract

Bleeding from the gastrointestinal tract and its management are associated with significant morbidity and mortality. The predisposing factors that led to the occurrence of these hemorrhagic instances are largely linked to the life style of the affected persons. Designing a new strategy aimed at educating the publics and improving their awareness of the problem could effectively help in eradicating this problem with no associated risks and in bringing the mortality rates down to almost zero.

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**Key words:** Gastrointestinal bleeding; Peptic ulcer; Esophageal varices; Helminthic infestation; Bowel cancer; Mortality; Morbidity; Predicting factors; Age; Sex

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### INTRODUCTION

Gastrointestinal (GI) bleeding involves any bleeding in the GI tract from the mouth, oesophagus, stomach, small intestines, large intestines, to the anus. The degree of bleeding can range from microscopic levels detected only by lab tests, to perceptible amounts of bleeding that can be seen in the stool or vomit. However, any level of bleeding can lead to serious problems. Microscopic levels of bleeding can lead to anaemia over time, and more massive amounts of bleeding can lead to death.

How to manage these instances more effectively and to prevent the happening of the co-incident adverse would be discussed by colleagues whom I invited to carry out this task. But in this review, I am going to assess how practically these haemorrhagic instances could be avoided.

### TRENDS ON GASTROINTESTINAL BLEEDING AND MORTALITY

Upper GI bleeding involves bleeding from the mouth to the duodenum (common causes of upper GI bleeding are listed in Table 1). But lower GI bleeding involves bleeding from the small intestines to the anus and can be caused by haemorrhoids, cancer, polyps and colitis, among other causes (Table 2). Upper GI bleeding has been estimated to account for up to 20 000 deaths annually in the United States (international records are not available). The overall incidence of acute upper GI haemorrhage has been estimated to be 50 to 100 per 100 000 persons per year. The trends of hospitalization for GI bleeding in the United States in 1998 and in 2006 have

**Table 1 Causes of acute upper gastrointestinal bleeding**

Common	Gastric ulcer Duodenal ulcer Esophageal varices Malory-Weiss tear
Less common	Gastric erosive/gastropathy Esophagitis Cameron lesions Dieulatory lesion Telangiectasias Portal hypertensive gastropathy Gastric antral vascular ectasia (watermelon stomach) Gastric varices Neoplasms
Rare	Esophageal ulcer Erosive duodenitis Aortoenteric fistula Hemobilia Pancreatic disease Crohn's disease

**Table 2 Causes of acute lower gastrointestinal bleeding**

Common	Colonic diverticula Angioectasia
Less common	Colonic neoplasms (including post polypectomy bleeding) Inflammatory bowel disease Colitis Ischemic Raditation Unspecified (infectious or non specific) Haemorrhoids Small bowel source Upper gastrointestinal source
Rare	Dieulatory lesion Colonic ulcerations Rectal varices

been summarised in Table 3.

And the hospitals' discharge rates of the admitted subjects for different causes of GI haemorrhage in 1998 and 2006 in the United States have also been listed (Table 4).

The incidence rate for upper GI bleeding appears to be, in general, decreasing (Table 3). This may be due to the prescription of proton pump inhibitors and the skilled efforts to eradicate *Helicobacter pylori* infections (Table 4). But, the risk of upper GI bleeding appears to be increasing in particular groups of patients, such as those with a history of oesophageal varices (Table 5).

Regarding bleeding from the lower GI tract, it appears that haemorrhage from rectum and anus and the incidences of diagnosis of occult blood in stool are increasing (Table 5).

When the total number of discharges for cases of GI bleeding was investigated per age of the discharged patient, it appeared that incidences of GI bleeding are increasing in certain subgroups. The incidences of GI haemorrhage was, for example, found increasing in those who were less than 20 years old (Table 3).

Oesophageal varices form less than 10% of the all causes of GI haemorrhages. However, patients with variceal haemorrhage have a mortality rate of at least 30% during their initial hospitalization, with a one year mortality rate approaches 60%<sup>[11]</sup>. Patients who have bled once from oesophageal varices have a 70% chance of rebleeding, and approximately one third of further bleeding episodes are fatal<sup>[2]</sup>. The risk of death is maximal during the first few days after the bleeding episode and decreases slowly over the first 6 wk. Oesophageal varices are present in approximately 40% of patients with cirrhosis and in as many as 60% of patients with cirrhosis and ascites<sup>[3]</sup>. In cirrhotic patients who do not have oesophageal varices at initial endoscopy, new varices will develop at a rate of approximately 5% per year. In patients with small varices at initial endoscopy, progression to large varices occurs at a rate of 10%-15% per year and is related chiefly to the degree of liver dysfunction<sup>[4]</sup>. On the other hand,

improvement in liver function in patients with alcoholic liver disease who abstain from alcohol is associated with a decreased risk, and sometimes even disappearance of the varices<sup>[5]</sup>. It has been estimated that up to 25% of the patients with newly diagnosed varices would bleed within two years<sup>[4]</sup>. The risk of bleeding in patients with varices less than 5 mm in diameter is 7% by two years, and in patients with varices greater than 5 mm in diameter is 30% by two years<sup>[4]</sup>. Mortality rates in the setting of surgical intervention for acute variceal bleeding are high<sup>[6]</sup>. Associated abnormalities in the renal<sup>[7]</sup>, pulmonary<sup>[7]</sup>, cardiovascular<sup>[8]</sup>, and immune systems in patients with oesophageal varices contribute to 20%-65% of mortality<sup>[9]</sup>. In Western countries, alcoholic and viral cirrhosis are the leading causes of portal hypertension and oesophageal varices. Thirty percent of patients with compensated cirrhosis and 60%-70% of patients with decompensate cirrhosis have gastroesophageal varices at presentation<sup>[9]</sup>. The *de novo* rate of development of oesophageal varices in patients with chronic liver diseases is approximately 8% per year for the first 2 years and 30% by the sixth year<sup>[9]</sup>. A recently published survey<sup>[10]</sup> on consumption of alcohol by teenagers in the North West of England revealed that almost 90% of the participant school children (aged 15 and 16) drink alcohol at least occasionally. Of those, 38.0% usually binge drink (5+ drinks in one session), 24.4% are frequent drinkers (drinking two or more times a week) and 49.8% drinks in public settings (such as bars, clubs, streets and parks). It is worth to note that excessive drinking by young people, for example, has seen a 20% rise in hospital admissions in England over the last five years. The number of people taken to Accident and Emergency with alcohol-related injuries has also doubled to 148 477 a year. Alcohol-related conditions such as liver disease have doubled in less than a decade, to 262 844 a year as well.

But in developing countries, hepatitis B is endemic in the Far East and Southeast Asia, particularly, as well as South America, North Africa, Egypt and other countries in the Middle East. Schistosomiasis is an important cause of portal hypertension in Egypt, Sudan and other African countries<sup>[8]</sup>. Those that have been affected with bilharsiasis, they almost have additional complications from hepa-

**Table 3 Trends of hospitalization for gastrointestinal bleeding in the United States in 1996 and 2006**

	Total number of discharges per 100 000 persons (principal diagnosis)			Total number of discharges per 100 000 persons (all diagnosis)		
	1998	2006	Percent changes (%)	1998	2006	Percent changes (%)
By bleeding site	189	182	-3.8	390	375	-3.7
Upper	96	82	-14	170	146	-14
Lower	43	44	+2	75	82	+8
Unspecified	50	56	+11	156	158	+1
By age (yr)						
< 20	7.4	7.5	+1.5	23	25	+8.6
20-29	25	23	-7	55	59	+6.1
30-44	65	59	-8.3	139	140	+0.6
45-64	187	181	-3.4	399	396	-0.9
65-84	859	806	-5.6	1731	1596	-7.8
> 85	2207	1871	-15.2	4257	4375	-18.4
By sex (%)						
Female	259 808 (51)	276 663 (51)				
Male	252 060 (49)	268 589 (49)				

**Table 4 Death rates for gastrointestinal bleeding inpatients**

	1998	2006	Percentage change (%)
Inpatient death number	20 013	16 344	-18
Inpatient death number/100 000	7	5	-26
Inpatient death rate (%)			
By bleeding site			
Upper	3.5	2.7	-23
Lower	3.5	2.9	-17
Unspecified	5	3.6	-28
By sex			
Male	4	3	-25
Female	3.8	3	-21
By age (yr)			
< 20	-	-	-
20-29	-	-	-
30-44	1.6	1.1	-31
45-64	2.7	2.2	-19
65-84	4.1	3	-27
> 85	6.4	5.2	-19

titis B and C<sup>[11-14]</sup>. The instances of acute lower GI bleeding are mainly self-limited and affected patients do not require hospitalization care, approximately 21 per 100 000 adults in the United States require hospitalization for severe lower GI bleeding every year<sup>[15]</sup> (international records are not available). The hospitalization rate for lower GI bleeding is approximately one third of that for upper GI bleeding<sup>[16]</sup> and in a survey by the American College of Gastroenterology, lower GI haemorrhage accounted for 24% of all GI bleeding occasions<sup>[17]</sup>. It has been estimated that detection of occult blood in stools formed 7% of all instances of GI bleeding in the United States in 2007. But it is expected that the incidence rates of detection of occult blood in the stools of patients in developing countries exceed this figure by many times. Helminthic infestation is a common cause for occult blood in stools in developing countries (Table 6). It has been estimated that 80% of the population of most countries in Asia, Africa and south America are infected with helminths, such as

Ascaris and widespread infection has been demonstrated throughout Europe, Particularly, Romania, Hungary, Portugal and Turkey<sup>[18]</sup>. When 312 children in the age group of 4-15 years were examined for different intestinal helminths in three schools located in rural areas in Kupwara, Kashmir, India<sup>[19]</sup>, 222 of 312 (71.15%) tested positive for various intestinal helminths<sup>[20]</sup>. The various helminth parasites included *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis* and *Taenia saginata*. The highest frequency of 69.23% (216/312) was noted for *Ascaris lumbricoides* followed by *Trichuris trichiura* 30.76% (96/312), *Enterobius vermicularis* 7.69% (24/312) and *Taenia saginata* 7.69% (24/312). Single infection was found in 33.65% (105/312) and mixed infection was seen in 37.5% (117/312) children. Again, Chandrasekhar MR and others in 2003<sup>[21]</sup> collected faecal samples from 1000 children below 6 years of age. Six hundred and eighty children (68.0%) were detected to have intestinal helminthic infection. The incidence of intestinal helminthiasis in urban group of children was 56.8% (284 out of 500 tested) while in rural group of children was 79.2% (396 out of 500 tested) both in rural and urban population *Ascaris lumbricoides* was the single predominant species, whereas a combination of *A. Lumbricoides* and *Trichuris trichiura* was common multiple infection. All cultures of faecal samples were positive for hook worm ova. In Pakistan, out of 200 children examined, 132 (66%) were found positive for various intestinal helminths infestation<sup>[22]</sup>. There were 6 different types of helminths found in the specimens examined.

It has become visible from the above review that the main causes for the occurrence of haemorrhage from the GI tract are strongly linked to the life style of the affected persons. Educating the public is thus expected to solve this problem. However, when the effect of health education in the control of bilharzias is assessed the results were disappointing. In 2001, Garba *et al*<sup>[23]</sup> carried out a survey on two groups of endemic villages in the Niger. In one group of villages, there were health educa-

**Table 5 Underlying conditions of gastrointestinal in 1998 and 2006 n (%)**

Underlying condition	1998	2006	Discharge percentage change (principal diagnosis)	1998	2006	Discharge percentage change (all diagnosis)
Upper GI: oesophageal varices, ulcer, perforation and other haemorrhages	23 007 (4)	35 058 (6)	52% and 38% after population adjustment	84 382 (8)	103 381 (9)	23% and 11% after population adjustment
Gastric, duodenal ulcers, gastrojejunal ulcers or perforation	156 29 (31)	131 225 (24)	-16% and -24% after population adjustment	215 912 (20)	179 032 (16)	-17% and -25% after population adjustment
Gastritis or duodenitis and haemorrhage	54 310 (11)	44 104 (8)	-19% and -27% after population adjustment	118 333 (11)	90 635 (8)	-23% and -31% after population adjustment
Angiodysplasia of stomach and duodenum with haemorrhage	9237 (2)	14 679 (3)	59% and 43% after population adjustment	15 061 (1)	23 032 (2)	53% and 38% after population adjustment
Haematemesis	16 466 (3)	21 230 (4)	29% and 16% after population adjustment	58 955 (6)	72 655 (6)	23% and 11% after population adjustment
Perforation of the large intestine	9117 (2)	10 066 (2)	10% and -0.3% after population adjustment	26 200 (2)	33 246 (3)	27% and 15% after population adjustment
Haemorrhage of rectum and anus	12 084 (2)	21 456 (4)	78% and 60% after population adjustment	52 974 (5)	85 592 (7)	56% and 41% after population adjustment
Diverticulosis and diverticulitis of the colon and haemorrhage	80 007 (16)	83 927 (15)	5% and -5% after population adjustment	101 000 (10)	104 516 (9)	3% and -7% after population adjustment
Diverticulosis and diverticulitis of the small intestine and haemorrhage	15 369 (3)	16 259 (3)	6% and -5% after population adjustment	26 933 (3)	27 433 (2)	2% and -8% after population adjustment
Unspecified GI bleeding (blood in stool)	31 044 (6)	38 284 (7)	23% and 11% after population adjustment	283 440 (27)	325 035 (29)	15% and 4% after population adjustment
Haemorrhage of GI tract (unspecified)	104 991 (21)	129 164 (24)	23% and 11% after population adjustment	283 440 (27)	325 035 (29)	15% and 4% after population adjustment

GI: Gastrointestinal.

**Table 6 Causes of occult gastrointestinal bleeding**

Mass lesions	Carcinoma (any site) Large > 1.5 cm adenoma (any site)
Inflammatory lesions	Erosive oesophagitis ulcer (any site) Cameron lesion Erosive gastropathy Celiac sprue Ulcerative colitis Crohn's disease Non specific colitis Caecal ulcer
Vascular lesions	Angiodysplasia (any site) Portal hypertensive gastropathy (colonopathy) Gastric antral vascular ectasia Hemangioma Dieulatory lesion
Infection	Hockworm Whipworm Stroyloidosis Ascariosis Tuberculous enterocolitis Amoebiasis Cytomegalo virus

tion campaigns but there were no education campaigns in the second group. The people in the targeted areas received information on Bilharziasis and on how to fight against it. However, 46.6% of interviewed people in the project area couldn't mention any means for controlling bilharziasis. Behaviours that favour the illness were ignored by 1/3 of interrogated people in the project area. Yet, there was an increase in knowledge about the illness in the program zone in comparison with the control area. Despite this increase in knowledge level, changes in behaviour in relation to the illness remained low. Risky be-

haviour continued in about 2/3 of interrogated people. Only 33% of persons of the project area declared having adopted at least a single good behaviour. This means that changes of behaviour may take time to have effect.

In addition, areas endemic for helminthic infestation worldwide suffer from poor economic growth, poor sanitation and lack of appropriate toilet facilities.

Multiple studies demonstrated that in endemic areas re-infection is exceedingly common and mass chemotherapy alone is insufficient to prevent the spread of these diseases<sup>[24]</sup>.

Relatively little attention has been focused on the impact of personal attitude on the development of haemorrhagic episodes from the GI tract. This review emphasizes on the importance of designing a new strategy aimed at preventing the happening of these episodes.

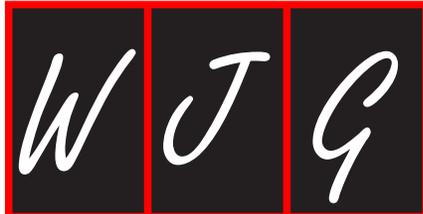
The occurrence of GI bleeding and its management are associated with significant harm; however, educating the publics through properly designed long term program and improving the general surrounding conditions are free from risk.

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## Management of non-variceal upper gastrointestinal tract hemorrhage: Controversies and areas of uncertainty

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### Abstract

Upper gastrointestinal tract hemorrhage (UGIH) remains a common presentation requiring urgent evaluation and treatment. Accurate assessment, appropriate intervention and apt clinical skills are needed for proper management from time of presentation to discharge. The advent of pharmacologic acid suppression, endoscopic hemostatic techniques, and recognition of *Helicobacter pylori* as an etiologic agent in peptic ulcer disease (PUD) has revolutionized the treatment of UGIH. Despite this, acute UGIH still carries considerable rates of morbidity and mortality. This review aims to discuss current areas of uncertainty and controversy in the management of UGIH. Neoadjuvant proton pump inhibitor (PPI) therapy has become standard empiric treatment for UGIH given that PUD is the leading cause of non-variceal UGIH, and PPIs are extremely effective at promoting ulcer healing. However, neoadjuvant PPI administration has not been shown to affect hard clinical outcomes such as rebleeding or mortality. The optimal timing of upper endoscopy in UGIH is often debated. Upon completion of volume resuscitation and hemodynamic stabilization, upper endoscopy should be performed within 24 h in all patients

with evidence of UGIH for both diagnostic and therapeutic purposes. With rising healthcare cost paramount in today's medical landscape, the ability to appropriately triage UGIH patients is of increasing value. Upper endoscopy in conjunction with the clinical scenario allows for accurate decision making concerning early discharge home in low-risk lesions or admission for further monitoring and treatment in higher-risk lesions. Concomitant pharmacotherapy with non-steroidal anti-inflammatory drugs (NSAIDs) and antiplatelet agents, such as clopidogrel, has a major impact on the etiology, severity, and potential treatment of UGIH. Long-term PPI use in patients taking chronic NSAIDs or clopidogrel is discussed thoroughly in this review.

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**Key words:** Hemorrhage; Proton pump inhibitors; *Helicobacter pylori*; Prokinetic agents; Hemostasis; Thienopyridines

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### INTRODUCTION

Fundamental changes have occurred over the past several decades in the management of upper gastrointestinal tract hemorrhage (UGIH). Pharmacologic gastric acid suppression, recognition of *Helicobacter pylori* (*H. pylori*) as a causative agent in peptic ulcer disease, and the wide-

**Table 1** Sensitivity, specificity, positive predictive value and negative predictive value for death and rebleeding using 6 common upper gastrointestinal tract hemorrhage scoring systems

Scoring system	Death	Rebleeding
Pre-endoscopy Rockall risk score		
Sensitivity	100 (43.8-100)	69.6 (49.1-84.4)
Specificity	18.5 (14.8-22.9)	17.5 (13.8-22.0)
PPV	1.0 (0.4-3)	5.5 (3.4-8.8)
NPV	100 (94.4-100)	89.2 (79.4-94.7)
Post-endoscopy Rockall risk score		
Sensitivity	33.3 (6.1-79.2)	87.0 (67.9-95.5)
Specificity	29.6 (25.1-34.6)	31.1 (26.4-36.3)
PPV	0.4 (0.1-2.2)	8.1 (5.3-12.1)
NPV	98.1 (93.4-99.5)	97.2 (92.0-99.0)
Blatchford scoring system		
Sensitivity	100 (83.89-100)	94.29 (81.40-98.42)
Specificity	1.83 (0.71-4.61)	0.98 (0.27-3.50)
PPV	8.51 (5.58-12.79)	14.04 (10.17-19.06)
NPV	100 (51.01-100)	50.00 (15.00-85.00)
Forest classification		
Sensitivity	85.00 (63.96-94.76)	71.43 (54.95-83.67)
Specificity	50.23 (43.66-56.79)	50.49 (43.68-57.28)
PPV	13.49 (8.6-20.54)	19.84 (13.81-27.65)
NPV	97.35 (92.49-99.10)	91.15 (84.77-95.12)
Cedars-Sinai Medical Center predictive index		
Sensitivity	95.00 (76.39-99.11)	80.00 (64.11-89.96)
Specificity	41.55 (35.22-48.17)	41.67 (35.12-48.53)
PPV	12.93 (8.44-19.31)	19.05 (13.52-26.15)
NPV	98.91 (94.09-99.81)	92.39 (85.12-96.26)
Baylor College scoring system		
Sensitivity	87.50 (52.91-97.76)	30.77 (12.68-57.63)
Specificity	58.49 (45.09-76.74)	47.92 (34.47-61.67)
PPV	24.14 (12.22-42.11)	13.79 (5.50-30.56)
NPV	96.88 (84.26-99.45)	71.88 (54.63-84.44)

95% confidence intervals are recorded in parentheses. PPV: Positive predictive value; NPV: Negative predictive value.

spread dissemination of flexible endoscopy and endoscopic hemostatic techniques have contributed to a paradigm shift in the treatment of complicated peptic ulcer disease, in particular, from predominantly surgical to predominantly endoscopic management. At the same time, an increasing proportion of patients presenting with UGIH are older or elderly patients<sup>[1]</sup>, and a significant number of patients with UGIH consume non-steroidal anti-inflammatory drugs (NSAIDs) and/or antiplatelet therapy to treat other medical comorbidities. Given the confluence of these factors, UGIH continues to have a considerable impact with respect to patient morbidity and mortality, as well as health care resource utilization. The annual incidence of UGIH has been estimated as low as 48 and as high as 165 cases per 100 000 population, and the mortality rate remains high somewhere between 7% and 14%<sup>[2-6]</sup>. UGIH accounts for > 300 000 annual hospitalizations in the United States, with an estimated cost of \$2.5 billion<sup>[3,5]</sup>. This article aims to review the management of UGIH, with an emphasis on discussion of areas of controversy or uncertainty in current practice.

## INITIAL ASSESSMENT

By definition, UGIH originates proximal to the ligament of Treitz. UGIH accounts for the preponderance of all gastrointestinal hemorrhage; estimated at 4-6 times more common than lower gastrointestinal (GI) hemorrhage<sup>[7]</sup>. Initial assessment of the pace and acuity of the bleeding episode has major bearing on the initial management of UGIH. For patients presenting with UGIH and evidence of hemodynamic compromise, the fundamental goal of initial management is adequate and appropriate volume resuscitation. Additional stratification of patients into high- and low-risk categories may be based on clinical and endoscopic criteria<sup>[8]</sup>. Predictors of poor prognosis include: age > 65 years, shock, poor overall health, comorbid conditions, low initial hemoglobin/hematocrit, active bleeding (red blood per rectum or hematemesis), sepsis, and elevated creatinine or serum transaminases<sup>[2,8,9]</sup>. Several scoring systems have been created and/or validated for this purpose, including APACHE II, Forrest Classification, Blatchford, pre-endoscopic Rockall, Baylor College, Cedars-Sinai Medical Center and Rockall indexes (Table 1 compares 6 commonly used scoring systems)<sup>[10,11]</sup>. Some of these may be cumbersome (APACHE II) or require data not immediately available based on initial clinical assessment (the Rockall Scoring System, for instance, requires endoscopic data) and therefore may be of limited utility in the acute setting<sup>[12]</sup>.

The role of nasogastric tube placement and aspirate inspection in the initial assessment of UGIH has fallen out of favor in many emergency room and acute care settings. In theory, the presence of bright red blood *via* nasogastric aspirate suggests active UGIH and should prompt urgent esophagogastroduodenoscopy (EGD)<sup>[3]</sup>. The absence of blood on nasogastric aspirate, however, does not exclude the presence of a culprit UGIH source. In a study by Aljebreen *et al*<sup>[13]</sup>, 15% of patients with UGIH and clear or bilious nasogastric aspirate were ultimately found to have an underlying high risk lesion during EGD.

## PHARMACOLOGIC THERAPY PRIOR TO ENDOSCOPY

Pharmacologic gastric acid suppression has changed the face of peptic ulcer disease (PUD) treatment, both by favoring hemostasis in the short term (platelet aggregation and clot formation are impaired at acidic gastric pH<sup>[14]</sup>), and enabling ulcer healing and remission over the longer term<sup>[3]</sup>. Proton pump inhibitors (PPIs) covalently bind to and inhibit the H-K ATPase pump of the gastric parietal cells, thus inhibiting the final common pathway of acid secretion. PPIs offer more durable and sustained acid suppression than histamine receptor antagonists, which are prone to tachyphylaxis<sup>[15]</sup>. For these reasons, PPIs have become the dominant acid suppressive therapy used in the treatment of UGIH.

An emerging area of importance is the optimal dose, route of administration, and timing of PPI therapy in patients presenting with UGIH. Administration of neoadjuvant PPI, prior to diagnostic endoscopy or endoscopic therapy, has become widespread, and this practice recommendation has been supported by consensus guidelines<sup>[2]</sup>. This approach may be particularly reasonable in instances when access to prompt EGD and/or availability of endoscopic therapeutic expertise is limited. Otherwise, however, it is uncertain whether neoadjuvant PPIs confer benefit with respect to meaningful clinical outcomes. In a controlled prospective study of patients with UGIH randomized to intravenous PPI *vs* placebo prior to endoscopy<sup>[16]</sup>, patients in the PPI arm were less likely to have active bleeding at the time of endoscopy and to require endoscopic hemostatic therapy. There was no difference in transfusion requirements, rates of rebleeding, requirement for surgery, or 30-d mortality when comparing patients receiving PPIs to those receiving placebo. A systematic meta-analysis, based on published controlled data in the medical literature, reported no benefit of neoadjuvant (pre-endoscopic) PPI therapy on rates of rebleeding, salvage surgery for failures of endoscopic hemostasis, or mortality<sup>[17]</sup>.

Adjuvant PPI therapy, administered following diagnostic and/or therapeutic EGD, has been proven effective, as well, leading to a decrease in recurrent PUD bleeding, need for blood transfusion, need for surgery, and duration of hospital stay<sup>[3,18,19]</sup>. Despite these major impacts, studies have not demonstrated an impact of PPIs on mortality due to UGIH<sup>[3,20,21]</sup>. Furthermore, whether in the neoadjuvant or adjuvant setting, the optimal dosing, route of administration, and duration of post-endoscopy PPI has not been clearly established. Current guidelines recommend the use of high-dose intravenous PPI therapy for 3 d following successful endoscopic hemostasis<sup>[2]</sup>. In many studies, high-dose PPI therapy is defined as an initial bolus (omeprazole 80 mg) followed by continuous infusion (omeprazole 8 mg/h) for up to 72 h. However, there has been limited direct comparison of this high-dose intravenous regimen in comparison to alternative regimens. Whether continuous intravenous infusion of PPIs is clearly superior to intermittent bolus dose is uncertain; similarly, whether intravenous PPIs are clearly superior to highly bioavailable oral PPIs in patients able to take oral medications is uncertain.

## TIMING OF ENDOSCOPIC EVALUATION

Endoscopic evaluation is an essential part of UGIH management. Urgent EGD has been proposed as the standard of care in patients with high-risk lesions, although the precise timing of urgent EGD has been variably defined. American Society for Gastrointestinal Endoscopy practice guidelines for the treatment of non-variceal UGIH suggest that early endoscopy (within 24 h) maximizes the impact on hospital length of stay and transfusion requirements, yet do not make formal recommendations regarding the optimal time for performing EGD within this 24-h window<sup>[22]</sup>.

In theory, the availability both of on-call physicians proficient in endoscopic hemostasis and on-call support staff with technical expertise in usage of endoscopic devices enable performance of EGD on a 24-h/7-d basis. Several studies have examined the timing of upper endoscopy and resultant impact on both patient outcomes and resource utilization. In a study by Sarin *et al*<sup>[23]</sup>, a retrospective review of > 500 patients who underwent upper endoscopy for non-variceal UGIH, the timing of endoscopy was stratified into three categories: < 6 h, 6-24 h and > 24 h. There was no significant difference in mortality or need for surgery between the < 6 h and 6-24 h groups. However, there was a difference between the two < 24 h groups and the > 24 h group. These findings were supported by a more recent retrospective review in 2007 that examined 169 patients with acute non-variceal UGIH with either systolic blood pressure < 100 mmHg or heart rate > 100 beats/min on presentation. Patients were divided into two groups: those who received endoscopy within 6 h *vs* 6-24 h. Again, there were no significant differences between the groups in any of the primary outcomes, including rebleeding, need for surgery, in-hospital mortality, or hospital readmission within 30 d<sup>[24]</sup>.

In a variation on this theme, Dorn *et al*<sup>[25]</sup> have examined the difference in clinical outcomes among patients admitted for UGIH on either a weekday or a weekend. Those admitted on a weekend had a slight but significant increase in mortality (hazard ratio of 1.09), hospital length of stay, and hospitalization cost. Although the weekend patients waited longer for EGD than their weekday counterparts, the entire effect on patient outcomes could not be accounted for by the delay in endoscopy timing alone. Further study is needed to elicit other potential causes of this weekend effect. Our practice is to perform EGD as soon as is feasible following hemodynamic resuscitation and stabilization of the patient, and within 12 h of clinical presentation.

In addition to offering hemostatic techniques to prevent continued or recurrent bleeding, a major benefit of endoscopy is the ability to risk stratify the lesion and triage patients to those who require inpatient or more intensive monitoring, *vs* those who are candidates for early hospital discharge. With respect to peptic ulcer hemorrhage, lesions at high risk for recurrent bleeding include ulcers > 2 cm, or ulcers with active bleeding, a visible vessel, or adherent clot. Such lesions warrant both endoscopic therapy, when feasible, and inpatient monitoring post-endoscopy. Conversely, clean-based ulcers or ulcers with flat pigmented spots are considered low risk and do not require endoscopic therapy<sup>[26]</sup>, and patients found to have such low-risk lesions may be candidates for early discharge, even from an emergency room setting.

Much improvement in current management of UGIH and resource allocation for treatment of UGIH could be achieved in this area. Numerous studies have shown that early endoscopy is not often followed by early discharge of appropriate low-risk patients. Bjorkman *et al*<sup>[27]</sup> have shown that EGD within 6 h of presentation to the emergency department versus 48 h did not change hospital utilization of resources, the study's primary endpoint. In

**Table 2** Areas requiring further investigation

Pharmacologic therapy prior to endoscopy	Determine optimal route and dosage of PPI in UGIH (continuous infusion <i>vs</i> intermittent IV bolus <i>vs</i> oral dosing)
Timing of endoscopic evaluation	Defining optimal timing of initial endoscopy, implementation of early discharge in low risk patients
Prokinetic agents as endoscopic adjuncts	Clearly define the role for prokinetics in UGIH with randomized controlled trials, specifically define the optimal agent, dose and timing prior to endoscopy
Long-term PPI management	Clarification of potential long-term sequelae of PPI including: osteoporosis, <i>c. difficile</i> infection and community-acquired pneumonia
PPI and thienopyridines	Consensus on the clinical importance of this interaction, a complete randomized controlled trial to support the truncated COGENT trial data
<i>H. pylori</i> and UGIH	<i>H. pylori</i> testing that allows accurate test results in the setting of acute UGIH

PPI: Proton pump inhibitor; *H. pylori*: *Helicobacter pylori*; UGIH: Upper gastrointestinal tract hemorrhage; IV: Intravenous; COGENT: Clopidogrel and the optimization of gastrointestinal events trial.

a recently published study, Chaparro *et al*<sup>[28]</sup> have formulated an early discharge algorithm using retrospective data and then examined this algorithm in a prospective cohort. Even at their institution using their algorithm, only 13/29 (45%) low-risk patients were discharged early. In view of these data, there is much room for improvement in clinical practice surrounding implementation of practice guidelines concerning early hospital discharge of low-risk UGIH patients and achieving the potential health care cost savings. Table 2 illustrates other areas in need of further investigation and improved recommendations.

## CHOICE OF ENDOSCOPIC HEMOSTATIC TECHNIQUE

Endoscopic therapies employed in the treatment of UGIH include: (1) pharmacologic therapies, including injection of epinephrine, sclerosants and even normal saline; (2) coagulation therapies, including monopolar or bipolar cautery and argon plasma coagulation; and (3) mechanical tamponade, including hemoclips and bands. Each of these therapies has demonstrated efficacy in different clinical settings. Often, the choice of which endoscopic therapy to employ is a clinical judgment based on provider preference and expertise.

Available data suggest that epinephrine injection plus a second endoscopic intervention is superior to epinephrine injection alone. Calvet *et al*<sup>[29]</sup> completed a systematic review and meta-analysis in 2004 which included 16 studies and > 1600 patients with UGIH secondary to PUD, and who underwent endoscopic therapy with epinephrine alone or epinephrine plus a second hemostatic modality. Adding an adjunct therapy reduced the rebleeding rate from 18.4% to 10.6% [odds ratio (OR): 0.53, 95% CI: 0.40-0.69], reduced the need for emergency surgery from 11.3% to 7.6% (OR: 0.64, 95% CI: 0.46-0.90), and reduced mortality from 5.1% to 2.6% (OR: 0.51, 95% CI: 0.31-0.84). Vergara *et al*<sup>[30]</sup> have confirmed these findings in a recent Cochrane review (2008). Rebleeding, need for surgical intervention, and mortality were all lower in groups receiving dual therapy. Additionally, there was no increased risk of significant complications or adverse events when dual therapy was used. In the above studies

and reviews, endoscopic therapy is usually reserved for high-risk lesions (active bleeding and visible vessels). Jensen *et al*<sup>[31]</sup> have additionally defined a role for removal of adherent clots in the treatment of PUD bleeding. In one study, removal of adherent clot and treatment with a second endoscopic intervention resulted in lower rebleeding rates when compared to not removing the adherent clot (35% *vs* 0%).

Even when endoscopy fails to provide durable and definitive hemostasis, endoscopy may provide a beneficial role in localization of the bleeding lesion to target salvage non-endoscopic intervention. Repeat endoscopy can assist both interventional radiology and/or surgery in the event that bleeding continues or recurs and alternative management is necessitated. Second-look endoscopy has also been shown to improve rebleeding rates in certain clinical situations, but without improvement in the need for surgery or mortality<sup>[3]</sup>. Current guidelines do not support routine use of second-look endoscopy.

## PROKINETIC AGENTS AS ENDOSCOPIC ADJUNCTS

The presence of retained blood in the UGI tract can limit the ability to identify definitively a bleeding source and/or deliver endoscopic hemostatic therapy. Prokinetic agents may promote UGI tract motility and facilitate gastric emptying of retained blood prior to endoscopy; however, the use of such agents may be highly physician dependent. American Society for Gastrointestinal Endoscopy guidelines<sup>[22]</sup> indicate that the use of erythromycin, when administered intravenously prior to EGD, may improve mucosal visibility. A recent meta-analysis published in *Gastrointestinal Endoscopy*<sup>[32]</sup> has shown that either erythromycin or metoclopramide given prior to endoscopy significantly reduced the need for repeat endoscopy to identify the bleeding source. However, use of a prokinetic agent led to no identifiable impact on total units of blood transfused, hospital stay, or need for surgical intervention<sup>[33]</sup>. As was germane to the discussion of pre-endoscopic PPI therapy, if there is a role of prokinetic agents in the endoscopic diagnosis and management of UGIH, the optimal agent,

dose and timing prior to endoscopy have not been defined. Metoclopramide has been assigned a “black box warning” by the United States Food and Drug Administration (FDA) due to risk of neurologic side effects, and caution should therefore be advised with use of this agent.

## LONG-TERM PPI MANAGEMENT

PPIs are recommended for 6-8 wk following UGIH and/or endoscopic treatment of PUD to allow for full mucosal healing. Several studies have shown a therapeutic benefit of PPIs in patients using NSAIDs chronically and/or patients with confirmed *H. pylori* infection<sup>[34,35]</sup>. After initial mucosal healing has been achieved, is there a benefit to long-term PPI use for secondary prophylaxis? Studies have shown that in patients who have PUD complicated by bleeding, there is a 33% risk of rebleeding in 1-2 years. Furthermore, there is a 40%-50% rebleeding risk over the subsequent 10 years following the initial episode of bleeding<sup>[13]</sup>. Randomized prospective trials have demonstrated a benefit to long-term acid-suppression therapy in two settings: chronic NSAID users and *H. pylori*-infected patients. As demonstrated in a 2001 *New England Journal of Medicine* article by Chan *et al*<sup>[34]</sup>, in patients taking NSAIDs other than acetylsalicylic acid (ASA) who were concomitantly infected with *H. pylori*, omeprazole provided added protection above bacterial eradication alone.

Although beneficial in both treatment and prevention of UGIH, PPI therapy is not without potential risks. Chronic PPI therapy has been associated with *Clostridium difficile* infection, community-acquired pneumonia, and calcium malabsorption resulting in osteoporosis and increased fracture risk<sup>[36]</sup>. These associations have originated largely from observational studies and no strong data exist to link PPI therapy directly as a causative factor in any of these outcomes. However, given these potential sequelae, long-term PPI therapy should only be used when justified by a clear medical indication<sup>[2]</sup>.

## PPIS AND THIENOPYRIDINES

Much recent attention has surrounded the concomitant use of PPIs and thienopyridines, particularly clopidogrel. This has included an FDA warning regarding combined use of these medications<sup>[36]</sup>. *In vitro* studies have suggested that omeprazole, which is metabolized predominately through the CYP2C19 isoenzyme of cytochrome P450, inhibits conversion of clopidogrel, a prodrug, to its active form, thus reducing its therapeutic margin<sup>[37]</sup>. Most of the original data were specific to omeprazole and subsequent experiments using pantoprazole, lansoprazole and esomeprazole have not shown an equal class effect<sup>[3,37,38]</sup>. Additionally, although a measurable change in platelet function occurred in patients concomitantly taking PPIs and clopidogrel, the data were mixed with regards to clinically significant cardiovascular outcomes.

Subsequent retrospective reviews and observational studies have attempted to define the true impact of PPIs on the therapeutic index of thienopyridines, but with mixed results. The French Omeprazole CLopidogrel As-

pirin study measured vasodilator-stimulated phosphoprotein phosphorylation; a measure of the inhibitory effect of clopidogrel on platelets *in vivo*. Patients taking both 75 mg ASA and 75 mg clopidogrel daily were randomized to either 20 mg omeprazole or placebo daily. One hundred and twenty-four patients were included and the results showed an 11.6% decrease in platelet inhibition by clopidogrel among those randomized to omeprazole, but no clinical outcomes were measured<sup>[38]</sup>. The TIMI Study Group at Brigham and Women’s Hospital in Boston have attempted to clarify the link between *in vitro* interactions, measures of platelet function, and clinical outcomes in patients on either of the thienopyridines and PPIs through the analysis of two trials: prasugrel in comparison to clopidogrel for inhibition of platelet activation and aggregation-TIMI 44, the primary endpoint of which was inhibition of platelet aggregation at 6 h, and trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel-TIMI 38, with a primary composite endpoint of cardiovascular death, myocardial infarction or stroke. The first study demonstrated a reduction in the inhibitory effect of thienopyridines on platelet aggregation in patients taking PPIs after loading doses of either clopidogrel (12% decrease) or prasugrel (7.1% decrease). However, in the second trial, > 13 000 patients with acute coronary syndrome were randomized to prasugrel or clopidogrel, of which 33% of 4500 patients were on PPIs at randomization. PPI was continued without change in dosing or frequency. There was no association between PPI use and the primary endpoint, a composite cardiovascular event<sup>[39]</sup>. Additionally, when the data were assessed by specific PPI (omeprazole, lansoprazole, pantoprazole or esomeprazole), there was no correlation.

Most recently, the only prospective randomized controlled trial to address this important clinical question, the Clopidogrel and the optimization of gastrointestinal events trial (COGENT) Trial, was published in the *New England Journal of Medicine*. COGENT randomized patients with an indication for dual antiplatelet therapy to clopidogrel 75 mg plus omeprazole 20 mg daily or clopidogrel alone. In addition, every patient was given either 81 or 325 mg enteric coated aspirin. The primary cardiovascular endpoint was a composite of death from cardiovascular causes, non-fatal myocardial infarction, revascularization, or stroke. Over 3700 patients were included in the final analysis of events at 180 d after randomization. There were 109 cardiovascular events; 4.9% in the clopidogrel plus omeprazole arm, and 5.7% in the clopidogrel plus placebo arm (HR: 0.99; 95% CI: 0.68-1.44). The primary gastrointestinal endpoint was a composite of overt or occult bleeding, symptomatic gastroduodenal ulcers or erosions, obstruction or perforation. There were 51 gastrointestinal events; 1.1% in the clopidogrel plus omeprazole group, and 2.9% in the clopidogrel plus placebo arm (HR: 0.13; 95% CI: 0.03-0.56)<sup>[40]</sup>. These data suggest no association between omeprazole and clinically meaningful adverse cardiovascular outcomes when used in conjunction with clopidogrel. However, there was a significant reduction in adverse gastrointestinal events in patients receiving omeprazole. A significant limitation of this study

was premature study termination due to loss of funding, thereby attenuating the statistical power of the study.

The ambiguity of this matter is further demonstrated in the following consensus statements. The American College of Cardiology Foundation/American College of Gastroenterology (ACG)/American Heart Association (AHA) joint guidelines released in 2008 state, "In the interest of patient safety, the AHA/ACC and the ACG advise that patients who are currently taking these medications should not change their medication regimen unless advised by their healthcare provider". A revised joint statement was released in December 2010 stating, "Pharmacokinetic and pharmacodynamic studies, using platelet assays as surrogate endpoints, suggest that concomitant use of clopidogrel and a PPI reduces the antiplatelet effects of clopidogrel. The strongest evidence for an interaction is between omeprazole and clopidogrel. It is not established that changes in these surrogate endpoints translate into clinically meaningful differences. Observational studies and a single randomized clinical trial have shown inconsistent effects on (cardiovascular) outcomes of concomitant use of thienopyridines and PPIs. A clinically important interaction cannot be excluded, particularly in certain subgroups, such as poor metabolizers of clopidogrel. The roles of either pharmacogenomic testing or platelet function testing in managing therapy with thienopyridines and PPIs has not yet been established"<sup>[41]</sup>.

## NON-ACID DIRECTED PHARMACOLOGIC THERAPY

The role of *H. pylori* in PUD has been clearly elucidated over the years, beginning with Marshall and Warren's pioneering work. Since that time, many studies have demonstrated the beneficial effects of *H. pylori* eradication in the treatment of PUD. In a 1997 *Lancet* study, Chan *et al.*<sup>[34]</sup> reported that clearance of *H. pylori* in patients taking naproxen for 8 wk, irrespective of PPI use, significantly reduced the presence of PUD at time of repeat endoscopy: 26% *vs* 7%. Current guidelines support the test and treatment mantra for *H. pylori* in the setting of UGIH<sup>[2]</sup>. However, the appropriate timing of *H. pylori* testing is unclear given the potential for false-negative test results in the setting of UGIH and false negative results due to PPI use. In a 2001 study by Griño *et al.*<sup>[42]</sup>, 78 patients with endoscopically documented UGIH secondary to PUD underwent diagnostic testing for *H. pylori* in the acute setting by one of four modalities: histology, rapid urease test, urea breath test and serology. The sensitivity/specificity for each respective diagnostic test was as follows: 48.5/100 for the rapid urease test, 91/77.8 for the breath test, 89.5/80 for serology and 86.3/100 for histology. Additional support for questioning immediate *H. Pylori* test results was provided by Guell *et al.*<sup>[43]</sup>, who have reported a 79% false-negative rate with rapid urease testing and a maximum sensitivity of only 86% if both a rapid urease test and histological examination were performed on each biopsy specimen. Based on these data, a recommendation to retest at a later date all patients with negative immediate *H. pylori* test in the setting of acute UGIH may be reasonable<sup>[42,44]</sup>.

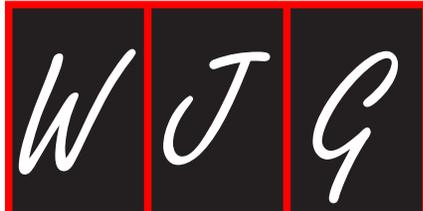
## CONCLUSION

UGIH requires early and accurate assessment, triage and resuscitation, in addition to well-coordinated care between generalist and sub-specialist to maximize patient outcomes. All patients with UGIH need upper endoscopy within 24 h of presentation to characterize further and potentially treat the bleeding source. Epinephrine plus a second modality of endoscopic therapy has proven superior to epinephrine alone. *H. Pylori* infection should be confirmed and treated when present. Negative *H. pylori* test in the acute setting should be followed by repeat testing to avoid false-negative results and minimize the risk of recurrent bleeding. Appropriate duration of PPI therapy is of critical importance to allow mucosal healing and to prevent rebleeding in the high-risk patient. Current data do not support a meaningful clinical interaction between PPIs and thienopyridines. Future clinical and research attention to the issues addressed in this review may serve to resolve current areas of uncertainty and controversy, and optimize clinical outcomes for patients presenting with UGIH.

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## Treatment of portal hypertension

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### Abstract

Portal hypertension is the main complication of cirrhosis and is defined as an hepatic venous pressure gradient (HVPG) of more than 5 mmHg. Clinically significant portal hypertension is defined as HVPG of 10 mmHg or more. Development of gastroesophageal varices and variceal hemorrhage are the most direct consequence of portal hypertension. Over the last decades significant advancements in the field have led to standard treatment options. These clinical recommendations have evolved mostly as a result of randomized controlled trials and consensus conferences among experts where existing evidence has been reviewed and future goals for research and practice guidelines have been proposed. Management of varices/variceal hemorrhage is based on the clinical stage of portal hypertension. No specific treatment has shown to prevent the formation of varices. Prevention of first variceal hemorrhage depends on the size/characteristics of varices. In patients with small varices and high risk of bleeding, non-selective  $\beta$ -blockers are recommended, while patients with medium/large varices can be treated with either  $\beta$ -blockers or esophageal band ligation. Standard of

care for acute variceal hemorrhage consists of vasoactive drugs, endoscopic band ligation and antibiotics prophylaxis. Transjugular intrahepatic portosystemic shunt (TIPS) is reserved for those who fail standard of care or for patients who are likely to fail ("early TIPS"). Prevention of recurrent variceal hemorrhage consists of the combination of  $\beta$ -blockers and endoscopic band ligation.

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**Key words:** Cirrhosis; Portal hypertension; Varices; Variceal hemorrhage; Primary prophylaxis; Secondary prophylaxis

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### INTRODUCTION

Portal hypertension is the increase in porto-systemic pressure gradient in any portion of the portal venous system. Although portal hypertension could result from pre-hepatic abnormalities (e.g., portal or splenic vein thrombosis), post-hepatic abnormalities (e.g., Budd-Chiari syndrome) or intrahepatic non-cirrhotic causes (e.g., schistosomiasis, sinusoidal obstruction syndrome), cirrhosis is by far the most common cause of portal hypertension and, as such, has been the most widely investigated. In cirrhosis, the portosystemic gradient is assessed by measuring the wedged hepatic venous pressure (a measure of sinusoidal hepatic pressure) and subtracting the free hepatic venous pressure (systemic pressure) thus obtaining the hepatic

venous pressure gradient (HVPG). A normal HVPG is 3-5 mmHg. An HVPG above 5 mmHg defines portal hypertension, however an HVPG of 10 mmHg or greater defines clinically significant portal hypertension as this pressure gradient predicts clinical course in patients with cirrhosis including development of varices<sup>[1]</sup>, clinical decompensation (i.e., development of ascites, variceal hemorrhage and encephalopathy)<sup>[2]</sup>, decompensation or death after liver resection<sup>[3]</sup>, and hepatocellular carcinoma<sup>[4]</sup>.

The complications that most directly result from portal hypertension are the development of varices and variceal hemorrhage. This review summarizes the current standard management for varices and variceal hemorrhage in the context of cirrhotic portal hypertension.

Over the last decades, research on animal models and clinical trials have evolved and have led to our current management recommendations. The field has moved forward in large part through consensus conference among experts where events and endpoints have been defined and the existing evidence has been carefully reviewed leading to practice recommendations. The first such conference took place in 1986 in Groningen, the Netherlands and since then consensus conferences have been alternating between Europe (Baveno conference) and the United States [American association for the study of liver diseases (AASLD) or AASLD single topic conference (STC)], and are briefly summarized below (Table 1).

## HISTORY OF CONSENSUS CONFERENCES ON PORTAL HYPERTENSION

Baveno is a small town in Northern Italy located on the west shore of Lake Maggiore. It has become the epicenter of the portal hypertension consensus workshops aimed to reach a consensus on the definitions of key events related to portal hypertension and variceal bleeding and to provide guidelines for future research as well as reviewing the evidence, eventually leading to clinical practice guidelines. The first Baveno consensus workshop was held in April 1990<sup>[5]</sup> in which significant advances in diagnosis and management of varices and variceal bleeding including vasoactive drugs and endoscopic sclerotherapy were reviewed. In addition to defining certain terms including size of varices, clinically significant bleeding and rebleeding; this workshop also provided recommendations on diagnostic modalities, imaging and directions for future clinical trials. The therapeutic recommendations included  $\beta$ -blockers for primary prophylaxis of large varices, sclerotherapy and vasoactive drugs for acute hemorrhage and endoscopic sclerotherapy,  $\beta$ -blockers or surgical shunt to prevent recurrent hemorrhage.

The Baveno II workshop was held in April 1995<sup>[6]</sup>. Definitions of key clinical events were revised and new definitions were proposed. Based on multiple randomized controlled trials, non-selective  $\beta$ -blockers (NSBB) were recommended to be the treatment of choice for primary prophylaxis of variceal hemorrhage, while isosorbide-5 mononitrate (ISMN) was recommended in patients who

did not tolerate  $\beta$ -blockers. Endoscopic sclerotherapy was not recommended in the prevention of first hemorrhage. Treatment of acute hemorrhage was mainly based on endoscopic therapy, terlipressin was deemed the most effective of the vasoactive agents, with somatostatin showing some efficacy. The transjugular intrahepatic portosystemic shunt (TIPS) was recommended in case of treatment failure of endoscopic and pharmacologic therapy. The recommendations to prevent recurrent hemorrhage included  $\beta$ -blockers or endoscopic variceal ligation (EVL) that had been shown to be better than sclerotherapy<sup>[7]</sup>. TIPS and surgical shunts were to be used only for patients with frequent repeated episodes of variceal hemorrhage.

In June 1996, the AASLD STC took place in Reston, Virginia, United States, with the objective of identifying important areas in the treatment of variceal hemorrhage and future research<sup>[8]</sup>. Guidelines for initial variceal screening and follow-up endoscopy were described in detail depending on severity of liver disease and the size of varices on first endoscopy. Areas of further research were identified as the role of sequential portal pressure measurements and their timing, and defining new predictors of first hemorrhage. Primary prophylaxis recommendations were the same as in the Baveno II conference, with  $\beta$ -blockers as the mainstay of treatment and EVL requiring further studies. Vasoactive drugs in combination with endoscopic treatment (sclerotherapy or EVL) became the established treatment for acute hemorrhage, recognizing the advantage of initiating vasoactive therapy prior to diagnostic endoscopy<sup>[9]</sup>. For secondary prophylaxis EVL or  $\beta$ -blockers were recommended. TIPS or surgical shunts were considered acceptable therapies for failure to control acute hemorrhage or recurrent hemorrhage despite standard treatments.

The Baveno III conference was held in April 2000<sup>[10]</sup>, and introduced the concept of clinically significant portal hypertension (CSPH) which was defined as HVPG of 10 mmHg or more. The presence of varices, variceal hemorrhage or ascites is indicative of the presence of CSPH. Non-selective  $\beta$ -blockers remained the treatment of choice to prevent first hemorrhage from large/medium varices, while EVL required further assessment. The goals of therapy with  $\beta$ -blockers were defined (25% reduction in baseline heart rate or a heart rate of 55 beats/min). ISMN, previously recommended as an alternative to  $\beta$ -blockers, was no longer recommended<sup>[11]</sup>. For treatment of acute hemorrhage, the early administration of vasoactive drugs and continued use for up to 5 d along with endoscopic therapy (EVL or sclerotherapy) were considered standard. Additional measures included use of antibiotics to prevent bacterial infection<sup>[12]</sup>, and lactulose to treat hepatic encephalopathy. With regard to prevention of rebleeding,  $\beta$ -blockers were considered first-line therapy<sup>[13]</sup> as was EVL, with TIPS reserved for treatment failures. The complications of treatment of portal hypertension were also defined for use in clinical settings and in research trials.

The Baveno IV conference was held in April 2005<sup>[14]</sup>, and some of the key criteria (failure to control bleeding,

Table 1 Portal hypertension consensus conferences in the last two decades

Title	Year	Venue
21st meeting of the European association for the study of liver	1986	Groningen, The Netherlands
Definitions, methodology and therapeutic strategies in portal hypertension. A consensus development workshop	1990	Baveno, Italy
Developing consensus in portal hypertension	1995	Baveno, Italy
Portal hypertension and variceal bleeding. AASLD single topic symposium	1996	Virginia, United States
Updating consensus in portal hypertension. Reports of the Baveno III consensus workshop on definitions, methodology and therapeutic strategies in portal hypertension	2000	Baveno, Italy
Evolving consensus in portal hypertension. Report of the Baveno IV consensus workshop on methodology of diagnosis and therapy in portal hypertension	2005	Baveno, Italy
Portal hypertension and variceal bleeding-unresolved issues. Summary of an AASLD and European association for the study of the liver single-topic conference	2007	Atlanta, United States
Revising consensus in portal hypertension: Report of the Baveno V consensus workshop on methodology of diagnosis and therapy in portal hypertension	2010	Baveno, Italy

AASLD: American association for the study of liver diseases.

failure of secondary prophylaxis) were revised. For primary prophylaxis,  $\beta$ -blockers remained the treatment of choice but endoscopic band ligation emerged as an excellent alternative for patients with medium or large varices, and contraindications or intolerance to  $\beta$ -blockers<sup>[15,16]</sup>. Isosorbide mononitrate as a single agent therapy was not recommended even in a combination of pharmacological therapies<sup>[17]</sup>. Primary prophylaxis of small varices could only be considered if they were high risk (red wale sign or Child C)<sup>[18]</sup>. There was no significant change in the recommendations of acute variceal hemorrhage from Baveno III. Small changes included the use of vasoactive drugs for at least 5 d, and the use of balloon tamponade only in massive bleeding as a temporary bridge until definitive treatment could be instituted. EVL was declared superior to sclerotherapy and as the endoscopic procedure of choice in the control of acute hemorrhage<sup>[16,19]</sup>. Secondary prophylaxis should be initiated 6 d after the index variceal bleed, and included the combination of EVL and  $\beta$ -blockers<sup>[20,21]</sup>. TIPS or surgical shunts were reserved for patients with failure of secondary prophylaxis.

The second AASLD STC was held in 2007 in Atlanta, Georgia<sup>[22]</sup>. The objective of this conference was to make clinical recommendations in areas that did not require further investigation and to identify research directions for the remaining areas. Compensated and decompensated cirrhosis were identified as separate entities to be studied separately both in clinical practice and in research<sup>[23]</sup>. The main differences compared with Baveno IV included the emergence of capsule endoscopy as a non-invasive alternative to esophagogastroduodenoscopy (EGD) for assessment of varices, a firm recommendation regarding use of  $\beta$ -blockers for primary prophylaxis of small varices with high-risk features, and consideration of  $\beta$ -blockers for small varices and no high-risk features<sup>[24]</sup>. EVL was considered as effective and safe as  $\beta$ -blockers for primary prophylaxis of medium to large sized varices. Early TIPS emerged as an option in patients at high risk of rebleeding<sup>[25]</sup>, but required further investigation.

The Baveno V conference in May 2010 revised the definitions of failure to control variceal bleeding, and fail-

ure of secondary prophylaxis<sup>[26]</sup>. Primary prophylaxis for small varices was the same as recommended in the 2007 AASLD STC. There was no significant change in the recommendations for primary prophylaxis of medium to large varices ( $\beta$ -blockers or EVL) with the choice of therapy dictated by local resources, expertise and patient preference<sup>[27]</sup>. The recommendations on the treatment of acute variceal bleeding were unchanged except that a stronger recommendation was made to consider early TIPS (within 72 h) in patients with high risk of treatment failure<sup>[28]</sup>. Recommendations for the prevention of recurrent hemorrhage, as in the AASLD STC, consisted of the combination of  $\beta$ -blockers and EVL.

Evidence-based guidelines endorsed by the AASLD<sup>[29]</sup> and the American College of Gastroenterology<sup>[30]</sup> as well as a recent comprehensive review<sup>[31]</sup> on the treatment of portal hypertension have been heavily based on these consensus conferences. These guidelines and review form the bases of the current recommendations that are described in the following section in which the advantages (pros) and disadvantages (cons) of these therapies are also discussed.

## CURRENT STANDARD TREATMENT OF PORTAL HYPERTENSION IN ADULTS

Therapy of varices and variceal hemorrhage in the adult patient with cirrhosis needs to be stratified depending on the different clinical stages in the natural history of portal hypertension: (1) the patient with cirrhosis and portal hypertension who has not yet developed varices and in whom the goal is to prevent the formation of varices (pre-primary prophylaxis); (2) the patient with gastroesophageal varices who has never had bleeding from them, and in whom the goal is to prevent their rupture (primary prophylaxis); (3) the patient with acute variceal hemorrhage in whom the goal is to stop the hemorrhage and prevent its early recurrence; and (4) the patient who has survived an episode of acute variceal hemorrhage, in whom the goal of therapy is to prevent late recurrence of hemorrhage (secondary prophylaxis).

### **Prevention of formation of varices (pre-primary prophylaxis)**

Every patient with a new diagnosis of cirrhosis should have an EGD to look for the presence and size of varices. In patients who do not have gastroesophageal varices, a large multicenter, randomized, controlled trial showed no differences between placebo and  $\beta$ -blockers in the prevention of varices<sup>[1]</sup>. Therefore, no specific treatment for portal hypertension is recommended in this setting. The main focus at this stage is to treat the underlying cause of cirrhosis which will reduce portal hypertension and may therefore prevent the development of clinical complications.

### **Prevention of first variceal hemorrhage (primary prophylaxis)**

First variceal hemorrhage occurs at an annual rate of about 15% and although current mortality from an episode of variceal hemorrhage is lower than in the past two decades, it still carries a significant mortality of 7%-15%<sup>[32-34]</sup>, and is still associated with significant morbidity and healthcare costs. Prevention of first hemorrhage, therefore, is an important part of treatment of portal hypertension. The size of varices, red wale signs on varices (visualized on EGD), and severity of liver disease (Child class C) identify the patients with highest risk of variceal hemorrhage<sup>[18]</sup>. Therefore, within this stage, patients need to be stratified by the risk of hemorrhage into (1) high-risk patients, i.e., those with medium/large varices or those with small varices that have red wale signs, or a Child C patient; and (2) low risk patients, i.e., those with small varices without red wale signs or occurring in a Child A or B patient.

In patients with medium/large varices, quality trials have shown that non-selective  $\beta$ -blockers (propranolol, nadolol) are as effective as EVL in preventing first variceal hemorrhage<sup>[35,36]</sup>, and the recommendation is to use therapy based on local resources, expertise and patient preference.

In patients with high-risk small varices the mainstay of treatment is NSBB because technically performing EVL in these varices may be challenging (although there is no clear evidence for this).

In patients with low-risk small varices, there is limited evidence that shows that their growth may be slowed by the use of NSBB<sup>[24]</sup>. Therefore, the use of NSBB in this setting is considered optional and should be discussed with the patient.

The doses are shown in Table 2, with therapeutic goals and follow-up procedures for each of the recommended therapies.

### **Pros**

NSBB decrease portal pressure through a reduction in portal blood flow. Their mechanism of action involves decreasing cardiac output *via*  $\beta$ -1 receptors and causing splanchnic vasoconstriction by blocking  $\beta$ -2 receptors, resulting in unopposed  $\alpha$ -1 activity. The latter is the most

important effect and therefore it is essential that NSBB (as opposed to selective  $\beta$ -blockers) be used. Advantages of NSBB include low cost, ease of administration and no requirement for specific expertise. As they act by decreasing portal pressure, NSBB may also reduce other complications of cirrhosis such as bleeding from portal gastropathy, ascites and spontaneous bacterial peritonitis<sup>[37,38]</sup>. In fact, a significant reduction in portal pressure has been related to an improvement in survival<sup>[38,39]</sup>. Additionally, once the patient is on NSBB there is no need for repeat EGD.

EVL has the advantage that the procedure can be done at the same time as screening endoscopy, although in some centers a screening EGD time slot will not allow for the performance of EVL, and a separate therapeutic EGD time slot is required. Also, there are relatively few contraindications to EVL and it has been associated with a lower incidence of side-effects compared with NSBB<sup>[15]</sup>.

### **Cons**

The main inconvenience of NSBB is that approximately 15% of patients may have absolute or relative contraindications to therapy, and that another 15% require dose-reduction or discontinuation due to its common side-effects (e.g., fatigue, weakness, shortness of breath) that resolve upon discontinuation but discourage patients from using these drugs<sup>[27]</sup>.

EVL requires specific expertise. The risks include that of the endoscopic procedure and conscious sedation (bleeding, aspiration, perforation and reaction to medications), plus the risk of bleeding from ligation-induced ulcers. In fact, although the quantity of side-effects is greater with NSBB than with EVL<sup>[15]</sup>, the severity of side-effects is greater with EVL. While no lethal side-effects have been reported with the use of NSBB<sup>[16]</sup>, three deaths resulting from EVL-induced bleeding ulcers have been reported<sup>[15,16]</sup>.

### **Recommendation**

The issue of which is the best treatment for primary prophylaxis (NSBB or EVL) has not yet been settled, and there are centers that perform predominantly EVL while others prefer the more rational approach of starting with NSBB and switching to EVL if there is intolerance to NSBB. Carvedilol is a NSBB with an added vasodilatory effect through anti- $\alpha$ -1 adrenergic activity that has recently been shown to be more effective than EVL in preventing first variceal hemorrhage<sup>[40]</sup>. Although considered a promising alternative, further research is necessary before it can be widely recommended.

## **MANAGEMENT OF ACUTE VARICEAL HEMORRHAGE**

Acute variceal hemorrhage is a medical emergency requiring intensive care. The basic medical principles of airway, breathing and circulation are followed to achieve hemodynamic stability. Blood transfusion is done conservative-

Table 2 Primary prophylaxis and secondary prophylaxis of variceal hemorrhage

Therapy	Starting dose	Therapy goals	Maintenance/follow-up
Propranolol	(1) 20 mg orally twice a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 320 mg	(1) Maximum tolerated dose; (2) Aim for resting heart rate of 50-55 beats per minute	(1) At every outpatient visit make sure that patient is appropriately $\beta$ -blocked; (2) Continue indefinitely; (3) No need for follow-up EGD
Nadolol	(1) 40 mg orally once a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 160 mg	As for propranolol	As for propranolol
EVL	Every 2-4 wk until the obliteration of varices	Obliteration of varices; Eradication of new varices following initial obliteration	First EGD performed 1-3 mo after obliteration and every 6-12 mo thereafter
Propranolol	(1) 20 mg orally twice a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 320 mg	(1) Maximum tolerated dose; (2) Aim for resting heart rate of 50-55 beats per minute	(1) At every outpatient visit make sure that patient is appropriately $\beta$ -blocked; (2) Continue indefinitely
Nadolol	(1) 40 mg orally once a day; (2) Adjust every 2-3 d until treatment goal is achieved; (3) Maximal daily dose should not exceed 160 mg	As for propranolol	As for propranolol
ISMN	(1) Only to be used in conjunction with propranolol or nadolol; (2) 10 mg orally at night every day; (3) Adjust every 2-3 d by adding 10 mg in am and then pm; (4) Maximal dose is 20 mg twice a day	(1) Maximal tolerated dose; (2) Systolic blood pressure remains over 95 mmHg	Continue indefinitely
EVL	Every 2-4 wk until the obliteration of varices	Obliteration of varices; Eradication of new varices following initial obliteration	First EGD performed 1-3 mo after obliteration and every 6-12 mo thereafter

Either one of the three therapies shown in the table are recommended. EGD: Esophagogastroduodenoscopy; EVL: Endoscopic variceal ligation; ISMN: Isosorbide-5-mononitrate.

ly for a target hemoglobin level between 7-8 g/dL<sup>[41]</sup>, because excessive blood volume restitution can increase portal pressure<sup>[42,43]</sup>. There are no definite recommendations on management of coagulopathy and thrombocytopenia, as randomized controlled trials of recombinant factor VIIa have not shown any advantages<sup>[44,45]</sup>. Antibiotic prophylaxis is provided by quinolones with consideration of iv ceftriaxone in patients with advanced cirrhosis or previous therapy with quinolones<sup>[12,46]</sup>. Safe vasoactive drugs are started as soon as possible, prior to diagnostic endoscopy. Endoscopy is done as soon as possible and not more than 12 h after presentation. If a variceal source is confirmed, EVL is the procedure of choice, but sclerotherapy is an option when EVL is technically difficult. TIPS is recommended in patients who fail standard combination therapy with endoscopic and pharmacological therapy, however salvage TIPS is accompanied by a very high mortality. Predictors of failure are Child class C, HVPG > 20 mmHg and active bleeding at endoscopy<sup>[47]</sup>. The use of early (pre-emptive) TIPS (within about 48 h of admission) in patients at high risk of failing standard therapy has been shown to reduce mortality<sup>[28]</sup>. These patients are specifically those who are Child C (score of 10-13 points) or are Child B with active hemorrhage (at the time of diagnostic endoscopy), and constitute < 20% of the patients admitted for variceal hemorrhage. In these patients it is recommended to consider early preemptive TIPS. All others should continue standard therapy with vasoactive drugs continued for 2-5 d depending on control of bleeding and severity of liver disease. Vasoactive drugs can be discontinued once the patient has been free of bleeding for at least 24 h. Balloon tamponade is

only used as a temporary measure (inflated for 12 h or less) to control bleeding while a definitive therapy (TIPS or endoscopic therapy) is planned. A new self-expanding esophageal stent is being tested that may replace balloon tamponade<sup>[48]</sup>.

Although there are pros and cons for each of these first-line therapies (pharmacological and endoscopic), the current recommendation is to use them jointly in the control of acute hemorrhage.

### Pros

Vasoactive agents improve the control of variceal hemorrhage when combined with endoscopic therapy and when compared to endoscopic therapy alone<sup>[49]</sup>. However there appears to be no significant difference among the different vasoactive agents regarding control of hemorrhage and early rebleeding. Vasopressin, a powerful vasoconstrictor, is associated with more adverse events<sup>[50]</sup>, and should not be considered a first-line vasoactive drug. Terlipressin is the only agent that, in small studies and when compared to no treatment, improved survival<sup>[50]</sup>. In practice, the choice of pharmacological agent is usually based on availability and cost. Octreotide, a somatostatin analogue, is the only safe vasoactive drug available in the United States. Doses and schedules for the different vasoconstrictors are shown in Table 3. Except for vasopressin that must be administered with nitroglycerin, the administration of these agents does not require any special procedure or expertise and can be started in the emergency room setting.

Endoscopic therapy in the acute setting is very effective in controlling variceal hemorrhage, particularly when

Table 3 Vasoactive agents used in the management of acute hemorrhage

Drug	Standard dosing	Duration	Mechanism of action
Somatostatin	Initial iv bolus 250 µg (can be repeated in the first hour if ongoing bleeding); continuous iv infusion of 250 to 500 µg/h	Up to 5 d	Inhibits vasodilator hormones like glucagon causing splanchnic vasoconstriction and reduced portal blood flow
Octreotide (somatostatin analogue)	Initial iv bolus of 50 µg (can be repeated in first hour if ongoing bleeding); continuous iv infusion of 50 µg/h	Up to 5 d	Same as somatostatin, longer duration of action
Vapreotide (somatostatin analogue)	Bolus: 50 µg; continuous iv infusion of 50 µg/h	Up to 5 d	Similar to somatostatin with higher metabolic stability
Vasopressin + nitroglycerine	0.2-0.4 units/min continuous iv infusion intravenously, may titrate to a maximum of 0.8 units/min; always use in combination with nitroglycerine	Maximum of 24 h at lowest effective dose	Causes direct vasoconstriction on splanchnic circulation resulting in decreased portal blood flow
Terlipressin (vasopressin analogue)	Initial 48 h: 2 mg iv every 4 h until control of bleeding; maintenance: 1 mg iv every 4 h to prevent re-bleeding	Up to 5 d	Splanchnic vasoconstriction; the active metabolite lysine-vasopressin is gradually released over several hours thus decreasing typical vasopressin side effects

a spurting varix is observed. However, in a meta-analysis comparing sclerotherapy *vs* vasoactive drugs, no differences in efficacy were observed between treatments, with more side-effects with sclerotherapy<sup>[51]</sup>. EVL has replaced sclerotherapy as the endoscopic procedure of choice due to more effective control of bleeding, obliteration of varices in fewer treatment sessions, a lower rebleeding rate, and lower mortality<sup>[19,33]</sup>. How EVL compares with vasoactive drugs alone remains to be determined. There is no added benefit of a combination of EVL and sclerotherapy over band ligation alone.

### Cons

Vasoactive drugs often require placement of central lines and require close monitoring for ischemic complications. Vasopressin is the most potent vasoconstrictor, but its use is limited by multiple side-effects related to splanchnic vasoconstriction (e.g., bowel ischemia) and systemic vasoconstriction (e.g., hypertension, myocardial ischemia). Terlipressin is an analogue of vasopressin that, although safer, is still accompanied by more side-effects than somatostatin<sup>[52]</sup>. The main side effects of the somatostatin analogs octreotide and vapreotide are sinus bradycardia, hypertension, arrhythmia, and abdominal pain.

Endoscopic therapy during acute hemorrhage carries the usual risks of endoscopic procedures, with increased risk of aspiration due to active bleeding and the emergency nature of the procedure. In the setting of active hemorrhage, the band ligator limits the visibility, and it becomes technically difficult to maneuver the endoscope back into the stomach. Elastic bands can slip or can cause ulcers that can result in rebleeding. As mentioned previously, EVL has less side-effects than sclerotherapy and is the endoscopic therapy of choice.

### Recommendation

The specific treatment of choice for acute variceal hemorrhage is the combination of vasoactive drugs (started prior to EGD) and emergency endoscopic therapy (at the time of initial diagnostic EGD). The pharmacological therapy of choice is terlipressin (lower mortality in small placebo-controlled studies) or somatostatin (fewer side-

effects), however the choice is dependent on availability and cost. Octreotide is the only vasoactive drug available in the United States. The endoscopic therapy of choice is EVL.

Recommendations may vary depending on the severity of liver disease. In patients who are Child C (or Child B with active hemorrhage), the risk of failing recommended treatment (vasoactive drugs and EVL) is high and therefore proceeding to a “rescue” therapy (i.e., TIPS) before failure occurs should be considered. In patients who are Child A, mortality with the treatment of choice is essentially nil<sup>[32,34]</sup>, and these patients may respond to vasoactive therapy alone, although this requires further exploration.

## PREVENTION OF RECURRENT VARICEAL HEMORRHAGE (SECONDARY PROPHYLAXIS)

The risk of rebleeding in patients who survive an episode of variceal hemorrhage is high (median rebleeding rate 60%), with a mortality of up to 33%. Prevention of rebleeding is therefore an essential part of the management of the patient with variceal hemorrhage. Patients who had a TIPS performed during the acute episode do not require specific therapy for portal hypertension or for varices but should be referred for transplant evaluation. TIPS patency should be checked through Doppler ultrasound every 6 mo. For the majority (patients who do not have a TIPS performed during the acute episode), secondary prophylaxis with NSBB should be started as soon as the intravenous vasoactive drug is discontinued. NSBB significantly reduce the risk of recurrent hemorrhage<sup>[13]</sup>. Although the addition of ISMN to NSBB has a greater portal pressure-reducing effect<sup>[53]</sup>, in clinical trials the combination of NSBB and ISMN is no different from NSBB alone regarding the rate of overall rebleeding or mortality, but has a higher rate of side-effects<sup>[54]</sup>. Sclerotherapy decreases rebleeding rates and mortality, but is associated with serious complications (e.g., esophageal strictures, bleeding from ulcers). Sclerotherapy has been replaced by EVL, since it has significantly better outcomes

(rebleeding, mortality and side-effects) compared with sclerotherapy. Studies comparing pharmacological therapy (NSBB plus ISMN) *vs* EVL show no differences in recurrent hemorrhage, but there is a suggestion of a beneficial effect on survival with pharmacological therapy in the long term<sup>[54,55]</sup>. The combination of pharmacological (NSBB alone or NSBB + ISMN) plus EVL is associated with lower rebleeding rates than either therapy alone<sup>[51,56]</sup>, and constitutes the treatment of choice.

In patients who experience recurrent variceal hemorrhage despite the combination of pharmacologic and endoscopic treatment, TIPS with polytetrafluoroethylene-covered stents<sup>[57]</sup> or, where expertise is available, surgical shunts<sup>[58]</sup> should be provided.

Table 2 presents the doses, therapeutic goals and follow-up procedures for the recommended therapies. The pros and cons of each of these first-line therapies (pharmacological and endoscopic) are the same as those described for primary prophylaxis, with some additional considerations described below.

### Pros

Pharmacologic agents provide protection against rebleeding during the initial phase after index hemorrhage while esophageal varices are being obliterated by EVL. NSBB alone or in combination with ISMN should be used. The choice will depend on patient tolerability. Patients who are not candidates for EVL should receive combination NSBB + ISMN.

The lowest rates of recurrent variceal hemorrhage (approximately 10%) are observed in individuals who have a hemodynamic response to pharmacologic therapy, defined as a decrease in HVPG to < 12 mmHg or a decrease of > 20% from baseline levels<sup>[39,59]</sup>. The more rational approach would thus be to guide therapy based on hemodynamic response and, in those who achieve a hemodynamic response, endoscopic therapy would not be necessary. However there are cons (see below) to this approach.

Patients who are intolerant or have contraindications to pharmacological therapy should receive EVL alone.

### Cons

A recent study suggests that NSBB are associated with a poorer survival in patients with refractory ascites<sup>[60]</sup>, a condition that may be present in patients in this clinical stage. However, the study is retrospective and the groups were disparate at baseline, with patients on NSBB having more advanced disease as shown by a higher prevalence of varices and variceal hemorrhage, and there is evidence that indicates the contrary, that is, that NSBB may be beneficial for these patients<sup>[13,61]</sup>. Therefore, unless stronger evidence arises, the use of NSBB in patients with refractory ascites should not be contraindicated.

The combination of NSBB + ISMN has a higher incidence of side effects because of the added ones associated with ISMN, specifically headache and lightheadedness. As mentioned above, the lowest rebleeding rates are in patients who experience a hemodynamic response. Although

HVPG-guided therapy would appear rational, a small trial showed that outcomes with HVPG-guided therapy are no different from those in patients treated with combined pharmacological and endoscopic therapy<sup>[62]</sup>. Until the best treatment for non-responders is settled, larger clinical trials are performed, and HVPG measurements are standardized across centers, HVPG-guided therapy cannot be currently recommended<sup>[63]</sup>.

As mentioned above, EVL is associated with bleeding from EVL-induced ulcers. Treatment with proton pump inhibitors post ligation reduces the size of these ulcers, with a trend towards a lower risk of bleeding<sup>[64]</sup>, and can be considered in this setting.

### Recommendation

The treatment of choice to prevent rebleeding is the combination of pharmacological therapy (NSBB ± ISMN) and EVL. Contrary to other clinical stages, risk stratification has not been tested in this setting. The main predictor of recurrent bleeding and death is the Child classification. It is conceivable that patients who are Child A would only require one or other therapy, while patients who have more advanced disease require the combination therapy. Patients who fail this therapy should be considered for TIPS placement and, in centers where expertise is available, for a surgical shunt. Patients with recurrent variceal hemorrhage are in a category of “further decompensation” of cirrhosis and, as such, should be evaluated for liver transplantation.

## CURRENT STANDARD TREATMENT OF PORTAL HYPERTENSION IN CHILDREN

The most common causes of portal hypertension in children are biliary atresia and portal vein thrombosis. Data regarding the prevalence of esophageal varices in children with portal hypertension is very limited and to date there have been no randomized controlled trials comparing different treatments for primary and secondary prophylaxis<sup>[65]</sup>.

Regarding primary prophylaxis, there is currently no recommended treatment<sup>[22,66]</sup>. In a recent gathering of experts at the AASLD annual meeting, it was concluded that, before a randomized trial could be performed in children, pediatric research should focus on addressing questions of the natural history and diagnosis of varices, prediction of variceal bleeding, optimal approaches to β-blocker and ligation therapy, and alternative study designs to explore therapeutic efficacy in children<sup>[65]</sup>.

Regarding acute variceal hemorrhage, management in children is based on limited data comparing EVL and sclerotherapy<sup>[67]</sup>, and expert pediatric opinion based on adult Baveno IV guidelines<sup>[66]</sup>. These include vasoactive agents, antibiotic prophylaxis and endoscopic variceal ligation.

EVL is also recommended for secondary prophylaxis of variceal hemorrhage but it has not been compared with β-blockers<sup>[22,66]</sup>. In children with portal vein throm-

bosis, meso-rex bypass appears to be the best option for secondary prophylaxis<sup>[22,66]</sup>.

## CONCLUSION

In the last two decades significant advances in the field of portal hypertension have improved the clinical care and survival of patients with cirrhosis and portal hypertension. In addition to better treatment strategies and improved therapeutic options, the issue of risk stratification has become more important so that, within each clinical stage, different patient subpopulations have been identified that require a different management. Clearly, further research is necessary to explore new pharmacological options that would allow a majority of patients to be hemodynamic responders, thereby foregoing the need for HVPG measurements and even the need for endoscopic therapy. The identification of different risk populations within each stage also requires further definition. It is expected that future trials and Baveno and AASLD conferences will continue to advance the field.

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## Management of portal hypertension in children

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### Abstract

Portal hypertension can be caused by a wide variety of conditions. It frequently presents with bleeding from esophageal varices. The approach to acute variceal hemorrhage in children is a stepwise progression from least invasive to most invasive. Management of acute variceal bleeding is straightforward. But data on primary prophylaxis and long term management prevention of recurrent variceal bleeding in children is scarce, therefore prospective multicenter trials are needed to establish best practices.

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### INTRODUCTION

Normal portal pressure is between 5 and 10 mmHg. Once portal pressure rises to 12 mmHg or greater, complications such as varices and ascites may occur.

The portal system drains the capillaries of the mesenteric and splenic veins and ends in the hepatic capillaries. The portal vein supplies partially oxygenated blood to the liver, supplementing the highly oxygenated blood of the hepatic artery to the liver. Blood flow to the liver is finely tuned; any disturbance of the flow in one of these vessels can be offset to a certain degree by increased flow through the other vessel. This is known as the arterial buffer response. Blood from both the portal venous system and the hepatic arterial systems combine within the sinusoids.

Portal hypertension occurs when there is increased portal resistance and/or increased portal blood flow. Generally, the portal venous system has a low baseline portal pressure of 7-10 mmHg and the hepatic venous pressure gradient (HVPG) ranges from 1 to 4 mmHg. Portal hypertension is defined as a portal pressure greater than 10 mmHg or gradient greater than 4 mmHg. Pressure gradients above 10 mmHg have been associated with esophageal varices formation, and those above 12 mmHg are associated with ascites and variceal bleeding in adults<sup>[1]</sup>. To measure the portal pressure gradient, a catheter can be wedged into the hepatic vein *via* the femoral or transjugular approach and a wedged hepatic venous pressure (WHVP) measurement obtained. If the catheter is then retracted into a free flowing hepatic vein, a free hepatic venous pressure (FHVP) can be measured. The HVPG is the difference between the WHVP and the FHVP. The cause of portal hypertension can be suggested by the

HVPG value. In pre-sinusoidal obstruction, the HVPG is normal but the WHVP is raised, whereas in cirrhosis both HVPG and WHVP are increased.

## HEMODYNAMIC CHANGES

Clinically, portal hypertension causes splenomegaly with resulting hypersplenism and the formation of a collateral circulation. Despite formation of a significant collateral network, portal hypertension persists. This is a result of an increase in cardiac output (result from increased venous return and diminished afterload); and a decrease in splanchnic arteriolar tone (mediated by several factors including glucagon and nitric oxide). Retention of sodium and water *via* a hepato-renal reflex increases the circulating blood volume. There is also production of vasodilatory factors that cause arterial vasodilation of the splanchnic circulation. Increases in the intrahepatic resistance are due to hepatocyte swelling, fibrosis and inflammation within the portal tracts. Clinical studies and animal models have demonstrated the hemodynamic events that occur; however, most of these investigations have not been performed in children or in pediatric models. The hyperdynamic circulatory state has not been well characterized in any cohorts of children.

## GASTROINTESTINAL BLEEDING

The clinical presentation of portal hypertension can be dramatic because it may be the first symptom of long-standing silent liver disease. In several large series of children with portal hypertension, approximately two thirds presented with hematemesis or melena, usually from rupture of an esophageal varix<sup>[2]</sup>. Gastrointestinal hemorrhage also may be associated with bleeding from portal hypertensive gastropathy, gastric antral vascular ectasia, or gastric, duodenal, peristomal, or rectal varices. Variceal hemorrhage is the result of increased pressure within the varix, which leads to changes in the diameter of the varix and increased wall tension. When the wall tension exceeds the variceal wall strength, physical rupture of the varix occurs. The majority of patients reported in the series had splenomegaly at the time of hemorrhage; thus, the combination of gastrointestinal bleeding and splenomegaly suggests portal hypertension until proven otherwise. The sentinel bleeding episode in children may occur in a wide range of ages, starting as early as 2 mo of age<sup>[3]</sup>. The risk of first-time bleeding from studies in children with cirrhosis is 22%, but rises to 38% in children with known varices over a 5 year period<sup>[4]</sup>. Bleeding occurs in 15%-25% of patients with biliary atresia in long term follow up<sup>[5,6]</sup>. The age of bleeding is dependent on the underlying etiology of cirrhosis. Patients who have surgically corrected but progressive biliary atresia bleed for the first time at a mean age of 3 years while children with cirrhosis due to cystic fibrosis bleed at a mean age of 11.5 years<sup>[7]</sup>.

Variceal bleeding in children often follows an acute

upper respiratory infection, fever, or aspirin ingestion<sup>[8]</sup>. The combination of factors including increased abdominal pressure from coughing or sneezing, increased cardiac output from fever, and ulceration from medications such as nonsteroidal antiinflammatory drugs or aspirin contribute to the rupture of varices. Prolonged gastroesophageal reflux can contribute to erosions over the varices that could result in bleeding.

Triger *et al.*<sup>[9]</sup> followed 44 children aged 12 years for a mean follow-up of 8 years. At the time of portal venous obstruction diagnosis, no child had either abnormal liver enzymes or abnormal liver function. The actuarial probability of bleeding was 49% at age 16 years and 76% at 24 years of age. If the child bled before 12 years of age, the probability of bleeding was higher than in those who had not bled before aged 12. Further, there was no evidence of variceal regression over time. Instead, progression of varices occurred in the majority of children suggesting that the previous hypothesis that variceal bleeding decreased in adolescence due to development of spontaneous porto-systemic collaterals was incorrect.

## SPLENOMEGALY

Splenomegaly is the second most common finding in children with portal hypertension after gastrointestinal bleeding. In many instances, an enlarged spleen is first discovered on routine physical examination. Many children will admit to a vague fullness in the left upper quadrant for many years prior to the diagnosis. Occasionally, manifestations of hypersplenism including thrombocytopenia, leukopenia, petechiae, or ecchymoses will prompt evaluation, leading to the discovery of portal hypertension. Hematologists should consider a biochemical liver profile and a Doppler ultrasonographic examination in the evaluation of any child with thrombocytopenia, especially if leukopenia is also present. Rarely will associated cytopenias lead to clinically relevant disease. Although splenomegaly is a common finding in patients with portal hypertension, splenic size does not correlate well with portal pressure<sup>[10,11]</sup>. Hypersplenism rarely requires surgical intervention. Exceptions include symptoms of symptomatic anemia and severe physical discomfort<sup>[12]</sup>.

## ABDOMINAL VENOUS PATTERNING

Specific cutaneous vascular patterns are observed with portal hypertension. Prominent vascular markings on the abdomen are the result of porto-collateral shunting through subcutaneous vessels. The direction of flow through these veins may be indicative of the site of obstruction. When the inferior vena cava is occluded, drainage is usually cephalad, but caudad below the umbilicus if the inferior vena cava is patent. Portal hypertension decompression through the umbilical vein results in prominent periumbilical collaterals, referred to as caput medusae. An audible venous hum (Cruveilhier-Baumgarten murmur) may occasionally be heard. Caput

**Table 1** Initial manifestation of portal hypertension

Reference	Mitra <i>et al</i> <sup>[60]</sup> (1978)	Pinkerton <i>et al</i> <sup>[62]</sup> (1972)	Spence <i>et al</i> <sup>[33]</sup> (1984)	Howard <i>et al</i> <sup>[61]</sup> (1988)
Patients	70	33	27	152
% Presenting with				
Hemorrhage	80	97	85	46
Splenomegaly	99	24	100	94
Ascites	17	21	8	7

medusae are rare in children, partly because of the high prevalence of portal vein obstruction associated with umbilical vein obliteration. Rectal varices are more common in children<sup>[13]</sup>. In children with short bowel syndrome, stomal varices which are a site of low resistance, are often present and a common site for hemorrhage<sup>[14]</sup>.

## ASCITES

Ascites arises when the hydrostatic and osmotic pressures within the hepatic and mesenteric capillaries result in a net transfer from blood vessels to lymphatics at a rate that overcomes the drainage capacity of the lymphatics. It is the presenting sign of portal hypertension in 7%-21 % of children (Table 1).

In patients with portal hypertension, increased sodium retention and raised portal pressure may cause accumulation of fluid within the abdomen. Impaired lymphatic drainage compounds the situation. Treatment includes salt and fluid restriction and the use of diuretics. Albumin infusions can be used to increase intravascular osmotic pressure, followed by diuretic dosing to facilitate urination. Paracentesis has been used safely in children and is reserved for use when the ascites is difficult to control resulting in respiratory compromise or if peritonitis is suspected for cell count and culture<sup>[15,16]</sup>.

## PULMONARY COMPLICATIONS

Hepatopulmonary syndrome (HPS) and portopulmonary hypertension are undoubtedly underdiagnosed in children. Barbé *et al*<sup>[17]</sup> reported on the presence of HPS in 29 pediatric patients of which 26 had cirrhosis and 3 had an extrahepatic cause of portal hypertension. HPS progresses more rapidly in patients with biliary atresia associated with polysplenia<sup>[18]</sup>. Patients with HPS have a higher incidence of dyspnea, cyanosis, clubbing and spider nevi<sup>[19-21]</sup>. There are two forms of HPS. In type I, the vessels enlarge such that the red blood cells traveling through the center of the vessel do not have significant contact time with the oxygen-rich alveoli. In type II HPS, the diffusion-perfusion mismatch is presumed to be due to arteriovenous communications completely bypassing alveoli<sup>[22,23]</sup>. HPS is thought to occur as a result of shunting of vasodilatory mediators from the mesentery away from the liver in portal hypertension. Liver transplantation reverses HPS in greater than 80% of patients. If large shunts are present and the arterial par-

tial pressure of oxygen is less than 50 mmHg on 100% oxygen, a poorer outcome may be expected.

Portopulmonary syndrome eventually leads to right-sided heart failure. Histologically there is pulmonary arteriopathy with concentric laminar intimal fibrosis consistent with a vasoconstrictive etiology. Pediatric cases have been reported<sup>[24,25]</sup>. The condition is defined by a pulmonary arterial pressure greater than 25 mmHg at rest and above 30 mmHg with exercise, raised pulmonary vascular resistance with pulmonary arterial occlusion pressure, or a left-ventricular end-diastolic pressure of less than 15 mmHg<sup>[26]</sup>. The most common symptom of pulmonary hypertension is exertional dyspnea. Other symptoms include fatigue, palpitations, and syncope or chest pains.

## THERAPY

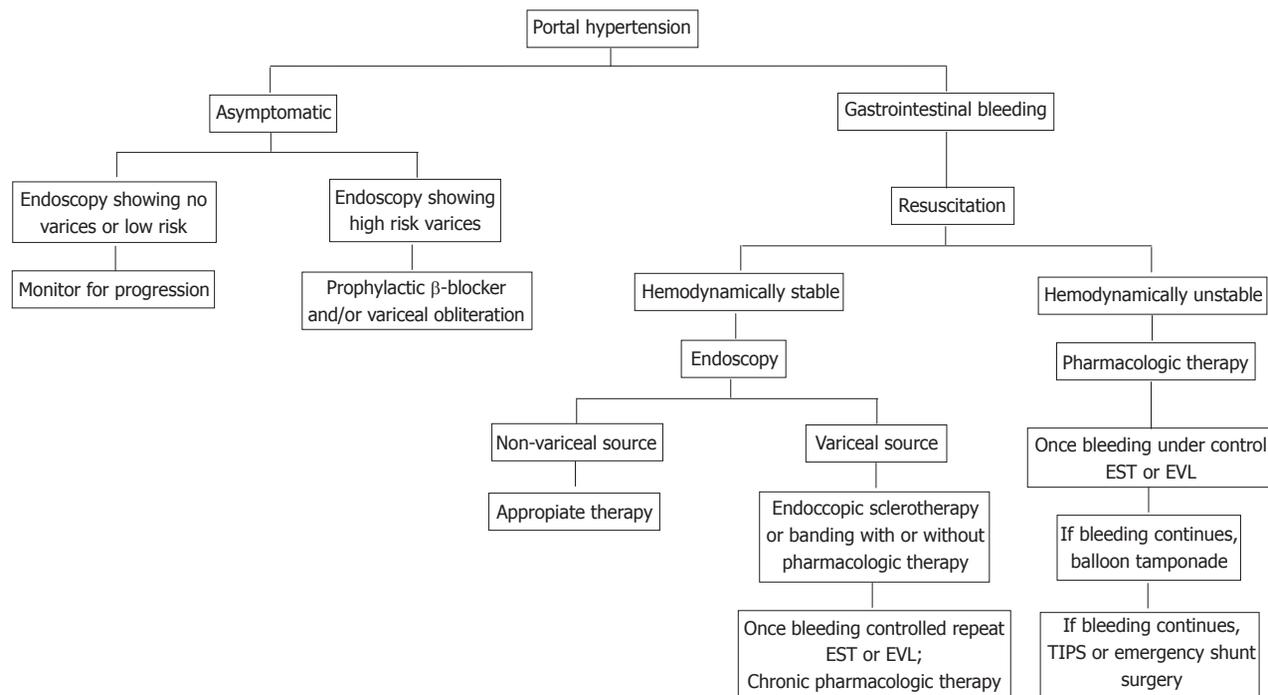
Therapy of portal hypertension is primarily directed at the management of its most dramatic manifestation, variceal hemorrhage. Variceal bleeding is a life threatening medical emergency, and patients with chronic liver disease should be instructed to seek immediate medical attention for any signs or symptoms of bleeding. The management can be divided into preprimary prophylaxis, prophylaxis (primary) of the first episode of bleeding, emergency therapy, and prophylaxis (secondary) of subsequent bleeding episodes. As with many other aspects of portal hypertension, almost all the modes of therapy are based on adult trials (Figure 1). Many of the trials are well-controlled randomized double-blinded studies, and comprehensive meta-analysis of these trials have been performed<sup>[27-30]</sup>. The literature on the management of variceal hemorrhage in children is predominantly descriptive and anecdotal. There have been few randomized trials of therapy for portal hypertension in children<sup>[31,32]</sup>.

### Preprimary prophylaxis

The concept is that early treatment of portal hypertension has the potential to delay or prevent the development of esophageal varices or other manifestations of portal hypertension. In a *S. mansoni* mouse model of portal hypertension, the administration of propranolol 5 wk into the infection resulted in a significant reduction in the development of portal hypertension, portosystemic shunting, and portal venous inflow<sup>[34]</sup>. A randomized controlled trial of timolol, a non-selective beta blocker, on the development of varices in adults did not show a significant benefit<sup>[35]</sup>. Currently, preprimary prophylaxis remains an interesting concept that is not applicable in clinical practice.

### Primary prophylaxis

The issue of prophylaxis of the first episode of variceal bleeding in children is controversial and is predicated on experience with adults who primarily have alcoholic cirrhosis. Surveillance endoscopy in children with liver disease and stigmata of portal hypertension is justified if the clinician anticipates recommending a prophylactic



**Figure 1 Portal hypertension.** EST: Endoscopic sclerotherapy; EVL: Endoscopic variceal band ligation; TIPS: Transjugular intrahepatic portosystemic shunt.

regimen. Prophylaxis may also be valuable in patients who live in remote areas far from emergency medical care. Given the unpredictable timing of the first episode of variceal bleeding, primary prophylaxis regimens need to be associated with relatively low morbidity and mortality. As such, beta blockade has been more extensively used in this setting. The improved risk-benefit ratio of endoscopic ligation therapy relative to sclerotherapy has led to reassessment of its role in primary prophylaxis<sup>[36,37]</sup>. Uncontrolled preliminary pediatric experience of variceal hemorrhage using beta blockade has recently been reported<sup>[38-40]</sup>. Beta blocker use in children has reduced the frequency of bleeding episodes, and in some trials has improved long-term survival in patients with esophageal varices. Initial randomized trials demonstrated efficacy in patients who had a previous bleeding episode<sup>[41]</sup>. Subsequently, propranolol was shown to be effective in patients with varices who had never bled. In a study of 230 subjects randomized to propranolol or placebo, the incidence of bleeding and mortality over a 14 mo period was reduced by almost 50%<sup>[42]</sup>. Several meta-analyses have demonstrated the success of propranolol<sup>[43-50]</sup>. It is clear that a goal of at least 25% reduction in resting heart rate needs to be achieved to realize these effects. In patients in whom HVPg drops below 12 mmHg, subsequent variceal bleeding is unlikely. Achieving such a large reduction in resting heart rate and achieving a HVPg below 12 mmHg may be problematic in children in whom baseline measurements may be difficult. A wide range of dosing (0.6-0.8 mg/kg per day) divided into two to four doses of propranolol has been required in children in order to observe a “therapeutic effect”.

Unfortunately, propranolol often does not reduce

HVPg below 12 mmHg, therefore a combination of beta blockade and vasodilatation therapies are now under investigation. Isosorbide-5-mononitrate, a long-acting vasodilator, may potentiate the effects of propranolol on the HVPg<sup>[51]</sup>. Combination pharmacologic agents, such as carvedilol, may have enhanced efficacy<sup>[52]</sup>. Unfortunately, there is little if any prospective data in children on the safety and effectiveness of beta blockade with or without vasodilators in patients with portal hypertension.

Endoscopic band ligation therapy has been used with greater frequency in adults with high risk varices<sup>[53,54]</sup>. As with beta blockade, endoscopic band ligation therapy cannot be recommended for routine use in children with varices. In fact, a small randomized trial of prophylactic endoscopic sclerotherapy in children showed no survival benefit<sup>[55]</sup>.

### **Emergency therapy of variceal bleeding**

The initial management of variceal bleeding is stabilization of the patient. Vital signs, particularly tachycardia or hypotension, can be especially helpful in assessing blood loss. Patients on beta blocker therapy may not manifest the usual compensatory tachycardia and are at higher risk of developing significant hypotension. Fluid resuscitation in the form of crystalloid initially, followed by red blood cell transfusion, is critical. One needs to administer these carefully to avoid overfilling the intravascular space and increasing portal pressure. Optimal hemoglobin levels in adults with variceal hemorrhage are between 7 and 9 g/dL<sup>[56]</sup>. Nasogastric tube placement is safe and may be an essential part of the management of these patients. It allows documentation of the rate of ongoing bleeding and removal of blood, a protein source that

may precipitate encephalopathy. In addition, blood in the stomach increases splanchnic blood flow and could aggravate portal hypertension and ongoing bleeding. Platelets should be administered for levels less than  $50 \times 10^9/L$ , and coagulopathy corrected with vitamin K and fresh frozen plasma. There may be a value to the use of recombinant factor VIIa in severe coagulopathy as the fluid requirements may be diminished<sup>[57]</sup>. Intravenous antibiotic therapy should be considered for all patients with variceal bleeding in light of the high risk of potentially fatal infectious complications<sup>[58,59]</sup>. Once the patient is stabilized, endoscopy should be performed to document that hemorrhage is indeed from variceal rupture. Continued bleeding at the time of endoscopy is a finding that portends poor prognosis. A significant percentage of both adults and children with chronic liver disease will have a source of bleeding other than varices, including duodenal or gastric ulceration<sup>[58]</sup>. Pharmacotherapy of acute hemorrhage should not be withheld until endoscopy can be performed. In fact, it may facilitate the procedure. At the time of initial endoscopy, management can begin in the form of sclerotherapy or band ligation. Bleeding that lasts more than 6 h or requires more than one red blood cell transfusion necessitates further investigation. A wide variety of therapeutic options exist in adults. Documentation of their efficacy in adults is fairly convincing, but data in children is scarce

### Pharmacology

The pharmacologic therapy of variceal bleeding usually consists of vasopressin or somatostatin (or their analogs) infusions. Vasopressin has the longest history of usage and acts by increasing splanchnic vascular tone and thus decreasing portal blood flow. Its use is often limited by the side effects of vasoconstriction, which include left ventricular failure, bowel ischemia, angina, and chest/abdominal pain<sup>[53]</sup>. In a study of 215 children with acute variceal hemorrhage, 184 had bleeding arrested by the combined use of fluid support and vasopressin. Vasopressin has a half-life of 30 min and is usually given as a bolus followed by continuous infusion. The recommended dose for children is 0.33 U/kg as a bolus over 20 min, followed by an infusion of 0.2 U/1.73 m<sup>2</sup> per minute (may be increased up to 3 times the initial rate). These recommendations are empiric, based on clinical practice, and derived from extrapolation of adult dosages. Terlipressin, a long-acting synthetic analogue of vasopressin, has shown similar effects and does not require continuous infusion<sup>[57]</sup>. Side effects appear to be reduced compared to vasopressin, but prospective data in children are lacking.

Alternatives to vasopressin have been investigated because of its poor side effect profile. Somatostatin and its synthetic homologue octreotide also have been shown to decrease splanchnic blood flow. Their effects on acute variceal hemorrhage appear to be similar to those of vasopressin, with fewer side effects<sup>[58,59]</sup>. Continuous infusion of 1-5 µg/kg per hour of octreotide appears to be effective but may need to be initiated by the ad-

ministration of a bolus. New longer-acting somatostatin analogues are currently under investigation<sup>[59]</sup>.

### Endoscopy

Approximately 15% of children will have persistent hemorrhage despite conservative management plus some form of splanchnic vasoconstriction. The most common secondary approach is endoscopic sclerotherapy or endoscopic band ligation. Endoscopic therapy is very effective in controlling bleeding, although it may be technically challenging. An extensive experience with emergency sclerotherapy exists in children, and it's rare for additional therapy to be required. A variety of agents have been used (sclerosants, chemically irritating compounds such as ethanolamine/tetradecyl sulfate). These sclerosants are injected either intra- or para-variceal, until bleeding has stopped. In the setting of emergency sclerotherapy it is important to be aware of the significant incidence of associated bacteremia and to consider antibiotic prophylaxis in most patients.

Endoscopic band ligation of varices may be a preferable approach because it is easier and safer. A randomized trial of band ligation versus sclerotherapy in adults demonstrated similar control of active bleeding and recurrence of hemorrhage with significantly lower overall complications and mortality). A potential concern of this technique in children (whose esophageal wall is thinner than adults), is entrapment of the full thickness of the esophageal wall by the rubber band with subsequent ischemic necrosis and perforation.

### Mechanical

The Sengstaken-Blackmore tube (SSBT) was designed to stop hemorrhage by mechanically compressing esophageal and gastric varices. The device consists of a rubber tube with at least two balloons. It is passed into the stomach, where the first balloon is inflated and pulled up snug against the gastroesophageal junction. Once the tube is secured in place, the second balloon is inflated in the esophagus at a pressure (60-70 mmHg) that compresses the varices without necrosing the esophagus. A channel in the rubber tube allows gastric contents to be sampled for evidence of bleeding. This therapy is very effective in controlling acute bleeding. Unfortunately, it is associated with significant number of complications and high incidence of re-bleeding when the tube is removed. Most patients find the treatment uncomfortable, and its use in children requires significant sedation. Use of the SSBT increases the risk of aspiration pneumonia, which can be a life threatening complication in a patient with liver failure. Re-bleeding has been reported in 33%-60% of patients. Given these problems it is reserved for severe uncontrollable hemorrhage and generally serves as a temporizing measure until a more definite procedure can be performed.

### Surgical and interventional radiology

Surgical therapy is usually a last resort approach to acute

**Table 2** Major complications of endoscopic sclerotherapy in children<sup>[61]</sup> (%)

Bleeding before treatment	39
Esophageal ulceration	29
Stricture formation	16
Recurrent varices	8

variceal hemorrhage. The reluctance to perform emergency surgery partly stems from its associated high mortality but also from concerns of an increased incidence of encephalopathy and greater difficulty for subsequent liver transplantation. The surgical procedures available can be divided into transection, devascularization, and portosystemic shunting. The first two techniques are rarely used and work by interrupting blood flow through the esophagus. Liver transplantation may be an effective means of treating esophageal variceal bleeding if an acceptable organ can be procured quickly enough. Variceal embolization *via* a percutaneous transhepatic or transsplenic approach has been advocated by some hepatologists as another method of controlling acute hemorrhage. Transjugular intrahepatic portosystemic shunt (TIPS) placement may be the optimal approach for intractable bleeding since it does not require surgery or puncture of an organ that is predisposed to hemorrhage. A catheter is inserted into the jugular vein and is advanced into the hepatic vein where a needle is used to form a tract between the portal vein and the hepatic vein. This tract is expanded with a balloon angioplasty catheter, and a stent is then placed forming a permanent portosystemic shunt. The experience in children is limited. Size limitations and local expertise may be limiting factors in some cases, but given the high risk associated with emergency surgery or the use of SSBT, TIPS may be the treatment of choice in the emergent setting, especially when liver transplantation is imminent.

**Secondary prophylaxis:** The long term management of portal hypertension in children with a previous episode of variceal bleeding is complex. One must take into consideration several factors; first the natural history; as discussed earlier, there are significant differences in the setting of minimal and inactive versus active and progressive hepatic disease. As a result, certain individuals may have the possibility of outgrowing their portal hypertension through the development of spontaneous portosystemic shunts, whereas other might be expected to develop end-stage liver disease and ultimately be candidates for liver transplantation. The second issue stems from the great diversity in therapeutic modalities. The physiologic goal of pharmacologic therapy varies from program to program (i.e, change in heart rate, hepatic portal venous gradient pressures, *etc*). Sclerotherapy may be administered with a wide variety of agents and by two different techniques (i.e, intra or para variceal). Endoscopic band ligation offers an important and generally safer alternative. Finally, at least six different portosys-

temic shunting procedures have been described, all with their own advantages and disadvantages.

**Sclerotherapy and ligation therapy:** Sclerotherapy and band ligation therapy work by physical obliteration of esophageal varices. Bleeding may occur during the several weeks required to complete the obliteration. Most importantly the principal problem of portal hypertension is not addressed. Despite these problems, endoscopic therapy has been a mainstay of the treatment of esophageal varices, and there is significant amount of clinical experience with these therapies in children.

The effectiveness of sclerotherapy has been studied for both prevention of initial and subsequent bleeding episodes. Sclerotherapy, which has in general been supplanted by band ligation, with the exception of very young or small children in which band ligation may not be feasible. Intravariceal, paravariceal, and some combination injection protocols have been used. A wide variety of sclerosing agents have been used without a clear cut difference in their efficacy or adverse side effects. A meta analysis of seven studies and 748 patients revealed mortality rates of 47% in the sclerotherapy group and 61% in the conservatively managed group. A variety of complications have been reported (Table 2).

Retrosternal pain, bacteremia, and fever post treatment are common. Esophageal ulceration may occur after sclerotherapy, and the associated symptoms may be ameliorated with sucralfate slurry therapy.

The range of complications associated with sclerotherapy has prompted the development of alternative endoscopic methods such as band ligation. This technique involves suctioning of a varix into the end of an endoscope so that a rubber band can be placed around the varix leading to thrombosis. Direct comparisons of endoscopic sclerotherapy and variceal ligation in adult patients have yielded results in favor of ligation. Similar results have been reported in children by Zargar *et al.* The major advantage of variceal ligation is avoidance of needle injection of varices, which appears to reduce the rate of complications. In addition, variceal ligation appears to lead to obliteration in fewer sessions and is associated with lower rate of rebleeding.

**Portosystemic Shunting:** A variety of procedures have been used to divert portal blood flow and decrease portal blood pressure: (1) Mesocaval Shunts: formed with the insertion of a graft between the superior mesenteric vein and the inferior vena cava; (2) Portacaval Shunts: formed by side-to-side anastomosis of the portal vein and the inferior vena cava; and (3) Distal Splenorenal Shunt: formed by end-to-side anastomosis of the splenic vein and the left renal vein.

The portacaval shunt diverts nearly all the portal blood flow into the subhepatic inferior vena cava. This is very effective decompressing the portal system, but also diverts a significant amount of blood from its normal hepatic metabolism, predisposing to the development

**Table 3** Results of portosystemic shunting in children<sup>[63,64]</sup>

Type of portal hypertension	No. of patients	Rebleeding	Mortality
Extrahepatic	292	45%	5%
Intrahepatic	76	50%	53%

of hepatic encephalopathy. Decreased hepatic blood flow theoretically also may lead to worsening of underlying liver disease. An intermediate shunt can be made by placing a graft between the mesenteric or portal vein and the vena cava. This decompresses the portal system while allowing a greater amount of portal blood flow into the liver. The use of grafts unfortunately is associated with increased risk of thrombosis and many times with worsening retrograde flow.

Another approach involves diversion of splenic blood flow into the left renal vein, which can be done nonselectively (central) or semiselectively (distal splenorenal shunt).

A substantial pediatric experience with surgical portosystemic shunting has been accumulated over the past 20 years. The results are clearly different in patients with extra- or intrahepatic portal hypertension (Table 3).

An alternative shunting procedure for children with extrahepatic portal vein thrombosis is the meso-Rex bypass, this procedure involves the placement of an autologous venous graft from the mesenteric vasculature to the left intrahepatic portal vein. One of the major advantages of this approach is the restoration of normal portal blood flow, which eliminates the risk of hepatic encephalopathy and should preserve hepatic function. The selection of patients for this procedure is not clear both from a clinical indication and surgical feasibility, and some have advocated that this procedure be considered in all children with portal vein thrombosis. Standard diagnostic imaging may not clearly indicate whether there is patency of the intrahepatic portal vein, and the potential in this group of children for hypercoagulable states must be kept in mind.

In stark contrast to the excellent results in portal vein thrombosis, there are generally poor results of portosystemic shunts in children with decompensated liver disease. The incidence of recurrent bleeding and death approaches 50%. Hepatic encephalopathy is a frequent and serious complication of portosystemic shunting in decompensated liver disease, and studies have failed to show improvement in long-term survival in patients with intrahepatic disease.

Overall, surgical portosystemic shunting is an excellent approach to the long-term management of children with intractable variceal bleeding in the setting of compensated cirrhosis. In addition, significant gastric variceal hemorrhage in children may be an indication to consider surgical shunting. TIPS may be an alternative shunting procedure for children with refractory bleeding and serves as an effective bridge to transplantation. The procedure is typically feasible, with published success in children as small as 14 kg, although special

procedural modifications must be undertaken in small children. Long term shunt occlusions limit the overall application of this efficacious therapy, although newer data with coated stents may improve long-term patency rates for TIPS.

## CONCLUSION

The approach to acute variceal hemorrhage in children is a stepwise progression from least invasive to most invasive. Surveillance endoscopy is predicated on the availability of an efficacious primary prophylactic therapy. Beta-blocker therapy is accepted primary prophylactic therapy in adults, and endoscopic ligation therapy is also gaining acceptance. Preliminary data in children appear to indicate that this approach is feasible, but further studies are needed before a wide-spread recommendation for children can be endorsed. Therefore, surveillance endoscopy and primary prophylaxis are not generally be indicated in children with portal hypertension who have not had a variceal bleed. Special medical and or social circumstances in which an initial bleeding episode may be particularly dangerous could justify this approach.

Management of acute variceal bleeding is more straightforward. Initial interventions should include stabilization of the patient, placement of a nasogastric tube, and institution of antibiotic therapy. Diagnostic and/or therapeutic endoscopy should be scheduled as soon as it is safe and feasible. In the interim, pharmacologic treatment with either a vasodilator or octreotide is indicated and may facilitate endoscopic therapy. Intractable and severe hemorrhage should be treated by TIPS.

The long term approach to prevention of recurrent variceal bleeding in children must be adapted for the etiology of portal hypertension, the needs of the specific patient, and the particular skills of the institution. The approach to extrahepatic portal vein obstruction is evolving. In general, the unpredictability of the timing of the sentinel bleeding episode and the low incidence of mortality associated with that episode make prophylactic therapy inadvisable. Enthusiasm for the utilization of the meso-Rex shunt is increasing because of the physiologic nature of the procedure, and should be considered for children with extrahepatic portal vein thrombosis and cavernous transformation and a normal liver. The long term management of portal hypertension in the child with biliary atresia is more complex. In patients with incomplete bile drainage, liver transplantation appears to be inevitable and should be the major focus of therapeutic intervention. Temporizing measures for these children may include band ligation therapy and TIPS. Biliary atresia patients who have a more successful response to Kasai portoenterostomy, have a more favorable long term outlook. Variceal hemorrhage may be followed by a relatively long term survival with medical intervention. Recurrent bleeding might be amenable to portosystemic shunting as opposed to transplantation.

The approach to patients with more slowly progressive intrahepatic disease is more difficult to generalize.

Well-conceived multicenter trials are required to determine whether the principals that have been developed in adults can be extrapolated to children.

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## Pros and cons of colonoscopy in management of acute lower gastrointestinal bleeding

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in the hospitalization, can decrease hospital length of stay, rebleeding and the need for surgery. However, results from available small trials are conflicting and larger, multicenter studies are needed. Compared to other management options, colonoscopy is a safe procedure with complications reported in less than 2% of patients, including those undergoing urgent examinations. The requirement of bowel preparation (typically 4 or more liters of polyethylene glycol), the logistical complexity of coordinating after-hours colonoscopy, and the low prevalence of stigmata of hemorrhage complicate the use of colonoscopy for LGIB, particularly in urgent situations. This review discusses the above advantages and disadvantages of colonoscopy in the management of acute lower gastrointestinal bleeding in further detail.

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### Abstract

Acute lower gastrointestinal bleeding (LGIB) is a frequent gastrointestinal cause of hospitalization, particularly in the elderly, and its incidence appears to be on the rise. Endoscopic and radiographic measures are available for the evaluation and treatment of LGIB including flexible sigmoidoscopy, colonoscopy, angiography, radionuclide scintigraphy and multi-detector row computed tomography. Although no modality has emerged as the gold standard in the management of LGIB, colonoscopy is the current preferred initial test for the majority of the patients presenting with hematochezia felt to be from a colon source. Colonoscopy has the ability to diagnose all sources of bleeding from the colon and, unlike the radiologic modalities, does not require active bleeding at the time of the examination. In addition, therapeutic interventions such as cautery and endoclips can be applied to achieve hemostasis and prevent recurrent bleeding. Studies suggest that colonoscopy, particularly when performed early

**Key words:** Colonoscopy; Acute lower gastrointestinal bleeding; Management; Diagnostic yield; Therapeutic intervention; Outcomes; Colon preparation; Stigmata of hemorrhage

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## INTRODUCTION

Acute lower gastrointestinal bleeding (LGIB) is a disorder frequently encountered by gastroenterologists, surgeons and internists. Hospitalization for LGIB in the 1990s was estimated to occur at an incidence of 20.5 per 100 000 person-years<sup>[1]</sup>. The incidence of LGIB may be rising concomitant with the aging of our population and the increasing use of aspirin/non-steroidal anti-inflammatory drugs and anticoagulants<sup>[2]</sup>. LGIB is associated with significant mortality, health care costs and increased length of hospitalization, emphasizing the need for effective evaluation and treatment<sup>[2-4]</sup>.

Currently, physicians managing LGIB have a number of different diagnostic and therapeutic options to choose from, ranging from radiographic interventions such as radionuclide scintigraphy and angiography to colonoscopy and flexible sigmoidoscopy. Although no modality has emerged as the gold standard in the management of LGIB, colonoscopy has several advantages and is generally regarded as the preferred initial test in the majority of cases. The advantages are as follows: (1) ability to identify bleeding source regardless of rate or presence of bleeding; (2) multiple therapeutic possibilities; (3) efficiency given diagnostic and therapeutic potential; (4) irrespective of initial testing, colonoscopy is required for definitive diagnosis; and (5) safety. Disadvantages are as follow: (1) requirement for colon preparation; (2) need for sedation, experienced staff and endoscopy facilities; (3) low prevalence of stigmata of hemorrhage; (4) invasive nature; and (5) rare but serious complications.

This article will explore the advantages and disadvantages of colonoscopy in the management of LGIB.

## PROS OF COLONOSCOPY

### **Potential for diagnosis and therapeutic intervention**

A major advantage of colonoscopy over other management options is the potential for diagnosis and therapeutic intervention even in the absence of ongoing bleeding. This is important given the often intermittent nature of LGIB and slow bleeding from diffuse mucosal sources such as colitis. In contrast, the radiographic alternatives including angiography, radionuclide scintigraphy and computed tomography (CT) require active bleeding at the time of the examination in order to identify and treat the source of bleeding, and are useful only in a subset of patients with severe ongoing bleeding. It is therefore not surprising that the diagnostic yield of colonoscopy exceeds that of the radiographic modalities.

Studies have shown a diagnosis is made in 74%-100% of patients with LGIB undergoing colonoscopy (Table 1)<sup>[5-11]</sup>. A pooled analysis by Strate *et al*<sup>[12]</sup> of six recent studies of colonoscopies following bowel preparation for LGIB found a composite diagnostic yield of 91% for colonoscopy. In comparison, in radionuclide scanning, a commonly used radiological diagnostic modality in LGIB, scans were positive in 40%-70% of patients with accuracy ranging from 35%-100%<sup>[13-17]</sup>. In small case series, multidetector

row CT scanning (MDCT) has shown promising results with localization of bleeding in 50%-100% of select cases<sup>[16,18,19]</sup>. Angiography is less sensitive than radionuclide or MDCT scan and therefore is infrequently utilized as a front line test. In a collected series of 247 patients reported by Browder *et al*<sup>[20]</sup>, angiography demonstrated a bleeding site in 72% of patients whereas a retrospective chart review study by Cohn *et al*<sup>[21]</sup> yielded only 35% positive angiographic findings. In addition, it is important to note that colonoscopy is generally required following radiographic tests, whether positive or negative, to confirm the source of bleeding and to exclude colorectal malignancy or other serious diagnoses. Therefore, colonoscopy is the most efficient initial test for the majority of patients with LGIB who can be stabilized and can undergo colon preparation.

Few studies have directly compared colonoscopy to radiographic interventions. In a study by Jensen *et al*<sup>[22]</sup>, emergency colonoscopy and upper endoscopy as well as emergency angiography were performed in 22 patients with severe bleeding. In the 17 patients with lower intestinal sources of bleeding, the diagnostic yield of colonoscopy was 82% *vs* 12% for angiography<sup>[22]</sup>. Similarly, in a retrospective study by Strate *et al*<sup>[23]</sup>, initial colonoscopy within 24 h of admission offered a diagnostic yield of 85% *vs* 45% for initial scintigraphy and angiography.

The timing of colonoscopy also may have an effect on diagnostic and therapeutic outcomes. Several studies indicate that urgent colonoscopy, or colonoscopy within 12-24 h of presentation, may improve the diagnostic and therapeutic yield of colonoscopy in LGIB. In a prospective study, Jensen *et al*<sup>[6]</sup> identified stigmata of hemorrhage in approximately 20% of patients with diverticular bleeding undergoing urgent colonoscopy. Subsequent endoscopic treatment significantly reduced the rate of rebleeding and surgery compared to historical controls. In a trial by Green *et al*<sup>[7]</sup> of colonoscopy following bowel preparation within 12 h *vs* elective colonoscopy (within 74 h), a definitive bleeding source was identified in 42% of patients undergoing urgent colonoscopy *vs* 22% in those undergoing elective colonoscopy. Most recently, in a trial of colonoscopy within 12 h *vs* colonoscopy in 36-60 h, Laine *et al*<sup>[24]</sup> found no differences in outcomes including the number of diagnoses. However, 78% of colonoscopies were diagnostic in the urgent group compared to 67% in the elective group, and the only 2 stigmata of hemorrhage were both identified in patients with diverticulosis undergoing urgent colonoscopy<sup>[24]</sup>.

For patients undergoing colonoscopy, once a diagnosis is made, endoscopic hemostasis can be carried out depending on the source. Epinephrine or saline injection, thermal contact, argon plasma coagulation, clipping and band ligation are available therapeutic modalities. A review of published series by Strate *et al*<sup>[12]</sup> showed that endoscopic therapy was applied to 10% to 40% of patients undergoing colonoscopy for LGIB and the most common intervention was thermal contact plus injection. Their review of 71 cases of diverticular bleeding treated with endoscopically placed hemoclips showed

Table 1 Comparison of management options for lower gastrointestinal bleeding (%)

Procedure	Diagnosis	Therapy	Early rebleeding	Major complications	Colon preparation	Requires active bleed
Colonoscopy <sup>[5-8]</sup>	74-100	8-37	0-24 <sup>1</sup>	0-2	Yes	No
Sigmoidoscopy <sup>[9-11]</sup>	About 10	0-20	0	Rare	Minimal	No
Angiography <sup>[20,21,26-29]<sup>2</sup></sup>	23-72	14-100	1-57	0-60	No	Yes
Radionuclide scan <sup>[13-15]</sup>	40-73	N/A	N/A	Rare	No	Yes
Multi-detector CT scan <sup>[16,18,19]</sup>	24-94	N/A	N/A	0%-11%	No	Yes

N/A: Not available; CT: Computed tomography. <sup>1</sup>Early rebleeding in patients who had undergone endoscopic therapy for diverticular bleeding; <sup>2</sup>Therapy, rebleeding and complications refer to superselective embolization only. It is difficult to determine the frequency of superselective embolization use because most series only report patients who receive therapy.

particular promise with a homeostasis success rate of 100% and with no complications.

### Improved outcomes

It is important to note that in most cases, diagnosis needs to be followed by therapeutic maneuvers in order to alter outcomes in patients with LGIB. Early colonoscopy has shown particular promise in this regard in patients with diverticular bleeding. In a study by Jensen *et al*<sup>[6]</sup>, patients undergoing endoscopic hemostasis for diverticular stigmata with epinephrine plus/minus thermocautery had significantly lower rates of rebleeding and surgery when compared to historical controls who did not receive endoscopic treatment. Indeed, none of the endoscopically treated patients experienced re-bleeding. In addition, length of hospital stay was significantly shorter in the colonoscopy treatment group ( $P < 0.001$ ).

Subsequent randomized trials for all sources of severe LGIB have yielded less positive results. However, these trials were small and the lack of significant findings may have been the result of inadequate power or other methodological issues. In the Green *et al*<sup>[7]</sup> trial, patients were randomized to urgent colonoscopy within 8 h or standard of care which was elective colonoscopy within 4 d of admission. As noted above, the diagnostic yield was superior in the urgent colonoscopy group. Other outcomes such as rebleeding, surgery and number of blood transfusions showed a trend in the favor of urgent colonoscopy but did not reach statistical significance. Also of note, only 36% of colon preparations were noted to be “excellent”, which may also have affected the efficacy of urgent colonoscopy. Laine *et al*<sup>[24]</sup> also did not find that urgent colonoscopy improved major outcomes compared to delayed colonoscopy. In addition to the small number of patients in their study (the study was stopped early with only 36 patients in each arm *vs* the planned 134 patients), patients in the urgent colonoscopy arm also appeared to have more severe bleeding which may have made it more difficult to detect a favorable difference.

A number of observational studies indicate that urgent colonoscopy reduces hospital length of stay. In a retrospective study of patients admitted with LGIB, Strate *et al*<sup>[25]</sup> looked at the impact of time to colonoscopy on hospital length of stay in patients presenting with LGIB. They found that earlier performance of colonos-

copy was associated with a shorter length of stay [hazard ratio (HR) 2.02, 95% CI: 1.5-2.6,  $P < 0.0001$ ] even when adjusted for other factors. Similarly, Schmulowitz *et al*<sup>[30]</sup> also showed that having a colonoscopy was associated with reduced hospital length of stay (HR 1.54, 95% CI: of 1.2-1.8), and mean length of hospital stay was significantly shorter in patients undergoing colonoscopy within 24 h compared to more than 24 h (5.4 d *vs* 7.2 d,  $P < 0.008$ ). Since hospital days are a major source of charges in LGIB this could have a significant economic impact. In a cost analysis study, Jensen *et al*<sup>[31]</sup> estimated that urgent colonoscopy was associated with an average savings of \$10 065 per patient compared with medical, angiography and surgical management; although this number likely overestimates the degree of cost savings due to other trends in care such as shorter hospital stay, and a recent randomized trial found no difference in hospital charges in patients undergoing urgent *vs* elective colonoscopy<sup>[24]</sup>. Overall, colonoscopy, particularly when performed early in the hospital course, has the potential to improve outcomes such as rebleeding, surgery and length of stay, but larger randomized trials are needed to better define its efficacy.

### Safety

Colonoscopy with or without intervention for LGIB is considered a safe procedure. In a review of 4 studies with 664 patients, there were 2 perforations for an overall complication rate of 0.3% for colonoscopy and 0.6% for colonoscopy performed urgently<sup>[12]</sup>. Other potential risks in addition to bowel perforation include congestive heart failure secondary to volume overload, electrolyte abnormalities and aspiration pneumonia. Also of note, in a review of 137 cases of endoscopic treatment for diverticular hemorrhage using a variety of modalities, there were no reported complications<sup>[12]</sup>. Complications are noted more frequently in patients undergoing angiography. The risks of bowel ischemia and cardiac arrhythmia seen with vasopressin infusions have been ameliorated with newer superselective embolization techniques. However, in a review of 20 studies utilizing superselective embolization for control of LGIB, minor complications were seen in 26% of patients and major complications requiring surgery or resulting in death were seen in 17%<sup>[12]</sup>. Another significant concern for both angiography and MDCT is renal compromise

secondary to contrast dye load. In the small amount of literature on MDCT there have been 2 reports of renal failure in patients with diabetes who also underwent angiography<sup>[19]</sup>.

## CONS OF COLONOSCOPY

### **Colon preparation**

Cleansing the colon of stool and blood prior to colonoscopy for management of LGIB is imperative, difficult to accomplish, and unique to endoscopic interventions for LGIB. Colonoscopy preparation is necessary for complete examination to the cecum. Studies of unprepped colonoscopy in LGIB report examinations to the cecum in only 55%-70% of cases<sup>[32-34]</sup>. Unprepped colonoscopy may increase the risk of perforation due to poor visualization. The identification of subtle bleeding sites, which are often among multiple lesions as is the case in diverticular hemorrhage, the most common source of LGIB, is contingent on removal of excess debris. In a prospective study by Jensen *et al*<sup>[6]</sup> on urgent colonoscopy in severe diverticular bleeding, active bleeding was detected in 21% of cases in the urgent colonoscopy arm and endoscopic hemostasis was achieved in 100% of the cases. The excellent results may have been due, in part, to their aggressive bowel preparation regimen. This regimen required 5-6 liters of sulfate purge over 3-4 h with 33% receiving the purge through a nasogastric tube. However, achieving consistently excellent bowel preparations, particularly in the urgent setting for LGIB, is difficult. The 4 or more hour time frame required for preparation may delay colonoscopy examinations until after-hours when nursing support and endoscopic facilities are not available. Thus, colon preparation makes urgent colonoscopy logistically complicated. Even under highly regulated study situations, colon preparations have been suboptimal in a high percentage of patients. In Green *et al*<sup>[7]</sup>'s study, endoscopic view was rated poor to fair in 62%-64% of patients (62% in elective arm and 64% in urgent arm). Laine *et al*<sup>[24]</sup> did not comment on the quality of the colon prep in their study but did report that 7% of their patients required a second colonoscopy due to poor bowel prep (2 in the urgent arm and 3 in the elective arm). In these two studies poor colon preparation has been cited as a reason for the lack of significant findings in favor of the colonoscopy arm<sup>[35,36]</sup>.

As mentioned above, aggressive bowel preparation requires a large volume of bowel preparation, often up to 6-8 liters. Rarely, this can lead to volume overload and electrolyte abnormalities such as hyponatremia<sup>[37]</sup>. There is also the risk for aspiration, especially when the prep is rapid or the patient is at risk for aspiration. The need for colon preparation makes colonoscopy for LGIB more complicated than esophagogastroduodenoscopy (EGD) for upper gastrointestinal bleeding, and is likely one of the main reasons it has not been equally embraced. Also, the need for colon prep clearly sets colonoscopy for management of LGIB apart from radiological interven-

tions for LGIB.

### **Sedation, experienced staff and procedure facilities**

Colonoscopy generally requires sedation given by trained professionals in a monitored location such as an intensive care unit or an endoscopy suite. Specialized support staff is also helpful to aid the gastroenterologist with the colonoscopy. These factors add to the complexity of coordinating the procedure, particularly on nights and weekends. However, angiography also requires sedation, an interventional radiologist, support staff and a specialized procedure suite. Indeed, in their study of utilization of early colonoscopy *vs* radiography in severe lower intestinal bleeding, Strate and Syngal found that the median times from admission to colonoscopy or angiography were similar (median of 17 h for colonoscopy *vs* 14 h for angiography)<sup>[23]</sup>.

### **Urgent vs delayed performance of colonoscopy**

The optimal timing of colonoscopy remains an area of controversy. As noted above, some studies suggest that performing colonoscopy within the first 12-24 h of hospital admission aids in the identification and treatment of the bleeding source. However, the two published randomized controlled trials of LGIB showed no difference in important clinical outcomes such as rebleeding and surgery<sup>[7,24]</sup>. Unfortunately, none of these studies was adequately powered to detect a statistically significant difference in important clinical outcomes, and the utility of urgent colonoscopy remains uncertain. The conflicting, albeit flawed, evidence paired with the practical issue of colon preparation make it difficult for clinicians to embrace urgent colonoscopy. Based on available literature, there is no specific time threshold for colonoscopy, but rather colonoscopy should be performed after a thorough bowel preparation, and more urgently in patients with signs of significant or ongoing bleeding as stigmata are rarely identified in delayed examinations<sup>[7,24]</sup>.

### **Low prevalence of stigmata of hemorrhage**

The identification and treatment of the bleeding source is the goal of urgent interventions for GI bleeding particularly when severe. Diagnostic interventions alone are unlikely to alter significant outcomes such as rebleeding and need for surgery. However, stigmata are infrequently identified in the colon. Studies report stigmata of hemorrhage in 7.7% to 43% of cases<sup>[5,30]</sup>. The colon has a large and complex surface area, often with multiple potential sources. In addition, bleeding tends to be intermittent in nature and it can be difficult to differentiate fresh blood from old blood and stool. Therefore, the effort entailed in orchestrating an urgent colonoscopy may be perceived as great in relation to the gain. Decision aids and predictive models have the potential to improve the utility of urgent colonoscopy for LGIB by identifying patients who are most likely to have ongoing bleeding and stigmata of hemorrhage and hence benefit from intervention. Three studies to date have aimed at

identifying high risk patients with LGIB with reasonable accuracy<sup>[38-40]</sup>. However, the use of these tools in routine practice has not been studied and they have not been widely embraced.

In addition to the low prevalence of stigmata, a substantial subset of patients (up to 20%) presenting with hematochezia and presumed LGIB will ultimately be found to have bleeding from the upper or small bowel<sup>[22,24]</sup>. An EGD or nasogastric lavage is necessary in briskly bleeding patients to begin to exclude these possibilities. In small bowel bleeding, colonoscopy and upper endoscopy serve only to exclude these areas of the bowel as bleeding sources, and these patients generally require a number of procedures for diagnosis<sup>[41]</sup>.

### Complications

As mentioned earlier, the complication rate of colonoscopy is low, 0.3% and 1.3% in two comprehensive reviews<sup>[12,42]</sup>. Safety is generally thought of as a relative strength of colonoscopy over other modalities. Radionuclide scanning and CT scanning are the only noninvasive option and these modalities do not provide therapeutic opportunities. However, complications of colonoscopy when they occur can be severe. Colon perforation is the most common complication and generally requires urgent surgical intervention. Other complications are mainly due to colon preparation and can be minimized with proper and careful administration of colon purge. For example, rapid preps should be avoided in patients with altered mental status, difficulty swallowing, delayed gastric emptying or bowel obstruction. Fleets Phosphosoda has been associated with renal compromise and is no longer available. Balanced electrolyte solutions are considered safer and less likely to result in fluid and electrolyte shifts<sup>[43]</sup>. However, there have been case reports of patients with decreased left ventricular systolic function experiencing exacerbation of their symptoms, thought to be secondary to fluid shifts in the setting of bowel preparation with polyethylene glycol (Golytely)<sup>[44]</sup>. These patients should hence be monitored closely while undergoing their prep.

### CONCLUSION

Colonoscopy offers many advantages in the management of acute lower gastrointestinal bleeding. All sources and severities of LGIB can be diagnosed with colonoscopy and the overall diagnostic yield of this procedure is high. Endoscopic hemostasis can be utilized to stop or prevent bleeding, and therefore colonoscopy offers the potential to improve important clinical outcomes such as rebleeding, although the data from existing small studies are conflicting. The need for bowel preparation, the logistical difficulty of coordinating the procedure after-hours and the infrequent identification of stigmata of hemorrhage deter the widespread use of urgent colonoscopy in LGIB. However, most of these difficulties also apply to other modalities such as angiography and radio-

nuclide scan. Albeit imperfect, current data indicate that colonoscopy offers more advantages than other management options and should be the initial modality in the majority of patients with LGIB. Further prospective randomized studies are needed to more clearly define the optimal timing of colonoscopy in LGIB and its role relative to other available options including radionuclide scanning, angiography and MDCT scanning.

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## Angiographic evaluation and management of acute gastrointestinal hemorrhage

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### Abstract

Although most cases of acute nonvariceal gastrointestinal hemorrhage either spontaneously resolve or respond to medical management or endoscopic treatment, there are still a significant number of patients who require emergency angiography and transcatheter treatment. Evaluation with noninvasive imaging such as nuclear scintigraphy or computed tomography may localize the bleeding source and/or confirm active hemorrhage prior to angiography. Any angiographic evaluation should begin with selective catheterization of the artery supplying the most likely site of bleeding, as determined by the available clinical, endoscopic and imaging data. If a hemorrhage source is identified, superselective catheterization followed by transcatheter microcoil embolization is usually the most effective means of successfully controlling hemorrhage while minimizing potential complications. This is now well-recognized as a viable and safe alternative to emergency surgery. In selected situations transcatheter intra-arterial infusion of vasopressin may also be useful in controlling acute gastrointestinal bleeding. One must be aware of the various side effects and potential complications associated with this treatment, however, and recognize the high re-bleeding rate. In this article we

review the current role of angiography, transcatheter arterial embolization and infusion therapy in the evaluation and management of nonvariceal gastrointestinal hemorrhage.

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**Key words:** Angiodysplasia; Aneurysm; Digital subtraction angiography; Contrast media; Hemorrhage; Radio-nuclide angiography; Therapeutic embolization

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### INTRODUCTION

Most cases of acute nonvariceal gastrointestinal hemorrhage resolve spontaneously and of those that do not, the majority respond to conservative medical management measures such as fluid resuscitation, correction of any coagulopathy and administration of blood products<sup>[1]</sup>. In those cases that are refractory to medical management, endoscopy is the mainstay for diagnosis and treatment. However, there is a subset of patients in whom endoscopic management is ineffective, and imaging evaluation with an accompanying alternative intervention is necessary for bleeding control because

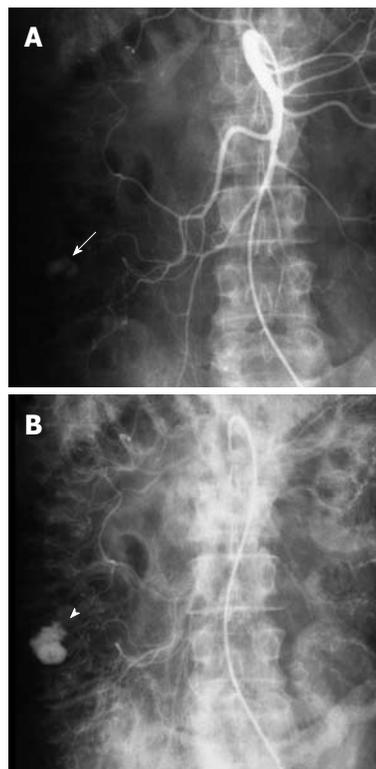
of recurrence or massive hemorrhage<sup>[1]</sup>. Localization and characterization of the bleeding source are important in determining the appropriate intervention, as treatment options range from minimally invasive catheter-directed therapy to extensive surgical resection. Although there has generally been a decline in the number of patients who present with acute gastrointestinal hemorrhage requiring angiography and/or transcatheter intervention, there are still patients who are unresponsive to either medical or endoscopic management and thus require emergency angiographic evaluation and possible transcatheter treatment.

The alimentary system is subdivided into the upper and lower gastrointestinal tracts and thus gastrointestinal hemorrhage is subcategorized according to the location of bleeding. The upper gastrointestinal system extends from the esophagus to the ligament of Treitz, while the latter includes the small bowel, colon and rectum. The distinction is important, as there are some characteristics that are relatively unique to each location and these may affect and determine the therapeutic approach to the particular bleeding source. Thus establishing the specific location of the bleeding as well as the etiology is critical to the treatment of patients with massive or recurrent gastrointestinal hemorrhage. Unfortunately the diagnosis can often be challenging because of the intermittent nature of gastrointestinal bleeding.

## PATIENT EVALUATION AND MANAGEMENT

The initial management of a patient with acute gastrointestinal hemorrhage should be directed at stabilization through administration of fluids and blood products, correction of coagulation abnormalities, placement of appropriate intravenous access lines and insertion of a nasogastric tube if needed. The vital signs should be closely monitored for signs of active bleeding that may manifest as tachycardia, hypotension and potential hypoxemia. Although persistent melena, hematochezia or hematemesis may provide direct clinical evidence of active hemorrhage, hemodynamic instability despite vigorous resuscitation is the best indicator of active bleeding that may be angiographically demonstrable.

Acute gastrointestinal hemorrhage far more frequently involves an upper than a lower gastrointestinal source, with the former most commonly due to either peptic ulcer disease or gastritis. The most common etiology of lower gastrointestinal hemorrhage, in young adults, is inflammatory bowel disease, while in patients older than 50 years, the cause is diverticulitis (Figure 1) and to a lesser extent, angiodysplasia. The colon is the source of lower gastrointestinal hemorrhage in 80% of individuals, with the bleeding site originating in the ascending colon in one-third, the transverse colon in another one-third, and the remainder in the descending colon and recto-sigmoid. A small bowel source of lower gastrointestinal hemorrhage occurs in only 20% of patients.



**Figure 1** Example of a bleeding colonic diverticulum. A: Arterial phase of a superior mesenteric artery arteriogram, obtained in a patient with acute lower gastrointestinal (GI) bleeding and a prior history of diverticulitis shows a rounded contrast collection (white arrow) arising from a branch of the right colic artery; B: In the later arterial phase the collection (white arrowhead) has increased in size but maintains the rounded configuration. The extravasated contrast medium is pooling in a colonic diverticulum, indicating that diverticular hemorrhage is the etiology of the lower GI bleeding.

If an upper gastrointestinal bleeding source seems most likely, endoscopy should be the initial diagnostic study, as the source can usually be identified and treated by the endoscopist. Unfortunately the endoscopic detection and treatment success rates in patients with lower gastrointestinal hemorrhage are generally less than with an upper source, particularly if rapid and significant bleeding is present.

## IMAGING PRIOR TO ANGIOGRAPHY

If a bleeding site cannot be identified endoscopically, or if a catheter-based intervention is being considered as a treatment option, obtaining imaging studies may be helpful. In order to successfully demonstrate bleeding by angiography, there must be active ongoing hemorrhage at the time of the examination. Bleeding rates of 0.5-1.0 mL/min have traditionally been considered necessary in order to angiographically demonstrate contrast extravasation<sup>[2]</sup>, but digital subtraction angiography (DSA) may be far more sensitive in detecting active extravasation than was previously thought<sup>[3]</sup>. Given the well-recognized intermittent nature of gastrointestinal bleeding, many advocate obtaining other imaging before proceeding directly to angiography unless there is massive ongoing



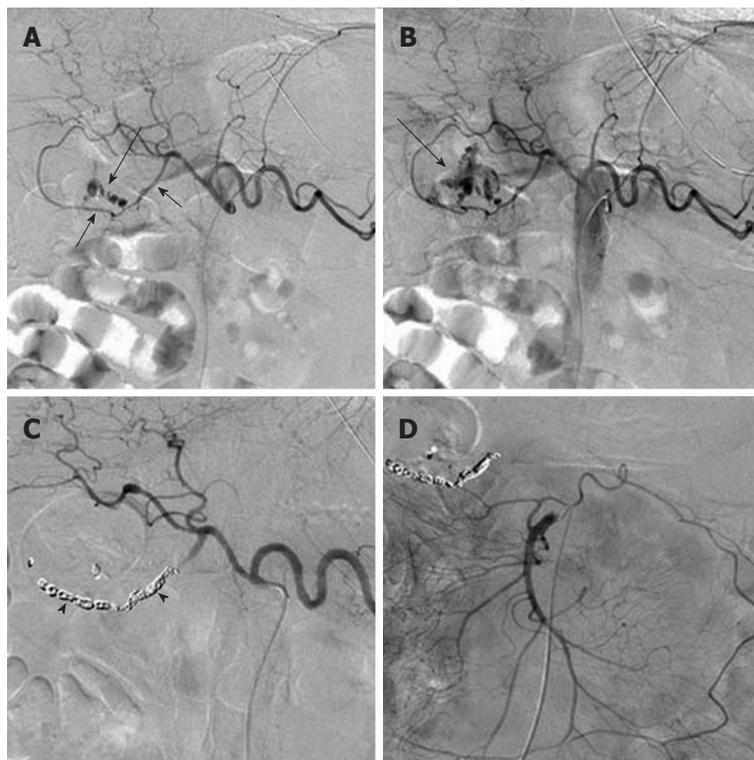
**Figure 2** A radionuclide Tc<sup>99m</sup>-labeled red blood cell scan obtained in a patient with acute lower gastrointestinal hemorrhage shows radioisotope accumulation in the left abdomen at 25 min (black arrow), which by 35 min has a small bowel location (black arrowhead) and by 50 min has distributed throughout much of the small intestine (short black arrows). The study demonstrates that the bleeding source is in the small bowel, and thus serves to appropriately direct either angiography or surgery.

hemorrhage or the patient is hemodynamically unstable. Thus a patient may be first evaluated with a radionuclide technetium<sup>99m</sup>-tagged red blood cell scan, as the nuclear scintigraphy study can demonstrate active bleeding at rates as low as 0.1 mL/min<sup>[4-6]</sup>. An arteriogram can be obtained following a positive bleeding scan (Figure 2), as a positive scintigram increases the likelihood of a positive angiogram from 22% to 53%<sup>[7]</sup>. The potential disadvantage of first obtaining a radionuclide study while there is clinical evidence of active bleeding is that prior to angiography the bleeding may cease during the delay necessitated by the nuclear medicine scan. A potentially useful algorithm to consider is to immediately perform angiography in hemodynamically unstable patients, and to first obtain nuclear medicine imaging in hemodynamically stable patients. This approach may potentially decrease the negative angiography examination rate.

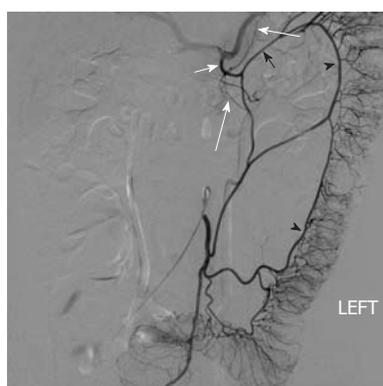
## ANGIOGRAPHIC EVALUATION OF ACUTE GASTROINTESTINAL HEMORRHAGE

Any angiographic evaluation of a patient with acute gas-

trointestinal hemorrhage should begin with the selective catheterization of the artery supplying the most likely site of bleeding, as determined by the available clinical, endoscopic and imaging data. Thus for suspected upper gastrointestinal hemorrhage, the celiac artery should first be evaluated (Figure 3), followed by the superior mesenteric artery (SMA), as the latter may contribute to a site of upper gastrointestinal hemorrhage through the pancreaticoduodenal arcade. The lower gastrointestinal tract, however, is the primary territory within the distribution of the SMA. It supplies the small bowel and the ascending and transverse portions of the colon, while the inferior mesenteric artery (IMA) supplies the splenic flexure, descending and sigmoid colonic segments as well as the rectum and anus. An additional arterial supply to the rectosigmoid and anus arises from the internal iliac arteries and this may become a dominant vascular pathway if there is IMA occlusion. The SMA becomes another collateral pathway in the presence of IMA occlusion and may provide the entire arterial supply to the descending and sigmoid colon *via* either the Arc or Riolan or the marginal artery of Drummond. One should be aware of the numerous mesenteric circulatory variations that may



**Figure 3** Angiographic diagnosis and transcatheter treatment of duodenal hemorrhage. A: Celiac digital subtraction angiography (DSA) arteriogram obtained in a patient with copious bleeding seen endoscopically in the duodenum shows focal contrast extravasation (black arrow) arising from the gastroduodenal artery (GDA); B: An image slightly later in the arterial phase of the DSA shows increasing extravasation (black arrow); C: The GDA was successfully coil embolized using microcoils (black arrowheads) through a microcatheter; D: An superior mesenteric artery DSA arteriogram was performed after the coil embolization in order to exclude any additional contribution to the duodenal hemorrhage from the pancreaticoduodenal arcade, as the duodenum has a rich collateral blood supply.

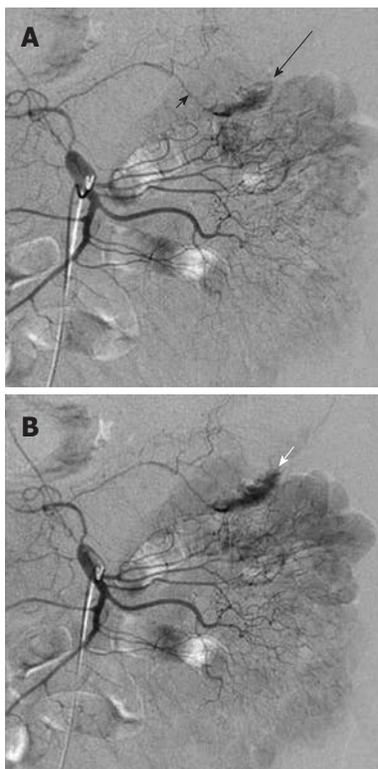


**Figure 4** Inferior mesenteric artery digital subtraction angiography arteriogram shows the marginal artery of Drummond (black arrowheads), which courses along the mesenteric border of the colon. There is retrograde filling of the left branch of the middle colic artery (black arrow), which arises from the dorsal pancreatic artery (white arrow) as an anatomic variant. There is also filling of pancreatic branches (long white arrows) along with partial opacification of the splenic and hepatic arteries.

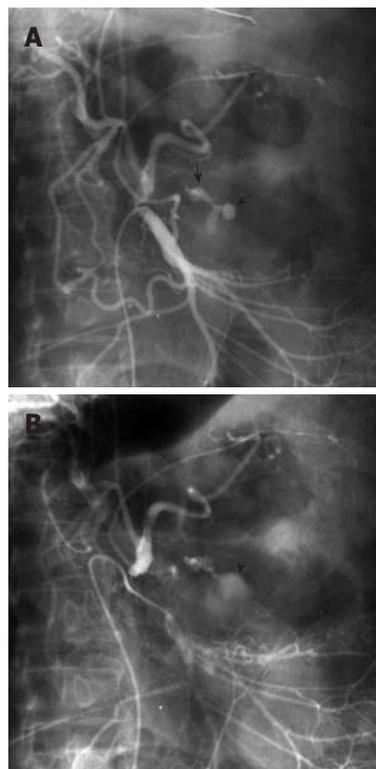
occur in the presence of occlusive disease. Furthermore, congenital variant vascular anatomy must be considered during the angiographic evaluation of gastrointestinal bleeding. For example, the entire middle colic artery may originate from the dorsal pancreatic artery in up to 2% of patients (Figure 4). If this situation exists, a celiac arteriogram may be required when investigating lower gastrointestinal bleeding, in order to fully evaluate the

colonic arterial supply, particularly in the presence of negative SMA and IMA arteriograms. If SMA, IMA, celiac, and internal iliac arteriography fail to either localize active hemorrhage or to demonstrate all the mesenteric vascular segments, then variant anatomy must be considered. There are known anomalous vessels that may arise directly from the abdominal aorta, such as an anomalous ileocolic artery or a middle mesenteric artery.

The classic angiographic finding denoting active gastrointestinal bleeding is extravasation of contrast material (Figure 5). Extravasation signifies a breach in the integrity of the arterial wall that permits the angiographic contrast to freely exit from the vessel. Extravasated contrast, therefore, does not have the typical tubular appearance of a vascular structure but instead distributes irregularly and often without a pattern. It may sometimes pool within the gastric rugae or within bowel folds or haustra so that the contrast assumes the appearance of a vein, the “pseudo-vein sign” (Figure 6). This may be differentiated from a true venous structure by the unusual location and appearance, as well as by the persistence beyond the venous phase of the contrast injection. Extravasation must also be differentiated from entities that mimic its appearance, such as a hypervascular bowel mucosa, adrenal gland vascular blush and DSA misregistration artifacts from bowel peristalsis or respiratory motion. There are also angiographic findings other than contrast extravasation that may be seen in certain patho-



**Figure 5 Contrast extravasation demonstrating location of lower gastrointestinal bleeding.** A: Superior mesenteric artery arteriogram shows an amorphous contrast collection (black arrow) arising from the left branch (long black arrow) of the middle colic artery; B: Later in the arterial phase the collection has increased and is layering dependently in the colon, assuming the configuration of the haustra. This extravasated contrast medium denotes the site of lower gastrointestinal bleeding.



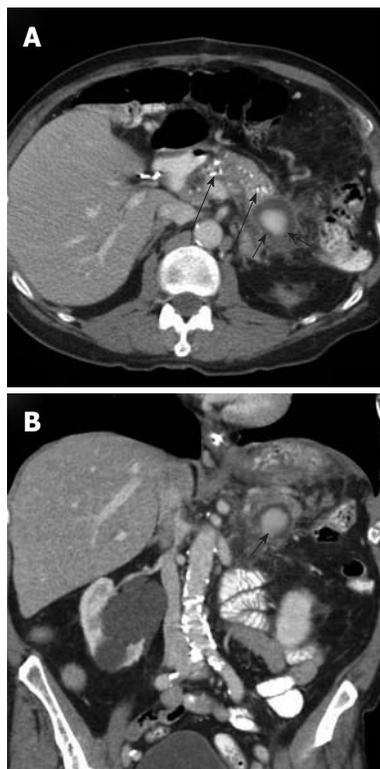
**Figure 6 The pseudovein sign in gastrointestinal hemorrhage.** A: Superior mesenteric artery arteriogram shows a branching contrast collection (black arrows), overlying the gastric air shadow. The collection has the appearance of a vascular structure such as a vein; B: A later image in the arterial injection shows that the collection (black arrowhead) has a more amorphous appearance, and represents extravasation. The former appearance is an example of the "pseudovein sign".

logic conditions and are suggestive of the cause and/or source of the gastrointestinal bleeding. In peptic ulcer disease, for example, small contrast collections may be seen within an ulcer crater, or may outline the gastric or duodenal mucosa. The angiographic demonstration of a gastric ulcer usually requires subselective catheterization of celiac arterial branches such as the left gastric artery, although the bleeding source may also potentially arise from the right gastric artery, the short gastric artery, or either the left or right gastroepiploic arteries. Additionally, the gastroduodenal artery may supply a bleeding pyloric or duodenal ulcer.

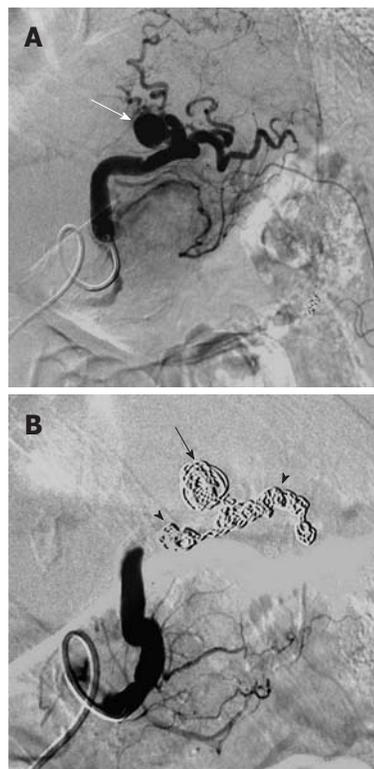
Arterial pseudoaneurysm is another angiographic abnormality that may be identified as a source of gastrointestinal hemorrhage. These occur most frequently in patients who have chronic pancreatitis. Hemosuccus pancreaticus refers to bleeding through the pancreatic duct, and may be caused by an arterial pseudoaneurysm that has resulted from chronic exposure of the arterial wall to the inflammatory effects of the pancreatic digestive enzymes<sup>[8]</sup>. The weakened arterial wall may permit the intermittent bleeding that characterizes hemosuccus pancreaticus, or the pseudoaneurysm may catastrophically rupture and cause acute massive, life-threatening intra-abdominal hemorrhage. Contrast enhanced computed tomographic angiography can be very effective in demonstrating these

characteristic pseudoaneurysms (Figure 7) and thus has an increasingly important role in the diagnosis of acute gastrointestinal hemorrhage that is secondary to pancreatitis, while angiographic transcatheter therapy provides the best treatment option in these patients (Figures 8 and 9). Given both the diffuse nature of the inflammatory process seen in pancreatitis, and the pancreatic arterial supply from both the celiac artery and SMA, pseudoaneurysms may be present involving either or both of these arteries or their branches. The angiographic evaluation must thus include both the celiac and SMA and transcatheter treatment may involve multiple sites. Surgical intervention is very difficult in these patients because of the extensive inflammatory process that characterizes this cause of bleeding (Figure 10).

Arterial pseudoaneurysms may also be seen in patients with chronic occlusive disease of the celiac artery, and involve the pancreaticoduodenal arteries (Figure 11). These pseudoaneurysms are rupture-prone and may cause massive acute upper gastrointestinal hemorrhage. Because of the occlusion of the origin of the celiac artery, the angiographic evaluation involves the selective catheterization of the SMA, in order to image the pancreaticoduodenal arcade. Any intervention, such as coil embolization, must typically also be performed *via* an SMA access, although there are cases in which a percutaneous route has been



**Figure 7** Upper gastrointestinal hemorrhage from pancreatitis related pseudoaneurysm. A: Axial contrast-enhanced computed tomography (CECT) scan, obtained in a patient who presented with hematemesis, shows pancreatic calcifications (long black arrows) indicating chronic pancreatitis and an enhancing mass (black arrows) in the pancreatic tail; B: Coronal CECT shows the rounded enhancing mass (black arrow) with surrounding inflammatory changes. This is suspicious for a pancreatitis-related pseudoaneurysm as the source of the upper gastrointestinal hemorrhage.



**Figure 8** Transcatheter treatment of a pancreatitis related pseudoaneurysm. A: Selective splenic artery digital subtraction angiography arteriogram shows that the pseudoaneurysm (white arrow) arises directly from the splenic artery; B: Coil embolization of the pseudoaneurysm (black arrow) and of the splenic artery (black arrowheads) both proximal and distal to the pseudoaneurysm was successful in controlling the hemorrhage. Arterial pseudoaneurysms associated with pancreatitis have a significant risk of rupture; if this occurs there is an extremely high mortality rate.

used to treat such an abnormality.

Angiodysplasia and arteriovenous malformations (AVMs) of the bowel are characterized by the early and prolonged opacification of a draining vein, accompanied by an abnormal tangle of vessels that may appear as a blush (Figure 12). The simultaneous filling of the feeding artery and the draining vein may give a characteristic “tram-track” sign. Although up to 80% of angiodysplastic lesions are in the right colon, other parts of the gut, particularly the lower small intestine, may be affected. Angiodysplasia is relatively common in older patients, aged 60 to 80 years, and may be an asymptomatic incidentally noted lesion identified at colonoscopy. Because the dilated vessels are superficial, however, they may bleed spontaneously and patients can either present acutely with overt hemorrhage or insidiously with iron deficiency anemia. Once bleeding has begun, recurrent episodic hemorrhage or persistent iron deficiency anemia requiring repeated transfusion is not uncommon. Symptomatic lesions may be effectively treated endoscopically with laser or heat coagulation or with sclerotherapy. These procedures are not risk-free in the thin cecal wall and have been known to cause serosal irritation and post-treatment bleeding. For patients with repeated bleeding from intestinal vascular malformations,

pharmacological treatment using estrogens (e.g., 0.05 mg ethinyl estradiol and 1 mg norethisterone) can be effective in reducing transfusion requirements. Although transcatheter arterial embolization of colonic angiodysplasia can be an effective treatment in emergencies<sup>[8-11]</sup>, it carries a significant risk of inducing colonic ischemia<sup>[12]</sup>. Definitive and curative treatment usually requires surgical resection.

## ANGIOGRAPHIC MANAGEMENT OF ACUTE GASTROINTESTINAL HEMORRHAGE

### ***Transcatheter arterial embolization***

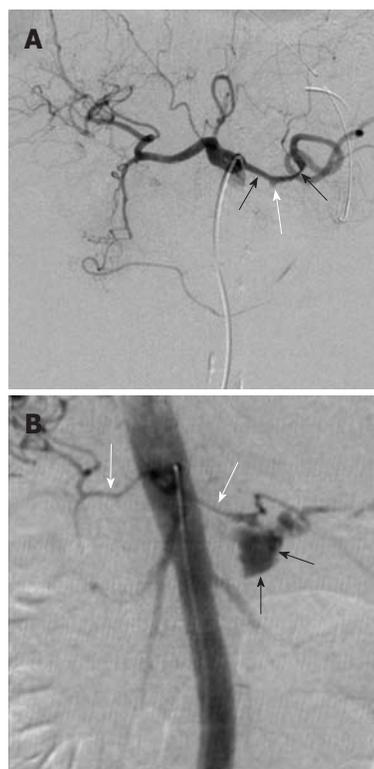
Transcatheter arterial embolization is an effective means of interrupting blood flow to the bleeding source, while maintaining bowel viability. Although there is a risk of bowel ischemia and/or infarction, the coaxial catheter systems and the variety of available embolic agents that are now used for embolotherapy allow for very selective and precise treatment, and thus have decreased the incidence of these complications. Additionally, the gastrointestinal tract has a rich collateral blood supply, with extensive vascular arcades that permit safe embolization



**Figure 9 Treatment of ruptured pseudoaneurysm with covered intravascular stent.** A: Digital subtraction angiography arteriogram obtained in a patient with upper gastrointestinal bleeding and a history of pancreatitis shows irregular dilatation of the proximal superior mesenteric artery (black arrows) and contrast extravasation (black arrowhead) consistent with pseudoaneurysm rupture; B: The hemorrhage was successfully controlled with covered stent (white arrows) placement.

if certain principles are observed. The objective of embolization therapy is to achieve a compromise between selective arterial inflow reduction and maintenance of collateral arterial blood flow. Arterial inflow must be sufficiently decreased to allow for hemostasis, but not to an extent that causes complete devascularization. Achieving a superselective embolization<sup>[11]</sup> in the presence of an intact coagulation cascade is key to attaining a successful outcome using this form of treatment.

In most of the early literature, embolization was performed proximally in the visceral arteries because microcatheters facilitating superselective embolization were not yet available. The technique that is currently used for transcatheter arterial embolization involves the initial placement of a larger caliber diagnostic catheter (4 or 5 Fr) into the main trunk of the feeding artery, followed by coaxial introduction of a microcatheter. The latter is the crucial step in modern embolization and requires the superselective catheterization of the target artery using a 3-Fr coaxial microcatheter over a 0.018 inch or 0.014 inch guidewire. The guidewire tip must be carefully advanced under fluoroscopic monitoring in a smooth and controlled fashion in order to avoid vasospasm, dissection, or vessel perforation. Vasodilators such as verapamil (100-200  $\mu$ g) or nitroglycerin (100-300  $\mu$ g) may be used to treat any vasospasm that may be induced by

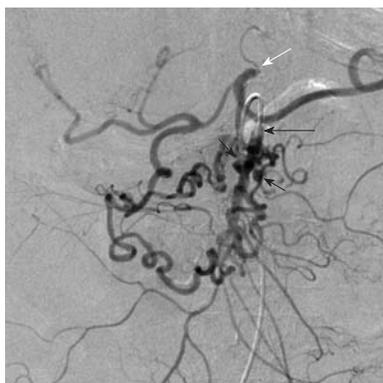


**Figure 10 Life-threatening hemorrhage from pancreatitis related pseudoaneurysm.** A: Celiac digital subtraction angiography arteriogram obtained in a patient with intermittent upper gastrointestinal bleeding and a history of chronic pancreatitis shows an irregular caliber to the splenic artery (black arrows) and a small pseudoaneurysm (white arrow). No intervention was performed at the time of this examination; B: Two weeks after the celiac arteriogram the patient presented with acute onset of severe abdominal pain and profound hypotension. Repeat angiography showed brisk contrast extravasation (black arrows) from the splenic artery. Note the marked vasoconstriction (white arrows) of the hepatic and splenic arteries due to the life-threatening hemorrhage.

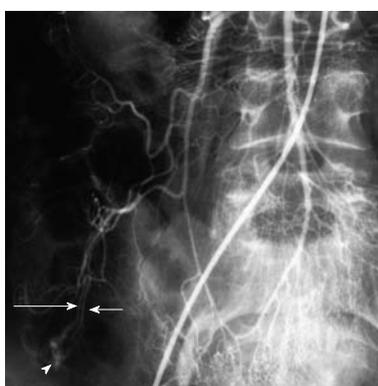
guidewire and/or microcatheter passage. Using the wire as a guide, the catheter should travel closely behind and eventually engage the target vessel.

The optimal level of embolization varies according to the bleeding site. In general, embolization should not be routinely attempted unless a microcatheter has been advanced close to the bleeding point so that the embolic agent can be deployed as selectively as possible. The risk of infarction is related to both the embolic agent and the proximity of embolization. In the colon, for example, ischemia and infarction may result from embolization of proximal branches supplying a large area of bowel or embolization of multiple distal arteries that do not have sufficient collateral flow. Submucosal collateral blood flow may be preserved only when arteries to a short segment of bowel are embolized, so one should attempt to occlude arteries to as limited a segment of bowel as possible. One must be aware that embolization in the setting of prior gastrointestinal surgery or radiation therapy may impose a greater risk of infarction because of the associated interruption of collaterals.

Various agents may be used for transcatheter embolization. The most commonly used agents include pledgets of absorbable gelatin sponge (Gelfoam<sup>®</sup>, Pfizer, Inc., NY,



**Figure 11** Digital subtraction angiography obtained with the catheter tip (long black arrow) in the superior mesenteric artery shows tortuous and dilated inferior pancreaticoduodenal arteries with at least two small pseudoaneurysms (black arrows). There is retrograde filling of the celiac arterial branches via the inferior pancreaticoduodenal arteries arcade, because the celiac origin is occluded (white arrow). The pseudoaneurysms are at risk of rupture.



**Figure 12** Magnified view from a superior mesenteric artery arteriogram shows an area of abnormal vascularity (white arrowhead) in the cecal area, with an early draining vein (long white arrow) paralleling the arterial inflow (white arrow). This simultaneous filling of the feeding artery and draining vein is known as the “tram-track” sign and is characteristic of angiodysplasia.

United States), particulate agents such as polyvinyl alcohol (e.g., Bead Block™, Biocompatibles International, Farnham, United Kingdom) and other spherical agents (e.g., Embospheres® BioSphere Medical, Inc., Rockland, MA, United States; Embozene™ microspheres Celonova BioSciences, Inc., Newnan, GA, United States) and microcoils of various sizes and configurations. Microcoils have the advantage of good radiopacity that allows for a precise deployment permitting reduction of the arterial perfusion pressure to the bleeding site while preserving sufficient collateral flow. The wide range of coil sizes allows one to appropriately match the coil to the target vessel diameter. Each microcoil is delivered sequentially, until hemostasis has been achieved. Intra-arterial microcoil placement is analogous to placement of a surgical ligature. The coil physically occludes the vascular lumen and causes a decreased perfusion pressure, while the attached synthetic fibers maximize thrombogenicity.

Gelfoam pledgets and particulate agents may also be used successfully, but are more difficult to control than

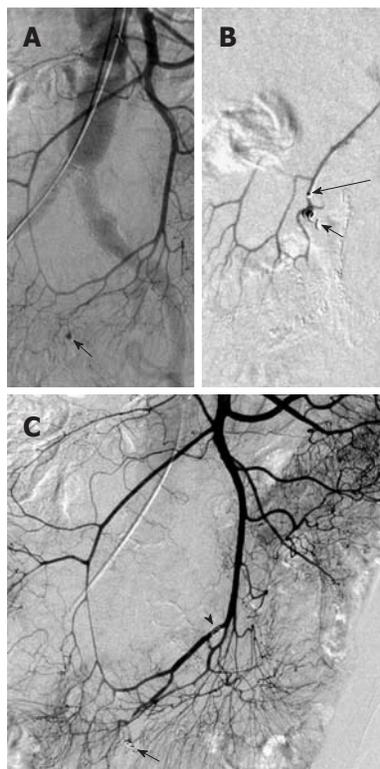
microcoils. Gelfoam is a temporary agent and often cannot easily be deployed superselectively. A disadvantage of the particulate agents is that small diameters may reach the intramural circulation distal to the collaterals, thereby risking bowel infarction, or may reflux into non-target arteries.

The liquid embolic agents n-butyl cyanoacrylate, known by the proprietary name Trufill® (Cordis Neurovascular, Miami Lakes, FL, United States), and liquid polyvinyl alcohol copolymer (Onyx®, ev3 Neurovascular, Irvine, CA, United States) have also been used successfully in treating gastrointestinal hemorrhage<sup>[13,14]</sup>. An advantage of liquid agents is that they may be used effectively in very small caliber vessels. However, the operator must be very familiar with the use of these agents, in order to achieve optimal outcomes and minimize complications.

### **Technical challenges of transcatheter arterial embolization**

Because of the intermittent nature of gastrointestinal hemorrhage, arteriography fails to demonstrate a distinct bleeding site in a considerable number of patients, and thus embolization is not possible. Furthermore, a negative arteriogram fails to guide emergency surgery and delays operative decision-making. In such circumstances, some investigators have advocated provocation of bleeding with vasodilators, anticoagulants, and/or thrombolytics in association with tagged red blood cell scans or angiography<sup>[15,16]</sup>. This may be appropriate for a patient who has undergone multiple blood transfusions and a prior exhaustive work-up that has failed to localize the occult bleeding site. Additionally there must be no contraindications to the administration of a thrombolytic agent. If provocative angiography is undertaken, one should arrange for surgical backup in the event that uncontrollable bleeding occurs.

Different methods of inducing bleeding and different rates of success have been reported. An optimal protocol has yet to be established and the procedure has also yet to become accepted by clinicians as part of the evaluation of gastrointestinal bleeding. The technique continues to evolve as experience and comfort with the use of thrombolytic agents in the setting of nonlocalized bleeding increases. One reported protocol used a combination of intravenous heparin, intra-arterial tolazoline, and intra-arterial tissue-type plasminogen activator (t-PA) to provoke bleeding. Doses used included 3000-10 000 U heparin, 25-100 mg intra-arterial tolazoline, and 10-50 mg intra-arterial t-PA (mean, 20.3 mg). The investigators also noted that more patients had bleeding provoked after smaller rather than larger doses of t-PA<sup>[16]</sup>. Tolazoline (Priscoline) was formerly used for a vasodilatory effect, but was withdrawn from the United States market in 2002 by Novartis Pharmaceuticals, the sole manufacturer of this drug. Alternative intra-arterial vasodilators that may be used include verapamil (100-200 µg) and nitroglycerin (100-300 µg), with the former showing the greater vasodilatory effects. In the authors' practice, we have occasionally used a similar transcatheter regimen, in



**Figure 13** Example of nontarget embolization during treatment of lower gastrointestinal bleeding. A: Superior mesenteric artery arteriogram obtained in a patient with lower gastrointestinal bleeding shows a focal contrast collection (black arrow) arising from a branch of the ileocolic artery; B: A microcatheter was introduced and was subselectively positioned with the tip (long black arrow) immediately proximal to the focal extravasation and a microcoil (black arrow) was placed; C: While attempting to place a second microcoil, the microcatheter tip was displaced proximally resulting in coil placement in a nontarget location (black arrowhead). Repeat digital subtraction angiography shows that the second coil is nonocclusive and the initially placed coil has successfully controlled the bleeding source.

which a vasodilator and a dose of 5000 U intra-arterial heparin and 5-10 mg intra-arterial t-PA were administered, with resultant provocation of bleeding that allowed for subsequent successful transcatheter embolization.

Superselective embolization of the arterial supply to the bleeding source may be technically demanding, particularly in older patients who may have significant atherosclerotic disease. The mesenteric vasculature and the various arterial arcades are often tortuous, and the smaller arteries are prone to vasospasm and care must be taken to avoid dissection.

An arterial bleeding site may receive a dual blood supply as a result of the rich collateral arcades that characterize the mesenteric circulation. One must therefore catheterize and inject both potential sources of perfusion to the lesion and be prepared to embolize two separate vessels if necessary. Although this dual approach will control the hemorrhage, it will also increase the risk of bowel ischemia<sup>[17]</sup>.

While angiodysplasia and AVMs may initially respond to embolization, recurrent hemorrhage is frequent and, as noted, surgical resection of the involved bowel segment is often required. A small bowel AVM is much more eas-

ily localized at surgery if an embolization coil has been placed distally in the arterial branch that supplies the lesion, so that it is palpable or visible to the surgeon<sup>[18]</sup>.

### **Results of transcatheter arterial embolization**

The current technique of embolization in the treatment of acute gastrointestinal hemorrhage successfully controls bleeding in about 80%-90% of patients<sup>[19-23]</sup>. Recurrent hemorrhage is infrequent, with the exception of angiodysplasia, AVMs and inflammatory lesions. Recurrences can usually be angiographically re-evaluated and, if a bleeding source is identified, treated with repeat embolization.

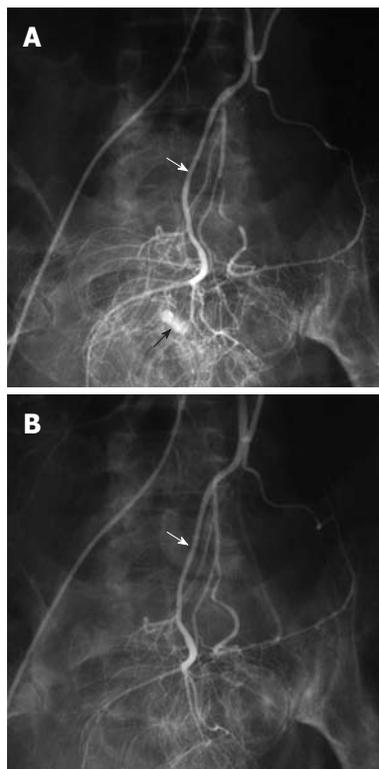
### **Complications of transcatheter arterial embolization**

Transcatheter arterial embolization for the treatment of acute gastrointestinal hemorrhage is safe, with major adverse events occurring in less than 2% of patients. A fraction of patients embolized superselectively will develop minor, asymptomatic and self-limited ischemic changes such as small ulcers that can only be detected incidentally *via* objective follow-up methods such as endoscopy, pathologic surgical specimen or by a radiographic imaging examination. Additionally, superselective microcoil embolization is unlikely to result in delayed infarction. If major bowel ischemia occurs several months to years later, it is more likely to be attributable to a new and acute insult such as thromboembolic disease affecting the mesenteric arterial bed.

Non-target embolization with microcoils is rare, as the coils are introduced only after a microcatheter has been successfully negotiated into the target vessel. One must carefully choose appropriate sized microcoils however, as a coil that is oversized relative to the target vessel may displace the microcatheter from its superselective position. This could lead to deployment of the microcoil in a non-target location (Figure 13). Similarly, undersized coils may fail to adequately occlude the target vessel or may lodge distal to the lesion that is to be treated.

### **Vasopressin infusion therapy**

Vasopressin (Pitressin) is a naturally occurring hormone that causes constriction of both the mesenteric arteries and of the smooth muscle of the bowel wall. The intra-arterial transcatheter infusion of vasopressin proximal to a mesenteric arterial bleeding site will reduce blood flow, thereby lowering the perfusion pressure and permitting clot formation at the lesion (Figure 14). There are several situations in which this form of treatment for gastrointestinal bleeding should not be used. These include bleeding that originates from a large diameter artery such as the gastroduodenal, splenic or proximal SMA or which occurs at a site with a dual blood supply such as the duodenum. It is also contraindicated in patients who have severe coronary artery disease, extreme hypertension, limb ischemia or cardiac arrhythmias. Superselective embolotherapy is now used preferentially over vasopressin infusion for treating gastrointestinal hemorrhage because embolization poses fewer risks and can



**Figure 14 Vasopressin infusion therapy for lower gastrointestinal hemorrhage.** A: Inferior mesenteric digital subtraction angiography arteriogram shows contrast extravasation (black arrow) in the rectosigmoid colon, arising from a branch of the superior hemorrhoidal artery (white arrow); B: Following trans-catheter arterial vasopressin infusion, there is no longer any evidence of active bleeding from the superior hemorrhoidal (white arrow) arterial distribution.

be completed more rapidly than a vasopressin infusion protocol. Vasopressin may still be useful in certain situations, despite the numerous side effects and the high re-bleeding rates. One may consider using vasopressin for treating lesions that are inaccessible to a microcatheter, for diffuse mucosal oozing, and for controlling multiple sites of hemorrhage in high-risk surgical patients.

#### **Technique of vasopressin infusion therapy**

Vasopressin is typically infused into the central vessel (e.g., celiac artery, SMA or IMA) that supplies the bleeding site *via* a catheter that has been placed proximally; to avoid bowel ischemia and potential infarction, a distal infusion should not be attempted. Vasopressin (100 U) is mixed in 500 mL of normal saline and the infusion is started at 0.2 U/min for 20 min using an arterial infusion pump set at 60 mL/min. If there is no cessation of bleeding, the dose is increased by 0.1 U/min up to a maximum of 0.4 U/min; each dosage change is followed by a repeat angiogram 20 to 30 min later to assess the effectiveness<sup>[24]</sup>. If the bleeding stops, the catheter is left in place for a 24-h infusion at the effective dosage, with monitoring in the intensive care unit. After another repeat angiogram shows control of bleeding, the vasopressin infusion is gradually reduced over 24 to 48 h and then vasopressin is replaced with an infusion of normal saline or 5% dextrose in water for 6-12 h.

#### **Results of vasopressin infusion therapy**

This form of therapy is particularly effective in controlling diverticular and gastric mucosal hemorrhage, with initial success rates ranging from 60%-90%<sup>[24-27]</sup>. As previously noted, however, there is a very high rate of re-bleeding that may be up to 50%.

#### **Complications of vasopressin infusion therapy**

Mild abdominal pain may occur at the initiation of the infusion and should be closely monitored, as persistence and/or worsening can be an indicator of bowel ischemia. If side effects develop during treatment, the vasopressin can be tapered to a lower dose or may need to be discontinued. Additional side effects and potential complications of vasopressin therapy include angina, myocardial infarction, hypertension, volume overload, abdominal cramps, and mesenteric ischemia. Some of the potential adverse effects may be treated or even pretreated. The simultaneous administration of intravenous, sublingual or transdermal nitroglycerin may prevent or reverse the cardiotoxic side effects of vasopressin infusion<sup>[28]</sup>.

#### **CONCLUSION**

Technical refinements and advances both in diagnostic angiography and in transcatheter arterial embolization have strengthened these options for the evaluation and management of acute gastrointestinal hemorrhage that is refractory to medical and endoscopic therapy. Highly sensitive noninvasive imaging modalities such as nuclear scintigraphy and contrast-enhanced computed tomography are extremely useful adjuncts to angiography as they often can localize and characterize the bleeding source, confirm active hemorrhage and aid in planning an appropriate transcatheter intervention. Superselective catheterization using a coaxial system that allows for microcoil embolization is an effective and safe alternative to emergency surgery. Other embolic agents such as gelatin sponge, spherical particles and liquids also have a role in transcatheter management of gastrointestinal bleeding as do transcatheter therapies such as covered stent placement and vasopressin infusion.

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## Management of acute nonvariceal upper gastrointestinal bleeding: Current policies and future perspectives

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### Abstract

Acute upper gastrointestinal bleeding (UGIB) is a gastroenterological emergency with a mortality of 6%-13%. The vast majority of these bleeds are due to peptic ulcers. Nonsteroidal anti-inflammatory drugs and *Helicobacter pylori* are the main risk factors for peptic ulcer disease. Endoscopy has become the mainstay for diagnosis and treatment of acute UGIB, and is recommended within 24 h of presentation. Proton pump inhibitor (PPI) administration before endoscopy can downstage the bleeding lesion and reduce the need for endoscopic therapy, but has no effect on rebleeding, mortality and need for surgery. Endoscopic therapy should be undertaken for ulcers with high-risk stigmata, to reduce the risk of rebleeding. This can be done with a variety of modalities. High-dose PPI administration after endoscopy can prevent rebleeding and reduce the need for further intervention and mortality, particularly in patients with high-risk stigmata.

### INTRODUCTION

Acute upper gastrointestinal bleeding (UGIB) is the most common gastroenterological emergency and has a considerable morbidity and mortality. Management strategies have changed dramatically over recent decades due to the introduction of acid suppressive therapy [histamine-2 receptor antagonists and especially proton pump inhibitors (PPIs)] and endoscopic therapy. This review deals with the current standards and future perspectives in management of acute nonvariceal UGIB.

### EPIDEMIOLOGY

The incidence rates of UGIB demonstrate a large geographic variation ranging from 48 to 160 cases per 100 000 population, with consistent reports of higher incidences among men and elderly people<sup>[1-5]</sup>. Possible

Table 1 Mortality rates in patients with upper gastrointestinal bleeding in various studies

	Czernichow <i>et al</i> <sup>[5]</sup>	Paspatis <i>et al</i> <sup>[4]</sup>	Van Leerdam <i>et al</i> <sup>[3]</sup>	Di Fiore <i>et al</i> <sup>[7]</sup>	Theocharis <i>et al</i> <sup>[11]</sup>	Hearnshaw <i>et al</i> <sup>[10]</sup>
Country	France	Greece	The Netherlands	France	Greece	United Kingdom
Year of publication	2000	2000	2003	2005	2008	2010
No. of patients	2133	353	769	453	353	6750
Mortality rate total (%)	14.3	5.6	13	7.2	6.5	7.4
Varices (%)	22.8	21.4	16	15.2	9	15
Peptic ulcer (%)	13.3	2.6	14	5	4.2	8.7

Table 2 Causes of upper gastrointestinal bleeding according to recent epidemiological studies<sup>[1,3-5,7,10]</sup>

	%
Peptic ulcer	31-67
Erosive	7-31
Variceal bleeding	4-20
Oesophagitis	3-12
Mallory-Weiss	4-8
Neoplasm	2-8
Other	2-8
None	3-19

explanations for the reported geographic variation in incidence are differences in definition of UGIB in various studies, population characteristics, prevalence of ulcerogenic medication, in particular aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* (*H. pylori*) prevalence. Some but not all time-trend studies have reported a significant decline in incidence of acute UGIB, especially peptic ulcer bleeding, in recent years<sup>[1,3,6]</sup>. This decline is likely due to a combination of factors, including decreasing prevalence of gastric colonization with *H. pylori*<sup>[1]</sup>, the use of eradication therapy in patients with ulcer disease, and the increased use of PPI therapy, both in general and in patients using aspirin and NSAIDs in particular.

Despite the introduction of therapeutic endoscopy and acid-suppressive therapy, the overall mortality of UGIB has remained stable over recent decades and is still 6%-14% in most studies (Table 1)<sup>[1,3-5,7]</sup>. The majority of deaths do not directly result from exsanguination, but are related to poorly tolerated blood loss and resultant shock, aspiration, and therapeutic procedures. As such, mortality from UGIB is strongly associated with advanced age and presence of severe comorbidity. The risk of mortality increases with rebleeding, which is thus another major outcome parameter<sup>[5]</sup>. The incidence of rebleeding in patients with UGIB shows a wide range from 5% to more than 20%, depending on several factors<sup>[3,4]</sup>. These firstly include the etiology of the bleeding, with rebleeding being more common in patients with variceal bleeding (25%) and uncommon in patients with small mucosal lesions such as Mallory-Weiss lesions. A second factor that determines the frequency of rebleeding is the timing and use of adequate endoscopic therapy. There is strong evidence that the risk of rebleeding is highest in the initial period of admission, and a 24-h time frame for endoscopic therapy is internationally

recommended as the optimal window of opportunity<sup>[8,9]</sup>. Mortality amongst those with recurrent bleeding is considerably higher, therefore, rebleeding must be prevented whenever possible<sup>[8]</sup>.

Peptic ulcer bleeding (PUB) is the most common cause of UGIB, accounting for 31%-67% of all cases, followed by erosive disease, variceal bleeding, esophagitis, malignancies and Mallory-Weiss tears (Table 2)<sup>[1,3-5,7,10]</sup>. In 2%-8% of cases, uncommon causes such as Dieulafoy's lesion, hemobilia, angiodysplasia, vasoenteric fistula, and gastric antral vascular ectasia have been found. In the remainder of this paper, we mainly focus on PUB, yet the approach to and treatment of any patient with nonvariceal UGIB is for the most part comparable. Possible differences will be discussed in the section on endoscopic therapy.

In the subgroup of patients with PUB, bleeding from duodenal ulcers is slightly more frequent than from gastric ulcers<sup>[1,4]</sup>. NSAID use and *H. pylori* infection are independent risk factors for UGIB, especially PUB<sup>[8,11]</sup>. The prevalence of *H. pylori* infection in PUB patients varies between 43 and 56%<sup>[12-14]</sup>, and treatment of *H. pylori* significantly reduces the rebleeding rate according to some randomized controlled trials<sup>[15,16]</sup>.

## PRE-ENDOSCOPIC MANAGEMENT

### Initial resuscitation and risk stratification

Patients with UGIB can present with various symptoms such as hematemesis, hematochezia, melena, or progressive anemia. Immediate evaluation and appropriate resuscitation is of major importance in these patients. Stratification of patients in low- and high-risk categories for rebleeding and mortality can be done using the Blatchford and initial Rockall scores (before endoscopy), or complete Rockall score (after endoscopy) (Table 3)<sup>[17,18]</sup>. The Blatchford score is more focused on clinical symptoms and laboratory results, whereas the Rockall score considers age as a parameter.

Resuscitation includes intravenous administration of fluids, and supplemental oxygen, correction of severe coagulopathy, and blood transfusion when needed. The threshold for blood transfusion depends on the underlying condition, rate of bleeding, and vital signs of the patient, but is generally set at a hemoglobin level of  $\leq 70$  g/L<sup>[19]</sup>. A recent meta-analysis regarding outcomes following red blood cell transfusion in patients with UGIB, however, suggests that red blood cell transfusion is associated with

**Table 3** Comparison of Blatchford and Rockall risk scoring systems

Risk factor	Blatchford score		Initial Rockall score	
	Parameter	Score	Parameter	Score
Age (yr)	-		60-79	1
			≥ 80	2
Systolic blood pressure (SBP) (mmHg)	100-109	1	< 100	2
	90-99	2		
	< 90	3		
Heart rate (bpm)	> 100	1	> 100 with SBP ≥ 100	1
Clinical presentation	Melena	1	-	
	Syncope	2		
Comorbidity	Hepatic disease	2	CHF, IHD, major comorbidity	2
	Cardiac failure	2	Renal or liver failure, or disseminated cancer	3
Blood urea, mg/dL (mmol/L)	18.2-22.3 (6.5-7.9)	2	-	
	22.4-27.9 (8-9.9)	3		
	28-69.9 (10-24.9)	4		
	≥ 70 (≥ 25)	6		
Hemoglobin, g/dL (mmol/L)	F: 10-11.9 (6.2-7.4)	1	-	
	M: 12-12.9 (7.5-8)			
	M: 10-11.9 (6.2-7.4)	3		
	F/M: < 10 (< 6.2)	6		
			<b>Complete Rockall score</b>	
Endoscopic diagnosis	-		Non-malignant, non-Mallory-Weiss diagnosis	1
			Upper GI tract malignancy	2
Evidence of bleeding	-		Blood, adherent clot, active bleeding	2

M: Male; F: Female; CHF: Congestive heart failure; IHD: Ischemic heart disease.

higher mortality and rebleeding rate. The conclusions of this study were limited by the small size of the studies and the large volume of missing data. In addition, the possibility that patients who present with more severe and active bleeding are more rapidly transfused, acted as a potential major confounder in these analyses<sup>[20]</sup>. This means that prospective studies need to be done with strict predetermined transfusion protocols, and that for now, the risks and benefits of blood transfusion must be carefully weighed individually.

**Pre-endoscopic pharmacotherapy**

Administration of PPIs before endoscopy has become common practice in patients suspected with PUB. A strongly acidic environment leads to inhibition of platelet aggregation and plasma coagulation as well as to lysis of already formed clots<sup>[21]</sup>. PPIs quickly neutralize intraluminal gastric acid, which results in stabilization of blood clots. In the longer term, antisecretory therapy also promotes mucosal healing. A recent systematic review has shown that pre-endoscopic PPI administration significantly reduces high-risk stigmata at index endoscopy (37% *vs* 46% respectively, OR: 0.67; 95% CI: 0.54-0.84) and need for endoscopic therapy (9% *vs* 12%

respectively, OR: 0.68; 95% CI: 0.50-0.93). However, no effect on clinically important outcome measures such as rebleeding, mortality and need for surgery was seen<sup>[22]</sup>.

Another pharmacotherapeutic approach includes the use of prokinetics before endoscopy, in particular, erythromycin or metoclopramide. A meta-analysis of five studies assessing a total of 316 patients with acute UGIB has found a significant reduction in the need for repeated endoscopy (OR: 0.55; 95% CI: 0.32-0.94) in the prokinetic treatment group compared to the reference group (placebo or no treatment). The groups did not differ in the need for blood products, hospital stay, and need for surgery<sup>[23]</sup>. Therefore, prokinetics are not routinely recommended, but can be useful in patients who are suspected of having substantial amounts of blood in the stomach<sup>[9]</sup>. Administration of PPIs and prokinetics should however not delay endoscopy.

**ENDOSCOPY**

**Time to endoscopy**

Endoscopy has become a valuable and indispensable tool for diagnosis and treatment of UGIB<sup>[24,25]</sup>. It allows for identification of the bleeding source and application of treatment in the same session. The optimal timing for endoscopy remains under debate. Emergency endoscopy allows for early hemostasis, but can potentially result in aspiration of blood and oxygen desaturation in insufficiently stabilized patients. In addition, extensive amounts of blood and clots in the stomach can hinder targeted treatment of the bleeding focus, which results in repeated endoscopic procedures. International consensus guidelines recommend early endoscopy within 24 h of presentation, because it significantly reduces the length of hospital stay and improves outcome<sup>[19]</sup>. Very early endoscopy (< 12 h) has so far not been shown to provide additional benefit in terms of reduction of rebleeding, surgery and mortality, compared with later endoscopy (within 24 h)<sup>[26-29]</sup>. However, emergency endoscopy should be considered in patients with severe bleeding.

**Endoscopic therapy for PUB**

The aim of therapeutic endoscopy is to stop any ongoing bleeding and prevent rebleeding. Several techniques, including injection therapy, ablative therapy and mechanical therapy have been studied over recent decades<sup>[24,30,31]</sup>. Depending on the appearance of the bleeding focus and the related risk for persistent or recurrent bleeding, a suitable technique should be chosen. In PUB, patients with active bleeding ulcers or a nonbleeding visible vessel in an ulcer bed are at highest risk of rebleeding and therefore need prompt endoscopic hemostatic therapy (Figures 1 and 2)<sup>[32]</sup>. Patients with low-risk stigmata (a clean-based ulcer or a pigmented spot in an ulcer bed) do not require endoscopic therapy.

The role of endoscopic therapy for ulcers with adherent clots has been a topic of debate<sup>[19]</sup>. The risk of rebleeding depends on underlying lesions, so that clot re-



Figure 1 Ulcer with visible vessel.

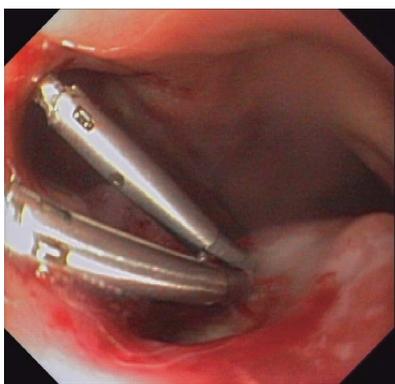


Figure 2 Ulcer with visible vessel after hemoclip placement.

removal should be attempted by vigorous irrigation. Stigmata revealed after clot removal are of high risk in about 70% of cases<sup>[33]</sup>. In a meta-analysis including 240 patients from six different studies, comparing endoscopic *vs* medical therapy for peptic ulcers with adherent clots, rebleeding was significantly lower in the endoscopic therapy group compared with the control group (8% *vs* 25%,  $P = 0.01$ )<sup>[34]</sup>. Another meta-analysis, however, has shown no benefit of endoscopic therapy for bleeding peptic ulcers with adherent clots<sup>[35]</sup>. These discrepancies could be attributed to inclusion of different studies and heterogeneity in statistical analysis. At present, endoscopic therapy should be considered, although intensive PPI therapy alone might be sufficient in ulcers with adherent clots<sup>[19]</sup>.

Epinephrine injection therapy promotes initial hemostasis by a combination of vasospasm and local tamponade. This effect declines after 20 min, and requires additional treatment with a more durable technique. In several meta-analyses, no superiority of one specific technique was proven; in particular, hemoclip placement, thermocoagulation (e.g., heater probe), and electrocoagulation (e.g., Gold probe, BICAP probe) all seem equivalent alternatives<sup>[24,30,31,36]</sup>. Patients with recurrent bleeding can usually be managed by endoscopic therapy. However, emergency surgery or angiographic embolization is required on occasion. There have been no randomized trials that have compared surgery and angiographic em-

bolization.

A new promising endoscopic application is the use of a chemical compound which, when sprayed as nanopowder on active bleeding, can lead to immediate hemostasis, with coverage of the bleeding ulcer with a powder layer. In a pilot study of 15 patients with active ulcer bleeding treated with this nanopowder, immediate hemostasis was achieved in 93%, and one patient had recurrent bleeding. No adverse events were reported during the 30-d follow-up<sup>[37]</sup>. Further studies with this product are ongoing and will elucidate if application is also beneficial for other causes of nonvariceal UGIB.

### Endoscopic therapy for other causes of nonvariceal UGIB

Treatment and prevention of (bleeding from) erosions depends upon the cause (e.g., drug-induced, mechanical, or inflammatory). Most cases respond well to PPIs. The offending agent should be discontinued whenever possible and, if present, *H. pylori* should be eradicated. Acute bleeding sometimes needs endoscopic therapy, similar to that for PUB<sup>[38]</sup>.

Hemorrhage due to neoplastic lesions is often difficult to manage because of the diffuse character of the bleeding and vulnerability of the mucosa. Primary endoscopic therapy is recommended, but additional surgical consultation is sometimes necessary. In cases with diffuse tumor bleeding in a palliative setting, radiotherapy is often the treatment of choice.

Most bleeding from Mallory-Weiss tears stops spontaneously. Patients with stigmata of active bleeding, however, might require interventional endoscopy<sup>[39]</sup>. Endoscopic therapy is the first choice in bleeding Dieulafoy's lesions and is usually performed with clipping or banding of the lesion<sup>[40]</sup>.

The current standard for endoscopic treatment of bleeding angiodysplasia consists of coagulation therapy. Sometimes, pharmacological agents such as estrogen and progesterone, octreotide or thalidomide are given, but their effects remain controversial.

Gastric antral vascular ectasia responds best to endoscopic ablation of the lesion.

## POSTENDOSCOPIC MANAGEMENT

### Antisecretory therapy

Pharmacotherapy plays a second major role in the treatment of UGIB. PPI therapy is superior over histamine-2 receptor antagonists<sup>[19]</sup>. PPIs can be administered orally or intravenously depending on the rebleeding risk. In a randomized placebo-controlled trial of 767 multiethnic PUB patients treated with endoscopic therapy because of high-risk stigmata, high-dose intravenous PPI (80 mg esomeprazole bolus, 8 mg/h continuous infusion for 72 h) significantly reduced rebleeding (5.9% *vs* 10.3%,  $P = 0.03$ ) and the need for endoscopic retreatment<sup>[41]</sup>. Similar results were found by meta-analysis; high-dose intravenous PPI after endoscopic therapy significantly reduced rebleeding [relative risk (RR): 0.40; 95% CI: 0.28-0.59], need for sur-

gery (RR: 0.43; 95% CI: 0.24-0.58) and mortality (RR: 0.41; 95% CI: 0.20-0.84) compared with placebo/no therapy<sup>[35]</sup>. These data support the guideline recommendation to give high-dose continuous intravenous PPI therapy to patients with PUB with high-risk stigmata.

Additionally, all patients with PUB should be discharged with a prescription for a single-daily-dose oral PPI to reduce the risk of recurrent bleeding. The duration and dose of the PPI depend on the underlying etiology and additional medication use<sup>[19]</sup>.

### ***H. pylori* eradication therapy**

Testing for *H. pylori* is recommended in all patients with PUB<sup>[19]</sup>. This should be followed by eradication therapy for those who are *H. pylori*-positive, with subsequent assessment of the effect of this therapy, and renewed treatment in those in whom eradication fails. The efficacy of eradication therapy and maintenance antisecretory therapy for the prevention of rebleeding has been assessed in a meta-analysis of randomized trials. This revealed a significantly lower risk of rebleeding in the *H. pylori* eradication group, that is, 1.6% *vs* 5.6% within a median follow-up of 12 mo. When only patients with successful *H. pylori* eradication were included, the rebleeding rate was even lower (1%)<sup>[42]</sup>. Therefore, confirmation of eradication is recommended. Diagnostics tests for *H. pylori* have a low negative predictive value in the setting of acute UGIB. This might be due to technical difficulties to collect a sufficient number of representative biopsies, or inaccuracy of the test in a more alkaline environment caused by the blood<sup>[43]</sup>. Initial negative results on biopsies obtained in the acute setting must therefore be interpreted with caution and repetition of the test during follow-up is recommended<sup>[19]</sup>.

### **CONCLUSION**

The management of UGIB has changed dramatically over recent decades. Endoscopic therapy and pharmacotherapy have become the mainstay in management. Early endoscopy within 24 h of presentation, or earlier in selected cases with signs of ongoing bleeding, improves outcome and reduces length of hospital stay. Endoscopic epinephrine injection in combination with another endoscopic technique reduces the risk for rebleeding and related mortality in patients with high-risk ulcers. Adequate *H. pylori* eradication and PPI therapy after discharge can bring the rebleeding and mortality rates further down.

Ongoing development is expected especially in the area of development of transfusion policies, and new tools for endoscopic hemostasis. Further studies are needed to clarify the optimal approach for patients with adherent clots. These developments should help to reduce the persistent high mortality rate of UGIB, a disease which nowadays in particular occurs in elderly patients with comorbidity and medication use.

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## Expression of HER2 and bradykinin B<sub>1</sub> receptors in precursor lesions of gallbladder carcinoma

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### Abstract

**AIM:** To determine the expression of HER2 and bradykinin B<sub>1</sub> receptors (B<sub>1</sub>R) in the two pathogenic models of gallbladder cancer: the metaplasia-dysplasia-carcinoma and the adenoma-carcinoma pathways.

**METHODS:** Receptor proteins were visualized by immunohistochemistry on 5- $\mu$ m sections of paraffin-embedded tissue. Expression of both receptors was studied in biopsy samples from 92 patients (6 males and 86 females; age ranging from 28 to 86 years, mean 56 years). High HER2 expression in specimens was additionally investigated by fluorescence *in situ* hybridiza-

tion. Cell proliferation in each sample was assessed by using the Ki-67 proliferation marker.

**RESULTS:** HER2 receptor protein was absent in adenomas and in normal gallbladder epithelium. On the contrary, there was intense staining for HER2 on the basolateral membrane of epithelial cells of intestinal metaplasia (22/24; 91.7%) and carcinoma *in situ* (9/10; 90%), the lesions that displayed a significantly high proliferation index. Protein up-regulation of HER2 in the epithelium with metaplasia or carcinoma *in situ* was not accompanied by *HER2* gene amplification. A similar result was observed in invasive carcinomas (0/12). The B<sub>1</sub>R distribution pattern mirrored that of HER2 except that B<sub>1</sub>R was additionally observed in the adenomas. The B<sub>1</sub>R appeared either as cytoplasmic dots or labeling on the apical cell membrane of the cells composing the epithelia with intestinal metaplasia (24/24; 100%) and carcinoma *in situ* (10/10; 100%) and in the epithelial cells of adenomas. In contrast, both HER2 (4/12; 33%) and B<sub>1</sub>R (1/12; 8.3%) showed a low expression in invasive gallbladder carcinomas.

**CONCLUSION:** The up-regulation of HER2 and B<sub>1</sub>R in precursor lesions of gallbladder carcinoma suggests cross-talk between these two receptors that may be of importance in the modulation of cell proliferation in gallbladder carcinogenesis.

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**Key words:** Bradykinin B<sub>1</sub> receptor; HER2; Gallbladder adenoma; Gallbladder dysplasia; Intestinal metaplasia; Gallbladder cancer

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## INTRODUCTION

Gallbladder carcinoma is an aggressive cancer of the gastrointestinal tract with incidence three times greater in the female population of Chile, India and Japan when compared to men<sup>[1,2]</sup>. The most common type of gallbladder cancer is adenocarcinoma. However, early diagnosis is difficult because most of the cases are detected only at an advanced stage following laparotomy.

Successive bouts of inflammation cause continuous and long lasting damage to the gallbladder epithelium, which thereby transforms into an architecturally abnormal regenerative epithelium very similar to low grade dysplasia. Injury and repair are also related to well known metaplastic changes of the mucosa, such as pyloric and intestinal metaplasia, and to other rare types of metaplasia<sup>[3]</sup>. Intestinal metaplasia usually develops into dysplasia and is frequently observed adjacent to carcinoma. Pyloric and intestinal metaplasias are associated with gallbladder carcinoma, but intestinal metaplasia show a greater relationship with malignancy<sup>[4,5]</sup>. Analysis of serial sections indicates the presence of microinvasion foci in the lamina propria next to carcinoma *in situ* and also that dysplasia and carcinoma *in situ* occur close to areas of intact mucosa in nearly all invasive carcinomas<sup>[6]</sup>.

Among the pathogenic models that explain the neoplastic transformation of gallbladder epithelium are the metaplasia-dysplasia-carcinoma and the adenoma-carcinoma pathways<sup>[5]</sup>. Morphological and molecular studies have shown that these two entities correspond to independent biological events. Therefore, we have used representative gallbladder samples of both putative pathways to investigate the expression of two receptor molecules involved in cell proliferation, namely the HER2 and bradykinin B<sub>1</sub> receptors (B<sub>1</sub>R). The HER2 (c-erbB-2) receptor is recognized to be of clinical importance because of its prognostic value in determining progression of some types of breast tumors. Similarly, B<sub>1</sub>R has recently been shown to induce the proliferation of estrogen-sensitive breast cancer cells by turning on the transactivation of the epidermal growth factor receptor (EGFR), a signal-transduction pathway involved also in the activation of HER2<sup>[7]</sup>. As with breast cancer, expression of HER2 in gallbladder carcinoma has been associated with progression of malignancy and linked to poor patient survival<sup>[8-11]</sup>. Furthermore, constitutive expression of HER2 in transgenic mice causes adenocarcinoma of the gallbladder<sup>[12]</sup> suggesting a key role for this member of the EGFR family in such neoplasia. Although the expression levels of HER2 have been

previously investigated in invasive gallbladder cancer<sup>[13]</sup>, so far no studies appear to have examined its expression in the putative precursor lesions of gallbladder carcinoma.

The B<sub>1</sub>R belongs to the family of G protein-coupled rhodopsin-like receptors which, upon stimulation by analogues that lack the Arg<sup>9</sup> from the carboxy terminus of the bradykinin molecule, trigger several second messenger signaling systems that control cell differentiation, proliferation and/or migration<sup>[14,15]</sup>. The kinin B<sub>1</sub>R agonists belong to a family of bioactive peptides produced locally and with paracrine activity, that are formed from precursor molecules by the proteolytic action of enzymes called kallikreins (kininogenases)<sup>[14,15]</sup>. So far, only a few studies have evaluated the role of B<sub>1</sub>R as well as the underlying molecular mechanisms that trigger its activation in cancer cells. Recent reports have suggested that the B<sub>1</sub>R is an important player in lung, prostate and breast cancer by regulating tumoral growth, migration and invasion<sup>[7,16,17]</sup>. In addition, some of the cellular actions of B<sub>1</sub>R stimulation are a consequence of EGFR transactivation<sup>[7]</sup>. However, the status of B<sub>1</sub>R in other neoplastic disorders such as gallbladder carcinoma has not been investigated previously. Thus, the primary aim of our study was to perform a comprehensive evaluation of the expression values for HER2 and B<sub>1</sub>R in the metaplasia-dysplasia-carcinoma and adenoma-carcinoma pathways.

## MATERIALS AND METHODS

### Ethics

This study was performed in accordance with the Declaration of Helsinki of the World Medical Association. The designated experiments were approved by the Ethical Committees of Hospital Base Valdivia, Universidad Austral de Chile and the National Fund for Development of Science and Technology in Chile (FONDECYT) that included guidelines for the protection of human subjects.

### Patient tissue

The study was performed on 92 routinely resected gallbladders retrieved from the surgical pathology archive of the Servicio de Patología, Hospital Base Valdivia, Chile. Of the 92 specimens, 6 were from males, and 86 were from females; the patients ranged in age from 28 to 86 years (mean, 56 years). The data on age, sex of patients bearing adenomas, gallbladder adenocarcinomas and their putative precursor lesions are summarized in Table 1. The following categories were recorded: (1) normal mucosa ( $n = 5$ ), gallbladders that were resected due to lithiasis and elective surgery; (2) pyloric-type adenoma ( $n = 15$ ); (3) intestinal-type adenoma ( $n = 6$ ); (4) pyloric metaplasia ( $n = 20$ ); (5) intestinal metaplasia ( $n = 24$ ); (6) carcinoma *in situ* ( $n = 10$ ); and (7) invasive carcinoma ( $n = 12$ ) (Table 1). Invasive adenocarcinomas examined histologically were classified into well-differentiated with a papillary pattern of growth (8 cases), poorly differentiated (2 cases), mucinous well-differentiated (1 case) and signet ring (1 case) adenocarcinomas.

**Table 1** Clinical and pathological data of patients with gallbladder adenomas, adenocarcinoma and its putative precursor lesions

Lesion	Sex	Age (yr, mean $\pm$ SD)
Normal mucosa ( <i>n</i> = 5)	F (5)	54.6 $\pm$ 9.0
Pyloric adenoma ( <i>n</i> = 15)	F (13)/M (2)	55.2 $\pm$ 3.5
Intestinal adenoma ( <i>n</i> = 6)	F (6)	68.5 $\pm$ 1.7
Pyloric-type metaplasia ( <i>n</i> = 20)	F (20)	51.5 $\pm$ 3.2
Intestinal metaplasia ( <i>n</i> = 24)	F (24)	50.0 $\pm$ 3.4
Carcinoma <i>in situ</i> ( <i>n</i> = 10)	F (9)/M (1)	65.8 $\pm$ 2.5
Invasive carcinoma ( <i>n</i> = 12)	F (9)/M (3)	66.0 $\pm$ 3.8

According to WHO classification. F: Female; M: Male.

Each gallbladder was fixed in 10% buffered formalin and embedded in paraffin according to conventional protocols. Sections from each block were stained with hematoxylin and eosin for precise histopathological classification before immunostaining. In all cases, two to four blocks were used for immunohistochemical evaluation.

### Immunohistochemistry

This method was performed as previously described by Molina *et al*<sup>71</sup>. Briefly, paraffin sections were incubated with primary antibodies overnight at 22 °C in a water bath that was used as a moist chamber. The primary antibody sources, dilutions, antigen retrieval and incubation conditions are listed in Table 2. Bound primary antibodies were localized by the biotin/streptavidin immunoperoxidase technique using the LSAB+ kit (Dako, Carpinteria, CA, United States). Peroxidase was developed with diaminobenzidine and hydrogen peroxide. Sections were counterstained with Harris hematoxylin. Negative controls included omission of primary antibody and its replacement by non-immune immunoglobulins of the same species or isotype matched immunoglobulins under identical conditions.

Immunolabeling was scored as positive (presence of staining) or negative (absence of staining) and then expressed as percentage of total number of normal mucosa, metaplasia, carcinoma *in situ* and invasive carcinoma cases examined. Staining was considered positive when over 30% of the gallbladder epithelium was immunostained. Only cell membrane immunoreactivity was considered positive for HER2 staining. Cell proliferation index was determined by recording the immunolabeled Ki-67 antigen in gallbladder carcinoma and its putative precursor lesions. For this purpose, nuclei were counted at x40 magnification in three different fields selected at random. The procedure was repeated twice by two independent observers for each lesion and the counts were averaged. The Ki-67 cell proliferation index was derived by dividing the average Ki-67 count by the average total number of nuclei in one field.

### Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) technique for

HER2 was performed on 3- $\mu$ m thick tissue sections according to the protocol of HER2 FISH PharmDx™ Kit (Dako). Hybridization was performed with a mixture of HER2-Texas Red and cen-17 labeled with fluorescein isothiocyanate. A breast cancer sample classified as HER2 (+3) was used as positive control. For each condition at least two samples were analyzed using a fluorescence Nikon Labophot-2, and at least 50 nuclei were visualized in each sample.

### Statistical analysis

Differences in the occurrence of immunolabeling were evaluated using Fischer's exact probability test with the aid of the software JMP Statistical Discovery Software 8.0 (SAS Institute, Cary, NC, United States). Probability values less than 0.05 were considered to be statistically significant.

## RESULTS

### Histopathological features

Normal, non-metaplastic epithelium was comprised of regular segments of tall columnar cells with basal nuclei and sporadic small apical vacuoles. Pyloric-type adenomas showed their typical morphology that was comprised of tightly packed pyloric or antral-type mucous glands similar to mucous glands of the stomach (Figure 1). Intestinal-type adenomas consisted of papillary structures lined by columnar epithelium with elongated and pseudostratified nuclei (Figure 1). Pyloric-type metaplasia was distinguished by glands lined by columnar cells with vacuolated cytoplasm and flattened nuclei located basally; the glands formed small lobular structures throughout the lamina propria. Epithelium with intestinal metaplasia was composed of tall columnar cells with a brush border and variable proportions of goblet cells. This type of metaplasia was often seen adjacent to invasive adenocarcinoma. Carcinoma *in situ* was characterized by the presence of epithelial cells that showed frequent mitosis and marked alterations in the size and shape of the nuclei such as hypercromasia, overlapping and crowding.

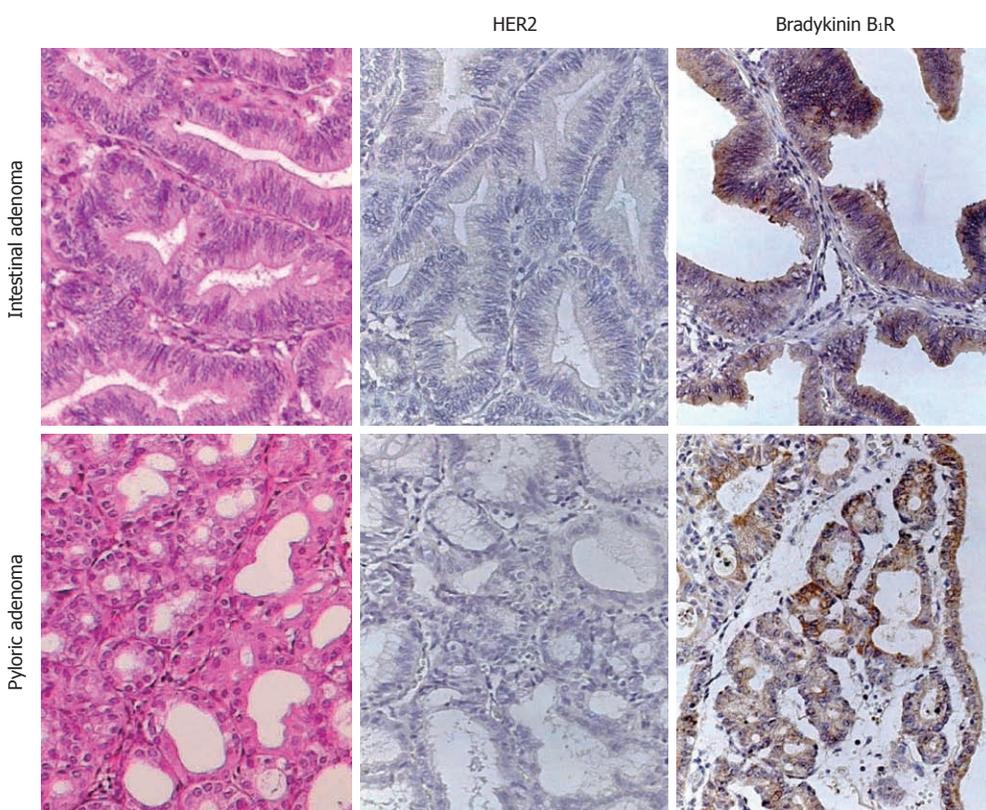
### Expression of HER2

No immunolabeled HER2 receptor was visualized in normal gallbladder epithelium and no significant expression of the protein was visualized in adenomas of either pyloric- or intestinal-type (Table 3; Figures 1 and 2). In contrast, an intense immunoreactivity was observed in the epithelium with intestinal metaplasia (22/24) and carcinoma *in situ* (9/10), with decreased staining in invasive carcinomas (4/12) (Table 3; Figure 2) and none in pyloric metaplasia and non-epithelial tissues (not shown). The HER2 immunolabeling was confined to the basolateral cell membrane region of all metaplastic epithelial cells, whereas the luminal membrane was devoid of staining (Figures 2 and 3). At a higher magnification, the boundary between the two segments was clearly delineated by the different immunoreactivity of the cells in the apical

Antibody	Source	Dilution/incubation	Microwave antigen retrieval
HER2	Novocastra, NCL-CBE-356	1:200/overnight	No
Bradykinin B <sub>1</sub> R	Santa Cruz, sc-25484	1:300/overnight	90 °C/7 min, Tris-HCl buffer pH 10
	Merck	1:500/overnight	No
Ki-67	Dako, A0047	1:200/overnight	90 °C/15 min, Citrate buffer pH 6

Receptor/marker	Normal epithelium	Pyloric adenoma	Intestinal adenoma	Intestinal metaplasia	Carcinoma <i>in situ</i>	Invasive carcinoma
HER2	0/5	0/14	1/6 (17)	22/24 (91.7) <sup>a</sup>	9/10 (90) <sup>a</sup>	4/12 (33)
Bradykinin B <sub>1</sub> R	0/5	10/15 (67) <sup>a</sup>	6/6 (100) <sup>a</sup>	24/24 (100) <sup>a</sup>	10/10 (100) <sup>a</sup>	1/12 (8.3)
Ki-67 <sup>†</sup>	0.05 ± 0.005 (1.1)	0.05 ± 0.01 (5.1)	0.10 ± 0.01 (10.8)	0.36 ± 0.03 <sup>b</sup> (36.7)	0.37 ± 0.05 <sup>b</sup> (37.5)	0.42 ± 0.03 <sup>b</sup> (42)

<sup>†</sup>Proliferation index estimated as immunoreactivity to Ki-67 antigen. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 *vs* normal epithelium. B<sub>1</sub>R: B<sub>1</sub> receptor.



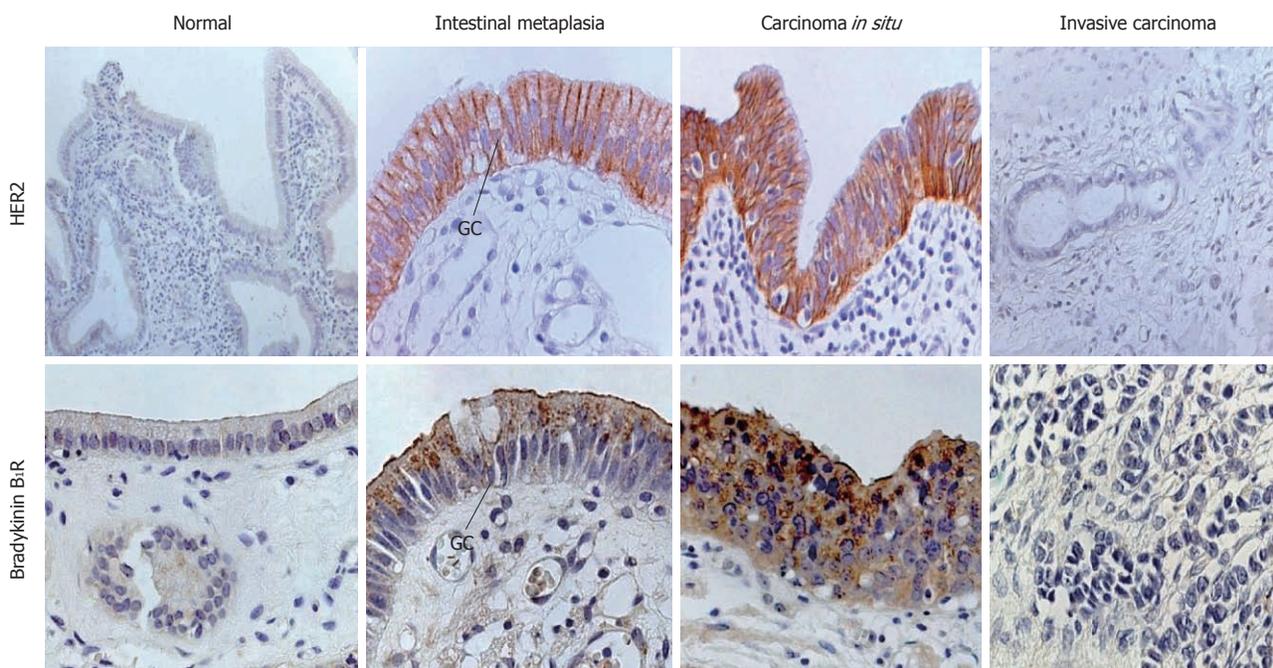
**Figure 1** Expression of immunoreactive HER2 and bradykinin B<sub>1</sub> receptor in pyloric- and intestinal-type adenomas. Tissue sections were incubated with each antibody and then the biotin/streptavidin-peroxidase technique was followed. B<sub>1</sub>R: B<sub>1</sub> receptor.

membrane when compared to those of the basolateral cell membrane (Figure 3). Loss of polarity in the epithelia with carcinoma *in situ* resulted in a complete membrane staining of some of the neoplastic epithelial cells. With FISH staining, *HER2* gene appeared as two red signals in normal and metaplastic epithelia and two to four signals in carcinoma *in situ* and invasive carcinoma, accompanied by the corresponding green signals pointing out the chromosome 17 centromeres (*HER2/CEN-17* < 1.8) that indicated lack of amplification (Figure 3C-F).

On the contrary, a clear amplification of *HER2* gene was observed in *HER2* (+3) breast cancer samples that were used as positive controls (Figure 3G).

**Expression of bradykinin B<sub>1</sub>R**

The B<sub>1</sub>R protein also followed a cell membrane distribution pattern. Immunolabeled B<sub>1</sub>R was visualized only in one case of the invasive carcinomas (Table 3). In contrast, samples with intestinal metaplasia and carcinoma *in situ* showed an intense staining for B<sub>1</sub>R that appeared as cy-



**Figure 2** Immunoreactive HER2 and bradykinin B<sub>1</sub> receptor receptors in normal gallbladder, invasive carcinoma and in epithelia with intestinal metaplasia and carcinoma *in situ*. Biotin/streptavidin-peroxidase technique. B<sub>1</sub>R: B<sub>1</sub> receptor; GC: Goblet cell.

toplasmic dots or labeling in the apical cell membrane region of metaplastic cells and in the epithelium with carcinoma *in situ* (Figures 2 and 3). Furthermore, B<sub>1</sub>R immunolabeling was observed in epithelial cells of pyloric- and intestinal-type adenomas, including the surface epithelium of adenomas (Figure 1 and Table 3).

### **Ki-67 cell proliferation marker**

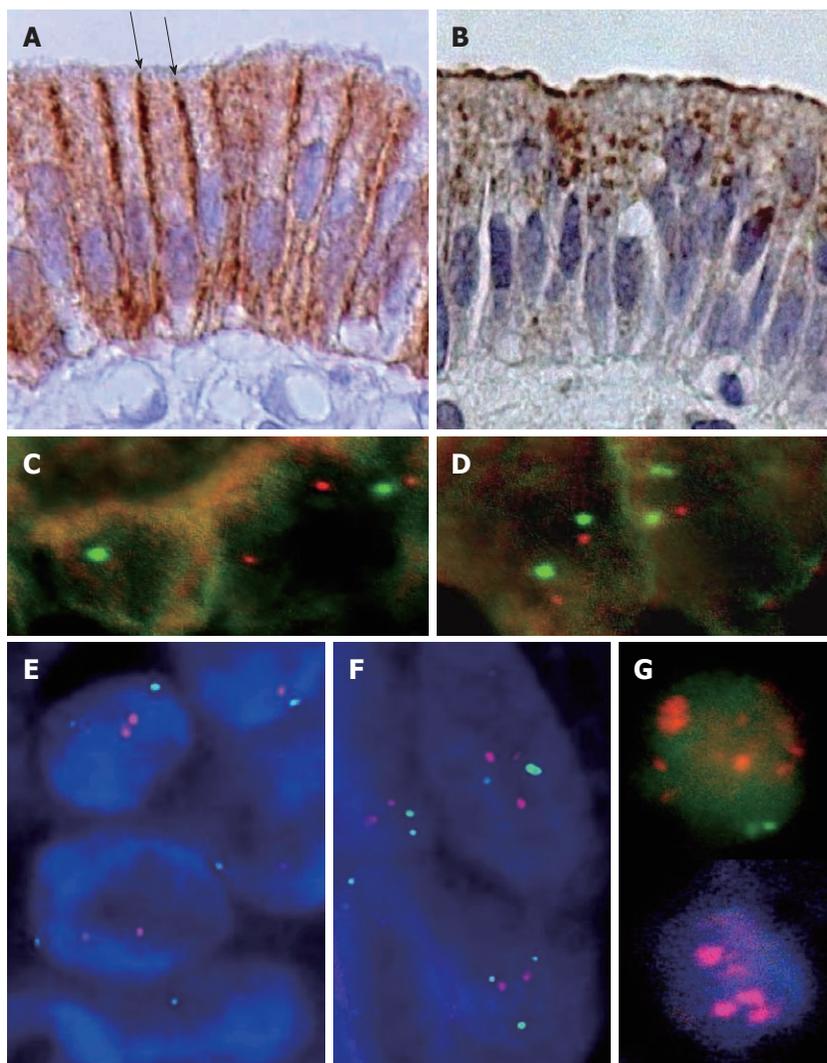
By using Ki-67, we confirmed that the observed cell proliferation was significantly higher in epithelia with metaplasia, carcinoma *in situ* and in cells of invasive gallbladder carcinomas than in normal epithelium and in epithelial cells of both pyloric- and intestinal-type adenomas (Table 3).

## **DISCUSSION**

Two pathogenic models designated as adenoma-carcinoma and metaplasia-dysplasia-carcinoma have been used to explain neoplastic transformation of the gallbladder epithelium. However, intestinal metaplasia is considered as the major precursor lesion that later progresses into carcinoma *in situ* and invasive adenocarcinoma. The question of whether expression of receptors such as HER2, a tyrosine kinase orphan receptor, and the B<sub>1</sub>R, a G protein-coupled receptor, are enhanced in such carcinogenic changes of the gallbladder epithelium formed the focus of the current study<sup>[7,15,16,18,19]</sup>.

Remarkably, the expression of HER2 and bradykinin B<sub>1</sub>R followed a similar pattern of distribution in invasive carcinoma and in its putative precursor lesions. Both receptors were absent in the normal epithelium but were strongly expressed in carcinoma *in situ* and the epithelia

with intestinal metaplasia. Absence of HER2 staining in normal gallbladder epithelium is in agreement with previous studies performed using normal breast tissue<sup>[20]</sup>. Two previous reports failed to demonstrate expression of HER2 protein in gallbladder dysplasia<sup>[15,21]</sup>. The discrepancy between these findings and our results may be explained by the use of different immunostaining procedures (e.g., monoclonal *vs* polyclonal antibodies and sensitivity of the technique), time of fixation, preservation of antigenic sites and number of samples analyzed. In our study, HER2 receptor protein stained intensely along the basolateral plasma membrane of the metaplastic and carcinoma *in situ* cells. A similar pattern of staining has been observed in apocrine metaplasia of the breast where HER2 immunoreactivity appeared restricted to the basolateral plasma membrane of the metaplastic cells<sup>[22]</sup>. It is well known that tight junctions morphologically divide cell membranes of non-neoplastic polarized epithelial cells into two regions: an apical one which faces the lumen and often has specialized features such as cilia or a brush border of microvilli; and a basolateral region, which covers the rest of the cell<sup>[23]</sup>. Further, tight junctions prevent proteins and lipids from diffusing between the basolateral and apical regions, so that not only the protein but also the lipid composition of the two membrane regions is different<sup>[23]</sup>. Therefore, the HER2 immunoreactivity, observed in the basolateral cell membrane domain of metaplastic gallbladder epithelium, is not comparable to that scored by the Hercep Test<sup>TM</sup> staining protocol, which is based on the immunoreactivity present on the cell membrane of invasive breast cancer cells that are not supported by a basement membrane, i.e., neoplastic non-polarized cells. Despite over-expression



**Figure 3** High magnification of gallbladder metaplastic epithelium showing immunoreactivity for HER2 (A) and bradykinin B<sub>1</sub> receptor (B). Arrows show the limit between apical and basolateral cell membrane domains. C-G: Fluorescence *in situ* hybridization for HER2. Tissue sections were hybridized with a mixture of HER2-Texas Red and cen-17 labeled with fluorescein isothiocyanate. C: Normal gallbladder epithelium; D: Epithelium with intestinal metaplasia; E: Carcinoma *in situ*; F: Invasive carcinoma; G: Positive control corresponding to a breast cancer sample classified as HER2 + 3.

of HER2 receptor protein in metaplastic epithelia and carcinoma *in situ*, our results using FISH revealed that this event was not accompanied by *HER2* gene amplification suggesting that this change may not be relevant for gallbladder cancer. Studies using FISH, but restricted to invasive carcinomas of the gallbladder, have shown amplification of the *HER2* gene only in approximately 10% of the cases investigated<sup>[8]</sup>. Absence of *HER2* gene amplification in our tissue samples with invasive carcinoma may be due to our low number of cases compared with other published studies<sup>[8]</sup>. Some authors suggest that *HER2* over-expression is due to gene deregulation rather than gene amplification because in some reports there is no strict correlation between receptor protein expression and gene amplification<sup>[24]</sup>. The HER2 receptor seems to be a key player since its constitutive expression leads to the development of gallbladder adenocarcinoma in 100% of transgenic mice<sup>[12]</sup>. Moreover, increased HER2/EGFR (erbB-1) heterodimer formation, hyperphosphorylation

of tyrosine residues of both HER2 and EGFR (but not erbB-3 or erbB-4) and activation of the mitogen-activated protein kinases (MAPK) signaling pathway were observed in the gallbladder epithelium of the transgenic mice<sup>[12]</sup>. Over-expression of the HER2 receptor protein, detected immunohistochemically in nearly all breast specimens showing carcinoma *in situ* of high grade, has been interpreted as an expression of rapid growth since its presence is related to cellular proliferation<sup>[25,26]</sup>. Our results show that the epithelia with intestinal metaplasia and carcinoma *in situ* display the higher values for Ki-67 cell proliferation marker. The fact that the *HER2* gene shows amplification only in 10% of the invasive gallbladder carcinomas<sup>[8]</sup> contrasts with the high levels of protein expression observed by us in intestinal metaplasia and carcinoma *in situ*, and suggests that major activity of HER2 may occur in these precursor lesions. Further, the expression of HER2 in other invasive tumors of the gastrointestinal tract such as gastric cancer has revealed

that less than 10% of all invasive tumors showed HER2 expression and that there is no relationship between its expression, patient survival or TNM stage<sup>[27]</sup>.

Members of the EGFR family are activated not only by direct binding of their corresponding ligands but also by transactivation triggered after stimulation of G protein-coupled receptors such as the B<sub>1</sub>R<sup>[13,19]</sup>. We have recently reported B<sub>1</sub>R binding sites in breast carcinoma and showed that B<sub>1</sub>R stimulation induces the proliferation of MCF-7 and ZR-75 breast cancer cells, an effect that depends on the transactivation of the EGFR and the subsequent activation of the MAPK signaling pathway<sup>[7]</sup>. Moreover, stimulation of the B<sub>1</sub>R increases the release of metalloproteases-2 and -9 from breast cancer cells<sup>[28]</sup>. Both metalloproteases are considered key enzymes for tumor invasion and metastasis, because they have the capacity to degrade type IV collagen, the major protein component of basement membranes. Here, we have described, for the first time, the expression of B<sub>1</sub>R in precursor lesions of gallbladder carcinomas (i.e., intestinal metaplasia and carcinoma *in situ*), conditions in which its expression is maximal and associated with high proliferation rates of the epithelial cells.

The kinin B<sub>1</sub>R agonists are short-lived peptides that exert most of their actions in a paracrine fashion for which the presence of kallikreins, kinin precursors and expression of the appropriate receptor is required<sup>[14]</sup>. In a previous report, we showed that both tissue kallikrein and the kinin precursors are present in the human gallbladder suggesting that kinin formation is feasible in this tissue<sup>[29]</sup>. Tissue kallikrein (*KLK1/hK1*, kininogenase) is expressed by gallbladder epithelial cells whereas the kininogens, substrates of hK1, diffuse from submucosal blood vessels to fill the interstitial space between epithelial cells<sup>[29]</sup>. A further source of tissue kallikrein is the neutrophil that infiltrates the inflamed gallbladder during acute episodes of cholecystitis and thereby releases hK1 in the vicinity of epithelial cells<sup>[29]</sup>. Additionally, the natural B<sub>1</sub>R agonist induces migration of glioma cells *via* up-regulation of cyclooxygenase-2 expression<sup>[30]</sup>; whether the B<sub>1</sub>R produces a similar effect in gallbladder cancer cells remains to be investigated using gallbladder cancer cell lines.

The identical pattern of expression observed for B<sub>1</sub> and HER2 receptors in precursor lesions of gallbladder carcinoma suggests a cooperative relationship, orientated towards promoting functional cell signaling that may result in an increased proliferation of the gallbladder epithelium.

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## COMMENTS

### Background

Gallbladder carcinoma is an aggressive malignancy of the gastrointestinal tract, and its early diagnosis is difficult, because most of the cases are detected at an advanced stage at laparotomy. It is well known that the HER2 receptor is involved in the carcinogenesis of many malignancies. Further, recent reports show that the bradykinin B<sub>1</sub> receptor (B<sub>1</sub>R) stimulates the proliferation of breast cancer cells. It is not known whether HER2 and B<sub>1</sub>R are overexpressed in precursor lesions of gallbladder carcinoma and whether they serve as biomarkers for early neoplastic transformation of gallbladder epithelium.

### Research frontiers

Most studies related to HER2 and gallbladder cancer have been focused on advanced stages of the disease, but not in their precursor lesions. So far, there have been no investigations on the expression levels of B<sub>1</sub>R in this neoplasm. Because HER2 has a low expression in invasive gallbladder cancer, it has been proposed by some authors that it is not relevant to gallbladder carcinoma.

### Innovations and breakthroughs

The novel finding of high expression of HER2 and B<sub>1</sub>R in precursor lesions of gallbladder carcinoma suggests that they enhance cell proliferation and play a significant role in gallbladder carcinogenesis.

### Applications

Because of the high HER2 and B<sub>1</sub>R expression data in precursor lesions of gallbladder carcinoma, and the therapeutic efficacy of anti-HER2 treatments in breast tumors, future studies should focus on HER2 and B<sub>1</sub>R as biomarkers for the detection of early neoplastic transformations in the gallbladder epithelium.

### Terminology

The HER2 is a tyrosine kinase receptor, member of the epidermal growth factor receptor family. Growth factors bind to receptors and initiate cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis. B<sub>1</sub>R belongs to the family of G protein-coupled receptors, which upon stimulation by analogues trigger cell proliferation and secretion of metalloproteases in cancer cells: proteases that assist cancer cells to invade normal human tissue. The results clearly indicate that both HER2 and the B<sub>1</sub>R play a significant role in transformation of epithelial cells through the metaplasia-dysplasia-carcinoma pathway.

### Peer review

This article has very well explored the HER2 and B<sub>1</sub>R in gallbladder carcinoma. While HER2 and B<sub>1</sub>R are scarcely expressed in invasive gallbladder carcinoma, their expression is upregulated in precursor lesions of this neoplasm which may help in early diagnosis and also provide some pathways of carcinogenesis. This study is worthy.

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## Proteomic analysis of gastric cancer and immunoblot validation of potential biomarkers

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### Abstract

**AIM:** To search for and validate differentially expressed proteins in patients with gastric adenocarcinoma.

**METHODS:** We used two-dimensional gel electrophoresis and mass spectrometry to search for differentially expressed proteins in patients with gastric adenocarcinoma. A set of proteins was validated with immunoblotting.

**RESULTS:** We identified 30 different proteins involved in various biological processes: metabolism, development, death, response to stress, cell cycle, cell communication, transport, and cell motility. Eight proteins

were chosen for further validation by immunoblotting. Our results show that gastrokine-1, 39S ribosomal protein L12 (mitochondrial precursor), plasma cell-induced resident endoplasmic reticulum protein, and glutathione S-transferase mu 3 were significantly underexpressed in gastric adenocarcinoma relative to adjacent non-tumor tissue samples. On the other hand, septin-2, ubiquitin-conjugating enzyme E2 N, and transaldolase were significantly overexpressed. Translationally controlled tumor protein was shown to be differentially expressed only in patients with cancer of the gastric cardia/esophageal border.

**CONCLUSION:** This work presents a set of possible diagnostic biomarkers, validated for the first time. It might contribute to the efforts of understanding gastric cancer carcinogenesis.

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**Key words:** Two-dimensional gel electrophoresis; Mass spectrometry; Gastric adenocarcinoma; Proteome; Biomarkers

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## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer worldwide<sup>[1]</sup>. However, incidence rates have steadily declined; this is largely determined by trends in the fundus and distal stomach<sup>[1]</sup>. Gastric cardia cancer, on the other hand, is on the increase, and is usually of the diffuse histological type<sup>[2]</sup>. However, despite declining incidence rates, GC is still the second leading cause of cancer death and thus remains a major health problem<sup>[1]</sup>. Radical surgery still offers the only chance of a cure for GC that invades the muscular layer, but only half of patients qualify for this at the time of their diagnosis<sup>[2]</sup>. The majority of patients are diagnosed at an advanced stage, where a systemic spread of the tumor cells has to be anticipated. The 5-year survival rate is very low and despite consistent improvements the prognosis remains poor<sup>[3]</sup>. On the other hand, the 5-year survival rate exceeds 90% for patients in an early stage of the disease<sup>[4]</sup>.

A definitive diagnosis of GC requires a gastroscopic or surgical biopsy<sup>[5]</sup>, which means there is a need for a non-invasive test, e.g., the detection of serum/plasma biomarkers. The most widely investigated serum markers include carcinoembryonic antigen, carbohydrate antigen (CA) 19.9, CA 72.4, cytokeratins and the  $\beta$  subunit of human chorionic gonadotropin. However, their low sensitivity and specificity precludes their use in screening and early diagnosis. Therefore, a search for more appropriate biomarkers is necessary.

One possible source of potential new biomarkers is the proteome. As opposed to static genomic data, the proteome is necessary for understanding the dynamic processes in cells<sup>[6]</sup>. It is also more complex, e.g., due to alternative splicing and post-translational modification, and the protein levels often do not correlate with the mRNA<sup>[7]</sup>. Proteomics is a rapidly developing field and is now applied to all areas of the life sciences<sup>[8]</sup>. Gel-based approaches, for example, belong to the most frequently used assays in protein separation and analysis<sup>[9]</sup>. Two-dimensional gel electrophoresis (2-DE) is still the method of choice for complex protein samples, although, due to its limitations with hydrophobic and large proteins, alternative or complementary methods based on mass spectrometry (MS) are gaining popularity.

2-DE, dating back to the 1970s<sup>[10,11]</sup>, in conjunction with MS, has allowed the identification of differentially expressed proteins in various diseases, including GC<sup>[12-17]</sup>. However, the process of translating basic proteomic discoveries to the clinic is very time consuming and expensive, and despite all the efforts put into biomarker research, very few diagnostic protein biomarkers have been approved by the United States Food and Drug Administration<sup>[8]</sup>. The lack of specific and sensitive biomarkers thus creates a persistent need for the expedited development of biomarkers and their use in improving the diagnosis and treatment of cancer. Furthermore, the validity of potential biomarkers, as found by proteome analysis, needs to be investigated and incorporated as the second step of the research, e.g., by immunohisto-

chemical and/or western blot analyses, in order to fulfill the criterion of a systematic investigation of the protein biomarkers in GC.

In this study, we used 2-DE and MS to search for differentially expressed proteins in patients with gastric adenocarcinoma: a total of 30 different protein alterations were found. Gastroskine-1 precursor (GKN1), 39S ribosomal protein L12 (mitochondrial precursor) (MRPL12), plasma cell-induced resident endoplasmic reticulum protein (PACAP), glutathione S-transferase mu 3 (GSTM3), septin-2 (SEPT2), ubiquitin-conjugating enzyme E2 N (UBE2N), transaldolase (TALDO1), and translationally controlled tumor protein (TPT1) were further validated with the immunoblot analysis, making them a set of possible biomarkers for gastric adenocarcinoma.

## MATERIALS AND METHODS

### Subjects and tissues

A total of 32 pairs of gastric adenocarcinoma tissues and adjacent control tissue from 32 patients were obtained from the tissue bank of the Institute of Oncology Ljubljana after the National Medical Ethics Committee's approval. They were stored at -70 °C until further use. The patients' and tumors' parameters are listed in Table 1.

### Sample preparation

The tissues were ground with a mortar and pestle in the presence of liquid nitrogen and then lysed with 7 mol/L urea, 2 mol/L thiourea, 40 g/L 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 20 mmol/L dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States). For every 10 mg of tissue, 50  $\mu$ L lysis buffer was added. After sonication on ice, three times for 10 s, the samples were incubated for 1 h on ice with occasional vortexing and then centrifuged for 1 h at 20 000  $\times g$  at 4 °C. The supernatant was collected and the protein concentration was determined according to a commercial Bradford reagent (Thermo Fisher Scientific, Waltham, MA, United States) with bovine serum albumin as the standard.

### 2-DE

2-DE was conducted on 12 pairs of tissues and repeated in triplicate. For the isoelectric focusing, 100  $\mu$ g protein was diluted in 450  $\mu$ L rehydration solution (7 mol/L urea, 2 mol/L thiourea, 20 g/L CHAPS, 20 mmol/L DTT, 0.5% immobilized pH gradient (IPG) buffer, pH 4-7), loaded by in-gel rehydration onto IPG strips with an acidic pH range of 4-7 (GE Healthcare, Stockholm, Sweden), and focused in an Ettan IPGphor II isoelectric focusing system (GE Healthcare) to a total of 63.5 kVh. Next, the IPG strips were equilibrated for 15 min in 6 mol/L urea, 30% glycerol, 20 g/L sodium dodecyl sulfate (SDS), 50 mmol/L Tris-HCl, pH 8.8, with 10 g/L DTT, and for another 15 min in the same solution with DTT replaced by 25 g/L iodoacetamide. The strips were then transferred to 12% polyacrylamide gels and the

Table 1 Patient and tumor characteristics

Characteristics	n (%)
Sex	
Male	19 (59.4)
Female	13 (40.6)
Lauren's classification	
Intestinal	20 (62.5)
Diffuse	11 (34.4)
Mixed	1 (3.1)
Location	
Corpus	8 (25)
Antrum	7 (21.9)
Cardia/gastroesophageal border	10 (31.2)
Several parts	7 (21.9)
Grade	
Well-differentiated	2 (6.2)
Moderately differentiated	12 (37.5)
Poorly differentiated	12 (37.5)
Undifferentiated	6 (18.8)
pT	
pT1b	1 (3.1)
pT2a	5 (15.6)
pT2b	15 (46.9)
pT3	11 (34.4)
pN	
pN0	8 (25)
pN1	16 (50)
pN2	6 (18.8)
pN3	2 (6.2)

second dimension was carried out in an Ettan DALTsix electrophoresis unit (GE Healthcare) under constant power in a Laemmli buffer system until the bromophenol blue reached the end of the gel.

### Silver staining

The gels were silver stained according to Mortz *et al.*<sup>18</sup>. Briefly, they were fixed overnight in 50% methanol, 12% acetic acid, and 0.05% formalin, washed for 2 × 20 min with 35% ethanol and sensitized for 2 min with 0.02% sodium thiosulfate. Next, they were washed for 3 × 5 min with water and stained for 20 min in 0.2% silver nitrate and 0.076% formalin. Then they were washed again for 2 × 1 min with water and developed with 6% sodium carbonate, 0.05% formalin and 0.0004% sodium thiosulfate. The reaction was stopped with 50% methanol and 12% acetic acid and the gels were left in 1% acetic acid until the scanning.

### Image acquisition and analysis

The gels were scanned with an ImageScanner II (GE Healthcare) at 300 dpi and analyzed with an ImageMaster 2D Platinum v7 (GE Healthcare). The spots were detected, matched and quantified by relative volume. The normalization of the spot values (relative spot volumes) was based on the total spot volume. Next, the data were analyzed with Excel. The spots of interest were determined using a pair-by-pair comparison. The spots were considered to be differentially expressed if they matched the following criteria: at least a twofold change in the relative spot volume, the occurrence of this change in at

least three patients, and statistical significance.

### In-gel digestion

The enzymatic digestion of the excised spots was performed using a Progest robot (Genomic Solutions, Holliston, MA, United States). Briefly, the protein spots were excised from the gel, placed into 96-well plates and washed with 50  $\mu$ L acetonitrile ( $\text{CH}_3\text{CN}$ ) for 3 min and then with 50  $\mu$ L 25 mmol/L ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) for 3 min. This washing step was repeated three times to eliminate SDS, Tris and glycine. After the washing procedure, the excised spots were treated with 100  $\mu$ L 10 mmol/L DTT for the reduction step and the reaction was left to proceed at 57 °C for 1 h. After DTT removal, 100  $\mu$ L 55 mmol/L iodoacetamide was added for the cysteine carbamidomethylation and the reaction was left to proceed at room temperature (RT) for 1 h. After removal of the supernatant, the washing procedure was repeated three times and the gel slices were dried with a SpeedVac (5 min). Based on the gel-slice volume, 5-10  $\mu$ L 12 ng/ $\mu$ L Porcine Trypsin (Promega, Madison, WI, United States) was added. The enzyme was freshly diluted in 25 mmol/L  $\text{NH}_4\text{HCO}_3$  and the digestion was performed overnight at RT. Trypsin was the protease of choice for the MS protein identification because of its reliability and its substrate specificity, yielding peptides with C-terminal basic residues (Arg and Lys), which facilitated positive ionization and subsequent MS detection. Finally, 10  $\mu$ L of 39%  $\text{H}_2\text{O}/60\%$   $\text{CH}_3\text{CN}/1\%$   $\text{HCOOH}$  was added and the samples were left for 3 h at RT to favor the extraction of peptides from the gel.

### MS and database search

Trypsin-generated peptide mixtures were analyzed by matrix-assisted laser desorption ionization coupled with a time-of-flight analyzer (MALDI-TOF) (Voyager, Applied Biosystems, Carlsbad, CA, United States). Samples that provided an ambiguous identification with MALDI-TOF were analyzed by tandem MS (MS/MS) using nanoliquid-chromatography electrospray ionization coupled with the quadrupole and time-of-flight analyzer (nanoLC-ESI-Q-TOF) (nanoAcquity, Q-TOF Premier; Waters, Milford, MA, United States).

For MALDI-TOF MS, the peptide extracts (0.5  $\mu$ L) were mixed with an equal volume of 2,5-dihydroxybenzoic acid (10 mg/mL; Sigma-Aldrich) dissolved in 20%  $\text{CH}_3\text{CN}$ . Trypsin-digested ovalbumin was used for external calibration. Crystals were obtained using the dried-droplet method on a 100-spots metallic plate and approximately 500 MALDI mass spectra were averaged per spot to optimize the signal-to-noise ratio. The laser fluency was adjusted to the threshold to achieve the best resolution and mass accuracy. The MS measurements were carried out at a maximum accelerating potential of 20 kV, in the positive reflectron mode. The acquisition range was set to  $m/z$  800-3500 with a low-mass gate at  $m/z$  700. For  $m/z$  values of about 1500, the mean mass resolution was 15 000.

Proteins were identified by peptide mass fingerprinting with two search programs: Mascot and ProFound. The following search parameters were applied: SWISS-PROT and NCBI were used as the protein-sequence databases; a mass tolerance of 50 ppm and one missed cleavage were allowed; the alkylation of cysteine by carbamidomethylation was considered complete; while the oxidation of methionine was considered as a possible modification.

For nanoLC-ESI-Q-TOF MS/MS analysis, peptide mixtures were SpeedVac-treated for 10 min to eliminate CH<sub>3</sub>CN, then 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub> was added before injection into the nanoAcquity/Q-TOF system equipped with a trapping column (Symmetry C18, 180 μm × 20 mm, 5 μm particle size) and an analytical column (BEH130 C18, 75 μm × 100 mm, 1.7-μm particle size) (Waters). The aqueous solvent (buffer A) was 0.1% formic acid and the organic phase (buffer B) was acetonitrile with 0.1% formic acid. A 2%-40% B gradient was set for 25 min. The MS parameters were as follows: positive ion mode; capillary voltage, 3 kV; cone voltage, 40 V; ion-source block temperature, 80 °C; and collision energy ramping from 15 to 40 eV. For the exact mass measurements, the glufibrinopeptide reference ( $m/z = 785.8426$ ) was continuously supplied during the nanoLC-MS/MS experiments using the lockspray device. The peptide mass measurements were corrected by the PLGS software (ProteinLynx Global Server; Waters) during data processing. Peak lists were generated by PLGS and the processed data were submitted to Mascot searching using the following parameters: data bank, NCBI; peptide tolerance, 15 ppm; fragment tolerance, 0.1 Da; number of missed cleavages, one; variable modifications, oxidation; and fixed modifications, carbamido methylation.

### Immunoblot analysis

To validate the differential expression from the 2-DE gels, an immunoblot analysis was performed on an expanded number of samples, on 27 pairs. For TPT1, additional four pairs of cardia/gastroesophageal border adenocarcinoma were used. A total of 30 μg protein per sample was loaded on 12% or any kD gels (Bio-Rad, Hercules, CA, United States), transferred onto PDVF membranes (Millipore, Billerica, MA, United States), and blocked in 50 g/L skimmed milk overnight at 4 °C. Primary antibodies were used in the following dilutions: anti-GKN1 at 0.5 μg/mL (WH0056287M1; Sigma-Aldrich), anti-MRPL12 at 1 μg/mL (WH0006182M1; Sigma-Aldrich), anti-PACAP at 1:1000 (ab96308; Abcam, Cambridge, United Kingdom), anti-GSTM3 at 0.75 μg/mL (ab74749; Abcam), anti-SEPT2 at 1 μg/mL (ab88657; Abcam), anti-UBE2N at 0.5 μg/mL (ab25885; Abcam), anti-TALDO1 at 1:1000 (ab67467; Abcam), and anti-TPT1 at 1 μg/mL (WH0007178M1; Sigma-Aldrich). Horseradish-peroxidase-conjugated secondary antibodies were used in the following dilutions: goat anti-mouse at 1:5000 (115-035-062; Jackson ImmunoResearch, Newmarket, Suffolk, United Kingdom) and goat anti-rabbit

at 1:10 000 (111-035-003; Jackson ImmunoResearch). The proteins were detected chemiluminescently with an LAS-4000 CCD camera (Fujifilm, Tokyo, Japan) at 10-s intervals. The blots were then quantified with Multi Gauge software (Fujifilm) and the intensity values were exported to Excel. Differential expression was determined after normalization to Ponceau S-stained membranes for loading and transfer differences and statistical significance was assessed.

### Statistical analysis

To assess the statistical significance of differential protein expression (tumor *vs* non-tumor) in 2-DE as well as in immunoblotting, Wilcoxon signed-rank test was used. The test was double-sided and values with  $P < 0.05$  and a 95% CI were considered to be statistically significant. To assess the correlation of the differential protein expression from immunoblotting with the histopathological parameters, repeated-measures analysis of variance was used. The values with  $P < 0.05$  and a 95% CI were considered to be statistically significant. Bonferroni post-test was used to narrow down where the differences were significant. All analyses were performed using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, United States) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, United States).

## RESULTS

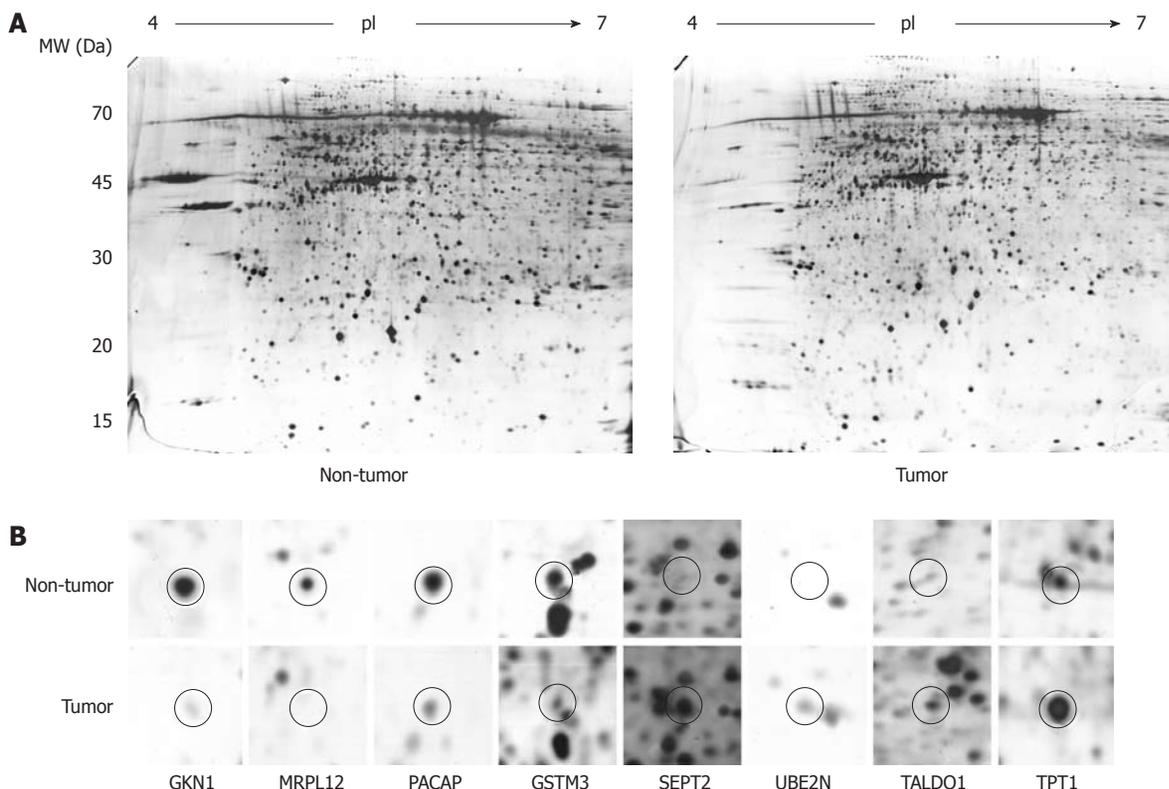
### Subject parameters

Altogether, we used 32 pairs of human gastric adenocarcinoma and adjacent normal tissue samples from 32 patients. The mean age of the patients was  $68.3 \pm 10.5$  (range 40-84) years. The subject group contained predominantly males (59.4%). According to Lauren's classification, the majority of tumors were intestinal (62.5%) and the largest part was located in the cardia/gastroesophageal border (31.2%). The tumors were mainly moderately (37.5%) and poorly (37.5%) differentiated. According to the TNM classification, most were pT2b (46.9%) and pN1 (50%). The data on pM were only available for some patients and were thus not included in any further analysis. For more details, refer to Table 1.

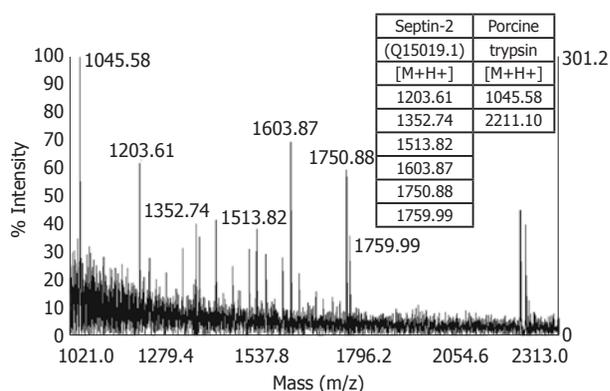
### 2-DE and MS

To determine the differentially expressed proteins in the gastric adenocarcinoma patients, we first performed 2-DE on 12 pairs of human gastric tissue samples. For good resolution we used large (24 cm) IPG strips with an acidic pH gradient (4-7). We performed the experiments in triplicate, which produced well-resolved spots and reproducible 2-DE patterns. On average,  $1197 \pm 150$  spots per gel were detected after silver staining. Figure 1 shows a typical image of a tumor- and a non-tumor-sample-derived gel. In the lower part of the figure, one can see differentially expressed spots from the tumor/non-tumor pairs, obtained from a pair-by-pair comparison.

After the proteolytic digestion of 32 excised spots,



**Figure 1** Results of two-dimensional gel electrophoresis from gastric adenocarcinoma patients. A: Examples of silver-stained two-dimensional gel electrophoresis gels for non-tumor and tumor tissue; B: Examples of differentially expressed spots (circled) for the non-tumor and tumor tissue that were chosen for immunoblot validation. MW: Molecular weight; GKN1: Gastrokine-1 precursor; MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); PACAP: Plasma cell-induced resident endoplasmic reticulum protein; GSTM3: Glutathione S-transferase mu 3; SEPT2: Septin-2; UBE2N: Ubiquitin-conjugating enzyme E2 N; TALDO1: Transaldolase; TPT1: Translationally controlled tumor protein.



**Figure 2** An example of mass spectrometry identification results for septin-2.

we identified 30 different proteins by MALDI-TOF MS or nanoLC-ESI-Q-TOF MS/MS. Eleven proteins were found to be underexpressed, while 19 were found to be overexpressed in the gastric adenocarcinoma relative to adjacent non-tumor tissue samples. These results are summarized in Table 2 and an example of the MS identification is shown in Figure 2.

The identified differentially expressed proteins could be classified into eight groups according to the biological process in which they are involved (Figure 3), by information from the GO Classification for *Homo sapiens* from European Molecular Biology Laboratory - European

Bioinformatics Institute (<http://www.ebi.ac.uk/integr8/GOAnalysisPage.do?orgProteomeID=25>). These groups were: metabolism (*HPGD, ECHS1, MRPL12, ACADS, NDUFV2, PBLD, PACAP, ATP5H, GSTM3, PGK1, ACTB, HSPA1, CBX3, UBE2N, TALDO1, MTPN, ENO1, NNMT, EIF2S1, NME1*), development (*HPGD, NDUFV2, GSTM3, SEPT2, SERPINB5, ACTB, NGFR, MTPN, NME1*), death (*PACAP, PKM2, NGFR, CTSD, ANXA4, TPT1, ANXA5, NME1*), response to stress (*ACTB, HSPA1, UBE2N, ENO1, EIF2S1, ANXA5*), cell cycle (*GKN1, HPGD, SEPT2, NGFR, HSPA1, NME1*), cell communication (*GSTM3, ACTB, NGFR, ANXA4, ANXA5*), transport (*ATP5H, TTR, SEPT2, TPT1*), and cell motility (*SERPINB5, ACTB*).

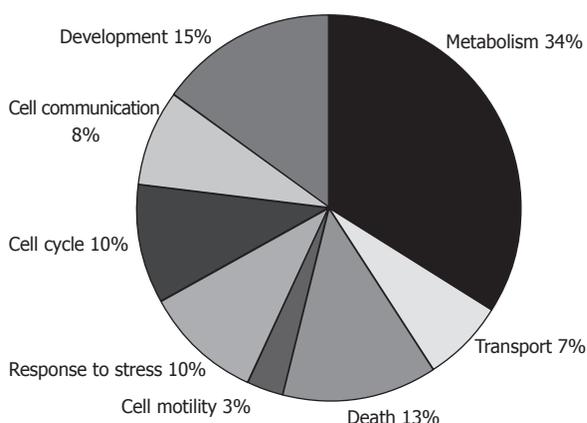
**Immunoblot analysis**

In order to validate the results from the 2-DE and to investigate the possibility of the identified proteins becoming relevant biomarkers, we performed an immunoblot analysis on an expanded group of samples: 11 that were used for 2-DE as well as an additional 16 (*n* = 27). The selection of proteins subjected to the analysis was based on their putative relevance. GKN1 was the most abundantly underexpressed protein and was already validated at the protein level<sup>[19]</sup>, so it was chosen as a general control in our 2-DE and immunoblot experiments. MRPL12 has, to the best of our knowledge, not yet been found

**Table 2** Results of mass spectrometry identification

Protein name	Gene name	Swiss-Prot ID	pI/MW	Frequency <sup>1</sup>	Fold <sup>2</sup>	P value	Coverage <sup>3</sup>	No. of pep. <sup>4</sup>
Down-regulated								
Gastrokine-1 precursor	<i>GKN1</i>	Q9NS71	5.3/21.0	75% (9/12)	15.17	0.000	16%	6
15-hydroxyprostaglandin dehydrogenase isoform 1	<i>HPGD</i>	P15428	5.6/25.2	66% (8/12)	3.85	0.016	23%	5
Enoyl CoA hydratase, mitochondrial precursor	<i>ECHS1</i>	P30084	5.8/27.9	66% (8/12)	3.38	0.001	49%	15
39S ribosomal protein L12, mitochondrial precursor	<i>MRPL12</i>	P52815	5.1/20.6	58.3% (7/12)	10.95	0.016	16%	4
Acyl-CoA dehydrogenase	<i>ACADS</i>	P16219	6.1/39.9	58.3% (7/12)	4.94	0.005	12%	5
NADH dehydrogenase (ubiquinone) flavoprotein 2, mitochondrial precursor	<i>NDUFV2</i>	P19404	5.7/24.2	50% (6/12)	2.26	0.027	58%	16
Phenazine biosynthesis-like domain-containing protein isoform a	<i>PBLD</i>	P30039	6.1/31.8	50% (6/12)	3.60	0.007	25%	5
Plasma cell-induced resident endoplasmic reticulum protein	<i>PACAP</i>	Q8WU39	5.2/22.0	41.6% (5/12)	4.41	0.042	39%	5
ATP synthase subunit d, mitochondrial	<i>ATP5H</i>	O75947	5.2/20.5	41.6% (5/12)	2.40	0.012	26%	5
Glutathione S-transferase mu 3	<i>GSTM3</i>	P21266	5.3/26.3	33.3% (4/12)	3.35	0.042	29%	4
Transthyretin	<i>TTR</i>	P02766	5.4/16.4	25% (3/12)	2.74	0.027	53%	7
Up-regulated								
Septin-2	<i>SEPT2</i>	Q15019	6.0/41.0	75% (9/12)	4.58	0.009	34%	7
Maspin	<i>SERPIN5</i>	P36952	5.6/40.2	58.3% (7/12)	4.99	0.007	14%	5
Phosphoglycerate kinase 1	<i>PGK1</i>	P00558	6.0/40.1	58.3% (7/12)	6.46	0.016	22%	6
Pyruvate kinase, muscle	<i>PKM2</i>	P14619	6.0/38.8	58.3% (7/12)	5.25	0.021	20%	8
Actin, cytoplasmic 1	<i>ACTB</i>	P60709	4.8/32.2	58.3% (7/12)	7.77	0.005	44%	12
Tumor necrosis factor receptor superfamily member 16 precursor	<i>NGFR</i>	P08138	5.2/35.5	50% (6/12)	3.94	0.002	17%	5
Heat shock 70 kDa protein 1A/1B	<i>HSPA1</i>	P08107	5.1/36.9	50% (6/12)	4.77	0.009	14%	8
Heterochromatin-like protein 1	<i>CBX3</i>	Q13185	5.2/22.0	50% (6/12)	4.12	0.027	19%	3
Ubiquitin-conjugating enzyme E2 N	<i>UBE2N</i>	P61088	5.7/16.4	41.6% (5/12)	4.94	0.001	40%	5
Transaldolase	<i>TALDO1</i>	P37837	5.9/38.5	41.6% (5/12)	3.12	0.003	18%	5
Cathepsin D precursor	<i>CTSD</i>	P07339	5.3/28.4	41.6% (5/12)	3.49	0.034	27%	11
Annexin A4	<i>ANXA4</i>	P09525	5.6/31.3	41.6% (5/12)	13.31	0.027	54%	26
Myotrophin	<i>MTPN</i>	P58546	5.1/13.5	41.6% (5/12)	2.45	0.000	22%	4
Enolase 1 variant	<i>ENO1</i>	P06733	5.5/42.6	41.6% (5/12)	3.95	0.034	19%	7
Nicotinamide N-methyltransferase	<i>NNMT</i>	P40261	5.4/28.5	41.6% (5/12)	4.24	0.000	9%	3
Eukaryotic translation initiation factor 2 subunit 1	<i>EIF2S1</i>	P05198	4.9/33.5	41.6% (5/12)	6.61	0.042	24%	8
Translationally-controlled tumor protein	<i>TPT1</i>	P13693	4.8/20.7	25% (3/12)	2.50	0.002	18%	4
Annexin A5	<i>ANXA5</i>	P08758	5.0/32.4	25% (3/12)	3.24	0.005	70%	25
Nucleoside diphosphate kinase A isoform a	<i>NME1</i>	P15531	5.7/19.0	25% (3/12)	3.11	0.016	33%	5

<sup>1</sup>Frequency shows the number of patients where the protein was differentially expressed in two-dimensional gel electrophoresis analysis; <sup>2</sup>Fold stands for the average fold change in the relative spot volume between non-tumor and tumor (downregulated) or tumor and non-tumor (upregulated) tissue; <sup>3</sup>Coverage stands for the sequence coverage in mass spectrometry (MS) analysis; <sup>4</sup>No. of pep. stands for the number of matched peptides in MS analysis. MW: Molecular weight.

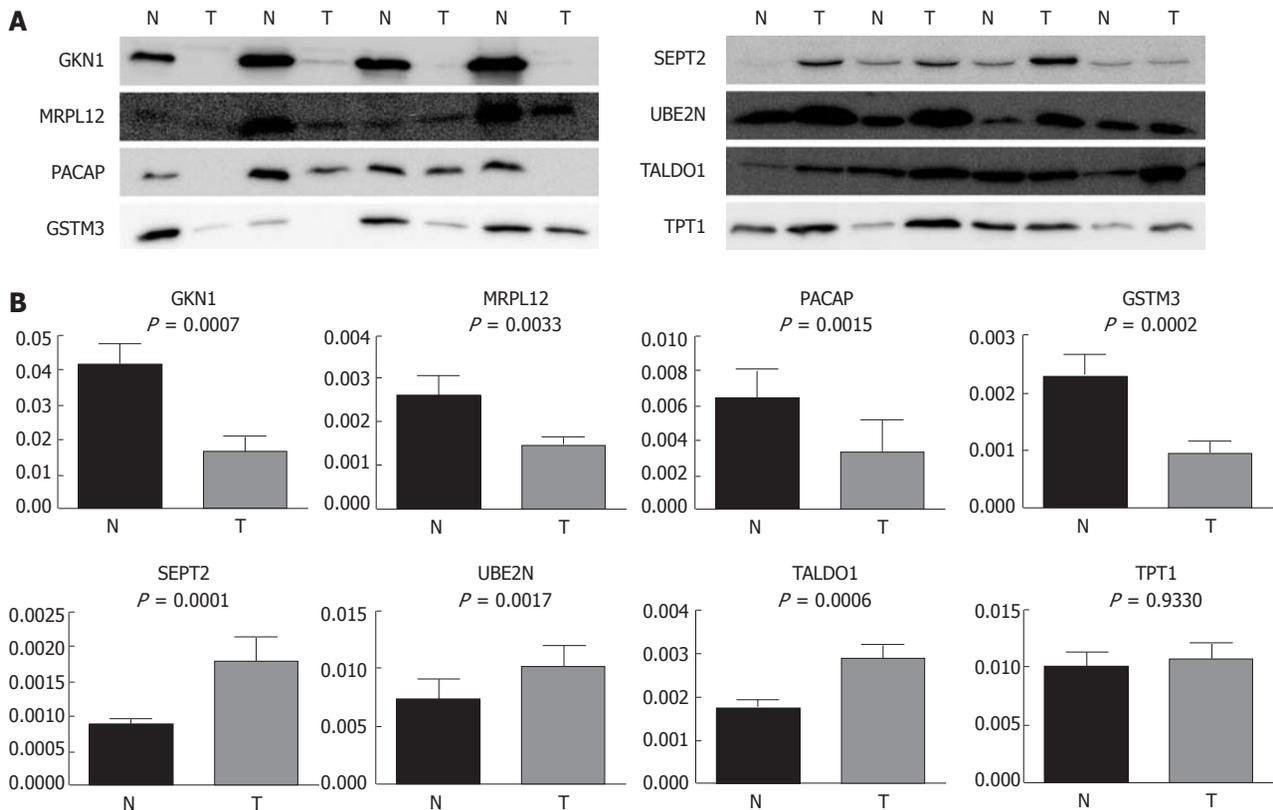


**Figure 3** Distribution of differentially expressed proteins according to the biological processes in which they are involved.

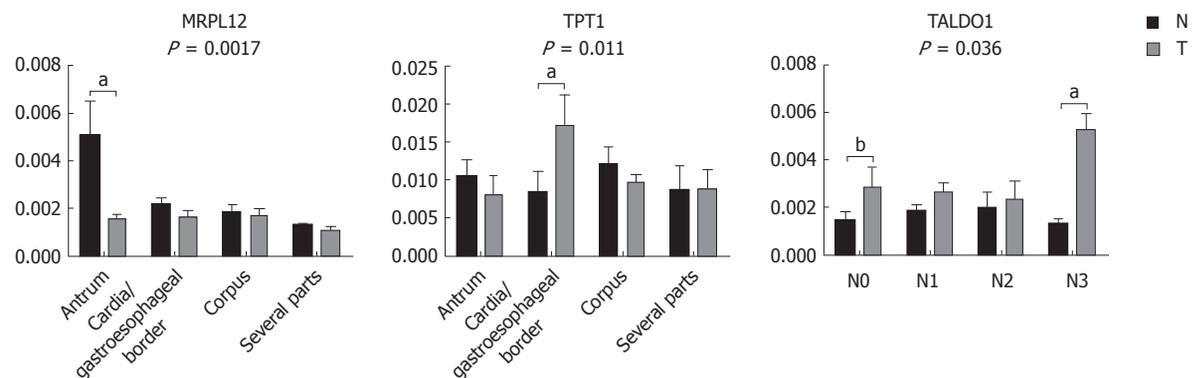
to be associated with gastric cancer, while SEPT2 (as the most abundantly overexpressed), UBE2N, TALDO1, TPT1, PACAP, and GSTM3 were already found to be

associated with gastric adenocarcinoma, but were not previously validated at the protein level.

Our results (Figure 4) show that GKN1 ( $P = 0.0007$ ), MRPL12 ( $P = 0.0033$ ), PACAP ( $P = 0.0015$ ), and GSTM3 ( $P = 0.0002$ ) were significantly underexpressed in gastric adenocarcinoma. SEPT2 ( $P = 0.0001$ ), UBE2N ( $P = 0.0017$ ), and TALDO1 ( $P = 0.0006$ ), on the other hand, were significantly overexpressed. The immunoblot results confirmed our results from the 2-DE analysis. TPT1, on the other hand, did not appear as generally differentially expressed in the tumor samples compared to the non-tumor samples. However, we also checked for a correlation of the differential expression with the histopathological parameters. We observed (Figure 5) significant differences in the MRPL12 expression and tumor location ( $P = 0.017$ ), as well as in TPT1 expression and tumor location ( $P = 0.011$ ). TALDO1 expression was observed to correlate with pN status ( $P = 0.036$ ). A higher rate of MRPL12 overexpression was found in the antrum ( $P < 0.001$ ) and a higher rate of TPT1 overexpression



**Figure 4** Examples of immunoblotting results on four pairs of non-tumor and tumor tissue for eight differentially expressed proteins (A) and densitometric results from immunoblotting for the same eight proteins (B). The x-axis represents non-tumor (N) and tumor (T) tissues and the y-axis represents relative band density. The *P* values are from the Wilcoxon signed-rank test. GKN1: Gastrin-1 precursor; MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); PACAP: Plasma cell-induced resident endoplasmic reticulum protein; GSTM3: Glutathione S-transferase mu 3; SEPT2: Septin-2; UBE2N: Ubiquitin-conjugating enzyme E2 N; TALDO1: Transaldolase; TPT1: Translationally controlled tumor protein.



**Figure 5** Densitometric results from immunoblotting for three proteins describing potential associations of differential protein expression and clinical parameters. The x-axis represents different clinical parameters and the y-axis represents relative band density. The *P* values are from the repeated measures analysis of variance. <sup>a</sup>*P* < 0.01 and <sup>b</sup>*P* < 0.05 are from the Bonferroni post-test. MRPL12: 39S ribosomal protein L12 (mitochondrial precursor); TPT1: Translationally controlled tumor protein; TALDO1: Transaldolase; N: Non-tumor; T: Tumor.

was found in the cardia/gastroesophageal border (*P* < 0.01). A higher rate of TALDO1 overexpression was found in pN0 (*P* < 0.05) and pN3 (*P* < 0.01) tumors.

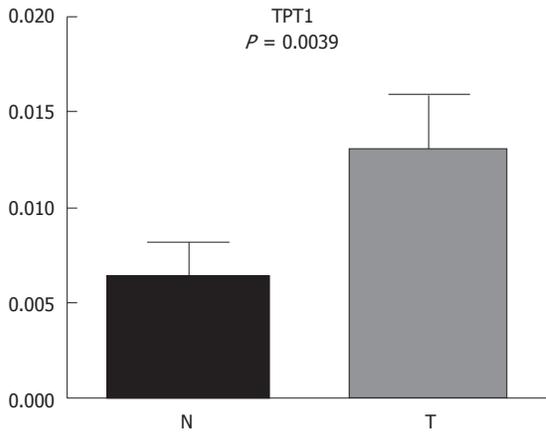
Due to the interesting results of TPT1 and cardia/gastroesophageal border correlation, we used additional tissue samples from four patients with cardia/gastroesophageal border adenocarcinoma to confirm it. The trend remained the same: TPT1 was upregulated. When combining tissues from all 10 patients with cardia/gas-

troesophageal border adenocarcinoma (six from before and four additional), Wilcoxon signed-rank test confirmed the differential expression (Figure 6).

## DISCUSSION

### General observations

Using a gel-based proteomic approach, we aimed to find and validate the differentially expressed proteins in a set



**Figure 6** Densitometric results from immunoblotting of 10 pairs of non-tumor and tumor tissue from cardia/gastroesophageal border adenocarcinoma patients. The x-axis represents non-tumor (N) and tumor (T) tissues and the y-axis represents relative band density. The *P* value is from the Wilcoxon signed-rank test. TPT1: Translationally controlled tumor protein.

of gastric adenocarcinoma patients. A total of 30 different proteins were identified in the study. They belonged to different biological processes, including metabolism, development, death, response to stress, cell cycle, cell communication, transport, and cell motility. The largest group, metabolism, contained 20 proteins with at least some role in cell metabolism.

A set of these proteins has already been found in similarly conducted experiments for GC. Among those downregulated in the tumor, enoyl CoA hydratase, mitochondrial precursor<sup>[20,21]</sup>, acyl-CoA dehydrogenase<sup>[22]</sup>, NADH dehydrogenase (ubiquinone) flavoprotein 2, mitochondrial precursor<sup>[22]</sup>, phenazine biosynthesis-like domain-containing protein isoform a<sup>[22]</sup>, and ATP synthase subunit d, mitochondrial<sup>[23]</sup> have been reported with the same alterations as in our study. In contrast to our observation, ATP synthase subunit d was reported to be upregulated in another study<sup>[22]</sup>. Among the proteins upregulated in the tumor, phosphoglycerate kinase 1<sup>[19]</sup>, pyruvate kinase isozymes M1/M2<sup>[24,25]</sup>, heat shock 70 kDa protein 1A/1B<sup>[24,25]</sup>, cathepsin D precursor<sup>[22,24,26]</sup>, annexin A4<sup>[26]</sup>, alpha-enolase<sup>[24,25]</sup>, nicotinamide N-methyltransferase<sup>[20,27,28]</sup>, annexin A5<sup>[20,29]</sup>, and actin, cytoplasmic 1 in rat GC metastases<sup>[30]</sup> have been found elsewhere with the same trend as in our study. However, other studies have reported that pyruvate kinase<sup>[22]</sup>, alpha-enolase<sup>[26]</sup>, and actin<sup>[26,31]</sup> have the opposite expression patterns compared to our results. This discrepancy could perhaps result from the heterogeneity that is often present in studies of human tumor tissue. In this paper, we present and discuss eight putative biomarkers for gastric carcinogenesis that were identified in our study, among which seven were validated for the first time by means of immunoblotting.

### Specific protein alterations

GKN1 has already been identified as a downregulated gene in GC<sup>[32]</sup>. It is suggested to maintain gastric mu-

cosal integrity and mediate repair after injury<sup>[33]</sup>. The protection of the mucosal barrier is thought to be due to the ability of GKN1 to alter the distribution of specific tight-junction proteins and to stabilize perijunctional actin<sup>[34]</sup>. It was later demonstrated to bind with F-actin in smooth muscle cells, suggesting a role in cell-cell adhesion and the assembly of actin stress fibers<sup>[35]</sup>. Recently, GKN1 has been shown to be a modulator of apoptotic signals<sup>[36]</sup>. It has been confirmed as a secreted protein and as being present in native and metaplastic gastric epithelium, but absent from the gastric carcinoma and the precursor lesion of intestinal metaplasia, making it a possible tumor suppressor in gastric carcinogenesis<sup>[37]</sup>.

GKN1 expression is downregulated in *Helicobacter pylori*-positive patients<sup>[38]</sup>. In another study, a loss of GKN1 occurred, especially in the diffuse-type tumor, but was associated with a significantly worse outcome in the intestinal type<sup>[39]</sup>. It has been found to be downregulated in GC, using 2-DE<sup>[20,23,25,27]</sup>, and these results have been validated at the protein and mRNA levels<sup>[19]</sup>. Consistently, we found that GKN1 was underexpressed in tumor tissue, using 2-DE, and we also validated the results by immunoblotting. Our result is in agreement with other reports and, like other research groups, we were also unable to find any correlation with histopathological parameters at the protein level. The overlapping of both steps of the biomarker identification with a number of other studies in this case contributed to the confidence in the approach for the analysis of additional biomarkers found in our proteomic analysis.

MRPL12 is the first cloned and characterized mammalian mitochondrial ribosomal protein encoded by the nucleus<sup>[40]</sup>. It accumulates in cells at the mRNA and protein levels upon growth-factor stimulation. The enhanced expression later contributes to transcriptional activation<sup>[41]</sup>. *MRPL12* mRNA levels have been detected in different organs, being especially high in the colon and skeletal muscle<sup>[41]</sup>. Besides being a component of the mitochondrial ribosome (its dimers bind the large ribosomal unit), MRPL12 binds to mitochondrial RNA polymerase and enhances transcription *in vitro*<sup>[42]</sup>. It has been speculated that it may either directly couple transcription and translation by binding simultaneously to polymerase and ribosomes, or alone bind to polymerase and activate its transcriptional activity in some way<sup>[43]</sup>. However, Litonin *et al*<sup>[44]</sup> have observed no such stimulation, so further experiments are necessary to clarify the possible role of this protein in transcription.

MRPL12 is differentially expressed, and it has previously been observed by 2-DE to be overexpressed in prostate cancer<sup>[45]</sup> and in hepatitis B virus (HBV)-associated hepatocellular carcinoma<sup>[46]</sup>. This is not in accordance with our results because we showed that MRPL12 was downregulated in gastric adenocarcinoma. We also observed its correlation with location: higher rates of underexpression were found in the antrum. In the context of cancer, the knockdown of MRPL12 decreases mitochondrial activity, increases glycolysis and accelerates

tumor growth<sup>[47]</sup>. We speculate that it may also be the case in gastric adenocarcinoma.

PACAP was found as an expressed sequence tag from a microarray analysis where it exhibited downregulation in intestinal-type GC<sup>[48]</sup>. The protein was found in the endoplasmic reticulum of lymphocytes, where it exhibited upregulation in the course of B-cell differentiation<sup>[49,50]</sup>. It was found to assist in the oxidative folding of Ig domains; however, both research groups were uncertain as to whether it acted as an oxidoreductase or as a chaperone. In a very recent report<sup>[51]</sup>, PACAP was described as helping to diversify peripheral B-cell functions by regulating Ca<sup>2+</sup> stores, antibody secretion and integrin activation.

A patent application<sup>[52]</sup> has disclosed the use of PACAP as a universal marker of different types of cancer, GC included. However, they claim that increased concentrations of the protein and/or its fragments are associated with cancer, whereas we discovered just the opposite for GC. The same was reported by Huang *et al.*<sup>[31]</sup> in a 2-DE experiment (although this was without validation), by Hasegawa *et al.*<sup>[48]</sup> and Katoh *et al.*<sup>[53]</sup>. However, in the latter two cases, this was at the non-protein level. We validated our results by immunoblotting. Due to its downregulation in the tumor, we strongly support the previous speculation that PACAP might be a candidate tumor-suppressor gene<sup>[53]</sup>.

GSTM3 is a glutathione S-transferase (GST) that belongs to the mu class<sup>[54]</sup>. It is rather unusual; it is not only about 70% identical in its protein sequence to the other mu-class transferases, but it is also considerably shorter and transcribed in the reverse orientation<sup>[55]</sup>.

Several polymorphisms have been found in GSTs, which can alter the susceptibility to carcinogens and toxins and influence the toxicity and efficacy of drug treatment<sup>[54]</sup>. They have been studied in relation to several cancers, GC included. For example, Martinez and colleagues have found no association with the *GSTM3* genotype and GC risk<sup>[56]</sup>, whereas Tatemichi and co-workers have described a possible association between *GSTM3* polymorphisms and Ig titer levels in serum against *H. pylori*<sup>[57]</sup>.

*GSTM3* is found in several normal tissues, including the stomach<sup>[58]</sup>. A comparison of the differential *GSTM3* expression in cancers is made rather difficult by the fact that many studies have focused on the whole GST family or class, but not on individual isoforms. For instance, antral GST enzyme activity has been found to be significantly lower in the stomach of *H. pylori*-infected patients<sup>[59]</sup>, but the contributions of separate isoforms has not been studied. For *GSTM3* specifically, the gene is highly expressed in a subgroup of patients with head and neck squamous cell carcinoma<sup>[60]</sup>. The protein is upregulated in neuroblastoma<sup>[61]</sup> and in polycystic ovary syndrome<sup>[62]</sup>. We found that *GSTM3* was downregulated in gastric adenocarcinoma. Our results are in agreement with a study reporting downregulation in the seminomatous germ cell tumor, where the changes were also reflected at the transcriptional level<sup>[63]</sup>. This reduced expression could be indicative of the decreased detoxification capacity of tumor cells<sup>[59]</sup>.

Septins belong to a family of conserved GTP-binding proteins<sup>[64]</sup>. They have been implicated in many cellular processes. SEPT2 is thought to be involved in cytokinesis,

as well as chromosome congression and segregation<sup>[65,66]</sup>. Its fibers appear to contact actin bundles and focal adhesion complexes physically, thereby linking it to a functional interaction with actin-based cytoskeletal systems in interphase cells<sup>[65]</sup>. It has also been found in the microtubule spindle during metaphase and is proposed to form a mitotic scaffold for different effectors to coordinate cytokinesis with chromosome congression and segregation<sup>[66]</sup>. Several other binding partners and functions have been proposed for SEPT2, such as the DNA damage response, the regulation of the efficiency of vesicular transport, and FCγR-mediated phagocytosis<sup>[64]</sup>. Recently, it has been reported that SEPT2 is part of a diffusion barrier between the primary cilia and the cell and it is essential for retaining receptor-signaling pathways in primary cilia<sup>[67]</sup>. Also, in response to physiological and pathological stimuli, SEPT2 redistributes and its interaction with actin increases, which allows for the dynamic modulation of the airway epithelial barrier function<sup>[68]</sup>. Despite all the progress, the exact molecular mechanisms, cellular, and physiological functions of septins are still poorly understood and interactome studies could help<sup>[69]</sup>.

Septins have been linked to diseases such as neurodegeneration and cancer<sup>[70]</sup>. It is proposed that altered SEPT2 expression can lead to disordered chromosomal dynamics and underlie the development of the aneuploidy common to cancers<sup>[66]</sup>. SEPT2, among others, has been found to be a fusion partner of the mixed lineage leukemia (*MLL*) gene in therapy-related acute myeloid leukemia<sup>[71]</sup>. Such fusion is associated with downregulation of *SEPT2* and *MLL* in myeloid neoplasia<sup>[72]</sup>. It has also been shown to be downregulated in glioblastoma<sup>[73]</sup>. On the other hand, it has been identified as upregulated in hepatoma carcinoma cells, where its phosphorylation on Ser218 by casein kinase 2 has been determined as crucial for hepatoma carcinoma cell proliferation<sup>[74]</sup>. In a 2-DE experiment, SEPT2 was, in agreement with our results, determined to be upregulated in GC<sup>[22]</sup>; however, no validation was carried out. The same expression pattern was found in renal cell carcinoma<sup>[75]</sup> and in late-stage human colon cancer tissue<sup>[76]</sup>, and it is abundantly expressed in several brain tumors and brain-tumor cell lines<sup>[77]</sup>. Taken as a whole, these results suggest its possible role as an oncogene.

Ubiquitination is a post-translational modification carried out in several steps<sup>[78]</sup>, one of them being conjugation of an activated ubiquitin to an ubiquitin-conjugating enzyme (E2) *via* a highly conserved catalytic cysteine residue. By directly influencing the type of lysine used to label the substrates, they influence the fate of the substrates. One of the E2s is UBE2N, which acts as part of a complex that enables the formation of the non-canonical Lys63-mediated polyubiquitin chains<sup>[79]</sup>. As opposed to Lys48-mediated ones, these do not target proteasome degradation but mediate other processes. Among other functions, it has been shown that UBE2N in a complex with Mms2 functions *via* Lys63-mediated polyubiquitination in DNA repair, whereas in a complex with Uev1A, it functions in activating nuclear factor-κB signaling<sup>[78,80]</sup>. Both of the partner proteins, however, are dispensable for the RNF8-dependent propagation of DNA damage signals *via* ubiq-

uitination<sup>[81]</sup>, although there are some questions as to the importance of this activity<sup>[82]</sup>.

UBE2N is differentially expressed between different types of leukemia and lymphoma cell lines<sup>[83]</sup>. It has significantly lower transcriptional expression levels in non-small-cell lung cancer and is correlated with pN and the stage of the disease<sup>[84]</sup>. In a breast-cancer metastatic model using iTRAQ technology, UBE2N was downregulated when comparing cells with the most metastatic potential and non-metastatic cells<sup>[85]</sup>. On the other hand, it was observed in a 2-DE experiment to be overexpressed in HBV-associated liver cancer<sup>[46]</sup>, which is consistent with our results. It has already been shown to be differentially expressed in GC<sup>[86]</sup>; however, it has not been validated whether it is up- or downregulated. UBE2N-dependent Lys63-mediated polyubiquitination regulates processes that often enhance cell survival in response to certain forms of stress<sup>[87]</sup>, therefore, our result supports its implication in the regulation of similar processes in gastric cancerogenesis.

TALDO1 is an almost ubiquitous cofactor-less enzyme of the pentose phosphate pathway<sup>[88]</sup>. Its activity is tissue specific and, in the brain, it is selectively expressed in oligodendrocytes, thus connecting it to different autoimmune diseases, such as multiple sclerosis<sup>[89]</sup>. Its expression is developmentally controlled<sup>[88]</sup>.

TALDO1 is the rate-limiting enzyme of the non-oxidative part of the pentose phosphate pathway<sup>[88]</sup> that catalyzes the reversible transfer of a three-carbon unit between various sugar phosphates (from ketose to aldose sugar phosphates). It has a role in regulating the balance between the two branches of the pentose phosphate pathway and its overall output, as measured by NADPH and glutathione production, and thus influences the sensitivity to cell-death signals<sup>[90]</sup>.

When comparing tumor and normal TALDO1, its activity is increased in neoplastic liver<sup>[91]</sup>. Its gene is highly expressed in a subgroup of patients with squamous cell carcinoma of the head and neck<sup>[60]</sup>. Furthermore, it is up-regulated in late-stage human colon-cancer tissue<sup>[76]</sup> and in the sera of colorectal cancer patients<sup>[92]</sup>. In metastatic, compared to non-metastatic GC cell lines<sup>[93]</sup> and in GC tissue<sup>[86]</sup>, TALDO1 was overexpressed, as shown by 2-DE. However, in both studies, again, no validation was performed. All these studies are consistent with our results, which were also validated by immunoblotting. We also found that TALDO1 correlated with pN status at stages pN0 and pN3. A higher TALDO1 expression in the tumor tissue could reflect an increased metabolism of glucose for the synthesis of nucleic acids in malignant cells<sup>[92]</sup>.

TPT1 is a ubiquitously expressed and highly conserved protein. It is associated with various cellular processes, such as cell-cycle progression, release of histamine and various interleukins, apoptosis, malignant transformation, and tumor reversion<sup>[64,95]</sup>. Very recently, it was also discovered as a glucose-regulated protein, important for the survival of pancreatic beta cells<sup>[96]</sup>.

It has been implicated in cancer, although it is not tumor-specific. It is upregulated in various tumor tissue cell lines when compared to normal tissue cell lines<sup>[97]</sup>, in breast<sup>[98]</sup> and colon cancer<sup>[99]</sup>. As for the gastric tissue,

TPT1 has been reported as cDNA present in libraries only from normal gastric tissues<sup>[100]</sup>. In our study, TPT1 was not validated as generally differentially expressed in the whole group of samples. Instead, its expression was location-correlated; TPT1 was upregulated in gastric adenocarcinoma from the cardia/gastroesophageal border. In contrast to the general worldwide decline of GC rates, an increasing incidence of gastric cardia cancer has been observed in several countries<sup>[1]</sup>. This suggests that it is a distinct clinical entity<sup>[26]</sup>. Therefore, it is possible that TPT1 is implemented only in gastric cardia/gastroesophageal border carcinogenesis.

Comparison of tumor and adjacent, non-tumor gastric tissues by means of proteome analysis, including differential 2-DE coupled to MS analysis, revealed 30 protein alterations. Some of the differentially expressed proteins had already been observed in GC in previous studies, which supports the reliability of our analysis. Several other proteins were found with the same trend of differential expression in other types of cancer, which could suggest that they are commonly involved in carcinogenesis. The high mortality rate from GC is due to delayed detection and surgical resection at advanced stages of the disease. A breakthrough in the early diagnosis of GC has not occurred yet and there are currently very few markers that are clinically in use; however, advances in proteomic research are facilitating the identification of novel diagnostic, prognostic, or therapeutic biomarkers. It is apparent that a collection of protein biomarkers will be necessary for reliable cancer detection and monitoring, as single biomarkers often have an inadequate predictive value<sup>[101]</sup>. There is, therefore, a need for the expedited development of new, validated biomarkers to be added to the list of clinically relevant tumor-associated proteins in the proteome databases of gastric tissue and cell lines. To the best of our knowledge, we are the first to observe aberrant expression of MRPL12 in gastric adenocarcinoma, and, in addition, aberrant expression of PACAP, GSTM3, SEPT2, UBE2N, TALDO1 and TPT1 for the gastric cardia/esophageal border were validated in gastric adenocarcinoma, also for the first time. Future experiments are planned to use these biomarkers in the design of a combinatory microarray and to translate the obtained results to blood samples, so the proteins would ultimately be useful as biomarkers for early detection.

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## COMMENTS

### Background

Gastric cancer (GC) is the fourth most common cancer worldwide. Despite declining incidence rates, it is still the second leading cause of cancer death and thus remains a major health problem. The majority of patients are diagnosed at an advanced stage when the 5-year survival rate is very low.

### Research frontiers

Current diagnosis is invasive, whereas blood biomarkers lack sensitivity and

specificity. This study investigated proteome changes in gastric cancerous vs non-cancerous tissue in the hope of discovering new biomarker candidates. It reports the validation of seven aberrantly expressed proteins, one of them being reported as a novel candidate biomarker.

### Innovations and breakthroughs

This is believed to be the first study that reports aberrant expression of 39S ribosomal protein L12 in gastric adenocarcinoma, and for the first time, validates aberrant expression of plasma cell-induced resident endoplasmic reticulum protein, glutathione S-transferase mu 3, septin-2, ubiquitin-conjugating enzyme E2 N, transaldolase, and translationally controlled tumor protein for the cardia/gastroesophageal border at the protein level.

### Applications

Future work will be focused on using the validated biomarkers in the design of a diagnostic protein microarray and on translating the research to blood samples, so that the proteins would ultimately be useful as biomarkers for early detection. By showing their differential expression, this work might contribute to the efforts to understand GC carcinogenesis, as well as present a set of possible diagnostic biomarkers for gastric adenocarcinoma.

### Peer review

Proteomic analysis was done in the present study. And several potential biomarkers were selected as an important protein for gastric cancer carcinogenesis. These fields are very important for further developments of the clinical treatment in patients with various malignancies including gastric cancer.

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## High-dose infliximab for treatment of pediatric ulcerative colitis: A survey of clinical practice

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### Abstract

**AIM:** To assess attitudes and trends regarding the use of high-dose infliximab among pediatric gastroenterologists for treatment of pediatric ulcerative colitis (UC).

**METHODS:** A 19-item survey was distributed to subscribers of the pediatric gastroenterology (PEDSGI) listserv. Responses were submitted anonymously and results compiled in a secure website.

**RESULTS:** A total of 113 subscribers (88% based in the United States) responded (101 pediatric gastroenterology attendings and 12 pediatric gastroenterology fellows). There were 46% in academic medical institutions and 39% in hospital-based practices. The majority (91%) were treating >10 patients with UC; 13% were

treating >100 patients with UC; 91% had prescribed infliximab (IFX) 5 mg/kg for UC; 72% had prescribed IFX 10 mg/kg for UC. Using a 5-point Likert scale, factors that influenced the decision not to increase IFX dosing in patients with UC included: "improvement on initial dose of IFX" (mean: 3.88) and "decision to move to colectomy" (3.69). Lowest mean Likert scores were: "lack of guidelines or literature regarding increased IFX dosing" (1.96) and "insurance authorization or other insurance issues" (2.34). "Insurance authorization or other insurance issues" was identified by 39% as at least somewhat of a factor (Likert score  $\geq 3$ ) in their decision not to increase the IFX dose. IFX 10 mg/kg was more commonly used for the treatment of pediatric UC among responders based in the United States (75/100) compared to non-United States responders (6/13,  $P = 0.047$ ). Induction of remission was reported by 78% of all responders and 81% reported maintenance of remission with IFX 10 mg/kg. One responder reported one death with IFX 10 mg/kg.

**CONCLUSION:** IFX 10 mg/kg is more commonly used in the United States to treat pediatric UC. Efficacy and safety data are required to avoid insurance barriers for its use.

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**Key words:** Immunosuppression; Inflammatory bowel disease; Ulcerative colitis; Children; Pharmacology

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## INTRODUCTION

In the last decade, infliximab has become an alternative treatment for moderate to severe ulcerative colitis (UC). The landmark ACT1 and ACT2 trials in adults showed that infliximab (IFX) successfully induced remission in patients with corticosteroid sensitive and corticosteroid resistant UC<sup>[1]</sup>. More recently, the Pediatric Inflammatory Bowel Disease Collaborative Research Group showed that children with corticosteroid-dependent or corticosteroid-refractory UC treated with IFX have a significant decrease in corticosteroid dependency and avoidance of colectomy in 72% after 1 year and 61% after 2 years of treatment<sup>[2]</sup>.

The Food and Drug Administration (FDA) has approved the use of IFX for treatment of moderate to severe UC for adult patients at a dose of 5 mg/kg<sup>[3]</sup>. However, in patients who lose responsiveness, the dose of IFX is commonly increased up to 10 mg/kg. The ACT1 and ACT2 trials demonstrated a response at this higher dose<sup>[1]</sup>. No studies have documented a benefit at this higher dose in pediatric patients.

Given the increased use of IFX for treatment of UC and the ongoing issues regarding efficacy, safety and insurance approval, we conducted a survey of pediatric gastroenterologists to collect information regarding dosing practices of IFX for the treatment of UC in the pediatric population. The results of this survey provide a glimpse into the attitudes of pediatric gastroenterologists regarding the use of high-dose IFX for pediatric UC.

## MATERIALS AND METHODS

An anonymous survey was created using the web-based survey software SurveyMonkey.com® (Portland, OR). The survey was distributed to all United States based and international pediatric gastroenterologists participating in the pediatric gastroenterology (PEDSGI) listserv with the approval of the listserv administrator. This survey included an electronic consent form and was free of identifiers. In addition, the survey forms were secured using secure sockets layer encryption to maintain the Health Insurance Portability and Accountability Act compliance. An "Opt Out" link was available at any point during the survey. This survey was approved by the University of California, San Francisco Committee on Human Research.

Demographic information was obtained by asking responders to provide data regarding their specialty (pediatric gastroenterology attending, pediatric gastroenterology trainee, or other specialty), practice type (academic, private, hospital based or other), practice size (< 10, 10-50, 51-100, 101-200 or > 200 patients) and location (Western United States, Midwest United States, Northeast United States, South United States, Europe, Middle East, Asia or "Other").

To determine the extent of IFX use among pediatric gastroenterologists, responders were asked, "Have you ever prescribed IFX at 5 mg/kg?" Those pediatric gastroenterologists that responded positively were asked, "What were the diagnoses for those patients receiving

IFX 5 mg/kg (Crohn's, UC, inflammatory bowel disease- unspecified (IBD-U) or other)?" Responders were then asked, "Have you ever prescribed IFX at 10 mg/kg?" Those that responded positively were again asked, "What were the diagnoses for those patients receiving IFX 10 mg/kg (Crohn's, UC, IBD-U or other)?" Responders who listed UC as a diagnosis were subsequently asked to specify all indications for which they prescribed IFX 10 mg/kg (steroid refractory UC, mild-to-moderate UC for induction or maintenance of remission, moderate-to-severe UC for induction or maintenance of remission, severe UC for induction or maintenance of remission).

Difficulty in achieving dose escalation was evaluated by asking those with experience in prescribing IFX 5 mg/kg: "... was an increase in IFX dosing considered at any point?" Responders who answered positively were subsequently asked: "...was an increase in IFX dose achieved?"

To further evaluate barriers to IFX dose escalation, a 5-point Likert scale was created whereby responders were asked to rank (scale of 1-5) factors that may have influenced their decision not to increase IFX to 10 mg/kg. The factors listed included: (1) patient improvement on the initial dose of IFX; (2) patient improvement on alternate medical therapy; (3) decision to move to colectomy; (4) lack of guidelines or literature regarding dose escalation; and (5) insurance authorization or other insurance issues. Responders were also asked to list other reasons that may have influenced their decision not to increase IFX dosing.

To evaluate the variation in administration of concomitant immunosuppressive medications, responders were asked to specify all medications instituted prior to IFX 10 mg/kg. In a separate question, responders were asked to specify all medications instituted concomitant with IFX 10 mg/kg for the treatment of UC. Answer choices included: corticosteroids, aminosaliclates, azathioprine (AZA), 6-mercaptopurine (6-MP), and methotrexate. Responders were also asked to specify any other medications that were not listed.

Methods for administration of IFX 10 mg/kg such as dosing interval and continuous or episodic treatment were not surveyed.

Responders were also asked to select any adverse events their patients experienced as a result of IFX 10 mg/kg. Answer choices included: worsening UC, headache, arthralgia, profound anemia, infection, oncologic process, neurologic event, antibodies against IFX or other autoimmune antibodies and infusion reactions. Responders were asked to specify any other adverse event that was not listed.

Finally, responders were asked to list all outcomes their patients experienced as a result of IFX 10 mg/kg (clinical remission, maintenance of remission, colectomy, continued or worsening colitis, death). Again, responders were asked to specify any other unlisted outcome.

Response frequencies were tabulated and expressed as percentages of total responses. Differences among groups with regard to frequency and Likert scale variables were assessed for significance using a Wilcoxon

Table 1 Indication for initiation of infliximab 10 mg/kg

Answer options	Percentage (n)
Steroid refractory UC	70.5 (55)
Severe UC for maintenance of remission	64.1 (50)
Moderate-to-severe UC for maintenance of remission	62.8 (49)
Severe UC for induction of remission	55.1 (43)
Moderate-to-severe UC for induction of remission	38.5 (30)
Mild-to-moderate UC for maintenance of remission	9.0 (7)
Mild-to-moderate UC for induction of remission	2.6 (2)
Other	6.4 (5)

78 respondents. UC: Ulcerative colitis.

Rank Sum test for comparing two groups, or a Kruskal-Wallis test for more than two groups as the data were not normally distributed. Differences between categorical variables were tested using  $\chi^2$  and Fisher exact tests. Differences were considered significant for  $P \leq 0.05$ .

## RESULTS

### Demographics

Our survey yielded 113 responders (5.7%) out of a total of 1993 subscribers to the PEDGI listserv. Of those responders, 101 were pediatric gastroenterology attendings and 12 were trainees in pediatric gastroenterology programs. Most responders identified themselves as based in an academic medical institution (46.0%) or a hospital-based practice (38.9%). A minority of respondents identified themselves as working in a private practice (12.4%). Most responders also identified themselves as practicing within the United States (88.5%). Ninety-one percent of responders reported practices that treat at least 10 patients with UC, 23.0% reported 50 to 100 patients, and 13.3% reported over 100 patients with UC.

### Initiation of IFX

Ninety-one percent (103/113) of respondents had prescribed IFX 5 mg/kg for treatment of UC, while 71.7% (81/113) had prescribed IFX 10 mg/kg for treatment of UC. The indications for prescribing IFX 10 mg/kg are listed in Table 1. A large majority of respondents selected steroid refractory UC as an indication for IFX 10 mg/kg (70.5%). In addition, a majority of respondents listed severe UC for induction and maintenance of remission as an indication for prescribing IFX 10 mg/kg (55.1% and 64.1%, respectively). A large number of respondents had also used IFX 10 mg/kg for treatment of moderate-to-severe UC, both for induction and maintenance of remission (38.5% and 62.8%, respectively).

The overall use of IFX 10 mg/kg to treat UC did not differ significantly when comparing responders in academic and non-academic practices. However, more responders working in non-academic practices had prescribed IFX 10 mg/kg for maintenance of remission for severe UC (29/39) compared with those in academic practices (19/39,  $P = 0.034$ ).

Responders based in the United States were more likely

Table 2 Therapies introduced prior to infliximab 10 mg/kg

Answer options	Percentage (n)
Corticosteroids	96.2 (75)
Infliximab 5 mg/kg	92.3 (72)
Aminosalicylates	89.7 (70)
Azathioprine <sup>1</sup>	76.9 (60)
6-mercaptopurine <sup>1</sup>	70.5 (55)
Methotrexate	25.6 (20)
Other	14.1 (11)
None	1.3 (1)

<sup>1</sup>Reported use by physicians included either azathioprine or 6-mercaptopurine in different patients. 78 respondents.

to use IFX 10 mg/kg to treat UC (75/100) compared with non-United States responders (6/13,  $P = 0.047$ ). However, the indication for initiation of IFX 10 mg/kg for UC did not differ between these two groups.

### Increasing the IFX dose

Pediatric gastroenterologists will often increase IFX dosing in patients that respond poorly to IFX 5 mg/kg. Respondents who had prescribed IFX 5 mg/kg for the treatment of UC were subsequently asked, "Have you ever considered an increase in IFX dosing above 5 mg/kg?" Overwhelmingly, 87.4% (90/103) of those using IFX had at least considered an increase in IFX dosing.

Using a 5-point Likert scale, respondents were asked to rank factors that may have influenced their decision not to increase IFX dosing in patients with UC (Figure 1). Among the 82 respondents, "improvement on initial dose of IFX" and "decision to move to colectomy" had the highest mean Likert scores (3.88 and 3.69, respectively). "Lack of guidelines or literature regarding increased IFX dosing" and "insurance authorization or other insurance issues" had the lowest mean Likert scores (1.96 and 2.34, respectively). Of note, however, 39.0% (32/82) identified "insurance authorization or other insurance issues" as at least somewhat of a factor (Likert score  $\geq 3$ ) in their decision not to increase the IFX dose in patients with UC.

Respondents were asked to list all medical therapies that may have been instituted prior to IFX 10 mg/kg (Table 2). Many of the respondents selected all the alternative medical therapies listed, and only one reported prescribing IFX 10 mg/kg without attempting any prior medical therapy. "Other" medications that were specified included tacrolimus, probiotics, metronidazole and other antibiotics.

Respondents were also asked what therapies they had instituted concurrently with IFX 10 mg/kg (Table 3). Most used corticosteroids with IFX 10 mg/kg. In addition, aminosalicylates were commonly administered with IFX 10 mg/kg. Conversely, fewer prescribed AZA or 6-MP along with IFX 10 mg/kg. A small minority administered IFX 10 mg/kg alone. Methotrexate was also noted to be used concurrently with IFX 10 mg/kg.

No significant differences in practice patterns with regards to medications initiated prior to and concurrent with IFX 10 mg/kg were observed between those in

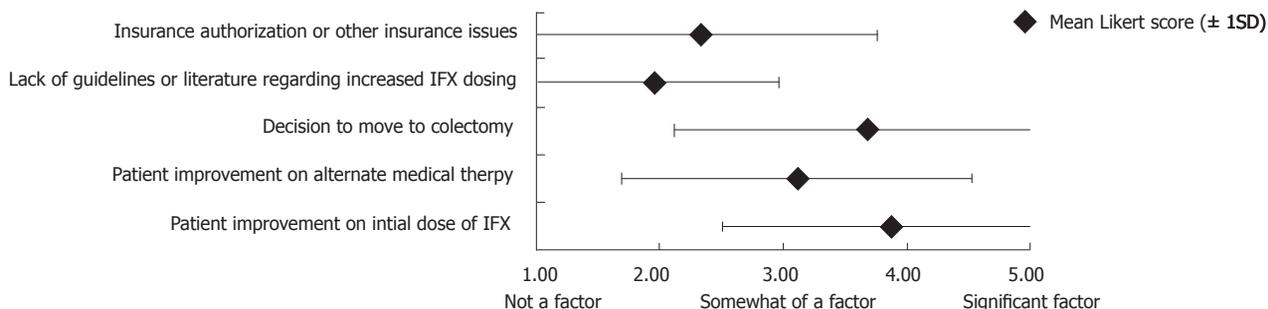


Figure 1 Factors that influenced pediatric gastroenterologists to not increase infliximab dosing depicted as a 5-point Likert scale. IFX: Infliximab.

Answer options	Percentage (n)
Corticosteroids	70.5 (55)
Aminosalicylates	61.5 (48)
Azathioprine <sup>1</sup>	39.7 (31)
6-mercaptopurine <sup>1</sup>	34.6 (27)
Antibiotics	44.9 (35)
None	11.5 (9)
Methotrexate	6.7 (5)
Other	1.3 (1)

<sup>1</sup>Reported use by physicians included either azathioprine or 6-mercaptopurine for different patients. 78 respondents.

Answer options	Percentage (n)
Maintenance of remission	80.8 (63)
Clinical remission	78.2 (61)
Colectomy	60.3 (47)
Continued or worsening colitis	51.3 (40)
Other	2.6 (2)
Mortality	1.3 (1)

78 respondents.

academic and non-academic practices.

A larger number of United States based responders had prescribed 6-MP prior to instituting IFX 10 mg/kg to treat UC (54/75, 72.0%) compared with non-United States based responders (1/6, 16.7%,  $P = 0.012$ ). In addition, more United States based responders had prescribed 5-aminosalicylates concurrently with IFX 10 mg/kg (47/75, 62.7%) compared with non-United States responders (1/6, 16.7%,  $P = 0.039$ ). Similarly, antibiotics was more commonly prescribed concurrently with IFX 10 mg/kg among United States responders (35/75, 46.7%) compared with non-United States responders (0/6,  $P = 0.034$ ).

### Outcomes with high-dose IFX

Table 4 lists the outcomes reported by respondents as a result of using IFX 10 mg/kg. A majority of pediatric gastroenterologists surveyed reported either clinical remission (61/78, 78.2%) or maintenance of remission

Answer options	Percentage (n)
None	64.1 (50)
Antibodies to infliximab	17.9 (14)
Infusion reaction or delayed hypersensitivity reaction	16.7 (13)
Worsening ulcerative colitis	14.1 (11)
Infection	12.8 (10)
Headache	9.0 (7)
Arthralgia	9.0 (7)
Development of autoimmune antibodies (e.g., antinuclear antibodies, anti-dsDNA antibodies)	5.1 (4)
Other	2.6 (2)
Profound anemia	1.3 (1)
Oncologic process	0 (0)
Neurologic event (e.g., neuritis, neuropathy)	0 (0)

78 respondents.

with this dose (63/78, 80.8%). Over half of the surveyed pediatric gastroenterologists who used 10 mg/kg IFX had patients who continued to suffer worsening colitis (40/78, 51.3%) or who moved to colectomy (47/78, 60.3%).

Most pediatric gastroenterologists (50/78, 64.1%) reported no adverse effects of the higher dose (Table 5). The most common adverse events reported by prescribers were antibodies to IFX (14/78, 17.9%) and infusion reactions (13/78, 16.7%); 14.1% (11/78) respondents reported discontinuing IFX 10 mg/kg as a result of these adverse reactions. No oncologic or major neurologic events were reported as a consequence of administering IFX 10 mg/kg. However, a single respondent reported one death while using IFX 10 mg/kg.

## DISCUSSION

This survey was designed to understand the attitudes and driving forces of pediatric gastroenterologists regarding the use of high-dose IFX for the treatment of UC. In our sample, a majority of the pediatric gastroenterologists surveyed had experience treating pediatric UC with IFX 10 mg/kg.

One of the strengths of this study is that the respondents were evenly split between academic institutions and non-academic practices, capturing the major practice settings of pediatric gastroenterologists.

One of the weaknesses of this study is the low response rate, which may represent a non-response bias. However, it should be noted that a large number of the 1993 PEDSGI listserv subscribers are not pediatric gastroenterologists. Many of the listserv subscribers are surgeons, pathologists, nurse practitioners and other healthcare professionals. Although the exact number of pediatric gastroenterologists that subscribe to the listserv is unknown, the response rate is likely much higher among this particular group. According to the American Board of Pediatrics, at the time this survey was conducted, 974 board certified pediatric gastroenterologists were registered in the United States<sup>[4]</sup>. Based on these numbers, our survey results represent the opinions of 9.3% (91/974) of the registered pediatric gastroenterologists practicing within the United States.

In addition, the results of this survey study are subject to recall bias. Respondents who have experienced more extreme outcomes with IFX or difficulty with insurance approval for higher doses of IFX are more likely to respond in an exaggerated manner.

While the results of this uncontrolled study are subject to various biases, they may also be indicative of the trends regarding the use of high-dose IFX among pediatric gastroenterologists.

IFX is effective for both induction and maintenance of remission for steroid-refractory and steroid-dependent UC<sup>[1,2,5]</sup>. Predictably, most of the pediatric gastroenterologists surveyed indicated steroid refractoriness as an indication for prescribing IFX 10 mg/kg. In addition, nearly all pediatric gastroenterologists surveyed indicated that both corticosteroids and IFX 5 mg/kg had been employed prior to increasing the IFX dose to 10 mg/kg. IFX 10 mg/kg may also be a corticosteroid-sparing agent as reflected in the response that fewer pediatric gastroenterologists reported using corticosteroids after the 10 mg/kg dose was instituted.

While nearly three-quarters of pediatric gastroenterologists surveyed have prescribed 6-MP or AZA therapy prior to instituting IFX, only about one-third of pediatric gastroenterologists surveyed have administered 6-MP or AZA concurrently with IFX 10 mg/kg. This practice may be in flux, as recent reports suggest improved disease responsiveness and lower risk of antibodies to IFX with concomitant immunomodulators, despite concerns of hepatosplenic T-cell lymphoma<sup>[6-10]</sup>. In our survey, no oncologic or serious neurologic adverse outcomes were reported. Future studies should further clarify the risk *vs* benefits of concomitant immunomodulator therapy.

Our results also suggest that pediatric gastroenterologists in the United States are more likely to prescribe IFX 10 mg/kg to treat UC compared with those working outside the United States. The only difference found for responders in academic compared with non-academic settings was an increased use of high-dose IFX for maintenance of remission for severe UC among non-academic pediatric gastroenterologists.

These differences highlight the lack of universal guidelines and need for further studies regarding the optimal use of IFX 10 mg/kg for the treatment of UC.

The most common reported side effects were antibodies to IFX and infusion reactions. Infections were also reported by 12.8% of responders. One respondent reported death as an adverse outcome. The events that lead to this death are not known. It is thought, however, that this fatality was not a result of oncologic, neurologic or infectious complications as the same respondent did not report experiencing any of these adverse effects while using IFX 10 mg/kg.

Most pediatric gastroenterologists surveyed reported improved outcome as a result of prescribing IFX 10 mg/kg, documenting a benefit of the higher dose in pediatric UC. However, over half of the respondents reported worsening disease and the need for colectomy, possibly due to loss of responsiveness, or in some cases a lack of any response to the increased dose.

IFX is approved by the FDA for the treatment of moderate to severe UC at a dose of 5 mg/kg<sup>[3]</sup>. Insurance companies have often rejected appeals for reimbursement for increased doses of IFX due to lack of published data showing efficacy and safety for pediatric patients with UC. Despite this, however, our survey suggests that insurance approval or other insurance issues did not uniformly prevent pediatric gastroenterologists from prescribing IFX 10 mg/kg.

Insurance approval for higher doses of IFX is achieved *via* an exception process or appeal process. An exception process involves an initial request for a health plan to consider coverage for a prescribed medication therapy or to reevaluate the possible benefits. An appeal is a request for an insurance carrier to reconsider a coverage decision that was initially denied<sup>[11]</sup>. The exception and appeal processes require letters of support from the prescribing physician. The time-consuming processes to initiate higher doses of IFX provide some patients with potentially lifesaving therapy. On the other hand, this litigious process is often unsuccessful and may delay alternative intervention for some patients.

Modification of UC treatment often becomes necessary in patients who lose responsiveness to an initial dose or standard maintenance regimen of IFX. Modification may consist of an increase in dose, decrease in dosing interval (not queried here), or addition of alternative medical or surgical therapy<sup>[12]</sup>. Treatment strategies with IFX should optimize drug pharmacokinetics to maximize dose response and minimize the development of antibodies to IFX. This may delay the need to increase IFX dose and reduce the risk of complications. Among pediatric gastroenterologists who prescribe IFX, IFX 10 mg/kg has a recognized role and perceived benefit in the treatment of some pediatric UC patients. Future prospective controlled clinical trials using high-dose IFX and other anti-tumor necrosis factor- $\alpha$  antibody agents, and testing of specific dose increases may provide more

standardized guidelines for the treatment of pediatric patients with UC.

## COMMENTS

### Background

In the last decade, infliximab (IFX) has become an alternative treatment of moderate to severe ulcerative colitis (UC) in both the adult and pediatric population. Based on current Food and Drug Administration recommendations the use of IFX for treatment of moderate to severe UC in adults is started at a dose of 5 mg/kg. However, in patients who lose responsiveness, the dose of IFX is commonly increased up to 10 mg/kg.

### Research frontiers

No studies have documented benefit at this higher dose in pediatric patients. The efficacy, safety and insurance approval for high-dose IFX have also been of growing concern in the medical community. The authors conducted a survey of pediatric gastroenterologists to collect information regarding the use of IFX for the treatment of UC in the pediatric population.

### Innovations and breakthroughs

Recently, the Pediatric Inflammatory Bowel Disease Collaborative Research Group (PIBDCRG) showed that children with corticosteroid-dependent or corticosteroid-refractory UC treated with IFX have a significant decrease in corticosteroid dependency and improved rates of colectomy. In the PIBDCRG study, dose escalation and decreased dose interval was at the discretion of prescribing physicians. The survey study conducted here is the first to report the extensive use of IFX 10 mg/kg by pediatric gastroenterologists for treatment of UC in the pediatric population.

### Applications

This study offers a glimpse in the attitudes and practice patterns of pediatric gastroenterologists regarding the use of high-dose IFX for the treatment of pediatric UC. The results highlight the recognized role of IFX 10 mg/kg for the treatment of UC in the pediatric population and the need for more standardized guidelines regarding dose escalation.

### Terminology

IFX is a biologic therapy used to treat autoimmune diseases such as UC. IFX is a monoclonal immunoglobulin G antibody that is genetically engineered to target tumor necrosis factor-alpha (TNF- $\alpha$ ). By selectively targeting specific players in the inflammatory cascade such as TNF- $\alpha$ , biologic agents may spare the need for treatment with systemic corticosteroids and thereby reduce the number of side effects associated with such treatment.

### Peer review

The authors examined the practice patterns of pediatric gastroenterologists regarding the use IFX for the treatment of UC. They found that a majority of those pediatric gastroenterologists surveyed have experience using IFX 10 mg/kg. In addition, significantly more pediatric gastroenterologists based in the United States have used IFX 10 mg/kg compared to those not based in the United States. Over one-third of pediatric gastroenterologists surveyed indicated insurance issues as a barrier to increasing IFX dosing.

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## Polymorphisms of the *TLR2* and *TLR4* genes are associated with risk of gastric cancer in a Brazilian population

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### Abstract

**AIM:** To investigate toll-like receptor 2 (*TLR2*) -196 to -174 del, and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms at risk of chronic gastritis and gastric cancer in a Brazilian population and association of gastric lesions with risk factors such as smoking, alcohol intake and *Helicobacter pylori* infection.

**METHODS:** In this case-control study, polymorphism at *TLR2* -196 to -174 del was investigated by using the allele-specific polymerase chain reaction (PCR) method, while the PCR-restriction fragment length polymorphism technique was carried out to identify the *TLR4* (rs4986790 and rs4986791) genotypes in 607 Brazilian individuals (208 with chronic gastritis-CG, 174 with gastric cancer-GC and 225 controls -C).

**RESULTS:** The single nucleotide polymorphisms *TLR4*+1196C/T was not associated with risk of chronic gastritis or gastric cancer and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. However, the frequency of *TLR2* -196 to -174 ins/del + del/del and *TLR4*+896AG

genotypes was significantly higher ( $P < 0.01$  and  $P = 0.01$ , respectively) in the cancer group (33.4% and 11.5%, respectively) than in the control group (16.9% and 4.5%, respectively). It was also observed that the G-C haplotype of the *TLR4*+896A/G+1196C/T ( $P = 0.02$ ) and the combination of variant alleles of the *TLR2*/*TLR4*+896G ( $P = 0.02$ ) are associated with susceptibility to gastric cancer. In addition, the multiple logistic regression showed that male gender [odds ratio (OR) = 2.70; 95% CI: 1.66-4.41;  $P < 0.01$ ], alcohol intake (OR = 2.93; 95% CI: 1.76-4.87;  $P < 0.01$ ), *TLR2* -196 to -174 del (OR = 2.64; 95% CI: 1.56-4.44;  $P < 0.01$ ) and *TLR4*+896G (OR = 3.19; 95% CI: 1.34-7.61;  $P < 0.01$ ) polymorphisms were associated with a higher susceptibility to developing this neoplasm.

**CONCLUSION:** Our data indicate that *TLR2* -196 to -174 del and *TLR4*+896G may increase the risk of gastric cancer in a Brazilian population.

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**Key words:** Polymorphisms; Toll-like receptor 2; Toll-like receptor 4; Gastric cancer; Gastritis

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## INTRODUCTION

Gastric cancer is one of the most serious health problems in many countries, including Brazil, which ranks third in incidence and mortality, and with an estimated incidence in 2010 of about 21 500 new cases, with an incidence of 14.25 per 100 000 males and 7.70 per 100 000 females<sup>[1]</sup>. The *Helicobacter pylori* (*H. pylori*) is the major etiological risk factor for this malignancy, which progresses through a multi-step process, developing from gastritis, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma<sup>[2]</sup>. It is widely accepted that chronic *H. pylori* infection induces a gastric atrophy and hypochlorhydria, which are precursors of all pathophysiological changes of gastric carcinogenesis<sup>[3]</sup>. However, colonization with *H. pylori* can lead to various outcomes. Nearly all *H. pylori* positive subjects have chronic gastritis, and only 1%-2% development of stomach cancer among infected<sup>[4]</sup>. Hence, other factors are likely to be involved in gastric tumorigenesis such as host genetic factors, as well as the diversity of *H. pylori* virulence genes.

Host genetic factors, as polymorphisms in inflammatory and immune response genes, are mainly related to the recognition of the bacteria by the immune system and the variation in the level of cytokine response<sup>[5]</sup>. Among host factors, several inflammatory proteins including cytokines, growth factors, and chemokines have been known to control immune response against *H. pylori* infection<sup>[6,7]</sup>. Therefore, many studies have focused on the analyses of polymorphisms in genes associated with the inflammatory response in the gastric mucosa and risk for malignancy<sup>[8-11]</sup>. Other mediators also have polymorphic variants that modulate the innate immune response pattern, as the toll-like receptors (TLRs), which provide first line of host defense against harmful pathogens<sup>[12]</sup>.

Among the TLRs, it has been reported that the TLR2, lipoproteins bacterial receptor and the TLR4, the lipopolysaccharide (LPS) receptor, are involved in the response to infection by *H. pylori* on gastric epithelial cells<sup>[3,13-15]</sup>. Both the TLR2 and the TLR4 promote transcription of genes involved in immune activation including nuclear factor kappa B (*NF-κB*) and also mitogen-activated protein (*MAP*) kinase pathways<sup>[16]</sup>. *TLR4* is up regulated in gastric epithelial cell lines infected with *H. pylori* and in macrophages and expression of TLR4 protein has been demonstrated in chronic active gastritis, in precancerous lesions, and also in gastric tumor cells. TLR2 activates *NF-κB* in epithelial cells, in response to *H. pylori* infection, causing the expression of interleukin (IL)-8, macrophage inflammatory protein-3α and growth-regulated oncogene alpha<sup>[7]</sup>. Thus, it is conceivable that functionally relevant polymorphisms in *TLR* genes can alter the host immune response to pathogens as infection induced by *H. pylori*.

Single nucleotide polymorphisms (SNPs) in *TLR2* have been associated with susceptibility to various infectious and inflammatory diseases such as leprosy<sup>[17]</sup>, increased risk of Gram-negative sepsis<sup>[18]</sup>, asthma<sup>[19]</sup>, recurrent bacterial infections<sup>[20]</sup> and sporadic colorectal cancer

susceptibility<sup>[21]</sup>. The specific polymorphism *TLR2* -196 to -174 del/del genotype has been reported to show decreased transcriptional activity of the *TLR2* gene<sup>[22]</sup>. Such fact aroused the interest for this polymorphism, and previous studies in the Japanese population demonstrated its association with increased susceptibility to non-cardia gastric cancer<sup>[23]</sup> and intestinal metaplasia<sup>[24]</sup>.

Likewise, the *TLR4* presents some polymorphisms implicated in increased susceptibility to various diseases such as atherosclerosis<sup>[25]</sup>, asthma<sup>[3]</sup>, malaria<sup>[26]</sup>, and also infection with the *H. pylori* associated with gastric cancer and its precursors<sup>[27]</sup>. Two SNPs in *TLR4*+896A/G (rs4986790) and +1196C/T (rs4986791) have received special attention in some studies, although the results are still controversial<sup>[6,28,29]</sup>.

Therefore, the aim of this study was to evaluate the influence of the 22-bp nucleotide deletion -196 to -174 del, in the promoter region of the gene *TLR2* and +896A/G and +1196C/T polymorphisms (Asp299Gly and Thr399Ile) respectively in *TLR4* gene on the risk of chronic gastritis and gastric cancer in a Brazilian population and whether there is an association of gastric lesions with risk factors such as smoking, alcohol intake and *H. pylori* infection.

## MATERIALS AND METHODS

### Subjects

This was a case-control study on chronic gastritis and gastric cancer, in which a total of 607 DNA samples from peripheral blood leukocytes were genotyped. The case groups comprised 208 individuals (102 men and 106 women) with a histopathologically confirmed diagnosis of chronic gastritis - CG (Sidney System)<sup>[30]</sup>, with a mean age of 52.8 + 14.5 years (range 19 to 84 years), and 174 individuals (134 men and 40 women) with a histopathologically confirmed diagnosis of gastric cancer - GC (Lauren's classification)<sup>[31]</sup>, with a mean age of 62.2 ± 12.2 years (range 28 to 93 years). All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. *H. pylori* infection was histologically established either by the Giemsa staining technique or by the urease test, performed at the Pathology Services of the Hospital de Base and the Pio XII Foundation. Results of *H. pylori* infection were obtained for the available cases. The cancer-free control group (C) with no previous history of gastric disease was composed of 225 healthy individuals (112 men and 113 women), mainly blood donors, with a mean age of 56.5 + 18.1 years (range 20 to 93 years). Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. All the individuals were ethnically classified on their visual appearance as Caucasians in the three groups evaluated. The few cases of African descent were excluded from the study (about 10%).

**Table 1** Primer sequences, restriction enzymes and fragment sizes for toll-like receptor 2 and toll-like receptor 4 gene polymorphisms and interleukin-1 $\beta$  gene

Genes	Primers	Enzyme T°/time	Fragment (bp)	Ref.
<i>TLR2</i> <i>del</i> -196 to -174	F: 5'-CACGGAGGCAGCGAGAAA-3' R: 5'-CTGGGCCGTGCAAAGAAG-3'	-	286 ins/ins: 286 ins/del: 286, 264 del/del: 264	[24]
<i>TLR4</i> +896A/G rs4986790	F: 5'-AGCATACTTAGACTACCACCTCGATG 3' R: 5'-GTTGCCATCCGAAATTATAAGAAAAG 3'	<i>Bst</i> XI 37 °C, 1 h	131 A/A: 131 A/G: 131, 108 G/G: 108	[34]
<i>TLR4</i> +1196C/T rs4986791	F: 5'-GGTTGCTGTCTCTCAAAGTGATTTTGGGAGAA-3' R: 5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3'	<i>Hinf</i> I 37 °C, 1 h	407 C/C: 407 C/T: 407, 378 T/T: 378	[33]
<i>IL1-<math>\beta</math></i>	F: CATGTGACCTGCTCGTCAGT R: CCCTAGGGATTGAGTCCACA	<i>Hinf</i> I 37 °C, 1 h	370 195, 175	[47]
<i>TLR2</i>	F: 5'-CACGGAGGCAGCGAGAAA-3' R: 5'-CTGGGCCGTGCAAAGAAG-3'	<i>Bst</i> XI 37 °C, 1 h	286 188, 98	[24]

TLR: Toll-like receptor; IL: Interleukin; T°: Temperature.

The National Research Ethics Committee approved this work, and written informed consent was obtained from all individuals.

### Genotyping

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. DNA was extracted according to a previous report<sup>[32]</sup>, and stored at -20 °C until use for genotyping.

Polymorphism at *TLR2* -196 to -174 del was investigated using the allele-specific polymerase chain reaction (PCR) method<sup>[24]</sup>, and the PCR-restriction fragment length polymorphism (RFLP) technique was carried out in order to identify the *TLR4* (rs4986790 and rs4986791) genotype<sup>[33,34]</sup> in cases and control groups. In brief, the procedure was carried in a total reaction volume of 25  $\mu$ L, containing 2.5  $\mu$ L 10  $\times$  PCR buffer, 2  $\mu$ L deoxyribonucleotide triphosphatess (1.25  $\mu$ mol/L), 0.5  $\mu$ L MgCl<sub>2</sub> (25 mmol/L), 1.25  $\mu$ L of each primer (25 mmol/L, Sigma-Aldrich, United States), 15.3  $\mu$ L dH<sub>2</sub>O, 2  $\mu$ L DNA (100 ng/ $\mu$ L), and 0.2  $\mu$ L Taq DNA polymerase (5 U/ $\mu$ L, Invitrogen, United States). PCR for *TLR2* -196 to -174 del was as follows: initial denaturation step at 95 °C for 5 min, amplification was carried out by 35 cycles at 95 °C for 30 s, at 60 °C for 40 s, and at 72 °C for 40 s, followed by a final elongation cycle at 72 °C for 7 min (Table 1). PCR for both *TLR4* polymorphisms were as follows: after an initial denaturation step at 94 °C for 3 min, amplification was carried out by 30 cycles at 94 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 30 s, followed by a final elongation cycle at 72 °C for 7 min. Then, 10  $\mu$ L of *TLR4* +896 A/G and *TLR4* +1196 C/T polymorphism PCR products were digested with 0.5  $\mu$ L (5 U/ $\mu$ L) of the *Bst*XI and *Hinf*I specific enzymes, respectively in a 10  $\mu$ L volume including 2.5  $\mu$ L 10  $\times$  buffer 1 (New England Biolabs, United States) and 7.0  $\mu$ L dH<sub>2</sub>O (Table 1). The products were then electrophoresed on a 3% agarose 1000 (Invitrogen, United States) gel, to allow

detection by ethidium bromide staining.

In order to confirm the veracity of the results, one confirmed polymorphic case was used as positive control for every RFLP procedure, to attest the good functioning of the restriction enzyme. For the *TLR4*+896A/G (rs4986790) and +1196C/T (rs4986791) polymorphisms, we also used other fragments of the *TLR2* and *IL-1 $\beta$*  gene that was known to have the enzyme recognition site to verify the correct functioning of the enzymes *Bst*XI and *Hinf*I (Table 1), considering we did not detect any polymorphic homozygous subjects.

### Statistical analysis

Fisher's exact test was used to compare the groups regarding genotype and allele frequencies, and the chi-square test for determining Hardy-Weinberg equilibrium. Multiple logistic regression models were used to determine the effects of the variables in gastric cancer and chronic gastritis. The models included age (reference: < 61 and 53 years old - median of the groups), gender (reference: female), smoking habits (reference: nonsmokers), drinking habits (reference: nondrinkers), and *H. pylori* infection (reference: *H. pylori*-negative). The results are shown as odds ratio (OR), showing 95% CI. ORs were calculated using a dominant model due to low frequency of polymorphic homozygous (i.e., combining heterozygous and homozygous for the minor allele *vs* homozygous for the major allele) for all SNPs. The haplotype frequencies of *TLR4* were inferred by Haploview program (4.0 version). Statistical analyses were performed using the GraphPad InStat, and SPSS (11.5 version) computer software programs. A probability level (*P*) of less than 0.05 was adopted as a significance criterion.

## RESULTS

The samples of the 607 subjects were genotyped for the *TLR2* -196 to -174 del and *TLR4* (+896A/G rs4986790

**Table 2** Genotype and allele frequencies of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791) polymorphisms in gastric cancer, chronic gastritis and control groups

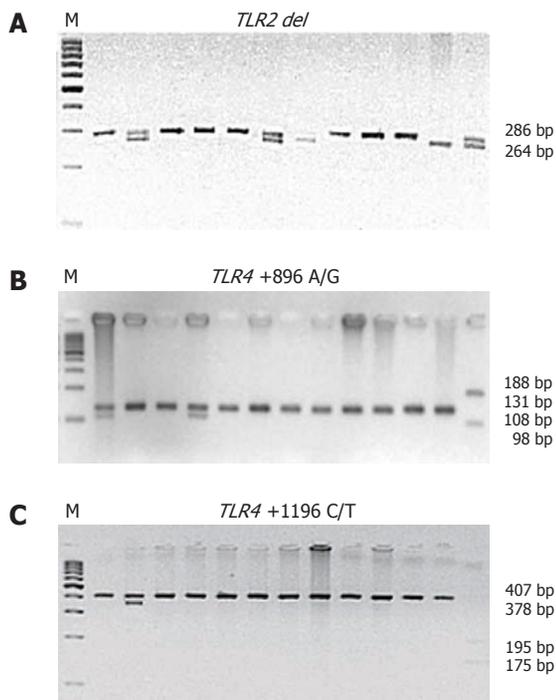
Genotypes/alleles	GC	C	CG
	n = 174 (%)	n = 225 (%)	n = 208 (%)
<b>TLR2 -196 to -174</b>			
<i>ins/ins</i>	116 (66.6)	189 (84.0)	160 (76.9)
<i>del/ins</i>	50 (28.7)	34 (15.1)	41 (19.7)
<i>del/del</i>	8 (4.7)	2 (0.9)	7 (3.4)
OR (95% CI)	2.62 (1.63-4.22)	1.57 (0.97-2.54)	
P value	< 0.01	0.06	
Alleles			
<i>ins</i>	81.0	91.5	87.0
<i>del</i>	19.0	8.5	13.0
OR (95% CI)	2.53 (1.65-3.88)	1.65 (1.06-2.55)	
P value	< 0.01	0.02	
<b>TLR4 +896A/G (rs4986790)</b>			
AA	154 (88.5)	215 (95.5)	187 (89.9)
AG	20 (11.5)	10 (4.5)	21 (10.1)
GG	0	0	0
OR (95% CI)	2.79 (1.27-6.13)	2.41 (1.10-5.25)	
P value	0.01	0.02	
Alleles			
A	94.0	97.0	95.0
G	6.0	3.0	5.0
OR (95% CI)	2.68 (1.23-5.81)	2.33 (1.08-5.02)	
P value	0.01	0.02	
<b>TLR4 +1196C/T (rs4986791)</b>			
CC	165 (94.8)	219 (97.3)	202 (97.1)
CT	9 (5.2)	6 (2.7)	6 (2.9)
TT	0	0	0
OR (95% CI)	1.99 (0.69-5.70)	1.08 (0.34-3.41)	
P value	0.28	1.00	
Alleles			
C	98.0	99.0	98.0
T	2.0	1.0	2.0
OR (95% CI)	1.96 (0.69-5.57)	1.08 (0.34-3.38)	
P value	0.29	1.00	

OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; C: Control.

and +1196C/T rs4986791). The genotype and allele frequencies for these polymorphisms are presented in Table 2. The genotype and allele frequency distribution of the three polymorphisms complied with Hardy-Weinberg equilibrium in both cases and control groups (data not shown). The banding patterns of these SNPs are represented in Figure 1.

For *TLR2* -196 to -174 *del*, the genotype (*ins/del* and *del/del*) and allele (*del*) frequencies were increased statistically ( $P < 0.01$ ) in gastric cancer group (33.4% and 19% respectively) than in control group (16.0% and 8.5% respectively). In addition, among the groups of chronic gastritis (13.0%) and control (8.5%), the allele frequencies (*del*) was statistically significant ( $P = 0.02$ ).

Similarly, for *TLR4*+896A/G (rs4986790), the genotypes (A/G) and allele (G) frequencies were increased statistically in gastric cancer group (11.5% and 6.0%, respectively;  $P = 0.01$ ) and chronic gastritis (10.1% and 5.0%, respectively;  $P = 0.02$ ) than in control group (4.5% and 3%, respectively). This results due to the higher



**Figure 1** Electrophoretic pattern of fragments generated by polymerase chain reaction-allele specific and polymerase chain reaction-restriction fragment length polymorphism for the polymorphisms. A: Toll-like receptor (*TLR2*) -196 to -174 *del*: *ins/ins* = 286 bp; *ins/del* = 286 + 264 bp; *del/del* = 264 bp; B: *TLR4* + 896A/G: A/A = 131 bp; A/G = 131 + 108 bp and positive control of enzyme *BstXI*, *TLR2* gene fragment of 286 bp with the enzyme cutting site: 188 + 98 bp (last lane); C: *TLR4* + 1196 C/T: C/C = 407 bp; C/T: 407 + 378 bp and positive control of enzyme *HinfI*, interleukin-1 $\beta$  gene fragment of 370 bp with the enzyme cutting site: 195 + 175 bp (last lane). M: Molecular weight marker of 100 bp.

**Table 3** Toll-like receptor 4 haplotype frequency distribution between gastric cancer, chronic gastritis and control groups

Haplotypes	GC (%)	C (%)	$\chi^2$	P value	CG (%)	C (%)	$\chi^2$	P value
<b>TLR4 +896/+1196</b>								
A-C	91.4	95.7	6.802	< 0.01	94.0	95.7	1.361	0.24
G-C	63.0	31.0	5.247	0.02	46.0	31.0	1.598	0.20
A-T	21.0	11.0	1.461	0.22	NF	NF	NF	NF

Haplotype G-T not found. NF: Not found; GC: Gastric cancer; CG: Chronic gastritis; C: Control.

frequency of polymorphic allele (*TLR4* +896G) in the gastric cancer and chronic gastritis groups.

In contrast, for *TLR4*+1196C/T (rs4986791), no significant difference was found between gastric cancer and control group (Table 2). Homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population. We also compared the genotype and allele frequencies between gastric cancer and gastritis groups and no significant difference was found for the polymorphisms studied (data not shown).

The *TLR4* haplotype analysis (Table 3) demonstrated higher frequency of both wild alleles (haplotype A-C) in control subjects compared with gastric cancer (95.7% and 91.4%, respectively;  $P < 0.01$ ). However, the opposite was observed for frequency of variant haplotype

**Table 4** Combined effect of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791) polymorphisms on risk of gastric cancer and chronic gastritis

Risk genotype	Groups								
	GC (n = 174)	C (n = 225)	OR (95% CI) P value	CG (n = 208)	C (n = 225)	OR (95% CI) P value	GC (n = 174)	CG (n = 225)	OR (95% CI) P value
Neither	113	187	1.00 (reference)	160	187	1.00 (reference)	113	160	1.00 (reference)
<i>TLR2</i> ins/del or del/del/ <i>TLR4</i> +896 AG	10	4	4.13 (1.26-13.50) 0.02	11	4	3.21 (1.00-10.29) 0.06	10	11	1.28 (0.52-3.13) 0.64
<i>TLR2</i> ins/del or del/del/ <i>TLR4</i> +1196 CT	4	1	6.61 (0.73-59.99) 0.07	1	1	1.16 (0.07-18.84) 1.00	4	1	5.66 (0.62-51.37) 0.16
<i>TLR4</i> +896 AG/+1196 CT	1	1	1.65 (0.10-26.73) 1.00	3	1	3.50 (0.36-34.05) 0.34	1	3	0.47 (0.04-4.59) 0.64

C: Control group; OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; TLR: Toll-like receptor.

**Table 5** Distribution of risk factors, genotypes of toll-like receptor 2 (-196 to -174 *del*) and toll-like receptor 4 (rs4986790 and rs4986791), and odds ratios for gastric cancer, chronic gastritis and control groups

Variables	F (GC/C) %	OR (95% CI)	P value	F (CG/C) %	OR (95% CI)	P value
Gender						
Female	23/49.7	Reference	< 0.01	51.0/50.3	Reference	0.96
Male	77/50.3	2.70 (1.66-4.41)		49.0/49.7	0.99 (0.66-1.48)	
Age (yr)						
< 61	40.9/56.0	Reference	0.30	52.8/43.5	(< 53 yr) Reference	0.03
≥ 61	59.1/44.0	1.26 (0.81-1.98)		47.2/56.5	(≥ 53 yr) 0.64 (0.43-0.95)	
Smoking						
Nonsmokers	30.5/34.3	Reference	0.13	44.8/34.3	Reference	0.01
Smokers	69.5/65.7	0.67(0.39-1.13)		55.2/65.7	0.57 (0.37-0.87)	
Alcohol						
Nondrinkers	46.6/74.3	Reference	< 0.01	68.2/74.3	Reference	0.06
Drinkers	53.4/25.7	2.93 (1.76-4.87)		31.8/25.7	1.54 (0.97-2.44)	
<i>TLR2</i>						
ins/ins	66.6/84.0	Reference	< 0.01	76.9/84.0	Reference	0.18
ins/del	33.4/16.0	2.64 (1.56-4.44)		23.1/16.0	1.39 (0.84-2.28)	
del/del						
<i>TLR4</i> +896A/G (rs4986790)						
AA	88.5/95.5	Reference	< 0.01	89.9/95.5	Reference	0.04
AG	11.5/4.5	3.19 (1.34-7.61)		10.1/4.5	2.29 (1.02-5.13)	
<i>TLR4</i> +1196C/T (rs4986791)						
CC	94.8/97.3	Reference	0.63	97.1/97.3	Reference	0.91
CT	5.2/2.7	1.33 (0.41-4.33)		2.9/2.7	0.93 (0.28-3.05)	

F: Frequency of individual (%); OR: Odds ratio; GC: Gastric cancer; CG: Chronic gastritis; C: Control; TLR: Toll-like receptor.

G-C, which was higher in the gastric cancer group compared with control group (63.0% and 31.0%, respectively;  $P = 0.02$ ).

In another statistical analysis, which evaluated the combined effect between the three polymorphisms (*TLR2* -196 to -174 *del*, *TLR4*+896A/G and +1196C/T), the combination of variant alleles of the polymorphisms *TLR2* ins/del and del/del with *TLR4* +896 AG showed a higher risk of gastric cancer compared to healthy individuals (OR = 4.13; 95% CI: 1.26-13.50;  $P = 0.02$ ). The other combinations of variant alleles did not show any significant difference (Table 4). The combination of the three variant alleles was found in only one individual of the group of chronic gastritis (data not shown).

The potential associations between the distributions of *TLR2* -196 to -174 *del* and *TLR4* (+896A/G and +1196C/T) genotypes adjusting for risk factors for gastric cancer and chronic gastritis in comparison of con-

trol group are presented in Table 5.

In the gastric cancer group, the multiple logistic regression shows that male gender (OR = 2.7; 95% CI: 1.66-4.41;  $P < 0.01$ ), alcohol intake (OR = 2.93; 95% CI: 1.76-4.87;  $P < 0.01$ ), *TLR2* -196 to -174 ins/del+del/del (OR = 2.64; 95% CI: 1.56-4.44;  $P < 0.01$ ) and *TLR4* +896AG (OR = 3.19; 95% CI: 1.34-7.61;  $P < 0.01$ ) were associated with a higher susceptibility to developing this neoplasm. The comparison between gastritis and control group showed that only *TLR4*+896AG polymorphism was associated with risk of chronic gastritis (OR = 2.29; 95% CI: 1.02-5.13;  $P = 0.04$ ), while age above 53 years (OR = 0.64; 95% CI: 0.43-0.95;  $P = 0.03$ ) and smoking (OR = 0.57; 95% CI: 0.37-0.87;  $P = 0.01$ ) were negatively associated with the development of gastritis. In another multiple logistic regression analysis considering also the individuals tested for *H. pylori* infection (95 with gastric cancer and 177 with gastritis), there was no statistically

significant association (OR = 0.73, 95% CI: 0.29-1.78;  $P = 0.49$ , data not shown in Table 5). There was also no association of three polymorphisms with *H. pylori* when evaluated negative and positive individuals within a group of gastric cancer and gastritis (data not shown).

## DISCUSSION

TLRs participate in *H. pylori* bacterium recognition in gastric mucosa, and SNPs in TLRs are associated with impaired immune response, inducing a potent inflammatory response. Therefore, it is relevant to carry out studies on host genetic factors that can be associated with susceptibility of gastric diseases. Hence, we investigated whether *TLR2* -196 to -174 del and *TLR4* (+896A/G rs4986790 and +1196C/T rs4986791) polymorphisms affect the risk of developing gastric cancer and chronic gastritis in a Brazilian population. Our results have demonstrated for the first time in this population, an association of *TLR2* -196 to -174 del and of *TLR4*+896 G polymorphisms with susceptibility to gastric cancer. The polymorphism *TLR4*+1196 T was not associated with risk to the gastric lesions evaluated, and the homozygous genotypes *TLR4*+896GG and *TLR4*+1196TT were absent in the studied population.

Some studies that investigated the association of *TLR2* -196 to -174 del polymorphisms at risk of developing diseases related to an inflammatory process have shown conflicting results. For instance, del allele or del/del genotype of *TLR2* -196 to -174 polymorphism was significantly associated with cervical cancer susceptibility<sup>[35]</sup> and risk of non-cardia gastric cancer in a Japanese population, but not for gastritis, gastric ulcer and duodenal ulcer<sup>[23]</sup>, while *TLR2* -196 to -174 ins allele was associated with more severe intestinal metaplasia in older patients<sup>[24]</sup>. However, Wang *et al.*<sup>[36]</sup> failed to show association of *TLR2* -196 to -174 del/del and ins/del carriers with ulcerative colitis.

The -196 to -174 del polymorphism in *TLR2* gene located on chromosome 4, causes a 22-bp nucleotide deletion that alters the promoter activity of gene. The *TLR2* del/del genotype is reported to show decreased transcriptional activity this gene<sup>[22]</sup>. In our study, we observed significantly higher frequencies of genotypes *TLR2* ins/del and del/del in the gastric cancer group compared to the healthy individuals, emphasizing its role in the gastric carcinogenesis.

The *TLR4* gene is mapped on chromosome 9 and consists of three exons. In exon 3, two non-synonymous SNPs *TLR4*+896A/G and +1196C/T allows the substitution of amino acids Asp299Gly and Thr399Ile, respectively. In the analysis by Haploview, the frequencies of *TLR4* G-C (299Gly-399Thr) and G allele were higher in patients with gastric cancer indicating an association of this haplotype with increased risk of gastric cancer to its carriers. The substitution of Asp299Gly amino acids disrupt the normal structure of the extracellular region of the TLR4 and may cause decreased ligand recogni-

tion or protein interaction, and decreased responsiveness to lipopolysaccharide, disrupting transport of *TLR4* to the cell membrane<sup>[13,37]</sup>. This change leads to an exaggerated inflammatory response with severe tissue destruction, likely due to a failure in stimulating regulatory cells and production of IL-10 cytokine<sup>[38]</sup>. Arbour *et al.*<sup>[13]</sup> were the first to report that individuals having either the Asp-299Gly and/or Thr399Ile polymorphisms had a blunted response towards inhaled LPS. Thus, during the cascade of progression of gastric carcinogenesis, the subjects with this polymorphism can have an increased risk of severe inflammation followed by development of hypochlorhydria and gastric atrophy, which are regarded as important precursor alterations of gastric cancer<sup>[12]</sup>.

Both SNPs in *TLR4* are presented in about 10% of Caucasian and African populations and are reported to have a positive correlation with susceptibility to infectious diseases, whereas studies in Asian populations have shown the absence of these polymorphisms<sup>[26,39]</sup>. However, in our study with a Southeastern Brazilian population, we found that both SNPs *TLR4* (+896A/G and +1196C/T) were present in heterozygous in about 4.5% to 11.5% and 2.7% to 5.2% respectively in the gastric cancer and control groups. Other studies in the Brazilian population found similar frequencies of heterozygous *TLR4*+896A/G polymorphism in Chagas disease (5.6%), ulcerative colitis (7.1%) and Crohn's disease (7%)<sup>[40,41]</sup>. But, to the best of our knowledge, there are not studies on *TLR2* and *TLR4* polymorphisms in gastric cancer of Brazilian population.

With regards to *TLR4*+896A/G and +1196C/T polymorphisms, Garza-Gonzalez *et al.*<sup>[29]</sup> showed no association with the risk of gastric cancer in the Mexican population, while Trejo de la O *et al.*<sup>[7]</sup> observed that both SNPs in *TLR4* had an association with duodenal ulcer and gastric cancer also in Mexican patients. Yet, other studies have demonstrated association with only one of these polymorphisms and risk of gastric cancer and precancerous lesions, either *TLR4*+1196C/T (Thr399Ile)<sup>[6,42]</sup> or *TLR4*+896A/G (Asp299Gly) polymorphisms<sup>[3]</sup> are associated with susceptibility to gastric carcinogenesis. In addition, in this study in Caucasian population<sup>[3]</sup>, homozygous polymorphic *TLR4*+896 GG was not found, corroborating our results.

Concerning *H. pylori* infection, we evaluated the association between *TLR2* and *TLR4* polymorphisms in the case groups with the available information in their medical records (95 with gastric cancer and 177 with gastritis) and no association was found. The reduced number of samples available for statistical analysis may have harmed these results. Rad *et al.*<sup>[8]</sup> studied the role of various TLRs (2/4/7 and 9) in response to *H. pylori* using mice mutants lacking these receptors. The results demonstrated the importance of the TLR2 in response to this bacterium, unlike the TLR4. TLR4-receptor lacking mice had little or no change in response to *H. pylori* compared with controls. Although the role that the effect of polymorphism in *TLR2* in the activity of this receptor in

gastric cells is not fully understood, this deletion is likely to alter their activity. Since *TLR2* has an important role in immune response against the *H. pylori* bacterium, the change of its function becomes relevant in carcinogenesis of the stomach<sup>[8]</sup>.

Besides the influence of the *H. pylori*, other risk factors as gender, age, smoking and alcohol intake were analyzed. There are statistically significant results for male gender and alcohol intake in the gastric cancer group compared with the control group. According to the National Cancer Institute the highest incidence of gastric cancer occurs in men around age 70 years, and about 65% of patients diagnosed with this type of cancer were over 50 years<sup>[1]</sup>. Another risk factor well established in the literature in relation to gastric carcinogenesis is the excessive consumption of alcohol<sup>[43-45]</sup>. Really, ethanol oxidation generates acetaldehyde, which presents carcinogenic effects, since it interferes with many DNA synthesis and repair sites, leading to tumor development<sup>[46]</sup>.

In conclusion, our findings indicate a significant role of both *TLR2* -196 to -174 del and *TLR4*+896G (Asp299Gly) polymorphic variant with susceptibility to gastric cancer in the Southeastern Brazilian population evaluated, whereas no association was observed for *TLR4* +1196T polymorphism. Thus, it is feasible to highlight that host genetic factors as the interaction of polymorphisms in genes of toll-like receptors can play an important role in gastric carcinogenesis.

## COMMENTS

### Background

Gastric cancer (GC) has high rate of incidence and mortality in Brazilian population. Thus is important to establish host genetic factors, as polymorphisms in genes related with inflammatory and immune response associated with higher risk of development of this neoplasia. In this study, authors have shown, for the first time association of toll-like receptor (*TLR*)2 -196 to -174 del and *TLR4*+896AG (Asp299Gly) polymorphisms with gastric cancer in a sample of Brazilian population.

### Research frontiers

Epidemiological studies on association of polymorphisms with susceptibility to disease as cancer frequently present conflicting results, possibly due to different factors as ethnicity, sample number, population sub-sampling, which can contribute to this discrepancy. Thus, are relevant studies in different populations that help clarify these aspects.

### Innovations and breakthroughs

In this study it was possible to make the combined analysis of three polymorphisms (*TLR2* -196 to -174 del, *TLR4*+896A/G and +1196C/T) and to show that the combination of single nucleotide polymorphisms *TLR2* ins/del and del/del with *TLR4* +896 AG led to a higher risk of gastric cancer.

### Applications

Considering the high incidence of gastric cancer in the Brazilian population, the data show that carriers of polymorphisms in genes involved with immune response as *TLR2* ins/del and del/del and *TLR4* +896 AG, together with other genetic and environmental factors constitute a risk group to gastric carcinogenesis.

### Peer review

This is a cross sectional study on the role of the *TLR2* and *TLR4* polymorphisms in GC in a Brazilian population. The main point that needs to be further clarified is the ethnic composition of the population. An important observation is that the *TLR4* variants are at similar frequencies on the African and Caucasian populations.

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## Adenoma incidence decreases under the effect of polypectomy

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polypectomy, the incidence of adenoma decreases with age.

**METHODS:** Consecutive patients with colonic adenomas identified at index colonoscopy were retrospectively selected if they had undergone three or more complete colonoscopies, at least 24 mo apart. Patients who had any first-degree relative with colorectal cancer were excluded. Data regarding number of adenomas at each colonoscopy, their location, size and histological classification were recorded. The monthly incidence density of adenomas after the index examination was estimated for the study population, by using the person-years method. Baseline adenomas were excluded from incidence calculations but their characteristics were correlated with recurrence at follow-up, using the  $\chi^2$  test.

**RESULTS:** One hundred and fifty-six patients were included (109 male, mean age at index colonoscopy  $56.8 \pm 10.3$  years), with follow-up that ranged from 48 to 232 mo. No significant correlations were observed between the number, the presence of villous component, or the size of adenomas at index colonoscopy and the presence of adenomas at subsequent colonoscopies ( $P = 0.49, 0.12$  and  $0.78$ , respectively). The incidence of colonic adenomas was observed to decay from 1.4% person-months at the beginning of the study to values close to 0%, at 12 years after index colonoscopy.

**CONCLUSION:** Our results suggest the sporadic formation of adenomas occurs within a discrete period and that, when these adenomas are removed, all neoplasia-prone clones may be extinguished.

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**Key words:** Colorectal cancer; Colorectal adenoma; Incidence; Age; Polypectomy

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### Abstract

**AIM:** To investigate whether, under the influence of pol-

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## INTRODUCTION

It has long been believed that initiation of sporadic colorectal cancer (CRC) increases over time, due to toxicity or loss of fidelity of DNA replication. Therefore, it is believed that the risk of adenoma formation is a function of age<sup>[1]</sup>, which justifies lifelong colonoscopy surveillance. However, current evidence has started to challenge these perceptions.

The data that have suggested that incidence of adenoma increases with age were mostly collected from autopsy studies<sup>[2,3]</sup>. Although relevant, these studies are prone to bias because the age-specific incidence rates do not adequately represent the time trend for newly formed lesions after removal of index lesions. Moreover, the rather important information of family aggregation of CRC was lacking in all of them.

More recent studies, based on surveillance colonoscopies<sup>[4-6]</sup>, have been mainly limited by short follow-up (3-5 years), which may lead to the inclusion, as new adenomas, of polyps that were missed at a previous examination. These time intervals are also likely to be too short to predict the lifelong dynamics of adenoma formation.

The notion that the formation of colon adenomatous polyps peaks at a certain age and then rapidly declines was first presented in 1975 by Henry *et al*<sup>[7]</sup>. He stated that: "some undefined stimulus to continuous polyp formation persists for up to 4 years in about one-third of patients who develop a colonic polyp. Thereafter, either the stimulus to neoplasia is no longer present, or the colonic mucosa adapts so that polyp formation does not persist." We propose that this undefined stimulus corresponds to the presence of a limited number of mutated clones that are scattered in the colonic epithelium and evolve through cumulative critical gene events selected over several decades. This sequence of events ends at a specific time window, in which most adenomas become endoscopically detectable and after which its incidence declines.

A study in Germany<sup>[8]</sup> has shown that individuals with negative findings at colonoscopy had a reduced risk of CRC for at least 20 years. Furthermore, when the examination was performed at 55-64 years of age and older, the risk of CRC was even lower. Additionally, according to the latest Guidelines for Colonoscopy Surveillance after Polypectomy issued by the American Gastroenterological Association (AGA)<sup>[9]</sup>, age is not considered to be a reliable predictor of subsequent advanced adenomas.

Taking these data into account and considering the

possibility of only a limited time window for sporadic adenoma expression, we aimed to study the temporal trend for adenoma formation, in a standard-risk population under colonoscopic surveillance. According to our hypothesis, which stated that a discrete number of mutated clones were scattered in the colon, we expected the adenoma incidence to decrease under the effect of polypectomy.

## MATERIALS AND METHODS

### *Patients and procedures*

Consecutive patients from three Portuguese hospitals (a tertiary oncology center, a tertiary general hospital and a regional hospital) with colonic adenomas who were identified at an index colonoscopy were retrospectively reviewed. These patients were included if they had undergone three or more complete colonoscopies that were at least 24 mo apart. The index colonoscopies were performed between 1978 and 2000, for screening or diagnostic purposes. The subsequent colonoscopies were performed according to the assistant physicians' choice, based on surveillance guidelines that changed during the time of the study (data were collected until 2007). Only colonoscopies reaching the cecum, with adequate bowel preparation and complete removal of all of the identified polyps were considered for inclusion in the present study.

The patient files were reviewed, and the patients were excluded if they had a previous history of CRC or adenomas, inflammatory bowel disease, hereditary non-polyposis CRC, familial adenomatous polyposis syndrome, a family history of CRC in any first-degree relative, or CRC at the index colonoscopy.

The location and size of all of the polyps had been recorded and the specimens had been sent for pathological evaluation and were classified according to the criteria of the World Health Organization<sup>[10]</sup>. All of the colonoscopies were performed by certified gastroenterologists, and sedation using intravenous midazolam (with or without pethidine association) or intravenous propofol (performed by an anesthesiologist) was administered on a case-to-case basis. The bowel preparation methods varied among the centers and over time, but were based on oral solutions that contained polyethylene glycol or senna.

The endoscopic and pathological reports were reviewed by the authors, for the results of the index colonoscopy, and of each colonoscopy reported thereafter. The left colon was defined as the splenic flexure and the segment distal to it.

The baseline adenoma characteristics were recorded and correlated with recurrence of adenomas at follow-up. However, these adenomas were specifically excluded from incidence ratio evaluation, because the time frame for their formation was unknown.

### *Data analysis*

The data regarding number of adenomas observed at each colonoscopy, along with their location, size and

Table 1 Outcomes of first four colonoscopies

Outcomes	Mean	SD
Index colonoscopy: 156 patients studied		
Adenomas	1.68	1.01
Tubular adenomas	1.49	1.13
Tubulo-villous/villous adenomas	0.14/0.05	0.35/0.22
Size of the larger adenoma (mm)	15.51	10.36
Adenomas in the left colon/remaining colon	1.34/0.35	0.93/0.69
Second colonoscopy: 156 patients studied (41.6 ± 21.0 mo since the first)		
Adenomas	0.54	0.86
Tubular adenomas	0.51	0.85
Tubulo-villous/villous adenomas	0.02/0.01	0.14/0.11
Size of the larger adenoma (mm)	8.02	7.39
Adenomas in the left colon/remaining colon	0.36/0.17	0.67/0.49
Third colonoscopy: 156 patients studied (83.7 ± 27.7 mo since the first)		
Adenomas	0.47	0.92
Tubular adenomas	0.46	0.92
Tubulo-villous/villous adenomas	0.02/0.0	0.18/0.0
Size of the larger adenoma (mm)	6.19	4.22
Adenomas in the left colon/remaining colon	0.21/0.26	0.47/0.71
Fourth colonoscopy: 44 patients studied (116.9 ± 34.1 mo since the first)		
Adenomas	0.32	0.74
Tubular adenomas	0.32	0.74
Tubulo-villous/villous adenomas	0.0/0.0	0.0/0.0
Size of the larger adenoma (mm)	5.78	1.92
Adenomas in the left colon/remaining colon	0.16/0.16	0.43/0.48

histological classification were recorded. The analysis of adenoma incidence over time was based on the person-years method. We assumed that the incidence rate of adenomas was constant between two consecutive colonoscopies in each individual and, in that time interval, the monthly incidence of adenomas in each patient was estimated by dividing the number of adenomas found at colonoscopy by the number of months that had elapsed since the previous examination. The incidence of adenomas in a given month after the index colonoscopy was determined for the entire sample by summing the incidence for that month across all of the patients that had been observed up to that time. The monthly incidence density of adenomas was then obtained by dividing the estimated incidence in that month by the population that was still at risk.

The statistical analysis was performed using Excel XP (Microsoft Inc) and Stata 10.0 (Stata Corporation, College Station, TX, United States). The baseline adenoma characteristics were correlated with recurrence at follow-up using the  $\chi^2$  test.

### Ethics

The present study was a retrospective observational study, in which no experimental intervention was used, and all of the data were kept anonymous. Therefore according to the local regulations, no approval by the Ethics Committee was necessary. All of the patients signed an informed consent document before each endoscopic examination.

## RESULTS

The present study included a total of 156 patients (109 male and 47 female). The mean age at the time of the

Table 2 Correlation between baseline adenoma characteristics and presence of adenomas in the second colonoscopy

Index colonoscopy	Second colonoscopy		$\chi^2$ test
	0 adenomas (n)	≥ 1 adenoma (n)	
> 1 adenoma	39	28	<i>P</i> = 0.24
≥ 3 adenomas	14	10	<i>P</i> = 0.57
≥ 1 TV adenoma	8	14	<i>P</i> = 0.004
≥ 1 villous adenoma	5	3	<i>P</i> = 0.95
≥ 1 TV or V adenoma	13	17	<i>P</i> = 0.01
≥ 1 adenoma > 1 cm	67	42	<i>P</i> = 0.43

TV: Tubulo-villous; V: Villous.

index colonoscopy was  $56.8 \pm 10.3$  years. No procedural complications were recorded. All 156 patients underwent three colonoscopies and 44 of them underwent a fourth examination. The outcomes of these colonoscopies are summarized in Table 1. The index colonoscopy was performed for screening in 31 patients and for diagnostic purposes in the remaining patients (and the symptoms were considered to be unrelated to the adenomas in the majority of cases).

The total number of adenomas and the numbers of each adenoma subtype (according to the histology or location) declined over the course of the four colonoscopies. Additionally, 12 patients underwent a fifth examination ( $162.3 \pm 32.7$  mo after the first), and two of these had a sixth examination ( $211.5 \pm 20.5$  mo after the first). No adenomas were found in any of these last examinations.

Of the initial 156 patients, who underwent three colonoscopies, 107 presently have scheduled colonoscopies, in agreement with the latest guidelines for surveillance after polypectomy; 27 patients have been released from follow-up due to advanced age or significant comorbidity; two patients have died of unrelated causes; and 20 have been lost from follow-up.

There was no significant correlation between the number of adenomas at the index colonoscopy and the presence or absence of adenomas at the second or all subsequent colonoscopies (*P* = 0.68 for the second colonoscopy, *P* = 0.49 for all subsequent colonoscopies). The presence of three or more adenomas at the index colonoscopy did not correlate with the presence of adenomas at the subsequent colonoscopies (*P* = 0.57 for the second, *P* = 0.21 for all subsequent colonoscopies), nor did the presence of adenomas > 1 cm at the index colonoscopy (*P* = 0.43 for the second colonoscopy, *P* = 0.78 for all subsequent colonoscopies). The presence of adenomas with a villous component at the index colonoscopy correlated with the presence of adenomas at the second colonoscopy (*P* = 0.01), but there was no significant correlation when all of the subsequent colonoscopies were considered together (*P* = 0.12) (Tables 2 and 3).

The presence of adenomas of the left colon at the index colonoscopy did not predict recurrence in the same segment in the second or all subsequent colonoscopies, and the same was true for the right colon (data not shown).

The incidence of colonic adenomas was found to

**Table 3** Correlation between baseline adenoma characteristics and presence of adenomas in all subsequent colonoscopies

Index colonoscopy	Subsequent colonoscopies		$\chi^2$ test
	0 adenomas ( <i>n</i> )	≥ 1 adenoma ( <i>n</i> )	
> 1 adenoma	31	36	<i>P</i> = 0.5
≥ 3 adenomas	9	15	<i>P</i> = 0.2
≥ 1 TV adenoma	7	15	<i>P</i> = 0.08
≥ 1 villous adenoma	4	4	<i>P</i> = 0.97
≥ 1 TV or V adenoma	11	19	<i>P</i> = 0.12
≥ 1 adenoma > 1 cm	53	56	<i>P</i> = 0.78

TV: Tubulo-villous; V: Villous.

decline from 1.4% person-months, at the beginning of the study, to values close to 0%, 12 years after the index examination (Figure 1).

Eight years after the index colonoscopy (with 65 patients evaluated), a peak in the incidence of adenomas was observed, that approached the baseline values, which was then followed by a steady decline until the end of follow-up (with 37 patients evaluated at 10 years and 19 patients evaluated at 12 years).

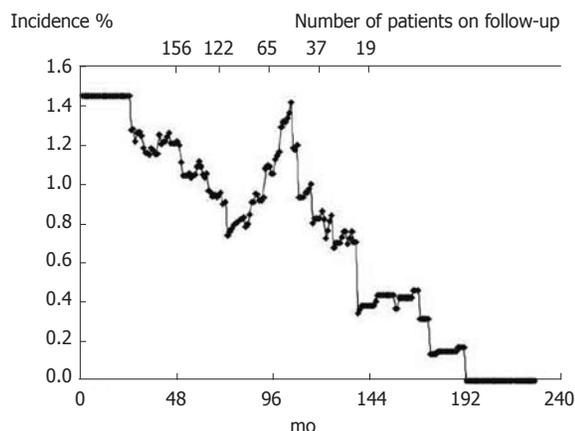
There were no reports of flat lesions of the colon in any of these examinations, and there were no colorectal adenocarcinomas reported in these patients during the study period.

## DISCUSSION

To explain the predominant CRC expression in the sixth and seventh decades of life, a necessary sequence of 4–7 known mutations fits a model of a stable mutation clock that ends in full-blown neoplasia at consistent time-intervals. The predominant molecular pathway responsible for CRC begins with the selection of cells with an *APC* gene loss or a  $\beta$ -catenin mutation, which is followed by the cumulative selection of subsequent critical events in other genes. Estimations of the time taken to acquire such a sequence of mutations suggest that full-blown neoplasia can take several decades to occur. According to recent estimations, to obtain the necessary sequence of mutations, the first event may need to occur at an early age<sup>[11–13]</sup>; most likely during the exponential phase of embryonic development, when *APC* is well known to play a key role<sup>[14]</sup>.

In 2007, human colon cancer stem cells were identified by two separate research groups<sup>[15,16]</sup>. More recently, the location of normal colon stem cells, at the crypt base, has also been demonstrated<sup>[17]</sup>, and these cells seem to be the origin of colon cancer stem cells<sup>[18]</sup>. It has been proposed that mutations in stem cells are much more likely to occur during the exponential phase of early growth, as opposed to later in life<sup>[19]</sup>.

Accordingly, the results of the present paper strongly suggest that the sporadic formation of adenomas occurs during a limited time period and, when these adenomas are removed, virtually all of the neoplasia-prone clones may be extinguished. If this model is shown to be true,



**Figure 1** Variation of colonic adenomas monthly incidence (person-month percentage) with time, since the index colonoscopy.

then the concept of a field carcinogenic defect, which progresses with continuous adverse environmental exposure and/or failure of the tight controls that assure DNA replication fidelity, should be replaced by a concept of limited colonic mosaicism. When the endoscopically visible neoplastic expression of this limited mosaicism is removed, the putative carcinogenic impulse no longer compromises colonic epithelia homeostasis.

Our study was limited mainly by its retrospective nature and by the small number of patients reaching longer follow-up. However, this sample was probably representative of the population at risk for sporadic CRC, because it included both symptomatic and asymptomatic individuals who had no relevant family histories, who underwent initial colonoscopy mostly during the sixth decade of life, and who were followed for at least 4 years.

In contrast to traditional beliefs, we did not observe a correlation between baseline adenoma characteristics and the risk of recurrence at follow-up. Although the AGA still takes baseline predictors of future adenomas or cancer into consideration, in their latest Guidelines for Colonoscopy Surveillance After Polypectomy<sup>[9]</sup>, several limitations of the available evidence have been raised. van Stolk *et al.*<sup>[20]</sup> have reported a study in which the number of adenomas at first colonoscopy was a significant predictor of having recurrent adenomas; however, these authors noted that missed polyps were a possible explanation for this relationship. Furthermore, that previous study only included 4 years of follow-up, and the presence of a family history of CRC was not taken into account at all. In another study, by Martínez *et al.*<sup>[21]</sup>, multiple adenomas at baseline, large adenomas (> 1 cm) or adenomas in the proximal colon were predictors of recurrence. However, the maximum follow-up of that study was 2 years, and the baseline colonoscopy was not the first examination for several of the patients. In addition, and although this relationship was not statistically significant, a family history of CRC in first-degree relatives was associated with a higher risk of recurrence in the study population. The initial National Polyp Study also included a follow-up of only 3 years, and investigated the incidence of colorec-

tal cancer but not adenoma<sup>[2,22]</sup>. The authors included patients regardless of their family history (other than established genetic syndromes)<sup>[2]</sup> and they admitted that, as a result of the short follow-up, three of the diagnosed cancers may have been missed polyps<sup>[23]</sup>. A more recent study, by Martinez *et al.*<sup>[24]</sup>, has revealed that the age of the patient and the number and size of prior adenomas were associated with the risk of advanced colorectal neoplasia after polypectomy. However, that study was also limited by a median follow-up of only 4 years and a maximum follow-up of < 6 years<sup>[24]</sup>. Our study had the advantages of a minimum follow-up of 4 years and exclusion of patients with any family history of CRC in first-degree relatives. This might explain why we did not find that baseline adenoma characteristics were predictive of recurrence, and why we were able to show a decreasing incidence of adenomas with time, after an age peak and under the influence of polypectomy.

At Digestive Disease Week 2007, Zauber *et al.* presented new data regarding the National Polyp Study population indicating that, after a mean follow-up of 14 years, CRC mortality was markedly reduced in all of the patients with adenomas at baseline, when compared to the general population. This was observed even when patients who refused follow-up were considered in the analysis. This finding supports the hypothesis that the major benefit is derived from the first colonoscopy and also brings into question the notion of increasing recurrence of adenomas over time.

With regard to the unexpected second peak that was observed for the incidence of adenomas in our study, after 8 years, we may speculate whether this peak is related to the type of the second hit in the *APC* gene. It has been reported that, when the first hit happens close to codon 1300, the second hit is most likely loss of heterozygosity, a faster and more efficient mutation process. In all other cases, point mutation is the most common mechanism for the second hit, thus driving a more sluggish development<sup>[25]</sup>. This confers different selective advantages to each colonic adenoma, according to the specific first hit-second hit combination and may explain why some adenomas appear earlier and why others are only apparent towards the end of the adenoma time window. We acknowledge that, during a limited period of embryonic development, a small number of stem cells may acquire different first mutations, and that these mutations are expressed along incidence waves during a restricted time window.

The results of the present study reinforce the importance of colonoscopy with polypectomy during the fifth to sixth decades of life, and the feasibility of increasing the time for re-examination after a normal examination. Furthermore, our study indirectly supports the concept of a relatively stable mutation clock, which is possibly initiated during the developmental phase of embryogenesis.

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## COMMENTS

### Background

Colorectal cancer (CRC) and adenomas are thought to have an increasing incidence with age, and this has led to almost lifelong surveillance colonoscopy. This view has recently started to be challenged.

### Research frontiers

*APC* is a crucial gene in colorectal carcinogenesis. If the first hit on this gene occurs in a colonic stem cell precursor early in life, it could lead to only a few colon crypts that are prone to originate adenoma and cancer, in a restricted time window. If these clones were removed, by polypectomy, the potential for colon cancer could be eradicated in that individual.

### Innovations and breakthroughs

Colon cancer stem cells have recently been identified, apparently originating from colonic stem cells, at the crypt base. Stem cells seem more prone to mutation during embryogenesis than later in life. Reports have shown that a single colonoscopy with polypectomy reduces CRC incidence. The study shows that, under the influence of polypectomy, adenoma incidence decreases with age.

### Applications

If the study results are confirmed, surveillance colonoscopy intervals may safely be lengthened, and some people may even be released from surveillance after a few examinations.

### Terminology

*APC* acts as a tumor suppressor gene by regulating the intranuclear concentration of  $\beta$ -catenin, a protein involved in the transcription of genes that promote proliferation. These genes and proteins are part of the Wnt pathway, which is involved in > 80% of sporadic CRC.

### Peer review

The authors do a nice job to expand on a current line of questioning the need for continuing surveillance and indirectly questioning the concept of age appropriateness for surveillance as well as cost-advantageous practice. This is a very relevant study and well written.

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## CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis

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### Abstract

**AIM:** To investigate how a complex network of CC chemokine ligands (CCLs) and their receptors influence the progression of tumor and metastasis.

**METHODS:** In the present study, we used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. We analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome.

**RESULTS:** We found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher tumor

node metastasis stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis.

**CONCLUSION:** These results suggest that overexpression of these two CC chemokine ligands is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

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**Key words:** CC chemokine; Chemokine ligand 7; Chemokine ligand 21; Gastric cancer; Lymph node metastasis; Poor prognosis

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### INTRODUCTION

Chemokine ligands (CCLs) belong to the small molecule chemoattractive cytokine family and are grouped into CC and CXC chemokines ligands on the basis of the characteristic presence of four conserved cysteine residues<sup>[1-3]</sup>. Chemokines mediate their chemical effect on target cells through G-protein-coupled receptors, which are characterized structurally by seven transmembrane spanning domains and are involved in the attraction and activation of mononuclear and polymorphonuclear leukocytes. CCLs and their receptors play an important role

in angiogenesis and tumor growth, however the role of CCLs in metastasis has only recently been explored<sup>[4,5]</sup>. CCL7 promoted the invasion and migration of oral squamous cell carcinoma<sup>[4]</sup>. CCL21 was significantly highly expressed in breast tumor cells with lymph node metastasis and prognosis<sup>[5]</sup>.

Gastric cancer is one of the commonest malignant tumors of the alimentary tract and is characterized by late clinical presentation, rapid progression, and poor survival<sup>[6]</sup>. The reason for this poor prognosis is that, at the time of diagnosis, gastric cancer usually shows extensive local tumor invasion and frequent spread to metastatic sites, particularly lymph nodes. Spread of malignant tumors is a multistep process and many of the stages of tumor invasion require degradation or breakdown of the extracellular matrix and connective tissue surrounding tumor cells<sup>[7,8]</sup>. The matrix metalloproteinases (MMPs) are a family of zinc containing enzymes which are involved in the degradation of different components of the extracellular matrix, and there is considerable evidence to indicate that individual MMPs have important roles in tumor invasion and tumor spread<sup>[9-11]</sup>. A recent study showed that increased levels of CCL recruit immature myeloid cells that carry the chemokine ligand receptor (CCR) from the blood to the tumor invasion front. These immature myeloid cells produced MMP9 and MMP2 and help the tumor cells to migrate and invade<sup>[12]</sup>.

In the present study, we used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. We analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome.

## MATERIALS AND METHODS

### Patients and specimens

A consecutive series of 194 tissue specimens were collected from patients with gastric cancer who received subtotal or total gastrectomy resection in Chang Gung Memorial Hospital (CGMH) in Taiwan. All operations were performed between January 2001 and December 2002. Written informed consent was obtained before sample collection and this study was approved by the Institutional Review Board of CGMH. There were 114 males and 80 females with a mean age of 62 years (range, 24-90 years). The age and gender of patients, tumor location, tumor size, cell differentiation, depth of wall invasion, status of lymph node metastasis, vascular invasion, lymphatic invasion and desmoplastic reaction were obtained from histopathology records. Stage of gastric cancer was described according to the 1997 tumor node metastasis (TNM) classification of malignant tumors by the American Joint Committee on Cancer. All patients were followed until December 2007 with a minimum 5 years of follow-up. All tissue specimens were formalin-fixed and paraffin-embedded. Formalin fixed tissue sections were stained with haematoxylin and eosin and classified by a pathologist. These results were compared with

the histopathology records from CGMH. Final pathology was determined by consensus and review if necessary.

### Immunohistochemistry

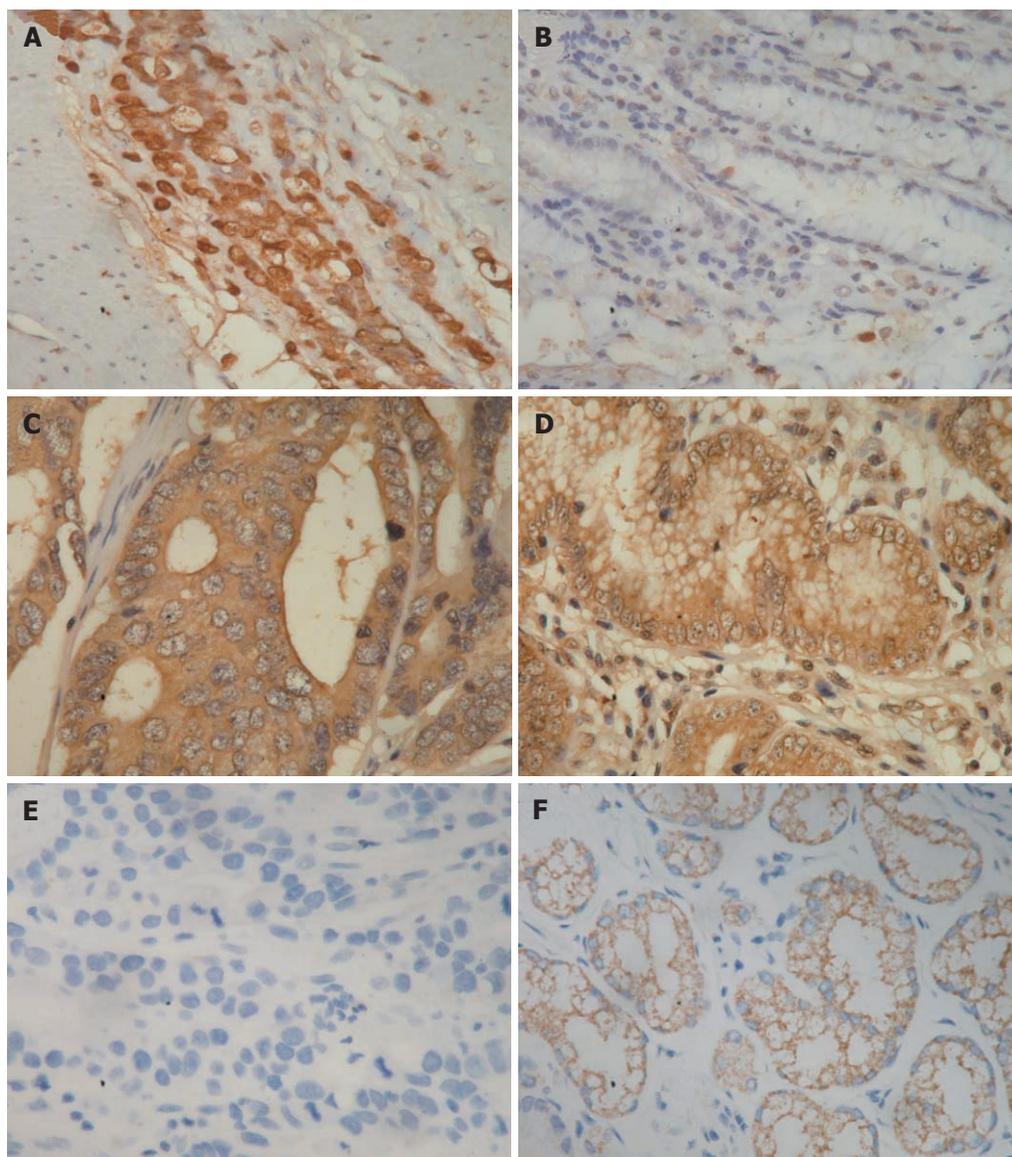
The tissue blocks were constructed according to the method of Schraml *et al.*<sup>[13]</sup> and the best representative morphological areas of tumors were used in this study. The specimen sections were deparaffinized, treated with 3% hydrogen peroxide and microwaved after pretreatment in 10 mmol/L citric acid to retrieve antigenicity. The sections were incubated with blocking solution containing phosphate buffered saline and 1% bovine serum albumin for 20 min at room temperature, and then incubated overnight at 4 °C with an anti-CCL7 antibody (1:100, R and D), an anti-CCL8 monoclonal antibody (1:50, R and D), or an anti-CCL21 monoclonal antibody (1:50, R and D), respectively. After washing 4 times with Tris Buffered Saline, the sections were incubated with biotinylated secondary antibody (Santa Cruz Biotechnology). The immuno-complex was visualized by the immunoglobulin enzyme bridge technique using the DAKO LSAB 2 System, HRP kit (DAKO corp. Carpinteria, CA) with 3,3' diaminobenzidine tetrachloride as a substrate. The sections were counterstained with haematoxylin, dehydrated with graded alcohols, cleared with xylene and mounted with a coverslip.

### Scoring of the immunohistochemical staining

The immunostaining results were scored as follows, according to a previous report<sup>[14]</sup>. The immunostaining reaction was evaluated by subjective assessments of the median staining intensity (0, no stain; 1, weak; 2, moderate; and 3, strong stain) and by the fraction of stained cells in percentage categories (0, 0%-9%; 1, 10%-49%; 2, 50%-89%; and 3, ≥ 90%). This scoring system was previously shown to be reproducible<sup>[15]</sup>. The scores of 0 to 3 were obtained as follows: percentage categories and staining were each ranked as indicated above. The ranks for percentage and staining intensity were multiplied by each other, divided by 3, and rounded up to the nearest whole number<sup>[15]</sup>. The results of immunostaining in tumor and normal tissues were divided into three groups, higher (rank of tumor tissue > rank of normal tissue), equal (rank of tumor tissue = rank of normal tissue), and lower (rank of tumor tissue < rank of normal tissue) (Figures 1-3).

### Statistical analysis

$\chi^2$  or Fisher's exact test was used to test for an association between CCL7, CCL8 and CCL21 expression and patient clinicopathologic parameters. Disease-free survival was defined as the time from surgery to the first relapse of cancer, occurrence of a second primary tumor, or death from any cause. Univariate survival analysis was assessed by the Kaplan-Meier method and significance of difference between groups was analysed using log rank test or log rank test for trend. Stepwise multivariate survival analysis was performed by the Cox proportional hazards model. All reported *P* values were two-sided and a *P* value < 0.05 was considered significant.



**Figure 1** Immunohistochemistry of chemokine ligand 7 in gastric cancer and adjacent normal tissues. Chemokine ligand 7 (CCL7) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL7 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL7 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification,  $\times 400$ ).

## RESULTS

### **CCL7, CCL8 and CCL21 expression in gastric cancer and adjacent normal tissues**

The percentages of the higher expression of CCL7, CCL8 and CCL21 in cancer tissues than in normal tissues were 42.3% (82 of 194), 29.9% (58 of 194) and 44.8% (87 of 194), respectively (Figures 1-3 and Table 1). The percentages of the equal expression of CCL7, CCL8 and CCL21 in cancer tissues and in normal tissues were 35.6% (69 of 194), 33% (64 of 194) and 32.5% (63 of 194), respectively (Figures 1-3 and Table 1). The percentages of the lower expression of CCL7, CCL8 and CCL21 in cancer tissues than in normal tissues were 22.2% (43 of 194), 37.1% (72 of 194) and 22.7% (44 of 194), respectively (Figures 1-3 and Table 1).

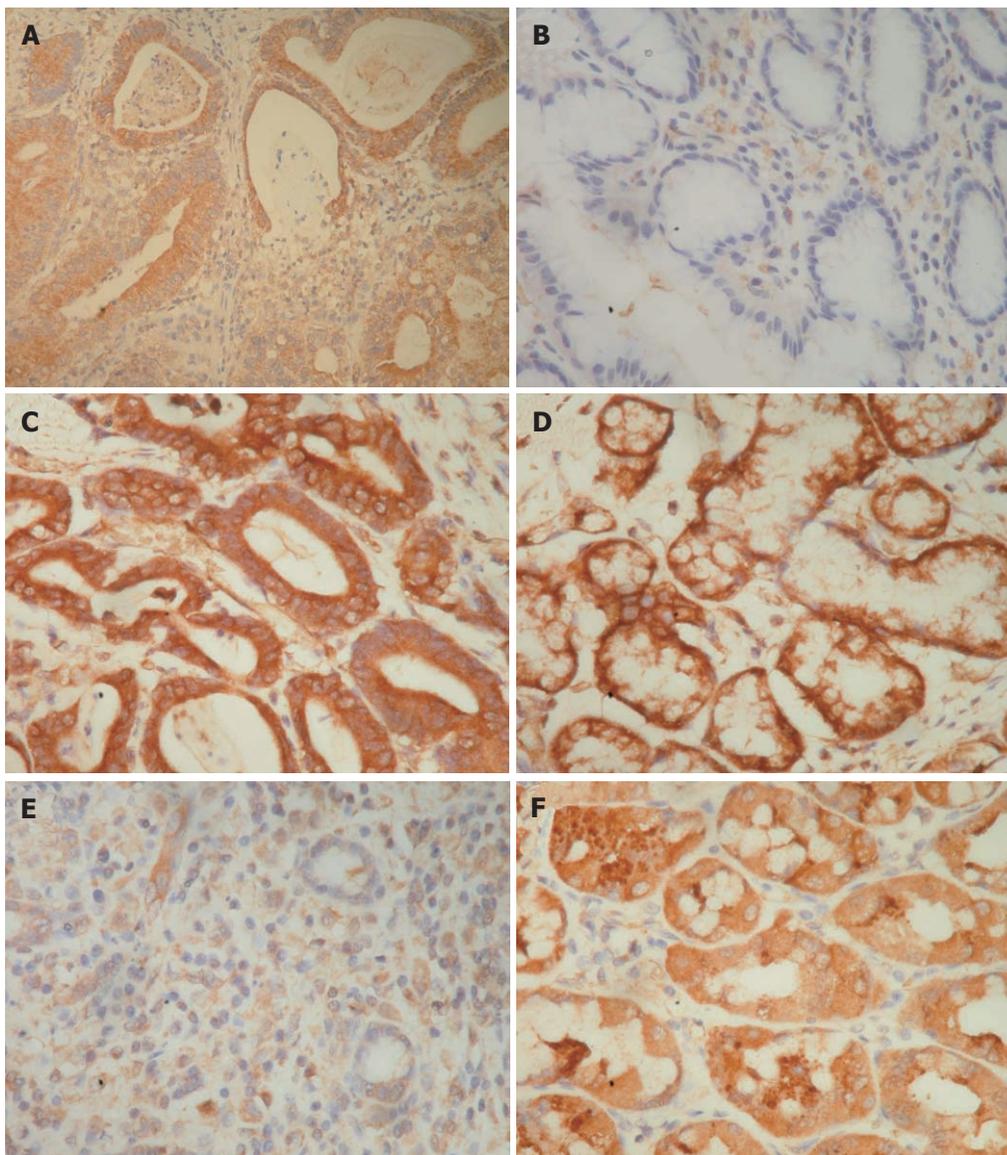
### **CCL7, CCL8 and CCL21 overexpression in relation to clinicopathologic parameters**

The overexpression of CCL7 in cancer tissues compared with normal tissues was significantly correlated with tu-

mor location ( $P = 0.025$ ) and tumor size ( $P = 0.001$ ). The overexpression of CCL7 was significantly higher in gastric cancer with advanced depth of wall invasion ( $P = 0.001$ ), lymph node metastasis ( $P = 0.020$ ), desmoplastic reaction ( $P = 0.006$ ) and higher TNM stage ( $P = 0.008$ ), but was not correlated with age, gender, differentiation, vascular invasion or lymphatic invasion (Table 1).

The overexpression of CCL8 was significantly correlated with age ( $P = 0.026$ ) and tumor location ( $P = 0.004$ ), but not with gender, tumor size, differentiation, depth of wall invasion, lymph node metastasis, vascular invasion, lymphatic invasion, desmoplastic reaction or TNM stage.

The overexpression of CCL21 was significantly higher in females than in males ( $P = 0.041$ ) and was correlated with tumor location ( $P = 0.026$ ), tumor size ( $P = 0.043$ ) and lymphatic invasion ( $P = 0.006$ ). As with CCL7, the overexpression of CCL21 was significantly higher in gastric cancer with an advanced depth of wall invasion ( $P < 0.0001$ ), lymph node metastasis ( $P = 0.003$ ), desmoplastic reaction ( $P < 0.0001$ ) and higher TNM stage ( $P < 0.0001$ ), but was not correlated with age, differentiation or vascular



**Figure 2** Immunohistochemistry of chemokine ligand 8 in gastric cancer and adjacent normal tissues. Chemokine ligand 8 (CCL8) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL8 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL8 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification,  $\times 400$ ).

invasion (Table 1).

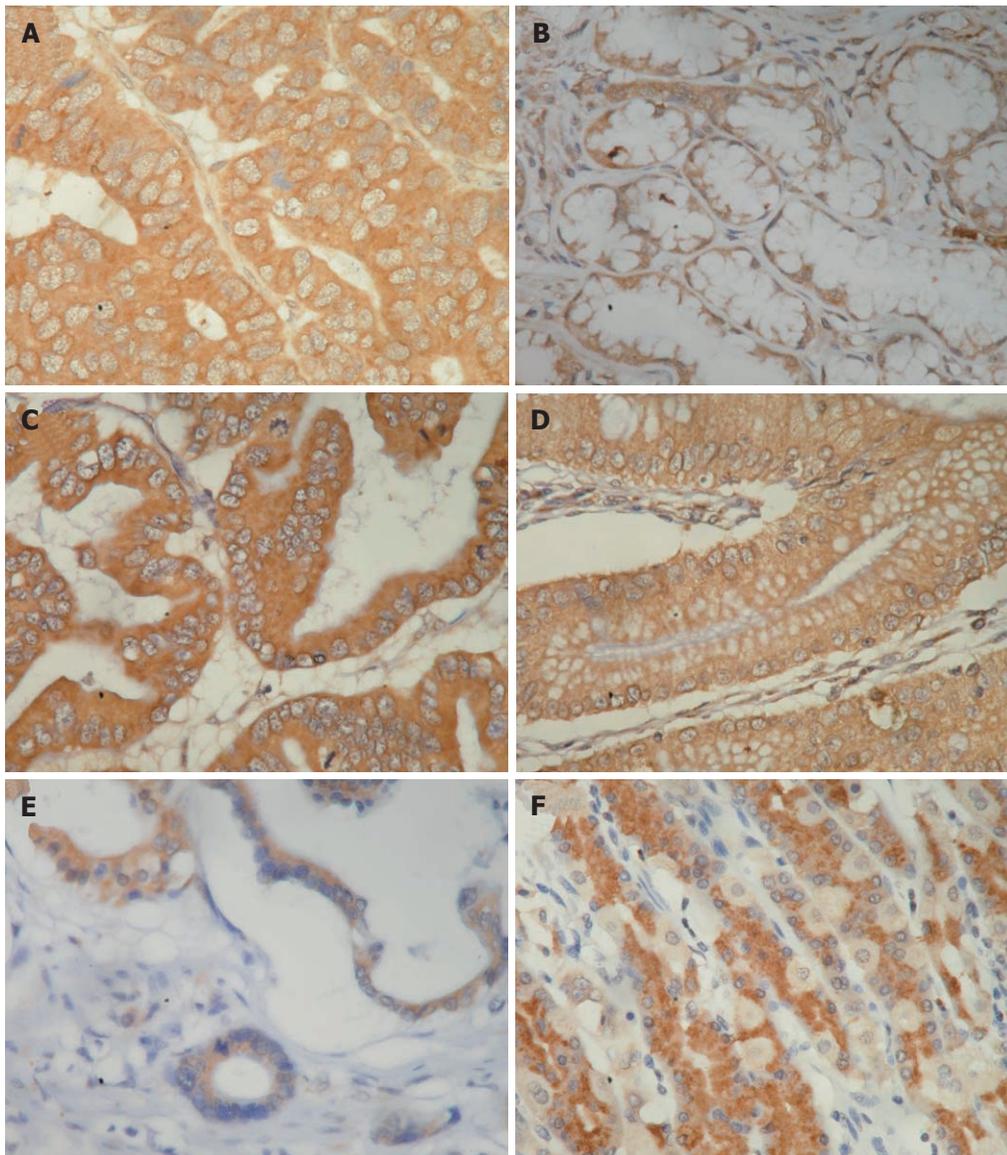
### Prognostic implications of CCL7, CCL8 and CCL21 overexpression in gastric cancer

CCL7 and CCL21 overexpression was correlated with a poor prognosis ( $P = 0.002$  and  $0.001$ , Table 2 and Figure 4A and C). CCL8 overexpression was not correlated with survival (Table 2, Figure 4B). Other significant prognostic factors were tumor location, tumor size, differentiation, depth of invasion, lymph node metastases, vascular invasion, lymphatic invasion, marked desmoplastic reaction and higher TNM stage. In multivariate analysis, depth of invasion, lymph node metastasis and desmoplastic reaction were independent prognostic factors (Table 3).

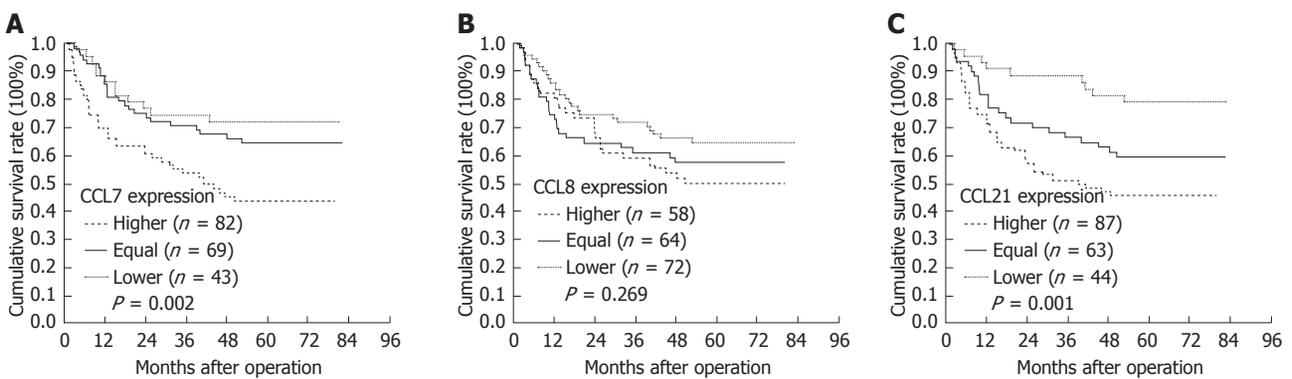
## DISCUSSION

In this study, CCL7, CCL8 and CCL21 expression levels were examined in 194 cases of gastric cancer for correlation with patient clinicopathologic factors. We found that the higher expression of CCL7 and CCL21 in cancer

tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher TNM stage. The mechanism for chemokine ligand promotion of tumor invasion and metastasis is not clear. Using a model of colorectal tumor progression, Kitamura *et al.*<sup>[12]</sup> showed that tumor-stromal interaction could promote tumor invasion. The colonic tumor can promote the production of CCL9. Increased levels of CCL9 recruited immature myeloid cells that carry the CCL9 receptor CCR1 from the blood to the tumor invasion front. The immature myeloid cells produce MMP2 and MMP9 and help the tumor epithelium to migrate and invade into the stroma. Jung *et al.*<sup>[4]</sup> also showed the importance of tumor-stromal crosstalk in invasion of oral squamous cell carcinoma (OSCC) *via* CCL7<sup>[4]</sup>. To identify key molecular regulators expressed by carcinoma-associated fibroblasts (CAF) that promote cancer cell invasion, Jung *et al.*<sup>[4]</sup> used microarrays to compare cocultured OSCC and CAF with monoculture controls. Microarray and real-time polymerase chain reaction analysis identified marked upregulation of CCL7 in cocultured CAF. Enzyme-linked



**Figure 3** Immunohistochemistry of chemokine ligand 21 in gastric cancer and adjacent normal tissues. Chemokine ligand 21 (CCL21) staining is higher in the cytoplasm of gastric cancer cells (A) than in the cytoplasm of adjacent normal cells (B); CCL21 staining is equal in the cytoplasm of gastric cancer cells (C) and in the cytoplasm of adjacent normal cells (D); CCL21 staining is lower in the cytoplasm of gastric cancer cells (E) than in the cytoplasm of adjacent normal cells (F). (magnification,  $\times 400$ ).



**Figure 4** Kaplan-Meier survival curves for disease-free survival of 194 patients with gastric cancer. A: Categorized by chemokine ligand 7 (CCL7) expression, survival was significantly worse for patients with higher CCL7 expression than those with equal or lower CCL7 expression ( $P = 0.002$ ); B: Categorized by CCL8 expression, no significant difference was observed among the three groups ( $P = 0.269$ ); C: Categorized by CCL21 expression, survival was significantly worse for patients with higher CCL21 expression than those with equal or lower CCL21 expression ( $P = 0.001$ ).

immunosorbent assay showed an elevated level of CCL7 secretion from CAF stimulated by coculture with OSCC cells. CCL7 promoted the invasion and migration of

OSCC cells, and the invasiveness was inhibited by treatment with CCL7 neutralizing antibody.

However, other studies have shown that CC chemokine

Table 1 Association of chemokine ligand 7, chemokine ligand 8 and chemokine ligand 21 expression with the clinicopathologic parameters

Factors	Cases	CCL-7 expression				CCL-8 expression				CCL-21 expression			
		Higher <i>n</i> = 82	Equal <i>n</i> = 69	Lower <i>n</i> = 43	<i>P</i> value	Higher <i>n</i> = 58	Equal <i>n</i> = 64	Lower <i>n</i> = 72	<i>P</i> value	Higher <i>n</i> = 87	Equal <i>n</i> = 63	Lower <i>n</i> = 44	<i>P</i> value
Age (yr)													
≤ 60	83	31	31	21	0.449	17	28	38	0.026	34	24	25	0.101
> 60	111	51	38	22		41	36	34		53	39	19	
Gender													
Male	114	52	40	22	0.412	36	40	38	0.429	47	45	22	0.041
Female	80	30	29	21		22	24	34		40	18	22	
Tumor location													
Upper	22	10	6	6	0.025	4	6	12	0.004	8	9	5	0.026
Middle	40	10	17	13		10	6	24		13	11	16	
Lower	124	58	46	20		42	48	34		60	43	21	
Whole	8	4	0	4		2	4	2		6	0	2	
Tumor size (cm)													
≤ 3	95	29	36	30	0.001	24	28	43	0.068	34	35	26	0.043
> 3	99	53	33	13		34	36	29		53	28	18	
Differentiation													
Well	18	6	8	4	0.177	5	6	7	0.422	7	7	4	0.130
Moderate	55	27	22	6		17	23	15		30	18	7	
Poor	52	25	15	12		19	13	20		27	12	13	
Signet ring cell	69	24	24	21		17	22	30		23	26	20	
Depth of wall invasion													
T1	47	12	19	16	0.001	8	15	24	0.070	9	18	20	< 0.0001
T2	37	8	18	11		13	12	12		9	18	10	
T3	101	58	29	14		35	31	35		64	24	13	
T4	9	4	3	2		2	6	1		5	3	1	
Lymph node metastasis													
N0	88	30	36	22	0.020	21	29	38	0.488	28	33	27	0.003
N1	47	16	16	15		16	17	14		20	17	10	
N2	23	13	7	3		7	6	10		13	5	5	
N3	36	23	10	3		14	12	10		26	8	2	
Vascular invasion													
No	168	70	60	38	0.800	50	58	60	0.561	71	57	40	0.163
Yes	26	12	9	5		8	6	12		16	6	4	
Lymphatic invasion													
No	104	37	39	28	0.086	27	34	43	0.325	36	38	30	0.006
Yes	90	45	30	15		31	30	29		51	25	14	
Desmoplastic reaction													
None	28	6	14	8	0.006	5	6	17	0.071	7	9	12	< 0.0001
Mild	64	22	20	22		18	21	25		17	26	21	
Moderate	75	43	25	7		23	30	22		44	24	7	
Marked	27	11	10	6		12	7	8		19	4	43	
TNM stage													
I	64	16	27	21	0.008	14	20	30	0.427	13	26	25	< 0.0001
II	44	17	17	10		13	14	17		18	18	8	
III	38	20	12	6		13	14	11		24	8	6	
IV	48	29	13	6		18	16	14		32	11	5	

CCL: Chemokine ligand; TNM: Tumor node metastasis.

ligands promote T cells to kill the tumor cells. Wu *et al.*<sup>[6]</sup> investigated the effect of exogenous CCL21 expressed in breast cancer MCF-7 cells on human monocyte-derived dendritic cells (DCs). Stimulation of CCL21-transfected MCF-7 cells prompted DC function: migration, antigen uptake and presentation. The stimulated DCs facilitated Th 1 type cytokine production, perforin-forming CD8<sup>+</sup> T cell transformation and final T cell-associated clearance of MCF-7 cells. Wetzel *et al.*<sup>[7]</sup> showed that human CCL7 can reduce tumorigenicity and augment infiltration of dendritic cells and neutrophils toward mouse mastocytoma; it also inhibits mouse melanoma growth through activation of T lymphocytes and natural killer cells.

The differences between the expression of CCL7 and CCL21 correlated with clinicopathologic parameters were gender and lymphatic invasion. The overexpression of CCL7 in gastric cancer was not correlated with gender and lymphatic invasion, but that of CCL21 was correlated with these two parameters. The overexpression of CCL21 was significantly higher in females than in males. The reason for the significance is not clear and more studies are necessary to clarify the significance. The overexpression of CCL21 was also correlated with lymphatic invasion. Recently, metastatic gastric carcinoma cells have been shown to express the receptor for chemokine CCL21, chemokine receptors CCR7, a prop-

**Table 2** Univariate analysis of the clinicopathologic parameters influencing the disease-free survival of 194 gastric cancer patients undergoing gastrectomy

Factors	Cases	Mean survival (mo)	95% CI of mean	5-year survival (%)	P value
Age (yr)					
≤ 60	83	57.33	50.22-64.44	60.2	0.516
> 60	111	53.34	46.93-59.76	56.1	
Gender					
Male	114	57.90	51.74-64.05	62.2	0.174
Female	80	51.09	43.61-58.56	51.7	
Type of gastrectomy					
Total	41	31.85	23.28-40.43	33.5	< 0.0001
Subtotal	153	60.21	55.14-65.48	64.5	
Tumor location					
Upper	22	26.01	15.81-36.21	27.3	< 0.0001
Middle	40	69.22	61.15-71.29	77.0	
Lower	124	56.56	50.70-62.43	59.5	
Diffuse	8	23.41	7.46-39.36	25.0	
Margin					
Negative	173	57.71	52.73-62.68	62.3	0.0001
Positive	21	27.41	17.26-37.56	12.6	
Tumor size (cm)					
≤ 3	95	70.94	65.97-75.92	80.7	< 0.0001
> 3	99	39.02	32.23-45.81	34.6	
Differentiation					
Well	18	65.58	59.43-71.73	94.4	< 0.0001
Moderate	55	43.11	35.16-51.06	54.4	
Poor	52	37.91	30.28-45.54	33.6	
Signet ring cell	69	62.16	54.89-69.43	67.9	
Depth of invasion					
T1	47	79.53	75.46-83.60	95.7	< 0.0001
T2	37	71.92	64.56-79.28	80.6	
T3	101	40.04	33.53-46.54	35.4	
T4	9	10.57	5.94-15.21	0.0	
Lymph node metastasis					
N0	88	73.64	69.17-78.11	83.8	< 0.0001
N1	47	57.67	47.75-67.59	64.9	
N2	23	31.61	21.57-41.65	23.9	
N3	36	15.39	10.83-19.95	0.0	
Vascular invasion					
No	168	60.36	55.48-65.23	65.4	< 0.0001
Yes	26	17.74	11.39-24.08	4.4	
Lymphatic invasion					
No	104	70.58	65.84-75.33	79.2	< 0.0001
Yes	90	36.48	29.43-43.53	32.1	
Perineural invasion					
No	109	67.57	62.27-72.88	76.3	< 0.0001
Yes	84	37.36	30.70-44.02	34.1	
Desmoplastic reaction					
None	28	67.55	56.87-78.24	78.6	< 0.0001
Mild	64	70.20	64.03-76.37	78.7	
Moderate	75	41.99	34.35-49.62	38.8	
Marked	27	36.67	24.49-48.85	36.7	
TNM stage					
I	64	78.86	75.66-82.07	92.1	< 0.0001
II	44	66.55	58.17-74.92	74.2	
III	38	46.52	36.06-56.98	45.7	
IV	48	14.71	10.94-18.48	0.0	
CCL-7					
Higher	82	45.62	37.97-53.28	43.6	0.002
Equal	69	60.09	52.62-67.57	64.7	
Lower	43	63.28	54.24-72.32	71.8	
CCL-8					
Higher	58	51.05	42.41-59.70	50.0	0.269
Equal	64	51.89	43.39-60.39	57.4	
Lower	72	59.97	52.60-67.34	64.6	
CCL-21					
Higher	87	44.96	37.83-52.10	45.8	0.001
Equal	63	56.60	48.30-64.90	59.5	
Lower	44	70.58	63.24-77.93	79.2	

CCL: Chemokine ligand; TNM: Tumor node metastasis.

**Table 3** Cox's proportional hazards analysis

Factors	Hazard ratio	95% CI upper-lower	P value
Depth of invasion			
T2/T1	7.850	1.454-42.390	0.017
T3/T1	23.200	4.733-113.716	0.000
T4/T1	65.052	10.830-390.730	< 0.0001
Lymph node metastasis			
N1/N0	1.856	0.865-3.982	0.112
N2/N0	3.520	1.597-7.758	0.002
N3/N0	7.227	3.349-15.596	< 0.0001
Desmoplastic reaction			
Mild/none	3.663	1.272-10.638	0.016
Moderate/none	3.623	1.304-10.101	0.014
Marked/none	4.926	1.590-15.152	0.006
CCL-7			0.801
CCL-8			0.620
CCL-21			0.084

CCL: Chemokine ligand.

erty that may allow them to access the lymphatic system and spread to regional lymph nodes<sup>[18]</sup>. Thus the “chemoattraction” theory of metastasis may be reflected by malignant cells expressing functional chemokine receptors that can respond to organ-specific chemoattractant molecules and migrate directionally along chemokine gradients to set up site-specific metastases in the target organs. Such chemotactic migration of tumors would mirror the physiologic mechanisms of lymphocyte homing into lymphoid organs.

Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis. If tumor-infiltrating leukocytes are able, in some instances, to promote cancer, then the local production of chemokines that attract leukocytes could be a poor prognostic sign. This is the case in human breast cancer, where levels of CCL5 and CCL2 correlate with tumor progression and there is a positive correlation between the extent of the macrophage infiltrate, lymph-node metastasis and clinical aggressiveness<sup>[19-21]</sup>. In esophageal squamous cell carcinoma, CCL2 expression has been associated with the extent of macrophage infiltration, tumor cell invasion and tumor vascularity<sup>[22]</sup>.

In conclusion, the higher expression of CCL7 and CCL21 in gastric cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher TNM stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis. These results suggest that overexpression of these two CC chemokine ligands is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

## COMMENTS

### Background

Gastric cancer is one of the commonest malignant tumors of the alimentary tract and is characterized by late clinical presentation, rapid progression, and

poor survival. The reason for this poor prognosis is that, at the time of diagnosis, gastric cancer usually shows extensive local tumor invasion and frequent spread to metastatic sites, particularly lymph nodes. Spread of malignant tumors is a multistep process and many of the stages of tumor invasion require degradation or breakdown of the extracellular matrix and connective tissue surrounding tumor cells.

### Research frontiers

The matrix metalloproteinases (MMPs) are a family of zinc containing enzymes which are involved in the degradation of different components of the extracellular matrix, and there is considerable evidence to indicate that individual MMPs have important roles in tumor invasion and tumor spread. A recent study showed that increased levels of chemokine ligand (CCL) recruit immature myeloid cells that carry chemokine ligand receptor from the blood to the tumor invasion front. These immature myeloid cells produced MMP9 and MMP2 and help the tumor cells to migrate and invade.

### Innovations and breakthroughs

In the present study, the authors used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. The authors analyzed their correlation with tumor metastasis, clinicopathologic parameters and clinical outcome. They found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher tumor node metastasis (TNM) stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis.

### Applications

These results suggest that overexpression of CCL7 and CCL21 is associated with tumor metastasis and serves as a prognostic factor in patients with gastric cancer.

### Peer review

The authors used immunohistochemistry to examine the expression of CCL7, CCL8 and CCL21 in 194 gastric cancer samples and adjacent normal tissues. They found that the higher expression of CCL7 and CCL21 in cancer tissues than in normal tissues was significantly correlated with advanced depth of wall invasion, lymph node metastasis and higher TNM stage. Moreover, Kaplan-Meier survival analysis revealed that CCL7 and CCL21 overexpression in cancer tissues was correlated with poor prognosis.

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## Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases

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### Abstract

**AIM:** To investigate the non-*Helicobacter pylori* (*H. pylori*) bacterial flora concurrent with *H. pylori* infection.

**METHODS:** A total of 103 gastric biopsy specimens from *H. pylori* positive patients were selected for bacterial culture. All the non-*H. pylori* bacterial isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

**RESULTS:** A total of 201 non-*H. pylori* bacterial isolates were cultivated from 67 (65.0%) of the 103 gastric samples, including 153 isolates identified successfully at species level and 48 at genus level by MALDI-TOF MS. The dominant species were *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, which differed from

the predominantly acid resistant species reported previously in healthy volunteers. The prevalence of non-*H. pylori* bacteria was higher in non-ulcer dyspepsia group than in gastric ulcer group (100% vs 42.9%,  $P < 0.001$ ). Six bacterial species with urease activity (*Staphylococcus epidermidis*, *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus aureus*, *Brevibacterium spp.* and *Klebsiella pneumoniae*) were also isolated.

**CONCLUSION:** There is a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* infection, and the non-*H. pylori* bacteria may also play important as-yet-undiscovered roles in the pathogenesis of stomach disorders.

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**Key words:** Non-*Helicobacter pylori*; Bacterial flora; Gastrointestinal diseases; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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## INTRODUCTION

Stomach is generally regarded as an environment that is not conducive to bacterial colonization. A notable exception is *Helicobacter pylori* (*H. pylori*), which may cause chronic gastritis, peptic ulcer and is correlated with gastric adenocarcinoma<sup>[1,2]</sup>. However, gastritis also occurs in *H. pylori* negative human subjects or mouse models, suggesting that *H. pylori* is just one of the organisms causing gastritis.

With the reduced level of acid induced by *H. pylori* dissipates or antiulcer medications, the stomach becomes more susceptible to the colonization of other organisms<sup>[3]</sup>, which may complicate the development of gastric disorders. In recent years, considerable interest has emerged in the interactions among *H. pylori*, non-*H. pylori* bacteria and acid-suppressive therapy. Some studies have shown that the co-infection of *H. pylori* and non-*H. pylori* bacteria enhances the development of atrophic corpus gastritis<sup>[4]</sup>. But up till now, there are only a limited number of studies regarding the stomach bacterial flora and all are based on a small size of a few patients or volunteers. The aim of this study was to investigate the non-*H. pylori* bacterial flora concurrent with *H. pylori* infection.

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a new technology for bacterial identification<sup>[5]</sup>. Its performance has been evaluated in different studies, and a higher accuracy was obtained compared with phenotypic methods<sup>[6,7]</sup>.

## MATERIALS AND METHODS

### Patients and materials

A total of 104 patients, who underwent upper gastrointestinal endoscopy because of dyspeptic symptoms and yield positive results in rapid urease test (RUT), were selected for the further *H. pylori* detection and bacterial examinations. Among the 104 patients, 103 were *H. pylori* positive and ultimately entered into this study, including 63 from Jiangxi Province and 40 from Beijing, China. No subject had received anti-secretory drugs, antibiotics, or probiotics two wk prior to entry into this study and no one had *H. pylori* eradication history. Diseases included gastritis ( $n = 23$ ), gastric ulcer ( $n = 21$ ), duodenal ulcer ( $n = 42$ ), reflux esophagitis ( $n = 4$ ), and non-ulcer dyspepsia (NUD) ( $n = 13$ ). There were 81 (78.6%) patients aged less than 50 years. The study was reviewed and approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention. Each subject gave informed oral consent before entering into the study.

Upper gastrointestinal endoscopy was performed after an overnight fast in all patients. Mucosal samples were taken from the greater curvature of gastric antrum and corpus using sterile disposable biopsy forceps. All the samples were placed into sterile brain-heart infusion broth for transport to the cultural laboratory.

### Bacterial culture and polymerase chain reaction detection for *H. pylori*

After biopsies were taken, the samples were dispersed using a homogenizer. Each homogenate was inoculated onto three plates, two Columbia blood agars (CM0331, OXOID) and a Campylobacter agar (CM0935, OXOID) supplemented with 5% sheep blood, polymyxin B, vancomycin, trimethoprim, and amphotericin B. One of the Columbia blood agar was incubated under aerobic condition at 37 °C, the other one and the Campylobacter agar were incubated under microaerophilic condition at 37 °C.

The *H. pylori* status was assessed with culture and polymerase chain reaction (PCR) detection. Small (0.5 to 2 mm) translucent colonies were selected to gram-stain and tested for urease, catalase and oxidase activity. Microscopy of gram-stained smears with curved gram-negative rods resembling *Helicobacter*, together with positive in all the tree enzyme activity tests were identified as *H. pylori*.

*H. pylori* culture-negative samples were further detected by *ureA*, *vacA* and *cagA* PCR analysis, using the primers described previously<sup>[8,9]</sup>. *H. pylori* status was defined as negative only if all the three PCR detections were negative.

### Mass spectrometry identification of non-*H. pylori* bacteria

**Preparation of samples:** Appropriate amount (5-10 mg) colony was scraped by inoculating loop and suspended in 300  $\mu$ L distilled water, and 900  $\mu$ L ethanol was added and mixed. Then the sample was centrifuged at 12 000 r/min for 2 min, and the pellets were dried. Fifty  $\mu$ L formic acid (70% in water) was added to the dried bacterial pellet and mixed thoroughly, and then added with 50  $\mu$ L acetonitrile. After centrifugation at 12 000 r/min for 2 min, 1  $\mu$ L of the supernatant containing the bacterial extract was transferred onto the MSP 96 target ground steel plate (Bruker Daltonics, Bremen, Germany) and dried, then 1  $\mu$ L of matrix solution (saturated solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile + 2.5% trifluoroacetic acid) was added and crystallized by air-drying at room temperature. Each isolate was analyzed on the day of isolation.

**Measurement with spectrometer:** Measurement was performed with Microflex LT mass spectrometer (Bruker Daltonics) equipped with a 200 Hz smart-beam laser. The parameter settings were as follows: delay 320ns; ion source 20 kV; ion source 18.5 kV; lens voltage 8.5 kV; and mass range 2-15 kDa. Each run was validated with an *E. coli* control sample where the presence of 10 specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analyzed by MALDI Biotyper 2.0 software (Bruker Daltonics) using the default settings. A list of peaks up to 100 was generated. The threshold for peak acceptance was a signal-to-noise (S/N) ratio of 3. After alignment, peaks with a mass-to-charge ( $m/z$ ) ratio difference of less than 250 ppm were considered to be identical. The peak lists generated were used for matches against the reference library, by directly

**Table 1** Non-*Helicobacter pylori* bacterial cultures of gastric biopsies

Micro-organism	Culture condition <sup>1</sup>			Colonized patients (n) <sup>2</sup>	Urease activity <sup>3</sup>	Gram stain
	I	II	III			
Streptococcus				(54)		G+
<i>Streptococcus pneumoniae</i>	3	10		11		
<i>Streptococcus salivarius</i>	10	9		16		
<i>Streptococcus anginosus</i>		3		3		
<i>Streptococcus oralis</i>		2		2		
<i>Streptococcus cristatus</i>	1			1		
<i>Streptococcus gordonii</i>	2			2		
<i>Streptococcus vestibularis</i>	1			1		
<i>Streptococcus spp.</i>	10	13		18		
Neisseria				(32)		G-
<i>Neisseria flavescens</i>	11	16	11	23		
<i>Neisseria perflava</i>	2	1	1	3		
<i>Neisseria macacae</i>		1		1		
<i>Neisseria sicca</i>	1			1		
<i>Neisseria spp.</i>	3	1		4		
Rothia				(29)		G+
<i>Rothia mucilaginosa</i>	6	10		15		
<i>Rothia dentocariosa</i>		3	1	4		
<i>Rothia aerea</i>	1			1		
<i>Rothia spp.</i>	4	7		9		
Staphylococcus				(21)		G+
<i>Staphylococcus epidermidis</i>	3	3		5	5	
<i>Staphylococcus aureus</i>	3	4		5	3	
<i>Staphylococcus capitis</i>		3		3	1	
<i>Staphylococcus warneri</i>	2	1		3	3	
<i>Staphylococcus cohnii</i>		2		2		
<i>Staphylococcus haemolyticus</i>		1		1		
<i>Staphylococcus spp.</i>		2		2		
Lactobacillus				(9)		G+
<i>Lactobacillus salivarius</i>			4	4		
<i>Lactobacillus oris</i>			2	2		
<i>Lactobacillus fermentum</i>			1	1		
<i>Lactobacillus spp.</i>		1	1	2		
Corynebacterium				(4)		G+
<i>Corynebacterium argentoratense</i>	2	1		3		
<i>Corynebacterium propinquum</i>	1	1		1		
Kingella				(3)		G-
<i>Kingella denitrificans</i>			2	2		
<i>Kingella spp.</i>			1	1		
Others						
<i>Capnocytophaga ochracea</i>			3	3		G-
<i>Haemophilus parainfluenzae</i>		1	1	2		G-
<i>Acinetobacter lwoffii</i>	2			2		G-
<i>Klebsiella pneumoniae</i>	1			1	1	G-
<i>Cardiobacterium sp</i>			1	1		G-
<i>Actinomyces spp.</i>		2		2		G+
<i>Micrococcus luteus</i>	1			1		G+
<i>Weissella confusa</i>			1	1		G+
<i>Aerococcus spp.</i>	1			1		G+
<i>Bacillus spp.</i>	1			1		G+
<i>Brevibacterium spp.</i>		1		1	1	G+
Total	72	99	30	168		

<sup>1</sup>The number of bacterial strains isolated in I: Columbia blood agar incubated under aerobic condition; II: Columbia blood agar incubated under microaerophilic condition; III: Campylobacter agar under microaerophilic condition; <sup>2</sup>The number of patients colonized by the same genus is shown in parentheses; <sup>3</sup>The number of bacterial isolates with urease activity.

using the integrated pattern matching algorithm of the software. All parameters were the same regardless of the bacteria analyzed. Spectra were obtained in the positive linear mode after 500 shots. A score was attributed to each identification. When this score was > 2.00, the identification was considered correct at the species level; between 1.7 and 1.999, the identification was considered correct at the genus level; and < 1.7, the identification was not similar enough to draw a conclusion.

#### Urease activity test of non-*H. pylori* bacteria

Each non-*H. pylori* bacterial culture was subjected to urease test. The medium used for the test was urea broth containing dipotassium hydrogen phosphate, 9.5 g/L; potassium dihydrogen phosphate, 9.1 g/L; urea 20 g/L; phenol red, 0.01 g/L; and yeast extract, 0.1 g/L, final pH 6.8. Any change in the indicator from pale yellow to pink in 24 h was taken as positive.

#### Statistical analysis

Continuous data were described with mean (minimum, maximum), categorical data with number and proportions. Difference between the groups for age and sex was analyzed using analysis of variance and  $\chi^2$  test. Fisher exact test was conducted to compare non-*H. pylori* prevalence data. *Post hoc* tests were conducted by the Bonferroni method.  $P < 0.05$  was considered as statistically significant. All analyses were performed with SPSS 12.0 software.

## RESULTS

A total of 92 (88.5%) samples were *H. pylori* culture positive. Only one of the 12 culture-negative cases was PCR negative. As result, 103 *H. pylori* positive patients were eventually enrolled into the analysis.

#### Non-*H. pylori* bacteria isolation

A total of 201 non-*H. pylori* bacterial isolates were cultivated from gastric samples of 67 (65.0%) of the 103 *H. pylori* positive patients. All the isolates were identified by mass spectrometry, including 153 identified at species level and 48 at genus level (Table 1). Some species isolated from more than two culture plates were shown in Table 1. A total of 168 isolates was obtained with the exception of the repeat identification of the same patient, and 39 patients harbored more than 2 non-*H. pylori* species. Overall, a total of 18 non-*H. pylori* bacterial genera (43 species) were isolated from the stomach biopsy specimen (Table 1), most of them were gram-positive bacteria (73.8%). The dominant bacterial species were *Neisseria flavescens* (13.7%), *Streptococcus salivarius* (9.5%), *Rothia mucilaginosa* (8.9%) and *Streptococcus pneumoniae* (6.6%). Twelve gram-negative bacilli isolates (7.14%) belonged to 6 genera obtained in this study, which were discovered from 10 patients (9.7% of all the patients and 14.9% of the non-*H. pylori* positive patients). Concurrent colonization by gram-positive and/or gram-negative

**Table 2** Demographic, non-*Helicobacter pylori* bacterial colonization and clinical characteristics of 103 patients

Diseases	Enrolled patients (n)	Age (yr) <sup>1</sup>	Gender (male:female)	Colonized patients
Duodenal ulcer	42	39 (20-56)	2:1	28 (66.7%)
Gastric ulcer	21	42 (20-62)	0.75:1	9 (42.9%)
Gastritis	23	39 (10-75)	0.44:1	13 (56.5%)
Non-ulcer dyspepsia	13	34 (22-57)	0.63:1	13 (100%) <sup>a</sup>
Reflux esophagitis	4	52 (41-63)	1:1	4 (100%)
Total	103	39 (10-75)	0.96:1	67 (65.0%)
P value		0.079 <sup>2</sup>	0.074 <sup>3</sup>	0.0028 <sup>4</sup>

<sup>1</sup>Data are expressed as median (range); <sup>2</sup>By analysis of variance; <sup>3</sup>By  $\chi^2$  test; <sup>4</sup>By Fisher exact test; <sup>a</sup> $P = 0.00006$  vs gastric ulcer group by Bonferroni method.

coccus occurs in 10 of them.

The demographic and relevant clinical characters of the patients are described in Table 2. The patients were subdivided into five groups based on the clinical diseases. There were no significant intergroup differences in age and sex ratio, but the prevalence of non-*H. pylori* flora was significantly different ( $P = 0.0028$ ) among different clinical diseases, as shown in Table 2. *Post hoc* tests were conducted and statistical difference was found between the gastric ulcer group (42.9%) and the NUD group (100%) ( $P = 0.00006$ ). In addition, significant difference was also presented between the 50-plus age group and the younger age (< 50 years old) group (45.5% vs 69.1%,  $\chi^2$  test,  $P = 0.048$ ).

### Urease activity of non-*H. pylori* isolates

All the 201 non-*H. pylori* isolates were screened for urease activity, 14 isolates belonged to 6 species (*S. epidermidis*, *S. warneri*, *S. capitis*, *S. aureus*, *Brevibacterium spp.* and *K. pneumoniae*) and were urease positive (Table 1), which could benefit in the bacterial overgrowth in the stomach. Interestingly, not all the isolates of the 6 species were urease-positive except *S. warneri*. Especially in a 26-year-old patient with non-ulcer dyspepsia, two *Staphylococcus epidermidis* isolates were isolated under aerobic and microaerobic conditions, respectively, but only the microaerobic isolate was urease-positive.

## DISCUSSION

It has been suggested that non-*H. pylori* bacteria and their by-products may act as a persistent antigenic stimulus, and thereby augment the inflammatory response induced by the *H. pylori* infection<sup>[4]</sup>. The current study documented a high prevalence (65.0%) of the non-*H. pylori* bacterial flora in the *H. pylori* positive patients, which indicate that the upper gastrointestinal disorders may enable non-*Helicobacter* bacteria to survive and colonize in the human stomach. Further studies should be done to elucidate the role of the co-infection of the stomach with *H. pylori* and non-*H. pylori* bacteria in the pathogenesis process.

Most of the non-*H. pylori* bacteria isolated in this

study were upper respiratory tract microflora, which was in agreement with other researches<sup>[10]</sup>. The major species were *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, that was different from the previous reports of healthy volunteers, which was predominantly acid resistant species - *Veillonella sp.*, *Lactobacillus sp.*, and *Clostridium sp.*<sup>[11]</sup>.

Gram-negative bacilli are uncommon in the upper gut of healthy individuals, but in patient populations with gastric hypochlorhydria, gram-negative bacilli are recovered in a minor proportion<sup>[12,13]</sup>. Gram-negative bacilli were discovered in 9.7% of the patients in this study. Concurrent colonization by gram-positive and/or gram-negative coccus occurs in 10 of the 12 gram-negative bacilli. *Acinetobacter lwoffii* was the only gram-negative bacilli species that was found as the only non-*H. pylori* bacteria in the patients. The two *A. lwoffii* carriers were a 42-year-old male patient with duodenal ulcer and a 25-year-old female patient with non-ulcer dyspepsia. *A. lwoffii* has been proved to cause the same histologic gastritis as *H. pylori* infection in a murine model<sup>[14]</sup>. And outer membrane protein (OmpA-like protein) of *A. lwoffii* was reported to activate interleukin 8 and gastrin promoter activity *in vitro*<sup>[15]</sup>. Moreover, a case of *A. lwoffii* bacteremia associated with acute gastroenteritis was reported recently<sup>[16]</sup>, which stimulate more interest in the contribution of *A. lwoffii* to gastrointestinal diseases.

It has been well known that gastric acid secretion declines with age which may increase the sensitivity of the stomach to the bacterial colonization. Surprisingly, a higher non-*H. pylori* prevalence was found in the younger age group (less than 50 years old,  $P = 0.048$ ). The result must be interpreted with caution.

The pathogenesis of NUD was not well understood and a number of hypotheses have been proposed. The role of *H. pylori* infection in NUD remains controversial too<sup>[17]</sup>. A high prevalence of non-*H. pylori* bacteria in NUD group observed in this study gave us a new clue that non-*H. pylori* bacteria may play a role in the pathogenesis of NUD.

Urease is a major virulent factor for *H. pylori*, which aids in neutralizing hydrochloric acid and allows *H. pylori* to colonize the gastric mucosa. Some rapid methods for *H. pylori* diagnosis, <sup>13</sup>C urea breath test (UBT) and RUT, were also based on the urease activity. But *H. pylori* is not the only bacteria which can produce urease. It has been reported that the colonization and overgrowth of urease-producing bacteria other than *H. pylori* induced false-positive UBT results<sup>[18,19]</sup>. All the 104 patients were RUT-positive, but one of them was *H. pylori*-negative based on the further detection and no urease-positive non-*H. pylori* bacteria was isolated. So role of the urease-positive non-*H. pylori* bacteria to cause false-positive results in RUT was not definite in this study.

Overall, a high prevalence of non-*H. pylori* bacteria obtained in this study, the major of which were *Streptococcus*, *Neisseria*, *Rothia* and *Staphylococcus*, differed from the previous report of healthy volunteers. It should be noted that this study only examined cultured bacteria. But not all flora can be cultivated due to the harsh culture condi-

tion, so the prevalence of the non-*H. pylori* bacteria may underestimate here. The remained question is whether these non-*H. pylori* species contribute to the pathogenesis of gastric disorders. Further studies should be done to elucidate it in the future.

## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) infection has been known to trigger a variety of gastric disorders. Recent years, non-*H. pylori* bacteria have also been reported to play roles in the development of upper gastrointestinal disease, but little is known about the stomach bacterial flora. Analysis of stomach bacterial flora was performed in order to stimulate future research in this area.

### Research frontiers

The pathogenesis of gastric disorders is complicated. Besides *H. pylori*, considerable interest has emerged in the co-infection in the stomach with non-*H. pylori* bacteria.

### Innovations and breakthroughs

To date, there has only a limited number of studies regarding the stomach bacterial flora and all are based on a few patients or volunteers. In this study, the authors enrolled 103 *H. pylori* positive patients with upper gastrointestinal diseases and employed forefront matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) bacterial identification technique, which is more accurate and rapid than the routine methods or genotypic identification. Furthermore, the authors confirmed a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* in the patients.

### Applications

A high prevalence of the non-*H. pylori* bacteria and the evaluation of the bacterial species found in gastric tissues from *H. pylori* infected patients would stimulate future research to elucidate the role of the mixed infection of the stomach in the pathogenesis of the upper gastrointestinal diseases.

### Terminology

MALDI-TOF MS fingerprinting is a fast and reliable method for the classification and identification of micro-organisms. The BioTyper™ MALDI-TOF MS fingerprinting system allows researchers to perform this process for the unambiguous identification of bacteria, yeasts and fungi in minutes by measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each species.

### Peer review

The study provided an interesting evaluation of the bacterial species found in gastric tissues from *H. pylori* infected persons.

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## Gastric cancer incidence and mortality in Zhuanghe, China, between 2005 and 2010

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### Abstract

**AIM:** To investigate the incidence and mortality of gastric cancer (GC) in Zhuanghe region, northeast China and the influencing factors for their changing trends.

**METHODS:** All new cancer cases and deaths registered from 2005 to 2010 in Zhuanghe County were reviewed. The annual GC cases, constituent ratio, crude rates,

age-standardized rates, their sex and age distribution and temporal trends were assessed. The method of annual percentage change (APC) was used to estimate the trends of GC.

**RESULTS:** Altogether 2634 new cases of GC and 1722 related deaths were registered, which accounted for 21.04% and 19.13% of all cancer-related incidence and deaths, respectively. The age-standardized incidence rate steadily decreased from 57.48 in 2005 to 44.53 in 2010 per 10<sup>5</sup> males, and from 18.13 to 14.70 per 10<sup>5</sup> females, resulting in a APC of -5.81% for males and -2.89% for females over the entire period. The magnitude of APC in GC mortality amounted to -11.09% and -15.23%, respectively, as the age-standardized mortality rate steadily decreased from 42.08 in 2005 to 23.71 in 2010 per 10<sup>5</sup> males, and from 23.86 to 10.78 per 10<sup>5</sup> females. Females had a significantly lower incidence (a male/female ratio 2.80,  $P < 0.001$ ) and mortality (a male/female ratio 2.30,  $P < 0.001$ ). In both genders, the peak incidence and mortality occurred in the 80-84 years age group. The age-standardized mortality/incidence ratio also decreased from the peak of 0.73 in 2005 to 0.53 in 2010 for males, and from 1.32 to 0.73 for females.

**CONCLUSION:** Encouraging declines of incidence and mortality of GC were observed in Zhuanghe region between 2005 and 2010, possibly due to the economic development and efficient GC control strategies.

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**Key words:** Gastric cancer; Incidence; Mortality; Trend; High-risk areas

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## INTRODUCTION

Gastric cancer (GC) is one of the most frequently occurring cancers globally; a total of 989 600 new GC cases and 738 000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total cancer-related deaths, respectively<sup>[1]</sup>. The geographical distribution of GC exhibits wide international variation and over 70% of new cases and deaths occur in developing countries<sup>[1,2]</sup>. Although a notable decreasing trend has been recently observed in the developed countries, the incidence of GC is still high<sup>[3]</sup> and it remains an important public health burden in China. Moreover, there is a significant regional disparity in the distribution of GC in China. People in rural areas are supposed to have a higher risk of GC than those in cities<sup>[4]</sup>, thus, strategy of GC control in China should be implemented specifically based on the different distribution patterns.

Zhuanghe is a county of northern China with a total population of approximate 900 000, of whom approximately 87.2% are agricultural residents. It is located along the coast of the Huanghai Sea and occupies an area of approximately 4034 km<sup>2</sup><sup>[5]</sup>. Zhuanghe has been recognized for its high GC mortality since 1984, the mortality rate being 49.55/10<sup>5</sup> in males and 22.23/10<sup>5</sup> in females<sup>[6]</sup>. The high risk of GC in this region aroused the interest of researchers, and a series of investigations regarding the potential risk factors and corresponding etiological intervention methods and screening measures were conducted in representative villages<sup>[7]</sup>. To date, the population in Zhuanghe County has been surveyed through census for 28 years. Various risk factors, such as salted pork, *Helicobacter pylori* (*H. pylori*) infection with a specific strain, and genetic polymorphisms in the etiology of GC in local residents have been found to play a role<sup>[8-12]</sup>. In addition, several pilot surveys regarding incidence and mortality were also conducted. The GC mortality rates fluctuated between 63.29/10<sup>5</sup> and 38.98/10<sup>5</sup> during the period of 1996-2003 and the incidence rate was 35.42-41.03/10<sup>5</sup> from 1998 to 2004<sup>[7,13,14]</sup>.

Despite some epidemiological surveys in pilot areas, a population-based investigation concerning the incidence and mortality of GC in Zhuanghe has not been previously reported. Monitoring and studying the incidence as well as mortality provides important information, enabling effective assessment of potential cancer prevention and control. Therefore, the aim of the present study is to examine the GC patterns and temporal trends of incidence and mortality at the population level in Zhuanghe region and to elucidate the factors that

influence the changing trends, in an attempt to establish future general and specific strategies for the prevention and control of cancers.

## MATERIALS AND METHODS

### Cancer registry

The preliminary cancer surveillance was conducted in Zhuanghe as a high-risk GC research base in 1984. However, previous cancer data were obtained from limited populations and sample surveys. Over the past two decades, a cancer registry was developed along with an expanding research scope, with coverage of only several villages initially in the 1980s which increased to 70 villages in the 1990s. Since 2001, a population-based cancer registry has been gradually established, in collaboration with the Cancer Registration Office of Liaoning Province, China that has collected information on all deaths and all cancer cases in Zhuanghe County and has functioned well since 2005.

The framework of this registry consisted of three parts: village clinics that served as basic units, rural administration units, and Center of Disease Prevention and Control (CDC) of Zhuanghe County. Village clinic doctors were required to report each new case of cancer using a standard card to the rural administration unit. The cards were then submitted to the CDC of Zhuanghe, registered and processed on a regular basis. These cards were checked, analyzed, coded and stored at the CDC. All data were coded according to the manual "National Criteria of Cancer Registry in China", which describes the cancer inclusion and exclusion criteria, and provides definitions and coding for cancer cases. The accuracy of the data was assured through the comprehensive training of doctors and registrars by the Cancer Registration Office of Liaoning Province, and by computerized consistency checks. At the end of each year, a sample survey was conducted to check the quality of the registration. The cancer occurrence data were compared one by one with the death registry database data to supplement cases of cancer death which were not registered for cancer occurrence.

### Data collection

Annual GC cases data were provided by the CDC of Zhuanghe County. All data were coded and checked for eligibility and validity prior to analysis. Data collected on each patient included demographics and native origins, primary cancer site/type, morphology, and pathological diagnosis confirmation, stage of the disease, and active patient follow-up. The 10th revision of International Classification of Diseases (ICD-10) was used for cancer classification. Data concerning age and sex in the Zhuanghe population were provided by the local police station.

### Statistical analysis

All of the data were checked and analyzed by SPSS 13.0 software (SPSS, Chicago, Illinois, United States). Incidence and mortality rates were calculated as the mean annual number per 100 000 residents. Age-standardized

Table 1 The top ten malignant tumors for incidence in Zhuanghe area, 2005-2010

Rank	Total				Males				Females			
	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR%	Cancer	ICD-10	Cases	CR %
1	LC	C33-34	2673	21.35	GC	C16	1950	26.41	LC	C33-34	1068	20.80
2	GC	C16	2634	21.04	LC	C33-34	1605	21.74	GC	C16	684	13.32
3	HCC	C22	1488	11.89	HCC	C22	1093	14.80	BC	C50	617	12.02
4	CRC	C18-21	1211	9.67	CRC	C18-21	719	9.74	CRC	C18-21	492	9.58
5	BC	C50	636	5.08	EC	C15	322	4.36	TC	C73	234	4.56
6	EC	C15	364	2.91	BLC	C67	246	3.33	CC	C53	212	4.13
7	TC	C73	317	2.53	PC	C25	151	2.05	EMC	C54	135	2.63
8	BLC	C67	286	2.28	CNC	C70-72	135	1.83	CNC	C70-72	111	2.16
9	PC	C25	253	2.02	PRC	C61	85	1.15	OC	C56	105	2.05
10	CNC	C70-72	246	1.97	KC	C64	83	1.12	PC	C25	102	1.99
	Total		10108	80.74	Total		6389	86.53	Total		3760	73.24

CR: Constituent ratio; ICD-10: The 10th revision of International Classification of Diseases; GC: Gastric cancer; LC: Lung cancer; HCC: Liver cancer; CRC: Colon/rectum cancer; BC: Breast cancer; EC: Esophageal cancer; TC: Thyroid cancer; BLC: Bladder cancer; PC: Pancreatic cancer; CNC: Cancers of brain and nervous system; PRC: Prostate cancer; KC: Renal cancer; CC: Cervical cancer; EMC: Endometrial carcinoma; OC: Ovarian cancer.

incidence rates (ASRs) and age-standardized mortality rates (ASMRs) were calculated using a direct method<sup>[15]</sup> by weighting age-specific incidence and mortality rates in accordance with the World Standard Population (World Health Organization, published in 2000) and the China Standard Population (2000). The Chi-squared test was used to determine whether the differences between sexes were statistically significant, and the test was also used to examine trends of annual change in crude rates.

The average annual percent change (APC)<sup>[16,17]</sup> was estimated by fitting a regression line to the natural logarithm of the rates using the calendar year as the regression variable, i.e.,  $y = mx + b$  in which  $y = \ln(\text{rate})$  and  $x = \text{calendar year}$ . The estimated APC =  $100(e^m - 1)$  and 95% CI of APC =  $100(e^{m \pm SE_m} - 1)$ , where the standard error of  $m$  (SEM) was obtained according to the fit of the regression line. To test the hypothesis that the APC equals zero is equivalent to the hypothesis that the slope of the regression line is zero, the  $t$ -distribution of  $m/SEM$  was used. The number of degrees of freedom is equivalent to the number of calendar years minus two. This calculation assumes that the rate increased/decreased at a constant rate over the entire period. Statistical significance was assessed by the two-sided Student's  $t$  test, and  $P < 0.05$  was considered statistically significant.

### Ethical consideration

Ethical approval for this study was obtained from the Human Ethics Review Committee of China Medical University (Shenyang, China). Written informed consents were obtained from the participants in accordance with the Declaration of Helsinki and its later revisions.

## RESULTS

### Incidence

From 2005 to 2010, a total of 2634 new cases of GC were registered in Zhuanghe, including 1950 males and 684 females. We analyzed the constituent ratios for incidence according to various types of cancer (Table 1).

GC ranked first among all types of cancers in males, whereas it ranked the second among females and both sexes combined. The ten most common types of cancer in the Zhuanghe population were lung, stomach, liver, colon/rectum, breast, esophageal, thyroid, bladder, pancreatic cancers, and cancer of brain and nervous system. These cancers comprised 80.74% of all cases of cancer. The five predominant primary cancer sites in males were stomach, lung, liver, and esophagus; whereas in females, the leading sites were lung, stomach, breast, colon/rectum, and thyroid.

The incidence rate of GC occurring over different years was analyzed. We initially calculated the annual crude incidence rate according to sex. The average crude incidence rate of GC in males was  $70.23/10^5$ , which was 2.80-fold higher than in females ( $25.12/10^5$ ). The difference was statistically significant ( $\chi^2 = 584.31$ ,  $P < 0.001$ ). Over the past 6 years, no obvious increasing or decreasing trend was observed concerning crude incidence ( $P = 0.404$  in males,  $0.061$  in females and  $0.101$  in both sexes) (Table 2).

To adjust for the effect of age differences over different time periods, age-standardized incidence rates were calculated in accordance with the China Standard Population (ASR1) and World Standard Population (ASR2). As shown in Figure 1, over the past 6 years, the incidence rate of GC has gradually decreased. For both genders, the ASR2 decreased from 37.67 to 29.83 per  $10^5$  with an APC of  $-4.82\%$  (95% CI:  $-3.58\%$  to  $-6.04\%$ ;  $P = 0.019$ ) and for males the rate decreased from 57.48 to 44.53 per  $10^5$  with an APC of  $-5.81\%$  (95% CI:  $-4.67\%$  to  $-6.94\%$ ;  $P = 0.008$ ). The ASR2 decreased from 18.13 to 14.70 per  $10^5$  with an APC of  $-2.89\%$  (95% CI:  $0.06\%$  to  $-5.75\%$ ) in females; however, the change had no statistical significance ( $P = 0.383$ ) (Table 2).

In both genders, GC was very rare among populations younger than 20 years, but the rate rose sharply to a peak at approximately 80 years of age, and it declined afterwards. The median age of GC patients was 67 years and the average age was 65.78 years. The cases above 50

**Table 2** Time trends for incidence rates of gastric cancer in Zhuanghe population, 2005-2010 (/100 000)

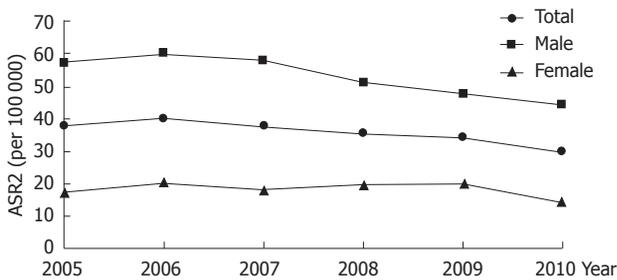
Year	Total				Males				Females			
	Cases	Crude	ASR1	ASR2	Cases	Crude	ASR1	ASR2	Cases	Crude	ASR1	ASR2
2005	409	44.74	33.41	37.67	312	67.51	47.92	57.48	97	21.46	16.97	18.13
2006	437	47.62	34.89	40.12	323	69.63	48.01	60.06	114	25.12	19.30	20.66
2007	411	44.60	32.88	37.77	314	67.42	46.89	57.86	97	21.28	16.68	18.17
2008	474	51.36	31.65	35.30	346	74.26	42.69	51.05	128	28.01	18.53	19.60
2009	476	51.98	30.55	34.04	337	72.97	39.58	47.66	139	30.63	19.53	20.30
2010	427	47.03	26.84	29.83	318	69.55	38.46	44.53	109	24.18	13.67	14.70
Total	2634	47.89	31.25	35.10	1950	70.23	43.28	51.95	684	25.12	17.17	18.26
$\chi^2$ trend test	$\chi^2 = 2.693, P = 0.101$				$\chi^2 = 0.696, P = 0.404$				$\chi^2 = 3.519, P = 0.061$			
APC	APC = -4.82%, $P = 0.019$				APC = -5.81%, $P = 0.008$				APC = -2.89%, $P = 0.383$			

Crude: The crude incidence rate; ASR1: The age standardized incidence of Chinese population in 2000; ASR2: The age standardized incidence of the world population in 2000; APC: Average annual percent change of incidence rates.

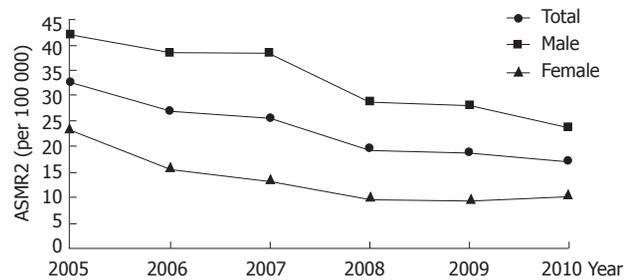
**Table 3** The top ten malignant tumors for mortality in Zhuanghe area, 2005-2010

Rank	Total				Males				Females			
	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR %	Cancer	ICD-10	Cases	CR%
1	LC	C33-34	2683	29.81	LC	C33-34	1560	27.89	LC	C33-34	1123	32.95
2	GC	C16	1722	19.13	GC	C16	1207	21.58	GC	C16	515	15.11
3	HCC	C22	1623	18.03	HCC	C22	1180	21.10	HCC	C22	443	13.00
4	CRC	C18-21	544	6.04	CRC	C18-21	305	5.45	CRC	C18-21	239	7.01
5	EC	C15	290	3.22	EC	C15	245	4.38	BC	C50	179	5.25
6	CNC	C70-72	289	3.21	CNC	C70-72	158	2.82	CNC	C70-72	131	3.84
7	PC	C25	252	2.80	PC	C25	143	2.56	LK	C91-95	118	3.46
8	LK	C91-95	251	2.79	LK	C91-95	133	2.38	PC	C25	109	3.20
9	BC	C50	185	2.06	BLC	C67	95	1.70	EMC	C54	101	2.96
10	LNC	C77	151	1.68	LNC	C77	93	1.66	LNC	C77	58	1.70
	Total		7990	88.77	Total		5119	91.52	Total		3016	88.48

CR: Constituent ratio; ICD-10: The 10th revision of International Classification of Diseases; GC: Gastric cancer; LC: Lung cancer; HCC: Liver cancer; CRC: Colon/rectum cancer; BC: Breast cancer; EC: Esophageal cancer; BLC: Bladder cancer; PC: Pancreatic cancer; CNC: Cancers of brain and nervous system; EMC: Endometrial carcinoma; LK: Leukemia; LNC: Secondary malignant tumors of lymph nodes.



**Figure 1** World standardized incidence rates of gastric cancer in Zhuanghe County during 2005-2010. ASR2: The age standardized incidence of the world population in 2000.



**Figure 2** World standardized mortality rates of gastric cancer in Zhuanghe County during 2005-2010. ASMR2: The age standardized mortality of the world population in 2000.

years old accounted for 88.65% of all GC cases.

**Mortality**

There were a total of 1722 GC deaths between 2005 and 2010 in Zhuanghe, including 1207 males and 515 females. Table 3 shows the constituent ratio of malignant tumors leading to death, in which GC accounted for 19.13% (21.58% in males and 15.11% in females) of the total cancer deaths, making it the second leading cause of cancer death.

The crude mortality rate during this period due to

GC in Zhuanghe was 31.31 per 10<sup>5</sup>; 43.47 per 10<sup>5</sup> in males and 18.91 per 10<sup>5</sup> in females. The difference in sex-specific incidence rates was statistically significant ( $\chi^2 = 264.82, P < 0.001$ ); a higher rate was observed in males than in females with a ratio of 2.30. A decreasing trend in crude mortality rate was observed over the past 6 years ( $P < 0.001$  in both sexes;  $P = 0.046$  in males,  $P < 0.001$  in females) (Table 4).

A progressive decrease in the world age-standardized mortality rate was noted in both genders (Figure 2 and Table 4); ASMR2 decreased steadily from 42.08/10<sup>5</sup> in

**Table 4** Time trends for mortality rates of gastric cancer in Zhuanghe population, 2005-2010 (/100 000)

Year	Total				Males				Females			
	Cases	Crude	ASMR1	ASMR2	Cases	Crude	ASMR1	ASMR2	Cases	Crude	ASMR1	ASMR2
2005	352	38.51	27.94	32.74	224	48.47	33.31	42.08	128	28.32	21.75	23.86
2006	293	31.93	23.11	27.00	204	43.97	30.16	38.67	89	19.62	14.55	15.91
2007	276	29.95	21.79	25.78	202	43.37	29.26	38.55	74	16.24	12.50	13.54
2008	268	29.04	17.06	19.61	199	42.71	23.22	29.02	69	15.10	9.54	10.32
2009	272	29.71	16.20	18.74	202	43.74	21.82	28.07	70	15.42	8.99	9.52
2010	261	28.74	14.92	17.28	176	38.50	18.86	23.71	85	18.86	9.92	10.78
Total	1722	31.31	19.54	22.65	1207	43.47	25.42	32.18	515	18.91	12.35	13.32
$\chi^2$ trend test	$\chi^2 = 12.949, P < 0.001$				$\chi^2 = 3.965, P = 0.046$				$\chi^2 = 12.736, P < 0.001$			
APC	APC = -12.23%, $P = 0.001$				APC = -11.09%, $P = 0.002$				APC = -15.23%, $P = 0.015$			

Crude: The crude mortality rate; ASMR1: The age standardized mortality of Chinese population in 2000; ASMR2: The age standardized mortality of the world population in 2000; APC: Average annual percent change of mortality rates.

**Table 5** Age-specific incidence and mortality of gastric cancer in Zhuanghe population (/100 000)

Age (yr)	Incidence			Mortality		
	Males	Females	Total	Males	Females	Total
0-4						
5-9						
10-14						
15-19				0.68	0	0.33
20-24	0.61	0.57	0.59	0	0	0
25-29	2.49	1.97	2.23	0.99	0.99	0.99
30-34	2.15	5.01	3.54	2.15	3.19	2.65
35-39	12.08	5.49	8.85	3.02	3.53	3.27
40-44	23.34	9.93	16.65	11.32	4.97	8.15
45-49	35.80	12.46	24.15	14.61	8.43	11.52
50-54	70.78	28.85	50.01	29.64	21.18	25.45
55-59	130.08	50.15	91.74	57.05	29.10	43.65
60-64	169.90	62.55	119.25	85.33	35.50	61.82
65-69	280.88	87.94	186.18	169.55	56.15	113.89
70-74	369.88	123.30	245.56	245.76	80.57	162.48
75-79	474.45	128.06	296.01	373.28	116.57	241.04
80-84	573.56	183.05	367.92	453.11	206.25	323.12
85+	341.65	129.97	224.60	414.00	175.46	282.10

2005 to 23.71/10<sup>5</sup> in 2010 for males, and from 23.86/10<sup>5</sup> to 10.78/10<sup>5</sup> for females, with an average reduction of -11.09% for males (95% CI: -9.65% to -12.50%;  $P = 0.002$ ) and -15.23% for females (95% CI: -11.71% to -18.62%;  $P = 0.015$ ) per year. In general, ASMR2 decreased from 32.74 to 17.28 per 10<sup>5</sup> with an APC of -12.23% (95% CI: -10.94% to -13.90%;  $P = 0.001$ ).

The minimum age group for GC death was 15-20 years. With increasing age, the mortality rate increased sharply, reaching a peak at approximately 80 years of age and declining thereafter. The median age was 71.00 years and the average age was 69.17 years. The cases in subjects over 50 years of age accounted for 91.70% of all deaths (Table 5).

In addition, we calculated the age-standardized mortality-incidence ratio (M/I ratio: ASR2/ASMR2) during different time periods. The M/I ratio decreased from 0.87 to 0.58 over a 6-year period. The worst ratio was recorded in 2005: 0.73 for males and 1.32 for females, followed by a steady decrease over the subsequent years with the M/I ratio falling to 0.53 for males and 0.73 for

**Table 6** Mortality to incidence ratio of gastric cancer

Year	Age-standardized mortality to incidence ratio <sup>1</sup>		
	Total	Male	Female
2005	0.87	0.73	1.32
2006	0.67	0.64	0.77
2007	0.68	0.67	0.75
2008	0.56	0.57	0.53
2009	0.55	0.59	0.47
2010	0.58	0.53	0.73
Total	0.65	0.62	0.73

<sup>1</sup>The age-standardized mortality to incidence ratio was computed according to ASR2 and ASMR2. ASR2: The age standardized incidence of the world population in 2000; ASMR2: The age standardized mortality of the world population in 2000.

females in 2010 (Table 6).

## DISCUSSION

GC is one of the most common cancers worldwide. There is substantial geographic variation regarding the incidence and mortality of GC. Studies specifically examining the population in high-risk areas should be very useful in potentially controlling this disease. Zhuanghe County, which is situated in the coastal area of the eastern Liaoning peninsula, is a rural area exhibiting a high GC mortality in north China. A series of epidemiological investigations and comprehensive preventive measures for GC have been conducted since 1984. Surveillance of cancer incidence and mortality rates has provided a guideline to identify the etiology and aid in the evaluation of the impact of intervention on the population. Our previous sampling data indicated the initial effects of GC intervention. However, the lack of an overall picture of GC remains due to the unavailability of cancer registry data covering the entire region prior to 2005. In this study, we reviewed the data regarding the incidence and mortality of GC in Zhuanghe County during the period of 2005-2010 based on the population-based registry in order to analyze the changing trends and to elucidate the underlying causes.

The results about the organ distribution of all can-

cers in Zhuanghe indicated that 80.74% of new cases originated from ten primary organ sites, the two major sites being lung (21.35%) and stomach (21.04%). Moreover, the ten major cancers accounted for 88.77% of all cancer-related deaths, with lung and stomach as the top two positions (29.81% and 19.13%). GC was the most common type of cancer in males and the second most common in females in the Zhuanghe region, and was the second leading cause of cancer death for both genders. All of the data indicated that GC remained a major health burden for the local population.

Globally, the incidence rate of GC is nearly twice as high in males as in females<sup>[1]</sup>; the male-to-female incidence ratio in Zhuanghe county was 2.80 ( $\chi^2 = 264.82$ ,  $P < 0.001$ ), and the mortality of GC in males was 2.30 times higher than in females ( $\chi^2 = 584.31$ ,  $P < 0.001$ ). There were gender differences in many types of cancer, which may be related to the different risk level for exposure to diet, smoking, drinking and occupation<sup>[18]</sup>. Investigation of genetic factors together with other intrinsic and extrinsic differences between the genders could potentially help reveal the relevant factors in oncogenesis, and lead to more effective prevention measures and disease control.

Regarding age-specific incidence and mortality, we found an overall increasing trend that corresponded with increasing age, peaking at age 80-84 years, and a declining trend for older men and women during this period. According to the data collected from 2001 to 2003, the peak in GC incidence and mortality occurred earlier<sup>[13]</sup> and the median and the average age were also increased. These differences were hypothesized to be related to preventive measures as well as the change of population composition such as population growth and aging.

In our study, we specifically analyzed the changing trends in GC rates in Zhuanghe, China, and found encouraging declines in both incidence and mortality. Between 2005 and 2010, GC incidence decreased with an APC of 4.82% in Zhuanghe. Moreover, a similar but more noticeable trend was observed in mortality (APC = -12.23%). The decreasing trends were in concordance with the results from various other reports in China. Wang *et al.*<sup>[19]</sup> found that the estimated annual percentage change in GC incidence in Yangzhong was -2.96% in males and -2.86% in females. The APC change for GC incidence in Changde was reported to be -3.44% in men and -2.21% in women<sup>[20]</sup>. The report by Cui *et al.*<sup>[21]</sup> demonstrated an APC of GC mortality in Kaifeng of approximately -2.92% in males and -3.37% in females. Although similar trends were observed, the amplitudes of the declines were not similar to those demonstrated by our data, which indicated a far more significant decrease among the comparable studies.

The descending trends of GC rates in other regions were mostly reported to be attributed to the “unplanned triumph” of improvement in sanitation, fresh fruit and vegetable consumption, and reduced *H. pylori* infection. It is commonly accepted that the incidence and mortality of GC in a region are related to both the economic sta-

tus of the population and dietary habits<sup>[22-24]</sup>. In the past, residents in Zhuanghe had a low standard of living and were accustomed to eating highly salted food. However, currently, with economic development and increased knowledge regarding tumor prevention, residents have changed their dietary habits, and this potentially plays an important role in the descending trends of the disease. In 2010, Gross Domestic Product of Zhuanghe average per capita reached 54 348 RMB *yuan* - a 5.2-fold increase since 2000. Meanwhile, the rural health investment including environmental sanitation for water and housing has been increased by 23.8 times in the past decade<sup>[25]</sup>, and the consequentially improved sanitary conditions may help reduce the prevalence of *H. pylori* infection, and the risk of GC development. Therefore, further research regarding preventive factors, such as eating habits, foods, prevalence of *H. pylori* infection, environmental factors, *etc.*<sup>[26-28]</sup> is warranted.

In addition to the above causes for the decreasing trends in GC, prevention and control measures were also taken in Zhuanghe, and these measures also play an important role in the encouraging declines of GC incidence in our study. In Zhuanghe, comprehensive control and etiological intervention through mass behavior interference and chemoprophylaxis in the high-risk populations have been conducted in pilot villages for many years by a research group from the First Affiliated Hospital of China Medical University (FAHCMU). With professional guidance by the researchers, knowledge of prevention and treatment of GC was disseminated for general residents by means of broadcasting, video, brochures and face-to-face conversation. In addition, the targeted high-risk population was treated promptly with antibiotics, Chinese herbal medicine and nutritional therapy based on *H. pylori* detection and gastroscopic and pathological examinations<sup>[29]</sup>. Our previous pilot survey demonstrated that these strategies are feasible and cost-effective for the treatment of gastric premalignant lesions and carcinoma in early stages, and a decrease in the mortality rate was observed in the intervened population at the initial period of prevention. The average mortality rate in the intervention groups (34.97/10<sup>5</sup>) during the post-intervention period (1997-2000) was significantly lower than (59.31/10<sup>5</sup>) in the pre-intervention period (1996) ( $P < 0.05$ )<sup>[30]</sup>. In the present study, we focused on trends at the population level. Interestingly, the same downward trend seen in the intervention groups was observed in the entire population. This study demonstrates that the preventive activities potentially contributed to alleviating the adverse effects of GC on the health and life of patients, even in the population as a whole.

Despite declines in both incidence and mortality of GC, we noted a dissociation phenomenon, i.e., an APC decrease was greater for mortality compared with incidence. It was hypothesized that early diagnosis and early treatment would play an important role in the dissociation phenomenon between incidence and mortality. The data suggested the need for further promotion of GC screening to enable early diagnosis. In this study,

we also analyzed the M/I ratio in Zhuanghe. The M/I ratio, which compares the number of deaths attributed to a specific cancer and the number of incident cases in the same time period, can be interpreted as an indirect indicator of general survival if registration is complete and there are no marked temporal changes in incidence rates<sup>[31]</sup>. The age-standardized M/I ratio steadily decreased in both genders, which indicated a prolonged survival; hence the decrease in mortality was not caused by the fall in incidence rate alone, but by other factors, including earlier diagnosis or improved treatment. Since 1997, more than 15 000 people have received GC screening by the research group from FAHCMU through a two-round GC screening strategy, and this large-scale population screening program extensively covered 70 villages of Zhuanghe region. Through unremitting efforts for years, the detection rate of early GC reached 60%-80% and has been increasing yearly. The GC patients screened with pathological confirmation will be sent to hospitals for treatment, and their postoperative five-year survival rate was above 90%<sup>[7]</sup>. Thus, such a population screening program may contribute to the above-mentioned dissociation phenomenon and prolonged survival of the patients. Furthermore, the age-standardized M/I ratio was lower in the male population, suggesting that the survival rate was higher in males than in female patients after treatment.

This study describes the changes in incidence and mortality rates in the entire population of Zhuanghe over the past 6 years, which may not fully reflect broader population trends; however, our observations of the short-term changes potentially form the basis for future analysis. In concert with our previous studies, we described a general picture of GC patterns and changing trends in Zhuanghe. In addition, despite a significant declining trend, the age-standardized incidence (ASR1:  $26.84/10^5$  vs  $17.06/10^5$ ) and mortality (ASMR1:  $14.92/10^5$  vs  $11.67/10^5$ ) remained higher than the national level in China<sup>[32,33]</sup>. Therefore, in future studies, continuous monitoring over a longer period of time is needed to not only identify whether the declining trends persist, but also to acquire a more comprehensive understanding of GC in this region. Accordingly, effective prevention and treatment strategies would be further developed, which will greatly benefit the residents in high-risk areas. Moreover, researches regarding the impact of genetics, the environment, and their interaction are warranted to fully elucidate the disease etiology.

## COMMENTS

### Background

Gastric cancer (GC) is one of the most common cancers worldwide. The geographical distribution of GC is characterized by wide regional disparity. Monitoring and studying the incidence as well as mortality at the population level in high-risk areas should be very helpful in controlling this disease in the future.

### Research frontiers

Surveillance of cancer incidence and mortality provides important epidemiological information, enabling development of cancer prevention and control strategies. Several population-based cancer registries provided data on cancer rates

in China, however, most of which were established in the affluent big cities rather than in the poor rural counties. In this study, the data regarding the incidence and mortality of GC in Zhuanghe County, a rural area of China, were reviewed based on the population-based registry, which provided a general picture of the GC distribution in a rural high-risk area.

### Innovations and breakthroughs

The distribution of GC has substantial geographic variation, thus, studies concerning the population in high-risk areas should be more meaningful in potentially controlling this disease. The present study, for the first time, examined all-cancers distribution and temporal trends of GC in Zhuanghe and explored the possible influencing factors. Based on the findings in the registry studies, the control strategies of GC could be further developed in local areas, which may be also instructive and meaningful to cancer prevention of other similar areas in China.

### Applications

There is a significant global variation in the distribution of GC. The findings of the study would be helpful for the development of general and specific cancer prevention and control strategies to benefit the residents in the high-risk areas both locally and nationally.

### Terminology

Age-standardized incidence rates and age-standardized mortality rates are used to compare the incidence or mortality rates of places without being skewed by the difference in age distributions from place to place.

### Peer review

The authors studied the patterns and time trends of gastric cancer in terms of incidence and mortality in Zhuanghe region at population level and investigated the influencing factors of the changing trends. Findings from this study showed a downward trend of both incidence and mortality that may be due to the raised economic level along with implementation of prevention and control measures. It enriched epidemiological data of GC and offered reference to the other high-risk areas of China to control the disease based on population-level evidences.

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## Esophageal duplication cysts: Endosonographic findings in asymptomatic patients

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### Abstract

Esophageal duplication cysts are rare inherited lesions usually diagnosed in early childhood. Most of them are found in the mediastinum and manifest themselves as separate masses along or in continuity with the native esophagus. Their prevalence remains unknown and they are treated either surgically or endoscopically. In this report we describe a series of four adult patients in whom esophageal duplication cysts were localised intramurally as masses pressing on the esophageal lumen and who were diagnosed with endoscopic ultrasonography. All patients were initially referred to other centres for upper gastroduodenoscopy due to non-specific dyspeptic symptoms. After finding suspicious lesions in the esophagus their endoscopists referred them for endoscopic ultrasound examination at our centre. In two of the cases lesions mimicked esophageal varices and the other two submucosal tumours. In all four patients endoscopic ultrasonography has shown esophageal duplication cysts. Patients had no symptoms suggesting disease of the esophagus

and required no treatment. As the true prevalence of esophageal cysts is unknown, it is very likely that in many patients, like in these four described by us, they may cause no symptoms, remain undetected and require no intervention. Increasing availability of new diagnostic modalities such as endoscopic ultrasonography may change the current view regarding the prevalence of esophageal duplication cysts and prove that they may, in fact, not be such rare findings.

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**Key words:** Esophageal cyst; Endoscopic ultrasonography; Endovascular surgery

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Wiechowska-Kozłowska A, Wunsch E, Majewski M, Milkiewicz P. Esophageal duplication cysts: Endosonographic findings in asymptomatic patients. *World J Gastroenterol* 2012; 18(11): 1270-1272 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i11/1270.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i11.1270>

### INTRODUCTION

Esophageal duplication cysts are congenital anomalies of the foregut resulting from aberration of the posterior division of the embryonic foregut at 3-4 wk gestation<sup>[1]</sup>. They represent either simple epithelial-lined cysts, or true esophageal duplication, which is a duplication of the muscularis mucosa and externa without epithelial duplication.

Esophageal cysts are considered rare, usually described in single case reports, and their true prevalence remains

unknown<sup>[2]</sup>. Up to 80% of cases are diagnosed during childhood<sup>[1-5]</sup>. Most of them are found in the mediastinum and manifest themselves as separate masses along or in continuity with the native esophagus. Intramural esophageal cysts are considered very uncommon in adults<sup>[6,7]</sup>.

Symptoms are caused by compression or displacement of surrounding mediastinal structures and comprise dysphagia, respiratory distress, failure to thrive and retrosternal pain<sup>[7,8]</sup>. Malignant progression is considered extremely rare<sup>[9]</sup>. Diagnosis is usually made by computed tomography scan or endoscopic ultrasonography (EUS).

Treatment of symptomatic esophageal cysts can be either surgical or endoscopic. Surgical resection of the cyst is usually carried out in childhood or in adults, in cases of lesions impossible to treat endoscopically. Surgical treatment is currently moving from thoracotomy to less-invasive procedures, such as video-assisted thoracoscopic surgery<sup>[7]</sup> and to endoscopic treatments which, however, still remain challenging interventions<sup>[10]</sup>.

We present a series of 4 patients with esophageal cysts which caused no symptoms and required no intervention.

## CASE REPORT

All patients were initially referred to other centres for upper gastroduodenoscopy due to non-specific, dyspeptic symptoms. They gave no history of dysphagia. After finding suspicious lesions in the esophagus their endoscopists referred them for EUS examination. Written informed consent was obtained from each patient.

### Case 1

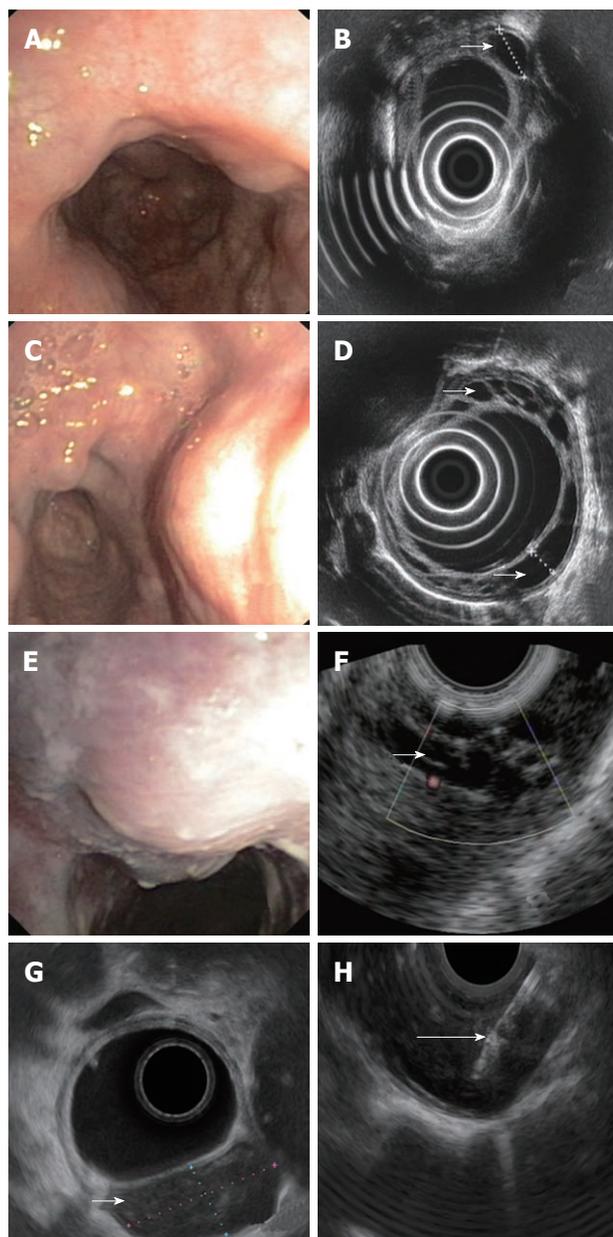
A 60-year old female: On endoscopy, multiple impressions, covered with normal mucosa and localized to the middle and lower third of the esophagus were seen (Figure 1A). They could be easily passed by the endoscope. No other abnormality was found on endoscopy. On EUS multiple cystic fluid-filled intramural lesions were detected (Figure 1B). They were avascular on Doppler study and their size varied from 3 mm × 5 mm to 11 mm × 25 mm.

### Case 2

A 51-year old male: Endoscopy revealed multiple impressions, covered with normal mucosa and localized in the lower third of the esophagus (Figure 1C). The prepyloric area showed features of atrophic gastritis subsequently confirmed with histology. On EUS, multiple cystic, fluid-filled, intramural lesions were detected (Figure 1D). Their size varied from 4 mm × 6 mm to 8 mm × 16 mm. They showed no vascular pattern on Doppler examination.

### Case 3

A 57-year old female: Endoscopy showed a solitary impression, localized 25 cm from the incisor teeth and covered with unchanged mucosa (Figure 1E). Examination was otherwise unremarkable. EUS revealed a cluster of anechoic fluid-filled intramural lesions showing no



**Figure 1** Endoscopic and endosonographic images of described patients.

A: Multiple impressions to middle and lower third of esophagus seen in endoscopy; B: Multiple cystic fluid-filled intramural lesions detected on endoscopic ultrasonography (EUS); C: On endoscopy multiple impressions, covered with normal mucosa, localized in the lower third of esophagus; D: On EUS multiple cystic, fluid-filled, intramural lesions; E: Solitary impression, localized 25 cm from the incisor teeth and covered with unchanged mucosa seen in endoscopy; F: On EUS a cluster of anechoic fluid-filled intramural lesions; G: On EUS an anechoic fluid-filled intramural lesion 24 x 12 mm large; H: Aspiration of the fluid with 19G needle. Small arrows show duplication cysts in D, F and G and big arrow shows the biopsy needle in H.

vascular pattern on Doppler examination (Figure 1F).

### Case 4

A 47-year old male: Endoscopy revealed a solitary lesion, size approximately 25 mm localized 30 cm from the incisor teeth and covered with unchanged mucosa (Figure 1G). No other abnormality was found on endoscopy. EUS demonstrated an anechoic fluid-filled intramural lesion

24 mm × 12 mm large. The lesion showed no vascular pattern on Doppler examination (Figure 1H). Aspiration with 18G needle revealed a clear fluid with no malignant or suspicious cells.

## DISCUSSION

In this paper we describe a series of 4 patients who were referred to our centre for further investigation of esophageal lesions found on routine gastroduodenoscopy. In two cases, where there were multiple lesions, referring doctors suspected unusual looking varices or vascular lesions. In view of our interest in EUS of portal hypertension<sup>[11-13]</sup> a more detailed study of both the lesions and intrinsic para- and peri-esophageal circulation was performed. However, these patients had no clinical, laboratory or imaging features suggesting liver cirrhosis or portal hypertension. In the other two cases the initial diagnosis was submucosal tumours. These findings were expected to be confirmed using EUS. All these patients turned out to have esophageal duplication cysts.

Esophageal cysts do not usually communicate with the esophageal wall but occur as a separate malformation along or in continuity with the native esophagus. In the presented cases they were localized intramurally as a mass pressing on the esophageal lumen and this is why they resembled esophageal varices or submucosal tumours.

Treatment of esophageal duplication cysts in asymptomatic patients is controversial with no clear clinical guidelines. As all our patients remained asymptomatic we arbitrarily decided to schedule them for follow up EUS/gastroduodenoscopy in a period of 2 years. An earlier intervention will be performed if they become symptomatic.

As the true prevalence of esophageal cysts is unknown<sup>[2]</sup>, it is very likely that in many patients, like in these four described by us, they cause no symptoms, remain undetected and require no intervention at all. Increasing availability of new diagnostic modalities such as EUS may change the current view regarding the prevalence of esophageal duplication cysts and prove that they are not rare findings.

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## Tumors with macroscopic bile duct thrombi in non-HCC patients: Dynamic multi-phase MSCT findings

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### Abstract

Non-hepatocellular carcinoma (non-HCC) with macroscopic bile duct tumor thrombus (BDTT) formation is rare, few radiological studies have been reported. In this case report, we retrospectively analyzed the imaging findings of three cases of non-HCC with macroscopic BDTT on dynamic enhanced multislice computed tomography (MSCT) scan. One case of primary hepatic carcinosarcoma was presented as a solitary, large well-defined tumor with significant necrotic changes. One case of liver metastasis from colon cancer was presented as a lobulated, large ill-defined tumor. One case of intraductal oncocytic papillary neoplasm involved the entire pancreas, presented as a cystic and solid mass with multilocular changes (the individual loculi were less than 5.0 mm in diameter). The bile duct was dilated due to expansible growth of the BDTT in all three patients. The BDTT was contiguous with hepatic or pancreatic

tumor, and both of them showed the same enhancement patterns on dynamic contrast-enhanced computed tomography scan: early enhancement in the hepatic arterial phase and a quick wash-out of contrast agent in the portal and equilibrium phases. Macroscopic BDTT in non-HCC patient is rare, dynamic enhanced MSCT scan may be valuable in the diagnosis of non-HCC with BDTT.

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**Key words:** Liver neoplasms; Carcinosarcoma; Metastasis; Pancreatic neoplasms; Oncocytic papillary neoplasm; Bile ducts tumor thrombus; Computed tomography; X-ray

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### INTRODUCTION

Hepatocellular carcinoma (HCC) associated with bile duct invasion and subsequent bile duct tumor thrombus (BDTT) formation occurs in only 0.79%-4% of patients with primary HCC<sup>[1,2]</sup>. Because HCC is one of the most prevalent malignant tumors, HCC with BDTT is not uncommonly encountered in clinical practice and its

clinical characteristics have been well described<sup>[3]</sup>. Non-HCC liver tumor can also invade the biliary tree, such as combined hepatocellular and cholangiocellular carcinoma and liver metastasis<sup>[4-10]</sup>. However, only few cases of non-HCC with BDTT have been reported in the radiological literatures<sup>[5-7]</sup>. In this study, we report three cases of macroscopic BDTT: one case of primary hepatic carcinosarcoma, one case of liver metastasis from colon cancer, and one case of intraductal oncocytic papillary neoplasm (IOPN) of the pancreas. The cases of primary hepatic carcinosarcoma or IOPN of the pancreas associated with macroscopic BDTT have not been previously reported.

## CASE REPORT

### Patients

All three non-HCC patients with macroscopic BDTT were treated in our hospital. They had jaundice on admission. Liver function tests showed that aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase and alkaline phosphatase were elevated. Total serum bilirubin (TBil) was significantly increased mainly due to direct bilirubin (DBil). Serum markers of hepatitis B and hepatitis C and tumor markers alpha-fetoprotein (AFP), carcino-embryonic antigen (CEA) and CA125 were all normal. However, CA19-9 was elevated in a patient with primary hepatic carcinosarcoma and a patient with IOPN of the pancreas. All three cases of non-HCC with macroscopic BDTT were pathologically confirmed. We retrospectively analyzed the dynamic enhanced multislice computed tomography (MSCT) findings in these three cases.

### Computed tomography imaging protocols

The computed tomography (CT) examination was performed using a 64-slice spiral CT scanner (Sensation 64, Siemens Medical Solutions), the technical parameters were as follows: 120 kVp tube voltage, 200 effective mAs, 64 mm × 0.6 mm beam collimation, pitch of 0.9, rotation time of 0.5 s, 5 mm reconstruction slice thickness. After acquisition of unenhanced images, nonionic iodinated contrast agent (iodipamide, 370 mg I/mL, Bracco) was injected through a dual-head injector at a rate of 3.5 mL/s and followed by a 20-mL saline flush, with a dose of 2.0 mL/kg body weight. To determine the timing for the hepatic arterial phase (HAP) scanning, a bolus-tracking technique was used with a region of interest in the descending aorta. After achieving enhancement of the descending aorta up to 100 HU, the HAP images were acquired with the scanning delay of 5 s. Portal venous phase (PVP) and equilibrium phase (EP) images were obtained with a delayed time of 30 s and 120 s, respectively.

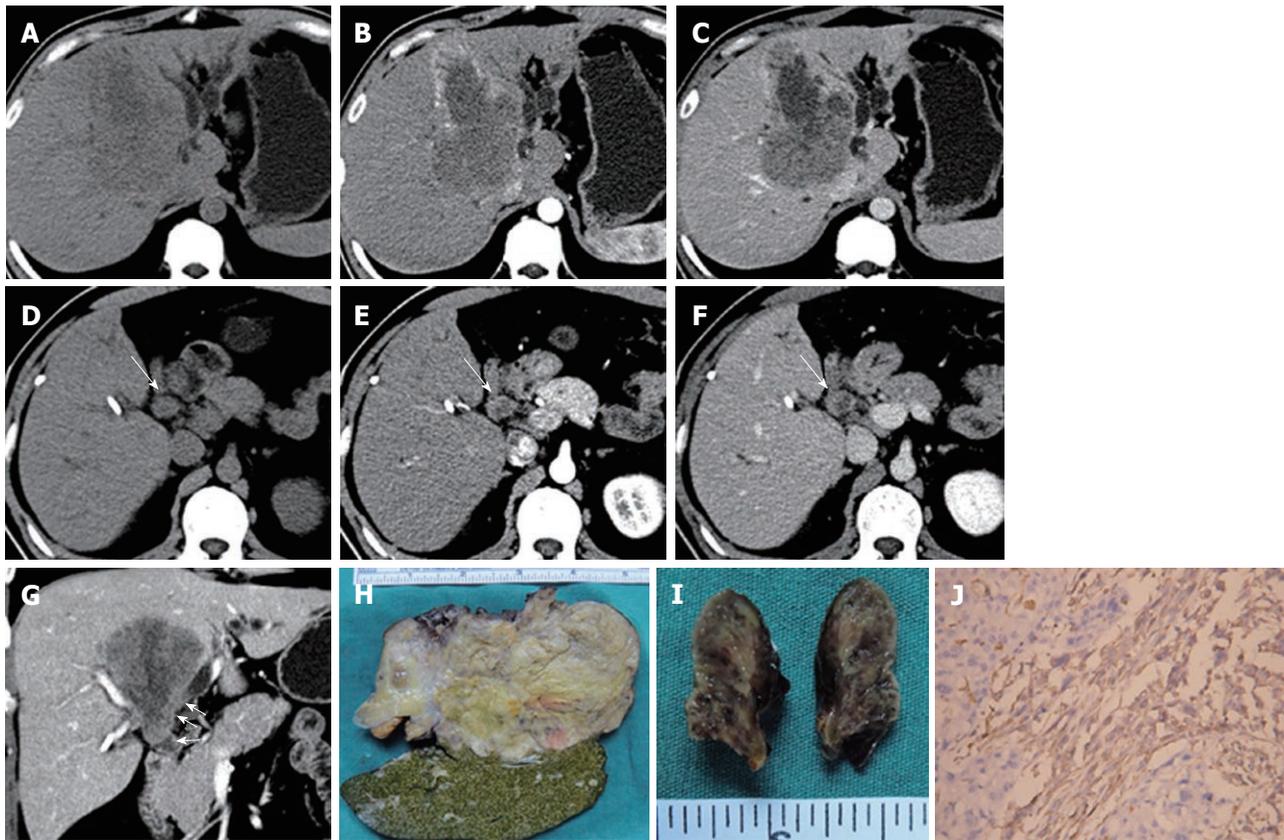
### Case 1

A 41-year-old man was admitted to our hospital on November 18, 2010 with a history of jaundice and right upper abdominal discomfort for one month. Laboratory examination showed that serum TBil was 282.1 μmol/L (normal range: 5-24 μmol/L), DBil was 221.4 μmol/L

(normal range: ≤ 11 μmol/L), and tumor marker CA19-9 was 953.4 U/mL (normal range: ≤ 35 U/mL). A well-defined tumor with a size of 10.8 cm × 6.9 cm in the middle hepatic lobe (Segments IV, V and VIII) was revealed on CT scan. The tumor showed hypo-attenuation (relative to normal liver parenchyma) on pre-contrast CT scan, early rim enhancement in the HAP images and hypo-attenuation in the PVP and EP images. A large necrotic area was noted in the tumor, and showed no enhancement after contrast administration. A tumor thrombus was revealed in the dilated left and right hepatic duct and the common hepatic duct, which was contiguous with the hepatic tumor. No bile duct wall invasion was noted. The enhancement pattern of the intraductal mass was consistent with intrahepatic tumor on dynamic enhanced CT scan. Biliary tree dilation was seen distal to the intraductal mass. The patient underwent left trisectionectomy and right posterior hepatic bile duct-jejunum anastomosis, and intraductal mass was removed through resection of the extrahepatic bile duct. The intrahepatic tumor was not encapsulated. The intraductal mass was not adhered to the bile duct wall, and was easily removed. Primary hepatic carcinosarcoma with BDTT was pathologically confirmed. The carcinomatous and sarcomatous elements were HCC and fibrosarcoma, respectively. The immunohistochemical results revealed that the sarcomatous elements were Vimentin positive, and negative results for cytokeratin, epithelial membrane antigen, hepatocyte, CD34, AFP, AAT, S-100, actin and CD117 (Figure 1). The patient died two months after surgery due to hepatorenal syndrome.

### Case 2

A 75-year-old woman was admitted on November 19, 2010 with epigastric pain and progressive jaundice for seven days. The patient underwent radical resection of colon cancer six years ago. Laboratory results showed significantly elevated serum TBil (196.5 μmol/L) and DBil (131.3 μmol/L). The tumor markers AFP, CEA, CA125 and CA19-9 were all in the normal range. A large lobulated tumor of 8.7 cm × 9.5 cm was displayed on CT scan in the left hepatic lobe with an ill-defined margin. The tumor demonstrated hypo-attenuation on precontrast CT scan, early mild enhancement in the HAP images and inhomogeneous hypo-attenuation in the PVP and EP images. An intraductal tumor thrombus was seen extending from the dilated left intrahepatic bile duct to the upper common bile duct, and was contiguous with intrahepatic tumor. The intraductal thrombus showed similar enhancement patterns with intrahepatic tumor on dynamic enhanced CT scans. Intrahepatic ductal dilation was found distal to the intraductal mass. Tumor thrombi were also seen in the inferior vena cava, the middle hepatic vein and the left hepatic vein. No enlarged lymph node was observed. The patient received percutaneous transhepatic biliary drainage instead of surgery due to poor liver reserve. However, jaundice was not effectively relieved, thus palliative thrombectomy through choledochotomy was performed. The intraductal tumor thrombus was easily removed as it was not adhered to the bile duct wall. Resection specimen



**Figure 1** A 41-year-old man with primary hepatic carcinosarcoma. A large hypodense tumor in the hepatic middle lobe appears on precontrast computed tomography (CT) scan (A), which shows early rim enhancement in the hepatic artery phase (B) and a low density in the portal phase with large areas of necrosis (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the intrahepatic tumor on pre-contrast CT scan (D), hepatic artery phase (E) and portal phase scan (F). Coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the intrahepatic tumor (G). The resected hepatic tumor (H) and BDTT (I) specimens. The sarcomatous component of the primary hepatic carcinosarcoma is vimentin positive on the immunohistochemical staining (J),  $\times 200$ .

revealed to be metastatic adenocarcinoma pathologically, and was positive for CDX2 and CEA, and negative for hepatocyte, AFP, HBsAg, and CD34 on immunohistochemical staining (Figure 2). The patient was still alive 10 months after the operation.

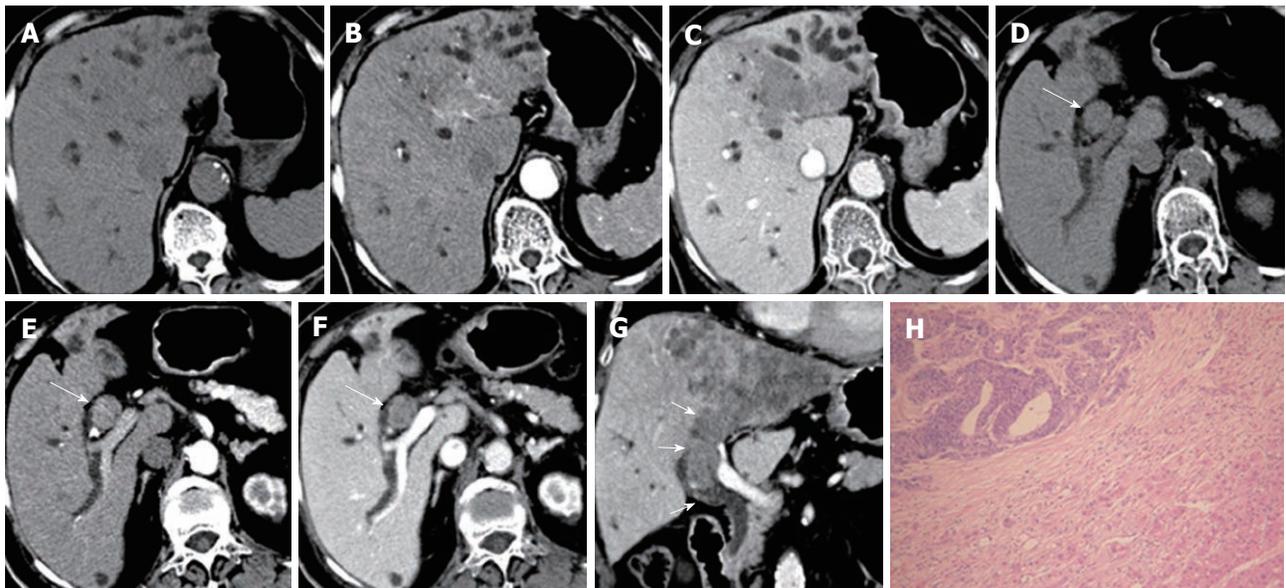
### Case 3

A 43-year-old woman was admitted on December 29, 2009 with a history of jaundice for 10 d. The laboratory results showed a high level of serum TBil (174.6  $\mu\text{mol/L}$ ) and DBil (141.3  $\mu\text{mol/L}$ ). The tumor marker CA19-9 was slightly elevated, with a level of 36.5 U/mL. CT scan showed enlargement of the pancreas which consisted of a mixed solid and cystic tumor. The solid components showed hypo-attenuation on precontrast CT scan, early enhancement on the HAP images and hypo-attenuation on the PVP and EP images. The cystic components were multilocular, with the loculi less than 5.0 mm in diameter, and showed no enhancement after contrast administration. The main pancreatic duct was mildly dilated and communicated with pancreatic tumor. An intraductal tumor thrombus appeared in the dilated right hepatic duct and the common bile duct on CT scan, and it was contiguous with the pancreatic tumor. The enhancement patterns of the intraductal thrombus were consistent with pancreatic tumor on dynamic enhanced CT scans. No en-

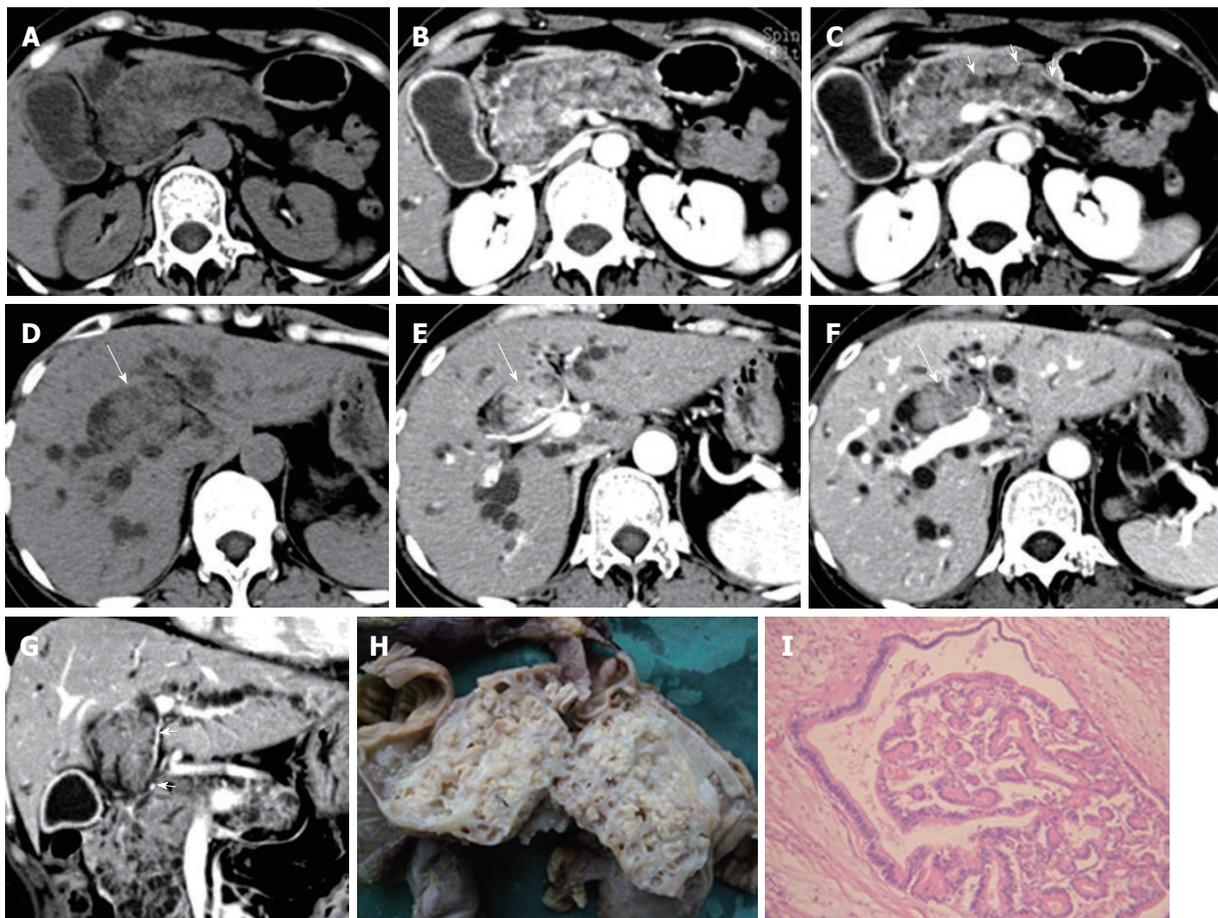
larged lymph node was detected. Thrombectomy through choledochotomy and pancreatic-duodenal resection was performed in this patient. IOPN of the pancreas with BDTT formation was confirmed pathologically. Tumor invasion was only observed in the lower segment of the common bile duct wall (Figure 3). The patient was still alive 21 mo after the operation.

## DISCUSSION

Macroscopic BDTT secondary to liver tumors is not commonly seen clinically and may occur in HCC, combined hepatocellular and cholangiocellular carcinoma and hepatic metastasis<sup>[4-10]</sup>. The mechanism of tumor thrombus formation in the bile duct is unclear. It is widely accepted that hepatic tumors (primary or secondary) can directly invade the bile duct, even extrabiliary malignant tumors can directly metastasize to the bile duct<sup>[10]</sup>. Consequently, tumors can develop along the bile duct and form intraductal thrombus. However, Peng *et al*<sup>[11]</sup> indicated that the tumor cells in HCC with BDTT might be originally derived from the canals of Hering and hepatic stem cells or primitive progenitor cells. We retrospectively analyzed three cases of BDTT secondary to primary hepatic carcinosarcoma, hepatic metastasis from colon cancer and IOPN of the pancreas and reviewed their



**Figure 2** A 75-year-old woman with liver metastasis from colon cancer. A lobulated, ill-defined hypodense tumor in the left hepatic lobe is noted on pre-contrast computed tomography (CT) scans (A), which shows early enhancement in the hepatic artery phase (B) and a low density in the portal phase (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the intrahepatic tumor on precontrast CT scan (D), hepatic artery phase (E) and portal phase scan (F). The coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the intrahepatic tumor (G). Intraductal tumor thrombus is proved to be metastatic adenocarcinoma pathologically (H), hematoxylin and eosin stain,  $\times 100$ .



**Figure 3** A 43-year-old woman with intraductal oncocytic papillary neoplasm of the pancreas. An inhomogeneous hypodense tumor involving the entire pancreas is noted on precontrast computed tomography (CT) scan (A), which shows early enhancement in the hepatic artery phase (B) and a low density in the portal phase with mild dilation of the pancreatic duct (arrows) and multilocular changes (C). The bile duct tumor thrombus (BDTT) (arrow) shows similar enhancement patterns with the pancreatic tumor on precontrast CT scans (D), hepatic artery phase (E) and portal phase scan (F). The coronal reconstruction image in the portal phase shows that BDTT (arrows) is contiguous with the pancreatic tumor (G). Resected pancreatic tumor specimen shows multilocular changes with papillary growth in the loculi (H). Pancreatic tumor is proved to be intraductal oncocytic papillary neoplasm pathologically (I), hematoxylin and eosin stain,  $\times 100$ .

clinical and imaging findings.

World Health Organization defined primary hepatic carcinomas as tumors containing both carcinomatous (either hepatocellular or cholangiocellular) and sarcomatous components and without obvious transition zone between them<sup>[12]</sup>. Primary hepatic carcinomas are extremely rare and aggressive. To our knowledge, only about 20 cases have been reported in the English literature, and the pathogenesis is still controversial<sup>[12,13]</sup>. This tumor occurs between 34 and 84 years of age and presented no specific clinical manifestations<sup>[12,13]</sup>. More than half of the patients had a background of hepatic cirrhosis or fibrosis<sup>[12-14]</sup>. Elevated serum AFP and CA19-9 could be observed in some cases<sup>[14]</sup>. Patients usually have poor prognosis due to the highly invasive and metastatic features of the tumors<sup>[14]</sup>.

The CT characteristics of primary hepatic carcinomas reported in the literature include a solitary mass with central necrosis and myxoid changes, round, oval or lobulated in shape, and without capsule formation. They show iso-attenuation or hypo-attenuation on precontrast CT scan. Dynamic enhanced CT scan revealed early rim or peripheral ring enhancement in the HAP images, hypo-attenuation in the PVP images and iso-attenuation in the EP images<sup>[12,13,15]</sup>. If the sarcomatous components were osteosarcoma or chondrosarcoma, dense rocky calcifications or bone formations may be presented within the mass radiologically<sup>[15]</sup>. The case of primary hepatic carcinoma described here has similar CT findings as reported in the literature. Interestingly, this case was also associated with BDIT, which has not been previously reported. The BDIT grew expansively without invasion of the bile duct wall, and was contiguous with the hepatic carcinoma. Both of them showed the same enhancement pattern on dynamic enhanced CT scan as they shared the same blood supply. We found similar phenomena in HCC with BDIT, i.e., the enhancement pattern of BDIT was the same as HCC on dynamic enhanced CT scan, intraductal tumor thrombus was continuous with the main intrahepatic HCC mass, no thickened bile duct wall adjacent to the thrombus was observed<sup>[16]</sup>. The differential diagnosis of primary hepatic carcinoma with BDIT from HCC with BDIT is difficult. In the presence of large amounts of tumor necrosis, tumor calcification or ossification and negative serum AFP, hepatic carcinoma should be considered in the differential diagnosis.

Macroscopic intrabiliary tumor growth is a peculiar mode of intrahepatic spread in patients with colorectal liver metastasis. Metastatic hepatic tumors from colorectal cancer demonstrated macroscopic BDIT in approximately 5.8%-12.1% of resected tumors<sup>[6,8,9]</sup>. The growth of BDIT included two components: intraluminal and intraepithelial extension<sup>[8]</sup>. The diagnosis of metastatic hepatic tumors from colorectal cancer associated with macroscopic BDIT is important because macroscopic BDIT is an independent indicator of favorable prognosis in such patients. Aggressive surgical treatment can improve the chances of long-term survival<sup>[8,9]</sup>. Currently, there are few radiological studies about hepatic metastasis from colorectal cancer with bile duct invasion<sup>[5-7]</sup>. Okano *et al*<sup>[6]</sup>

reported the following radiological findings on CT scan in detecting the presence of intrabiliary tumor growth in patients with liver metastases from colorectal cancer: intrahepatic bile duct dilatation, thickened portal tract and wedge-shaped area with enhancement. A thickened portal tract around the tumor corresponded to intrabiliary thrombus itself, and the presence of this sign depended on the length of the intraductal thrombus. If the length of the thrombus is larger than 30 mm, thickened portal tract will likely be observed<sup>[6]</sup>. However, in our cases and in 8 cases of hepatic metastasis with BDIT from colon cancer reported by Lee *et al*<sup>[5]</sup>, no such sign was observed. This discrepancy was likely due to the use of an obsolete CT scanner as reported by Okano *et al*<sup>[6]</sup>, which was limited by low spatial resolution and thicker slice scanning (7-10 mm), thus the intraductal thrombus itself could not be clearly detected. The wedge-shaped area with enhancement is assumed to be caused by reduced portal flow and a compensatory increase in arterial blood flow caused by tumor compression or arteriportal shunt in the involved portal venous branch<sup>[6]</sup>. Jinzaki *et al*<sup>[7]</sup> reported four cases of liver metastasis from colorectal cancer with intraductal invasion. Two of the cases revealed hypo-dense hepatic tumors with bile duct dilation on an enhanced CT scan without presence of intraductal thrombus, while in the other two cases, intraductal thrombus itself appeared on CT scans, which were contiguous with the main intrahepatic tumors. The intraductal thrombus was isodense on unenhanced and enhanced CT compared with the main intrahepatic tumor. In our case of liver metastasis from colon cancer with macroscopic BDIT, the BDIT showed similar expansible growth pattern, and was contiguous with the hepatic metastatic tumor. Both of them showed the same enhancement pattern on CT scan as they shared a common blood supply.

In clinical practice, it is important to differentiate metastatic tumor from primary intraductal cholangiocarcinoma in patients with a history of colorectal cancer when an intraductal mass is presented in the bile duct. The expansible growth of intraductal mass and its direct connection with hepatic tumors are very valuable features in the differential diagnosis<sup>[5]</sup>. The diagnosis of liver metastasis with BDIT should also be differentiated from HCC with BDIT. Hepatic cirrhosis background and a rise in AFP level may aid in the diagnosis of HCC<sup>[16]</sup>.

The term "intraductal oncocytic papillary neoplasm" was proposed by Adsay *et al*<sup>[17]</sup> in 1996 to describe a rare type of pancreatic tumor. Currently, only about 20 cases of IOPN of the pancreas have been reported, but the accurate incidence and etiology of these cases are still unknown<sup>[18,19]</sup>. One of the distinguishing features of IOPN of the pancreas is the abundant, finely granular, eosinophilic cytoplasm of the cells. The tumors usually form complex, branching, and arborizing papillaries<sup>[17,18]</sup>. IOPN of the pancreas is often found in the elderly (mean age, 63.9 years), with no gender predilection and specific clinical features<sup>[18]</sup>. Tumors mainly involve the main pancreatic duct. They are more commonly observed in the head of the pancreas although they can occur in any part of the pancreas. Multiple sites involved have also been reported

in the literature<sup>[17-20]</sup>. In general, IOPN of the pancreas usually presents as either unilocular or multilocular cystic tumor with individual loculi ranging from 0.2 to 4.0 cm. The cystic loculi are filled with mucin and have prominent papillary growth. Cystic tumors usually communicate with the pancreatic duct system<sup>[17-20]</sup>. Most IOPNs of the pancreas exhibit severe dysplasia histopathologically to warrant the term intraductal oncocytic papillary carcinoma, and therefore they should be surgically resected completely<sup>[17]</sup>.

Radiologically IOPN of the pancreas can be presented as a well-defined mass with unilocular or multilocular cystic changes and with mural or solid nodules. The tumors always communicated with the dilated pancreatic duct<sup>[18-20]</sup>. Mural nodules or solid tumor components may show enhancement on CT or magnetic resonance imaging<sup>[19,20]</sup>. Adsay *et al.*<sup>[17]</sup> reported one case of IOPN of the pancreas with common bile duct involvement, and Fischer *et al.*<sup>[19]</sup> reported one case of IOPN of the pancreas with dilation of the common bile duct and intrahepatic ducts. However, no further description has been mentioned in both cases. Currently, no case of IOPN of the pancreas with macroscopic BDTT has been reported. The case we reported here has the following features: tumor involved the entire pancreas with multilocular, mixed cystic and solid changes, and the individual loculi were less than 5.0 mm in diameter. The pancreatic tumor invaded the lower segment of the common bile duct, and hence intraductal thrombus was formed, extending upward along the bile duct with expansible growth pattern. On dynamic enhanced CT scans, pancreatic tumor and BDTT have the same enhancement patterns, which might be due to direct connection of BDTT with main pancreatic tumor, and both sharing the same blood supply. Intraductal papillary mucinous neoplasm (IPMN) of pancreas has similar radiological features as IOPN. A fluorodeoxyglucose (FDG) positron emission tomography scan can help differentiate between IOPN and IPMN of the pancreas. A strong uptake of FDG was reported in IOPN of the pancreas, which suggested high glucose metabolic activity of tumor cells<sup>[19,20]</sup>.

In conclusion, non-HCC with macroscopic BDTT is a rare clinical entity. All three cases in this study shared the following imaging features: expansible growth of the BDTT; the intrahepatic tumor or pancreatic tumor contiguous with the BDTT, and both showing the same enhancement patterns on CT scans; and intrahepatic bile duct dilation distal to the BDTT. MSCT may be a valuable modality in the diagnosis of non-HCC with BDTT.

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## Events Calendar 2012

January 13-15, 2012  
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Meeting 2012  
Kuala Lumpur, Malaysia

January 19-21, 2012  
American Society of Clinical  
Oncology 2012 Gastrointestinal  
Cancers Symposium  
San Francisco, CA 3000,  
United States

January 19-21, 2012  
2012 Gastrointestinal Cancers  
Symposium  
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January 20-21, 2012  
American Gastroenterological  
Association Clinical Congress of  
Gastroenterology and Hepatology  
Miami Beach, FL 33141,  
United States

February 3, 2012  
The Future of Obesity Treatment  
London, United Kingdom

February 16-17, 2012  
4th United Kingdom Swallowing  
Research Group Conference  
London, United Kingdom

February 23, 2012  
Management of Barretts  
Oesophagus: Everything you need  
to know  
Cambridge, United Kingdom

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Canadian Digestive Diseases Week  
2012  
Montreal, Canada

March 1-3, 2012  
International Conference on  
Nutrition and Growth 2012  
Paris, France

March 7-10, 2012  
Society of American Gastrointestinal  
and Endoscopic Surgeons Annual  
Meeting  
San Diego, CA 92121, United States

March 12-14, 2012  
World Congress on  
Gastroenterology and Urology  
Omaha, NE 68197, United States

March 17-20, 2012  
Mayo Clinic Gastroenterology and  
Hepatology  
Orlando, FL 32808, United States

March 26-27, 2012  
26th Annual New Treatments in  
Chronic Liver Disease  
San Diego, CA 92121, United States

March 30-April 2, 2012  
Mayo Clinic Gastroenterology and  
Hepatology  
San Antonio, TX 78249,  
United States

March 31-April 1, 2012  
27th Annual New Treatments in  
Chronic Liver Disease  
San Diego, CA 92121, United States

April 8-10, 2012  
9th International Symposium on  
Functional GI Disorders  
Milwaukee, WI 53202, United States

April 13-15, 2012  
Asian Oncology Summit 2012  
Singapore, Singapore

April 15-17, 2012  
European Multidisciplinary  
Colorectal Cancer Congress 2012  
Prague, Czech

April 18-20, 2012  
The International Liver Congress  
2012  
Barcelona, Spain

April 19-21, 2012  
Internal Medicine 2012  
New Orleans, LA 70166,  
United States

April 20-22, 2012  
Diffuse Small Bowel and Liver  
Diseases  
Melbourne, Australia

April 22-24, 2012  
EUROSON 2012 EFSUMB Annual

Meeting  
Madrid, Spain

April 28, 2012  
Issues in Pediatric Oncology  
Kiev, Ukraine

May 3-5, 2012  
9th Congress of The Jordanian  
Society of Gastroenterology  
Amman, Jordan

May 7-10, 2012  
Digestive Diseases Week  
Chicago, IL 60601, United States

May 17-21, 2012  
2012 ASCRS Annual Meeting-  
American Society of Colon and  
Rectal Surgeons  
Hollywood, FL 1300, United States

May 18-19, 2012  
Pancreas Club Meeting  
San Diego, CA 92101, United States

May 18-23, 2012  
SGNA: Society of Gastroenterology  
Nurses and Associates Annual  
Course  
Phoenix, AZ 85001, United States

May 19-22, 2012  
2012-Digestive Disease Week  
San Diego, CA 92121, United States

June 2-6, 2012  
American Society of Colon and  
Rectal Surgeons Annual Meeting  
San Antonio, TX 78249,  
United States

June 18-21, 2012  
Pancreatic Cancer: Progress and  
Challenges  
Lake Tahoe, NV 89101, United States

July 25-26, 2012  
PancreasFest 2012  
Pittsburgh, PA 15260, United States

September 1-4, 2012  
OESO 11th World Conference  
Como, Italy

September 6-8, 2012  
2012 Joint International

Neurogastroenterology and Motility  
Meeting  
Bologna, Italy

September 7-9, 2012  
The Viral Hepatitis Congress  
Frankfurt, Germany

September 8-9, 2012  
New Advances in Inflammatory  
Bowel Disease  
La Jolla, CA 92093, United States

September 8-9, 2012  
Florida Gastroenterologic Society  
2012 Annual Meeting  
Boca Raton, FL 33498, United States

September 15-16, 2012  
Current Problems of  
Gastroenterology and Abdominal  
Surgery  
Kiev, Ukraine

September 20-22, 2012  
1st World Congress on Controversies  
in the Management of Viral Hepatitis  
Prague, Czech

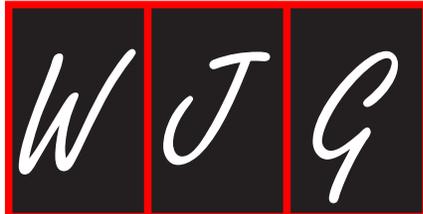
October 19-24, 2012  
American College of  
Gastroenterology 77th Annual  
Scientific Meeting and Postgraduate  
Course  
Las Vegas, NV 89085, United States

November 3-4, 2012  
Modern Technologies in  
Diagnosis and Treatment of  
Gastroenterological Patients  
Dnepropetrovsk, Ukraine

November 4-8, 2012  
The Liver Meeting  
San Francisco, CA 94101,  
United States

November 9-13, 2012  
American Association for the Study  
of Liver Diseases  
Boston, MA 02298, United States

December 1-4, 2012  
Advances in Inflammatory Bowel  
Diseases  
Hollywood, FL 33028, United States



## GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

### Name of journal

*World Journal of Gastroenterology*

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ISSN 1007-9327 (print)

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## SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only

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**Author contributions:** The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections.

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AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g.  $6.92 \pm 3.86$  vs  $3.61 \pm 1.67$ ,  $P < 0.001$ ; CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: [http://www.wjgnet.com/1007-9327/g\\_info\\_20100315215714.htm](http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm).

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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. Immunolog-

ic 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]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harden 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*

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## Risk for gastric neoplasias in patients with chronic atrophic gastritis: A critical reappraisal

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### Abstract

Chronic atrophic gastritis (CAG) is an inflammatory condition characterized by the loss of gastric glandular structures which are replaced by connective tissue (non-metaplastic atrophy) or by glandular structures inappropriate for location (metaplastic atrophy). Epidemiological data suggest that CAG is associated with two different types of tumors: Intestinal-type gastric cancer (GC) and type I gastric carcinoid (T I GC). The pathophysiological mechanisms which lead to the development of these gastric tumors are different. It is accepted that a multistep process initiating from *Helicobacter pylori*-related chronic inflammation of the gastric mucosa progresses to CAG, intestinal metaplasia, dysplasia and, finally, leads to the development of GC. The T I GC is a gastrin-dependent tumor and the chronic elevation of gastrin, which is associated with CAG, stimulates the growth of enterochromaffin-like cells with their hyperplasia leading to the development of T I GC. Thus, several events occur in the gastric mucosa before the development of intestinal-type GC and/or T I GC and these take several years. Knowledge of

CAG incidence from superficial gastritis, its prevalence in different clinical settings and possible risk factors associated with the progression of this condition to gastric neoplasias are important issues. This editorial intends to provide a brief review of the main studies regarding incidence and prevalence of CAG and risk factors for the development of gastric neoplasias.

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**Key words:** Chronic atrophic gastritis; Gastric neoplasia; Intestinal-type gastric cancer; Type I gastric carcinoid; Prevalence; Incidence; Risk factors

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### INTRODUCTION

Chronic atrophic gastritis (CAG) is an inflammatory condition characterized by the loss of gastric glandular structures which are replaced by connective tissue (non-metaplastic atrophy) or by glandular structures inappropriate for location (metaplastic atrophy)<sup>[1]</sup>. Epidemiological data suggest that CAG is associated with two different types of tumors: Intestinal-type gastric cancer (GC) and type I gastric carcinoid (T I GC). The pathophysiological mechanisms which lead to the development of these gastric tumors are different. It is accepted that a multistep process initiating from *Helicobacter pylori* (*H. pylori*)-related chronic inflammation of the gastric mucosa progresses to CAG, intestinal metaplasia, dysplasia, and finally leads to

the development of GC<sup>[2]</sup>. T I GC is a gastrin-dependent tumor and the chronic elevation of gastrin, which is associated with CAG, stimulates the growth of enterochromaffin-like (ECL) cells with their hyperplasia leading to the development of T I GC<sup>[3-5]</sup>.

Considering that several events occur in the gastric mucosa before the development of GC and/or of T I GC, and that these events take several years, the knowledge of CAG incidence from superficial gastritis, its prevalence in different clinical settings and possible risk factors associated with the progression of this condition to gastric neoplasias are important issues.

## EPIDEMIOLOGY OF CHRONIC ATROPHIC GASTRITIS

A recent systematic review was performed with the aim of evaluating the CAG incidence in patients free of CAG at moment of inclusion in the study<sup>[6]</sup>. From published studies, the authors selected only 14 follow-up studies in which CAG diagnosis was carefully made by histology (12 studies) or by serum pepsinogen (PG) levels (2 studies). The CAG incidence rates ranged from 0% to 10.9% per year. This wide CAG incidence range is explained by the particular settings in which the CAG diagnoses were made. In fact, the lowest incidence rates (0%) were found in patients with reflux esophagitis<sup>[7]</sup> and in patients successfully treated for *H. pylori* infection<sup>[8]</sup>. The highest incidence rate was observed in an older study conducted on patients who underwent vagotomy because of ulcer disease<sup>[9]</sup>. Regarding *H. pylori* infection, the CAG incidence rate was higher in *H. pylori*-positive patients than in *H. pylori*-negative ones<sup>[7,10-13]</sup> and the meta-analysis on the association between *H. pylori* infection and CAG incidence presented a rate ratio of 5 (95% CI: 3.1-8.3).

The prevalence of CAG was evaluated by serological screening using surrogate markers of gastric function (PG I or PG I/PG II ratio) or by gastroscopy/histology. In the vast majority of cases, the serological and histological screenings were both made in a general population. Serological studies reported CAG prevalence rates between 3% and 7%, which were lower than those reported by histological ones. Studies on CAG prevalence subdivided on the basis of diagnostic tools used for CAG diagnosis (histology or serology) are shown in Table 1<sup>[14-23]</sup>. The observed differences between serological and histological studies could be explained by the fact that it is likely that symptomatic patients accepted more easily to undergo gastroscopy. Higher rates of CAG prevalence found in the Asian countries may be justified by the fact that these areas are at higher risk of GC and by the fact that the definition of CAG diagnosis may be different between Western and Asian countries. In studies reporting from Asian countries, CAG diagnosis included all atrophic lesions irrespective of the atrophy localization in the gastric mucosa (antrum and/or corpus); in the vast majority of the studies conducted in Western countries, CAG diagnosis included only patients with a corpus atro-

phic involvement such as corpus-atrophic gastritis or a multifocal atrophic gastritis (i.e., patchy areas of atrophic-metaplastic changes in the antral and corpus mucosa), because it is maintained that only corpus atrophic changes can lead to the development of gastric cancer.

## ATROPHIC GASTRITIS AND GASTRIC CANCER

Nowadays, GC represents one of the most challenging tumors due to the fact that its diagnosis is often late and, in the advanced stage, the therapeutic options are scarce with consequent high rate of mortality<sup>[24]</sup>. In fact, although a reduction of global incidence for this neoplasm is reported, it remains the second cause of cancer-related death. The knowledge of precursor lesions for the development of intestinal-type GC could contribute to anticipating GC diagnosis at an early stage when surgery or chemotherapy offers a better prognosis. Several studies have estimated the risk of GC in patients with CAG<sup>[25-33]</sup>. Although the vast majority of these were performed on small numbers of patients and were based on older histological classifications, the progression rate of CAG to GC fluctuates from 0% to 10% with annual incidence (person-year) lower than 1% (Table 2). It is interesting to observe that, although the incidence rate of CAG in patients with superficial gastritis is higher in populations with higher risk of GC (Table 1), the progression rate of CAG towards GC is similar irrespective of different geographic areas.

Some studies have attempted to identify risk factors linked with the progression of precancerous lesions (CAG or intestinal metaplasia) towards GC to select those patients who should undergo endoscopic surveillance.

### Age

Age has been identified as a possible risk factor in several studies. In the study by Leung *et al.*<sup>[40]</sup>, *H. pylori*-positive patients with intestinal metaplasia were followed up for 5 years to evaluate the progression or the improvement of histological lesions after *H. pylori* eradication treatment compared with placebo. At multivariate analysis, the presence of age > 45 years showed an approximate two-fold increased risk of progression of intestinal metaplasia compared to younger subjects<sup>[40]</sup>. This same age limit had already been identified in a screening survey performed on 3386 subjects from a rural Chinese population that showed an approximate three-fold increased risk of progression to GC<sup>[28]</sup>. In a large cohort study, increasing age at initial diagnosis was associated with higher hazard ratio (HR) for the progression to GC (for age > 55 years, HR > 2.38)<sup>[32]</sup>. In a recent work, patients with CAG who were aged > 50 years at the moment of initial diagnosis presented HR = 8.8 for the progression to gastric neoplastic lesions<sup>[33]</sup>.

### Pernicious anemia

Although the vast majority of the older studies on CAG

Table 1 Prevalence of chronic atrophic gastritis

Author	Year	Country	Study type	Patients	Age (yr)	CAG (%)
Serology						
Sipponen <i>et al</i> <sup>[14]</sup>	2003	Finland	General population	12 252 (men)	51-65	5.2
Green <i>et al</i> <sup>[15]</sup>	2005	New Zealand	General population	466	> 65	6.7
Weck <i>et al</i> <sup>[16]</sup>	2007	Germany	General population	9444	50-74	6
Telaranta-Keerle <i>et al</i> <sup>[17]</sup>	2010	Finland	General population	4256	18-92	3.5
Histology						
Oksanen <i>et al</i> <sup>[18]</sup>	2000	Finland	Endoscopic cohort	207	19-83	13 <sup>1</sup>
Borch <i>et al</i> <sup>[19]</sup>	2000	Sweden	General Population	501	35-85	9.4 <sup>2</sup>
Asaka <i>et al</i> <sup>[20]</sup>	2001	Japan	General Population	2455	< 20 to > 70	55.5 <sup>3</sup>
Red�en <i>et al</i> <sup>[21]</sup>	2003	Sweden	General Population	488	37-85	9
Storskrubb <i>et al</i> <sup>[22]</sup>	2008	Sweden	General Population	976	20-80	6.6 <sup>4</sup>
Zou <i>et al</i> <sup>[23]</sup>	2011	China	General Population	1022	18-80	63.8 <sup>3</sup>

<sup>1</sup>This percentage refers to patients ( $n = 27$ ) with atrophic body gastritis; <sup>2</sup>this percentage refers to patients ( $n = 47$ ) with atrophic pangastritis and corpus- predominant (gastritis); <sup>3</sup>these percentages included chronic atrophic gastritis (CAG) diagnosis irrespective of the atrophy localization in the gastric mucosa (antrum and/or corpus); <sup>4</sup>this percentage refers to patients ( $n = 54$ ) with multifocal atrophic gastritis and atrophic corpus- limited gastritis.

Table 2 Incidence of gastric cancer in patients with chronic atrophic gastritis or pernicious anemia

Author	Year	Country	Study type	Patients	Age, median or range (yr)	GC	Annual incidence of GC, person-year (%)
Patients with chronic atrophic gastritis							
Walker <i>et al</i> <sup>[25]</sup>	1971	Australia	Retrospective	40	40-64	4 (10)	0.6
Ectors <i>et al</i> <sup>[26]</sup>	1986	United Kingdom	Retrospective	225	-	3 (1.3)	0.1
Tatsuta <i>et al</i> <sup>[27]</sup>	1993	Japan	Retrospective	654	-	22 (3.4)	0.2
You <i>et al</i> <sup>[28]</sup>	1999	China	Prospective	2082 <sup>1</sup>	35-64	19 (0.9)	0.2
Whiting <i>et al</i> <sup>[29]</sup>	2002	United Kingdom	Prospective	1042	> 40	12 (11.5)	1.1
Dinis-Ribeiro <i>et al</i> <sup>[30]</sup>	2004	Portugal	Retrospective	1771	-	4 (2.2)	0.7
Lahner <i>et al</i> <sup>[31]</sup>	2005	Italy	Prospective	106	22-74	1 (0.9)	0.1
de Vries <i>et al</i> <sup>[32]</sup>	2008	Netherlands	Retrospective	84 072 <sup>2</sup>	65.7	1035 (1.2)	0.2
Vannella <i>et al</i> <sup>[33]</sup>	2010	Italy	Retrospective	300	18-78	3 (1)	0.2
Patients with pernicious anemia							
Borch <i>et al</i> <sup>[34]</sup>	1986	Sweden	Prospective	61	-	0	0
Kokkola <i>et al</i> <sup>[35]</sup>	1998	Finland	Prospective	62	20-73	2 (3.2)	1.10
Sj�blom <i>et al</i> <sup>[36]</sup>	1993	Finland	Prospective	56	27-78	2 (3.5)	1.20
Armbrecht <i>et al</i> <sup>[37]</sup>	1990	United Kingdom	Prospective	27	26-81	0	0
Bresky <i>et al</i> <sup>[38]</sup>	2003	Spain	Prospective	68	-	0	0
Ye <i>et al</i> <sup>[39]</sup>	2003	Sweden	Retrospective	21 265	74.3	177 (0.8)	0.10
Vannella <i>et al</i> <sup>[33]</sup>	2010	Italy	Retrospective	129	23-74	2 (1.5)	0.30

<sup>1</sup>This number refers to biopsies taken in 144 patients and includes chronic atrophic gastritis (CAG) with type I, II, III intestinal metaplasia; <sup>2</sup>this number refers to CAG patients with or without intestinal metaplasia. GC: Gastric cancer.

included patients with pernicious anemia, the risk of GC in this particular clinical setting seems to be generally low (Table 2). In fact, this clinical condition is often associated with corpus-restricted gastritis and, as a consequence, with less extensive atrophy in the gastric mucosa. In a recent study, the presence of atrophic pangastritis increased the risk of progression to gastric neoplastic lesions by 4.5 times, in keeping with previous works<sup>[33,41,42]</sup>. The apparent contrast between older and more recent works about pernicious anemia can be explained by the difficulty in comparing studies with methodological differences linked to adopted gastritis classification or small number series. It is interesting to underline the fact that studies on the relationship between pernicious anemia and GC are lacking in Asian countries where the risk of GC is higher, thus it remains to be established whether pernicious ane-

mia has low prevalence in the Asian geographic area or if this condition is overlooked.

### Intestinal metaplasia

Parallel with more extensive atrophy in the gastric mucosa, the extensive replacement of this by intestinal metaplasia is considered a hallmark of severity of CAG. In the literature, the intestinal metaplasia extension was widely related to a higher risk of GC<sup>[32,33,40,42]</sup>. In particular, type III intestinal metaplasia was associated with an increased risk of GC in some studies<sup>[43,44]</sup>, but subsequent studies showed conflicting findings<sup>[45,46]</sup>, thus the clinical utility of different subtyping of intestinal metaplasia is limited.

### Helicobacter pylori

The role of *H. pylori* infection in progression from CAG

to GC is controversial. In the Leung study, *H. pylori*-positive patients who had not undergone eradication therapy had a progression rate of intestinal metaplasia higher than cured patients<sup>[40]</sup>. However, in this study, the vast majority of patients had only a superficial gastritis at baseline and, after 5 years of follow-up, the rate of patients with intestinal metaplasia increased significantly. It is maintained that the effect of eradication therapy on the progression to GC in patients with precancerous lesions is limited. A previous large prospective study demonstrated that *H. pylori* eradication may be beneficial in arresting the progression to GC only in patients without CAG or intestinal metaplasia<sup>[47]</sup>. Two recent meta-analyses showed a beneficial long-term effect of *H. pylori* eradication therapy on atrophic gastritis, but not on intestinal metaplasia<sup>[48,49]</sup>. Up till now, although the possibility of histological improvement of CAG is accepted after *H. pylori* cure, the efficacy of *H. pylori* eradication in reducing GC incidence needs to be demonstrated.

## ATROPHIC GASTRITIS AND TYPE I GASTRIC NEUROENDOCRINE TUMOR

T I GC derives from ECL cells which are localized in the gastric fundus and corpus. ECL cells are specialized in the secretion of histamine that, in turn, stimulates acid secretion by parietal cells<sup>[50]</sup>. Gastric carcinoids have been classified into three subgroups, type I to type III, with different outcomes<sup>[51-53]</sup>. Type I lesions are associated with atrophic gastritis and constitute up to 80% of all gastric carcinoids<sup>[54]</sup>. Gastrin, released by G-cells in the gastric antrum, stimulates the release of histamine and produces trophic effects upon ECL cells<sup>[5]</sup>. In CAG, the loss of appropriate glands in the body leads to achlorhydria, and the consequent chronic hypergastrinemia stimulates ECL hyperplasia and sometimes the development of T I GC<sup>[4,5]</sup>.

The prevalence rate of T I GC in patients with CAG is reported to be between 1% and 12.5% in different studies<sup>[36,37,55-58]</sup>. The wide range of the prevalence rates of T I GC among several studies can be explained by different settings where patients were selected, such as type of hospital (secondary, tertiary center) or symptoms/signs of presentation. CAG can have a wide range of clinical presentations such as dyspepsia, iron deficiency anemia or pernicious anemia<sup>[59]</sup>. In particular, in a recent observational study in which the T I GC incidence and prevalence were evaluated, pernicious anemia was present in almost 50% of patients, while previous studies included exclusively patients with this condition<sup>[60]</sup>.

Long-term observational studies assessing incidence of T I GC in CAG patients are scarce<sup>[35,56,61]</sup>. We recently followed up a cohort of CAG patients for 1463 person-years reporting an annual incidence rate (person-year) for T I GC of 0.4%<sup>[60]</sup>. An old study by Kokkola *et al.*<sup>[35]</sup> reported an annual incidence of 2%, observing 8 new cases of T I GC in 416 patient-years. Sjöblom *et al.*<sup>[61]</sup> studied 196 patients with pernicious anemia and after

1397 patient-years, 2 new cases of T I GC were reported in hospital registries among the initial group of patients. This figure should correspond to an annual incidence rate of 0.1%, but in this study only 70 patients (35.7%) underwent gastroscopy and the incidence rate can only be obtained indirectly. Furthermore, although there are small fluctuations in the reported incidence rates, only a small group of CAG patients develop T I GC showing that factors other than gastrin are necessary for the progression of ECL cells to T I GC.

Few studies have attempted to identify risk factors associated with the development of T I GC. In a recent work, we found higher baseline levels of gastrin and chromogranin A in CAG patients with T I GC compared to those without T I GC. However, all patients with CAG present high plasma values of chromogranin A<sup>[62]</sup> and gastrin, thus these markers have limited clinical utility because of low specificity<sup>[63]</sup>.

An accepted risk factor for T I GC is the presence of ECL dysplasia, which is often associated with T I GC. This lesion is considered as the true gastric carcinoid precursor lesion and it can represent the sign of a concomitant carcinoid lesion<sup>[56,64]</sup>. CAG patients with a diagnosis of ECL cell dysplasia could benefit from a shorter endoscopic follow-up time to exclude concomitant T I GC lesions or to identify newly arisen lesions in the gastric mucosa.

Although T I GC lesions can also be present on flat mucosa, in the vast majority of cases they are associated with the presence of body polyps. In CAG patients, hyperplastic or adenomatous polyps are very common; however, the presence of body polyps increases the risk of having a T I GC<sup>[60]</sup>. Unfortunately, no feature of endoscopic appearance of the gastric polyps (size, number, sessile/pedunculated presentation) seems useful to differentiate histology of polyps, thus all polyps should be removed and histologically examined<sup>[65,66]</sup>.

## CONCLUSION

The risk of development of GC or T I GC appears higher in CAG patients with respect to the general population. In geographic areas with low risk of GC, a surveillance program for all CAG patients may be not cost-effective considering that the vast majority of CAG patients will not develop a gastric neoplasm<sup>[67]</sup>. A subset of CAG patients at higher risk for GC should be identified allowing the selection of those CAG patients in whom gastroscopic/histologic surveillance may be warranted. Recently, an international consensus developed evidence-based guidelines on the management of precancerous conditions and lesions of the stomach, recommending an endoscopic surveillance every 3 years after diagnosis in all patients with extensive atrophy and/or intestinal metaplasia in the antrum and corpus<sup>[68]</sup>. New systems for histopathological staging (OLGA, OLGIM) have been developed with the aim of combining pathological findings with the risk of GC for the patient and to iden-

tify a subgroup of those at higher risk<sup>[69,70]</sup>. The OLGA system includes gastritis grading and staging<sup>[69]</sup>. Grading measures the severity of acute and chronic inflammatory infiltrate in the antrum and body. Staging refers to the extent of atrophy with or without intestinal metaplasia. The OLGIM system is based on intestinal metaplasia which is considered a more reproducible histopathological diagnosis with respect to atrophy diagnosis. Further studies are necessary to validate these new classifications and to establish their real clinical value. Regarding T IGC, although risk factors for its development have not been identified, ENETS guidelines suggest an endoscopic follow-up every 6-12 mo after T IGC diagnosis. This interval allows the identification of recurrent lesions or new lesions (incidence-case) at an early stage when they can easily be removed by polypectomy without complications<sup>[71]</sup>. This approach seems safe for T IGC, a neoplasm with an excellent outcome<sup>[60,72]</sup>.

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## Mouse models of pancreatic cancer

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### Abstract

Pancreatic cancer is one of the most lethal of human malignancies ranking 4th among cancer-related death in the western world and in the United States, and potent therapeutic options are lacking. Although during the last few years there have been important advances in the understanding of the molecular events responsible for the development of pancreatic cancer, currently specific mechanisms of treatment resistance remain poorly understood and new effective systemic drugs need to be developed and probed. *In vivo* models to study pancreatic cancer and approach this issue remain limited and present different molecular features that must be considered in the studies depending on the purpose to fit special research themes. In the last few years, several genetically engineered mouse models of pancreatic exocrine neoplasia have been developed. These models mimic the disease as they reproduce genetic alterations implicated in the progression of pancreatic cancer. Genetic alterations such as activat-

ing mutations in KRas, or TGF $\beta$  and/or inactivation of tumoral suppressors such as p53, INK4A/ARF, BRCA2 and Smad4 are the most common drivers to pancreatic carcinogenesis and have been used to create transgenic mice. These mouse models have a spectrum of pathologic changes, from pancreatic intraepithelial neoplasia to lesions that progress histologically culminating in fully invasive and metastatic disease and represent the most useful preclinical model system. These models can characterize the cellular and molecular pathology of pancreatic neoplasia and cancer and constitute the best tool to investigate new therapeutic approaches, chemopreventive and/or anticancer treatments. Here, we review and update the current mouse models that reproduce different stages of human pancreatic ductal adenocarcinoma and will have clinical relevance in future pancreatic cancer developments.

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**Key words:** K-Ras; Mouse models; Transgenic; Pancreatic cancer; Xenografts

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### INTRODUCTION

Infiltrating ductal adenocarcinoma of the pancreas (PDAC) accounts for over 85% of all pancreatic malignancies and has a poor prognosis as less than 5% of patients survive 5 years after diagnosis with a median survival period of 4-6 mo<sup>[1-3]</sup>. During the last few years there have been important advances to better understand the molecular mechanisms regulating the development of PDAC<sup>[4,5]</sup>. However, prog-

ress in prevention, early diagnosis and treatment needs major advances<sup>[6]</sup>.

Some of the recent advances have been possible by employing mouse models which have provided an important model system to better understand the molecular mechanism underlying pancreatic cancer. However, in stark contrast to the successful murine models of most common human tumors, the generation and use of appropriate mouse models of pancreatic cancer has remained an area of significant frustration and not always well established. Currently, there are several different genetically modified mouse tumors and xenograft models available that offer the possibility of experimental and preclinical model systems to evaluate different strategies for targeting this disease, early detection, chemoprevention, treatment and finally improve the outcome for pancreatic cancer patients<sup>[7]</sup>.

These models use a variety of approaches to target the expression of mutant or endogenous specific genes and as a result they develop a broad spectrum of pathologic changes, some of them mimic human disease while others are not equivalent to human pancreatic neoplasia. According to the cancer progression model postulated by Fearon and Vogelstein<sup>[8]</sup> in 1990, at least 4-5 genetic events are required for the progression from normal epithelium to carcinoma. Since, the genetic basis of pancreatic ductal adenocarcinoma was revealed, with activation of *Kras* and inactivation of the p16INK4a, p53 and Smad4 tumor suppressors<sup>[9]</sup>, several mouse models of invasive pancreatic cancer have been developed and modified. Also, regarding the role of pancreatic intraepithelial neoplasia (PanIN) as a direct noninvasive neoplastic precursor to human pancreatic cancer<sup>[10]</sup>, different mouse models are currently available, some of these models reproduce only PanIN lesions and others progress to invasive pancreatic carcinoma. Most of these models were previously presented and evaluated at the International Workshop sponsored by the National Cancer Institute and the University of Pennsylvania in 2004. Twelve genetically engineered mouse models were included and have been considered models for the study of pancreatic disease including PanINs and carcinomas<sup>[11-18]</sup>. Since then, several new models have been introduced in the basic and translational research fields and previous models have been re-evaluated. Here, we will focus only on pancreatic cancer mouse models as PanIN lesions are considered preinvasive.

Since an activating mutation of the *Kras* oncogene is the most frequent genetic alteration associated with pancreatic cancer, having been identified in up to 90% of all pancreatic adenocarcinomas<sup>[19-21]</sup>, most of the genetically engineered mouse models are based on the *Kras* oncogene. As mice expressing mutant *Kras* develop early and advanced forms of the most common pancreatic cancers in humans, these *Kras*-based models provide preclinical model systems to analyze the molecular biology of this disease and measure the benefit of new therapies<sup>[7,22]</sup>.

In these review, we update and describe the most common genetically engineered mouse and xenograft models of PDAC that could be useful for assessing the

role of genes and pathways, environmental conditions, co-morbidities and response to new adjuvant, neoadjuvant and anti-metastatic therapies.

## TRANSGENIC MOUSE MODELS

As *Kras* mutations are not sufficient to induce progression to the invasive stage of pancreatic adenocarcinoma, different transgenes have been used to generate combined models that progress to invasive PDAC and metastatic disease.

The common genetically engineered models are based on *Kras* mutations and also include PDX-1-Cre/Lox-Stop-Lox (LSL)-*Kras* or p48/LSL-*Kras* mice which have been modified with deletions or mutations of *Ink4*<sup>[23]</sup>, *p53*<sup>[24]</sup>, *Mist*<sup>[25]</sup>, *Smad4*<sup>[26]</sup> or *TGFβ*<sup>[27]</sup> (Table 1).

These *Kras*-mutated models can be induced using inducible alleles of Cre recombinase, such as estrogen receptor-Cre fusion genes (*CreER* or *CreERT*) and cycline-responsive Cre expression alleles (TRE-Cre) which are temporally expressed and initiate the expression in adult pancreata reflecting the somatic mutation as it occur in humans<sup>[28,29]</sup>. Also, some models that only develop PanIN lesions are available as *Ela-LSL-Kras*<sup>G12D</sup><sup>[12]</sup>, *Nestin-Cre, LSL-Kras*<sup>G12D</sup><sup>[30]</sup>, *PDX-1-CRE<sup>ERT</sup>, LSL-Kras*<sup>G12D</sup>, *R26Notch<sup>NIC</sup>*<sup>[31]</sup> and *PDX-1-CRE, LSL-Kras*<sup>G12D</sup>, *Tif1β<sup>lox/lox</sup>*<sup>[32]</sup>, however, these are not the purpose of our review.

### ***PDX1-Cre, LSL-Kras<sup>G12D</sup> and P48<sup>+/Cre</sup>, LSL-Kras<sup>G12D</sup> transgenic model***

After different studies identified PDX-1 and p48 as critical transcription factors in the developmental program of the pancreas<sup>[21,33]</sup>, these genes have been used in almost all transgenic mouse models to study pancreatic cancer. It is well known that the first identifiable pancreatic progenitor cell in the pancreas arises in the dorsal and ventral endoderm at embryonic day 8 in the fetal mouse: expression of PDX-1 occurs around E8.5<sup>[34]</sup> and P48 is expressed slightly later and is required to commit cells to a pancreatic fate<sup>[35]</sup>.

In addition, Ptf1a, a component of the pancreas transcription factor 1 complex (Ptf1) which plays an important role in mammalian pancreatic development has been used in some mouse models. Pdf1a determines whether cells allocated to the pancreatic buds continue towards pancreatic organogenesis or revert to duodenal fates<sup>[36,37]</sup>. To target the expression of oncogenic *Kras* in pancreatic progenitor cells, a conditionally expressed allele was constructed as previously described by Jackson *et al*<sup>[38]</sup>.

Briefly, the targeting vector contains genetic elements inhibiting transcription and translation flanked by functional LoxP sites. This Lox-Stop-Lox (LSL) construct was inserted into the mouse genomic *Kras* locus upstream of locus 1 to contain G-A transition in codon 12 (G12D). This transition mutation results in a glycine to aspartic acid substitution in the expressed protein that activates constitutive downstream signaling of Ras effector pathways and is one of the most common mutations found in human pancreatic tumors.

Table 1 Mouse models of pancreatic adenocarcinoma

Genotype (reference)	Time of expression	Time to tumor development (mo)	Pancreatic cancer phenotype	Survival (mo)
PDX-1-Cre; LSL-Kras <sup>G12D</sup> [22]	E8.5	6	PDAC; penetrant PanIN; age dependent increase severity; occasionally PDAC with long latency	16
P48 <sup>+/-Cre</sup> ; LSL-Kras <sup>G12D</sup> [22]	E9.5	8	PDAC; penetrant PanIN; age dependent increase severity; occasionally PDAC with long latency	16
PDX-1-Cre; LSL-Kras <sup>G12D</sup> ; LSL-Trp53 <sup>R172H/-</sup> [8]	E8.5	2-3	PDAC	5-6
Mist1 <sup>KrasG12D/+</sup> [9]	E10.5	2	Accelerated PanIN; well differentiated PDCA	10.8
KPCB <sup>wt/wt</sup> [25]	E8.5	2-3	PDAC	5.6
KPCB <sup>Tr/wt</sup> [25]	E8.5	3	PDAC	4.8
KPCB <sup>Tr/<math>\Delta</math>11</sup> [25]	E8.5	1.5	PDAC; mixed	2.8
CKB <sup>wt/<math>\Delta</math>11</sup> [24]	E8.5	6	PDAC	12
CKB <sup>wt/wt</sup> [24]	E8.5	6	PDAC	13.5
CPB <sup><math>\Delta</math>11/<math>\Delta</math>11</sup> [24]	E8.5	3-5	PDAC; mixed	10
Pdx1-Cre; Kras <sup>G12D</sup> Ink4a/Arf <sup>fllox/fllox</sup> [7]	E8.5	2	PDAC; accelerated development of PanIN; poorly differentiated PDAC	2-3
Pdx1-Cre; Kras <sup>G12D</sup> Smad4 <sup>fllox/fllox</sup> [32]	E8.5	2-3	IPMN; PDAC	2-6
Ptf1a <sup>cre/+</sup> ; LSL-Kras <sup>G12D/+</sup> ; Tgfb2 <sup>fllox/fllox</sup> [11]	E9.5	1	PDAC; accelerated PanIN; PDAC development	2

PDAC: Ductal adenocarcinoma of the pancreas; PanIN: Pancreatic intraepithelial neoplasia; IPMN: Intraductal papillary mucinous neoplasia.

Hingorani *et al.*<sup>[39]</sup> developed a mouse model expressing a Cre-activated Kras<sup>G12D</sup> allele inserted into the endogenous Kras locus, and these mice were crossed with mice expressing Cre recombinase in pancreatic tissue, either by virtue of a PDX-1 promoter-driven transgene or by Cre knockin at the Ptf1-p48 locus. Prior lineage studies suggest that both of these lines express Cre in a common endocrine/exocrine precursor cell during development, while expression in adults is retained in mature islet cells in the case of PDX-1-Cre transgenics and in mature acinar cells in the case of the Ptf1-p48+/Cre knockin<sup>[35]</sup>.

The subsequent recombination resulted in interbreeding LSL-Kras<sup>G12D</sup> mice with animals that express Cre recombinase from the pancreatic-specific promoters PDX-1 or P48 is a heterozygous mutant condition (KRAS<sup>+G12D</sup>). Note that only genomic DNA isolated from pancreata and not from tails evidence the recombination. The mutant mice PDX-1-Cre, LSL-Kras<sup>G12D</sup> and P48<sup>+/-Cre</sup>, LSL-Kras<sup>G12D</sup> have increased Kras oncogenic protein and their pancreata are larger than their wild type littermate controls.

The pancreata of compound mutant mice develop ductal lesions identical to all three stages of human PanINs. PanIN-1A lesions are observed in compound mutant mice as young as 2 wk old. As the mice age, higher-grade PanINs were observed with increasing frequency and in many of the older mice, the pancreata contained extensive ductal lesions and the acinar parenchyma was replaced by stromal or desmoplastic fibroblasts and inflammatory cells. This fibroinflammatory reaction is highly reminiscent of that seen in human pancreatic cancers. PanIN lesions show evidence of histologic progression and it has been demonstrated that these PanINs activate quiescent pathways such as Notch. These mice have increased Hes1 and Cox2, components of the prostaglandin pathway involved in the inflammatory response and increased ma-

trix metalloproteinase-7. Finally, at low frequency these mice progress to invasive and metastatic ductal adenocarcinoma within one year. In these mice, profuse hemorrhagic ascites was noted, the pancreas was large, firm and fibrotic and nodular densities were observed in liver, diaphragm, pleural surfaces and adrenal cortex.

This model developed by Hingorani *et al.*<sup>[39]</sup> shows progressive PanIN lesions and low-frequency progression to invasive and metastatic adenocarcinoma following activation of oncogenic K-Ras in mouse pancreas. The physiopathology and the sites of metastases observed in these mice are precisely found in human pancreatic ductal adenocarcinoma and further underscore the applicability of this model to study the human disease.

#### **PDX-1-Cre, LSL-Kras<sup>G12D</sup>, LSL-Trp53<sup>R172H/-</sup> transgenic model**

This mouse model was generated based on the previously described PDX-1-Cre, LSL-Kras<sup>G12D</sup> mouse. Using similar methods, Hingorani *et al.*<sup>[24]</sup> generated a conditionally expressed point mutant allele of the Li-Fraumeni human ortholog, Trp53<sup>R175H</sup><sup>[40]</sup>. Activation of both the Kras<sup>G12D</sup> and the Trp53<sup>R172H</sup> alleles occurs in tissue progenitor cells of the developing mouse pancreas through interbreeding with PDX-1-Cre transgenic animals. The presence of each rearranged, activated allele can be detected in the pancreata but not in tails. Thus, tissues not expressing Cre recombinase (non-pancreatic tissue) remain functionally heterozygous for these loci.

Four to six weeks old mice PDX-1-Cre, LSL-Kras<sup>G12D</sup>, LSL-Trp53<sup>R172H/-</sup> present early PanIN lesions similar to what it is observed in single PDX-1-Cre, LSL-Kras<sup>G12D</sup> mice. A significant disease burden is observed in animals by ten weeks of age at the earliest and the full spectrum of preinvasive lesions is apparent. Histological analyses reveal a predominant moderately well-differentiated to well-differentiated morphology organized as is observed

in the human disease. The carcinomas express CK19 and frequently contain mucin. Metastasis to the liver and lungs are similar to the pancreatic primaries. Finally, PDX-1-Cre, LSL-Kras<sup>G12D</sup>, LSL-Trp53<sup>R172H/-</sup> mice have dramatically shortened median survival of approximately 5 mo, significantly less than wild type, PDX-1-Cre, LSL-Trp53<sup>R172H/-</sup> and PDX-1-Cre, LSL-Kras<sup>G12D</sup>.

The triple mutant mice succumb earlier than PDX-1-Cre, LSL-Kras<sup>G12D</sup> animals which spontaneously develop PDA with a proscribed latency after manifesting preinvasive neoplasia. These triple mutant animals develop cachexia, abdominal distension, and hemorrhagic ascites. They also present metastasis in the liver, diaphragm and adrenals and all of them die before 12 mo.

### **PDX-1-Cre, Brca2<sup>F11</sup>, LSL-Kras<sup>G12D</sup>, Trp53 F2-10 transgenic model**

This transgenic mouse is a conditional Brca2<sup>F11</sup>, LSL-Kras<sup>G12D</sup>, Trp53 F2-10 and PDX-1-Cre and has been used as a model of pancreatic cancer, although the role of Brca2 in pancreatic cancer development is still unclear<sup>[41,42]</sup>. Brca2 plays a key role in the maintenance of genomic integrity, particularly through regulation of DNA repair by homologous recombination repair<sup>[43]</sup>, a process that is also controlled by another tumor suppressor protein, Brca1<sup>[44]</sup>. However, the significance of Brca2 in pancreatic cancer is not clear<sup>[45]</sup>.

While Rowley *et al.*<sup>[41]</sup> demonstrated that the inactivation of Brca2 promotes Trp53-associated but inhibits Kras<sup>G12D</sup>-dependent pancreatic cancer development in mice, Skoulidis *et al.*<sup>[42]</sup> showed that Brca2 heterozygosity promotes Kras<sup>G12D</sup>-driven carcinogenesis in the murine model of familial pancreatic cancer. In this model, the mouse expressed a functional wild type *Brca2* gene, in which exon 11 of Brca2 is flanked by loxP sites (B2<sup>F11</sup>). Conditional rearrangement of this allele in the developing pancreas in response to PDX-1-Cre expression results in the deletion of Brca2 exon 11, and the generation of a functionally null Brca2 allele (B2<sup>Δ11</sup>). These authors crossed CB2<sup>Δ11/Δ11</sup> mice with conditional Trp53F2-10/F2-10 (P) mice, in which exons 2 and 10 are flanked by loxP sites to generate Trp52 null CPB2<sup>Δ11/Δ11</sup>, CPB2<sup>wt/Δ11</sup> and CPB2<sup>wt/wt</sup> mice.

CPB2<sup>Δ11/Δ11</sup> mice develop pancreatic cancer at high frequency and their median survival is 300 d, showing substantially reduced pancreatic cancer-free survival relative to CB2<sup>wt/Δ11</sup>. However, in contrast, CB2<sup>Δ11/Δ11</sup>, CB2<sup>wt/Δ11</sup> and CB2<sup>wt/Δ11</sup> mice expressing wild type Trp53 alleles failed to develop pancreatic cancer.

This mouse model shows that the inactivation of Brca2 alone does not promote pancreatic cancer, but the disruption of Trp53 signaling in combination with the inactivation of Brca2 promotes pancreatic cancer formation. CPB2<sup>Δ11/Δ11</sup> mice display severe acinar cell dysplasia and a reduced number of islets. The pancreas is atrophic with acini replaced by mature adipose tissue, inflammatory infiltrates and little evidence of fibrosis. In contrast, in CPB2<sup>wt/Δ11</sup> and CPB2<sup>wt/wt</sup> mice the dysplasia, atrophy

and chronic inflammatory infiltrate is less severe and frequent<sup>[41]</sup>. The mouse model combining Brca2<sup>F11</sup> and LSL-Kras<sup>G12D</sup> (K) shows that CKB2<sup>Δ11/Δ11</sup>, CKB2<sup>Δ11/Δ11</sup> and CKB2<sup>wt/wt</sup> mice display normal development although CKB2<sup>wt/Δ11</sup> and CKB2<sup>wt/wt</sup> present PanINs and metaplastic lesions at 8 mo but not CKB2<sup>Δ11/Δ11</sup>. This mouse model showed that the loss of Brca2 tumor suppressor inhibits the development of premalignant lesions and pancreatic tumors that are induced by activated Kras. Only 13% of CKB2<sup>Δ11/Δ11</sup> mice develop tumors, whereas 66% of CKB2<sup>wt/Δ11</sup> and 61% of CKB2<sup>wt/wt</sup> develop pancreatic tumors with an average latency of 366 and 406 d, respectively<sup>[41]</sup>.

Skoulidis *et al.*<sup>[42]</sup> described a mouse model PDX-1-Cre-Kras<sup>G12D</sup> with two distinct mutant alleles of Brca2. The first encodes a germline truncating allele Brca2<sup>Tr</sup> (Tr), that mimics Brca2 human mutations in pancreatic cancer, and the second is a conditional deletion (F11) in which LoxP sites flank Brca2 exon 11 and emulates the loss of heterozygosity observed in human cancers.

Homozygous Brca2 inactivation in KPCB2<sup>Tr/Δ11</sup> mice displays pancreatic cancer in high penetrance with rapid and predictable clinical decline. The median survival was 84 d compared with the KPCB cohort whose median survival was 168 d. Mice with germline heterozygosity for Brca2<sup>Tr</sup> display pancreatic carcinogenesis, as even KCB<sup>Tr/wt</sup> mice with wild type Trp53 and mutant Kras-G12D in which pancreatic cancer is reported to develop less readily<sup>[39]</sup>. There is a reduction in PDAC-free survival of KCB<sup>Tr/wt</sup> mice in comparison with KCB controls with wild type Brca2. The pancreatic tumors observed in these mice display histological features similar to human pancreatic cancers with desmoplastic stroma. These tumors evolved with pancreatic intraepithelial neoplasia and metastatic behavior.

Interestingly, the KPCB<sup>Tr/Δ11</sup> mice which carry biallelic Brca2 mutations uniquely develop an acinar cell carcinoma component in 18% of cases, not observed in the other cohorts with Brca2 heterozygosity. This model shows that Brca2 inactivation promotes Kras-driven pancreatic malignancies<sup>[42]</sup>.

### **Mist1<sup>KrasG12D/+</sup> transgenic model**

To generate this transgenic model, Tuveson *et al.*<sup>[25]</sup> used homologous recombination to target the expression of Kras<sup>G12D</sup> to the Mist1 locus, a gene known to be expressed at earlier stages of pancreatic exocrine development. Mist1 is a basic helix-loop-helix transcription factor that is expressed at low levels in the embryonic pancreas at day 10.5<sup>[43,46,47]</sup> and in the adult, Mist protein is restricted to mature pancreatic acinar cell and is not found in ductal or islet cells<sup>[48,49]</sup>. Mist1<sup>KrasG12D/+</sup> mice have a diminished median survival of 10.8 mo compared with 24.2 mo in control wild type mice. Newborn mice show acinar hyperplasia with an increased proliferative index and acinar adenomas at 2 mo known as “acinar-ductal metaplasia”. Metaplastic ductal structures with mucinous cytoplasm that resemble murine PanIN-IA are found in the pancreas in close association with metaplastic acini. These metaplastic ducts are

characterized by the presence of CK19 and acidic mucin staining with alcian blue. At three months of age they become cachectic with pancreatic tumors and metastasis. Most of these tumors are acinar although some of them are cystic papillary neoplasms with acinar differentiation. Surprisingly, these mice also develop early and advanced hepatocellular carcinoma and some of them succumb before invasive pancreatic carcinoma.  $Mist1^{KrasG12D/+}$  mice die of advanced pancreatic exocrine carcinoma.

#### **PDX1-Cre, $Kras^{G12D}$ , $Ink4a/Arf^{flox/flox}$ transgenic model**

As the loss of function of the G1 cyclin-dependent kinase inhibitor, INK4A, appears to be a near universal event in pancreatic adenocarcinoma when there is an alternate reading frame or distinct first exon in the INK4A/ARF locus<sup>[50-52]</sup>, transgenic mice with this modification have been studied.

It was shown that mice with a constitutive deletion of both or either component of the  $Ink4a/Arf$  locus do not develop spontaneous pancreatic cancer<sup>[53]</sup>. Aguirre *et al.*<sup>[23]</sup> demonstrated the cooperative interaction between  $Ink4$  and  $Kras$  using mice engineered with Cre-mediated activation of mutant  $Kras$  ( $Kras^{G12D}$ ) and the deletion of a conditional  $Ink4/Arf$  tumoral suppressor allele.

In this model, the LSL- $Kras^{G12D}$  allele is expressed at the endogenous level after Cre mediates the expression of a transcriptional stopped element. The conditional  $Ink4a/Arf$  allele ( $Ink4/Arf^{flox}$ ) was engineered to sustain Cre-mediated excision of exon 2 and 3, thereby eliminating p16<sup>Ink4</sup> and p19<sup>Arf</sup> proteins. The double engineered mouse expressed the  $Kras^{G12D}$  allele and lack of both copies of the conditional  $Ink4/Arf$  allele specifically in the pancreas after using the PDX-1-Cre transgene. Between 7 and 11 wk of age, PDX-1-Cre,  $Kras^{G12D}$   $Ink4a/Arf^{flox/flox}$  mice show weight loss, ascites, jaundice and pancreatic tumors ranging in diameter from 4 to 20 mm. These pancreatic tumors are highly invasive, frequently involving the duodenum, stomach and spleen but no liver or lung metastasis. Furthermore, invasion of the lymphatic and vascular system is detected, an observation suggestive of metastatic potential of these neoplasms.

Consistent with a ductal phenotype, the tumors are positive for CK-19, DBA lectin and show stromal collagen deposition. In contrast, they do not show reactivity for amylase and insulin.

In conclusion,  $Kras^{G12D}$  expression in combination with  $Ink4a/Arf$  deficiency resulted in an earlier appearance of PanIN lesions and these neoplasms progressed rapidly to highly invasive and metastatic cancers, resulting in death in all cases by 11 wk.

#### **PDX1-Cre, $Kras^{G12D}$ , $Smad4^{flox/flox}$ transgenic model**

Although selective SMAD4 has no discernable impact on pancreatic development or physiology, when combined with the activated  $KRAS^{G12D}$  allele, SMAD4 deficiency enabled rapid progression of  $Kras^{G12D}$ -initiated neoplasms including pancreatic tumors. The combination of  $Kras^{G12D}$  and SMAD4 deficiency resulted in the rapid development

of tumors resembling intraductal papillary mucinous neoplasia (IPMN), a precursor to PDAC in humans. The SMAD4 tumor suppressor gene encodes a transcription factor that is a central effector of transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>[50]</sup> and inactivating mutations in this gene are common in PDAC<sup>[54]</sup>. Bardeesy *et al.*<sup>[55]</sup> generated a conditional knockout allele of  $Smad4$  ( $Smad4^{lox}$ ) harbouring loxP sites flanking exons 8 and 9 in the mouse germline. They crossed  $Smad4^{lox}$  homozygous mice to either the PDX1-Cre or Ptf1a-Cre transgenic mice. Mice with a homozygous deletion of  $Smad4$  in the pancreas showed no evidence of any gross anatomic or physiological abnormalities, and exhibited normal pancreatic cytoarchitecture and differentiation.

In contrast, LSL- $Kras^{G12D}$ - $Smad4^{lox/lox}$  mice showed low-grade PanINs and acinar-ductal metaplasia from 4 wk of age, an abdominal mass between 7 and 12 wk and reached terminal morbidity between 8 and 24 wk of age and a tumor-free survival of 13-15 wk. The pancreatic tumors were positive for cytokeratin 19, Shh, Hes1, phospho-stat3, mucin, Muc1, Muc4 and Muc5AC, but lacked acinar (amylase) and islet (insulin) marker expression. Mice showed palpable abdominal masses between 7 and 12 wk of age, and reached terminal morbidity between 8 and 24 wk of age.

Since the combination of  $Kras^{G12D}$  expression and  $Smad4$  deletion showed a rapid onset of IPMN and advanced PanIN lesions, but exhibited only moderate pancreatic malignant progression, and since SMAD4 loss occurs with concurrent INK4A loss and  $Kras$  activation in human PDAC, the authors developed a transgenic mouse PDX1-Cre,  $Kras^{G12D}$   $Ink4a/Arf^{flox/lox}$   $Smad4^{lox/lox}$ . These mice have significantly reduced survival, around 8 wk associated with PDAC and a small number of them also have IPMN and liver metastasis.

#### **Ptf1a<sup>cre/+</sup>, LSL- $Kras^{G12D/+}$ , $Tgfb2^{flox/flox}$ transgenic model**

TGF- $\beta$  signaling plays an important role in PDAC progression, as indicated by the fact that  $Smad4$ , which encodes a central signal mediator downstream from TGF- $\beta$ , is deleted or mutated in 55% of human PDAC<sup>[54,56-58]</sup>. Pancreas-specific  $Tgfb2$  knockout mice have also been generated, alone or in the context of active  $Kras^{G12D}$  expression. Ijichi *et al.*<sup>[27]</sup> crossed the LSL- $Kras^{G12D/+}$  mice with  $Tgfb2$  knockout mice<sup>[59]</sup> (previously developed) and generated mice of the genotype Ptf1a<sup>cre/+</sup>, LSL- $Kras^{G12D/+}$ ,  $Tgfb2^{flox/flox}$ . These mice had active  $Kras^{G12D}$  expression plus  $Tgfb2$  knockout both in a pancreas epithelium-specific manner.

Ptf1a<sup>cre/+</sup>,  $Tgfb2^{flox/flox}$  mice did not have pancreas development effects or discernable pancreatic cancer phenotype during 1.5 years.

In contrast, Ptf1a<sup>cre/+</sup>, LSL- $Kras^{G12D/+}$ ,  $Tgfb2^{flox/flox}$  mice had abdominal distension due to ascites, weight loss, and jaundice at 6-7 wk of age. Finally, these mice developed well-differentiated PDAC with 100% penetrance and a median survival of 59 d. Tumors are always accompanied by a whole panel of mPanINs and acinar-ductal metapla-

sia lesions from 3.5 wk and mice frequently have liver and lung metastases, direct invasion to the duodenum, and peritoneal dissemination.

While Ptf1a<sup>cre/+</sup>, LSL-Kras<sup>G12D/+</sup>, Tgfr2<sup>fllox/+</sup> mice show normal pancreas histology, tumors from Ptf1<sup>acre/+</sup>, LSL-Kras<sup>G12D/+</sup>, Tgfr2<sup>fllox/fllox</sup> mice exhibited uniformly well-differentiated glandular architecture, which occupied the entire pancreas, resulting in almost complete loss of normal pancreatic tissue. Tumoral cells show positive ductal markers, CK19 and mucin, and are negative for the acinar and islet markers, amylase and insulin, indicating ductal adenocarcinoma. In addition, these tumors are rich in stromal component, positive for vimentin and smooth muscle actin staining.

In conclusion, Tgfr2 knockout mice combined with Kras<sup>G12D</sup> expression developed well-differentiated PDAC with 100% penetrance and a median survival of 59 d. Moreover, a distinct and important feature of this mouse model is that the Ptf1a<sup>cre/+</sup>, LSL-Kras<sup>G12D/+</sup>, Tgfr2<sup>fllox/fllox</sup> tumors did not show sarcomatoid architecture, which was seen in one-third of the KrasG12D, Ink4a/Arf knockout model<sup>[23]</sup>.

## XENOGRAFT MOUSE MODELS

Tumor xenograft mouse models have been commonly used in preclinical studies for the last few years<sup>[60-62]</sup>. Human tumor xenograft models are created by the injection of human tumor cells grown from culture into a mouse or by the transplantation of a human tumor mass into a mouse. The xenograft may be readily accepted by immunocompromised mice such as athymic nude mice or severely compromised immunodeficient mice<sup>[63]</sup>. Xenografts show different advantages as they mimic genetic and epigenetic abnormalities that exist in tumors, can be used in the development of individualized molecular therapeutic approaches and can be implanted into the same organ to reproduce the organ microenvironment or the tumor<sup>[63]</sup>.

There are two main types of human xenograft mouse models used for pancreatic cancer research, heterotopic and orthotopic, defined by the location of the implanted xenograft.

### Heterotopic xenograft model

For heterotopic subcutaneous models, the xenograft is implanted between the dermis and underlying muscle and is typically located on the flank, on the back or the footpad of the mice. For many years, the subcutaneous xenograft model has been the most widely used preclinical mouse model for cancer research because it is rapid, inexpensive, reproducible, and has been considered sufficiently preclinical to test anti-cancer drugs. The subcutaneous model also has the advantages of providing visual confirmation that mice used in an experiment have tumors prior to therapy; and provides a means of assessing tumor response or growth over time, compared to intracavitary models where animal survival is the sole measure

of response<sup>[64]</sup>.

Different studies have used tumor engraftment in nude mice to study the possible response to chemotherapy treatment such as gemcitabine<sup>[65]</sup> or new pharmacological blocking agents<sup>[66]</sup> obtaining good results and suggesting new potential treatment options for pancreatic cancer.

One of the disadvantages of the heterotopic model is that it was observed that drug regimens that are curative in these models often do not have a significant effect on human disease as the subcutaneous microenvironment is not relevant to that of the organ site of primary or metastatic disease. Additionally, subcutaneous tumor models rarely form metastases. These observations suggest that heterotopic tumor models that do not represent appropriate sites for human tumors are not predictive when used to test responses to anti-cancer drugs<sup>[60,67,68]</sup>.

### Orthotopic xenograft model

Orthotopic tumors are transplanted to the appropriate organ in the mouse. For example, human pancreatic cancer cells are injected into the mouse pancreas and not into the skin on the mouse's back. Advantages of orthotopic models include use of the relevant site for tumor-host interactions, the development of metastases, the ability to study site-specific dependence of therapy, organ-specific expression of genes and the clinical scenario can be replicated. Major disadvantages are that orthotopic tumor xenograft generation is labor intensive, technically challenging, expensive, requires longer healing and recovery time and that monitoring tumor volume requires relatively lower throughput imaging methods<sup>[67]</sup>. Nonetheless, orthotopic tumor models are emerging as the preferred model for cancer research due to the increased clinical relevance.

To study pancreatic cancer, the standard procedure uses anesthetized mice 6-8 wk old. The abdominal skin and muscle are incised just off the midline and directly above the pancreas to allow visualization of the pancreatic lobes; the pancreas is gently retracted and positioned to allow direct injection of tumoral cells. The pancreas is replaced within the abdominal cavity; and both the muscle and skin layers are closed with surgical glue. Following recovery from surgery, mice are monitored and weighed daily to evaluate the tumor or response to treatment<sup>[61]</sup>.

These models have been employed to study gene expression profiling of liver metastases and tumour invasion in pancreatic cancer<sup>[69]</sup> in basic research. In translational medicine, orthotopic models have been used to evaluate the antitumor efficacy of gemcitabine plus emodin<sup>[70]</sup>.

In conclusion, different *in vivo* models of pancreatic cancer have been developed for the evaluation of multiple chemotherapeutic drugs and to study the molecular mechanisms implicated in resistance to different treatments.

These models are now available to investigate basic and translational aspects, but multiple considerations should be kept on mind for model selection depending on the purpose. The optimal model system should investigate

**Table 2** Comparison of mouse models for the clinical approach in pancreatic cancer

Mouse model	Cost	Time consuming	Clinical approach	Clinical reproducibility (human disease)
Transgenic engineered	++++	++++	+	++++
Xenograft heterotopic	+	+	++++	+
Xenograft orthotopic	++	++	+++	++

+: Low; ++: Medium; +++: High; ++++: Very high.

invasiveness or metastasis, the criteria for assessing response and altered molecular pathways, expression of markers and time expression and tumor development are some of the most important factors (Table 2).

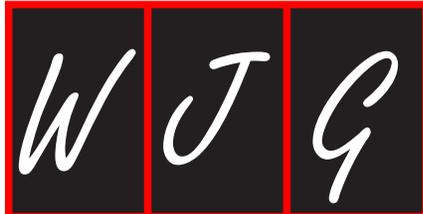
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## Magnifying endoscopy in upper gastroenterology for assessing lesions before completing endoscopic removal

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### Abstract

Any prognosis of gastrointestinal (GI) cancer is closely related to the stage of the disease at diagnosis. Endoscopic submucosal dissection (ESD) and *en bloc* endoscopic mucosal resection (EMR) have been performed as curative treatments for many early-stage GI lesions in recent years. The technologies have been widely accepted in many Asian countries because they are minimally invasive and supply thorough histopathologic evaluation of the specimens. However, before engaging in endoscopic therapy, an accurate diagnosis is a precondition to effecting the complete cure of the underlying malignancy or carcinoma *in situ*. For the past few years, many new types of endoscopic techniques, including magnifying endoscopy with narrow-band imaging (ME-NBI), have emerged in many countries because these

methods provide a strong indication of early lesions and are very useful in determining treatment options before ESD or EMR. However, to date, there is no comparable classification equivalent to "Kudo's Pit Pattern Classification in the colon", for the upper GI, there is still no clear internationally accepted classification system of magnifying endoscopy. Therefore, in order to help unify some viewpoints, here we will review the defining optical imaging characteristics and the current representative classifications of microvascular and microsurface patterns in the upper GI tract under ME-NBI, describe the accurate relationship between them and the pathological diagnosis, and their clinical applications prior to ESD or *en bloc* EMR. We will also discuss assessing the differentiation and depth of invasion, defying the lateral spread of involvement and targeting biopsy in real time.

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**Key words:** Magnifying endoscopy with narrow-band imaging; Upper gastroenterology; Assessment; Endoscopic submucosal dissection; Endoscopic mucosal resection

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### INTRODUCTION

Gastrointestinal (GI) cancer is a major medical and economic burden worldwide. Esophageal and gastric cancers remain a considerable source of morbidity and mortality in Asian countries. For instance, in Linxian, Henan province (China), cancer of the upper GI tract is endemic.

Mortality rates for esophageal cancer in Linxian exceed the American average (for white men) one hundredfold<sup>[1]</sup>. The prognosis of GI cancer is closely related to the stage of disease at diagnosis, and most cases are still detected at advanced stages and result in a relevantly poor outcome<sup>[2]</sup>. Early detection of these neoplasms or their precursors may be the only chance to reduce this high mortality.

Early GI cancers - such as Barrett's esophagus (BE) with high-grade dysplasia and early gastric cancer (EGC)- whose invasion is limited to the mucosa or submucosa regardless of the size or the presence of regional lymph-node and distant metastasis<sup>[3]</sup>, confer a survival rate of greater than 90% in 5 years in many centres<sup>[4,5]</sup>.

The screening program for gastric cancer in Japan indicates that 53% of diagnosed gastric cancers are localized lesions. Additionally, the accumulated clinical experience and formal outcome studies have shown that the majority of early-stage neoplastic lesions is localized with a low risk of lymph node metastasis. Recent data from 3261 patients who underwent gastrectomy with meticulous D2-level lymph node dissection over a 30-year period show that lymph node invasion was observed in only 2.7% of mucosal tumors and 18.6% of EGC invading the submucosa<sup>[6]</sup>. Clinical experience suggests that complete resection of the cancer is possible, and a cure can be achieved as long as the potential for metastatic spread is definitively excluded<sup>[7,8]</sup>.

Based on the above knowledge, the doctors began to try to use endoscopes for local excision with GI early tumors *in situ*, invading lamina propria or submucosa. More than a decade ago, endoscopic mucosal resection (EMR) technique emerged first in Japan as a critical tool in the management of patients with both high-grade dysplasia and superficial carcinomas<sup>[9]</sup>. But the indication of EMR is generally limited to mucosal tumors less than 2 cm in size even with the series of improvements that have been most widely used in recent years, such as using a transparent cap-fitted endoscope to suck targeted lesions into the cap and resect them with a snare (EMR-C) or a ligation device (EMR-L). All above EMR technologies are difficult to resect *en bloc* tumors larger than 2 cm in size, which is required for accurate and reliable pathological examination. However, though some endoscopists adopt piecemeal EMR techniques in order to cure the larger lesions, further investigation has revealed it involves problems such as remnants or high recurrent rates due to incomplete resections<sup>[10]</sup>. Thus, to overcome the problem of EMR techniques, a recent key issue in the field of therapeutic endoscopy is the development of a new therapeutic strategy for early GI cancers using endoscopic submucosal dissection (ESD). In this procedure, submucosal dissection is carried out by using an electrocautery knife to acquire a single-piece specimen, which is the gold-standard technique for offering *en bloc* resection of large superficial tumors in the GI tract, especially when R0 resection cannot be performed with other resection techniques. Within only a few years, ESD has become widespread in Asian countries - such as Japan, Korea and China - where there is a large volume of early upper GI

lesions that need endoscopic treatment. However, there are hardly any reports about long-term results after ESD, and the procedure involves a much higher complication rate and requires much higher skills<sup>[11,12]</sup>.

The two endoscopic local procedures are increasingly accepted by many patients and doctors mainly because they (1) **provide new alternatives for minimal invasiveness**; (2) **are perhaps the first approximations to true intraluminal resection of superficial malignant GI neoplasms**; and (3) **yield results that are comparable to surgery**. They also result in lower morbidity rates, lower costs and better quality of life than traditional surgery because of tissue preservation. But the difficulty lies in achieving *en bloc* or R0 resection and getting improved survival that precisely assesses resection margins and the depth of malignant invasion prior to performing EMR or ESD. The lesions with undifferentiated histology, lymphatic or vascular involvement and submucosal invasion were excluded due to possible lymph node metastases<sup>[5]</sup>.

Therefore, a thorough preoperative endoscopic examination is considered necessary for selecting the appropriate therapeutic modality. Due to this requirement, endoscopic equipment has improved markedly with respect to resolution in recent years. However, in 1967, Okuyama *et al*<sup>[13]</sup> produced a magnification endoscope for viewing the gastric mucosa. At present, magnification endoscopes have the ability to enlarge the image from 1.5 × to 150 × and produce images that have pixel densities as high as 850 000, allowing the discrimination of objects that are only 10-71 μm in diameter<sup>[14]</sup>. The newest magnification endoscopes permit magnification without loss of resolution<sup>[15]</sup>. Nevertheless, it was reported recently that some GI disorders, such as intestinal metaplasia, often appear translucent when observed with magnification endoscopy alone. Thus, the mucosal surface cannot be easily examined without staining<sup>[16]</sup>. Methylene blue, Lugol's iodine, and indigo carmine are several topical stains or pigments that have been used in conjunction with magnification endoscopy to improve tissue localization, characterization, or diagnosis during endoscopy<sup>[17]</sup>. The technique known as magnification chromoendoscopy (MCE) has been applied in a variety of clinical settings and throughout the GI tract for more than 10 years. In addition, other newer technologies, including narrow band imaging (NBI), that have proved particularly helpful during gastrointestinal endoscopic examinations have been developing in recent years. This shows that the two techniques have a similarly high sensitivity for detecting early neoplasia in the upper GI tract<sup>[18,19]</sup>. However, compared with MCE, the "electronic dyeing endoscopy," such as NBI, that are based upon the phenomenon that the depth of light penetration depends on its wavelength, are more user-friendly because their filters can be manually enabled and disabled during endoscopy, making it easy to switch them between the standard mode and the "electronic dyeing" mode, and no staining agents are required. Beyond these practical advantages, NBI reveals the superficial capillary network with a high contrast due to absorption of the blue light by hemoglobin, whereas the vascular pattern is

often less visible in chromoendoscopy<sup>[20]</sup>. When magnifying endoscopy is combined with narrow band imaging (ME-NBI), the combination has been shown to enhance visualization of the micromucosal and microcirculatory structure for a more detailed assessment of the early lesions<sup>[21]</sup>.

Hence, in many institutions, especially in Japan, MCE or the ME-NBI technique has been extensively included in standardized procedure and is performed in addition to conventional white-light endoscopy prior to ESD or EMR<sup>[22]</sup>. For the colorectum, “Kudo’s Pit Pattern Classification” has begun to be widely adopted by many endoscopists because it appeared valuable in the histological prediction from the observation of five-types pit patterns by MCE or NBI-although the microvascular observation is helpful as well<sup>[23]</sup>. In the upper GI, despite numerous studies from investigators around the world and especially in some Asian countries, there is still no consistent classification diagnosis system for ME-NBI before the endoscopic removal of esophageal and gastric lesions; each medical institution tends to adopt its own classification<sup>[24-36]</sup>. Therefore, here we will comprehensively review the literature in recent years on the main characteristics of microsurface (MS) and microvascular patterns, introduce their classifications that have become relatively popular in some Asian countries under ME-NBI, describe the accurate relationship between them, the pathological diagnosis for early lesions in the upper GI tract, and their clinical utility in ESD or *en bloc* EMR. We do this to help build consensus on observation flowcharts of ME-NBI and to help endoscopists recognize the classification of early upper GI lesions more clearly so that they can select the most appropriate therapeutic intervention.

## DEFINING OPTICAL IMAGING CHARACTERISTICS VISUALIZED UNDER MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING IN UPPER GASTROINTESTINAL

In general, the doctor inspects the patient first under white-light endoscopy without magnification. He then slowly moves the scope, washes the tissue well, and pays special attention to areas containing slight differences. The key endoscopic finding by using white light (WL) has been reported to be a change of color (slight redness) and pallid mucosa<sup>[37]</sup>. However, the margin is difficult to identify by conventional WL. Then, the NBI model was employed to make it easier to detect the change in colors and structure of the mucosa. Moreover, with magnification, the microvascular (MV) pattern and MS pattern can be evaluated. So, what will be seen under ME-NBI if the cancerous lesion is suspected within the area?

### Esophagus

**Brownish area:** A brownish area can often be recognized by NBI observation as distinct boundaries are formed between the tumor lesion and normal epithelium (Fig-

ure 1)<sup>[38]</sup>. An intraepithelial papillary capillary loop (IPCL) appears as brown dots under NBI-enhanced observation. For example, in the esophagus, if the lesion appears brownish under magnifying NBI observation, it will predict the possibility of mucosal squamous-cell carcinoma as a result of assessing the morphologic changes in the IPCL. The brownish areas in the esophagus visualized by NBI generally correspond to the Lugol chromoendoscopy displayed the lesions as unstained areas<sup>[39]</sup>.

**Intraepithelial papillary capillary loop:** It is well known that angiogenesis plays a critical role in the transition from premalignant to malignant lesions. Consequently, early detection and diagnosis based on morphological changes to the microvessels are crucial<sup>[40]</sup>. Superficial blood vessels in the esophageal mucosa consist of branching vessels and IPCL. However, in some cases, only the former can be observed under the WL that extend to the horizontal plane and exist immediately above the muscularis mucosa while IPCL that rises perpendicularly from a branching vessel can be observed through ME-NBI (Figure 2)<sup>[41]</sup>. In these cases, Muto *et al.*<sup>[43]</sup> have reported that a well-demarcated brownish area or an area of scattered brownish dots under NBI is connected with the proliferation of IPCL. This is a useful indicator for early esophageal squamous-cell carcinoma or high-grade intraepithelial neoplasia.

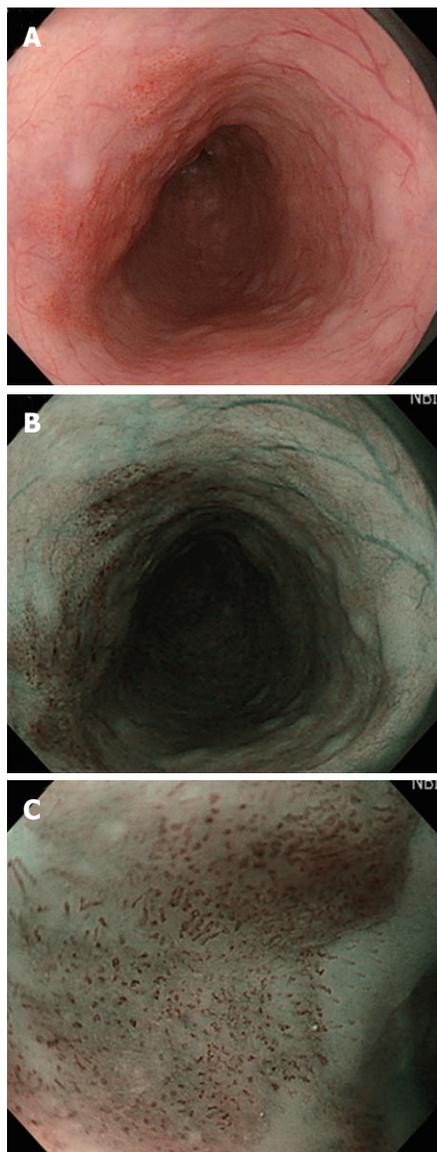
### Stomach

Besides the MV architecture, the imaging characteristics of the MS structure of mucosa the so-called pit or crypt patterns can be obtained by ME-NBI in the stomach (Figure 3).

### Subepithelial capillary network and collecting venule:

By ME-NBI, the subepithelial capillary network (SECN) and the collecting venule (CV) can be clearly visualized. A polygonal-shaped subepithelial capillary loop surrounding each pit forms a network in a regular arrangement, and this capillary network drains into a CV. SECN and CV are basic anatomical components for analysis of the MV architecture. The SECN shows two distinct patterns depending on the region of the normal stomach being imaged: The body mucosa demonstrates a regular honeycomb-like SECN pattern with a CV, whereas the gastric antrum shows a coil-shaped SECN but the CVs are rarely observed. This might be because the CVs in the antral mucosa are relatively deeper from the surface epithelium than those of the gastric body mucosa<sup>[44]</sup>.

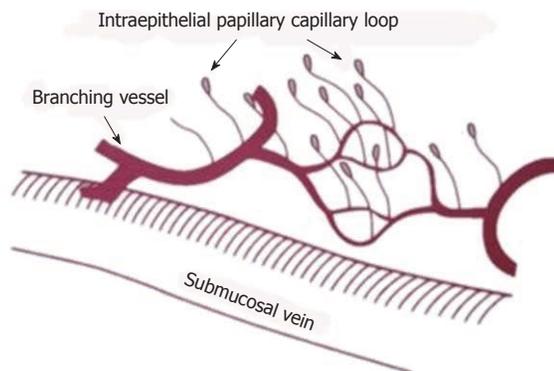
For the abnormal stomach, there are two characteristics of MV that can be identified by ME-NBI: the first is a relatively regular “fine network pattern” (Figure 3A), which is more likely to be observed in well-differentiated adenocarcinoma and appears as mesh and abundant microvessels connected with each other; the second is a “corkscrew pattern” (Figure 3B) as with isolated and tortuous microvessels, which often represents the low density of MV and corresponds to poorly-differentiated, depressed (0-IIc), early gastric adenocarcinoma<sup>[45]</sup>.



**Figure 1** The carcinoma visualized in esophagus. A: Carcinoma in esophagus is difficult to identify by conventional white light; B: Carcinoma in esophagus can be easily recognized by narrow-band imaging (NBI) as well-demarcated brownish area; C: Intraepithelial papillary capillary loop can be observed by magnifying endoscopy with NBI at the edge of the tumor.

Intrastructural irregular vessel (ISIV) (Figure 3C) also has an irregular MV pattern but often appears in the superficial flat gastric lesion (0-II b) as well as the marginal flat area of an elevated or a depressed lesion. This is a cancerous indication. Differing from the fine network pattern and corkscrew pattern shown in the areas where fine mucosal structure (FMS) disappear or are unclear in 0-II c gastric lesions, the ISIVs are found enclosed in villous or papillary FMSs and have characteristics of dilation, heterogeneity, abrupt caliber or tortuousness of shape<sup>[46]</sup>.

**Microsurface:** Applying ME-NBI is helpful for clearly visualizing not only some of the MV characteristics introduced above but also the gastric mucosal MS structures, namely pit or crypt opening patterns. The MS structures

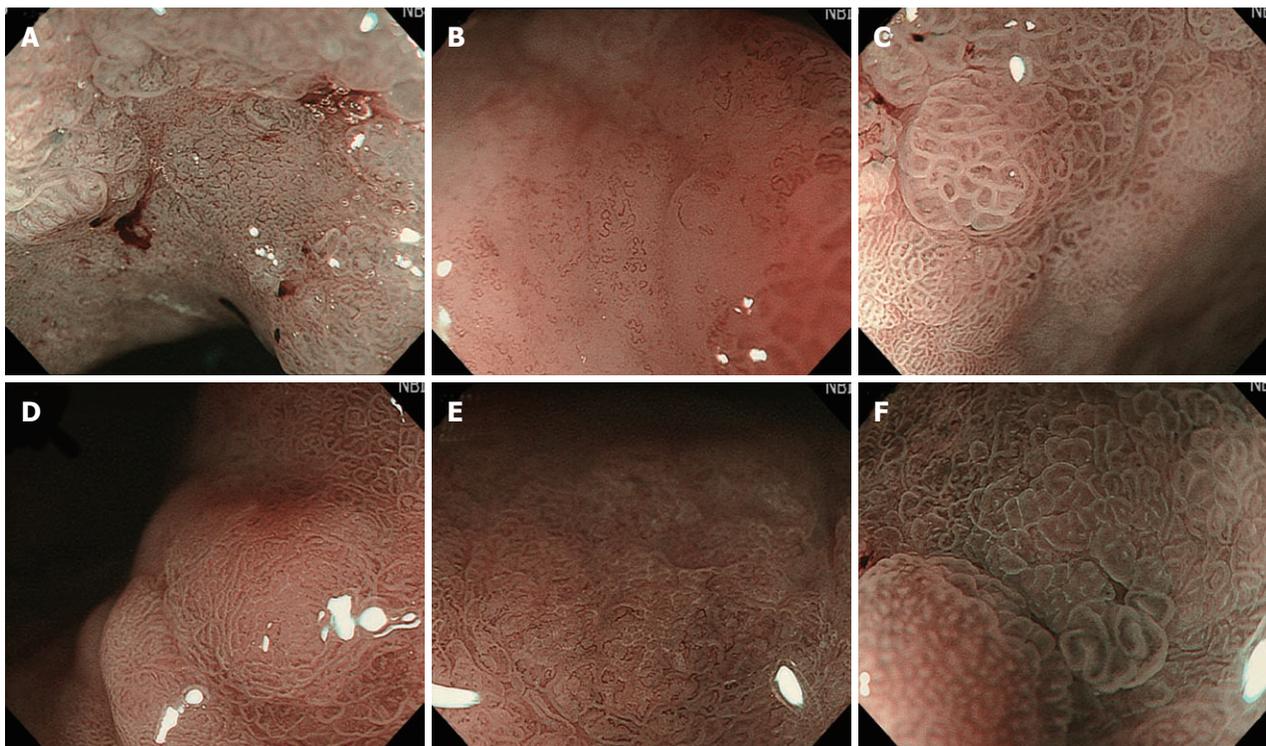


**Figure 2** The superficial blood vessels in the squamous esophagus (from Inoue *et al*<sup>[42]</sup>; with permission, making a little change for the original graph), the intraepithelial papillary capillary loop rises from the branching vessel and terminates in a diffuse.

include the FMS in a normal stomach as well as the irregular or loss of pit pattern that occurs with early gastric carcinomatous lesions.

Although it is necessary to assess a neoplasm in the stomach by the MV and MS patterns simultaneously, it is sometimes impossible to visualize the subepithelial MV pattern on account of overcurtaining by the white opaque substance (WOS). In most adenomatous lesions, the WOS is frequently observed more clearly under ME-NBI than WL and is speculated to be some intracellular component within the neoplastic epithelium of the intervening part between the crypts, obscuring the morphology of the subepithelial MV and causing difficulty in assessing the MV pattern. In such cases, rather than assessing the MV pattern, the morphology of the WOS could be an alternative new optical microstructure sign for distinguishing adenomas from adenocarcinomas. Yao *et al*<sup>[47]</sup> reported that only about 6% of the WOS was found in II b and II c lesions. For 0-II a type neoplasms, the WOS was more frequently visualized in adenomas (78%) than in carcinomas (43%) and showed a well-organized and symmetrical distribution of the dense WOS of a regular reticular/maze-like/speckled pattern (Regular WOS) (Figure 3D) within adenomas (100%), but showed a disorganized and asymmetrical distribution of the fine WOS of irregular reticular/speckled pattern (Irregular WOS) (Figure 3E) within carcinomas (83%). That is to say, the regular WOS is characteristic of adenomas, whereas its irregular distribution is characteristic for carcinomas.

Similar to the WOS, the light blue crest (LBC) (Figure 3F) is another characteristic optical microstructure under ME-NBI caused by the dense reflection of 400 to 430 nm short-wavelength light at the ciliated tissue. The LBC is defined as a fine, blue-white line on the crests of the epithelial surface/gyri, just at the edge of crypts. It has been suggested that the appearance of the LBC on the epithelial surface of the gastric mucosa may be a distinctive endoscopic finding associated with the presence of histological intestinal metaplasia in high sensitivity (89%), high specificity (93%), and high accuracy (91%)<sup>[48]</sup>. The LBC was also demonstrated to have a significant associa-



**Figure 3** Some typical microvascular and microsurface imaging characteristics visualized in stomach under magnifying endoscopy with narrow band imaging. A: Fine network pattern, mostly corresponding to well-differentiated adenocarcinoma (0-II c, gastric); B: Corkscrew pattern, mostly corresponding to the poorly-differentiated adenocarcinoma (0-II c, gastric); C: Intrastructural irregular vessel, enclosed in villous or papillary fine mucosal structure, had irregular shape characters such as dilation, heterogeneity, abrupt caliber or tortuosity (0-II b, gastric); D: Regular white opaque substance (WOS), that shows well-organized and symmetrical distribution with a regular reticular pattern and obscures the subepithelial microvascular (MV) pattern (0-II a adenoma, gastric); E: Irregular WOS, that is present within the cancerous epithelium with an irregular speckled pattern and makes the subepithelial MV pattern cannot be clearly visualized (0-II a cancer, gastric); F: Light blue crest, defined as a fine, blue-white line on the crests of the epithelial surface in the gastric mucosa may be a distinctive endoscopic finding associated with the presence of histological intestinal metaplasia.

tion with gastric atrophy and a high occurrence of gastric cancer<sup>[49]</sup>.

As noted above, the strategies for diagnosing upper GI lesions by ME-NBI are specific to different organs. Under a magnifying endoscope, an esophageal neoplasia could be diagnosed solely according to the findings from the MV pattern, namely IPCL, because the esophageal squamous epithelium does not show FMS. In contrast, a gastric neoplasia could be diagnosed with the findings of the MV pattern as well as the MS pattern<sup>[50,51]</sup>. Of course, sometimes the WOS or LBC is more useful for the diagnosis.

## CURRENT REPRESENTATIVE CLASSIFICATIONS OF MICROVASCULAR AND MICROSURFACE PATTERNS IN THE UPPER GASTROINTESTINAL UNDER MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING

### *Classifications of intraepithelial papillary capillary loops in the esophagus*

IPCLs beneath the basement membrane of the esophageal squamous epithelium can be observed by ME-NBI.

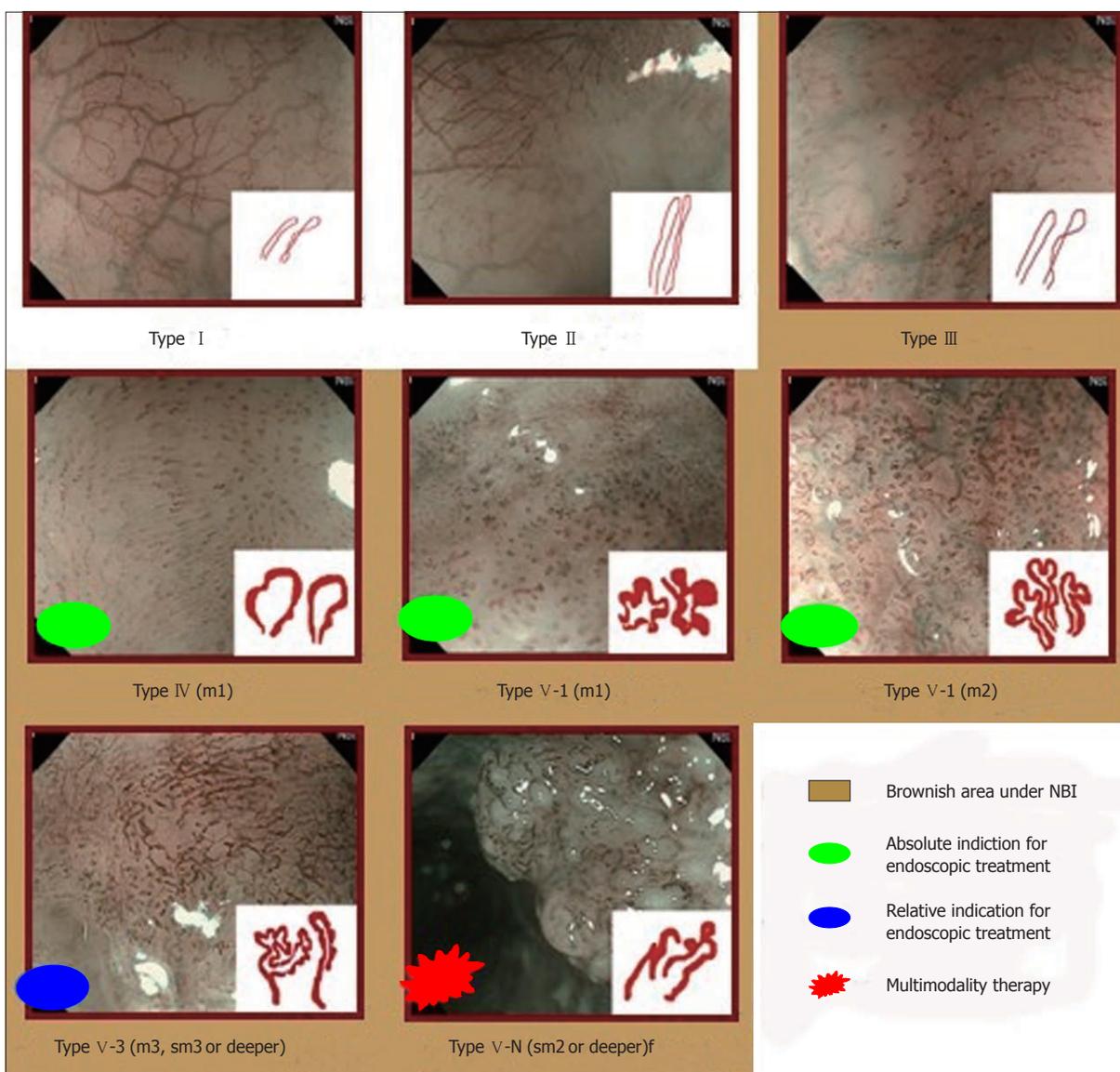
It has been shown that identifying IPCL changes is very important in predicating early lesions of the esophagus. Regarding the classifications of IPCLs, there have been several systems adopted by different researchers<sup>[26-28]</sup>, but in Japan, Inoue's classification and Arima's classification of IPCLs have been relatively popular.

**Inoue's classification of intraepithelial papillary capillary loop:** Under NBI, the IPCLs are easily recognized as brown spots, and the normal patterns appear as a smooth-running, small-diameter capillary vessel in the normal epithelium. The abnormal shapes appear as four typical changes: **Dilation, tortuous weaving, irregular caliber and form variation.** Inoue *et al.*<sup>[24,52]</sup> classed them into five types and several subtypes from type I to type V-N as below (Table 1 and Figure 4). IPCLs in type I is no different from the normal pattern. IPCLs in type II has one or two different characteristics: elongation and/or dilation is often seen. IPCLs in type III have no or few differences from the normal pattern, but this type differs from type I mainly in the features of color changes under NBI and iodine staining. Under NBI, the lesions of type I and type II often show no change or negligible change, but the types between type III and type V-N appear brownish. **In addition, type I and type II lesions are often positively stained with iodine while the types from**

**Table 1** Inoue's classification of intra-papillary capillary loop in esophagus

Typing	IPCL	Iodine staining	Under NBI	Pathological assessing	Treatment
Type I	Smooth running small diameter capillary vessel with no difference from the normal pattern	Stained		Normal epithelium	
Type II	Elongation and/or dilation capillary is often seen	Slightly stained		Esophagitis or re-generative tissue	
Type III	No or minimal change from the normal	Unstained	Brownish	HGIEN	Further follow-up
Type IV	Showing two or three of four patterns among dilation, meandering, caliber changes and different shapes	Unstained	Brownish	HGIEN or m1 carcinoma <i>in situ</i>	ESD/ <i>en bloc</i> EMR
Type V	Demonstrating all four characteristic changes: dilation, tortuous weaving, irregular caliber and form variation	Unstained	Brownish	M1 carcinoma <i>in situ</i>	
Type VI	Elongation basing on the shapes of type V IPCL, keeping IPCL partly	Unstained	Brownish	M2 carcinoma <i>in situ</i>	
Type VII	Destructuring dramatically and running on horizontal plane	Unstained	Brownish	M3-Sm1 deeper carcinoma	Relatively indicated for ESD/EMR
Type VIII	New tumor vessel appear	Unstained	Brownish	Sm2 deep carcinoma	Surgery, chemoradio-therapy

IPCL: Intraepithelial papillary capillary loop; NBI: Narrow-band imaging; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; HGIEN: High-grade intraepithelial neoplasia.



**Figure 4** The case examples of Inoue's intraepithelial papillary capillary loop classification from type I to type V-N. NBI: Narrow-band imaging.

type III to type V-N are negatively stained. IPCLs in type IV appear to have two or three of the four abnormal characteristic changes. IPCLs in type V-1 demonstrate all the four typical changes. IPCLs in type V-2 are elongated on the base of the four shapes and only keeping part of the original IPCL. IPCLs in type V-3 are further degraded and run on a horizontal plane. As for type V-N, the most remarkable feature is the appearance of new tumor vessels.

According to the grade of the changes of IPCL, the depth of invasion can be assessed. Type I mainly appears in normal epithelium. Type II corresponds to inflammatory changes or regenerative tissue. Type III often reflects low-grade intraepithelial neoplasia. Type IV is linked to with high-grade intraepithelial neoplasia (HIN) or M1 carcinoma *in situ*. Type V-1 is definitively diagnosed as M1 carcinoma *in situ*. The appearance of Type V-2 strongly suggests m2 carcinoma. Type V-3 often indicates m3 to sm1 deep lesions. Type V-N is often associated with sm2 invasion cancer. In short, type I to type V-1 demonstrate the characterization for flat lesions while type V-1 to type V-N reflect invasive cancers.

With treatment, lesions of type III IPCLs need further follow-up, and type IV to type V-2 should be considered for ESD or *en bloc* ESD. Type V-3 lesions are thought to be an indication for ESD or EMR because of the depth of invasion ranges between m3 and sm1. A complete biopsy should be applied before deciding on a treatment strategy. For type V-N, it is taken for granted that the surgical treatment or chemoradiotherapy should be recommended to counteract the significantly increasing risk of lymph node metastasis.

**Arima's classification of intraepithelial papillary capillary loop:** In 2005, Arima *et al*<sup>[25]</sup> reported another classification of the microvasculature of esophageal IPCLs under magnifying endoscopy. The microvascular patterns are categorized into four types (Figure 5). The thin, liner capillaries in subepithelial papillae are recognized as type I, resembling the shapes in normal mucosa. The vessels of type II become distended and dilated in subepithelial papillae, and the structure of capillaries is preserved. Most of them are usually found in lesions with inflammatory changes and are also associated with intraepithelial neoplasia. Spiral vessels with an irregular caliber and crushed vessels with red spots are characteristics of type III, which are often seen in m1 or m2 cancers. Type IV usually appears to be irregularly multilayered, irregularly branched, reticular vessels with an irregular caliber as generally observed in cancers with an m3 invasion or deeper. Avascular areas as well as stretched vessels are seen in cancers with downward growth. In addition, reticular vessels are commonly seen in poorly differentiated cancers, and the size of a vascular area surrounded by distended vessels is related to the depth of tumor invasion.

Comparing to the above two classification systems on the morphologic changes of IPCL and predicting the depth of the tumor invasion, it can be argued that type I

of Arima's classification partly corresponds to type I -type III of Inoue's classification. Furthermore, type II of Arima's classification partly corresponds to Inoue's type IV, Arima's type III partly to Inoue's type V-1 or V-2, and Arima's type IV partly to Inoue's type V-3 or V-N. However, the two systems do not always have such clear corresponding links. The invasion depth diagnosis by Inoue's classification is possible for most lesions, and the correct ratio is about 78%<sup>[24,52]</sup>. By contrast, when using Arima's type III and type IV classifications as diagnostic criteria for HIN and cancers, the rate of differential diagnosis goes up to 99%<sup>[25]</sup>. Recently, it has been reported<sup>[53]</sup> that some flat areas are not able to be predicted by Inoue's classification. However, combining the two classification systems could result in greater accuracy of the preoperative diagnosis, which is proved by the pathological diagnosis after ESD. Therefore, it is recommended for clinical endoscopists using Inoue's classification and Arima's classification together to make an invasion depth diagnosis of esophageal cancer under ME-NBI.

### Stomach

As for the MS of the stomach, in 1978, Sakaki *et al*<sup>[54]</sup> described the gastric pit appearances under magnifying endoscopy and classified them into five types: (1) foveolar pattern; (2) foveo-intermediate pattern (FIP); (3) foveolousulciform pattern; (4) sulciform pattern; and (5) mesh pattern. Although "Sakaki's classification" is still currently the most widely adopted classification by many Japanese endoscopists, not all gastric pathological changes can be expressed by this system because it is not consistent with structural changes under some pathological conditions<sup>[55]</sup>, which were found to have round and long elliptical gastric pits. The width of the FIP band seems to be related to the severity of atrophic gastritis, and the FIP is considered to indicate the position of the atrophic border.

Therefore, in 2002, Yagi *et al*<sup>[56]</sup> first reported a new modified classification system named the "A-B classification system," which is useful to describe typical micromucosal structures related to the development of *Helicobacter pylori* (*H. pylori*) gastritis. They classified the morphological changes in the glandular structure and microvascular architecture obtained by WL magnifying endoscopy into four types: (1) type Z-0: Gastric round pits resembling pinholes surrounded by a regular arrangement of collecting venules with SECN forming a network; (2) type Z-1: Irregular true capillaries but no collecting venules observed; (3) type Z-2: White gastric pits and sulci with neither collecting venules nor true capillaries being seen; and (4) type Z-3: Dilated pits with surrounding redness. Type Z-0 specifically indicated the *H. pylori*-negative mucosa and differed significantly from types Z-1, Z-2 and Z-3 with regard to the grade of inflammation, activity and presence of *H. pylori*.

More recently, with the development of brand new optical techniques, such as ME-NBI, which can clearly visualize not only the glandular structure but also the mucosal microvascular architecture in units as small as the capillary, the prior diagnostic classification system seemed

less able to meet clinical needs, especially for early diagnosing of premalignant lesions and assessing the relationship between microvessel patterns, pit patterns and histological patterns ahead of endoscopic *en bloc* resection. In recent years, many researchers modified the above classifications but varied individually<sup>[45,57-61]</sup>, and there is still no set of consistent classification guidelines. Nonetheless, the key characteristic findings of all the current classifications for ME-NBI with respect to early gastric carcinomatous lesions are based on the types of abnormal MV patterns and irregular MS patterns. Among these, the representative diagnostic system is advocated by Yagi *et al.*<sup>[62]</sup>, who established a flowchart for ME-NBI diagnosis in early gastric cancerous lesions as below: first, the “white zone” should be imaged, which is Yagi’s term for the border of the uniform or heterogeneous papillae in the mucosal MS structure that appears as a bold white line. Next, microvessels should be observed. A regular MV pattern means the microvessels appear regular in shape and arrangement and look like closed or open loops of uniform size caliber. An irregular MV pattern means the microvessels appear irregular in shape and arrangement, looking like tortuous or irregular branches of various sizes or abnormal caliber<sup>[47]</sup>. Then, according to the white zone, the MV pattern, the WOS, and the LBC, the histological imaging of entire mucosa should be done. (1) Fine network patterns and loop patterns are mostly associated with well- or moderately-differentiated adenocarcinoma; (2) irregular MV patterns, namely ISIVs, enclosed in villous or papillary FMSs can often be observed in II b gastric cancerous lesions; (3) corkscrew patterns or wavy microvessels mostly correspond to the poorly-differentiated adenocarcinoma; (4) regular WOSs often appear in II a gastric adenoma lesions while irregular WOSs often present in II a gastric cancerous lesions; (5) LBC is mostly connected with intestinal metaplasia<sup>[47,48,63]</sup>.

As a matter of course, with regard to the classification of early gastric lesions under ME-NBI, more in-depth studies are needed to address the more morphologically-complex microstructures of the stomach relative to the other parts of the GI system. Some features described previously are not general enough to apply to each lesion, and the number of cases in the studies is limited as well. At present, it is reasonable to use ME-NBI as a supplementary diagnostic tool to normal endoscopy with chromoendoscopy in the stomach before deciding on therapy strategies. The current strategies require new additions and some modifications.

### Barrett’s esophagus

BE is thought to be a complication of longstanding gastroesophageal reflux and a condition of the distal esophagus where normal squamous lining is replaced by columnar epithelium containing specialized intestinal metaplasia (SIM), which has the tremendous potential for developing esophageal adenocarcinoma with generally poor prognoses and a median survival rate of less than one year. Short BE is defined as < 3 cm and long BE as ≥ 3 cm<sup>[64]</sup>.

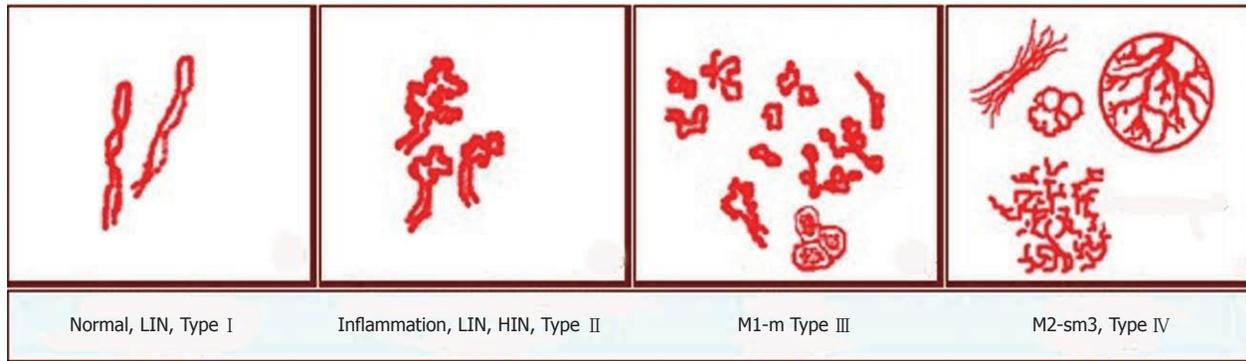
Using ME-NBI allows clear visualization of micro-mucosal and vascular patterns in BE. Now, depending on which targeted biopsy technique can be performed, improved distinction of nondysplastic SIM from HIN is possible. Recently, several pieces of literature<sup>[16,33,65,66]</sup> have reported their own classification systems, of which the principal features are summarized as follows: SIM is characterized by the mixing of villous, tubular and linear patterns with mostly regular arrangements and having regular vascular patterns or appearing as long, branching vessels in a flat mucosa. In addition, absent microstructural patterns also have a very high correlation to and predictive power for SIM. HIN is characterized by irregular/disrupted microstructural and irregular microvascular patterns, and the frequency of abnormalities shows a significant rise with increasing grades of dysplasia.

## USEFULNESS OF MAGNIFYING ENDOSCOPY WITH NARROW BAND IMAGING PRIOR TO ENDOSCOPIC SUBMUCOSAL DISSECTION OR EN BLOC ENDOSCOPIC MUCOSAL RESECTION

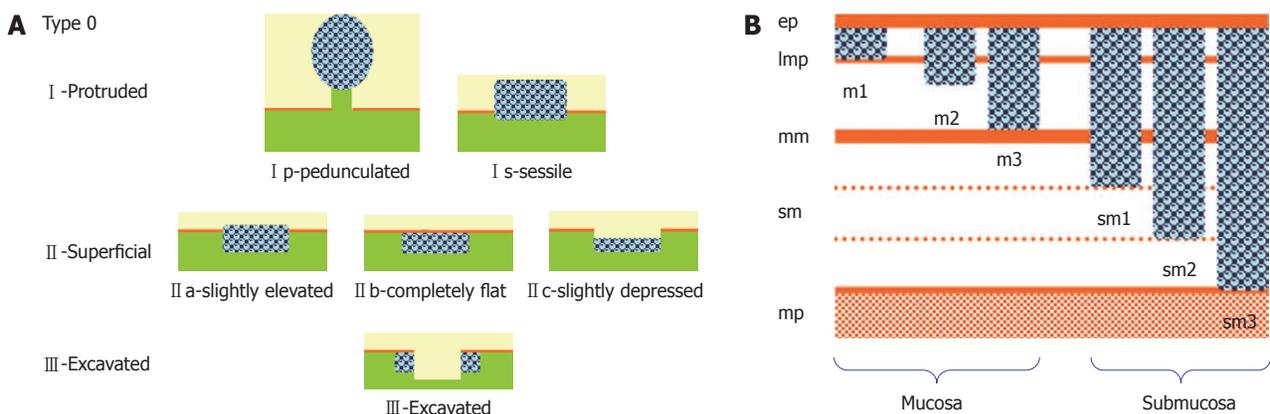
### To assess the differentiation and depth of invasion

**Criteria for endoscopic submucosal dissection/endoscopic mucosal resection:** Only some differentiation and invasion limited to sm1 lesions should be considered for endoscopic removal. Nowadays, in Asian countries, one of the widely adopted guidelines for ESD or *en bloc* EMR is that the histology of the tissues must be intramucosal, well-differentiated, early carcinoma, and the minute invasion of submucosal lesions must be limited to sm1—namely, with a depth less than 200 μm in the squamous epithelium of the esophagus and less than 500 μm in the stomach. If the lesion is recognized as undifferentiated, surgery should be recommended<sup>[6,67]</sup>.

Japan’s data show that the five-year cancer-specific survival rates of EGC limited to the mucosa and submucosa are 99% and 96%, respectively<sup>[67]</sup>. In other words, *en bloc* endoscopic treatment should be mainly applied to some category 0 superficial GI neoplastic lesions with the invasion limited to the mucosa or submucosa. These are divided into three subtypes according to the “Paris classification”: 0-I include I p and I s, referring to polypoid pedunculated and sessile respectively; 0-II are non-polypoid and non-excavated, and they are further subdivided into 0-II a for slightly elevated lesions, 0-II b for completely flat lesions, and 0-II c for slightly depressed lesions; 0-III are non-polypoid with an ulcer (Figure 6, left side)<sup>[68]</sup>. In order to get a more precise evaluation for choosing the appropriate therapy, endoscopists classify early GI cancer into the following subdivisions according to the depth of invasion: M1, carcinoma with questionable invasion carcinoma limited to the epithelium; m2, cancer invasion to the lamina propria; m3, cancer infiltration into the muscularis mucosa; sm1, to the upper third of submucosa; sm2, to the middle third; and sm3, to



**Figure 5** The morphology of Arima's intraepithelial papillary capillary loop classification in esophagus. LIN: Low-grade intraepithelial neoplasia; HIN: High-grade intraepithelial neoplasia.



**Figure 6** The Paris classification of early lesion of gastrointestinal tract (A) and the depth of tumor infiltration (B). ep: Epithelium; Imp: Lamina propria; mm: Muscularis mucosa; sm: Submucosa; mp: Muscularis propria.

the lower third. (Figure 6, right side)<sup>[69]</sup>. The distribution of subtypes in category 0 differs in the esophagus and stomach. As an example, the respective proportions of subtypes 0- I and 0- II c are 16% and 45% in the squamous epithelium of the esophagus, and they are 17% and 78% in the glandular epithelium of the stomach, respectively<sup>[70]</sup>.

Presently, the most critical factor in the decision of whether to perform ESD or *en bloc* EMR is the probability of unexpected lymph node metastasis. Studies have shown that early cancer without lymphovascular involvement could be cured by endoscopic removal. Intramucosal, moderately- or well-differentiated early carcinomas that have been proved do not have submucosal lymphovascular involvement. In contrast, poorly differentiated squamous-cell carcinoma, adenocarcinoma and/or signet-ring cell carcinoma have a high incidence of lymph node metastasis. M1 and m2 carcinomas have no metastasis, whereas less than 10% of m3 carcinomas and about 15%-20% of sm1 have lymph node metastasis. The risk increases to more than 50% of sm2 and sm3 carcinomas<sup>[7,67,71,72]</sup>. Therefore, before performing ESD or EMR, an accurate histological evaluation of the resected specimens is essential to avoid recurrence.

Magnified images obtained with the ME-NBI system could be a useful, non-invasive method of histologically predicting for early lesions in clinical practice, especially

with regard to the IPCL pattern in the esophagus and MV and MS patterns in the stomach, based on which alone usually could help us perform a successful endoscopic therapy. Many researchers focused on the relations between the ME-NBI classifications categories with the characteristics of the histopathological types. For example, regarding the depth of superficial esophageal cancer, the accuracy rate of diagnosis is about 83.3%, according to the Inoue's classification of IPCL<sup>[73]</sup>. And in the stomach, differentiated-type adenocarcinomas are mainly observed as fine-network patterns in about 15.7% of cases or loop patterns in about 83.8% of cases. Undifferentiated-type lesions are primarily characterized by the cork-screw pattern in approximately 58.8% of cases<sup>[57]</sup>. For HIN of BE, without the need for staining, the ME-NBI images have a sensitivity of 94% and a specificity of 76% as well as a positively predictive value of 64% and a negatively predictive value of 98%<sup>[65]</sup>.

Comparing the diagnostic accuracy of ME-NBI and endoscopic ultrasonography (EUS) for estimating the depth of invasion of early cancers before removal, some endoscopists conclude that the overall accuracy of ME-NBI is a little higher than EUS, but the difference is not statistically significant. However, ME-NBI is at least as accurate as EUS for preoperative locoregional staging of early cancers. On the other hand, EUS can be used for observing lymph nodes, but the diagnostic capability of

EUS for lymph nodes is less reliable, which can affect therapy-related decisions before ESD. Regarding this point, a consensus is still required. For some cases that are difficult to diagnose, it is even necessary to combine two stool tests with computed tomography before ESD or *en bloc* EMR<sup>[74-76]</sup>.

### To define the margin and size of involvement

**Criteria for endoscopic submucosal dissection/endoscopic mucosal resection:** For differentiated lesions, a size of  $\leq 2$  cm in diameter is an indication for EMR; a size of  $\leq 3$  cm of mucosal cancer with ulcers or sm1 submucosal cancers, and any size of mucosal cancer without ulcer are indication for ESD.

In Japan and a few other Asian countries, another current guideline for ESD or *en bloc* EMR regarding well-differentiated lesions is based on data relating the size of the early lesion and the rate of lymph node metastasis. For mucosal cancers with ulcers or sm1 submucosal cancers, lesions that are 3 cm or smaller present a negligible risk of venous or lymphatic involvement. These are indications for ESD. For larger lesions, surgery should be recommended. For lesions confined to the mucosa but without ulcers, the risk of lymph node metastasis is not affected by the size of the tumor, so there is no consensus on a maximal size, although circumferential lesions in the esophagus are usually avoided because of the potential for strictures. Because a 2 cm diameter is the upper limit for resection by EMR in one piece, if the lesions simultaneously meet the conditions of ESD and are not more than 2 cm large, these should also be reasonable indications for *en bloc* EMR treatment because this technique is easier than ESD<sup>[6,67,77,78]</sup>.

Therefore, prior to endoscopic treatment, it is absolutely necessary to accurately identify the full lateral spread of the margins of the lesion, which leads to the determination of the lesion's final size and contributes to the next step of making well-reasoned treatment decisions. In the upper GI, *en bloc* endoscopic removal needs to be carried out 2 mm outside the margin outlined by the spots. This is the key to ensuring that the complete R0 resection has a negative margin for the tumor cells and that the risk of local recurrence is reduced.

ME-NBI allows a more detailed observation of the mucosal changes of microstructures and microvessel patterns of GI carcinoma and is extremely useful, not only for identifying EGC itself, but also for differentiating the borders of cancerous tumors from background non-cancerous mucosa. By ME-NBI, the following points can help determine precise horizontal margins in clinical practice<sup>[44]</sup>: (1) **recognize a demarcation line** by the difference between an irregular MV or MS pattern and the surrounding regular normal mucosa. This has been proven to correspond to the tumor margins determined by histopathological examination; (2) **pay close attention** to the areas disappearing from the regular SECN pattern as well as the appearance of an ISIV pattern. Sometimes, WOSs are helpful for identifying tumor margins that have not been determined. Also, LBC is a specific indicator for

tumors derived from intestinal metaplasia by ME-NBI.

However, for IIb flat reddened lesions that have the same color as the surrounding normal mucosa, it is still occasionally difficult to detect the margins. On the other hand, accurate marking of tumors by ME-NBI also relies on an operator's skill. Therefore, in order to improve the accuracy rate of marking margins, many endoscopists combine ME-NBI with conventional chromoendoscopy. For example, Lugol's solution can dramatically outline the boundaries of a squamous cell esophageal cancer in the esophagus. Although one recent article has concluded that tumor margins can be identified more clearly by ME-NBI than by indigocarmine chromoendoscopy in the stomach<sup>[79]</sup>, it is likely that in the majority of cases, a combination of these two methods prior to ESD or EMR will ensure there are no residual lesions.

### To perform a target biopsy in real time

Before local endoscopic *en bloc* resection, the histopathologic diagnosis is very important for making therapy decisions. For a surveillance biopsy to detect early tumors, multiple random biopsies under conventional WL endoscopy are quite time-consuming and may miss a small lesion<sup>[80]</sup>. For example, for monitoring BE so far, the present recommended strategy is to perform random four-quadrant biopsies at every 2 cm. However, this approach is still prone to sampling errors, inconsistent histopathological interpretations, and delays in diagnosis<sup>[81]</sup>.

Ultimately, the higher-accuracy pathological diagnosis as well as the ultrarapid *in vivo* diagnosis would be preferred in clinical practice<sup>[82]</sup>. It has been reported that chromoendoscopy could provide a good validity score for early cancer targeted biopsies<sup>[83]</sup>. However, it still has its limitations, including spending time lost in spraying and washing out the dye. Moreover, some dyes-such as methylene blue-might induce DNA damage in columnar cell-lined mucosa<sup>[84]</sup>.

To this end, in recent years, many researchers suggest using the ME-NBI technique as an "optical biopsy" to better target biopsies in real time. Because this approach can provide better details of mucosa MV and MS patterns that significantly correlate with pathological diagnosis, it has the potential to reduce the need for histological examination of mucosal biopsy specimens<sup>[47,49,85,86]</sup>. Additionally, some endoscopists even think that ME-NBI can sometimes be substituted for a biopsy before endoscopic therapy because a biopsy might only focus on some suspected, poorly-differentiated lesions under magnifying endoscopy. However, to date, ME-NBI cannot always replace biopsies for histological assessment. In addition, ESD or *en bloc* EMR can supply specimens that are resected in one piece and provide more accurate histopathological diagnosis for determining whether the patient should receive an operation or other treatments<sup>[36,87-89]</sup>.

## CONCLUSION

In conclusion, ME-NBI is a very promising endoscopic technique that can clearly reveal detailed micromorpho-

logical differences corresponding to histology and provide some information about layer, origin, size, and extramural extension of GI early lesions. All of these benefits may augment the endoscopic R0 resection of early cancers in the GI tract and help guide targeted biopsies in the surveillance of certain high-risk conditions<sup>[19]</sup>. To some extent, ME-NBI has now become an indispensable tool in ultra-rapid *in vivo* diagnosis and immediate clinical decision-making, such as when performing ESD or EMR.

In this topic review, most representative references come from the experience of Japanese endoscopists because Japan remains the country with the most ESD cases reported around the world by far. Outside Asia, more recently, techniques such as magnification, NBI and ESD have been increasingly used although viewpoints differ between Eastern and Western cultures, especially regarding extending indications for ESD, the classifications of MV and MS under ME-NBI in the upper GI tract, and partly substituting EUS or biopsy with ME-NBI. However, current data is limited, and we would need long-term outcome data to unify some assessments in order to conduct multicenter trials to develop clear, internationally accepted classification systems. This system review was intended to make a small contribution to some of the aforementioned debates.

Additionally, besides ME-NBI, it is necessary to combine various endoscopic techniques including EUS and chromoendoscopy in some difficult cases before *en bloc* endoscopic resection. It is important to emphasize here that the first step should always be to look carefully for the suspected area by conventional WL endoscopy before switching to the ME-NBI model.

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## Probiotic modulation of dendritic cells co-cultured with intestinal epithelial cells

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### Abstract

**AIM:** To investigate cytokine production and cell surface phenotypes of dendritic cells (DC) in the presence of epithelial cells stimulated by probiotics.

**METHODS:** Mouse DC were cultured alone or together with mouse epithelial cell monolayers in normal or inverted systems and were stimulated with heat-killed probiotic bacteria, *Bifidobacterium lactis* AD011 (BL), *Bifidobacterium bifidum* BGN4 (BB), *Lactobacillus casei* IB5041 (LC), and *Lactobacillus acidophilus* AD031 (LA), for 12 h. Cytokine levels in the culture supernatants were determined by enzyme-linked immunosorbent assay and phenotypic analysis of DC was investigated by flow cytometry.

**RESULTS:** BB and LC in single-cultured DC increased the expression of I-Ad, CD86 and CD40 (I-Ad, 18.51 vs 30.88, 46.11; CD86, 62.74 vs 92.7, 104.12; CD40, 0.67 vs 6.39, 3.37,  $P < 0.05$ ). All of the experimental probiot-

ics increased the production of inflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$ . However, in the normal co-culture systems, LC and LA decreased the expression of I-A<sup>d</sup> (39.46 vs 30.32, 33.26,  $P < 0.05$ ), and none of the experimental probiotics increased the levels of IL-6 or TNF- $\alpha$ . In the inverted co-culture systems, LC decreased the expression of CD40 (1.36 vs -2.27,  $P < 0.05$ ), and all of the experimental probiotics decreased the levels of IL-6. In addition, BL increased the production of IL-10 (103.8 vs 166.0,  $P < 0.05$ ) and LC and LA increased transforming growth factor- $\beta$  secretion (235.9 vs 618.9, 607.6,  $P < 0.05$ ).

**CONCLUSION:** These results suggest that specific probiotic strains exert differential immune modulation mediated by the interaction of dendritic cells and epithelial cells in the homeostasis of gastrointestinal tract.

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**Key words:** Dendritic cells; Intestinal epithelial cells; Probiotics; Co-culture; Immune modulation

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### INTRODUCTION

The gastrointestinal (GI) tract is an immunologic organ

with continuous antigen exposure in the form of food, normal bacteria and pathogens. Despite numerous antigenic challenges, the complicated mucosal immune system maintains GI homeostasis *via* the concerted actions of the various mucosal immune cells. Dendritic cells (DC), dedicated antigen-presenting cells, modulate the immune balance in the GI tract<sup>[1]</sup>. DC can take up antigens directly by extending their dendrites into the lumen or indirectly after transport of the antigens by M cells overlying Peyer's patch<sup>[2,3]</sup>. Antigen-carrying DC may traffic through the lymphatics to the mesenteric lymph nodes<sup>[4]</sup>, mediating the homing of activated effector/memory T cells and IgA-secreting B cells<sup>[5,6]</sup> and inducing regulatory T cells to produce interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ <sup>[7,8]</sup>. These roles depend on the regulation of cell surface expression of co-stimulatory molecules and production of inflammatory chemokines and cytokines<sup>[9-11]</sup>.

DC can recognize and present microbial components using pattern receptor system which includes toll-like-receptor (TLR). TLR can interact with microorganism-associated molecules such as peptidoglycan, lipoprotein, and lipopolysaccharide<sup>[12-16]</sup>. *Bifidobacterium* and *Lactobacillus* are major components of the commensal microbes of the GI tract and are frequently used as probiotics<sup>[17,18]</sup>. Probiotics, defined as live microorganisms which, when consumed in appropriate amounts in food, confer a health benefit on the host<sup>[19]</sup>, exert various host physiological responses such as immunomodulatory effect<sup>[20]</sup>. Recent experiments reported that DC could be modulated by probiotics. Several *Lactobacillus* species could regulate DC surface expression and cytokine production<sup>[21]</sup>. In addition, the probiotics mixture VSL No. 3 upregulated the expression of major histocompatibility complex (MHC) class II and co-stimulation molecules<sup>[22]</sup>.

DC are often located close to epithelial cells, populating the subepithelial dome of Peyer's patches, immediately adjacent to the follicle-associated epithelium and the lamina propria<sup>[23,24]</sup>. Intestinal epithelial cells secrete many mediators, including functional peptides such as defensins, mucins, chemokines, and cytokines such as IL 8<sup>[25-27]</sup>. TLR5 on the epithelium is a key mediator of pro-inflammatory responses to flagella from commensal bacteria<sup>[28,29]</sup>. Flagella also stimulate the maturation of responsive DC<sup>[30]</sup>.

Interaction between DC and epithelial cells is integral to the intestinal immune system. We hypothesized that epithelial cells stimulated by probiotics could regulate the maturation of DC. Accordingly, the present study investigated the pattern of cytokine production and the surface phenotype of DC in the presence of epithelial cells polarized by heat-killed probiotic bacteria.

## MATERIALS AND METHODS

### Preparation of probiotic bacteria

*Bifidobacterium bifidum* BGN4 (BB) was isolated from healthy infant fecal matter and identified in our laboratory<sup>[31]</sup>. *Bifidobacterium lactis* AD011 (BL), *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA) were provided

by the Research Institute of Bifido Co. Ltd. (Hongchun, Gangwondo, South Korea). Four probiotic bacteria were anaerobically propagated in de Man, Rogosa, and Sharpe (Difco, Detroit, MI, United States) broth containing 0.05% L-cysteine (Sigma, St. Louis, MO, United States) at 37 °C until mid-log phase was reached. Subsequently, probiotics were inoculated at 1% and anaerobically cultured in de Man, Rogosa, and Sharpe (Difco) broth containing 0.05% L-cysteine (Sigma) at 37 °C. *Lactobacillus* species were incubated for 16 h, and *Bifidobacterium* species were incubated for 24 h to late log phase. The bacteria were collected by centrifugation at 1000 × *g* for 15 min at 4 °C and washed twice with phosphate-buffered saline (PBS). After washing, the bacteria were resuspended in 1 mL of PBS and incubated at 95 °C for 30 min to prepare heat-killed bacteria cells. The killed bacteria were collected by centrifugation at 1000 × *g* for 15 min and then lyophilized (Combi-514R, Hanil Science Industrial, Seoul, South Korea).

### Generation of CMT-93 monolayers

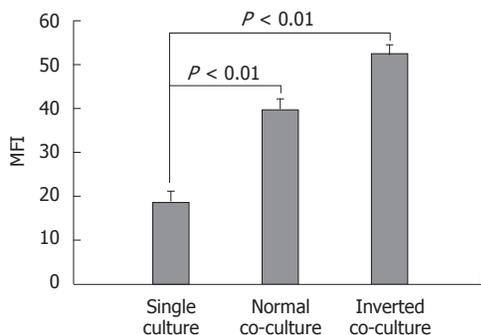
CMT93 was derived from carcinomas of C57BL mouse large intestine. The cells have an epithelial morphology and forms acini, junctional complexes, and microvilli with attached glycoprotein<sup>[32]</sup>. CMT-93 cells were maintained in DMEM (Gibco Life Technologies, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, United Kingdom) and 1% penicillin/streptomycin (Invitrogen), and were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Monolayers were grown in 24-well Corning Costar Transwell plates (Corning Inc., United States) with 3 μm pore-size filter inserts. In the normal co-culture system, 5 × 10<sup>5</sup> cells were seeded into the inserts, and the wells were filled with 1 mL medium. In the inverted co-culture system, inserts were removed and inverted in tissue culture dishes, and the cells of the same volume were seeded to the exposed filter membrane. The culture dishes were filled with enough medium to sink the inserts. The transwell inserts were cultured for 3-4 d until CMT-93 established monolayers. Confluence of the cells was confirmed when the trans-epithelial electrical resistance (TEER; Millicell ERS Ohmmeter, Millipore, Eschborn, Germany) exceeded the cut-off point of 250 Ω/cm<sup>2</sup>.

### JAWS II cell preparation

JAWS II, mouse bone marrow-derived immature DC<sup>[33]</sup>, were maintained in α-MEM (Gibco) supplemented with 5 ng/mL GM-CSF (Sigma, St. Louis, MO, United States), 20% heat-inactivated FBS (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The mixture was incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were cultured at a 1/2 subcultivation ratio for 5-6 d in complete medium.

### Co-culture experiment model

The co-culture experiment model is shown in Figure 1. JAWS II cells were harvested, washed, and resuspended in RPMI1640 complete medium (Gibco) containing 5 ng/mL GM-CSF (Sigma), 10% heat-inactivated FBS



**Figure 1** Effect of non-stimulated intestinal epithelial cells on surface phenotype of dendritic cells. Fluorescence activated cell sorter analysis of dendritic cells (DC) cultured alone or co-cultured with non-stimulated epithelial cell monolayers for 12 h showing DC surface phenotype by staining with I-A<sup>d</sup>. Data are shown as the mean fluorescent intensity (MFI) ± SEM of three representative experiments. Significant difference between the single culture and co-culture as determined by Student's *t*-test ( $P < 0.01$ ).

(Invitrogen), and 1% penicillin/streptomycin (Invitrogen). A total of  $1 \times 10^6$  JAWS II cells were added into lower chambers, and the normal and inverted cultured CMT-93 monolayer inserts were placed in the JAWS II seeded transwell plates. One hundred  $\mu\text{g}/\text{mL}$  of the experimental bacteria or 10  $\mu\text{g}/\text{mL}$  of LPS (Sigma) were added to the CMT-93 monolayer inserts. For comparison, JAWS II cells were also plated at the same concentration in 24 well tissue culture plates (Corning Inc.), and the same amount of the bacteria or LPS were added to the cells. The single- or co-cultured cells were incubated with 1 mL RPMI1640 complete medium at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 12 h.

### Flow cytometry analysis

Incubated JAWS II cells were harvested and washed three times in cold FACS buffer (Dulbecco's PBS; Gibco, 2% FBS) and then stained with the appropriate monoclonal antibodies: PE-conjugated anti-I-A<sup>d</sup>, anti-CD80, anti-CD86, and anti-CD40 at a final concentration of 10  $\mu\text{g}/\text{mL}$  for 30 min at 4 °C in the dark. Isotype control antibodies were hamster IgG2 k, rat IgG2a k, and mouse IgG2b. The stained cells were analyzed immediately by FACSCalibur (Becton Dickinson, San Diego, CA, United States). All of the antibodies used in this flow cytometry analysis were purchased from Pharmingen (San Diego, CA, United States).

### Cytokine measurement

JAWS II cell supernatants were harvested from the lower chamber of the Transwell or from the JAWS II cultured-alone plate following incubation, and were assayed for levels of IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)- $\alpha$  and TGF- $\beta$  using enzyme-linked immunosorbent assay. Briefly, Nunc-Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 2  $\mu\text{g}/\text{mL}$  of rat anti-mouse IL-6 and TGF- $\beta$  capture antibodies in coating buffer (1.6 g/L Na<sub>2</sub>CO<sub>3</sub>, 7.1 g/L NaHCO<sub>3</sub>, pH 9.5, or 2  $\mu\text{g}/\text{mL}$  of rat anti-mouse IL-10, IL-12p70, and TNF- $\alpha$  capture antibodies in coating buffer (11.8 g/L Na<sub>2</sub>HPO<sub>4</sub>, 16.1 g/L NaH<sub>2</sub>PO<sub>4</sub>, pH 6.5, overnight at 4 °C. After

washing and blocking, 100  $\mu\text{L}$  of 1:100 diluted (IL-6) or undiluted (IL-10, IL-12p70, TNF- $\alpha$  and TGF- $\beta$ ) supernatant was added to individual wells and incubated overnight at 4 °C. Plates were washed, and biotinylated rat anti-mouse IL-6, IL-10, IL-12p70, TNF- $\alpha$  and TGF- $\beta$  monoclonal antibodies (2  $\mu\text{g}/\text{mL}$ ) and HRP-conjugated streptavidin were added to the plates for cytokine detection for 1 h at room temperature. The reactions were developed with the 3,3',5,5'-tetramethylbenzidine substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 2 N H<sub>2</sub>SO<sub>4</sub> and analyzed at 450 nm. Equivalent levels of IL-6, IL-10, IL-12p70, TNF- $\alpha$  and TGF- $\beta$  were measured for comparison with a reference curve generated using standards of these cytokines.

### Statistical analyses

Data are presented as the mean  $\pm$  SE, indicated by bars in the figures. All statistical analyses were performed using SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, United States). Differences between the single culture and co-culture were determined by Student's *t*-test, and differences between cytokine levels were analyzed by analysis of variance followed by Duncan's multiple range test. The *P* values  $< 0.05$  were considered to be statistically significant.

## RESULTS

### Development of stable CMT-93 epithelial cell monolayers

To obtain stable CMT-93 intestinal epithelial cell monolayers, we monitored the culture every day for TEER using a Millicell-ERS ohmmeter for a period of 7 d. On day 3, normal insert monolayer integrity was obtained at 300-500  $\Omega/\text{cm}^2$ , and inverted insert monolayer integrity was obtained at 250-350  $\Omega/\text{cm}^2$ . In addition, the generation of epithelial cell monolayers was observed on the surface of the inserts by microscope (data not shown). Monolayers between day 3 and 4 were used for co-culture experiments. After co-culture with DC for 12, the integrity of CMT-93 monolayer was evaluated by TEER. There was no difference between before and after co-culture in terms of the resistances within the margin of error.

### Dendritic cells phenotype modulation during co-culturing with epithelial cells

DC surface phenotypes were compared in the presence and absence of epithelial cells. The expression of MHC class II I-A<sup>d</sup> on the normal and the inverted co-cultured DCs was upregulated compared with that of the single-cultured DC (single culture, 18.51  $\pm$  2.86; normal co-culture, 39.46  $\pm$  2.53; inverted co-culture, 52.03  $\pm$  2.41; Figure 1). Co-culture with epithelial cells did not alter the DC surface expression of CD80, CD86 and CD40 (data not shown).

### Effect of probiotics on the expression of major histocompatibility complex class II and costimulatory molecules

We performed flow cytometry analyses to examine the

effects of BL, BB, LC, LA, LPS and control on single- or co-cultured immature DC surface phenotypes. In the DC single culture, the expression of MHC class II I-A<sup>d</sup> was significantly increased by stimulation with BL, BB, and LC compared with the control (Figure 2). In the normal co-culture, the expression of I-A<sup>d</sup> was significantly decreased by the stimulation of LC and LA compared with the control. In the inverted co-culture, none of the experimental probiotics modulated the expression of I-A<sup>d</sup>.

BL and LA significantly downregulated the expression of CD80 in the single-cultured DC. However, none of the experimental probiotics regulated CD80 in the normal and inverted co-cultured DC (Figure 3A).

BB and LC upregulated the expressions of CD86 and CD40 in the single-cultured DC, whereas none of the experimental probiotics regulated the expression of CD86 in the normal or inverted co-cultured DC (Figure 3B). LC significantly downregulated the expression of CD40 in the inverted co-cultured DC compared with medium alone (Figure 3C).

#### **Cytokine profiles in dendritic cells supernatant by co-culturing with epithelial cells**

The levels of IL-6, IL-12p70, TNF- $\alpha$  and TGF- $\beta$  from the co-cultured system were significantly reduced compared with those from the single-cultured DC (Figure 4A and C-E); however, the production of IL-10 showed no decrease in the co-cultured DC.

#### **Effect of probiotics on the cytokine production in the co-culture system**

We quantified the cytokine levels in the single- and co-cultured DC supernatants to investigate the effect of the experimental probiotics on the production of cytokines. LPS was used as a stimulator control to compare with non-treated naïve control. In the single-cultured DC, stimulation with the experimental probiotic bacteria markedly increased the production of IL-6 and TNF- $\alpha$  compared with the control (Figure 4A and D). In the normal co-cultured DC, the levels of IL-6 stimulated by BB and LC and the level of TNF- $\alpha$  stimulated by LA were lower than those of non-stimulated control. In the inverted co-cultured DC, all of the experimental bacteria significantly decreased the production of IL-6 compared with the control, but had no significant effect on the production of TNF- $\alpha$ .

In the single-culture, the level of IL-10 in DC stimulated by BL, LC, and LA was higher than that in the control DC (Figure 4B). The levels of IL-10 from the normal co-cultured DC stimulated by BL, BB and LC were lower. IL-10 from the inverted co-cultured DC stimulated by BL was higher than that from the control.

In the single-cultured DC all of the experimental probiotics decreased the production of IL-12p70. In the normal co-cultured DC only BL increased the production of IL-12p70. In the inverted co-cultured DC the levels of IL-12p70 stimulated by all of the experimental probiotics were similar to that of control.

BL, BB and LC in the single-cultured DC decreased

the production of TGF- $\beta$ . The levels of TGF- $\beta$  in all of the treated groups in the normal co-culture system were similar to that from the non-stimulated control but in the inverted co-cultured system LC and LA significantly increased the production of TGF- $\beta$  (Figure 4C and E).

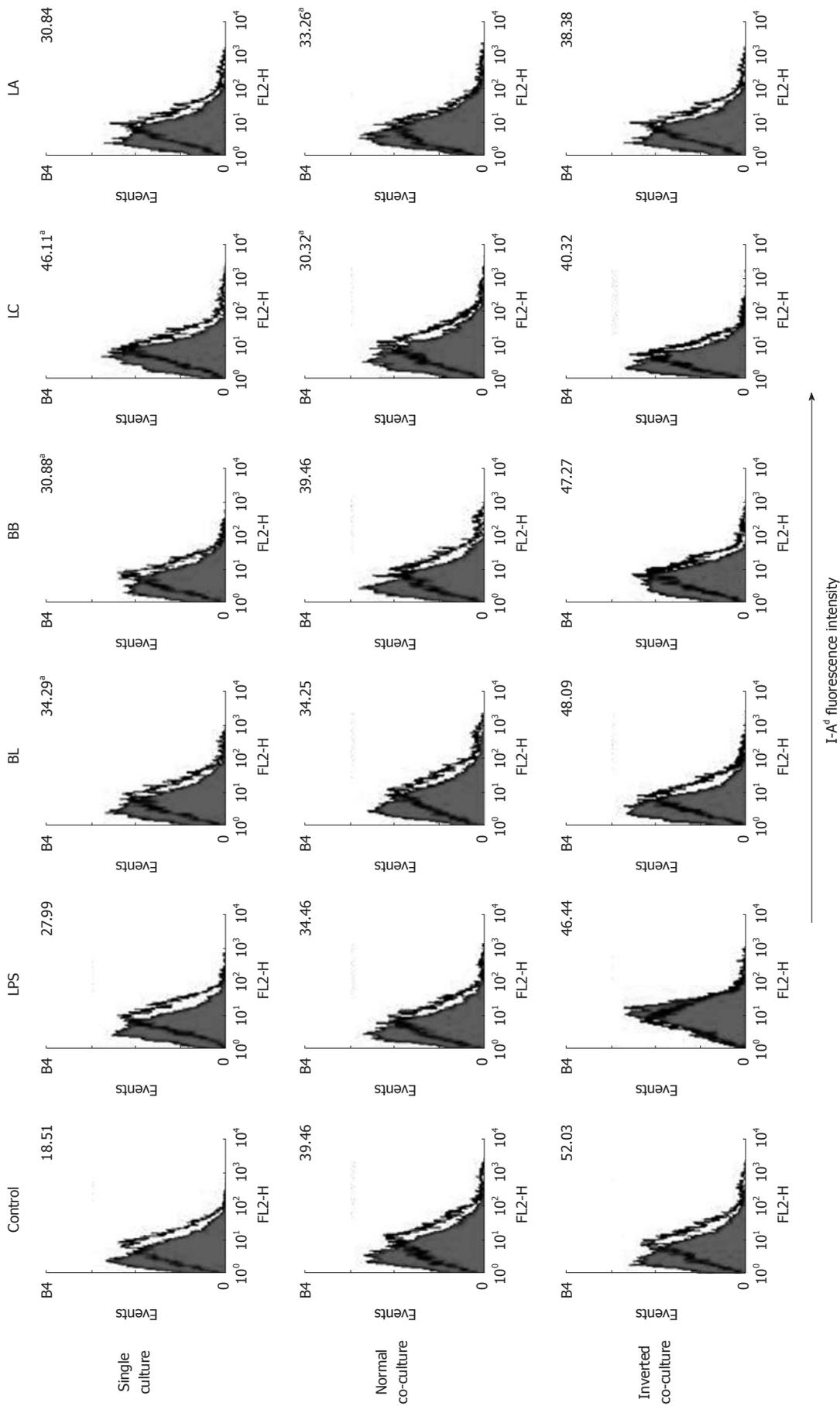
## **DISCUSSION**

The modulatory effect of probiotics on the host immune system was reported in *in vivo* experiments and clinical trials<sup>[34]</sup>. However, the exact mechanism of the immunomodulatory effect of probiotics, especially with respect to the interaction between DC and epithelial cells in the presence of probiotics, has not been well elucidated. In the present study, we investigated the effect of heat-killed BL, BB, LC and LA on the modulation of JAWS II (DC) using an *in vitro* co-culture model. *In vitro*, live bacteria grown by geometric progression exhausted culture nutrients, produced various acid metabolites, and induced necrosis of the cultured animal cells within a few hours. Therefore, the treatment of the host cells with live bacteria was inappropriate. The adhesive properties of heat killed bacteria might be differentially modified by the heat treatment depending on the specific strain of the experimental bacteria. However, a previous study reported that oral administration of heat-killed and lyophilized BB could suppress the occurrence of allergy by the immune regulatory actions in the mouse allergy model<sup>[35]</sup>, which implied that heat-killed and lyophilized bacteria could maintain their immunomodulatory effects.

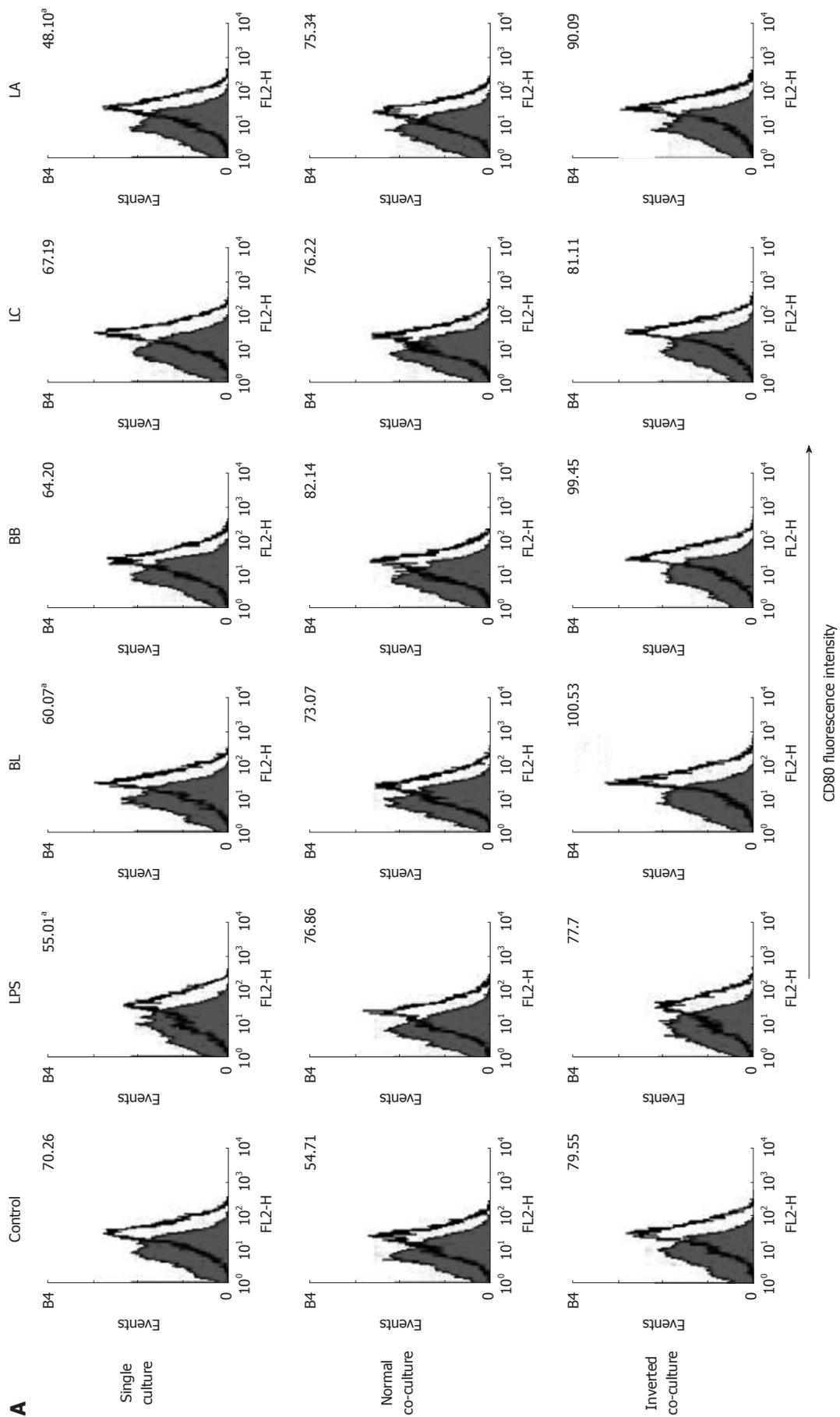
To simulate the interaction between DC and epithelial cells in the intestinal environment, we used a transwell co-culture system with CMT93 epithelial cell lines<sup>[32]</sup>. CMT93 was derived from the same mouse origins as JAWS II, C57BL mouse, and forms junction complexes. A previous study reported that there was a gap junctional communication between murine lymphocytes and CMT93 epithelial cells, and gap junctional communication might regulate cell functions<sup>[36]</sup>. Nonpathogenic intestinal bacteria can induce DC migration into the epithelial layer and recruit DC uptake of bacteria and apoptotic fragments derived from apoptotic epithelial cells to maintain peripheral self-tolerance<sup>[2,37]</sup>. In the normal co-culture system, there was an insert membrane and a gap of 1 mm between JAWS II and CMT93. On the other hand, an inverted co-culture model was established by the generation of a CMT93 monolayer on the underside of the inverted insert. A previous study demonstrated that DCs directly interacted with luminal bacteria using CX<sub>3</sub>CR1-mediated trans-epithelial dendrites<sup>[38]</sup>.

DC interact with microbes and distinguish gram positive, negative, or closely related organisms using TLR and present the processed antigens through MHC class II<sup>[12,16,39]</sup>. DC then mediate T cell activation, which is regulated by MHC class II molecules, co-stimulatory molecules such as CD80 and CD86, and cytokines<sup>[40]</sup>.

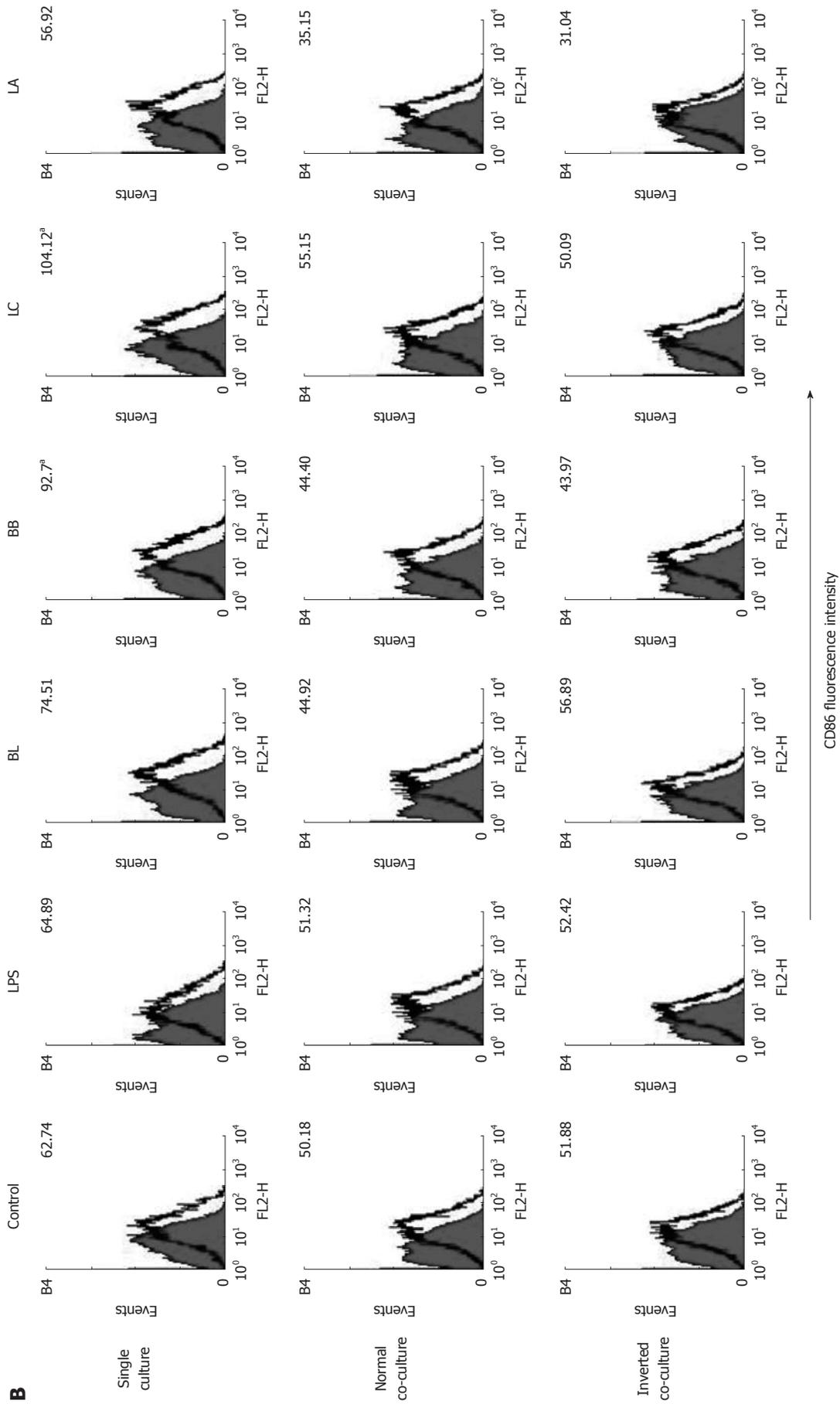
In the single-cultured systems BB and LC upregulated the expression of MHC class II I-A<sup>d</sup>, CD86, and CD40, while all of the experimental probiotics attenuated IL-

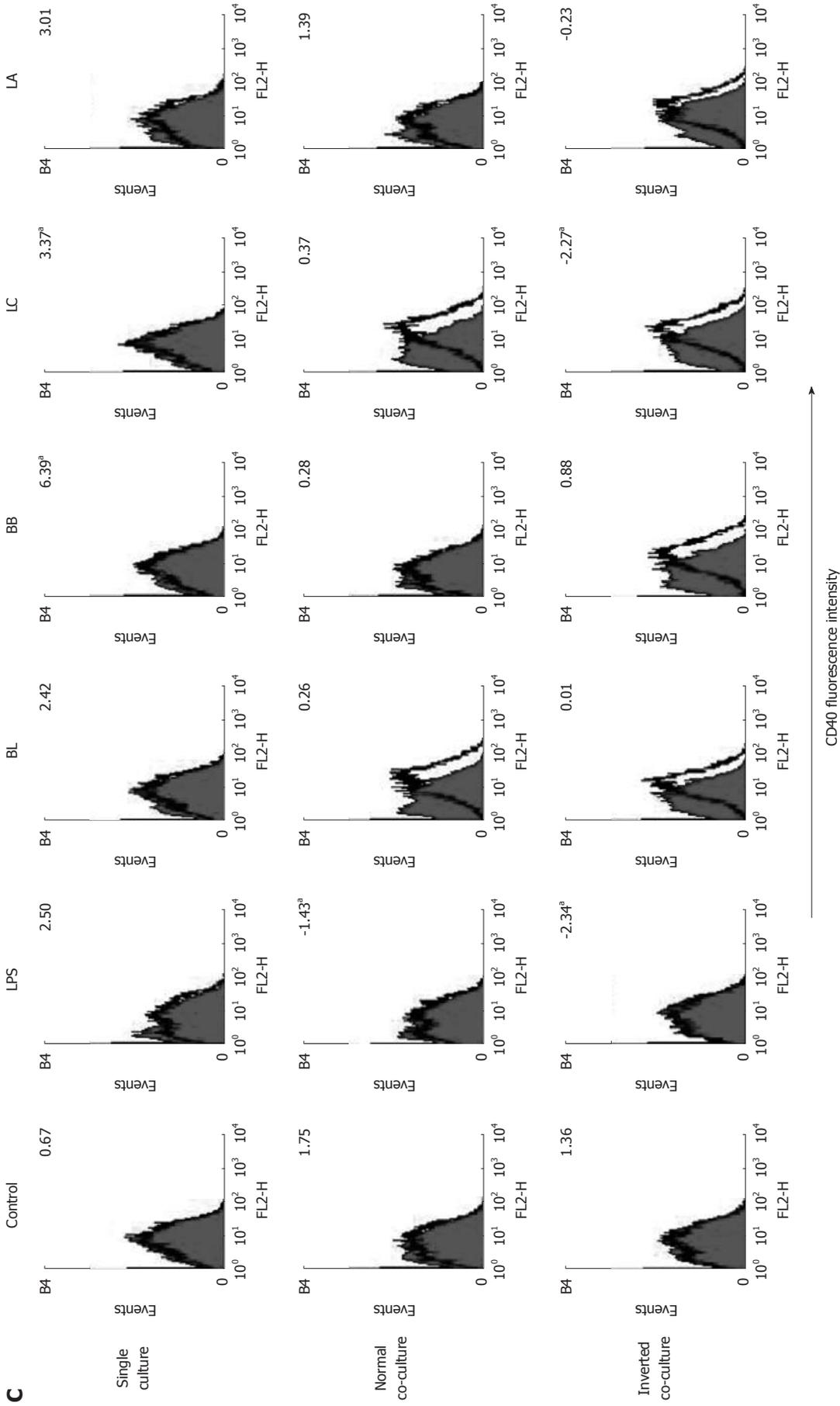


**Figure 2 Effect of probiotics on I-A<sup>d</sup> of single- or co-cultured dendritic cells.** Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histogram show staining for I-A<sup>d</sup>. Numbers indicate the mean fluorescent intensity of three representative experiments. <sup>a</sup>Significant difference among the control, lipopolysaccharides and probiotics as determined by analysis of variance ( $P < 0.05$ ). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011, BB: *Bifidobacterium bifidum* BGN4, LC: *Lactobacterium casei* IBS041, LA: *Lactobacillus acidophilus* AD031.

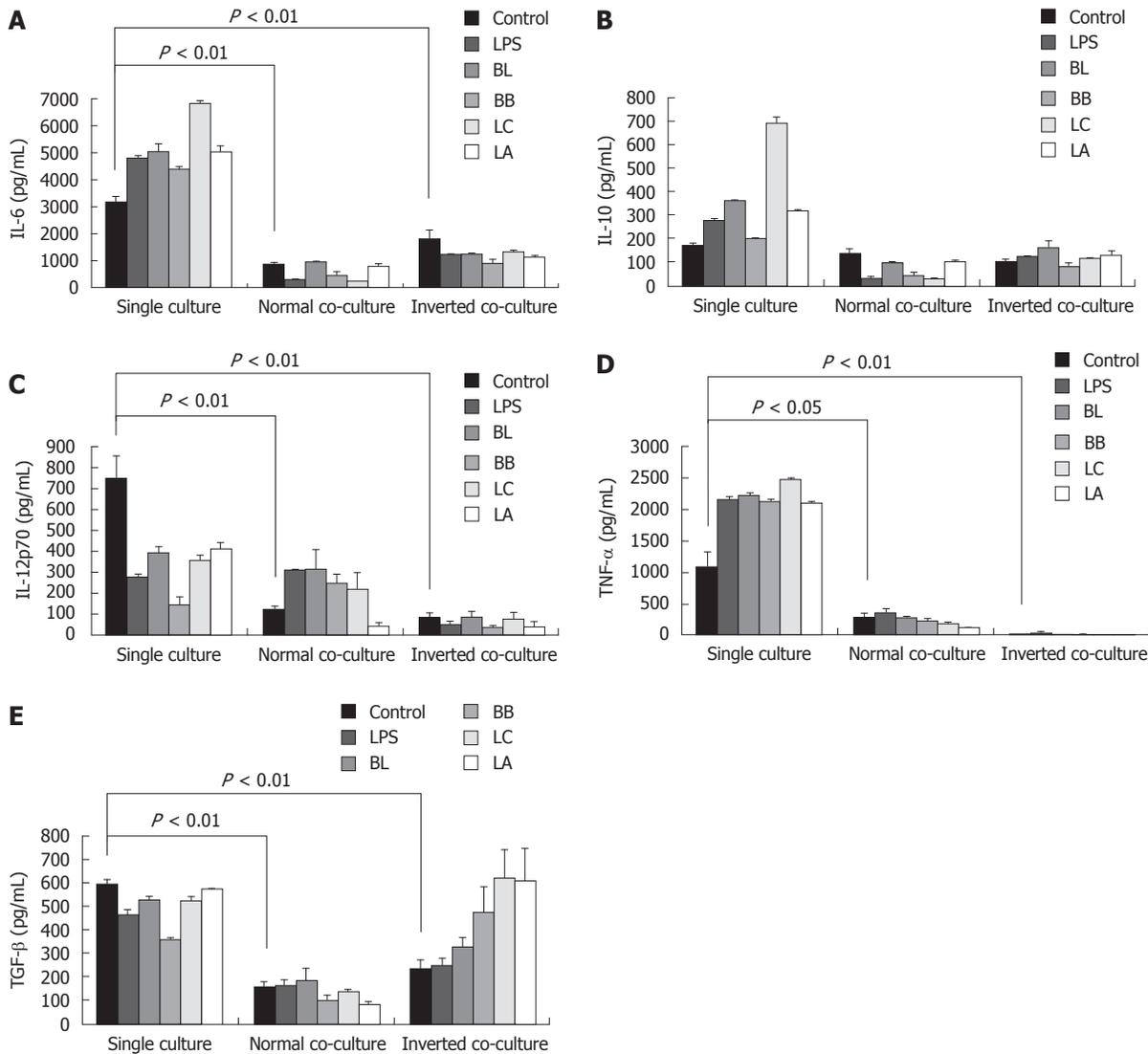


**A**





**Figure 3 Effect of probiotics on the CD80, CD86 and CD40 of single- or co-cultured dendritic cells.** Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histograms shows staining for CD80 (A), CD86 (B) and CD40 (C). Numbers indicate the mean fluorescent intensity of at least three representative experiments. <sup>a</sup>Significant difference among the control, lipopolysaccharides, and probiotics as determined by analysis of variance ( $P < 0.05$ ). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011; BB: *Bifidobacterium bifidum* BGN4; LC: *Lactobacillus casei* IBS041; LA: *Lactobacillus acidophilus* AD031.



**Figure 4** Effect of probiotics on the production of cytokines from single- or co-cultured dendritic cells. Supernatants were obtained from probiotic-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Levels of interleukin (IL)-6 (A), IL-10 (B), IL-12p70 (C), tumor necrosis factor (TNF)- $\alpha$  (D), and transforming growth factor(TGF)- $\beta$  (E) were determined by enzyme-linked immunosorbent assay. Data are shown as mean  $\pm$  SE of three representative experiments. Different letters indicate significant differences among the control, lipopolysaccharides (LPS), and probiotics determined by Duncan's multiple range test ( $P < 0.05$ ). Significant difference between the single culture and co-culture as determined by Student's *t*-test ( $P < 0.05$ ). BL: *Bifidobacterium lactis* AD011; BB: *Bifidobacterium bifidum* BGN4; LC: *Lactobacillus casei* IBS041; LA: *Lactobacillus acidophilus* AD031.

IL-12p70 secretion. IL-12 directed the differentiation of T cells to a Th1 phenotype<sup>[41]</sup>. Nier reported that *Bifidobacterium bifidum* enhanced the expression of CD86 and MHC class II in human neonatal DC, which led in turn to the polarization of IFN- $\gamma$ -producing T cells<sup>[42]</sup>. Mohamadzadeh *et al*<sup>[43]</sup> showed that *Lactobacillus gasseri*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri* upregulated the expression of MHC class II, CD40, CD80 and CD86 in human myeloid DC and increased the level of IL-12p70 which induced the polarization from CD4(+) and CD8(+) T cells to T helper 1 and Tc1 cells. Meanwhile, Drakes *et al*<sup>[22]</sup> showed that probiotic products containing *Lactobacillus* and *Bifidobacterium* upregulated the expression of MHC class II, CD40, CD80 and CD86, and did not induce the production of IL-12p70 in mouse DC. Additionally, mouse bone marrow-derived DC treated with *Lactobacillus reuteri* induced Th2 immune response<sup>[21]</sup>. Taken together,

the results of these earlier studies suggested that probiotics upregulated the expression of MHC class II and differently modulated co-stimulatory molecules such as IL-12p70 and T cell polarization, depending on the DC origin and the strain of probiotics.

Interestingly, in the present study the effects of probiotics on cytokine production and the surface phenotype in co-cultured DC with epithelial cells were markedly different from those in single-cultured DC. All of the experimental probiotics induced the production of pro-inflammatory cytokines, IL-6 and TNF- $\alpha$ , in the single cultured DC. TNF- $\alpha$  mediated various immune responses<sup>[44]</sup>, and over-production of TNF- $\alpha$  could play a role in tissue damage and intestinal pathologies<sup>[45,46]</sup>. In contrast with the results from the single system the experimental probiotics reduced or did not affect the expression of I-A<sup>d</sup>, CD86 and CD40 or the production of IL-6, IL-12p70

and TNF- $\alpha$  in inverted co-cultured DC. These findings suggest that epithelial cells are essential components of the immune system to be considered in assessing the effects of probiotics on the regulation of the gastrointestinal immune system. Consequently, previous studies which employed only DC cells without epithelial cells might have provided only partial pictures or sometimes misleading information about the interaction of the probiotics with DC cells.

Previously, Haller *et al.*<sup>[47]</sup> showed that *Lactobacillus johnsonii* increased the production of TGF- $\beta$  in human epithelial cell lines co-cultured with leucocytes. In our study, BL, LC and LA induced IL-10 secretion from single-cultured DC. In an inverted co-culture system, BL increased IL-10 secretion, and LC and LA increased TGF- $\beta$  secretion. IL-10 was known to activate regulatory T cells<sup>[48]</sup>. TGF- $\beta$  which is an important factor in enhancing the differentiation of regulatory Th3 cells was reported to have wide-ranging immunomodulatory properties<sup>[49,50]</sup>. Th3 cells suppress Th1 and other immune responses and maintain oral tolerance<sup>[40,50,51]</sup>. Conceivably, enhanced secretion of IL-10 or TGF- $\beta$  observed in the co-culture systems by BL, LC and LA might contribute to the activation of regulatory T cells in the intestinal tracts. The present study is novel since we assessed the effect of probiotics on immunomodulation in a co-culture model. We suggest that a co-culture model better reflects the environmental status of the *in vivo* immune system. Our model supports the hypothesis that the interaction of DC and epithelial cells stimulated with probiotics may help maintain intestinal homeostasis by downregulating the production of inflammatory cytokines and expression of MHC class II in DC.

## COMMENTS

### Background

It is known that dendritic cells (DCs) modulate the immune balance in the intestinal tract by mediating the activation of different subsets of T cells. The functions and differentiation of the DCs may be modulated by probiotics. To better understand the role of the probiotics in the intestinal immune system the interactions of probiotics in the context of epithelial cells and DCs need to be assessed.

### Research frontiers

Intestinal epithelial cells secrete various immunological mediators, but the interactions between probiotics, intestinal epithelial cells, and DCs were not fully known. The present study investigated the pattern of cytokine production and the surface phenotype of DCs in the presence of epithelial cells polarized by heat-killed probiotic bacteria.

### Innovations and breakthroughs

The present study showed the differential effects of probiotics between the single cultured and the co-cultured DCs. We suggest that a co-culture model better reflects the environmental status of the *in vivo* immune system. The interaction of DCs and epithelial cells polarized with probiotics may contribute to the homeostasis of the immune system in the intestinal tracts.

### Applications

The employment of the co-culture system may facilitate the development of probiotic bacteria with immunomodulatory effects for people with hypersensitive or imbalanced immune symptoms.

### Peer review

The study is well-conducted and results are interesting. However, the results are curiously reported in a confusing manner. Moreover, some controls are needed and the positive control used should be more justified.

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## Enhancement of CTLs induced by DCs loaded with ubiquitinated hepatitis B virus core antigen

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### Abstract

**AIM:** To investigate whether hepatitis B virus (HBV) could induce a hepatitis B virus core antigen (HBcAg)-specific cytotoxic T lymphocyte (CTL) response *in vitro* by dendritic cells (DCs) transduced with lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg).

**METHODS:** Recombinant LV-Ub-HBcAg were transfected into highly susceptible 293 T cells to obtain high virus titres. Bone marrow-derived DCs isolated from BALB/c mice were cultured with recombinant granulocyte-macrophage colony-stimulating factor and recombinant interleukin (IL)-4. LV-Ub-HBcAg, lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg), lentiviral vector (LV) or lipopolysaccharide were added to induce DC maturation, and the DC phenotypes were analyzed by flow cytometry. The level of IL-12 in the supernatant was detected by enzyme-linked immunosorbent assay (ELISA). T lymphocytes were proliferated using Cell Counting Kit-8. DCs were cultured and induced to mature using different LVs, and co-cultured with allogeneic T cells to detect the secretion levels of IL-2, IL-4, IL-10

and interferon- $\gamma$  in the supernatants of T cells by ELISA. Intracellular cytokines of proliferative T cells were analyzed by flow cytometry, and specific CTL activity was measured by a lactate dehydrogenase release assay.

**RESULTS:** LV-Ub-HBcAg-induced DCs secreted more IL-12 and upregulated the expression of CD80, CD86 and major histocompatibility class II. DCs sensitised by different LVs effectively promoted cytokine secretion; the levels of IL-2 and interferon- $\gamma$  induced by LV-Ub-HBcAg were higher than those induced by LV-HBcAg. Compared with LV-HBcAg-transduced DCs, LV-Ub-HBcAg-transduced DCs more efficiently stimulated the proliferation of T lymphocytes and generated HBcAg-specific cytotoxic T lymphocytes.

**CONCLUSION:** LV-Ub-HBcAg effectively induced DC maturation. The mature DCs efficiently induced T cell polarisation to Th1 and generated HBcAg-specific CTLs.

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**Key words:** Ubiquitin; Hepatitis B virus core antigen; Lentiviruses; Dendritic cells; Cytotoxic T lymphocytes

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### INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem, particularly in Asia and South Africa<sup>[1]</sup>. Notably,

an effective T cell response is critical for virus clearance, and defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection<sup>[2]</sup>. Moreover, the defective CTL response was ascribed to the impaired dendritic cell (DC) function<sup>[3]</sup>. Promoting and improving DC function is a promising approach to combating persistent HBV infection.

Various methods have been attempted to modify DC function, including the use of protein antigens, cytokines, costimulatory molecules, and signalling pathway ligands known to activate the immune response<sup>[4-7]</sup>. Nevertheless, these methods may be insufficient to induce a strong antigen-specific immune response. Further enhancement of the immune response to HBV-specific CTL may be more conducive to clear the HBV. Thus, a novel therapeutic approach is needed to activate T cell expansion and induce a strong antigen-specific T cell response.

Ubiquitin (Ub) is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes<sup>[8]</sup>. The Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses. Accordingly, Ub-mediated processing of antigens has been widely used in chronic infection and cancer studies to improve immune response. Wang *et al.*<sup>[9]</sup> found that an Ub-fused *Mycobacterium tuberculosis* antigen ESAT-6 DNA vaccine significantly increased the antigen-specific cellular immune response in BALB/c mice. That study confirmed that the Th1-type immune response and CTL activity were enhanced by changing the antigen processing. Zhang *et al.*<sup>[10]</sup> reported that Ub-fused melanoma antigens induced antigen proteins to execute proteasome-dependent degradation and created epitopes of major histocompatibility complex (MHC) class I, resulting in the preferential activation of antigen-specific CD8<sup>+</sup> T cells.

Retroviral and adenoviral vectors have been the focus of many studies because of their high efficiency. Lentivirus vectors (LVs) transfect both dividing and relatively quiescent cells and have been widely used to modify DCs<sup>[11,12]</sup>. The aim of this study was to investigate the capacity of DCs transfected with LVs encoding the ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg-DC) to stimulate lymphocyte proliferation and to generate antigen-specific CTLs. The results may provide effective approaches to the control of persistent HBV infection.

## MATERIALS AND METHODS

### Animals

BALB/c mice (H-2<sup>d</sup>), 6-8 wk old, were purchased from the Shanghai Experimental Animal Centre of the Chinese Academy of Sciences and maintained under pathogen-free conditions. Mice were cared for and treated in accordance with the guidelines established by the Shanghai Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

### Cell lines

HEK293T cells were cultured in Dulbecco's modified

Eagle's medium (Invitrogen, Gaithersburg, MD, United States) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, United States), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO<sub>2</sub>. The H-2<sup>d</sup> mastocytoma cell line P815/c (expressing the HBV core antigen) was maintained in our lab.

### Construction of lentiviral vectors

The plasmid pcDNA3.1(-)-Ub-HBcAg was constructed and maintained in our lab. The *Ub-HBcAg* gene was amplified by polymerase chain reaction (PCR). The primers used were: Ub-HBcAg: forward: CGTGGGATC-CATGCA GATCTTCGTGAAG, reverse: CGCAGC CGTCTAACATTGAGATTCCCGAG from plasmid pcDNA3.1(-)-Ub-HBcAg. The purified Ub-HBcAg fragment was cloned into the pWPLXd vector (provided by Prof. Jianming Li, Nanjing, China) using BamH I and Mlu I restriction sites. The recombinant pWPLXd-Ub-HBcAg plasmid was confirmed by restriction enzyme digestion and DNA sequencing. LV-Ub-HBcAg was derived by a combined transfection of three elements: 10 µg pWPLXd-Ub-HBcAg backbone plasmid, 5 µg psPAX2 packaging plasmid, and 5 µg PMD2.G envelope plasmid. We transiently transfected 293T cells with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States). Two days after the transfection, the viral supernatant was collected and filtered through a 0.45-µm filter. Concentrated vectors for the *in vitro* studies were prepared by ultracentrifugation at 25 000 rpm and 4 °C for 90 min. Viral pellets were resuspended in 2 mL sterile phosphate-buffered saline (PBS) and stored at -80 °C.

The control plasmid was constructed by inserting the HBcAg fragment into the BamH I and Mlu I site of the pWPLXd plasmid and named pWPLXd-HBcAg. LV particles (LV-HBcAg) were produced by Lipofectamine transfection into 293T cells.

To determine the titre of the green fluorescent protein (GFP)-expressing vector, 293T cells (1 × 10<sup>6</sup> cells/well) were infected with serially diluted viral supernatant. On day 2, the infected cells expressing GFP were counted by flow cytometry. The titre was calculated as: transduction units per mL (TU) = the number of infected cells/volume of virus supernatant.

### Western blotting

The 293T cells were seeded in six-well plates at 1 × 10<sup>6</sup> cells/well. LV-Ub-HBcAg, LV-HBcAg or LV was added at an multiplicity of infection (MOI) of 1. In some experiments, a specific inhibitor of proteasomes, MG132, was used at 10 µmol. The cells were harvested 48 h after infection, washed twice with PBS, gently dispersed into a single-cell suspension and homogenised using RIPA lysis buffer. Protein concentrations were determined using the Pierce BCA Protein Assay Reagent kit (Rockford, IL, United States). Homogenates were diluted to the desired protein concentration with 2 × SDS-PAGE loading buffer (Invitrogen). Samples were boiled and loaded onto polyacrylamide mini-gels (Invitrogen) for electrophoresis. Proteins from the gels were transferred to Im-

mobilon-PVDF membranes (Millipore Corp., Bedford, MA, United States) using a semi-dry apparatus (Bio-Rad, Hercules, CA, United States). A mouse anti-human HBcAg monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used as the primary antibody, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin-G antibody was used as the secondary antibody.

### **Dendritic cell generation**

Femurs and tibiae of Balb/c mice were removed and purified from the surrounding muscle tissues. Thereafter, intact bones were left in 70% ethanol for 5 min for disinfection and then washed with PBS. Both ends were cut with scissors and the marrow was flushed with PBS using a syringe with a 0.45-mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. Bone marrow cells were cultured at  $2 \times 10^6$  cells/mL in complete RPMI 1640 culture medium (containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) in the presence of 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, United States) and 10 ng/mL murine IL-4 (mIL-4; PeproTech). Nonadherent single cells were gently removed, and fresh medium containing murine GM-CSF and mIL-4 was added on day 3 after beginning culture.

### **Dendritic cell immunophenotyping**

On day 5, immature DCs were cultured for an additional 96 h in the presence of LV-Ub-HBcAg, LV-HBcAg or LV (MOI = 20), and lipopolysaccharide (LPS, 0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) was used as a control group. On day 9, non-adherent and loosely adherent cells were harvested as DCs. The expression of DC surface molecules was analyzed by incubation with allophycocyanin-labelled anti-mouse CD11c, CD80, CD86 and MHC class II (eBioscience, San Diego, CA, United States). The stained cells were analyzed by flow cytometry.

### **Interleukin-12 production**

On day 5, immature DCs were infected with LV-Ub-HBcAg, LV-HBcAg, or LV (MOI = 20) for 72 h. On day 8, the IL-12 levels in harvested supernatants of mature DCs were measured using a standard sandwich enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions.

### **Mixed leukocyte reaction**

On day 9, harvested mature DCs were pre-treated with 25  $\mu$ g/mL mitomycin C and 5% CO<sub>2</sub> for 30 min at 37 °C. Mouse spleens were dissociated on 200-gauge nylon mesh. Splenocytes were collected and treated with lysis buffer to eliminate red cells, washed, and resuspended in RPMI-1640 with 10% FBS. Lymphocytes were derived from splenocytes using nylon wool columns. Single-cell

suspensions of lymphocytes ( $5 \times 10^5$  cells/well) were grown in 96-well plates. Lymphocytes were co-cultured with mature DCs at different responder/stimulator (T cell/DC) ratios (5:1, 10:1 or 20:1) for 72 h. The cells were incubated in a final volume of 200- $\mu$ L complete RPMI 1640 for 72 h, and 10- $\mu$ L Cell Counting Kit-8 solution (Beyotime Institute of Biotechnology, Haimen, China) was added to the plates for 4 h at 37 °C. The absorbance was finally read at 450 nm.

### **Cytokine production**

Splenocytes from mice were cultured in 96-well culture plates in the presence of mature DCs for 4 d at a T-cell to DC ratio of 10:1, and the supernatants were collected. The levels of different cytokines [interferon (IFN)- $\gamma$ , IL-2, IL-4 and IL-10] in the supernatants of proliferating T cells were measured using commercial ELISA kits according to the manufacturer's protocol (R and D Systems). Data were expressed as pg/mL.

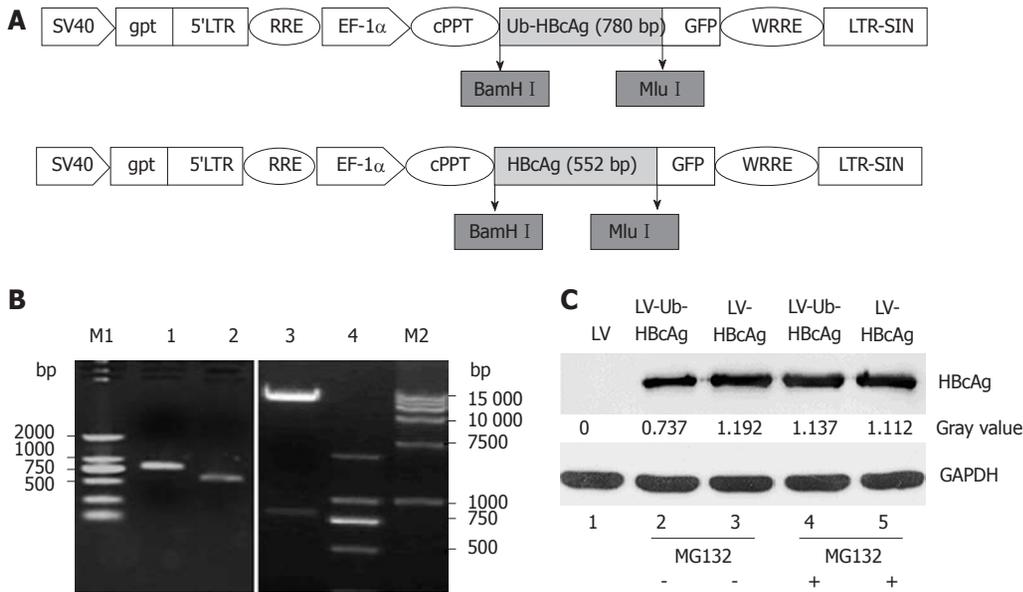
IFN- $\gamma$  production was detected by intracellular staining and flow cytometry. The above proliferative T cells were suspended in complete RPMI 1640 and stimulated for 6 h in the presence of 25  $\mu$ g/mL phorbol 12-myristate 13-acetate, 1  $\mu$ g/mL ionomycin and 1.7  $\mu$ g/mL monensin (Sigma). After washed with PBS, the cells were stained with FITC-conjugated anti-CD8 $\alpha$  mAb (eBioscience) for 30 min at 4 °C, washed with PBS, fixed with 4% paraformaldehyde, and permeabilised with PBS containing 0.5% saponin (both from BD, Shanghai, China). Cells were incubated with PE-labelled anti-INF- $\gamma$  McAb (eBioscience) for 30 min at 4 °C, washed with PBS, and analyzed by flow cytometry.

### **Hepatitis B virus core antigen-specific cytotoxic T lymphocytes activity**

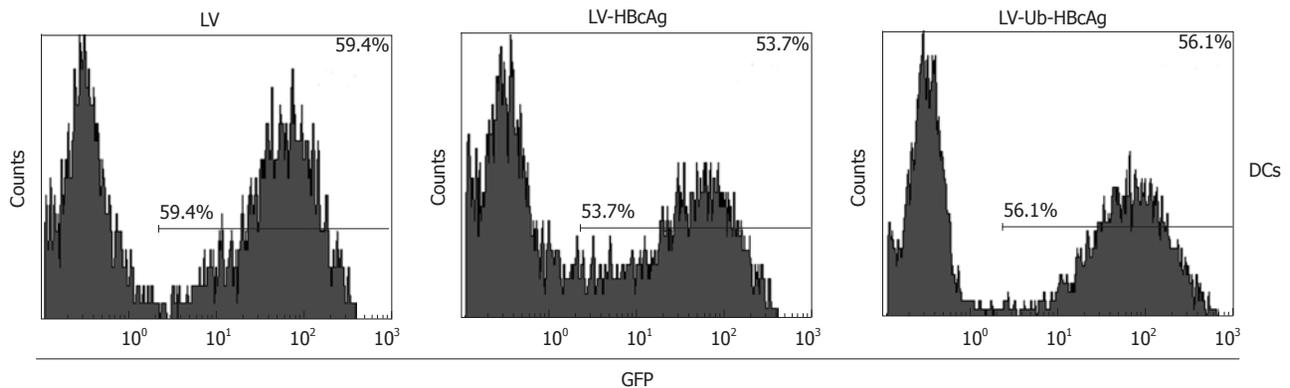
The former stimulated splenocytes ( $5 \times 10^6$ /mL) were used as effectors, and the P815/c cell line was used as target cells. P815/c cells were seeded at a density of  $5 \times 10^4$  cells/well in 96-well plates. Effector cells were incubated with P815/c at different effector and target (E/T) ratios (12.5:1, 25:1 or 50:1) at 37 °C under 5% CO<sub>2</sub> for 4 h. The HBcAg-specific CTL activity was measured using a Cyto-Tox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega, Madison WI, United States) for lactate dehydrogenase (LDH) release according to the manufacturer's instructions. The absorbance values of the supernatants were recorded at optical density 450 nm. Percent cytotoxicity was calculated as follows: [(Experimental release - Effector spontaneous release - Target spontaneous release)/(Target maximum release - Target spontaneous release)]  $\times$  100%.

### **Statistical analysis**

Results were expressed as mean  $\pm$  SD. Differences between groups was determined using Student's *t* test, and the differences between two or more groups were determined using a one-factor analysis of variance. Data were considered statistically significant at  $P < 0.05$ .



**Figure 1** Schematic diagram, electrophoresis of ubiquitinated hepatitis B virus core antigen and HbCag genes, pWPXLd-Ub-HBcAg digested by BamH I and Mlu I, and HbCag protein expression (about 21 kDa). A: Schematic diagram of pWPXLd vector; B: Lane 1, ubiquitinated hepatitis B virus core antigen (Ub-HBcAg) polymerase chain reaction (PCR) product (780 bp); lane 2, HbCag PCR product (552 bp); lane 3, The digested products pWPXLd-Ub-HBcAg by BamH I and Mlu I; lane 4 and lane M1, DNA marker 2000; lane M2, DNA marker 15 000; C: 293T cells were transduced with lentiviral vector (LV), LV-Ub-HBcAg or LV-HBcAg and cultured for 48 h. MG-132 (10 mmol) was added for 24 h before harvesting the cells. Cell lysates (10 mg) were analyzed by immunoblotting with an anti-HBc antibody. Relative expression of HbCag was calculated by a gray value.



**Figure 2** Transduction of dendritic cells with lentiviral vectors expressing green fluorescent protein was evaluated by flow cytometry. Dendritic cells (DCs) were seeded in six-well plates at  $1 \times 10^6$  cells/well. Lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg), lentiviral vectors hepatitis B virus core antigen (LV-HBcAg) or lentiviral vector (LV) was added at an multiplicity of infection of 20. GFP: Green fluorescent protein.

## RESULTS

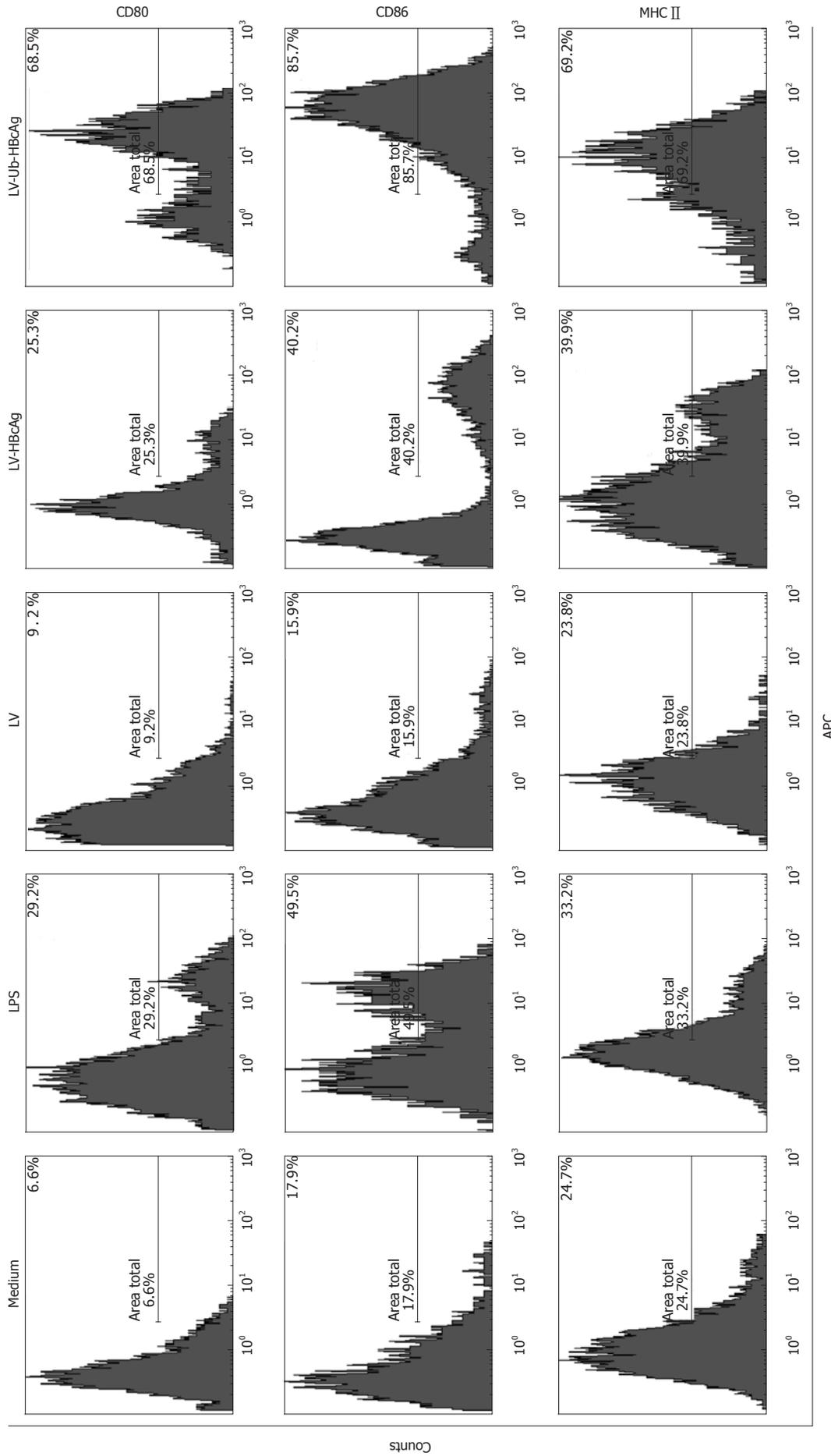
### Construction of lentiviral vectors and transduced dendritic cells

A 780 bp fragment of the *Ub-HBcAg* gene was cloned into pWPXLd (Figure 1B) and packed into LVs. The *HbCag* gene was similarly assembled as a control. After concentration, all vectors in the study achieved a titration of approximately  $7.5 \times 10^8$  transducing units/mL. The construction procedure is shown in Figure 1A. As expected, Ub-HBcAg expression was lower than that of HbCag and recovered to the same level as that of HbCag when MG-132 was added to the culture (Figure 1C). The transduction efficiency of LVs into DCs was evaluated using flow cytometry by detecting GFP expression

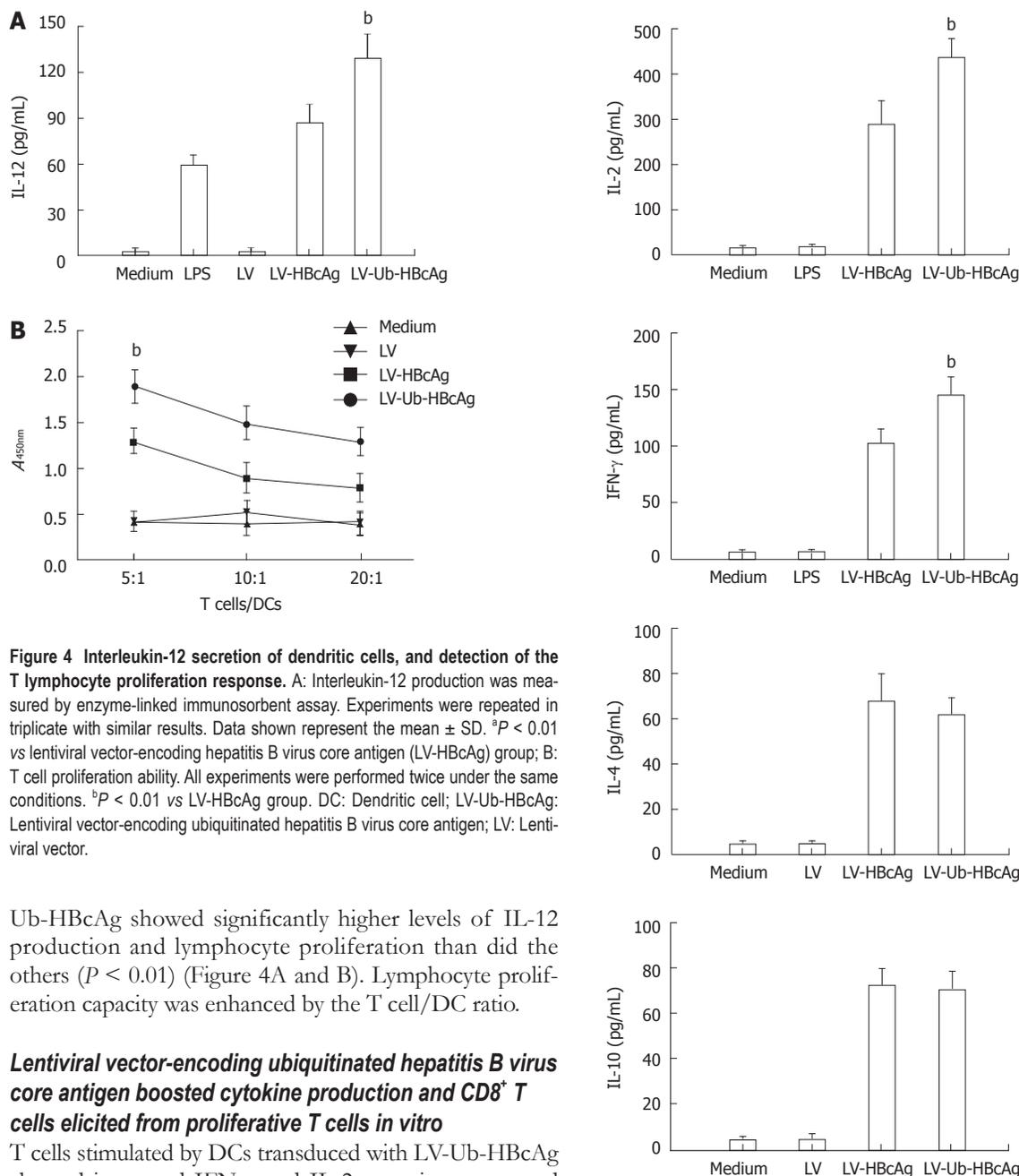
(Figure 2). On day 4 after infection, 56.1% of GFP-expressing DCs were detected.

### Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen-induced dendritic cell maturation increased IL-12 production and enhanced lymphocyte proliferation

At the end of the treatment, the amount of DCs (CD11c<sup>+</sup>) was 75% by fluorescence-activated cell sorting analysis. MHC II, CD80 and CD86 molecules, which are characteristic of DCs, were used to evaluate DC differentiation and maturation. These molecules were highly expressed in DCs transduced with LV-Ub-HBcAg compared with those transduced with the alternatives (Figure 3). DC function was evaluated by IL-12 secretion and promotion of lymphocyte proliferation. DCs transduced with LV-



**Figure 3 Percentage of dendritic cell surface molecules.** In the lentiviral vectors ubiquitinated hepatitis B virus core antigen (LV-Ub-HBcAg) group, the expression of major histocompatibility complex (MHC) class II, CD80 and CD86 molecules characteristic of dendritic cells (DCs) was significantly higher than that in the lipopolysaccharide (LPS) or lentiviral vectors hepatitis B virus core antigen (LV-HBcAg) group. The results represent one of three experiments. LV: Lentiviral vector; APC: Antigen-presenting cell.



**Figure 4 Interleukin-12 secretion of dendritic cells, and detection of the T lymphocyte proliferation response.** A: Interleukin-12 production was measured by enzyme-linked immunosorbent assay. Experiments were repeated in triplicate with similar results. Data shown represent the mean  $\pm$  SD. <sup>a</sup> $P < 0.01$  vs lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group; B: T cell proliferation ability. All experiments were performed twice under the same conditions. <sup>b</sup> $P < 0.01$  vs LV-HBcAg group. DC: Dendritic cell; LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector.

Ub-HBcAg showed significantly higher levels of IL-12 production and lymphocyte proliferation than did the others ( $P < 0.01$ ) (Figure 4A and B). Lymphocyte proliferation capacity was enhanced by the T cell/DC ratio.

**Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen boosted cytokine production and CD8<sup>+</sup> T cells elicited from proliferative T cells in vitro**

T cells stimulated by DCs transduced with LV-Ub-HBcAg showed increased IFN- $\gamma$  and IL-2 secretion compared with DCs transduced with LV-HBcAg (Figure 5). No significant difference between the two groups was observed for IL-4 and IL-10 production. CTLs were analyzed by intercellular IFN- $\gamma$  and CD8 $\alpha^+$  levels. The levels and intensities of IFN- $\gamma$  expression were higher in the LV-Ub-HBcAg than in the LV-HBcAg samples (Figure 6A and B), suggesting that DCs transduced with LV-Ub-HBcAg were effective for inducing CTLs.

**Enhancement of cytotoxic T lymphocyte activity in dendritic cells transduced with Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen**

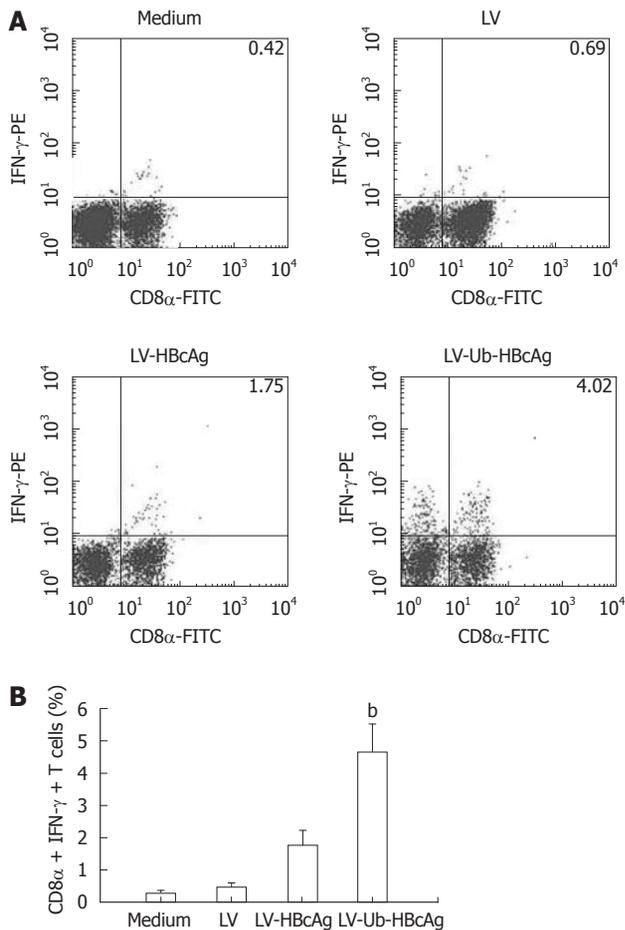
The LDH relaxation index was determined to evaluate the specific cytotoxicity of T lymphocytes in response to different LV-transduced DCs. HBcAg-specific CTL activities with different effector/target ratios are shown in Figure 7. T lymphocytes from the LV-Ub-HBcAg-

**Figure 5 Cytokine production.** Cytokine secretion of proliferative T cells. Data represent the mean  $\pm$  SD. <sup>b</sup> $P < 0.01$  vs lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector; INF- $\gamma$ : Interferon- $\gamma$ ; IL: Interleukin.

transduced DCs killed  $55.0\% \pm 4.3\%$  target cells at an effector and target ratio of 50:1, which was significantly higher than that of the LV-HBcAg-transduced DC group ( $32.4\% \pm 5.2\%$ ) ( $P < 0.01$ ). Accordingly, these results indicated that LV-Ub-HBcAg-transduced DCs induced strong specific CTL responses.

**DISCUSSION**

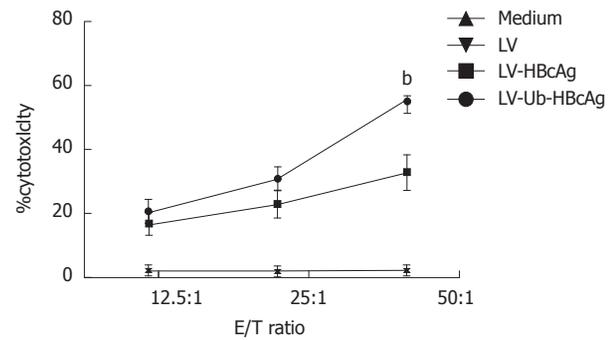
The development of novel immunotherapies has been highly anticipated because HBV infection is one of the



**Figure 6 Intracellular cytokine analysis.** A: The proliferative T cells were suspended in complete RPMI 1640 ( $2 \times 10^6$ /mL), intracellular cytokine analysis by flow cytometry using CD8 $\alpha$ -FITC and Interferon (IFN)- $\gamma$ -PE antibodies. The results are representative of one of three experiments; B: The presence of CD8 $\alpha$  and IFN- $\gamma$ + cells. <sup>b</sup>*P* < 0.01 vs lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV-HBcAg: Lentiviral vector-encoding hepatitis B virus core antigen; LV: Lentiviral vector.

leading causes of cancer or hepatocellular carcinoma-related death. Several studies have demonstrated that the main cause of viral persistence during HBV infection is an inadequate antiviral immune response to the viral antigens<sup>[13,14]</sup>. The viral-specific CD8 $^+$  T cell response plays an important role in the process of viral clearance. Patients with **chronic hepatitis B (CHB) or therapeutic failure** show deficient Th1 immunity associated with inefficient CD8 $^+$  T cell cytotoxicity<sup>[15]</sup>. Therefore, induction of CTL responses specific to HBV represents a promising strategy to protect against HBV infection.

DCs are key antigen-presenting cells that induce primary and memory immune responses. Impaired DC function is found in chronic HBV infection, in which patients are generally in an immunocompromised state of immune tolerance<sup>[16-18]</sup>. Considerable effort has been made to introduce antigens into DCs in the forms of peptides, proteins, or transgenic protein antigens using viral vectors<sup>[19,20]</sup>. Various viral vectors, including poxvirus and adenovirus, have been used to genetically modify DCs, but low transduction efficiencies have limited their ap-



**Figure 7 Cytotoxic response of proliferative T cells.** Effector cells were incubated with P815/c at different effector/target (E/T) ratios (12.5:1, 25:1 or 50:1) for 4h. Experiments were repeated three times with similar results. Data represent the mean  $\pm$  SD. <sup>b</sup>*P* < 0.01 vs lentiviral vector-encoding hepatitis B virus core antigen (LV-HBcAg) group. LV-Ub-HBcAg: Lentiviral vector-encoding ubiquitinated hepatitis B virus core antigen; LV: Lentiviral vector.

plication. We chose LVs as a gene transfer vector because they can transduce non-dividing, monocyte-derived DCs and bone marrow-derived DCs with very high transduction efficiencies<sup>[21,22]</sup>. Several reports have demonstrated that immunizing mice with LVs by delivering viral or tumor model antigens mice elicited broad and long-lasting specific immune responses. For example, lentiviral transduction of DCs expressing ovalbumin effectively processed and presented the ovalbumin antigens and induced ovalbumin-specific T cell responses<sup>[23]</sup>. Our results confirmed that lentivirus-mediated gene transfer could offer the unique opportunity to investigate the biologic activity of DCs.

The ubiquitin-proteasome system (UPS) is a highly selective adenosine-5'-triphosphate-dependent proteolytic system present in all eukaryotic cells and plays a key role in antigen presentation<sup>[8]</sup>. It is well established that short antigenic peptides must be presented on MHC class I molecules of target cells to be recognized by specific CTLs. Proteasomes are responsible for the proteolysis of intracellular proteins, including viral antigens, to generate MHC class I ligands.

Attachment of Ub to a protein is the initial signal for targeted protein degradation. To prevent fusion gene (*Ub-HBcAg*) cleavage by deubiquitination enzymes, we constructed a pWPXLd vector encoding HBcAg fused with Ub, in which the Ub C-terminal glycine was replaced with alanine<sup>[24]</sup>. Additionally, HBcAg with a modified N-terminal Met residue was replaced by Arg. By this method, the fusion protein can be quickly recognized by the UPS, resulting in a promotion of HBcAg degradation<sup>[25-27]</sup>. In our study, Western blotting analyses identified efficient expression of HBcAg from the 293T cells transduced with recombinant LVs. Ub-fused HBcAg was converted into an excellent substrate for the UPS. We found that the 293T cells transduced with LV-Ub-HBcAg showed low levels of protein expression in the absence of MG-132.

Immature DCs expressed low levels of surface MHC molecules, producing almost no expression of CD40, CD80 or CD86. Fully matured DCs showed strong surface expression of MHC class II and co-stimulatory mo-

lecules (CD80 and CD86). In our study, the surface molecules CD80, CD86, and MHC class II DCs were markedly upregulated by LV-Ub-HBcAg stimulation, whereas no significant change was observed after LPS or LV-HBcAg stimulation. An important sign of mature DCs is IL-12 secretion. Mature DCs secrete high levels of IL-12 that promote activation of effector cells (e.g., natural killer cells, lymphokine-activated killer cells, tumor-infiltrating lymphocytes and macrophages) and induce a variety of cytokines (e.g., IFN- $\gamma$ , GM-CSF, IL-2 and IL-8)<sup>[28]</sup>. IL-12 is produced by mature DCs in response to infection by various intracellular pathogens. This response plays a critical role initiating a specific T cell-mediated immune response and drives Th1 cell activation and differentiation<sup>[29,30]</sup>. We examined IL-12 production after adding different maturation factors to the culture medium. As expected, IL-12-induced LV-Ub-HBcAg production of DCs was markedly elevated compared with that produced by other treatments. In this study, LV-Ub-HBcAg-transduced DCs not only promoted DC surface molecule expression, but also promoted further secretion of IL-12, which helped stimulate the immune response.

Higher rates of intracellular antigen traffic should increase the number and varieties of peptides available for MHC class I binding, which may result in an increase in the cell immune response to the expressed antigen. DCs pulsed with HBV antigens effectively abrogated CTL tolerance in HBV transgenic mice. Chen *et al.*<sup>[4]</sup> demonstrated that DCs loaded with HBcAg not only induce the production of HBV-specific T cells but also restore the impaired function of such cells. The DCs generated by transfection of LV-Ub-HBcAg were able to stimulate proliferation of naive allogeneic T lymphocytes and to increase the number of antigen-specific CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells *in vitro*. Th1 cells primarily secrete IL-2 and IFN- $\gamma$ , whereas Th2 cells secrete type II cytokines IL-4 and IL-10. Th1/Th2 immune balance plays a key role in the outcome of HBV infection. Dominant Th1 cells tend to lead to an acute self-limited HBV infection; dominant Th2 cells tend to occur with a chronic persistent HBV infection. In our study, we observed that the LV-Ub-HBcAg group had higher levels of both IL-2 and IFN- $\gamma$  in the lymphocyte supernatant compared with those in the LV-HBcAg group. This was further supported by enhanced levels of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells. These results clearly indicate that the immune responses were directed toward a Th1 type rather than a Th2 type. Th1 cells are correlated with the induction of CTL activity, which is beneficial for viral or tumor eradication<sup>[31,32]</sup>. In this study, the LV-Ub-HBcAg-transfected DCs stimulated T lymphocytes and generated antigen-specific cytotoxic T lymphocytes more efficiently than those of the LV-HBcAg-transfected DCs. Thus, LVs carrying Ub-fused HBcAg effectively activated antigen-specific CD8<sup>+</sup> T cells. Inadequate endogenous antigen presentation by MHC class I molecules to CD8<sup>+</sup> T cells is one of the reasons for the failure of the immune system to eliminate pathogens. Patients with CHB or therapeutic failure showed deficient Th1 immunity associated with inefficient CD8<sup>+</sup> T cell cytotoxicity. In

our study, enhanced antigen presentation increased the number of antigen-specific CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in the LV-Ub-HBcAg-transfected DC group. Ub-fused HBcAg was rapidly degraded by the proteasome, resulting in efficient production of a variety of peptides, including many CTL epitopes that may be presented by many types of MHC class I molecules.

In summary, we have successfully transfected murine bone marrow-derived DCs with LVs encoding the Ub-HBcAg fusion gene. The Ub-HBcAg-transfected DCs proliferated and generated HBcAg-specific CTLs more efficiently than did the HBcAg-transfected DCs. Therefore, this novel strategy may have therapeutic value that can be applied to the treatment of infectious diseases.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection is a serious public health problem. Defective cytotoxic T lymphocytes (CTLs) may lead to persistent HBV infection, and the defective CTL response was ascribed to the impaired dendritic cell (DC) function. Promoting and improving DC function is a promising approach to combating persistent HBV infection. Ubiquitin (Ub) is a highly conserved small regulatory protein, and the Ub-mediated processing of antigens is rapid and efficient and stimulates cell-mediated immune responses.

### Research frontiers

Ub-fused melanoma antigens could induce antigen proteins to execute proteasome-dependent degradation and created epitopes of major histocompatibility complex (MHC) class I, resulting in the preferential activation of antigen-specific CD8<sup>+</sup> T cells.

### Innovations and breakthroughs

The study reported for the first time the capacity of DCs transfected with lentivirus encoding the ubiquitinated hepatitis B virus core antigen (Ub-HBcAg) to stimulate lymphocyte proliferation and to generate antigen-specific CTLs.

### Applications

The authors found that lentivirus encoding Ub-HBcAg effectively could induce DC maturation. The mature DCs efficiently induced T cell polarization to Th1 and generated HBcAg-specific CTLs. These results may be helpful in seeking novel approaches to the control of persistent HBV infection.

### Terminology

Ubiquitin is a highly conserved small regulatory protein, ubiquitous in eukaryotes, that usually serves as a signal for the target protein that is recognized and degraded in proteasomes.

### Peer review

The topic is novel, with very few articles published in this field till now. The manuscript is well organized with objectives, methods, results being adequately described, and the conclusions are based on the results found. Tables and figures are appropriate. Statistical analysis needs better description by providing *P* values through the text where comparisons between groups are present.

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## Loss of Wnt5a and Ror2 protein in hepatocellular carcinoma associated with poor prognosis

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### Abstract

**AIM:** To investigate the expression and clinical significance of Wnt member 5a (Wnt5a) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) in hepatocellular carcinoma (HCC).

**METHODS:** In HCC tissues obtained from 85 patients, the protein expressions of Wnt5a, Ror2,  $\beta$ -catenin, and Ki-67 *via* immunohistochemical staining using the Envision Plus System. The antibody binding was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) before brief counterstaining with Mayer's hematoxylin. The degree of immunohistochemical staining was recorded using a semiquantitative and subjective grading system. The mRNA expression of Ror2 was examined by real-time reverse transcription polymerase chain reaction, including nineteen of the 85 HCC and three nor-

mal liver tissues. The ratios of Ror2 to the housekeeping gene GAPDH represented the normalized relative levels of Ror2 expression. To determine the prognostic factor, the outcome of the 82 patients was determined by reviewing their medical charts. The overall and disease-free survival rates were estimated using the Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with univariate and multivariate Cox regressions models.

**RESULTS:** Compared to nontumorous (hepatitis or cirrhotic) tissues, Ror2 mRNA expression was clearly decreased in HCC. Ror2 and Wnt5a protein expressions in the majority of HCC patients (63% and 77%, respectively) was significantly less in tumor tissues, as compared to adjacent nontumorous tissues, and this reduction was correlated with increasing serum  $\alpha$ -fetoprotein and tumor stage. In 68% (58/85) of the HCC cases, the expression of  $\beta$ -catenin in tumor tissues was either downregulated in the cellular membrane, upregulated in the cytoplasm, or both. Survival analysis indicated that Wnt5a and Ror2 protein expressions could be regarded as independent prognostic factors for HCC; HCC patients with decreased Wnt5a or Ror2 protein expression had a poorer prognosis than those with elevated Wnt5a and Ror2 expression ( $P = 0.016$ ,  $P = 0.007$ , respectively).

**CONCLUSION:** Wnt5a and Ror2 may serve as tumor suppressor genes in the development of HCC, and may serve as clinicopathologic biomarkers for prognosis in HCC patients.

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**Key words:** Hepatocellular carcinoma; Wnt5a; Receptor tyrosine kinase-like orphan receptor 2;  $\beta$ -catenin; Prognosis

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring tumors worldwide. It develops mostly in cirrhotic livers, and risk factors include chronic infection by the hepatitis B and C viruses (HBV and HCV), as well as nonviral liver diseases<sup>[1,2]</sup>. Unfortunately, the cellular mechanisms of hepatocarcinogenesis remain poorly understood. Recent advances have shown that apart from autocrine stimulation by growth factors such as insulin-like growth factor-II and transforming growth factor- $\alpha$ , the dysregulation of at least four different growth regulatory pathways is frequently involved in hepatocarcinogenesis<sup>[3,4]</sup>. These signaling pathways include the retinoblastoma, the transforming growth factor- $\beta$ , the tumor protein 53, and the wingless-type murine-mammary-tumor virus integration site family (Wnt). These pathways also interfere with each other at different levels<sup>[2,5,6]</sup>.

The Wnt family of genes encodes a large and diverse group of signaling molecules involved in the patterning, proliferation, and differentiation of a variety of organs and cell types<sup>[7,8]</sup>. The Wnt ligand binds to its receptor Frizzled and the low-density lipoprotein receptor-related proteins (Lrp) 5 and 6 to activate the canonical Wnt/ $\beta$ -catenin signaling pathway, or functions through  $\beta$ -catenin-independent (noncanonical) pathways which include the planar cell polarity and Wnt/ $\text{Ca}^{2+}$  pathways<sup>[9]</sup>. Wnt ligands are typically classified into canonical and non-canonical Wnts by the pathways they work through<sup>[9-11]</sup>.

The Wnt member 5a (Wnt5a) is one of the most highly investigated non-canonical Wnts and has been implicated in almost all aspects of non-canonical Wnt signaling<sup>[12-14]</sup>. In terms of cancer developmental research, Wnt5a has lived in the shadow of its better-characterized relatives. This was largely because of its apparent inability to transform cells or signal through the canonical  $\beta$ -catenin pathway that is so important in cancer<sup>[15-18]</sup>. Recent work with a wide of human tumors has indicated that Wnt5a has a critical role in malignant progression, but there is conflicting evidence as to whether that role is tumor-promoting or tumor-suppressing<sup>[17-22]</sup>. We have shown that Wnt5a has a tumor suppressing effect in HCC and is probably associated with HBV infection<sup>[23,24]</sup>. Emerging evidence suggests that the functions of Wnt5a can be drastically altered depending on the availability of key receptors<sup>[17,18,25]</sup>. It was recently reported that an alternative Wnt receptor, receptor tyrosine kinase-like orphan

receptor 2 (Ror2), an orphan tyrosine kinase, mediates Wnt5a-initiated noncanonical signaling and is required for the Wnt5a-mediated inhibition of canonical signaling<sup>[25,26]</sup>.

The Ror2 receptor belongs to the receptor tyrosine kinase superfamily<sup>[25]</sup>. This large protein family is involved in regulating diverse cellular processes such as the cell cycle, cell migration, proliferation and differentiation<sup>[27]</sup>. In addition, the Ror2 protein and its homolog Ror1 play essential roles during development. Mutations of the Ror2 receptor, resulting in protein misfolding or premature truncation, have been associated with human diseases such as dominant Brachydactyly type B and recessive Robinow syndrome<sup>[28]</sup>. Currently, investigations to elucidate the role of Ror2 in cancer have shown paradoxical results, indicating that Ror2 was overexpressed in oral and renal cell cancer and metastatic melanoma, but downregulated in colon cancer<sup>[29-31]</sup>. These different effects appear to be dependent on the cancer type and signaling pathway<sup>[32]</sup>.

Here, we investigate the expression and clinical significance of Ror2, Wnt5a and  $\beta$ -catenin in HCC.

## MATERIALS AND METHODS

### Patients and specimens

We collected tumors from 85 consecutive patients who had undergone surgery for HCC at the Jinan Military General Hospital from January 2006 to September 2010. The Ethics Committee of the Jinan Military General Hospital approved the protocol of this study. Among the 85 patients, 55 had serum  $\alpha$ -fetoprotein (AFP)  $\geq 30$   $\mu\text{g/L}$ , and 73 were sera positive for hepatitis B surface antigen (HBsAg). On gross examination, 3 cases had tumor sizes that were  $\leq 2$  cm, and 82 had tumor sizes  $> 2$  cm (median tumor size, 6.1 cm; range, 1.0-16 cm). Histopathological diagnoses were made according to the pathological classification system of the World Health Organization (2000), and the tumor was staged following the tumor-node-metastasis classification of the International Union Against Cancer<sup>[33]</sup>. Nine cases were well differentiated; 60 cases were moderately differentiated; and 16 cases were poorly differentiated. In total, 56 HCC cases had liver cirrhosis; 25 cases had chronic hepatitis; and 4 cases had basically normal liver tissue. We also collected 3 cases of lung metastasis. Furthermore, tissues of comparative normal liver obtained during surgery for liver cholelithiasis ( $n = 3$ ) and HBV-infected liver biopsies ( $n = 5$ ) were studied.

Nineteen of the 85 cases included chronic ( $n = 8$ ) and cirrhotic ( $n = 11$ ) HCC. From these, fresh tissues were obtained immediately after resection, including HCC tumor and adjacent nontumorous liver tissues. In addition, normal liver tissues ( $n = 3$ ) with no HBV infection were obtained during surgery for liver cholelithiasis. In these 22 cases, one portion of the fresh tissue was snap frozen in liquid nitrogen immediately and stored at  $-80$   $^{\circ}\text{C}$ ; the remainder portion was fixed in 10% buffered formalin and embedded in paraffin.

The available patient clinicopathological information

included gender, age, serum AFP, serum HBsAg, tumor size, tumor stage, histological grade and cancer-specific survival time.

### **Extraction of RNA and real-time reverse transcription-polymerase chain reaction**

Total RNA was extracted from 10-mm frozen HCC tissue sections. To isolate the RNA from defined areas containing  $\geq 80\%$  tumor cells, all tumors were manually microdissected under direct visual control through a dissecting microscope. Total RNA in the frozen tissues was extracted using Trizol (Invitrogen) following the manufacturer's recommendations. Total RNA was digested with DNase I (Invitrogen) and was used for the first-strand cDNA reaction. The reaction mixture consisted of 5  $\mu\text{g}$  of DNase I-treated RNA, 1  $\times$  reverse transcriptase buffer, 2.5 mmol dNTP mix, 3.5  $\mu\text{mol}$  oligo primer, and 2.5 U/mL MultiScribe™ reverse transcriptase (PE Applied Biosystems). Each sample was handled using the same protocol, with the exception that reverse transcriptase was added to exclude the presence of interference from genomic DNA.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) was carried out using SYBER green dye in a Rotor Gene 3000 Detection System (Corbett Research, Sydney, Australia). Each SYBER green reaction (25  $\mu\text{L}$ ) contained one microliter diluted cDNA and 10.5  $\mu\text{L}$  SYBR Green PCR Master Mix, as well as 5 pmol forward and reverse primer (Ror2: forward 5'-AGGT-GCCTATGCAAGTTCA-3', reverse 5'-TGTGCGAG-GTTTAAGGTCTA-3'). Samples were activated by incubation at 95 °C for 5 min and denatured at 95 °C for 20 s. This was followed by annealing at 60 °C for 20 s and extension at 72 °C for 20 s, for 40 cycles. In all of the cDNA samples, gene expressions of Ror2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-GAA-GATGGTGATGGGATTC-3'), an internal quantitative control, were determined by SYBR green fluorescence using the Rotor-Gene 3000; the ratios of Ror2 to the housekeeping gene *GAPDH* represented the normalized relative levels of Ror2 expression. A non-template negative control was also included in each experiment. Analyses of all tumor samples were carried out at least twice, and the mean value was calculated.

### **Immunohistochemistry**

Immunohistochemical staining was performed on thin sections (4  $\mu\text{m}$ ) of paraffin-embedded archival tissue. The samples were dewaxed with xylene/ethanol before antigen retrieval (i.e., pressure cooked for one minute at full pressure, 15 psi, in 0.001 mol/L EDTA buffer, pH 8.0). The primary antibodies used were: Wnt5a (LS-C47384, Lifespan, 1:200), Ror2 (PAB3386, Abnova, 1:200),  $\beta$ -catenin (C19220, BD Transduction Laboratories, 1:400) and Ki-67 (MIB-1, Dako, 1:100). Immunohistochemical staining of antibodies was done using the Dako Envision Plus System (K5007, Dako). The anti-

body binding was visualized with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) before brief counterstaining with Mayer's hematoxylin. For monoclonal antibodies of mouse origin, negative controls were obtained using isotypic mouse immunoglobulin in the same dilution as the primary antibody of concern. All control experiments gave negative results.

### **Evaluation of immunostaining**

Two authors (Cao YC and Jiang H) who had no knowledge of the patients' clinical status reviewed all of the immunostained sections. Cases with discrepant results were re-evaluated jointly until agreement was reached. For expression of Wnt5a, Ror2 and  $\beta$ -catenin protein, in cases with multiple areas of low intensity that occurred during evaluation of immunostaining, five areas were selected at random and scored.

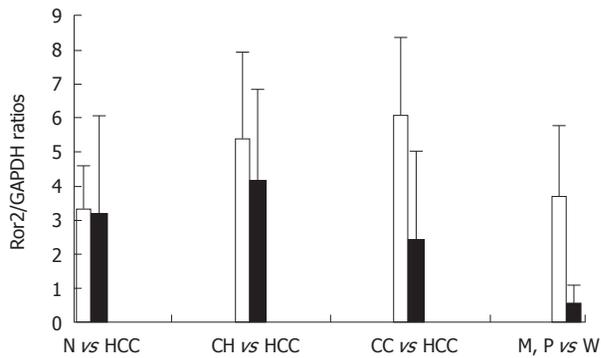
The degree of immunohistochemical staining was recorded using a semi-quantitative and subjective grading system that considered both the intensity of staining and the proportion of tumor cells that had an unequivocal positive reaction. Grades for stain intensity were: 0: No staining; 1: Weak staining; 2: Positive staining; and 3: Strong staining. For rating stained areas: 0: No staining; 1: Positive staining in  $< 10\%$  of tumor cells; 2: Positive staining in 10% to 50% of tumor cells; 3: Positive staining in  $> 50\%$  of tumor cells. The staining index was calculated as the staining intensity multiplied by the positive area.

Ki-67-positive cells were counted by viewing  $\geq 200$  HCC cells from  $\geq 10$  randomly selected fields. The percentage of antigen-positive nuclei among the total number of nuclei counted was calculated to obtain the nuclear labeling index (LI).

In the subsequent statistical analysis, the cutoff points for the staining index categories were mainly based on median values, as well as each marker's frequency distribution curve and the size of the subgroups. Therefore, cytoplasmic Wnt5a and Ror2 and membranous  $\beta$ -catenin staining indices were categorized by their median value as high ( $> 4$ ) or low (0-4), and the cytoplasmic  $\beta$ -catenin staining index was categorized as high ( $> 3$ ) or low (0-3). However, nuclear  $\beta$ -catenin expression was categorized based on the absence (staining index = 0) or presence (staining index  $\geq 1$ ) of staining. The Ki-67 labeling indices were divided into two groups (LI  $< 10\%$  and LI  $\geq 10\%$ ).

### **Follow-up and statistical analysis**

To determine the prognostic factor, the outcome of the 82 patients was determined by reviewing their medical charts. The follow-up period ranged from one to 54 mo (average: 31.3 mo; median: 27.0 mo). The end point in the analysis was HCC-related death. The overall and disease-free survival rates were estimated using the Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with univariate and multivariate Cox regressions models.



**Figure 1** Real-time reverse transcription-polymerase chain reaction analysis of *Ror2* gene (mRNA) expression in hepatocellular carcinoma, chronic hepatitis, cirrhotic and normal liver tissue. Ror2: Receptor tyrosine kinase-like orphan receptor 2; HCC: Hepatocellular carcinoma; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Bars: mean; Columns: SD; N: Normal; CH: Chronic hepatitis; CC: Cirrhosis; M, P, W: Moderately, poorly, and well differentiated tumor tissues, respectively.

The differences in *Ror2* mRNA expression between HCC and nontumorous liver tissue was statistically analyzed using Student's *t*-test and one-way analysis of variance (ANOVA) for multiple comparisons. The correlations between the clinicopathological parameters and Wnt5a, *Ror2* and  $\beta$ -catenin protein expression were analyzed using the  $\chi^2$  or Fisher's exact tests.

Pearson's correlation was used to determine the correlation between mRNA and protein expression, as well as between the expressions of different proteins. All statistical calculations were carried out using SPSS software (for Windows, version 13.0). A significant difference was defined at  $P < 0.05$ .

## RESULTS

### *Ror2* gene and protein expression in hepatocellular carcinoma

The *Ror2* gene (mRNA) expression levels relative to that of GAPDH in normal, HCC, chronic hepatitis, cirrhotic liver and adjacent nontumorous liver tissues are shown in Figure 1. *Ror2* mRNA levels were elevated in chronic hepatitis ( $5.420 \pm 5.492$ ,  $n = 11$ ) and cirrhotic liver tissues ( $6.128 \pm 5.252$ ,  $n = 8$ ) compared to that of normal ( $3.381 \pm 1.182$ ,  $n = 3$ ) and HCC ( $3.189 \pm 3.856$ ,  $n = 19$ ). Based on Student's *t*-test, statistically significant differences were found between the *Ror2* mRNA levels in HCC *vs* adjacent nontumorous (chronic hepatitis or cirrhotic) liver tissues ( $P = 0.029$ ), but not between HCC and normal liver tissues ( $P = 0.934$ ) or normal and adjacent nontumorous liver tissues ( $P = 0.094$ ). The *Ror2* mRNA level in moderately and poorly differentiated tumor tissues ( $n = 16$ ) was greater by 7.2-fold ( $P = 0.014$ ) than the level in well-differentiated tumor tissues ( $n = 3$ ). No significant differences were found between *Ror2* gene expression levels and other clinicopathological findings such as age, serum AFP concentration, tumor size, and HCC tumor stage.

Immunohistochemistry was performed to evaluate *Ror2* protein expression in tumor and non-tumorous liv-

**Table 1** The relationship between receptor 2 expression and clinicopathological features in hepatocellular carcinomas

Variables	n	Ror2 immunoreactivity		P
		Low	High	
Gender				0.890
Male	77	56	21	
Female	8	6	2	
Age (yr)				0.725
< 53 (median)	38	27	11	
$\geq 53$	47	35	12	
Serum AFP level ( $\mu\text{g/L}$ )				< 0.001
< 30	30	14	16	
$\geq 30$	55	48	7	
HBsAg				0.862
Positive	73	53	20	
Negative	12	9	3	
Tumor size (cm)				0.116
$\leq 2$	3	1	2	
> 2	82	61	21	
Histological grade				0.090
Well differentiated	9	7	2	
Moderately differentiated	60	40	20	
Poorly differentiated	16	15	1	
Liver cirrhosis				0.553
Present	56	42	14	
Absent	29	20	9	
T classification				< 0.001
T1	3	1	2	
T2	30	14	16	
T3	40	36	4	
T4	12	11	1	
Total	85	62	23	

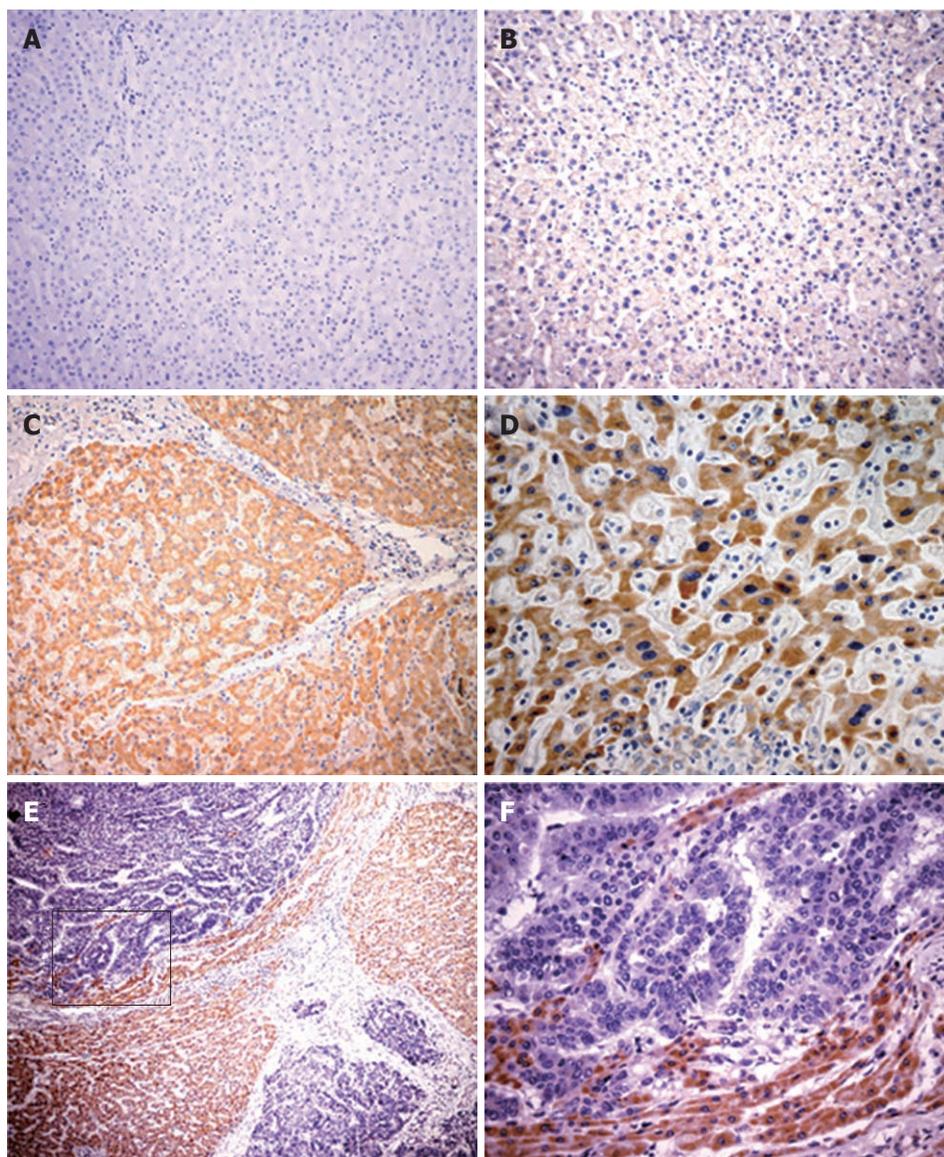
AFP:  $\alpha$ -fetoprotein; HBsAg: Hepatitis B surface antigen; Ror2: Receptor tyrosine kinase-like orphan receptor 2.

er cells. In non-tumorous liver cells and HCC tumor cells, *Ror2* protein expression was displayed in the cytoplasm, but in stromal cells *Ror2* protein was not observed. In comparative normal liver cells *Ror2* was negative or weakly expressed (Figure 2A and B), whereas all chronic hepatitis, cirrhotic, and dysplastic liver cells exhibited positive immunostaining for *Ror2* (Figure 2C and D). In 62/85 (72.9%) of the HCCs, *Ror2* immunostaining was reduced or absent (Figure 2E and F).

A significant correlation was found between the normalized *Ror2* gene expression ratio and the protein expression level in normal, tumor and non-tumorous liver tissues ( $r = 0.254$ ,  $P = 0.021$ ). Furthermore, statistical comparisons between *Ror2* mRNA expression and patients' clinicopathological features revealed a significant negative association between *Ror2* mRNA and tumor stage ( $P < 0.001$ ), and between *Ror2* mRNA and serum AFP ( $P < 0.001$ ). However, there were no significant differences between *Ror2* protein expression and the other clinicopathological findings in HCC (Table 1).

### Wnt5a protein expression in hepatocellular carcinoma

Wnt5a protein expression was observed in the cytoplasm of non-tumorous liver and tumor cells, but nowhere in stromal cells. There was little or no Wnt5a seen in normal liver cells. However, all chronic hepatitis, cirrhosis and



**Figure 2** Immunohistochemical staining for Ror2 in hepatocellular carcinoma. Patient-matched normal liver cells showed negative (A) or weak expression (B) of receptor tyrosine kinase-like orphan receptor 2 (Ror2). Liver cirrhosis cells (C) and dysplastic liver cells (D) exhibited strong positive immunostaining for Ror2. Tumor cells (E, F) showing negative Ror2 staining in hepatocytes, while strong cytoplasmic staining is seen in adjacent nontumorous cells. Original magnification, 200 × in A; 400 × in B and D; 100 × in C.

dysplastic liver cells exhibited strong positive immunostaining for Wnt5a. In contrast, in 65/85 (76.5%) of HCC patients, Wnt5a immunostaining was reduced or absent compared to the levels in adjacent nontumorous (hepatitis and cirrhotic) tissues (Figure 3A). There was a significant negative correlation between Wnt5a expression and tumor stage ( $P < 0.001$ ), and between Wnt5a and serum AFP ( $P = 0.016$ ). However, there were no significant associations between Wnt5a protein expression and the other clinicopathological features of HCC patients.

#### ***β-catenin protein expression in hepatocellular carcinoma***

In non-neoplastic liver tissue, a thin membranous β-catenin signal delineated the hepatocytes, and strong membranous and pale cytoplasmic staining of bile ductules

was observed. As shown in Figure 3B and C, altered expressions of β-catenin were found in 68.2% (58/85) of HCC cases. These alterations included reductions in the cellular membrane, increases in the cytoplasm, or both, and nuclear accumulation (in 7%, 6/85). However, no evidence of altered β-catenin expression was found in cirrhotic nodules or dysplastic liver cells in adjacent non-cancerous liver tissue. In tumor tissues, altered β-catenin expression was significantly associated with a worsening histopathological tumor grade ( $P = 0.041$ ) and was not significantly associated with the other clinicopathological parameters.

#### ***Correlations among the protein expressions of Wnt5a, Ror2 and β-catenin***

Associations among the protein expression levels of Wn-

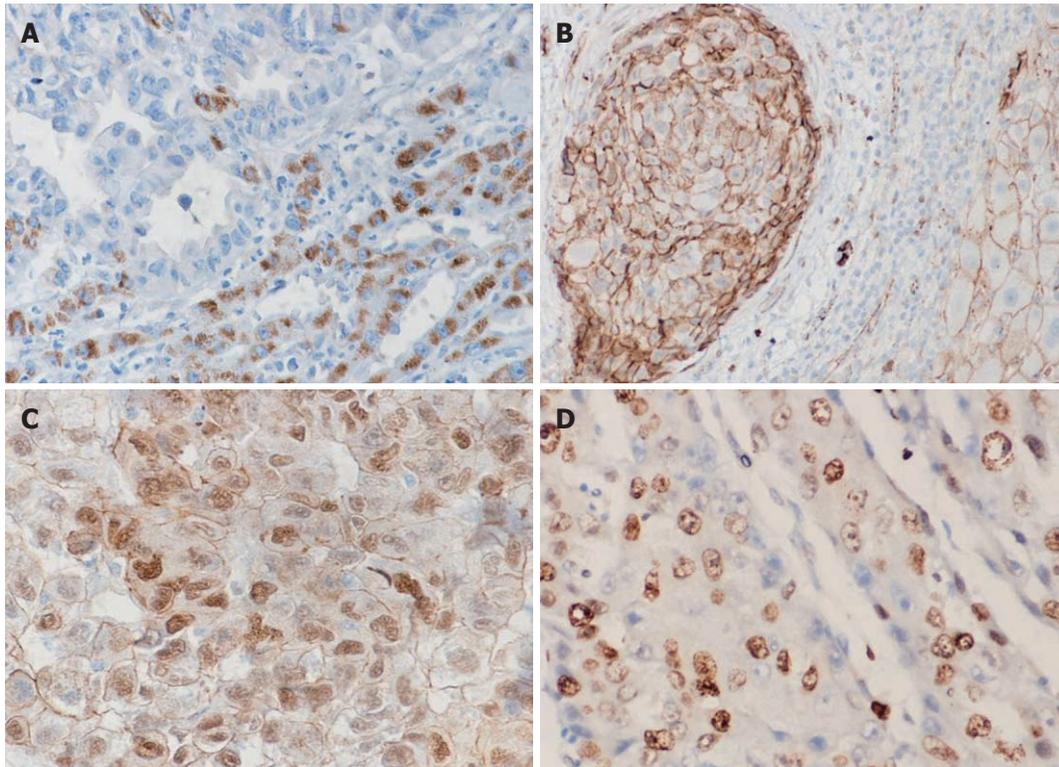


Figure 3 Immunohistochemical staining for Wnt member 5a (A),  $\beta$ -catenin (B, C) and Ki-67 (D) in hepatocellular carcinoma. Original magnification, 400  $\times$ .

Table 2 Correlation between the expression levels of Wnt member 5a, receptor 2 and  $\beta$ -catenin

Variable	n	Wnt5a			$\beta$ -catenin		
		Low	High	P-value	N	A	P-value
Ror2							
Low	62	54	8	< 0.001	15	47	0.014
High	23	11	12		12	11	
$\beta$ -catenin							
N	27	16	11	0.019			
A	58	48	10				

N: Normal membranous staining; A: Abnormal non-membranous staining; Wnt5a: Wnt member 5a; Ror2: receptor tyrosine kinase-like orphan receptor 2.

t5a, Ror2 and  $\beta$ -catenin are shown in Table 2. Low cytoplasmic Wnt5a expression was positively associated with low cytoplasmic Ror2 expression ( $r = 0.411$ ,  $P < 0.001$ ) and abnormal  $\beta$ -catenin expression ( $r = 0.254$ ,  $P = 0.019$ ) in HCC tissue. Similarly, there was a statistically significant correlation between low cytoplasmic Ror2 expression and abnormal  $\beta$ -catenin expression ( $r = 0.267$ ,  $P = 0.014$ ).

#### Tumor cell proliferation in hepatocellular carcinoma

To investigate the biological functions of proteins in HCC, the Ki-67 LI was assessed in relation to Ror2, Wnt5a and  $\beta$ -catenin status. A strong correlation between a high Ki-67 LI and the reductive loss of Ror2 ( $r = -0.344$ ,  $P = 0.002$ ), or Wnt5a ( $r = -0.278$ ,  $P = 0.010$ ), but not  $\beta$ -catenin ( $r = 0.095$ ,  $P = 0.386$ ) was found (Figure 3D).

#### Immunohistochemistry for tumor tissues from patients with lung metastasis of hepatocellular carcinoma

A previous study reported that Wnt5a and Ror2 were expressed predominantly in metastatic but not primary lesions of metastatic melanoma, suggesting that Wnt5a and Ror2 might be closely correlated with tumor invasiveness and metastasis<sup>[31,34]</sup>. To determine whether a similar phenomenon occurs in the metastasis of HCC, three cases of lung metastasis of HCC were included in this study. Immunohistochemical analysis showed that Wnt5a and Ror2 were not expressed in either primary or metastatic lesions (Figure 4A and B), whereas  $\beta$ -catenin-positive staining were detected in the cellular membrane (Figure 4C and D). The Ki-67 LI in tumor tissues was 10%.

#### Statistical analysis

The median follow-up was 27.0 mo for survivors (range, 1-54 mo). Three patients were lost to follow-up after surgery and were excluded from the survival analyses. The overall survival curve for the remaining 82 HCC cases is shown in Figure 5A. The estimated 1- and 3-year overall rate of survival was 75% and 44%, respectively. Kaplan-Meier analysis was used to compare the survival rates of HCC patients with tumors expressing low or high levels of Wnt5a and Ror2 and normal or abnormal  $\beta$ -catenin (Figure 5B-D).

In a univariate Cox proportional hazard regression model analysis (Table 3), tumor stage ( $P < 0.001$ ), serum AFP ( $P = 0.036$ ), and the expressions of Wnt5a ( $P = 0.024$ ) and Ror2 ( $P = 0.011$ ) were significantly associated

Table 3 Univariate Cox and multivariate Cox regression analysis overall survival

Covariate	P-value	Risk ratio	95% CI
Univariate			
Sex (male, female)	0.130	0.482	0.187-1.240
Age (< 53 yr, ≥ 53 yr)	0.166	0.640	0.341-1.203
Serum AFP level (< 30 μg/L, ≥ 30 μg/L)	0.036 <sup>a</sup>	2.162	1.051-4.449
HBsAg (positive, negative)	0.506	1.621	0.390-6.732
Tumor size (≤ 2 cm, > 2 cm)	0.467	2.089	0.286-15.239
Histological grade (well, moderately, poorly differentiated)	0.268	1.388	0.777-2.482
Liver cirrhosis (present, absent)	0.738	1.123	0.568-6.732
T classification (T1-T4)	< 0.001 <sup>a</sup>	2.339	1.487-3.679
Wnt5a (low, high)	0.024 <sup>a</sup>	3.288	1.167-9.263
Ror2 (low, high)	0.011 <sup>a</sup>	0.323	0.134-0.774
β-catenin (normal, abnormal)	0.052 <sup>a</sup>	1.966	0.995-3.885
Ki-67 (mitosis ≤ 10%, > 10%)	0.273	1.479	0.734-2.981
Multivariate			
Sex (male, female)	0.017 <sup>a</sup>	0.240	0.074-0.776
Age (< 53 yr, ≥ 53 yr)	0.075	0.538	0.272-1.065
Serum AFP level (< 30 μg/L, ≥ 30 μg/L)	0.343	1.476	0.661-3.296
HBsAg (positive, negative)	0.515	1.731	0.332-9.026
Tumor size (≤ 2 cm, > 2 cm)	0.711	1.535	0.159-14.827
Histological grade (well, moderately, poorly differentiated)	0.298	1.462	0.715-2.993
Liver cirrhosis (present, absent)	0.858	0.928	0.408-2.111
T classification (T1-T4)	0.001 <sup>a</sup>	2.119	1.347-3.336
Wnt5a (low, high)	0.020 <sup>a</sup>	0.288	0.101-0.824
Ror2 (low, high)	0.144	0.509	0.205-1.259
β-catenin (normal, abnormal)	0.013 <sup>a</sup>	3.233	1.286-8.130
Ki-67 (mitosis ≤ 10%, > 10%)	0.494	0.839	0.507-1.387

<sup>a</sup>P < 0.05 vs over survival. AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; Wnt5a: Wnt member 5a; Ror2: Receptor tyrosine kinase-like orphan receptor 2

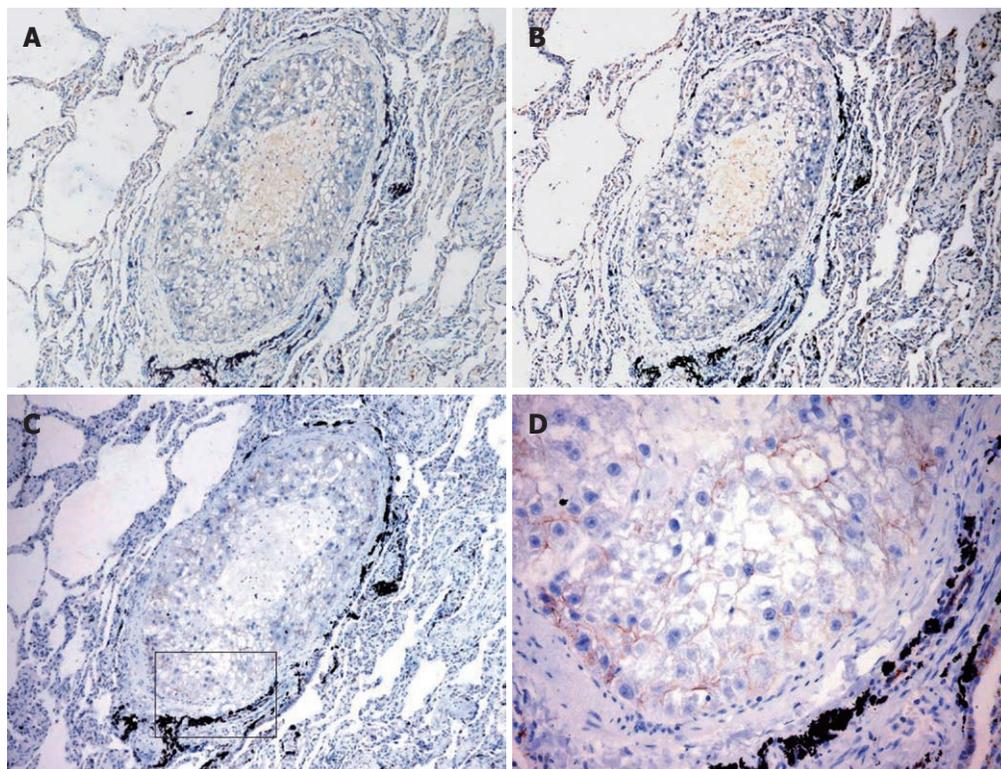
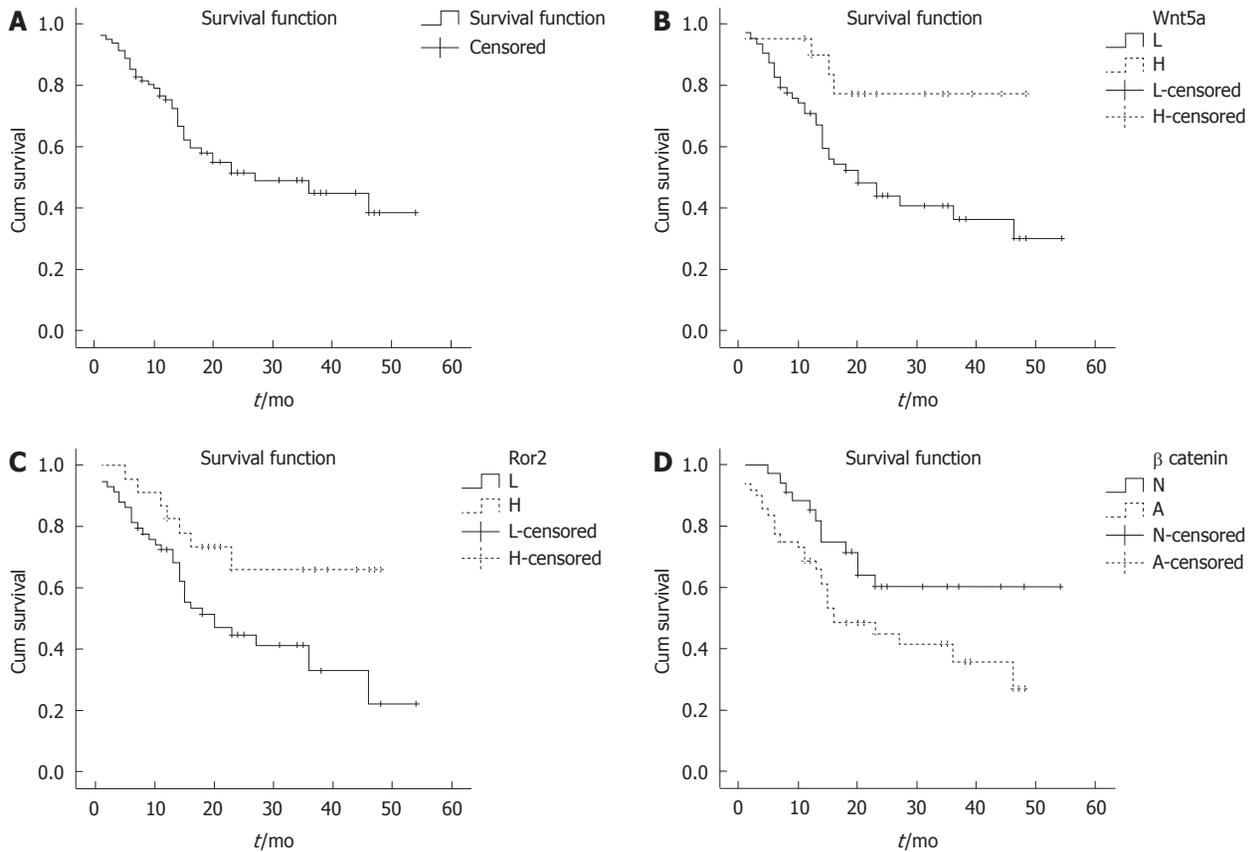


Figure 4 Immunohistochemical staining for Wnt member 5a (A), receptor 2 (B), β-catenin (C, D) in lung metastasis tissues. Original magnification: 400 × in D; 100 × in the others.



**Figure 5 Survival curves of 82 hepatocellular carcinoma patients.** A: Overall survival curves of 82 hepatocellular carcinoma (HCC) patients; B: Survival curves of 82 HCC patients with tumors expressing low or high levels of Wnt member 5a (Wnt5a) (log-rank test,  $P = 0.016$ ); C: Survival curves of 82 HCC patients with tumors expressing low or high levels of receptor 2 (Ror2) (log-rank test,  $P = 0.007$ ); D: Survival curves of 82 HCC patients with tumors expressing low or high levels of  $\beta$ -catenin (log-rank test,  $P = 0.045$ ). L: Low expression; H: High expression; N: Normal expression; A: Abnormal expression.

with overall survival. Therefore, patients with tumors having a low expression of Wnt5a and Ror2 had a poorer prognosis than those with tumors of high Wnt5a and Ror2 expression.

Multivariate Cox regression analysis (Table 3), the expression levels of Wnt5a ( $P = 0.020$ ), and  $\beta$ -catenin ( $P = 0.013$ ) showed a significant association with overall survival. However, a significant correlation between the expression levels of Ror2 and overall survival ( $P = 0.144$ ), serum AFP and overall survival ( $P = 0.343$ ) were not demonstrated.

## DISCUSSION

Consistent with previous reports<sup>[23,24]</sup>, in this study immunohistochemical analysis showed that the loss of Wnt5a protein expression in HCC tumors frequently occurred in patients with HCC (71%-81%), and this also correlated with increased AFP and poor histologic grade. Wnt5a may act as a tumor suppressor gene in the development of HCC. Similar results were obtained in colon carcinoma, breast cancer and thyroid carcinoma<sup>[17,18,35,36]</sup>. We also performed a survival analysis for 82 patients with HCC. Our results demonstrated that HCC patients with low expression of Wnt5a had a poorer prognosis than those with high Wnt5a expression, and Wnt5a was an indepen-

dent prognostic factor for HCC.

Recent studies have indicated that the upregulation of Wnt5a was associated with tumor invasiveness and metastasis in metastatic melanoma, gastric cancer, and non-small-cell lung carcinoma<sup>[17-19]</sup>. Wnt5a was expressed predominantly in the metastatic but not primary lesions of metastatic melanoma<sup>[34]</sup>. Therefore, three cases with lung metastasis of HCC were recruited in the present study. Immunohistochemical analysis with anti-Wnt5a antibody showed that Wnt5a was not expressed in either primary or metastatic lesions, which confirmed our hypothesis that Wnt5a acts as a tumor suppressor gene in HCC. These observations suggested that the complex Wnt5a-regulated signal pathways and the functional role of Wnt5a depends on cell type as well as stimulus factors during the development of HCC tumor.

Previous reports showed that Ror2 shared a similar structure with the Wnt receptor<sup>[25]</sup>. Mikels *et al.*<sup>[25,37]</sup> revealed that Wnt5a suppressed Wnt/ $\beta$ -catenin activity via the Ror2-mediated signal pathway, and confirmed that the Ror2 receptor required tyrosine kinase activity to mediate Wnt5A signaling. He *et al.*<sup>[38]</sup> demonstrated that Wnt5a levels correlated with those of Ror2 during mammalian palate development. Similar to Wnt5a, Ror2 plays different roles in different human tumor tissues. There is evidence that the enhanced expression of Ror2 is associ-

ated with tumor invasiveness and metastasis in metastatic melanoma, renal cell carcinoma, and squamous cell carcinoma<sup>[29-31]</sup>. In contrast, the mRNA and protein expression of Ror2 was reduced in colon cancer tissues compared with adjacent nontumorous liver tissue, which might be due to the hypermethylated Ror2 promoter<sup>[32]</sup>.

Our current study showed that *Ror2* gene transcription and protein translation were both suppressed in tumor tissues of HCC as compared with tissue adjacent to the tumor. This reduced expression of Ror2 in tumor tissues was correlated with decreased Wnt5a expression ( $P < 0.001$ ), a high Ki-67 LI, increased AFP, high differentiation, and poor prognosis. The consistency of Wnt5a and Ror2 expression in tumor tissues as well as in lung metastasis of HCC implies that Ror2 may be active downstream of Wnt5a and participate in the regulation of the noncanonical Wnt signal pathway. Moreover, the mRNA and protein expression of Ror2 is increased in chronic hepatitis livers and is greatly enhanced in cirrhotic livers as compared with normal liver tissues, suggesting Ror2 may play important roles in regulation of cell repair. The expression of Ror2 is reduced in tumor tissues and is associated with poor prognosis, indicating the impaired regulatory effect of Ror2 in cells, and Ror2 may also serve as an anti-tumor gene. In addition, in this present study, the mRNA expression of Ror2 was decreased in highly differentiated HCC as compared with moderately or poorly differentiated HCC ( $P < 0.05$ ), whereas similar results were not obtained in the protein expression of Ror2. The underlying mechanism needs to be further elucidated. However, due to the limited sample size in highly differentiated HCC (3 cases), future study will be continued by enlarging the sample size.

$\beta$ -catenin is recognized as the key mediator in the canonical Wnt signal pathway. Evidence indicates that Wnt5a inhibits the abnormal expression of  $\beta$ -catenin through the Ror2-mediated pathway<sup>[25,39,40]</sup>. The involvement of  $\beta$ -catenin in tumorigenesis has been intensively researched. In colon carcinoma, the nuclear localization of  $\beta$ -catenin induced by gene mutation contributes to tumorigenesis. However, in HCC associated with HBV infection,  $\beta$ -catenin mutations are rarely seen, and  $\beta$ -catenin mainly accumulates in the cytoplasm<sup>[41,42]</sup>. Consistent with these observations, statistically reduced membrane expression and elevated cytoplasmic expression of  $\beta$ -catenin were detected in HCC tumor tissue, compared with the  $\beta$ -catenin expression in cell membranes in the adjacent liver tissue. Among 85 HCC cases, 6 exhibited condensed nuclear staining of  $\beta$ -catenin, suggesting that this protein is involved in the development of HCC. Nevertheless, we could not rule out the possibility that the loss of Wnt5a and Ror2 protein expression may decrease  $\beta$ -catenin degradation, which contributes to disease progression. Additionally, the lining shape of  $\beta$ -catenin expression in cell membranes was observed in lung metastasis of HCC, which was different from their cytoplasmic expression in the primary lesion. Since  $\beta$ -catenin not only acts as the key mediator of the canonical Wnt signal pathway, but also binds to E-cadherin and together they contribute to

the cell adhesion and migration process<sup>[43]</sup>, we hypothesize that the lung metastasis expression of  $\beta$ -catenin benefits the accumulation and adhesion of tumor cells in metastatic lesions.

In summary, in patients with chronic hepatitis or cirrhosis, loss of Wnt5a and Ror2 protein expression in HCC tumor tissue frequently occurs during the progression of HCC and is associated with patient prognosis. We hypothesize that Wnt5a acts upstream of Ror2. Wnt5a and Ror2 synergistically execute an anti-tumor effect during the development of HCC. The decreased expression of Wnt5a and Ror2 in HCC tissues may be directly or indirectly correlated with the abnormal activity of  $\beta$ -catenin. It is possible that the Wnt5a-mediated noncanonical Wnt signal pathway and the  $\beta$ -catenin-mediated canonical signal pathway contribute to the pathogenesis and progression of HCC. These critical mediators may be novel promising targets for gene therapy. Our study showed that HCC patients with reduced Wnt5a and Ror2 expression had poorer prognosis, indicating that protein expression of Wnt5a and Ror2 might be used as clinicopathological biomarkers for prognosis of HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Understanding the molecular biological features of HCC is necessary for early diagnosis and better prognosis. The potential role of Wnt member 5a (Wnt5a) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) in human HCC is receiving increasing attention.

### Research frontiers

Recent work in a wide of human tumors has indicated that Wnt5a and Ror2 have a critical role in malignant progression. However, little is known about the association of Wnt5a expression with Ror2 and canonical Wnt in HCC. In this study, the authors demonstrate that Wnt5a, in conjunction with Ror2 and  $\beta$ -catenin, may take part in the progression of HCC.

### Innovations and breakthroughs

The loss of Wnt5a and Ror2 protein expression in HCC tumor tissue frequently occurs during the progression of HCC and is associated with patient poor prognosis. Wnt5a and Ror2 synergistically execute an anti-tumor effect during the development of HCC. The loss of Wnt5a and Ror2 protein expression was shown to be associated with abnormal  $\beta$ -catenin expression. This is the first study to report an association of Wnt5a expression with Ror2 and  $\beta$ -catenin in HCC.

### Applications

The study results suggest that protein expression of Wnt5a and Ror2 may be used as clinicopathological biomarkers for prognosis of HCC.

### Terminology

Wnt5a is a non-canonical member of the Wnt family of secreted glycoproteins that acts through the family of frizzled G-protein-coupled receptor, Ror2, to mediate important events during development and cancer.

### Peer review

This paper reported that the loss of Wnt5a and Ror2 protein expression in HCC was associated with poor patient prognosis. Based on reduction in tumors, the authors conclude these markers could be tumor suppressor genes and good prognostic markers for HCC patients. The work is purely descriptive and relevance to clinical practice is significant.

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## Chronic hepatitis C: Treat or wait? Medical decision making in clinical practice

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chronic hepatitis C virus (HCV) infection are treated or not.

**METHODS:** This prospective cohort study included 7658 untreated patients and 6341 patients receiving pegylated interferon  $\alpha$ 2a/ribavirin, involving 434 physicians/institutions throughout Germany (377 in private practice and 57 in hospital settings). A structured questionnaire had to be answered prior to the treatment decision, which included demographic data, information about the personal life situation of the patients, anamnesis and symptomatology of hepatitis C, virological data, laboratory data and data on concomitant diseases. A second part of the study analyzes patients treated with pegylated interferon  $\alpha$ 2a. All questionnaires included reasons against treatment mentioned by the physician.

**RESULTS:** Overall treatment uptake was 45%. By multivariate analysis, genotype 1/4/5/6, HCV-RNA  $\leq$  520 000 IU/mL, normal alanine aminotransferase (ALT), platelets  $\leq$  142 500/ $\mu$ L, age > 56 years, female gender, infection length > 12.5 years, concomitant diseases, human immunodeficiency virus co-infection, liver biopsy not performed, care in private practice, asymptomatic disease, and unemployment were factors associated with reduced treatment rate. Treatment and sustained viral response rates in migrants (1/3 of cohort) were higher than in German natives although 1/3 of migrants had language problems. Treatment rate and liver biopsy were higher in clinical settings when compared to private practice and were low when ALT and HCV-RNA were low.

**CONCLUSION:** Some reasons against treatment were medically based whereas others were related to fears, socio-economical problems, and information deficits both on the side of physicians and patients.

### Abstract

**AIM:** To analyze the decision whether patients with

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**Key words:** Hepatitis C virus; Interferon, Ribavirin; Liver cirrhosis; Migrants; Treatment barrier

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## INTRODUCTION

Approximately 170 million humans worldwide are estimated to have a chronic hepatitis C virus (HCV) infection including 400 000 in Germany<sup>[1,2]</sup>. More than 20 % of these patients will progress to cirrhosis, hepatocellular carcinoma, liver transplantation or death<sup>[3,4]</sup>. Therefore, all patients are candidates for antiviral therapy<sup>[5]</sup>. Its benefits need to be determined based on the individual's disease stage and on the likelihood of adherence and success<sup>[5,6]</sup>. Probably only 20 % of HCV-infected subjects know of their infection<sup>[3]</sup>. This diagnostic deficit is caused by various factors; e.g., physicians do not follow guidelines to screen for HCV infection when alanine aminotransferase (ALT) is elevated<sup>[7,8]</sup>. In addition only 11%-41% of known infections are treated<sup>[9-12]</sup>. Only some reasons for this therapeutic deficit have been identified including comorbidity, drug abuse and psychosocial factors<sup>[9,12-15]</sup>. Considering that therapy cures the disease in 50% of patients, treatment rate should be increased. The present study evaluates which factors influence the treatment decision in daily German practice.

## MATERIALS AND METHODS

The study which is ongoing was started in March 2003; the present data analyzes the treatment decision in patients included between March 2003 and May 2008. Throughout Germany 434 physicians (377 in private practice and 57 in hospital settings) contributed a mean number of 35 patients with chronic hepatitis C. The study included only one academic center. Basic data of the cohort have been published<sup>[16]</sup> and are only briefly mentioned here. The study was approved by health authorities and ethical committees. Due to its observational character it did not affect individual medical decisions. A structured questionnaire had to be answered prior to the treatment decision; a second part of the study analyzes patients treated with pegylated interferon  $\alpha$ 2a (Pegasy<sup>®</sup>, Roche Pharma AG) and ribavirin. This part is not fully analyzed here; only those aspects are analyzed which are relevant to the treatment decision. All questionnaires included rea-

**Table 1** Demographic data and basic characteristics

Characteristics	Not treated (n = 7658)	Treated (n = 6341)
% of the 13 999 patients	55.7	45.3
Genotypes 1/4/5/6 (%)	69.8	59.4
Genotypes 2/3 (%)	30.2	40.6
Age (yr, median)	44.0	41.0
BMI (kg/m <sup>2</sup> , median)	24.2	24.3
Gender (male %)	56.6	61.1
Regular employment (%)	35.3	50.2
Infection length (yr, median)	11.0	10.0
Ultrasound performed (%)	76.8	87.6
Liver biopsy performed (%)	12.8	30.2
Fibrosis score F 0-1	72.8	58.6
Fibrosis score F 2-4	27.2	41.4
Active drug or alcohol abuse (%)	28.3	13.8
HIV co-infection (%)	6.7	3.7
Psychiatric disease (%)	14.8	9.2
Severe language problems (%)	9.6	10.0
Initial HCV-RNA (IU/mL, median)	482 500	500 000
ALT (U/L, median)	61.0	78.0
Thrombocytes (/ $\mu$ L, median)	217 000	218 000
At least on concomitant disease (%)	62.3	42.6

BMI: Body mass index; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; ALT: Alanine aminotransferase.

sons against treatment mentioned by the physician. After July 2004 questionnaires also asked why patients denied therapy ( $n = 7658$ ). Language skills were assessed after January 2006. Fibrosis was staged according to Desmet and Scheuer from F0 to F4<sup>[17]</sup>. Among the total 15 137 patients 7658 subjects did not receive any treatment ("untreated patients") while 6341 received pegylated interferon  $\alpha$ 2a and ribavirin ("treated patients") and 1138 alternative treatments. Details on alternative therapies (92.5% silymarin, 2.8% ursodesoxycholic acid, 4.9% other interferons) are not given because their characteristics were similar to the group receiving pegylated interferon  $\alpha$ 2a/ribavirin. Thus, in the following text the total cohort consists of 13 999 patients separated by the treatment decision into "treated patients" ( $n = 6341$ ) and "untreated patients" ( $n = 7658$ ). Specific procedures were not mandatory for inclusion except for documentation of chronic hepatitis C. There were no exclusion criteria except for patients below age 18 years and those with Child B/C cirrhosis. Thus, the study represents a real life scenario of a rather unselected cohort including a significant fraction of all patients diagnosed with hepatitis C in Germany.

## Statistical analysis

For continuous variables, receiver operating characteristic analyses estimated the best cut-off point for treatment decision; these cut-off points were 56 years for age, 520 000 IU/mL for basal HCV-RNA,  $\geq$  one concomitant disease,  $\geq$  12.5 years for infection length, and 142 500/ $\mu$ L for platelets. Categorical variables were used for continuous variables using these cut-off points. Association of various factors with treatment decision and sustained virological response (SVR = negative HCV-RNA 24 wk after end of therapy) were analyzed in an

Table 2 Treatment and sustained virological response rates in various subgroups

	Treatment rate %	SVR %	Number	Fischer's exact test, two-sides <i>P</i> value	
				Treatment rate	SVR
Total	45.3	49.6	13 999		
Genotypes 1/4/5/6	41.4	42.7	9114	< 0.0001	< 0.0001
Genotypes 2/3	52.7	59.8	4885		
Clinical setting	63.9	49.8	1298	< 0.0001	NS
Private practice	43.4	49.6	12 701		
Male	47.2	47.9	8214	< 0.0001	< 0.01
Female	42.6	52.3	5785		
Age ≤ 56 yr	49	51.3	11 497	< 0.0001	< 0.0001
Age > 56 yr	28.2	36.7	2502		
BMI ≤ 23 (kg/m <sup>2</sup> )	44.3	51.8	4762	< 0.01	< 0.05
BMI > 23 (kg/m <sup>2</sup> )	46.9	48.6	8846		
No employment	38.9	47.3	8113	< 0.0001	< 0.001
Regular employment	54.1	52	5886		
Bad German language skills	47	52.5	824	NS	NS
Good German language skills	45.8	47.8	7565		
Migrants	53.3	52.6	2663	< 0.0001	< 0.0001
German natives	41.7	45.4	5465		
Infection length ≤ 12.5 yr	62.8	51.6	3639	< 0.0001	< 0.01
Infection length > 12.5 yr	37.2	48	3165		
Ultrasound not performed	30.7	47.5	2568	< 0.0001	NS
Ultrasound performed	48.6	50	11 431		
Liver biopsy not performed	39.9	50.1	11 100	< 0.0001	NS
Liver biopsy performed	66.1	48.5	2899		
Fibrosis scores F0-1	60.9	52.4	1766	< 0.0001	< 0.01
Fibrosis scores F2-4	74.6	44.1	1017		
Clinical symptoms absent	42.2	47.8	4430	< 0.0001	NS
Clinical symptoms present	46.7	50.4	9569		
No concomitant disease	55.7	51.8	6527	< 0.0001	< 0.0001
At least one concomitant disease	36.2	46.8	7472		
Psychiatric disease absent	46.9	49.8	12 281	< 0.0001	NS
Psychiatric disease present	34.1	48.4	864		
Active drug or alcohol abuse absent	49.9	49.7	10 960	< 0.0001	NS
Active drug or alcohol abuse present	28.7	49.4	3039		
HIV co-infection absent	46.1	50	13 254	< 0.0001	< 0.01
HIV co-infection present	31.4	39.3	745		
Good quality-of-life	43.8	49.5	11 348	< 0.0001	NS
Reduced quality-of-life	51.8	50.1	2651		
ALT normal (< 50 U/L for men, < 30 U/L for women)	34.8	50.8	3297	< 0.0001	NS
ALT elevated (U/L)	49.6	49.7	10 105		
Thrombocytes ≥ 142 500 /μL	48	51.6	11 284	< 0.0001	< 0.0001
Thrombocytes < 142 500 /μL	38.9	36.2	1816		
HCV-RNA ≤ 520 000 IU/mL	45.4	54.8	6810	< 0.0001	< 0.0001
HCV-RNA > 520 000 IU/mL	49.7	43.3	5904		
No concomitant disease	55.7	51.8	6527	< 0.0001	< 0.0001
At least one concomitant disease	36.2	46.8	7472		
HIV co-infection absent	46.1	50	13 254	< 0.0001	< 0.01
HIV co-infection present	31.4	39.3	745		

SVR: Sustained virological response; BMI: Body mass index; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; NS: Not significant.

univariate fashion using Fisher's exact test. Only those variables which were significant in the univariate analysis were included in the multivariate analysis.

## RESULTS

### Effects of various factors on treatment rate by univariate analysis

Basic characteristics of treated *vs* untreated patients are shown in Table 1. Many characteristics were similar for genotypes 1 (*n* = 8625), 4 (*n* = 440), 5 (*n* = 22) and 6 (*n*

= 27) and for genotypes 2 (*n* = 1000) and 3 (*n* = 3885) (data not shown); thus, further analyses were done in two subgroups, i.e., genotypes 1/4/5/6 *vs* 2/3. Table 2 summarizes treatment and SVR rates in the total cohort (45.3% and 49.6%, respectively) and in treated *vs* untreated patients.

By univariate analysis reduced treatment uptake and reduced SVR were seen in these groups: (1) genotypes 1/4/5/6 *vs* 2/3; (2) age > 56 years *vs* ≤ 56 years; (3) platelets ≤ 142 500/μL *vs* > 142 500/μL; (4) disease duration >12.5 years *vs* ≤ 12.5 years; (5) human im-

**Table 3** Treatment and sustained virological response rates *vs* socio-economic problems and concomitant diseases

Characteristics	Treatment rate %	SVR %	<i>n</i>
Drug abuse absent and employed without psychiatric disease or HIV co-infection	58.2	52.7	4382
Drug abuse absent and employed without psychiatric disease	58.2	52.4	4560
Drug abuse absent and employed	57.1	52.6	4929
Drug abuse absent	49.2	49.6	10 839
Drug abuse present	32.0	49.9	3160
Drug abuse present and unemployed	29.1	51.6	2203
Drug abuse present and unemployed with psychiatric disease	25.1	50.8	470
Drug abuse present and employed with psychiatric disease and HIV co-infection	7.1	0.0	56

HIV: Human immunodeficiency virus.

munodeficiency virus (HIV)/HCV co-infection *vs* HCV mono-infection; (6) presence *vs* absence of concomitant diseases; (7) German natives *vs* migrants; and (8) absence *vs* presence of regular employment.

Treatment uptake was reduced but SVR was higher in the following groups: (1) women *vs* men; (2) fibrosis F0-1 *vs* F2-4; and (3) basal HCV-RNA > 520 000 IU/mL *vs* ≤ 520 000 IU/mL.

Treatment uptake was reduced while SVR was similar in the following groups: (1) normal *vs* elevated ALT; (2) good *vs* reduced quality of life; (3) treatment in private practice *vs* clinical setting; (4) presence *vs* absence of psychiatric disease; (5) presence *vs* absence of alcohol or drug abuse; and (6) liver biopsy (and ultrasound) not performed *vs* performed.

History of *i.v.* drug abuse was the most frequent mode of infection (44.6%) followed by history of blood transfusions (17.0%). By multivariate analysis infection mode did influence neither treatment uptake nor SVR (data not shown). In the total cohort only 20.7 % of patients had a liver biopsy. Biopsy was done more often in genotypes 1/4/5/6 when compared to genotypes 2/3 (23.6% *vs* 15.3%,  $P < 0.001$ ) and in patients with elevated ALT (75.4% had elevated ALT) when compared to those with normal ALT (21.6% *vs* 18.4%,  $P < 0.05$ ). Biopsy rate was three-times higher in hospital settings when compared to practitioners (53.4% *vs* 17.4%,  $P < 0.001$ ). Alcohol or drug abuse was a frequent treatment barrier in particular in patients with psychiatric diseases or HIV co-infection and in jobless people (Table 2). Treatment rates were similarly low in drug abusers with or without substitution (data not shown). Patients with alcohol or drug abuse refused therapy less often compared to patients without abuse (50.2% *vs* 67.9%,  $P < 0.001$ ). Thus, the decision not to treat was made primarily by the physician. About 1/3 of all patients were migrants among whom 1/3 had severe language problems. Nevertheless, treatment and SVR rates were higher in migrants than in German natives while language problems did not affect treatment and SVR rates. Treatment uptake decreased with an increasing number of socio-economical and psychiatric problems; HIV infection on top of other problems reduced treatment uptake to 7 % (Table 3). SVR was unaffected even by presence of several socio-economical problems but was drastically reduced when there was a HIV co-infection on top of other problems.

### Multivariate regression analysis

Gender, age, genotype, HCV-RNA, ALT, platelets, symptoms, infection length, occupational status, concomitant diseases, HIV co-infection, alcohol and drug abuse, performance of liver biopsy and ultrasound, and quality-of-life significantly affected the treatment decision in the multivariate analysis (Figure 1). In patients with genotypes 1/4/5/6 the same factors as for the total cohort affected the treatment decision except for presence of symptoms; in patients with genotypes 2/3 the same factors as for the total cohort affected the treatment decision except for symptoms, platelets, employment, and performance of liver biopsy (data not shown). SVR was associated with various factors in the univariate analysis (Table 2). By multivariate analysis SVR was associated only with gender, genotype, HCV-RNA, age, platelets, symptoms, employment and HIV co-infection (data not shown).

### Analysis of specific reasons against treatment

The analysis looked at reasons mentioned by physicians and patients (Figure 2). The patients' wish was the most common reason against treatment (62.9 %). Among these patients lack of understanding the need of therapy, fear of side-effects, and problems with family and job were frequent reasons. Fear of side-effects was mentioned more often in women than in men (29.9% *vs* 18.8%,  $P < 0.001$ ). Alcohol or drug abuse and concomitant diseases (most commonly depression) were also frequent treatment barriers. Among patients who did not see a need for therapy reasons included lack of liver disease, symptoms, fibrosis and bad prognosis as well as normal ALT. In patients with normal ALT minor disease activity was mentioned by the physician as a reason to wait in 24.1% whereas this reason was mentioned in only 6.6% when ALT was elevated ( $P < 0.001$ ). In contrast, a similar percentage of patients mentioned the lack of disease activity as a treatment barrier irrespective of whether ALT was normal or elevated (27.1% *vs* 24.4%; NS). In patients with a HCV-RNA ≤ 520 000 IU/mL minor disease activity was mentioned by the physician as a reason to wait in 15.8% whereas this reason was mentioned in only 6.7% when HCV-RNA was > 520 000 IU/mL ( $P < 0.01$ ). The percentage of patients mentioning lack of disease activity as a treatment barrier was similar when looking at high or low HCV-RNA (data not shown). In patients who had liver biopsy minor disease activity was mentioned by the

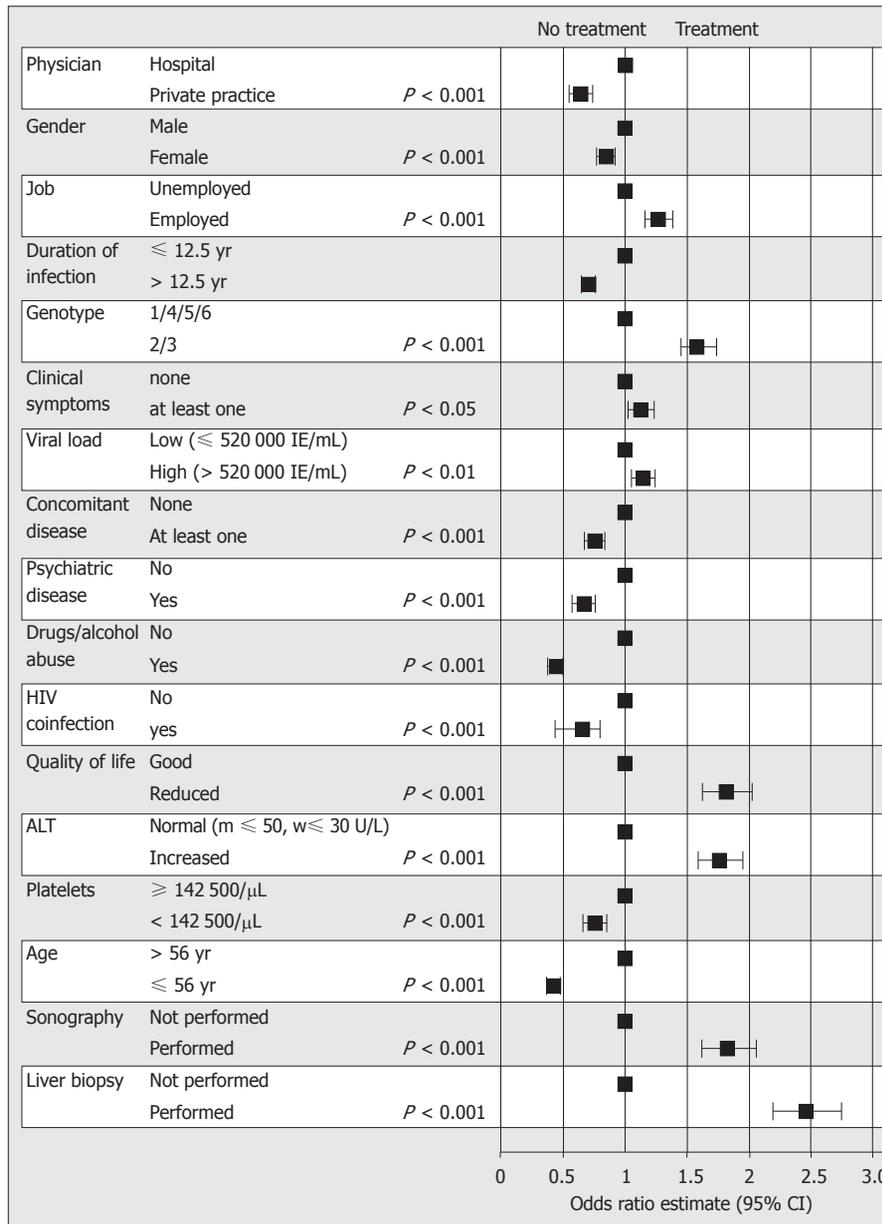


Figure 1 Multivariate regression analysis of treatment rates vs various factors. HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase.

physician as a treatment barrier in 21.4 % whereas this reason was mentioned in only 10.3 % of patients without a liver biopsy ( $P < 0.01$ ). Patients mentioned fear of side effects and lack of understanding the need for therapy less often when treated in hospital settings as compared to private practice (18.5% vs 24.1% and 17.4% vs 25.9%,  $P < 0.01$ , respectively). In patients with drug/alcohol abuse, this abuse was the main treatment barrier mentioned by physicians (48.1 %). In contrast, patients with abuse refused therapy less often than those without (50.2% vs 67.9%,  $P < 0.001$ ). In HIV co-infection concomitant diseases and drug/alcohol abuse were more frequent treatment barriers than in mono-infection (25.0% vs 16.6% and 25.2% vs 16.4%,  $P < 0,01$ ). HIV co-infected patients refused therapy less often than mono-infected patients (59.1% vs 63.2%,  $P < 0.05$ ). Similarly, in patients with psychiatric diseases, the psychiatric disease was the main

treatment barrier (46.2%); among patients with psychiatric disease drug and alcohol abuse was another common barrier (24.5% vs 15.7% in patients without psychiatric disease;  $P < 0.001$ ). Older age was associated with a reduced treatment rate (49.0% vs 28.2% in patients ≤ 56 years vs patients > 56 years) (Table 2; Figure 1); in patients aged between 65 and 70 years treatment rate was 26.3% (158/600) and thus similar to the rate of 28.2% seen at ages > 56 years.

## DISCUSSION

Treatment uptake in the present cohort (45%) is one of the highest reported in the literature. Since the cohort included a significant fraction of all HCV-infected patients in Germany, the high treatment rate is probably not due to selection bias. In the literature treatment uptake

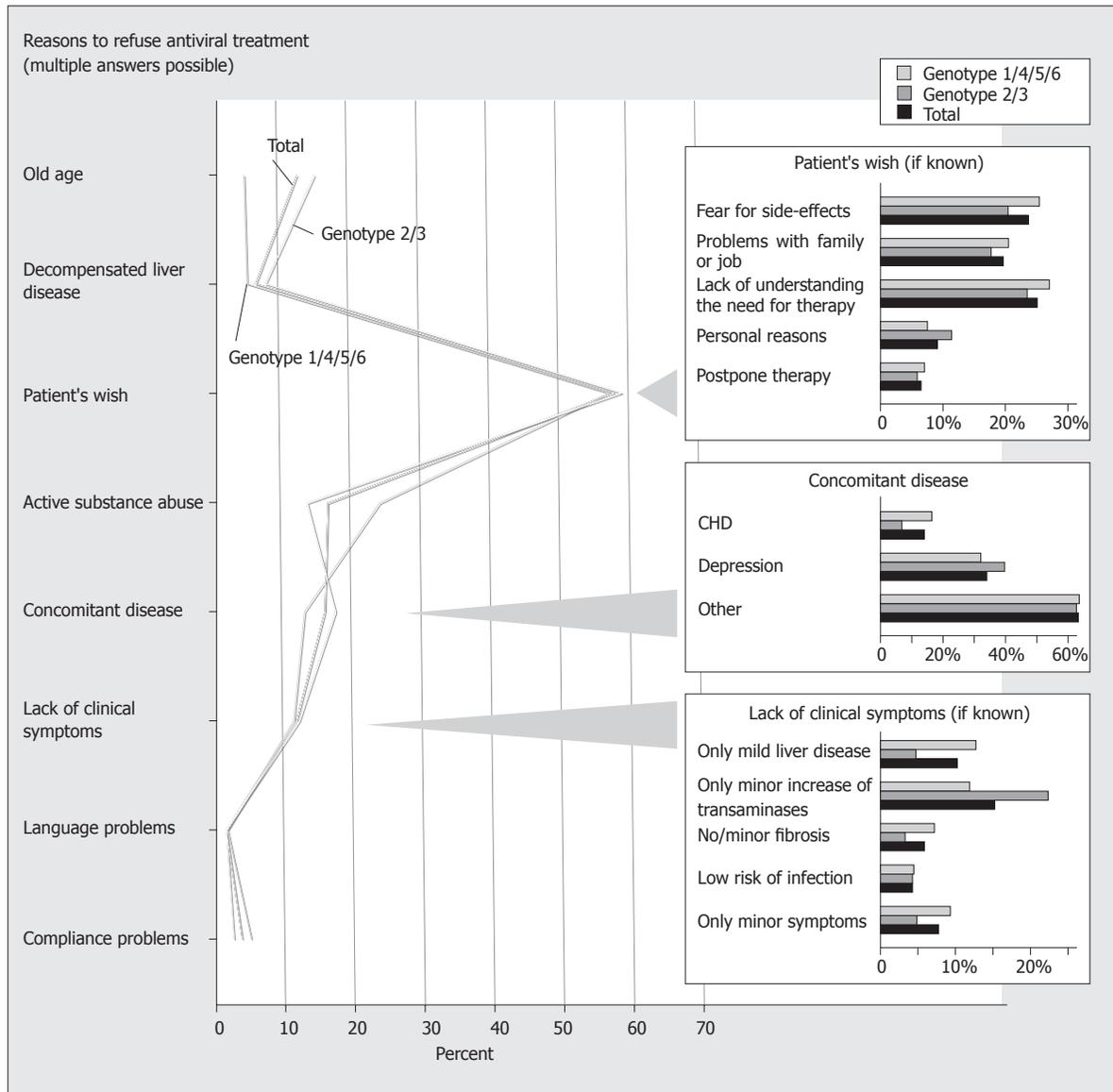


Figure 2 Reasons to refuse antiviral treatment.

tends to decrease with increasing number of subjects studied<sup>[9,12-15,18]</sup> with the lowest rate of 12% reported for the largest group of subjects studied<sup>[15]</sup>. There is little pre-selection in the present cohort; only patients with Child B/C cirrhosis were excluded as well as those under age 18 years. The present study did not include a relevant number of academic centers where most previous studies had been done. The community-based character of the present cohort incorporating 434 physicians and hospitals throughout the country reflects daily life in Germany probably better than looking at academic centres. However, one needs to keep in mind that most of the 434 physicians were not general practitioners, but gastroenterologists or at least physicians who treat hepatitis C. In general practitioners treatment rates may be lower than the 45% reported here. In the general United States community only 11% of all HCV-infected subjects had been treated<sup>[15]</sup>. This low treatment uptake suggests that therapeutic deficits are located on level of the general practitioner or

the health care system itself<sup>[7,8]</sup>. Recent studies show that knowledge deficits and misperceptions are main treatment barriers<sup>[19-21]</sup>. A high treatment rate might therefore reflect good knowledge among physicians and patients. In Germany most physicians who treat hepatitis C in private practice are organized in the Association of German Gastroenterologists (“bng”). Via their association gastroenterologists have been involved in the development of national HCV guidelines<sup>[6,22]</sup>. Many of them are members of the national “hepatitis competence network”. Recent studies have also shown that German patients with hepatitis C are well informed and better than patients with hepatitis B<sup>[23-25]</sup>. However, some practice aspects did not meet standards in the present cohort including the use of liver biopsy and interpretation of HCV-RNA values. Also, there were misperceptions among patients. Patients’ refusal was a common treatment barrier in the present cohort and in previous studies<sup>[9-11]</sup>. One of the highest treatment rates (41%) was published by Delwaide *et al.*<sup>[9]</sup>;

in that study only 17% of patients declined therapy. Thus, a high treatment uptake may be associated with low rate of refusal by patients<sup>[9]</sup>. This association may partly be explained by information deficits. In some subgroups, e.g., in patients with HIV co-infection and those with drug and alcohol abuse, the decision against treatment was often made by the physician whereas patients were rather willing to receive therapy.

Genotype and viral replication are major factors for estimating the chance for SVR and are therefore considered in the treatment decision. Correspondingly treatment rate and SVR were higher for genotypes 2/3 when compared to genotypes 1/4/5/6. In accordance with most previous studies<sup>[5,11,15,22]</sup> older age was associated with both reduced treatment uptake and reduced SVR in the present cohort. These results are in contrast to a recent study<sup>[18]</sup> in which being elderly was not associated with a low SVR. Surprisingly, treatment rate was low in patients with low HCV-RNA. This is a paradox because SVR is low at high replication in the present study and in the literature<sup>[26-28]</sup>. Thus, there may be misperceptions that high viral load indicates bad prognosis. All evidence shows this is not the case<sup>[22,29,30]</sup>. Further analyses suggested that physicians (and not patients) carry this misperception.

For many years normal serum aminotransferases were a common treatment barrier because they were thought to indicate good prognosis and reduced efficacy of therapy. In the meantime it has been shown that up to 30% of patients with normal ALT have major fibrosis and that SVR is not associated with ALT as also seen in the present study<sup>[22,29-31]</sup>. Despite this data, treatment rate was markedly lower in patients with normal ALT when compared to those with elevated ALT. We have reported a similar misperception of ALT for the decision to do HCV antibody tests<sup>[8]</sup>; many physicians just tested for HCV infection if ALT was markedly increased although most infections were associated with normal or slightly elevated ALT. Thus, ALT values are overestimated both in diagnostic<sup>[8]</sup> and treatment decisions<sup>[9,12]</sup>.

In contrast to academic trials, only 20% of patients had a liver biopsy in daily German practice. According to guidelines liver biopsy should be considered when the results will influence the treatment decision and in particular when treatment is not initiated<sup>[5,22]</sup>. However, treatment rate in patients with a liver biopsy was twice that seen in patients without a biopsy; according to guidelines it should be the other way around. Only a single previous study has also shown a positive association between performance of liver biopsy and treatment uptake<sup>[32]</sup>. It may be speculated that patients who refused liver biopsy may have a general problem to accept medical means. However, further analyses support other explanations. Biopsy rate in hospital settings was more than three-times higher than that in private practice. Although non-invasive means of assessing fibrosis are entering clinical routine, only a minority of community-based physicians use serum markers or sonographic stiffness in daily clinical routine as yet. Thus, physicians in private practice underestimate the value of liver biopsy more often than physicians in hospital

settings. The lack of immediate availability of biopsy may explain the low biopsy rate among practitioners. Also, treatment uptake was markedly lower for patients treated in private practice when compared to hospital settings. The analysis of specific reasons against treatment may partly explain this difference: patients mentioned fear of side effects and lack of understanding the need for therapy less often when treated in clinical settings when compared to private practice.

The treatment rate of HCV infection was considerably lower in HIV co-infected patients when compared to HCV mono-infection. Although SVR rates were also somewhat lower in co-infected patients, they were still in an acceptable range considering that end-stage liver disease is a common cause of death in HIV/HCV co-infection<sup>[33-35]</sup>. When compared with the literature the present rates of treatment and SVR (31% and 39%) look favorable. In other studies SVR ranged from 8% to 40% in co-infected patients<sup>[36-38]</sup>. Nevertheless HIV co-infection was a main treatment barrier also in the present cohort. Among co-infected patients drug and alcohol abuse as well as fear of side-effects were frequent treatment barriers. The present analysis also shows that HIV/HCV co-infected patients refused therapy less often than mono-infected patients; thus the low treatment rate is probably mainly caused by physicians and not by patients. In previous studies only 12%-33% of HIV co-infected patients initiated HCV therapy<sup>[36,39-40]</sup>; main barriers were non-adherence, patients' refusal, drug abuse and psychiatric problems. The present results demonstrate that the HIV infection on top of psychiatric and socio-economical problems may not only reduce treatment uptake but almost eliminates chances for SVR.

Recently it has been shown that HCV infection can successfully be treated in patients with drug and alcohol abuse and in those with HIV co-infection provided that there is a good management<sup>[35-38,41-43]</sup>. This is of great importance because alcohol abuse and co-infections accelerate fibrosis<sup>[34,35,44,45]</sup>. Although a history of drug abuse did not reduce treatment rate in the present cohort, active alcohol and drug abuse were associated with a markedly reduced treatment uptake as reported previously<sup>[10,11,14,15]</sup>; SVR was not affected by abuse. In 50% of abusers, physicians specified the abuse as the main treatment barrier. In contrast, patients with alcohol or drug abuse refused therapy less often than did patients without abuse. Thus, the decision not to treat was made primarily by the physician. A survey of 320 American Society of Addiction Medicine physicians showed that even among these specialists only a minority were providing HCV treatment or willing to provide treatment<sup>[46]</sup>. Treatment rates are even lower in the general community and may approach values of less than 1% in unselected drug addicts<sup>[47]</sup>.

Treatment rate was lower in unemployed patients when compared to those with a job while SVR was similar between these groups. Since jobless people tend to have a low educational state, these results fit to recent United States data showing that psychosocial factors and low education were associated with reduced treatment up-

take<sup>[12,14,48]</sup>. In the present cohort 1/3 of HCV infected patients were migrants among whom 1/3 had severe language problems. Unexpectedly, treatment uptake was not lower but higher in migrants when compared to German natives. These results can not be explained easily. Along this line women had a lower treatment rate when compared to men in this cohort as well as in another previous study<sup>[10]</sup>. This is also unexpected because men have a lower use of medical services than women both in the United States<sup>[49]</sup> and in Germany<sup>[50]</sup>. Thus, good knowledge and care about health issues *per se* do not necessarily increase treatment uptake for hepatitis C.

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## COMMENTS

### Background

In recent surveys only 20% of hepatitis C virus (HCV)-infected subjects know of their infection and only 20% of the latter are treated. Considering that therapy cures the disease in 50% of patients, treatment rate should be increased.

### Research frontiers

Bio-epidemiological research focuses to identify treatment barriers in patients with chronic hepatitis C. As yet only some reasons for the current large therapeutic deficit have been identified including co-morbidity, drug abuse and psychosocial factors. The present study evaluates which factors influence the treatment decision in daily German practice.

### Innovations and breakthroughs

Treatment uptake in the present cohort (45%) is one of the highest reported in the literature. A high treatment rate usually reflects good knowledge among physicians and patients. In Germany many physicians who treat hepatitis C are members of the national "hepatitis competence network" which is aimed to implement practice guidelines in the broad medical community. Despite the obvious success of the German hepatitis competence network some practice aspects did not meet standards in the present cohort including the use of liver biopsy and interpretation of HCV-RNA and alanine aminotransferase (ALT) values. Liver biopsy and thus knowledge about fibrosis stage were too low in particular in patients treated in private practice and in those with normal ALT. Also, there were misperceptions among patients as their refusal was a common treatment barrier. Unexpectedly, therapy uptake was higher in migrants despite language problems. Some further reasons against treatment appeared medically based whereas others seemed to be based on fears, socioeconomical problems and information deficits both on the side of physicians and patients.

### Applications

The present cohort study includes a significant fraction of all HCV-infected patients in Germany. The community-based character of the present cohort incorporating 434 physicians and hospitals throughout the country reflects daily

life in Germany probably better than looking at specialized academic centres.

### Terminology

Treatment barrier: Reasons why patients with chronic hepatitis C are not treated with antiviral drugs.

### Peer review

This is an important paper with a large HCV patient cohort from Germany including both academic and non-academic centres detailing reasons for treating and not treating HCV.

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## Celiac disease: Management of persistent symptoms in patients on a gluten-free diet

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### Abstract

**AIM:** To investigate all patients referred to our center with non-responsive celiac disease (NRCD), to establish a cause for their continued symptoms.

**METHODS:** We assessed all patients referred to our center with non-responsive celiac disease over an 18-mo period. These individuals were investigated to establish the etiology of their continued symptoms. The patients were first seen in clinic where a thorough history and examination were performed with routine blood work including tissue transglutaminase antibody measurement. They were also referred to a specialist gastroenterology dietician to try to identify any lapses in the diet and sources of hidden gluten ingestion. A repeat small intestinal biopsy was also performed and compared to biopsies from the referring hospital where possible. Colonoscopy, lactulose hydrogen breath testing, pancreolauryl testing and computed tomography scan of the abdomen were undertaken if the symptoms persisted. Their clinical progress was followed over a minimum of 2 years.

**RESULTS:** One hundred and twelve consecutive patients were referred with NRCD. Twelve were found to have celiac disease (CD). Of the remaining 100 patients, 45% were not adequately adhering to a strict gluten-free diet, with 24 (53%) found to be inadvertently ingesting gluten, and 21 (47%) admitting non-compliance. Microscopic colitis was diagnosed in 12% and small bowel bacterial overgrowth in 9%. Refractory CD was diagnosed in 9%. Three of these were diagnosed with intestinal lymphoma. After 2 years, 78 patients remained well, eight had continuing symptoms, and four had died.

**CONCLUSION:** In individuals with NRCD, a remediable cause can be found in 90%: with continued gluten ingestion as the leading cause. We propose an algorithm for investigation.

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**Key words:** Celiac disease; Non-responsive celiac disease; Refractory celiac disease; Gluten; Gluten-free diet

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### INTRODUCTION

Celiac disease (CD) is induced by ingestion of gluten and related proteins with consequent intestinal injury and varied clinical manifestations. The defining feature is the expectation that the intestinal lesion improves with strict

exclusion of gluten from the diet. However, a proportion of individuals do not respond to a gluten-free diet (GFD), in terms of clinical or histological recovery. Early analysis has indicated that as many as 30% of individuals prescribed a GFD do not experience symptomatic improvement<sup>[1]</sup>. Non-responsive CD (NRCD) is defined as continued symptoms (including lethargy, abdominal pain and diarrhea) in patients on a GFD. There have been no recent studies to provide robust epidemiological data to assess the incidence of NRCD, although in clinical practice it is a common occurrence, based on the authors' experience and several publications<sup>[2-5]</sup>. The investigation of NRCD has been reported<sup>[6]</sup>, however, there are no data on the management and longer term follow-up of these subjects. Most patients with CD experience a rapid symptomatic recovery with a strict GFD. In 30% of cases there may be a protracted ( $\geq 12$  mo) or incomplete phase of mucosal recovery<sup>[7]</sup>. An arbitrary period of 6-12 mo on a GFD before reassessment has been suggested but the urgency of further investigation is often dictated by the severity of continued symptoms or clinical manifestations. In this context, we define NRCD as failure of expected symptomatic response to a GFD. Accordingly, NRCD is not intended to be a diagnostic term but rather a clinical description to allow a pragmatic and systematic approach to be followed to evaluate and investigate these patients. The practical management of NRCD depends on establishing a cause for continued symptoms. The commonest reason for persistent symptoms in a previous study of 55 patients was failure to comply with a GFD<sup>[6]</sup>. Imposition of a strict gluten-free dietary regimen appears to abolish symptoms in the majority of CD patients with continued symptoms<sup>[8]</sup>.

Refractory CD (RCD) describes a distinct clinical entity and represents a subset of non-responsive patients. RCD is defined by symptomatic and persistent villous atrophy in patients despite a strict GFD<sup>[8]</sup>. RCD can be diagnosed after primary failure of GFD or occur as a secondary phenomenon in previously treated CD. It can be subdivided into types I and II. This clinical definition has been refined by the discovery that 80% of individuals with true RCD possess an abnormal population of intraepithelial lymphocytes (IELs) detectable in their small intestinal mucosa (CD103<sup>+</sup>, intracellular CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup>, surface CD3<sup>+</sup>)<sup>[8]</sup>. These IELs may demonstrate a monoclonal T cell receptor (*TCR*)- $\gamma$  gene rearrangement, detectable by polymerase chain reaction (PCR) analysis of biopsy specimens. The presence of this aberrant T cell phenotype has been termed type II RCD (as opposed to type I RCD in which this anomaly is not present). Studies have shown that type II RCD is associated with a significantly greater mortality than type I RCD; 41% *vs* 14% at 2 years<sup>[9]</sup>, 42% *vs* 4%<sup>[10]</sup> and 56% *vs* 7% 5-year mortality<sup>[11]</sup>, with the major cause of death attributed to the development of enteropathy-associated T cell lymphoma (EATL). This is characterized by malignant lymphoid tissue with the same immunophenotype as described in type II RCD.

It has been postulated that the presence of this type II RCD T cell phenotype may represent a cryptic T cell lymphoma. In 41 patients with RCD, over 50% developed EATL during a mean of 2 years follow-up<sup>[9]</sup>. Survival from EATL remains abysmal. Thus, there are compelling clinical reasons to investigate CD patients with continued symptoms despite a GFD, in order to establish a treatable cause or identify cases of RCD or intestinal lymphoma. NRCD and RCD may both be present with weight loss, diarrhea, or malabsorption; all of which warrant expeditious investigation.

## MATERIALS AND METHODS

We maintain a prospective database of patients diagnosed with CD. We selected patients who were referred to our institution with a diagnosis of NRCD (defined as failure of expected symptomatic response to a GFD) between April 2002 and October 2003.

Initial evaluation included an appraisal of the original diagnosis of CD, history of symptoms (including lethargy, increased bowel frequency and weight loss), clinical examination, routine blood tests and assessment of dietary intake and GFD compliance. Patients were then investigated according to our usual clinical practice and subsequent findings; thus, some patients were investigated differently to others, however, all patients were followed for a minimum of 2 years; those who developed further symptoms were reinvestigated. Unless an obvious cause was immediately apparent, we undertook a further small bowel biopsy, which was performed by the authors to ensure a standard quality of biopsy specimen. Jumbo endoscopy forceps were used to obtain four samples that were carefully placed, mucosal surface upwards, onto paper to ensure optimal orientation.

Following standard preparation, histological examination was performed by our histopathology department, although in borderline or ambiguous cases, we often elected additionally to examine the slides within our department. An excess above 20 IELs per 100 enterocytes defined a pathological increase and villous atrophy was defined as being unequivocally present if the villous height to crypt depth ratio was below 2<sup>[12]</sup>. Direct visual comparison was made with any previous small intestinal specimens for the same patient. If there were any concerns regarding the validity of the diagnosis of CD, a gluten challenge was carried out. This involved ingestion of 10 g gluten (equivalent to four slices of white bread daily) for a minimum of 2 wk before repeat duodenal biopsy<sup>[13]</sup>. If colonoscopy was performed, random colonic biopsies were taken. In the diagnosis of microscopic colitis, we defined this condition as  $> 20$  lymphocytes per 100 epithelial cells in the superficial colonic mucosa in patients with diarrhea<sup>[14]</sup>.

Tests for small bowel bacterial overgrowth (SBBO) involved a lactulose hydrogen breath test. A positive test was indicated by an early rise in breath hydrogen  $>$

20 ppm from baseline after ingestion of 10 g lactulose. We note the low sensitivity and specificity of breath tests for bacterial overgrowth, including hydrogen and labeled carbon tests<sup>[15]</sup>. In order to validate a diagnosis of SBBO, we additionally required that symptoms resolved following rotating antibiotic treatment (ciprofloxacin 250 mg bd for 2 wk followed by metronidazole 200 mg tds fortnightly for 4 mo).

Lactose intolerance was diagnosed on dietary exclusion alone as tests are also unreliable. Exclusion of dairy products carries no risk and, if symptoms resolve, is reliable in establishing a confident diagnosis of lactose intolerance. Non-invasive testing of pancreatic function was performed in a number of patients (pancreolauryl test). False positives may occur in CD<sup>[16]</sup>, so the diagnosis could only be confirmed with symptomatic improvement with oral pancreatic supplements.

RCD was suspected in those with severe, symptomatic NRC with demonstrated villous atrophy, particularly those with pronounced weight loss. Urgent and extensive investigation was arranged in these individuals. This included computed tomography scanning of the abdomen and pelvis, colonoscopy and small bowel imaging. Video capsule endoscopy was not routinely performed at the outset of this study, although this now forms part of our assessment of suspected RCD. If appropriate, serological testing for anti-enterocyte antibody was performed to exclude autoimmune enteropathy.

Additionally, tissue analysis for IEL immunophenotyping and PCR reaction amplification for TCR clonality were undertaken; DNA was analyzed by a series of multiplex PCR assays, which amplified *TCR β* and *γ* gene rearrangements. PCR primer sequences were those used by the Biomed-2 consortium and have been shown to detect clonal signals in approximately 95% of all T cell clonal cases<sup>[17,18]</sup>.

The presenting symptoms, investigation process, results and outcome of subsequent management were recorded. We followed up patients for a minimum of 2 years and observed if patients remained symptom-free or suffered further relapses or related adverse events such as death or identification of malignancy. Any other tests deemed necessary, based on clinical history and examination, were performed, which resulted in a number of other diagnoses.

## RESULTS

One hundred and twelve patients were referred to our center and underwent assessment for NRC. The mean age of this group was 48.5 years (range 19-72 years) and 69% were female; CD had been diagnosed at a mean age of 31 years. The commonest presenting symptoms were diarrhea (65%), lethargy (43%), abdominal pain (27%) and weight loss (23%). The demographic details and clinical symptoms are shown in Table 1. The results are summarised in Figure 1

Twelve out of the 112 patients had been wrongly diagnosed with CD. Due to the doubt over the diagno-

**Table 1** Demographics and distribution of symptoms in 112 patients referred to our institution with continued symptoms on a gluten-free diet (%)

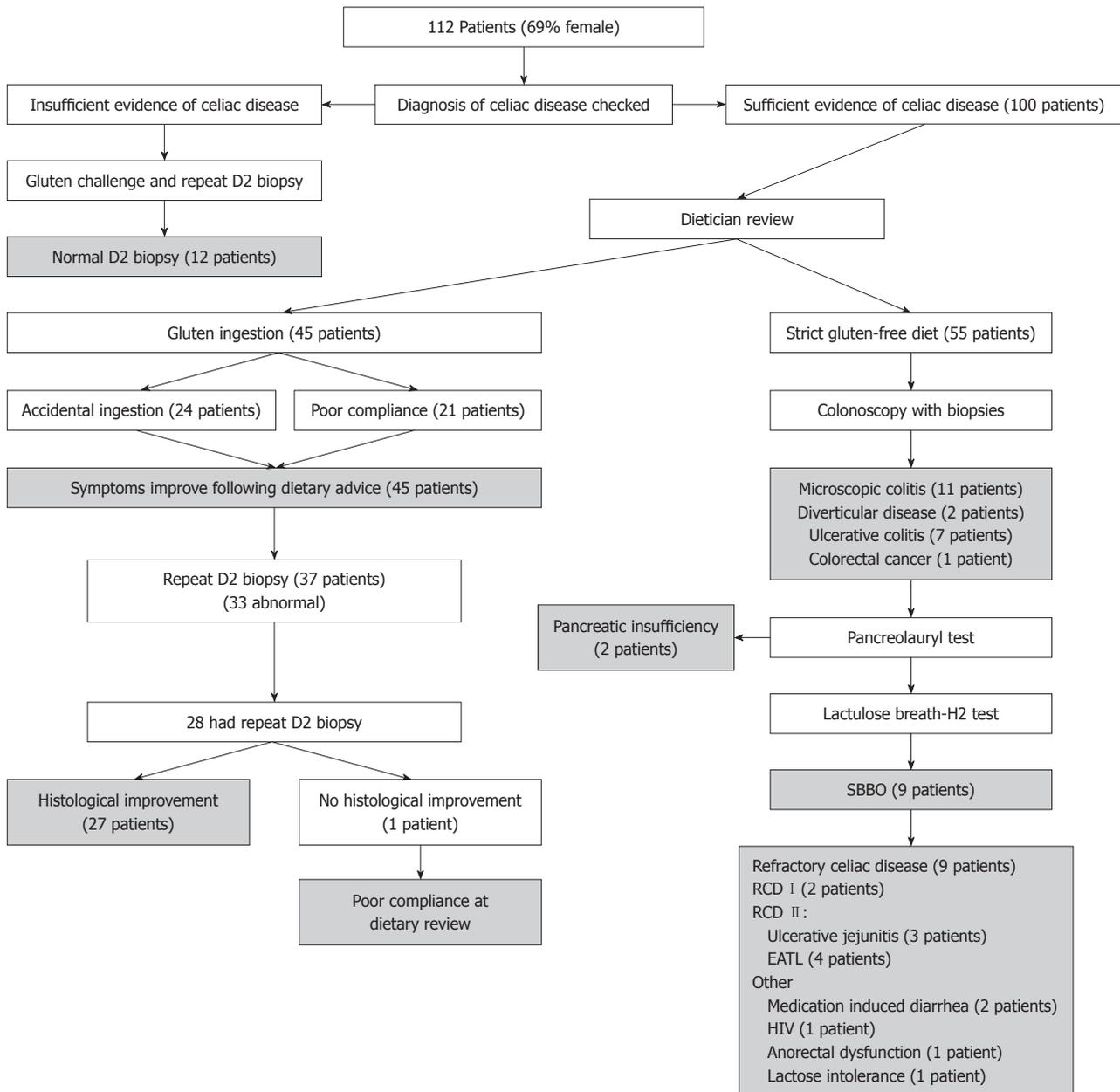
Male	31
Female	69
Mean age (yr)	48.5
Primary non-responsive	72
Secondary non-responsive	28
Mean years since diagnosis of CD (yr)	3 (range 1-12)
Diarrhea	65
Lethargy/fatigue	43
Abdominal pain	37
Weight loss	23
Nausea and vomiting	10
Symptoms of anemia	10
Two symptoms	49
Three symptoms	20

CD: Celiac disease.

sis, these 12 patients underwent gluten challenge and repeat biopsy which was normal in all cases. Additionally, anti-endomysial antibody (EMA) tests were all negative, although four patients had anti-gliadin antibodies detected. In four cases, initial duodenal biopsy had not been performed previously and diagnosis had been made based on dramatic reduction in symptoms with initial wheat exclusion. In the remaining eight, we were able to examine the original histology in five patients. Four of these were sufficiently normal to exclude CD in tandem with the subsequent negative gluten challenge. One patient did have villous atrophy on their original biopsy, which was felt to have been due to bacterial overgrowth, which had subsequently improved with antibiotic treatment. We were not able to examine previous specimens from three patients but the negative gluten challenge was deemed sufficient to exclude a diagnosis of CD. In total, six out of 12 patients had been previously shown to have supportive positive serology for CD in other institutions (mainly anti-gliadin antibody). Seven patients were diagnosed with irritable bowel syndrome; three with primary SBBO; and one each with anorexia nervosa and IgE-mediated wheat allergy. These individuals were subsequently removed from the analysis.

Forty-five of the remaining 100 patients were found to be ingesting sufficient gluten to cause their symptoms. Of these, 24 were discovered to be consuming gluten accidentally, and 21 admitted poor compliance with aspects of their prescribed diet. In total, 37 (23/24 accidental group and 14/21 poor compliance group) patients underwent repeat duodenal biopsy in order to establish this information. Of these specimens, 33/37 were abnormal (Marsh III a-c) which assisted in correlating the continued ingestion with the persisting histological abnormalities.

The majority (28/37) proceeded to have a further duodenal sample taken that showed comparative improvement in all but one case. In this case, further gluten ingestion was admitted on further questioning. In summary, all 45 patients in this group reported symptomatic improve-



**Figure 1** Flow chart showing the investigation and diagnoses of the patient cohort. RCD: Responsive celiac disease; SBBO: Small bowel bacterial overgrowth; EATL: Enteropathy-associated T cell lymphoma.

ment on a strict GFD, with 27/45 having demonstrable histological improvement.

Eleven patients were treated successfully for microscopic colitis. Diagnosis was made based on the presence of diarrhea and typical colonic histological features. All of these patients underwent simultaneous small bowel biopsy which was abnormal in 7/11 (64%) cases, mainly with an isolated intra-epithelial lymphocytosis. No alternative cause was established on enquiry or testing. These individuals were treated with a combination of mesalazine, loperamide, prednisolone and azathioprine (1-2.5 mg/kg). Five out of 11 required azathioprine for resolution of symptoms. Three patients suffered a relapse of diarrhea within 2 years; again treated successfully with oral steroids. When abnormal, patients had comparative improvement in their

duodenal histology following resolution of symptoms. We performed a total of 75 colonoscopies in NRCD patients with diarrhea and found significant lymphocytic infiltration in 15. This included four patients defined as having RCD who did not show histological or clinical improvement with immunosuppressive treatment.

Nine patients were successfully diagnosed and treated for bacterial overgrowth with sustained resolution of symptoms. There have been two relapses both in the same patient within 2 years; responding on each occasion to further courses of antibiotic treatment (metronidazole and ciprofloxacin). Interestingly, one patient was found to have combined variable immunodeficiency as an underlying cause for bacterial overgrowth and was referred for immunoglobulin infusions as part of further management.

Ten patients had normal investigations (all had duodenal biopsy and colonoscopy). This group was reassured and treated symptomatically for irritable bowel syndrome. At review after 2 years, 5/10 had continued functional symptoms with no new positive investigations. One patient had been diagnosed empirically with lactose intolerance. The remaining four patients were symptom free.

Lactose intolerance was diagnosed in six individuals; all of whom had dramatic symptomatic resolution when a lactose-free diet was commenced. All of these patients had primary NRCD.

We identified seven patients with coexisting inflammatory bowel disease (IBD); all of whom were suffering from ulcerative colitis. The predominant pattern was proctitis in five patients, and two had sigmoid colitis. Six responded to 5-ASA therapies, and one required azathioprine to control their IBD. All remained well and no surgical intervention has been required at 2 years follow-up.

After initial assessment and duodenal biopsy, 20 patients were considered to have a high suspicion of RCD. All of these patients had weight loss and diarrhea and a history of positive correlative celiac serology. After exhaustive investigation and assessment according to the United European Gastroenterology Week guidelines<sup>[11]</sup> (median duration 5 mo), a firm diagnosis of RCD was made in 9/20 patients; all of whom had a raised IEL count (> 20 per 100 enterocytes). Furthermore, all had marked villous atrophy (Marsh IIIa-c). None of this group was found to have a positive anti-enterocyte antibody. An alternative and remediable explanation for symptoms was identified in 11 patients (seven continued gluten ingestion; three with bacterial overgrowth; and one with microscopic colitis). RCD may be divided into those without aberrant T cells (type I) and those with aberrant T cells or ulcerative jejunitis (type II)<sup>[11]</sup>. Of the nine refractory patients, seven had type II RCD with positive clonality by  $\gamma$  TCR PCR. Three had ulcerative jejunitis; four were found to have or developed an enteropathy-associated intestinal lymphoma, two of whom have subsequently died, one from proven EATL and the other from suspected EATL (a post-mortem was refused by the relatives); both survived less than 1 year from diagnosis. The other two patients remain alive; one is on immunosuppressive medication and the other has been successfully treated with surgery. The remaining patients have continued to have symptoms over the follow-up period of 2 years (median 33 mo).

Of the two patients with type I RCD, one has died but we have no information available as to the precise cause of death, and the other patient has continued to have symptoms over the follow-up period of 2 years. In summary 3/9 (33%) patients diagnosed with RCD in our study have died.

Other diagnoses that were established are listed in Table 2. A diagnosis was only included if the symptoms were clearly attributable and symptomatic improvement occurred with appropriate treatment. Ten patients had more than one diagnosis established during the study

**Table 2** Summary of established diagnosis in 100 patients referred to our center with non-responsive celiac disease

Diagnosis	<i>n</i>
Continued dietary gluten	45
Microscopic colitis	11
Bacterial overgrowth	9
Lactose intolerance	7
Inflammatory colitis	7
Irritable bowel syndrome	10
Refractory celiac disease	9
Type I RCD	2
Type II RCD	7
Anorexia nervosa	2
Pancreatic insufficiency	2
Diverticular disease	2
Medication-induced diarrhea	2
Combined variable immunodeficiency	1
Human immunodeficiency virus	1
Colorectal cancer	1
Anorectal dysfunction	1
Incorrect diagnosis of celiac disease	12

RCD: Responsive celiac disease.

period (median 33 mo). This was largely as a result of ongoing investigation for additional symptoms during the study period.

Further assessment of patients' symptoms was conducted 2 years after their initial evaluation. Overall, four patients had died, with one from an unrelated cause. The vast majority (78%) reported being symptom-free at 2 years. A total of eight patients reported continued symptoms, with four describing them as moderate or severe. Those with continued symptoms included four diagnosed with RCD, two with irritable bowel syndrome and two with microscopic colitis. Ten patients could not be contacted.

In the 100 patients with NRCD, 73% had detectable anti-tissue transglutaminase (tTG) antibodies at varying titers. There was no statistical correlation between presence of antibodies, antibody titer and the established cause of NRCD. However, it was noted that 9/20 patients with RCD had positive celiac serological tests.

## DISCUSSION

Evaluation of patients referred to us with continued symptoms on a GFD concluded that 12 out of 112 patients did not actually have CD. The diagnosis of CD might appear straightforward but this indicates that errors are still made in clinical practice. The main difficulties appear to be basing the diagnosis on serology alone; where available tTG and EMA should be tested because these are most sensitive and specific<sup>[19,20]</sup>. DQ2/8 HLA typing may be useful to exclude CD in patients when tTG is negative but villous atrophy is present, and there is doubt over the diagnosis. In this study, DQ2/8 was not performed given difficulty in availability; furthermore, it adds little in patients who have a positive tTG and villous atrophy. In experienced hands, serology testing is highly specific but,

there can be discordant results between different laboratories. The limitations of celiac serology have previously been reported<sup>[21]</sup>. Accordingly, duodenal biopsy remains mandatory for a clear diagnosis of CD to be made and this is supported by current recommendations. We feel it is important to reassess the initial biopsy, as failure to orientate the small intestinal mucosal biopsy can result in a false interpretation of villous atrophy.

When the diagnosis of CD is secure, investigation of continued symptoms yields a remediable cause in 90% of cases, with continued gluten ingestion as the leading diagnosis. This parallels the findings of a previous study in an NRCD group<sup>[6]</sup>. In our study, the commonest culprit for inadvertent intake was malted breakfast cereals, although beer, cooking sauces, pizza, and biscuits - the latter two of which were clearly labeled as containing gluten - were also identified as sources of continued gluten ingestion. The diagnosis of continued gluten ingestion was only accepted, if after dietary modification, the patients' symptoms were reported to have resolved at a later follow-up appointment. It is of interest that nearly half of those failing to adhere to a GFD were aware that their compliance was suboptimal but withheld this information at initial assessment. It appears that some CD patients are reluctant to acknowledge that a minor intake of gluten could account for their continued symptoms. It is therefore important that appropriate dietary advice is provided at the outset to avoid unnecessary investigation at a later date. Celiac societies have a useful role in advising on GFD. However, some patients in our study were following a recommended GFD but improved when certain "safe" foods were removed from their diet. There has been considerable debate as to the acceptable safe threshold for gluten in foods, with 200 ppm being initially recommended. Some individuals do appear to suffer ongoing symptoms with persistent duodenal injury, even with trace quantities of gluten ingested in certain foods. Therefore, a lower limit of 20 mg/kg (20 ppm) has been accepted for labeling of gluten-free foods with 100 mg/kg labeled as gluten-reduced. These regulations will be introduced in 2012<sup>[22]</sup>. We advise patients that appear to be exquisitely sensitive to traces of gluten to adhere to a wheat-free GFD. This involves avoidance of products that are made by extraction of wheat proteins from flour because this process is usually incomplete to some degree, with traces of residual gluten remaining.

The association of microscopic colitis has been reported in CD patients<sup>[6,13]</sup>. It has been postulated that the lymphocytic infiltrate is part of the same autoimmune pathogenesis that is seen in the small bowel and that this infiltrate improves with a GFD. Similarly, microscopic colitis appears to be linked with RCD, which again suggests an aberrant immunological process. In our study, we suggest that there may have been an overlap between the groups diagnosed with microscopic colitis and RCD. It may be difficult to differentiate the two conditions, especially when duodenal abnormalities are marked. We based our diagnosis on the predominant abnormality be-

tween colonic and duodenal histology, severity of clinical manifestations, and the response to treatment. In practice, once lymphoma has been exhaustively excluded, the management of resistant symptoms may be largely similar with recourse to immunosuppressive therapy. Treatment of microscopic colitis is currently suboptimal but overall, the natural history is benign. We do not attempt here to discuss the validity of treatments for microscopic colitis in CD; only that sustained symptomatic improvement was achieved in these cases. In our experience, a trial of oral mesalazine may prove sufficient, although this is frequently ineffective. Following this, moderate-dose oral systemic steroids (20 mg/d prednisolone) usually provides rapid complete symptomatic response. The dose should be tapered gradually, although in a few cases it may be necessary to maintain 5-7.5 mg/d; in such instances, azathioprine as a steroid-sparing agent should be considered.

SBBO is associated with CD and is probably underdiagnosed<sup>[23]</sup>. The mucosal abnormalities may theoretically disrupt the innate defenses of the small intestine and predispose to this condition. In our study, patients all responded to antibiotic therapy but relapse was common. A second longer course of rotating antibiotic therapy was prescribed, which appeared to eradicate symptoms in the long term. If suspected, the diagnosis can be difficult to confirm, either by duodenal aspiration or breath test because these tests have a low sensitivity and specificity<sup>[15]</sup>. The duodenal histology may be normal, abnormal or exhibit patchy changes that are difficult to detect<sup>[24]</sup>. Treatment may be reasonably advised empirically if this diagnosis is suspected<sup>[23]</sup>. Although there is minimal data from clinical trials, it is our practice to treat patients with ciprofloxacin 250 mg twice daily, rotating fortnightly with metronidazole 200 mg three times daily for 3 mo. Symptomatic response is assessed to determine success of treatment. Duration of treatment depends on the conviction that SBBO is the underlying course. In our experience, patients may require treatment with alternating antibiotics for up to 4 mo if symptoms are resistant or recur after discontinuation of a short course of empirical antibiotics.

Acquired lactose intolerance is widely recognized to be a potential problem in CD. Exclusion of dairy produce is often recommended in the first 3-6 mo of GFD to allow disruption of the brush-border disaccharidase activity to recover. IBD can coexist with CD, because both are common and not mutually exclusive. One study has previously reported an increased incidence of IBD in patients with CD compared with the general population<sup>[25]</sup>. Two patients in our cohort had evidence of concomitant pancreatic insufficiency. Abnormal exocrine function, as tested with fecal elastase, was demonstrated in 13 (42%) subjects in one series of 31 CD patients, although only in three was this clinically significant<sup>[26]</sup>. A trial of treatment with pancreatic supplements may be advisable in those in whom pancreatic insufficiency is suspected.

Continued symptoms in CD patients may be functional because the symptoms are often indistinguish-

able<sup>[27]</sup>. In 10% of our NRCD patients, further investigations, including duodenal biopsy, were normal and the symptom pattern was consistent with standard criteria for irritable bowel syndrome. It is possible that the original symptoms at presentation were functional and that the CD was an incidental diagnosis. Additionally, a GFD frequently fails to provide adequate fiber intake that may exacerbate constipation and symptoms of irritable bowel syndrome, for which we advise supplementary fiber with either an ispaghula or psyllium seed husk preparation. Clearly, the GFD should be continued if the diagnosis of CD has been confirmed. Patients with CD may also suffer from a range of other conditions that affect the general population and they should be investigated accordingly. It is not satisfactory to attribute any subsequent symptoms to a previous diagnosis of CD, particularly in cases in which symptoms initially responded to a GFD.

### Responsive celiac disease

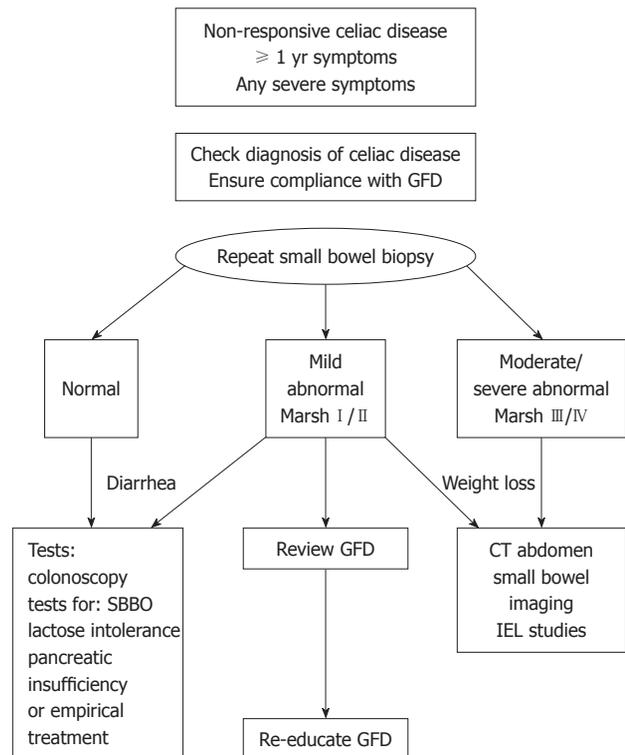
In our study of NRCD, nine patients were characterized as having RCD. Three were diagnosed with intestinal lymphoma, but one survived following treatment. At 2 years, 3/9 had died (33%), which is comparable to pre-existing cohorts<sup>[9,28]</sup>. There are no controlled trials but there are reports of symptomatic improvement with use of oral steroids and azathioprine. It is our practice to manage RCD and ulcerative jejunitis with moderate-dose prednisolone (20 mg/d), with initiation of azathioprine as a steroid-sparing agent (2-2.5 mg/kg). The steroid dose is tapered according to symptomatic response. We continue to monitor for the development of EATL. It is our practice to repeat duodenal biopsy after 4-6 mo to assess the small bowel inflammation and correlate this to ongoing symptoms and treatment.

### Celiac serology

We test for serum IgA EMA and tTG antibodies in all patients with suspected CD, because these are the most sensitive and specific. We also test for IgA deficiency because this is over-represented in CD patients and can lead to a false-negative EMA result. We no longer recommend using anti-gliadin antibody testing because of poor specificity<sup>[19]</sup>. Initial reports have suggested that celiac serology is a good indicator of response to GFD<sup>[29]</sup>. However, a further study has indicated that serology correlates poorly with histological recovery<sup>[30]</sup>. In our experience of NRCD, there was a high rate of low titer positive serology and this disappointingly failed to correlate with specific causes. Although celiac antibody testing should be performed routinely in symptomatic CD, we do not feel that this should deter further investigation of the non-responsive patient.

### Prognosis at two years

We have followed up this group of NRCD patients to provide information on longer-term outcome of NRCD. Only eight patients reported continued symptoms after 2 years, which included patients with RCD, as one might



**Figure 2** Algorithm for investigating non-responsive celiac disease. Moderate to severe abnormalities were defined by villous atrophy (Marsh III-a-c, or IV)<sup>[20]</sup>. GFD: Gluten-free diet; SBBO: Small bowel bacterial overgrowth; IEL: Intraepithelial lymphocytes; CT: Computed tomography.

expect, and microscopic colitis. This is reassuring in that NRCD has a good prognosis if evaluated and managed appropriately.

### Investigation algorithm

This algorithm (Figure 2) has been used as a basic guide in the investigation of patients referred to our institution with NRCD. It reflects the pivotal role of repeat duodenal biopsy. It recognizes that mild histological abnormalities are more likely to indicate continued trace gluten intake or be present in the context of a secondary diagnosis. More severe histological changes or significant weight loss warrant more urgent investigation for RCD or intestinal lymphoma. In our study, all nine patients with RCD had significant weight loss and severe histological abnormalities on duodenal biopsy.

The management of NRCD depends on confirming the diagnosis of CD and establishing a cause for the symptoms, which should be possible in 90% of cases. We suggest that those with RCD should be evaluated for lymphoma and subsequently managed with immunosuppressive therapy. Alternative strategies involving treatment with cyclosporine<sup>[31]</sup>, cladribine<sup>[32]</sup>, or fludribine and melaphan, stem cell transplantation for type II RCD<sup>[33]</sup> have been reported, although their use is not generally accepted. Continued gluten ingestion accounts for 45% of persistent symptoms in patients with CD and a thorough and honest dietary assessment should be encouraged. Microscopic colitis and SBBO are impor-

tant causes of persistent ongoing symptoms that should respond to treatment. The longer-term prognosis of NRC D is good, with a 90% prospect of sustained symptom resolution.

## COMMENTS

### Background

Celiac disease (CD) is a common disease that affects approximately 1% of Northern Europeans and North Americans. It is an inflammatory condition predominantly involving the proximal small bowel in genetically susceptible individuals. Treatment involves a life-long gluten-free diet (GFD) with avoidance of dietary gluten present in wheat, rye and barley. Thirty percent of CD patients fail to improve or may relapse while on a GFD, which is termed non-responsive CD (NRC D).

### Innovations and breakthroughs

The investigators report that the commonest cause of NRC D is continued gluten ingestion, either deliberately or by accidental ingestion. This cause is easily remediable by simple dietary measures. The authors also describe how other diagnoses can also contribute to the persistent symptoms: this includes microscopic colitis, a disease that causes diarrhea with a normal visual examination of the large bowel, and small bowel bacterial overgrowth; both of which occur more commonly in CD than previously reported.

### Applications

This article helps investigation of NRC D through provision of an investigative algorithm for physicians to investigate the persistent symptoms in individuals with CD who have been prescribed a GFD. It also highlights that continued gluten intake and other diagnoses can be concomitant, such that they should be considered in the diagnostic work up.

### Peer review

This is a good descriptive study in which authors investigate all patients referred to our centre with non-responsive celiac disease to establish a cause for their continued symptoms. The results are interesting and suggest that an algorithm for managing patients with non-responsive celiac disease.

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## Second-line therapy for gemcitabine-pretreated advanced or metastatic pancreatic cancer

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a second-line therapy among 206 patients who had initially received first-line treatment with a gemcitabine-based regimen. Median number of cycles was 4 (range: 1-12) and the median duration of treatment was 2.6 mo (range: 0.3-7.4). The overall disease control rate was 40.0%. The median overall survival and progression-free survival from the start of second-line therapy were 5.8 (95% CI: 4.1-6.6) and 3.4 mo (95% CI: 2.4-4.2), respectively. Toxicity was generally acceptable. Median overall survival of patients with a CA 19-9 level declining by more than 20% was 10.3 mo (95% CI: 4.5-11.6) vs 5.2 mo (95% CI: 4.0-6.4) for others ( $P = 0.008$ ).

**CONCLUSION:** A large proportion of patients could benefit from second-line therapy, and CA 19-9 allows efficient treatment monitoring both in first and second-line chemotherapy.

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**Key words:** Second-line; Chemotherapy; Pancreatic cancer; Gemcitabine; Carbohydrate antigen 19-9

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Altwegg R, Ychou M, Guillaumon V, Thezenas S, Senesse P, Flori N, Mazard T, Caillo L, Faure S, Samalin E, Assenat E. Second-line therapy for gemcitabine-pretreated advanced or metastatic pancreatic cancer. *World J Gastroenterol* 2012; 18(12): 1357-1364 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i12/1357.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i12.1357>

### Abstract

**AIM:** To investigate second-line chemotherapy in gemcitabine-pretreated patients with advanced or metastatic pancreatic cancer [(frequency, response, outcome, course of carbohydrate antigen 19-9 (CA 19-9)].

**METHODS:** This retrospective study included all patients with advanced or metastatic pancreatic cancer (adenocarcinoma or carcinoma) treated with second-line chemotherapy in our center between 2000 and 2008. All patients received first-line chemotherapy with gemcitabine, and prior surgery or radiotherapy was permitted. We analyzed each chemotherapy protocol for second-line treatment, the number of cycles and the type of combination used. The primary endpoint was overall survival. Secondary endpoints included progression-free survival, response rate, grade 3-4 toxicity, dosage modifications and CA 19-9 course.

**RESULTS:** A total of eighty patients (38%) underwent

### INTRODUCTION

Pancreatic cancer is the tenth most common cause of cancer in the United States and the fourth leading cause

of cancer death, with an estimated 42 000 new cases and 35 000 associated deaths in 2009<sup>[1]</sup>. In France, over 7200 patients were diagnosed with a pancreatic cancer in 2008, and almost the same number died from their disease<sup>[2]</sup>. At the time of diagnosis, most of patients present with advanced or metastatic pancreatic cancer, thereby precluding surgical resection<sup>[3]</sup>. Gemcitabine has been considered as the standard treatment for advanced pancreatic cancer ever since a randomized trial demonstrated significant improvement in survival and clinical benefit over 5-FU<sup>[4]</sup>. However, its efficacy remains moderate with median overall survival (OS) times ranging from 5 to 8 mo, and one-year survival rates varying between 17% and 25%. Numerous studies have attempted to increase efficacy of chemotherapy by combining gemcitabine with other drugs, but most of the regimens evaluated in phase III trials failed to show any improvement in overall survival<sup>[5-18]</sup>. Only one randomized trial<sup>[6]</sup> ( $n = 569$  patients) comparing gemcitabine alone *vs* gemcitabine combined with erlotinib showed a modest but significant increase in OS in the erlotinib arm (6.2 mo *vs* 5.9 mo,  $P = 0.025$ ). Actually, the rate of patients receiving second-line chemotherapy varied from 16% to 57% in the trials evaluating a gemcitabine-based combination therapy<sup>[7-18]</sup>. This difference can be explained by both the deterioration in performance status after gemcitabine and the absence of recommended standard treatment in second-line<sup>[19]</sup>. Despite limited clinical data in this situation, a phase II trial comparing oxaliplatin/folinic acid/5-FU (OFF) combination *vs* best supportive care as second-line treatment in gemcitabine-pretreated patients with advanced pancreatic cancer showed substantial benefit in the chemotherapy arm, with an overall survival prolonged by 2.6 mo ( $P = 0.008$ )<sup>[20]</sup>.

Serum carbohydrate antigen 19-9 (CA 19-9), the sialylated Lewis blood group antigen defined by the monoclonal antibody 1116 NS 19-9<sup>[21]</sup>, is the most common tumor marker in Europe and in the United States for patients with pancreatic cancer, both as a prognostic factor and an early marker of response to treatment. To date, the reliability and prognostic value of CA 19-9 levels to monitor first-line chemotherapy of advanced pancreatic cancer patients is well established<sup>[3]</sup>.

In this context, this study aimed to describe the frequency of gemcitabine-pretreated patients with advanced or metastatic pancreatic cancer receiving second-line chemotherapy, their overall survival and progression-free survival. We also investigated response rates, outcome and potential correlations between the level and course of CA 19-9 and survival.

## MATERIALS AND METHODS

### Patients

This retrospective study included all adult patients with an advanced or metastatic histologically proven pancreatic cancer (adenocarcinoma or carcinoma) initially treated with gemcitabine in our center between 2000 and 2008. All patients received first-line chemotherapy with gemcitabine at a dose of 1000 mg/m<sup>2</sup> once weekly for 7 wk

followed by 1 wk of rest; thereafter, gemcitabine was given once weekly for 3 wk followed by 1 wk of rest until progression of disease. Prior surgery or radiotherapy for local disease was permitted. All patients' medical records were registered within a computerized database [following national registry council (CNIL) authorization]. While there was no standard treatment used in second line, the treatment decision regarding a second-line therapy was systematically made by a multidisciplinary oncology committee according to the performance status, age and comorbidities.

### Methods

We assessed each second-line chemotherapy protocol for the duration, the number of cycles and the type of drug combinations. The primary endpoint was OS. Secondary endpoints included progression-free survival (PFS), response rates, grade 3-4 toxicity, dosage modifications and CA 19-9 course. We stratified overall survival and progression-free survival according to the response to gemcitabine treatment (duration of treatment  $\geq$  or  $<$  4 mo) and the performance status (0-1 *vs* 2-3). Response rates and disease progression were evaluated after 2 mo of treatment by Response Evaluation Criteria in Solid Tumors<sup>[22]</sup> and clinical examination. Toxicity was assessed at every visit using the National Cancer Institute Common Toxicity Criteria v.2.0 (CTC AE v2.0). The CA 19-9 levels were determined from serum samples collected at baseline (maximum one month before starting treatment) and at final treatment evaluation. A value of 60 IU/mL was accepted as the upper limit of normal. A reduction in CA 19-9 level was considered as relevant when serum concentrations decreased by more than 20% after the completion of treatment.

### Statistical analysis

In this retrospective study, information relating to identification, treatment, available biological material, surgery, response to therapy and outcome were collected for each patient. The primary objective was to evaluate the efficacy of a variety of second-line regimens in a large series of advanced pancreatic adenocarcinoma after first-line treatment with a gemcitabine-based regimen. Categorical variables were reported by contingency tables. Continuous variables were expressed as medians and ranges. The objective response rate was presented with a 95% CI. Survival rates and median values were estimated according to the Kaplan-Meier method. Patients alive at the time of analysis were censored at their last follow-up examination. Overall survival duration was measured from the date of first infusion until death from any cause. Progression-free survival duration was calculated from the date of first infusion until the first disease progression. Survival curves were drawn, and the log rank test was performed to assess differences between groups. All reported  $P$  values are two-sided. For all statistical tests, differences were considered as significant at the 5% level. Statistical analyses were performed using the STATA 9.0 software.

**Table 1** Baseline patient characteristics in second line therapy

Clinical features	80 patients
Sex	
Male	38
Female	42
Median age (yr)	61.0
Histological diagnosis	67 (83.8)
OMS	
0	31 (38.7%)
1	40 (50.0%)
2	7 (8.8%)
3	2 (2.5%)
Presence of primary tumor	55 (68.8%)
Gemcitabine	
Median number of cycle	3.0 (1.0-12.0)
Median duration (mo)	3.3 (0.5-18.9)
Duration ≥ 4 mo	29 (36.2%)
Metastatic disease	77 (96.3%)
Hepatic	70.1%
Peritoneal	29.9%
Nodal	23.4%
Pulmonary	16.9%
Carbohydrate antigen 19-9	
Initial median concentration (IU/mL)	741 (2.0-> 2000)
Elevated (> 60 IU/mL)	57 (89.1%)

Data are expressed as median values (range).

## RESULTS

### Patient characteristics

Baseline characteristics of the study population are detailed in Table 1. Of 206 patients receiving a first-line gemcitabine-based treatment for advanced or metastatic pancreatic cancer, 80 patients (38%) underwent a second-line therapy between January 2000 and May 2008. The median age was 61 years (range 36-81 years), and 38 patients were male (47.5%). The diagnosis of cancer was histologically confirmed in 67 patients (83.8%). Thirty-seven patients had undergone surgery including a pancreatoduodenectomy (*n* = 25) and palliative operation (*n* = 12) before first-line chemotherapy. Three other patients had received external radiation therapy. An endoscopic biliary prosthesis had been inserted prior to chemotherapy in eight patients. All patients received first-line chemotherapy with gemcitabine, with a median of 3 cycles (range: 1-12) and a median duration of 3.3 mo. Twenty-nine patients (36.2%) were treated for more than 4 mo. A total of 77 patients (96.3%) had evidence of metastatic disease, for most of them localized in the liver (70.1%). Despite the advanced stage of disease, patients generally showed good performance status before initiating second-line treatment, the WHO PS was of 0-1 in 71 patients (88.7%) and ≥ 2 in nine patients (11.3%).

From the CA 19-9 analyses performed in 64 patients, fifty-seven (89.1%) showed an elevated level, and initial median serum concentration was 741.5 IU/mL (range: 2-2000 IU/mL).

### Treatment

The median number of second-line chemotherapy cycles

**Table 2** Treatment regimens in second line *n* (%)

Groups of chemotherapy	Patients
Cisplatin group	23 (28.8)
LV5FU2-CDDP	23 (28.8)
Irinotecan group	22 (27.5)
FOLFIRI	12 (15.0)
XELIRI	10 (12.5)
Oxaliplatin group	21 (26.2)
GEMOX	13 (16.2)
FOLFOX	8 (10.0)
Other group	14 (17.5)
5-FU alone	3 (3.7)
Gemcitabine + erlotinib	4 (5.0)
Gemcitabine + capecitabine	3 (3.7)
Capecitabine	1 (1.2)
5-FU + CDDP + RT	3 (3.7)

LV5FU2-CDDP: Folinic acid 400 mg/m<sup>2</sup>, 5-FU bolus 400 mg/m<sup>2</sup>, 5-FU 2400 mg/m<sup>2</sup> over 46 h and cisplatin 50 mg/m<sup>2</sup> on day 2, every 2 wk; FOLFIRI: Irinotecan 180 mg/m<sup>2</sup>, folinic acid 400 mg/m<sup>2</sup>, 5-FU bolus 400 mg/m<sup>2</sup>, 5-FU 2400 mg/m<sup>2</sup> over 46 h, every 2 wk; XELIRI: Irinotecan 240 mg/m<sup>2</sup> and capecitabine po 2000 mg/m<sup>2</sup> J2-J15 every 3 wk; GEMOX: Gemcitabine 1000 mg/m<sup>2</sup> J1 and oxaliplatin 100 mg/m<sup>2</sup> J2, every two weeks; FOLFOX: Oxaliplatin 85 mg/m<sup>2</sup>, folinic acid 400 mg/m<sup>2</sup>, 5-FU bolus 400 mg/m<sup>2</sup>, 5-FU 2400 mg/m<sup>2</sup> over 46 h, every 2 wk; 5-FU alone: 5-FU 250 mg/m<sup>2</sup> every day as continuous infusion; Gemcitabine + erlotinib: Gemcitabine 1000 mg/m<sup>2</sup> weekly X 7 for 8 wk then weekly X 3 out of 4 wk plus either erlotinib 100 mg po daily; Gemcitabine + capecitabine: Gemcitabine 1000 mg/m<sup>2</sup> weekly X 3 for 4 wk and capecitabine 1600 mg/m<sup>2</sup> J1-J21; Capecitabine: 2500 mg/m<sup>2</sup> weekly X 2 for 3 wk; 5-FU + CDDP + RT: 60 Gy in 6 wk, 2 Gy/fraction, concomitant with 5-FU 300 mg/m<sup>2</sup> per 24 h as a continuous infusion, day 1-5 every week and cisplatin 20 mg/m<sup>2</sup> per day, day 1-5 at week 1 and 5.

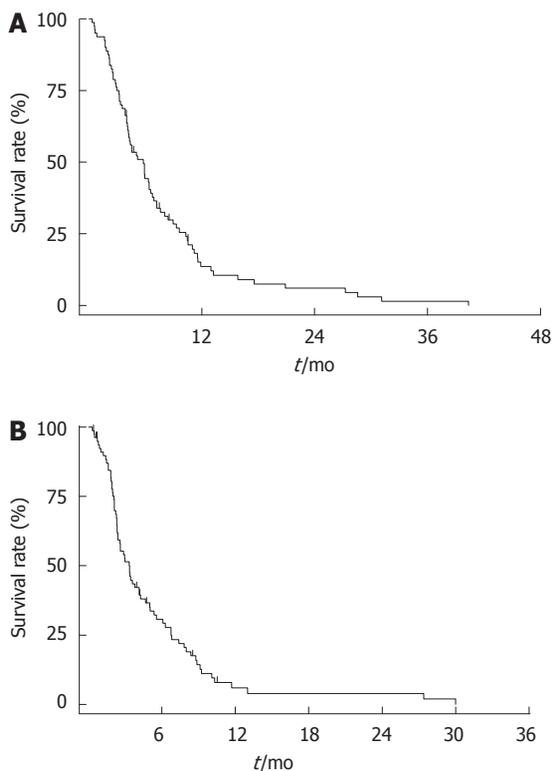
**Table 3** Chemotherapy regimens in second line

	Cisplatin group	Irinotecan group	Oxaliplatin group	Other group	<i>P</i> value
Number of patient	23 (28.8%)	22 (27.5%)	21 (26.3%)	14 (17.5%)	NS
Median number of cycle	5.0 (1.0-10.0)	5.0 (1.0-12.0)	4.0 (1.0-12.0)	2.0 (1.0-5.0)	NS
Median duration of treatment (mo)	2.7 (0.5-6.9)	3.2 (0.3-7.4)	2.3 (0.6-7.1)	2.3 (0.3-7.4)	NS
Disease control rate	10 (43.5%)	9 (40.9%)	9 (42.9%)	4 (28.6%)	NS
OS (mo)	6.7 (3.2-9.3)	4.5 (3.2-6.4)	4.5 (2.6-9.6)	5.2 (3.8-15.8)	NS
PFS (mo)	4.1 (1.9-6.7)	3.0 (2.0-6.1)	2.6 (1.8-5.4)	2.4 (2.1-10.1)	NS

OS: Overall survival; PFS: Progression-free survival; NS: Not significant.

was 4 (range: 1-12) and the median duration of treatment was 2.6 mo (range: 0.3-7.4).

All treatment regimens are described in Table 2. Different drug combinations were used in second-line. Twenty-three patients (28.8%) received a treatment with cisplatin (cisplatin group), 22 patients (27.5%) with irinotecan (irinotecan group) and 21 patients (26.3%) with oxaliplatin (oxaliplatin group). Fourteen patients (17.5%) were given other treatment, including a single agent for four of them. The duration of treatment did not significantly differ between groups (Table 3).



**Figure 1** Survival from the start of second-line therapy. A: Overall survival; B: Progression-free survival.

**Response and survival**

There was no complete response. Six patients (7.5%) achieved a partial response, 26 patients (32.5%) a disease stabilisation, 44 patients (55.0%) experienced disease progression and 4 patients could not be assessed. The overall disease control rate (complete response, plus partial response, plus stable disease) was 40.0% (median follow-up was 6.0 mo).

The median OS from the start of second-line therapy was 5.8 mo (95% CI: 4.1-6.6 mo). The 1-year and 2-year OS rates were 13.6% (95% CI: 6.9-22.7 mo) and 6.1% (95% CI: 2.0-13.5 mo), respectively (Figure 1A). The median PFS from the start of second-line therapy was 3.4 mo (95% CI: 2.4-4.2 mo). The one-year and two-year PFS rates were 6.0% (95% CI: 1.8-13.9 mo) and 4.0% (95% CI: 0.8-11.5 mo), respectively (Figure 1B). There was no significant difference between the four chemotherapy groups for overall disease control rates, overall survival and progression-free survival ( $P > 0.05$ ) (Table 3).

The median OS was 6.3 mo (95% CI: 4.3-7.2 mo) in patients with a performance status of 0-1 (71 patients) *vs* 1.8 mo (95% CI: 0.3-5.9 mo) in patients with a PS  $> 1$  (9 patients) ( $P < 0.001$ ). The one-year OS rates were 16.0% and 0%, respectively. The median PFS was 3.4 mo (95% CI: 2.6-4.9 mo) in patients with a performance status of 0-1 *vs* 2.1 mo (95% CI: 0.5-3.0 mo) in patients with a PS  $> 1$  ( $P = 0.004$ ). The one-year PFS rates were 7.0% and 0%, respectively.

The median OS times were 7.2 mo (95% CI: 4.5-10.5 mo) in patients treated for more than 4 mo with gem-

citabine as first-line therapy (29 patients) and 4.2 mo (95% CI: 3.2-5.9 mo) in those treated less than 4 mo (51 patients) ( $P = 0.046$ ). The one-year PFS rates were 10.0% and 4.0%, respectively.

**Toxicity and dosage modifications**

Toxicity was generally acceptable. The incidence of severe adverse events (grade 3-4) is reported on Table 4. Twenty-seven patients (33.7%) experienced at least one grade 3-4 toxic event. Neutropenia was the most frequent haematological toxicity, occurring in 14 patients (17.1%). There were 5 chemotherapy-related deaths. Two deaths were attributed to sepsis, and three to a combination of cancer and treatment-related complications. There was no difference in the incidence of toxicity and treatment-related deaths between the four chemotherapy groups (Table 5).

Forty-one patients (51.3%) had dosage modifications, including treatment suppression for 7 patients, dose reduction for 17 patients and cycle delay for 33 patients. Dose reductions were caused by haematological (9 patients, 53%) or clinical toxicities (8 patients, 47%) (Table 6). In thirty-one patients (41.3%), the chemotherapy was discontinued before evaluation because of disease progression (74.2%), toxicity (9.7%) or death (16.1%). There was no significant difference between groups for dose modification and chemotherapy discontinuation before evaluation.

**Carbohydrate antigen 19-9 measurement and survival**

Reduction in CA 19-9 levels during treatment was associated with improved survival. The median OS was significantly higher in patients whose level of CA 19-9 declined by more than 20% when compared to other patients 10.3 mo (95% CI: 4.5-11.6) *vs* 5.2 mo (95% CI: 4.0-6.4) ( $P = 0.008$ ) (Figure 2A). In this subgroup of patients, the median PFS was 6.7 mo (95% CI: 3.3-8.8 mo) *vs* 3.4 mo (95% CI: 2.6-4.2 mo) ( $P = 0.031$ ) (Figure 2B). All patients who experienced a CA 19-9 reduction  $> 20\%$  achieved disease control (3 partial responses and 5 cases of stable disease).

**DISCUSSION**

If gemcitabine-based chemotherapy is the current standard of care for first-line treatment of advanced pancreatic cancer, there are limited data to support a standard second-line chemotherapy regimen<sup>[23]</sup>. Indeed, the true survival benefit from first-line therapy is small, and few patients can endure a second line as their performance status deteriorates with disease progression. In our study, the rate of patients treated with second-line chemotherapy was 38.8%, in accordance with most published data regarding gemcitabine-pretreated pancreatic cancer (16%-57%). Median overall survival from the start of second-line setting was 5.8 mo (4.1-6.6 mo), and median progression-free survival was 3.4 mo (2.4-4.2 mo). These results are similar to those obtained in first-line with gemcitabine by Burris *et al*<sup>[4]</sup> or Heinemann *et al*<sup>[1]</sup>.

Table 4 Toxicity, dosage modifications and chemotherapy discontinuation <i>n</i> (%)	
	Patients
Clinical toxicity grade 3-4	
Nausea	3 (3.7)
Vomiting	5 (6.2)
Diarrhea	2 (2.4)
Stomatitis	1 (1.2)
Fever	6 (7.5)
Infection	6 (7.5)
Haematological toxicity grade 3-4	
Anemia	2 (2.4)
Neutropenia	14 (17.1)
Thrombocytopenia	1 (1.2)
Dosage modifications	
Type	41 (51.3)
Treatment suppression	7 (17.1)
Dose reduction	17 (41.5)
Delay of cycle	33 (80.5)
Discontinuation before evaluation	
Progressive disease	23 (41.3)
Toxicity	3 (9.7)
Chemotherapy-related deaths	5 (16.1)

Table 5 Toxicity for chemotherapy groups <i>n</i> (%)					
	Cisplatin group	Irinotecan group	Oxaliplatin group	Other group	<i>P</i> value
Clinical toxicity grade 3-4					
Nausea	0	0	3 (14.3)	0	NS
Vomiting	2 (9.1)	0	2 (9.5)	1 (5.3)	NS
Diarrhea	1 (4.5)	1 (5.6)	0	0	NS
Fever	0	4 (22.3)	1 (4.8)	1 (5.3)	NS
Infection	0	3 (16.7)	3 (14.3)	0	NS
Haematological toxicity grade 3-4					
Anemia	0	1 (5.6)	1 (4.8)	0	NS
Neutropenia	6 (27.3)	6 (33.4)	2 (9.5)	0	NS
Thrombocytopenia	1 (4.5)	0	0	0	NS

NS: Not significant.

Table 6 Dose reduction <i>n</i> (%)	
Dose reduction	Patients
Neutropenia grade 2 or 3-4	5 (6.2)
Thrombocytopenia grade 2	4 (5.0)
Hand-foot skin reaction grade 2	5 (6.2)
Neuropathy grade 2	2 (2.4)
Diarrhea grade 3-4	1 (1.2)

Moreover, patients with good performance status (0-1) and who had benefited from gemcitabine chemotherapy in first line (duration of treatment  $\geq$  4 mo) had a significantly greater duration of overall survival than those who had not (6.3 mo *vs* 1.8 mo,  $P < 0.001$ ; and 7.2 mo *vs* 4.2 mo,  $P = 0.046$ , respectively). The rate of grade 3-4 toxicity was determined to be 33.7% (27 patients), but there were no unexpected side effects. Consequently, our experience demonstrates that a selected population of

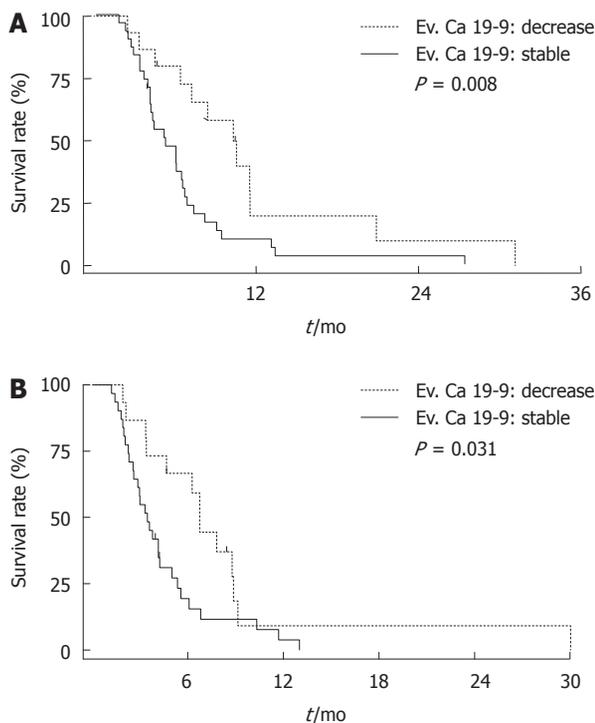


Figure 2 Overall survival and carbohydrate antigen 19-9 evolution in second line. A: Overall survival; B: Progression-free survival. Ev. Ca 19-9: Course of carbohydrate antigen 19-9.

patients with good performance status can benefit from second-line chemotherapy after first-line gemcitabine-based treatment, with appreciable overall and progression-free survivals. This retrospective study included a large population, while most of data published over the last ten years involved relatively small samples in monotherapy (from 13 to 52 patients) as well as in bithery (from 12 to 46 patients)<sup>[24]</sup>. The disease control rate was 40%, as described by many authors for both monotherapy and bithery regimens, and median overall and progression-free survivals were superior to those reported in monotherapy studies, but were not different from bithery<sup>[24]</sup>.

In daily practice, second-line therapies are regularly used in gemcitabine-pretreated patients with pancreatic carcinomas, but the efficacy and benefit in terms of survival or quality of life have never been validated. A randomized phase III trial conducted in second line was presented by Pelzer *et al*<sup>[25]</sup>. One hundred and sixty-five gemcitabine-pretreated patients with pancreatic cancer were randomly assigned to receive either FF (5-FU 2 g/m<sup>2</sup> for 24 h plus folinic acid or leucovorin 200 mg/m<sup>2</sup> on days 1, 8, 15 and 22) or OFF (FF plus oxaliplatin 85 mg/m<sup>2</sup> on days 8 and 22). Median overall survival and progression-free survival were significantly improved with OFF protocol (20 wk *vs* 13 wk,  $P = 0.014$ ; and 13 wk *vs* 9 wk,  $P = 0.012$ , respectively), with an acceptable tolerance profile. This study illustrated the effectiveness of this protocol which may become the standard second-line therapy. Currently, the National Comprehensive Cancer Center pancreatic cancer guidelines encourage the participation of patients with satisfactory performance status in

clinical trials, and recommend the use of oxaliplatin and fluoropyrimidine if enrolment in trials is not possible<sup>[26,27]</sup>. Finally, the XELOX regimen<sup>[28]</sup> showed comparable efficacy to FOLFOX (or OFF) regimen, while offering the advantage of oral fluoropyrimidine treatment. Even so, more large randomized controlled trials are required in second line before a new standard of care can be established.

Interestingly, the CA 19-9 measurement was correlated with OS and PFS in our study. Patients whose level of CA 19-9 declined by more than 20% had a significantly greater duration of survival. The prognostic value of CA 19-9 level and course is well established for patients with pancreatic cancer treated with surgery<sup>[29-31]</sup>, radiotherapy and chemoradiotherapy<sup>[32,33]</sup>. Some studies also correlated the level and the course of CA 19-9 with OS and PFS of pancreatic cancer patients treated with gemcitabine as first-line chemotherapy<sup>[3,34-36]</sup>. These studies showed improved median OS for patients with a decrease of CA 19-9 > 20% after two months of treatment with gemcitabine. Saad *et al.*<sup>[37]</sup> reported an increase in the median OS for patients with a reduction of CA 19-9 at any time after treatment. In second-line, only one study demonstrated that a CA 19-9 value > 400 IU/mL was a significant independently negative prognostic factor<sup>[38]</sup>. To our knowledge, it was the second report which showed a correlation between OS and CA 19-9 course<sup>[39]</sup>, and the first report for PFS and CA 19-9 course in second-line chemotherapy for gemcitabine-pretreated patients with pancreatic cancer.

In summary, treatment of metastatic pancreatic cancer remains a major challenge and requires new chemotherapeutic and targeted agent combination to be compared to gemcitabine in first-line. It should be noted that a new therapeutic alternative could merge in first-line for selected patients according to the recent results obtained in a randomized Phase III study comparing FOLFIRINOX regimen to gemcitabine<sup>[40]</sup>. A significant longer overall survival, progression-free survival, and higher response rates were obtained with FOLFIRINOX than with gemcitabine alone, associated with manageable toxicities.

The present study focused on second-line therapy in gemcitabine-pretreated patients with advanced pancreatic cancer. From our experience, second-line chemotherapy is a valuable treatment option after progression on gemcitabine-based regimen, because 30% to 40% of patients could benefit from this therapy, especially those with good performance status (1-2) and who gained benefit from first-line therapy. Further randomized clinical trials are necessary to provide a standard treatment in this situation. Additionally, measurement of the CA 19-9 level was confirmed to be an efficient marker for treatment monitoring in first-line as well as in second-line treatment.

## COMMENTS

### Background

Most of patients have advanced or metastatic pancreatic cancer at the time of

diagnosis, and cannot benefit from surgery. Gemcitabine-based chemotherapy is the standard treatment in first-line, but there are limited data to support standard second-line chemotherapy.

### Research frontiers

In practice, second-line therapies are regularly used in gemcitabine-pretreated pancreatic carcinomas, but the efficacy and benefit in terms of survival or quality of life have never been validated. Most of published studies in second line involved small samples, in monotherapy as well as in bitherapy.

### Innovations and breakthroughs

In our study, the rate of patients treated with second-line chemotherapy was 38.8%, and median overall and progression-free survivals from the start of second-line were similar to those obtained in first-line with gemcitabine. Carbohydrate antigen 19-9 (CA 19-9) course was correlated with prolonged overall and progression-free survival.

### Applications

Second-line chemotherapy is a valuable treatment option after progression on gemcitabine-based regimen, because 30% to 40% of patients could benefit from this therapy. Measurement of the CA 19-9 level was confirmed to be an efficient marker for treatment monitoring, in first-line as well as in second-line treatment.

### Peer review

The study is very interesting because there is no consensus about second-line therapy after disease progression while patients are receiving gemcitabine. The paper is well written. However, the authors should revise several points in the entire text.

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## Post-cholecystectomy symptoms were caused by persistence of a functional gastrointestinal disorder

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**METHODS:** One hundred and fifty three patients with a clinical and ultrasonographic diagnosis of gallstones filled out a structured questionnaire on abdominal pain symptoms and functional gastrointestinal disorder (FGID) before and at six months after cholecystectomy. Symptom frequency groups (SFG) were categorized according to frequency of pain attacks. According to certain pain characteristics in gallstone patients, a gallstone symptom score was accorded on a scale from one to ten. A visual analogue scale was used to quantify pain. Operative specimens were examined for size and magnitude of stone contents as well as presence of bacteria. Follow-up took place after six months with either a consultation or via a mailed questionnaire. Results were compared with those obtained pre-operatively to describe and analyze symptomatic outcome.

**RESULTS:** SFG groups were categorized as severe (24.2%), moderate (38.6%), and mild (22.2%) attack frequency, and a chronic pain condition (15%). Pain was cured or improved in about 90% of patients and two-thirds of patients obtained complete symptom relief. Patients with the most frequent pain episodes were less likely to obtain symptom relief. FGID was present in 88% of patients pre-operatively and in 57% post-operatively ( $P = 0.244$ ). Those that became asymptomatic or improved with regard to pain also had most relief from FGID ( $P = 0.001$ ). No pre-operative FGID meant almost complete cure.

**CONCLUSION:** Only one third of patients with FGID experienced postoperative relief, indicating that FGID was a dominant cause of post-cholecystectomy symptoms.

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### Abstract

**AIM:** To classify gallstone disease as a basis for assessment of post-cholecystectomy symptoms.

**Key words:** Gallstone symptoms; Functional gastrointestinal disease; Cholecystectomy; Post-cholecystectomy symptoms

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## INTRODUCTION

It is commonly accepted that removal of the gallbladder is the best treatment for symptomatic gallstone disease. However, less focus has been on patient selection and typical or common symptoms of this disease in order to understand prevailing symptoms after surgery<sup>[1-4]</sup>. Although disease severity has been used<sup>[5-7]</sup>, these efforts have not been united into useful and widely accepted working terms for preoperative clinical use and outcome assessment. As a consequence, the indication for cholecystectomy is sometimes vague and assessment of outcome suffers accordingly<sup>[8]</sup>.

Pain is a key element in gallstone symptoms but pain is a general symptom. Therefore pain characteristics and additional symptoms reported in classical descriptions of the disease has expanded the interpretation<sup>[9-11]</sup>. Functional gastrointestinal disorder (FGID) is quite common in the population and the two diseases often appear together<sup>[12]</sup>. FGID may go away or appear more distinct to the patient after cholecystectomy and thus distort the sense of postoperative relief. Up to 30% of patients have some symptoms following cholecystectomy<sup>[13,14]</sup>. No consistent physiological substrate for such pain has been documented<sup>[2]</sup>. It is somewhat unclear to what degree post-cholecystectomy symptoms resemble the exact symptoms before removal of the gallbladder<sup>[13]</sup>. Most studies are retrospective with follow-up periods commonly ranging from a few weeks to a couple of years<sup>[6,14,15]</sup>. A recent, prospective study lacked clinically useful working terms with a mixture of both pain associated symptoms and FGID<sup>[3]</sup>.

Our aim was to categorize gallstone disease according to the severity of clinical symptoms, pain characteristics, and quantify FGID to define postoperative outcome in terms of new or persistent symptoms.

## MATERIALS AND METHODS

One hundred and fifty-three patients with an ultrasonographic diagnosis of gallstones admitted for elective laparoscopic treatment of symptomatic, uncomplicated gallstone disease in a Norwegian ( $n = 100$ ) and a US ( $n = 53$ ) institution.

### Questionnaire

The patients filled out a structured questionnaire on pain

characteristics and functional abdominal symptoms before and at six months after surgery. The questionnaire was assembled after a large experience with pre-operative interviews in two randomized trials and modeled as a simplified version of the Rome II questionnaire<sup>[16,17]</sup>. Symptoms were classified according to appearance ranging from never to almost always in four steps: never, occasionally, very often, and almost always. Only those symptoms that were present more than 50% of the time (i.e. the last two) was counted as a positive answer (Table 1).

Follow-up was conducted at the outpatient clinic for all Norwegian patients at six months at which time the questionnaire was filled out. The American patients were mailed the questionnaire for logistic reasons.

Gallstone pain attacks were categorized as symptom frequency groups (SFG) according to the frequency experienced during the last three months. Patients that were unable to define exact time periods for pain attacks or had a dominant pattern of ubiquitous pain or had symptoms dominated by severe nausea or food intolerance were classified as a chronic symptom group.

A visual analogue scale score (VAS) was used to quantify the severity of pain in the symptom questionnaire. A 100 mm long horizontal line was to be marked vertically at the point consistent with the pain experienced by the patient. The left end was marked "No pain" and the right end "Unbearable pain".

### Gallstone symptom score

According to certain pain characteristics in gallstone patients, a Gallstone symptom score (GSS) was accorded from 1 to 10 (Tables 2 and 3).

In our practice, patients were found to have symptomatic, uncomplicated gallstone disease if ultrasonography detected gallbladder stones and the patients had relevant clinical symptoms. Endoscopy was neither a routine part of the pre-operative work-up nor planned as a diagnostic aid in case of persistent symptoms.

### Pathology

Operative specimens were prepared for examination of bacterial contents, stone size and routine histology on the back table immediately after the operation finished.

Bile was aspirated with a syringe from the gallbladder and sent for culture together with a piece of the wall. Stone size was measured with a caliper after the gallbladder had been opened on the back table. Finally the specimen was put on formalin and sent to the pathologist for routine (hematoxylin and eosin) staining and histological assessment.

### Ethics

The Regional Ethical Committee of Western Norway and The National Data Inspectorate approved the study. The Institutional Review Board (IRB) of Cleveland Clinic approved the study (IRB 7000/04). The study was registered with [clinicaltrials.gov](http://clinicaltrials.gov) as part of NCT01190280.

**Table 1** Assessment of functional abdominal symptoms (functional gastrointestinal disorder)

Perspiration
Intolerance to food
Acid regurgitation
Heartburn
Difficulty swallowing, food sticking in the lower esophagus
Nausea
Loss of appetite (anorexia)
Feeling full after eating very little (early satiety)
Feeling of abdominal fullness or bloating
Abdominal distension, which requires loosening of the belt
Frequent loose bowel movements (or more often than usual)
Constipation (or less bowel movements than usual)
Alternating constipation and loose bowel movements
Difficulty passing stools with straining, urgency or feeling of incomplete evacuation
Abdominal pain or discomfort is relieved by bowel movements (passing of stool)

Rate the frequency of the following symptoms associated with abdominal pain during the last 3 mo or longer, using the following scale: 0: Not at all or rarely (less than 10% of the episodes); 1: Occasionally (less than 50% of the episodes); 2: Very often (more than 50% of the episodes); 3: Almost always (more than 80% of the episodes).

### Statistical analysis

The  $\chi^2$  test was used to compare the level of improvement between groups, and to compare the presence of FGID between patients with different symptom alleviation before and after operation. Logistic regression for dependent paired data was used to analyze the difference in FGID before and after surgery between different GSS-groups. The statistical software used was PASW Statistics version 18.0 and Intercooled Stata 9.2 for Macintosh.

## RESULTS

The patient demographics are shown in Table 4.

### Symptom frequency groups and visual analogue scale score

Four SFG were categorized according to frequency of pain attacks: severe (24.2%):  $\geq 1$  pain attack per week, moderate (38.6%):  $\leq 3$  pain attacks per month, mild (22.2% of the patients):  $\leq 2$  pain attacks per 3 months, or chronic pain condition (15%): no discernable pain attack pattern.

The VAS was equally distributed between all patients, mean VAS preoperatively was 82.8 with variation from 17 to 100 (Table 5).

### Gallstone symptom score

Mean preoperative GSS in pair-wise comparisons showed a significant difference preoperative between chronic and moderate disease patients ( $P = 0.022$ ). There was a non-significant trend towards a greater rate of cure or symptom relief measured with GSS among patients with less severe disease ( $P = 0.651$ ). Patients in the most severe SFG had the highest GSS and experienced more remaining symptoms, for details see Tables 5 and 6.

**Table 2** Assessment of pain symptoms

Had an abdominal pain attack at least once for the last 3 mo or longer?
Experienced either pain or discomfort in the abdomen of a continuous steady nature at least once per week for the last 3 mo or longer?
For women: Did the onset of pain begin during pregnancy or soon after pregnancy?
Evaluated in the Emergency Department or seek medical attention for the abdominal pain?
Admitted to the hospital for the abdominal pain?
Estimate how often pain medications are required for the pain:
Not at all or rarely (less than 10% of the episodes)
Occasionally (less than 50% of the episodes)
Very often (more than 50% of the episodes)
Almost always (more than 80% of the episodes)
Time-interval during which the pain most often occurs:
7 am - 12 pm
12 pm - 6 pm
6 pm - 11 pm
11 pm - 7 am
Highly variable and unable to predict time of onset
Rate how often the pain occurs in the following abdominal areas:
Right upper quadrant <sup>1</sup>
Left upper quadrant <sup>1</sup>
Right lower quadrant <sup>1</sup>
Left lower quadrant <sup>1</sup>
Midline or center of the upper abdomen <sup>1</sup>
Is there often an area where the pain is strongest (able to point with one or two fingers):
Right upper quadrant
Left upper quadrant
Right lower quadrant
Left lower quadrant
Midline or center of the upper abdomen
Highly variable and unable to predict one area
No
Experience discomfort in the right upper quadrant when bending forward?
Abdominal pain radiates from where it started?
If yes, where does it radiate most often?
Right upper back beneath the right shoulder blade
Upper back between the shoulder blades
Lower back
None of these places mentioned
Highly variable and unable to predict a dominant area
Estimate the number of pain attacks over the last 3 mo
Estimate the usual duration of a pain attack in hours and minutes
Experience urge to move around during a pain attack <sup>1</sup>
Choose one of four patterns describing pain attacks (depicted by graphs):
Low-grade warning pain followed by a steady rise to a maximal constant pain, gradually getting better after a while
Low-grade warning pain followed by a steady rise to a maximal degree with occasional waves of pain, gradually getting better after a while
Pain begins suddenly with maximal intensity and improves over time
Pain begins suddenly with maximal intensity and persists with waves of pain until it goes away
Rate level of maximal pain intensity by 100 mm visual analogue scale score-scale

Pain attacks are defined as suddenly appearing pain that is distinct from, and stronger than any continuous, steady pain or discomfort. <sup>1</sup>The pain occurrence in each area is rated as: not at all or rarely (less than 10% of the episodes), occasionally (less than 50% of the episodes), very often (more than 50% of the episodes) or almost always (more than 80% of the episodes).

### Functional gastrointestinal disorder symptoms

A FGID was present in 87.6% before surgery and in 57.6%

**Table 3** Assignment of a clinical gallstone symptom score to different preoperative symptom frequency groups (%)

Symptom	Score	Percent of patients with symptoms according to pain presentation			
		Severe	Moderate	Mild	Chronic
Pain in upper abdomen: Pain most common in right upper quadrant or intensifies when bending forward or lying on the right side	2	100	96.6	94.1	88.2
Pain attacks commonly last more than one hour	1	73.0	66.7	76.5	46.2
Pain in a "plateau fashion"	1	62.2	72.9	67.6	64.3
Urge to move during pain attacks	1	51.4	69.0	58.8	84.6
Pain commonly occurs at night	1	43.2	61.0	50.0	29.4
Pain radiation to the back	1	40.5	47.5	38.2	58.8
Nausea during pain attacks	1	61.1	48.3	52.9	50.0
Use of analgesics in > 50% of pain attacks	1	54.0	54.3	44.1	41.2
Perspiration during pain attacks	1	36.1	41.4	41.2	60.0

**Table 4** Demographics of the study population of 153 patients and 115 follow-up responders *n* (%), mean age (range, yr)

Symptom frequency group	Females	Males	Total
All groups	122 (79.7), 47 (17-81)	31 (20.3), 51 (28-85)	153 (100), 48 (17-85)
Severe disease	31, 45 (17-81)	6, 44 (25-64)	37 (24.2), 45 (17-81)
Moderate disease	47, 44 (20-72)	12, 53 (39-70)	59 (38.6), 46 (20-72)
Mild disease	26, 53 (25-78)	8, 52 (34-85)	34 (22.2), 53 (25-85)
Chronic disease	18, 53 (23-81)	5, 55 (30-80)	23 (15.0), 54 (23-81)
Responders to follow-up	89 (77.4), 49 (20-81)	26 (22.6), 52 (25-85)	115 (75.2), 50 (20-85)

$\chi^2$  for gender;  $P = 0.889$ .

**Table 5** Changes in gallstone severity score by symptom frequency group in 115 responding patients from the study population of 153 patients *n* (%)

Preoperative SFG	Patients	Preoperative		Responders	Postoperative		mean % reduction in GSS
		mean GSS	mean VAS		mean GSS	mean VAS	
Severe disease	37 (24.2)	6.11	81.1	29 (78.4)	1.76	33.0	69.1
Moderate disease	59 (38.6)	6.47	86.6	41 (69.5)	1.32	15.8	78.7
Mild disease	34 (22.2)	6.09	81.3	26 (76.5)	1.04	12.8	87.0
Chronic disease	23 (15.0)	4.35	76.8	19 (82.6)	1.00	8.9	62.7

SFG: Symptom frequency groups; GSS: Gallstone symptom score; VAS: Visual analogue scale score.

at follow-up after surgery. No difference was seen between the different SFG ( $P = 0.244$ ). There was a trend that patients with FGID before surgery were less likely to experience improvement of their pain or complete relief. Likewise, patients without FGID after surgery were more likely to report improvement or complete relief of pain (Table 7).

#### Gallbladder specimen examination

Histology of the gallbladder showed that 85% had chronic and 10% subacute inflammation while 5% were normal. Bacteriological examination in 79 patients discovered bacteria in 12 (15.2%) without difference between the groups. The distribution of bacteria was: gram-negatives 3.8%, gram-positive cocci 8.9%, and mixed cultures 2.5%. Stone type was not examined.

The number of stones was measured in 66 patients and size in 64 patients. The mean number was 2.5 (range 1-9) with variation between SFG from 2.3 to 2.8. The

stone size was mean 13 mm (range 1-40) with variation between groups from 12.5 to 13.2 mm. There were no statistically significant differences between the groups.

## DISCUSSION

Gallstone symptoms are still classified simply as biliary colic long after a variety of pain characteristics have been described for these pain attacks<sup>[9,10]</sup>. Thus, studies of outcome of gallstone disease are usually hampered by lack of scientifically acceptable definitions and designs<sup>[3,5,6,16]</sup>. This includes an inadequate definition of gallstone symptoms, lack of proper recognition of FGID as a concomitant complaint, prospective design and defined follow-up methods. Freedom of pain attacks is a major outcome measure after cholecystectomy. Complete cure of a biliary type pain in contrast to a persisting dull aching pain, has been reported as a reasonable goal for surgery<sup>[18]</sup>. Previous studies have reported that so-called

Table 6 Symptomatic improvement in 115 patients after cholecystectomy *n* (%)

	Groups			<i>P</i> value <sup>1</sup>
	Asymptomatic but improved	Symptomatic or worse	Unchanged	
Patients				0.651
All patients	76 (66.1)	28 (24.3)	11 (9.6)	
Severe disease	15 (51.7)	10 (34.5)	4 (13.7)	
Moderate disease	27 (65.8)	9 (22.0)	5 (12.2)	
Mild disease	20 (76.9)	5 (19.2)	1 (3.9)	
Chronic disease	14 (73.7)	4 (21.0)	1 (5.3)	
Age				0.490
< 60	54 (64.3)	23 (27.4)	7 (8.4)	
> 60	22 (71.0)	5 (16.1)	4 (12.9)	
Gender				0.573
Women	56 (62.9)	24 (27.0)	9 (10.1)	
Men	20 (76.9)	4 (15.4)	2 (7.7)	

<sup>1</sup>*P* values from  $\chi^2$  calculation.

Table 7 Presence of pre- and post-operative functional gastrointestinal disorder in 115 patients with different *n* (%)

Presence of FGID	Patients	Asymptomatic	Symptomatic, improved	Unchanged or worse	<i>P</i> value <sup>1</sup>
None pre-operative	13 (11.3)	11 (84.6)	2 (15.4)	0	0.449
Present pre-operative	102 (88.7)	65 (63.7)	26 (25.5)	11 (10.8)	
None post-operative	49 (42.6)	42 (85.7)	7 (14.3)	0	0.001
Present post-operative	66 (57.4)	34 (51.5)	21 (31.8)	11 (16.7)	
Total	115 (100)	76 (66.1)	28 (24.3)	11 (9.6)	

FGID: Functional gastrointestinal disorder.<sup>1</sup>*P* values from  $\chi^2$  calculation.

biliary colic remained in only 8%-9% of patients in contrast to non-colicky pain in 18%-32%<sup>[13,19]</sup>. Others have found an incidence of around 20% of persistent pain of the same character as before the operation<sup>[20,21]</sup>. Lublin and coworkers<sup>[6]</sup> reported presence of pain in 25% and non-pain symptoms in 43%. It seems that distinct or marked pain is present in up to 4%-9%<sup>[12,13]</sup> whereas pain or "discomfort" connected with dyspeptic symptoms are not clearly categorized<sup>[22]</sup>. Around 25% of our patients improved without being completely cured after removal of the gallbladder. This corresponds to previous figures of 18%<sup>[13]</sup> and the frequency of more diffuse intestinal symptoms found by others during post-operative examination<sup>[12]</sup>. One author mentioned similar findings without giving figures but did not find interference with quality of life measurements<sup>[23]</sup>. Up to 93% satisfaction has been reported after removal of the gallbladder<sup>[13,15,22,24,25]</sup>.

FGID consists of two main subgroups, functional dyspepsia and irritable bowel syndrome (IBS), with overlapping features making them both symptomatic of an irritable or dysfunctional gut<sup>[26,27]</sup>. The criteria in the Rome II and the more recent Rome III questionnaire give a formal definition of FGID<sup>[17,28]</sup>. In the West, there tends to be a female predominance. FGID appears as a real condition of gallstone disease<sup>[3,12,22,29]</sup>. The pathological connection is still obscure but a common dysfunctional trait has been shown<sup>[30]</sup>. A diagnostic problem arises only when gallstone disease becomes vague with regard to pain expression<sup>[3,5,22]</sup>. Lublin and coworkers<sup>[6]</sup> reported that 80% of patients had so-called non-pain symptoms pre-opera-

tively in accordance with an 88% incidence of FGID in our patients. In our practices, nearly all gallstone patients coming to surgery have upper abdominal pain either in the right upper quadrant or epigastrium although a small percentage has intolerable nausea or food intolerance that dominates over pain. FGID was therefore judged a concomitant condition in most cases. Our outcomes are quite similar to those of others who have attempted to classify pre-operative symptoms<sup>[5,6]</sup>. It could be perceived that freedom of pain with an attack pattern was the decisive factor when cure or relief was achieved, whereas FGID of various intensities caused the bulk of the persistent symptoms, because FGID persisted in 57% of the patients. The post-operative GSS and VAS were markedly decreased and it is therefore likely that the patients were cured of the pain attacks that led to cholecystectomy. Besides, even so-called biliary colic, even if it resembles pre-operative symptoms, needs a substrate when the gallbladder has been removed. It has not been proved that this stems from the common bile duct (CBD) or the sphincter of Oddi, even though symptoms caused by CBD disease, such as a stone, may be quite similar. Therefore, we will argue that there is reasonable evidence pointing to FGID as a cause of persisting symptoms after surgery.

Some investigators have reported that patients with the most severe, frequent or bothersome pre-operative symptoms are less likely to be cured<sup>[5,6,13,22]</sup>. The present study corroborated this as results showed that only frequency of pain attacks expressed as SFG separated the patients with regard to severity in the pre-operative evalu-

ation. GSS only separated the pain attack groups against the chronic group. This is broadly correlated with a Swedish study but differed insofar that we amalgamated what were their two most severe groups into one<sup>[5]</sup>. Lublin and coworkers<sup>[6]</sup> used frequency without a more specific definition. The disease may wax and wane and this may influence the response to the questionnaire<sup>[5,5]</sup>. A minority of 15% had chronic symptoms with daily occurrence as the rule. We suspect that some of the patients with daily symptoms reported by Halldestam and coworkers<sup>[5]</sup> might have been classified as a chronic symptom group by our definition. This would distort comparison of outcome because these two groups responded differently to operative treatment in our study. It is also difficult to ascertain the meaning of “atypical” or multiple locations of pain<sup>[5]</sup>. Pain in the right upper quadrant or epigastrium is a core issue in the diagnosis of gallstone disease but admittedly in a small minority of patients other symptoms dominate. However, as long as these symptoms can be assigned to gallstone disease, they are not a contraindication to surgery in such cases.

Although patients with the highest pre-operative mean GSS had the largest relative score reduction, this group retained a higher post-operative score and had the highest VAS score. The reason for that was largely assumed as being caused by persistent symptoms of FGID even though this could not be established with certainty because of overlapping symptoms in gallstone patients. It was, however, consistent with the observation that the severe SFG had more patients with no relief and also had a slightly larger GSS burden and consequently higher post-operative GSS and VAS score, indicating that a larger disease burden or more symptoms was in concert with a higher VAS. This may be interpreted as more persistent pain. One study found that patients with the most bothersome symptoms before surgery had less chance of cure<sup>[22]</sup>. The highest odds ratio for persistence was obtained by “gas/flatulence”, a common symptom of IBS or FGID. This could easily be interpreted as caused by FGID but it has been unusual to explicitly label post-cholecystectomy symptoms as FGID even though many symptoms fit this diagnosis<sup>[22]</sup>. One explanation may be that these symptoms has for too long been discerned as part of a wider range of gallstone symptoms while we will argue that they are two concomitant disease expressions with many overlapping features making it difficult to separate them.

Compared with measurements before surgery VAS has reached levels of around 68 (of 100) pre-operatively to levels of 35 to 45 post-operatively<sup>[13,15,19,21]</sup>. In the present study, VAS was similar across all four GSS groups and it fell after surgery to a mean of 18 (range 9 to 33). Therefore, it could not by itself be used to distinguish between the patients before or after surgery. Our post-operative median score value indicates no more than mild to moderate pain<sup>[31]</sup>.

Theoretically, a bile duct focus might cause pain quite similar to that originating in the gallbladder but only

about 2% has common bile duct stones after removal of the gallbladder<sup>[6,13]</sup>. Psychometric testing has shown that a psychosomatic disturbance may influence outcome after cholecystectomy<sup>[8,32]</sup>. It has been observed that women tend to have more postoperative pain<sup>[33]</sup> while some have reported that gender is irrelevant<sup>[15,20,34]</sup>. Women under the age of 60-years have been found to have significantly more pain of the diffuse, more continuous type that is also described in functional dyspepsia, and satisfaction has been greater for men<sup>[15]</sup>. We found that women were less likely to become asymptomatic. Age of the patient has not influenced outcome<sup>[20,34]</sup>, whereas the opposite was found when 50 or 55-years-of-age was used as cut off value<sup>[4,22]</sup>. In contrast to previous studies, patients more than 60-years-of-age fared slightly worse in the present study<sup>[5,13]</sup>. Stone size and number, bacteriology, or histology<sup>[2]</sup> did not impact the symptom presentation in this study.

We recommend a follow-up period of 6 mo before assessing outcome after cholecystectomy<sup>[8,22]</sup>. Whether qualified personnel should interview a study object or a questionnaire be used, remains open for discussion<sup>[8,16,22,29]</sup>. It may be a point of concern whether a self-assessment questionnaire will make the patient report more complaints than will be revealed by a professional interview<sup>[35]</sup>.

Approximately 10% of patients did not improve or even got worse whereas the condition of 25% improved and the rest was cured. Patients with the most SFG were less likely to be completely cured and this group also had a higher pre-operative symptom score (GSS). Post-operative FGID persisted in 57% of patients and indirect evidence suggests that persistent symptoms were caused mainly by FGID. The main indication for elective cholecystectomy in uncomplicated gallstone disease should be pain attacks. Patients should be informed about the chance of persistent symptoms.

## COMMENTS

### Background

Patients with gallstones often have various abdominal symptoms that may be caused by the gallstones or are present as a separate condition but with a common physiology. The accompanying abdominal symptoms are called functional gastrointestinal disorders (FGID). Because of its common nature and presence of pain or discomfort it is difficult to separate a functional condition from the gallstone disease itself. Lack of a clear distinction between the two and a poor understanding of the physiology that causes both conditions, especially FGID, makes it difficult to treat these symptoms if they remain after the operation. The article characterizes symptoms caused by gallstone disease in order to define which symptoms remain after removal of the gallbladder. Hope of improving understanding of their character and origin will subsequently have a potential bearing on treatment.

### Research frontiers

Current treatment methods may not be satisfactory due to limited insight in physiological mechanisms. Therefore, FGID causes a major health problem with a large amount of sick-leave days. Because of this burden on both patient and society it is important to conduct proper research to gain insight in disease mechanisms and offer effective treatment.

### Innovations and breakthroughs

The study tried to characterize gallstone disease according to intensity and frequency of pain attacks as well as concomitant functional symptoms. The pre-

operative condition has then been compared to persisting symptoms after surgery. Such methodical studies of the character of gallstone disease are scarce.

### Applications

An understanding of disease expression may give better insight into why complete symptom relief does not occur in some patients after cholecystectomy. Thus, it may be possible in the future to decide which patients will have the greatest chance of cure as well as offer efficient treatment of persisting symptoms after cholecystectomy.

### Terminology

Gallstone disease is characterized by bouts of pain or pain attacks in about 85% of patients. The rest have a combination of more consistent pain, strong food intolerance or nausea. FGID is present in about 88% of gallstone patients. This condition may have particular symptoms but a clear-cut physiologic mechanism or organic origin has not been decisively described for it. The diagnosis is sometimes made by exclusion of other diseases. It is difficult to separate clinically from gallstone disease when both are present in the same patient.

### Peer review

The authors have nicely analyzed the existing preoperative functional disorder in patients of gallstones to substantiate its correlation with post-operative symptoms.

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## Three benefits of microcatheters for retrograde transvenous obliteration of gastric varices

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### Abstract

**AIM:** To evaluate the usefulness of the microcatheter techniques in balloon-occluded retrograde transvenous obliteration (BRTO) of gastric varices.

**METHODS:** Fifty-six patients with gastric varices underwent BRTOs using microcatheters. A balloon catheter was inserted into gastrosplenic or gastrocaval shunts. A microcatheter was navigated close to the varices, and sclerosant was injected into the varices through the microcatheter during balloon occlusion. The next morning, thrombosis of the varices was evaluated by contrast enhanced computed tomography (CE-CT). In patients with incomplete thrombosis of the varices, a second BRTO was performed the following day. Patients were followed up with CE-CT and endoscopy.

**RESULTS:** In all 56 patients, sclerosant was selectively injected through the microcatheter close to the varices. In 9 patients, microcoil embolization of collateral veins

was performed using a microcatheter. In 12 patients with incomplete thrombosis of the varices, additional injection of sclerosant was performed through the microcatheter that remained inserted overnight. Complete thrombosis of the varices was achieved in 51 of 56 patients, and the remaining 5 patients showed incomplete thrombosis of the varices. No recurrence of the varices was found in the successful 51 patients after a median follow up time of 10.5 mo. We experienced one case of liver necrosis, and the other complications were transient.

**CONCLUSION:** The microcatheter techniques are very effective methods for achieving a higher success rate of BRTO procedures.

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**Key words:** Balloon-occluded retrograde transvenous obliteration; Gastric varices; Microcatheter; Portal hypertension; Ethanolamine oleate

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### INTRODUCTION

Balloon-occluded retrograde transvenous obliteration (BRTO) is a treatment for gastric varices that has a high success rate<sup>[1-5]</sup>. However, there are three major problems with BRTO procedures such as overdose of the sclerosant,

leakage of the sclerosant into the systemic circulation, and incomplete thrombosis of large gastric varices<sup>[6-10]</sup>. We introduced the microcatheter techniques<sup>[11-13]</sup> in 1999 to solve these problems, and we have been using three major beneficial techniques for BRTO procedures such as selective injection of the sclerosant, microcoil embolization of collateral veins and additional injection of the sclerosant. Since 1999 we have collected a great deal of data and are now able to accurately report on the long-term results of these techniques in a large number of cases.

## MATERIALS AND METHODS

BRTO using 2.9Fr microcatheters was performed in 56 patients with liver cirrhosis-related gastric varices between August 1999 and December 2008. The subjects consisted of 35 males and 21 females, with a mean age of 65.3 years (range: 33-83 years). Liver cirrhosis was associated with hepatitis B in 3 patients, hepatitis C in 29 patients, alcohol in 15 patients, and unknown factors in 9 patients. According to the Child-Pugh classification, liver function was evaluated as A in 19 patients, B in 31 patients, and C in 6 patients. Prophylactic BRTO was performed in 31 patients with large tumor-like gastric varices or growing varices in danger of rupture. Elective BRTO was performed in 12 patients with a history of hemorrhage related to gastric varices. Emergency BRTO<sup>[16,17]</sup> was performed in 13 patients within 24 h after hematemesis or tarry stool. Informed consent for BRTO was obtained from all patients.

Gastric varices were confirmed by endoscopy. The presence and diameter of gastroduodenal shunt or gastrocaval shunt were evaluated by contrast enhanced computed tomography (CE-CT). An 8Fr sheath (Cobra type; Medikit, Tokyo, Japan) was inserted into the left renal vein or inferior vena cava through the right internal jugular vein or right femoral vein, and a 6Fr balloon catheter (Cobra type; Clinical Supply, Gifu, Japan) was inserted into the gastroduodenal shunt or gastrocaval shunt. The balloon diameter was 13 or 20 mm. In patients with a shunt diameter of 13 mm or more, a balloon measuring 20 mm in diameter was used. A 2.9Fr microcatheter was navigated close to the gastric varices. A sclerosant, 5% ethanolamine oleate iopamidol (EOI), was infused slowly and intermittently through a microcatheter during balloon occlusion. 5% EOI was prepared by making a 20 mL solution consisting of 10 mL contrast medium and 10 mL of 10% ethanolamine oleate (Oldamin; Grelan Pharmaceutical, Tokyo, Japan). The infusion of 5% EOI was continued until the entire gastric varices and feeding veins were opacified. The mean volume of sclerosant (5% EOI) was 22.9 mL per one procedure (range: 1.5-47 mL). The balloon occlusion time ranged from 12 to 48 h. To fix the sheath and catheters, sterilized tape (Hogy Medical, Tokyo, Japan) was used. The next morning after the BRTO procedure, thrombosis of gastric varices was evaluated by CE-CT. In patients with incomplete thrombosis after the first BRTO, a second BRTO was performed the following day. After complete thrombosis of gastric varices

was confirmed on CE-CT, all catheters were removed. To prevent renal damage due to EOI-induced hemolysis, 4000 units of haptoglobin (Mitsubishi Pharma, Osaka, Japan) was administered intravenously during and after the infusion of EOI in all patients<sup>[18,19]</sup>. Patients were followed up with endoscopy and CE-CT 1 d, 1 wk and 1, 3, 6 mo after the procedure and every 6 mo thereafter.

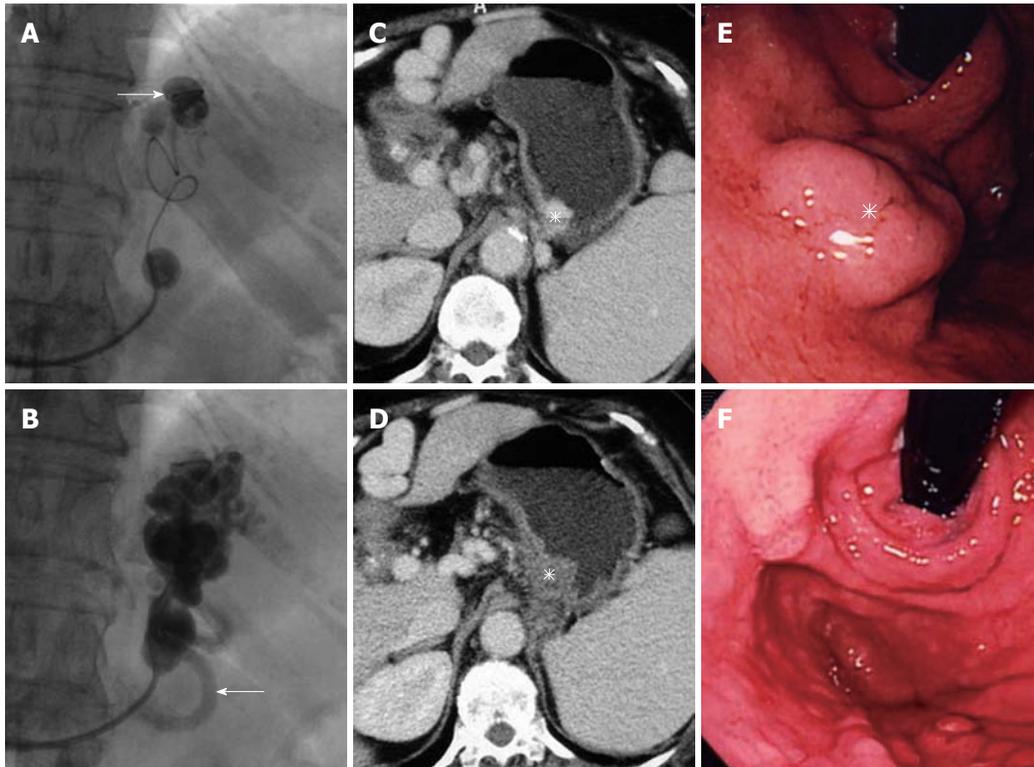
## RESULTS

In all (100%) of 56 patients, the sclerosant was selectively injected through the microcatheter close to the gastric varices (Figures 1 and 2). In 9 (16%) of 56 patients, microcoil embolization of dilated collateral veins was performed using the microcatheter (Figure 3). In 12 (21%) of 56 patients, CE-CT the next day after the first BRTO showed incomplete thrombosis of the varices, and additional injection of the sclerosant was performed in the second BRTO through the microcatheter which remained inserted overnight (Figure 4). Complete thrombosis of the varices was achieved in 51 of 56 patients after all BRTO procedures, and the remaining 5 patients showed incomplete thrombosis of the varices. Endoscopic treatments were performed in 4 of the 5 patients<sup>[20-24]</sup>, and a surgical treatment was performed in the other patient. No cases of recurrence or variceal bleeding of the gastric varices were found in the successful 51 patients after a median follow up time of 10.5 mo (range one day-7 years). Esophageal varices with red color sign appeared in 5 of the 51 patients<sup>[25-28]</sup>. Red color sign indicates a high risk of variceal bleeding<sup>[29]</sup>. These patients' varices were treated by endoscopic treatment.

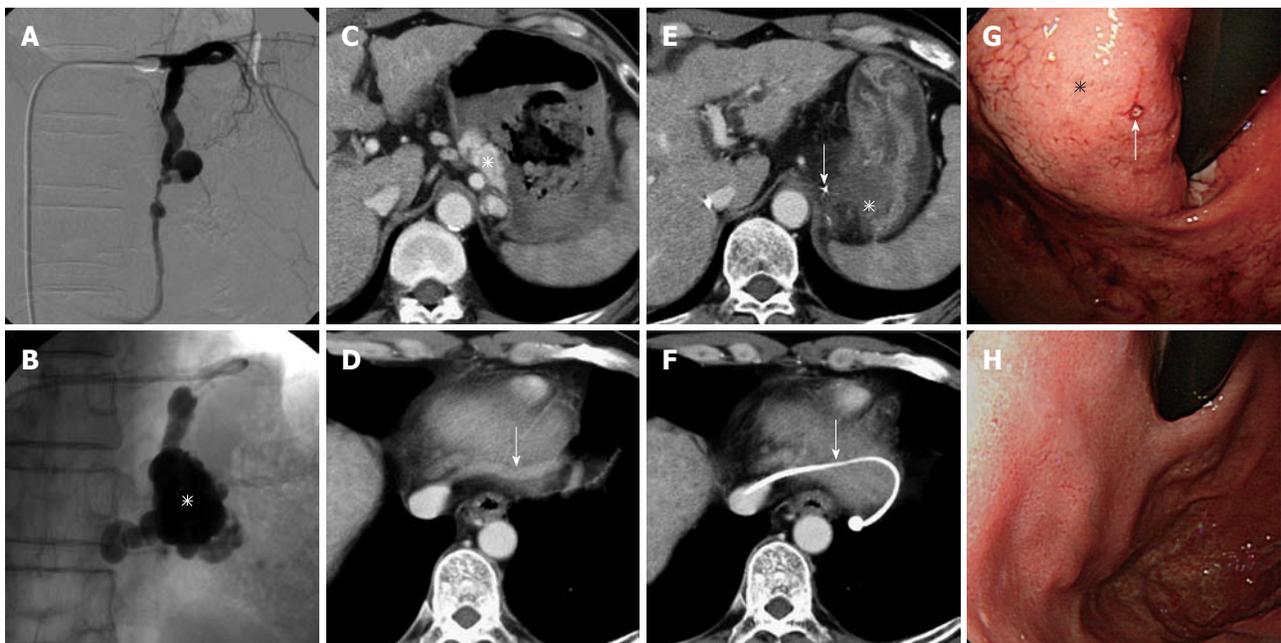
Most complications were transient and minor. These include: hematuria due to the sclerosant (8 of 56 patients), high fever (8 of 56), abdominal pain (5 of 56), elevation of blood pressure during infusion of the sclerosant (3 of 56), pleural effusion (35 of 56), ascites (33 of 56)<sup>[30]</sup>, and extravasation of the sclerosant during the procedure (3 of 56). In the three patients with extravasation, BRTO was continued, and complete thrombosis of the varices was achieved in 2 patients. We experienced one case of liver necrosis after the BRTO procedure<sup>[31]</sup>. No other major complications such as renal failure, pulmonary embolism, or acute respiratory distress syndrome (ARDS) were experienced.

## DISCUSSION

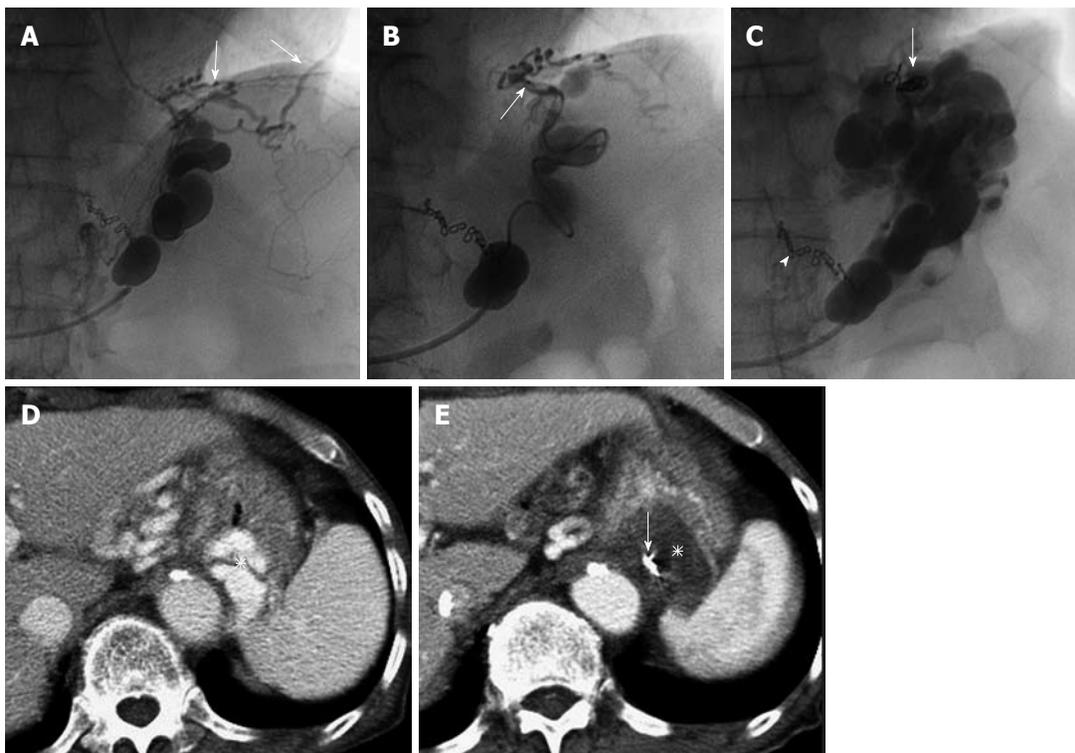
Microcatheters have three major benefits in BRTO for gastric varices. The first benefit is a selective injection of the sclerosant through a microcatheter<sup>[11-13]</sup>. Infusion of the sclerosant with a microcatheter, which is inserted close to the gastric varices, enables a decrease in the dose of the sclerosant, preventing sclerosant-related complications. We consider that the optimal volume of the sclerosant used for one BRTO procedure is 40 mL or less. To decrease the sclerosant volume of 5% EOI, 50% glucose solution may be infused before injection of 5% EOI during BRTO<sup>[32]</sup>.



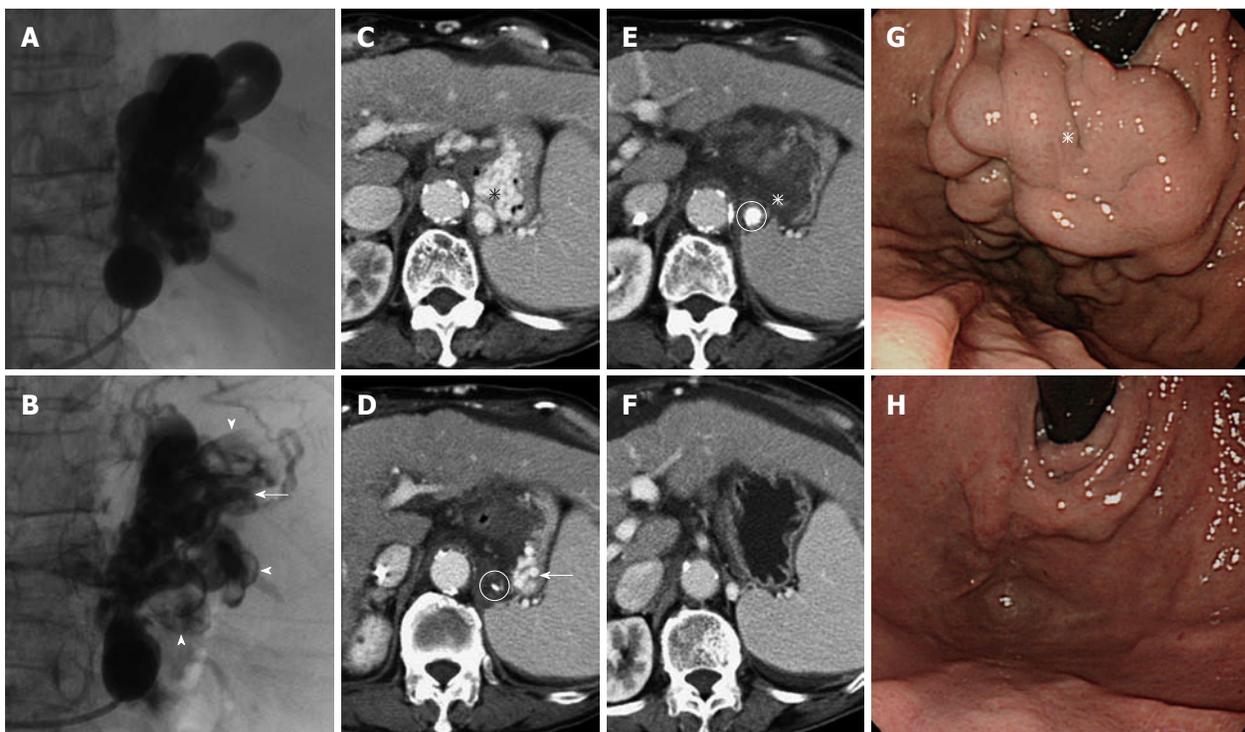
**Figure 1** Selective injection of the sclerosant. A: A microcatheter is navigated close to the gastric varices, and the sclerosant is selectively injected through the microcatheter (arrow); B: The gastric varices and gastrorenal shunt are fully filled with the sclerosant with contrast medium, and the afferent vein (arrow) is opacified; C: Contrast-enhanced computed tomography (CE-CT) before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (asterisk); D: CE-CT one week after BRTO shows complete thrombosis of the varices (asterisk); E: Endoscopy before BRTO shows tumor-like varices (asterisk) in the fornix of the stomach; F: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.



**Figure 2** Selective injection of the sclerosant. A: A balloon catheter is inserted into the gastrocaval shunt. Balloon-occluded venography shows no gastric varices; B: The balloon catheter is advanced further into the shunt, and the sclerosant is selectively injected through the microcatheter which is navigated close to the gastric varices. The varices (asterisk) are opacified sufficiently; C: Contrast-enhanced computed tomography (CE-CT) shows the varices (asterisk) and a large amount of hematomas in the stomach; D: The gastrocaval shunt (arrow) flows into the inferior vena cava; E: CE-CT next day shows complete thrombosis of the gastric varices (asterisk) and the tip of the microcatheter (arrow) close to the varices; F: CE-CT shows the balloon catheter in the shunt (arrow); G: Endoscopy before balloon-occluded retrograde transvenous obliteration (BRTO) shows large gastric varices (asterisk) with a bleeding site (arrow); H: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.



**Figure 3** Microcoil embolization of collateral veins. A: Pericardiophrenic veins (arrows) develop as collateral draining veins; B: A microcatheter (arrow) is navigated into the pericardiophrenic vein and microcoil embolization is performed; C: The sclerosant is selectively injected through the microcatheter which is withdrawn a little, and the gastric varices are opacified sufficiently. Microcoils (arrow) from embolization and surgical clips from previous operation of gastric cancer. (arrowhead) are seen; D: Contrast-enhanced computed tomography (CE-CT) before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (asterisk); E: CE-CT next day after BRTO shows complete thrombosis of the varices (asterisk) and microcoils close to the varices (arrow).



**Figure 4** Additional injection of the sclerosant. A: Fluoroscopic image obtained during the first balloon-occluded retrograde transvenous obliteration (BRTO) shows full opacification of the gastric varices and gastrorenal shunt; B: Fluoroscopic image obtained during the second BRTO (next day) shows partial opacification of the varices and shunt, suggesting residual varices (arrow) and thrombosis of the varices and shunt (arrowheads); C: Contrast-enhanced computed tomography (CE-CT) before BRTO shows large varices (asterisk); D: CE-CT after the first BRTO shows residual varices (arrow) in the lateral portion of the stomach. The microcatheter tip (circle) is in the gastrorenal shunt close to the varices; E: CE-CT after the second BRTO shows complete thrombosis of the varices (asterisk). The sclerosant with contrast medium (circle) is detected in the gastrorenal shunt; F: CE-CT 3 mo after BRTO shows complete disappearance of the varices; G: Endoscopy before BRTO shows bulky varices (asterisk); H: Endoscopy 3 mo after BRTO shows complete disappearance of the varices.

The second benefit is a microcoil embolization of dilated collateral veins<sup>[33]</sup> using a microcatheter<sup>[11,12,14]</sup>. Obliteration of collateral veins prevents renal failure, pulmonary embolism, and ARDS induced by leakage of the sclerosant into the systemic circulation. Haptoglobin was intravenously administered as a counteragent of ethanolamine oleate, which is a sclerosant that damages the endothelial cell of the vessel and induces thrombus formation in the vessel.

The third benefit is an additional injection of the sclerosant through the microcatheter that remained inserted overnight<sup>[15]</sup>. To achieve complete thrombosis of gastric varices, the balloon occlusion time was prolonged from 30 min (original BRTO) to 12 h or more<sup>[1]</sup>. After a complete thrombosis of gastric varices was confirmed on CE-CT done the next morning after the first BRTO, all catheters were removed. When complete thrombosis of gastric varices was not achieved, a second BRTO was performed, and additional sclerosant was injected through the microcatheter. Insertion of a microcatheter close to the gastric varices until the next day allows for an additional injection of the sclerosant into the varices through the microcatheter, even when occlusion of a shunt occurs.

Another minor benefit is that microcatheters can be a safer and more accurate guidance tool for balloon catheters than the 0.035 inch guidewires. The stiff guidewires sometimes induce venous damage. On the other hand, it's easy to insert a soft microcatheter into the shunts and advance a balloon catheter into the shunts over the microcatheter and microguidewire, because we can check the position of the microcatheter tip by test injection of the contrast material.

In the Kanagawa *et al*<sup>[1]</sup> on use of BRTO without the microcatheter technique, complete eradication of gastric varices was not achieved after a single BRTO procedure in 7 (22%) of 32 patients. This is compatible with our results that show 21% of patients with incomplete thrombosis of the varices and 16% of patients having microcoil embolization.

BRTO procedures for gastric varices may be difficult to conduct when varices lack a gastroduodenal shunt<sup>[34-37]</sup>. However, gastric varices without the gastroduodenal shunt are rare.

We experienced one case of liver necrosis. It is supposed that the liver necrosis was due to leakage of the sclerosant into the portal vein through afferent veins. So we must be careful in order to prevent leakage of the sclerosant into the portal vein.

Esophageal varices with red color sign appeared in 5 patients<sup>[25-28]</sup>. Occlusion of a gastroduodenal shunt and/or gastroduodenal shunt may have induced esophageal varices as another collateral route. Esophageal varices can be readily treated by endoscopic treatment. Therefore, the status of esophageal varices should be endoscopically checked at 6-month intervals after BRTO.

Three major beneficial techniques of microcatheters for BRTO of gastric varices are selective injection of the sclerosant, microcoil embolization of collateral veins and additional injection of the sclerosant. Microcatheters are

useful for achieving a higher success rate of BRTO procedures.

## COMMENTS

### Background

Gastric varices have a larger blood flow compared with esophageal varices, so when they are ruptured, there is a high mortality rate. Therefore, prophylactic treatment is necessary in patients with gastric varices in danger of rupture. Balloon-occluded retrograde transvenous obliteration (BRTO), is a treatment for gastric varices that is minimally invasive and has a high success rate. However, there are three major problems with BRTO procedures such as overdose of the sclerosant, leakage of the sclerosant into the systemic circulation, and incomplete thrombosis of large gastric varices. We introduced the microcatheter techniques to solve these problems

### Innovations and breakthroughs

Microcatheters have three major benefits in BRTO for gastric varices. The first benefit is a selective injection of the sclerosant through a microcatheter. The second benefit is a microcoil embolization of dilated collateral veins using a microcatheter. The third benefit is additional injection of the sclerosant through the microcatheter that remained inserted overnight. When complete thrombosis of gastric varices was not achieved, a second BRTO was performed, and additional sclerosant was injected through the microcatheter.

### Applications

Patients with large gastric varices and/or dilated collateral veins can be treated with BRTO procedures using the microcatheter techniques.

### Peer review

In this study, the authors described three major beneficial techniques of microcatheters for BRTO of gastric varices. Microcatheters are useful for achieving a higher success rate of BRTO procedures.

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## Branched-chain amino acid treatment before transcatheter arterial chemoembolization for hepatocellular carcinoma

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### Abstract

**AIM:** To examine the significance of branched-chain amino acid (BCAA) treatment before transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC).

**METHODS:** This study included 99 patients who underwent TACE therapy for HCC at our hospital and were followed up without treatment for at least 6 mo between January 2004 and January 2010. They were divided into 2 groups: those receiving BCAA granules ( $n = 40$ ) or regular diet ( $n = 59$ , control). Data obtained were retrospectively analyzed (prior to TACE, and 1 wk, 1, 3, and 6 mo after TACE) in terms of nutritional condition and clinical laboratory parameters (serum albumin level and Child-Pugh score), both of which are determinants of hepatic functional reserve.

**RESULTS:** The BCAA group comprised 27 males and 13 females with a mean age of  $69.9 \pm 8.8$  years. The patients of the BCAA group were classified as follows: Child-Pugh A/B/C in 22/15/3 patients, and Stage II/III/IVA HCC in 12/23/5 patients, respectively. The control

group comprised 32 males and 27 females with a mean age of  $73.2 \pm 10.1$  years. In the control group, 9 patients had chronic hepatitis, Child-Pugh A/B/C in 39/10/1 patients, and Stage I/II/III/IVA HCC in 1/11/35/12 patients, respectively. Overall, both serum albumin level and Child-Pugh score improved significantly in the BCAA group as compared with the control 3 and 6 mo after TACE ( $P < 0.05$ ). Further analysis was performed by the following categorization: (1) child-Pugh classification; (2) liver cirrhosis subgroup with a serum albumin level  $> 3.5$  g/dL; and (3) epirubicin dose. A similar trend indicating a significant improvement of all variables in the BCAA group was noted ( $P < 0.05$ ).

**CONCLUSION:** Treatment with BCAA granules in patients who have undergone TACE for HCC is considered useful to maintain their hepatic functional reserve.

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**Key words:** Hepatocellular carcinoma; Branched-chain amino acid granules; Transcatheter arterial chemoembolization; Liver function; Improvement; Cirrhosis; Protein-energy malnutrition

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common

carcinoma worldwide<sup>[1]</sup>. Treatment for HCC varies depending on the disease stage and liver function, and includes radiofrequency ablation, percutaneous ethanol injection therapy, hepatic resection, liver transplantation, transcatheter arterial chemoembolization (TACE), and molecular target therapy<sup>[2-4]</sup>.

TACE is a procedure whereby an embolizing agent is injected into the hepatic artery to deprive the tumor of its major nutrient source *via* embolization of the nutrient artery, resulting in ischemic necrosis of the tumor. Hepatic arterial embolization, which had been used until early in the 1990s, is divided into two treatment methods: injection of an embolizing agent after intra-arterial injection of an anticancer drug and intra-arterial injection of a mixture of an embolizing agent and an anticancer drug<sup>[5,6]</sup>. Subsequently, it was revealed that an oil contrast medium or iodized oil (Lipiodol) accumulates within the tumor after injection. This led to introduction of TACE, in which an embolizing agent is injected after injection of a mixture of Lipiodol and an anticancer drug (Lipiodol emulsion)<sup>[7,8]</sup>. Until the middle of the 1990s TACE had been performed in a large majority of patients with unresectable HCC. With the subsequent introduction of local treatment, however, TACE is now mainly indicated for treatment of an HCC measuring 3 to 5 cm in diameter or treatment of 4 or more HCCs less than 3 cm in diameter that are both unresectable and not indicated for local treatment.

Takayasu *et al.*<sup>[9]</sup> reported that independent prognostic factors in relation to survival in patients who underwent TACE include (1) degree of hepatic damage, (2) tumor staging and (3) serum  $\alpha$ -fetoprotein level, and recommended TACE, which can sufficiently maintain the volume ratio of a chemoembolized tumorous liver to the entire tumor-free liver as well as of residual hepatic functional reserve, while emphasizing the importance of maintenance of hepatic functional reserve in these patients.

Branched-chain amino acids ( BCAAs) are three amino acids possessing branched side chains (*i.e.*, valine, leucine, and isoleucine). Patients with liver cirrhosis are known to have decreased plasma BCAA levels, which can lead to protein-energy malnutrition (PEM). PEM is associated with a high morbidity and mortality due to an increased risk of life-threatening complications, resulting in poor survival and quality of life (QoL)<sup>[10]</sup>.

A considerable proportion of patients with HCC have concurrent liver cirrhosis. In those patients with underlying PEM, interventional therapy such as TACE may further worsen their nutritional condition and even occasionally cause development of ascites and jaundice, resulting in an irreversible outcome<sup>[11]</sup>.

Supplementation with BCAAs in patients with liver disorder has been attracting attention. BCAA treatment can correct malnutrition associated with liver cirrhosis in animals and humans<sup>[12-14]</sup>, and long-term nutritional BCAA supplementation may also be useful for prevention of hepatic failure while it also improves surrogate markers in patients with advanced cirrhosis<sup>[15,16]</sup>. BCAA

supplementation is also effective in down-regulating protein metabolism in liver cirrhosis patients by reducing ammonia (NH<sub>3</sub>) level, thus improving the nitrogen balance and resulting in better clinical outcomes<sup>[17,18]</sup>. The mechanism underlying these beneficial effects of BCAAs might be mediated by stimulation of hepatocyte growth factor activity that induces liver regeneration<sup>[19]</sup>. Therefore, nutritional support may play an important role in management of liver cirrhosis in patients with unresectable HCC. Studies dealing with the effect of treatment with BCAA granules before TACE in patients with HCC, nevertheless, are few as yet to our knowledge. This study was thus performed to investigate the significance of BCAA treatment in HCC patients who had undergone TACE.

## MATERIALS AND METHODS

### Patients

This retrospective study included 99 patients who underwent TACE alone for treatment of HCC at our hospital and were followed up thereafter without treatment for at least 6 mo between January 2004 and January 2010. Patients were divided into two groups: those receiving BCAA treatment ( $n = 40$ ) or regular diet ( $n = 59$ , control). BCAA therapy had been started at least one month before the day TACE was performed, and treatment compliance was good in all patients receiving BCAAs.

### Diagnosis of hepatocellular carcinoma

Dynamic computed tomography (CT) and abdominal echography were performed in all patients. A lesion visualized as a tumor blush in the early phase scan and as a defect area in the late phase scan on dynamic CT was diagnosed as HCC. It has been verified that such lesions appear as blushes on CT hepatic angiography and as defect areas on CT arterial portography during TACE. Two radiologists proficient in diagnostic imaging of the liver made a diagnosis of HCC. No pathological examination was conducted.

### Branched-chain amino acid granules

BCAA granules, containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine per sachet, were orally administered to subjects at a dose of one sachet three times daily after meals. The control patients received no such treatment.

### Transcatheter arterial chemoembolization procedure

Written informed consent was obtained from each patient prior to TACE. The protocol for TACE was approved by the independent ethics committee of the hospital. TACE for HCC was performed in conformity with Japanese guidelines for this therapy<sup>[20]</sup> and consisted of catheterization *via* the femoral artery with super-selective cannulation to the hepatic artery feeding the target HCC. Farnorubicin (epirubicin hydrochloride, Pfizer) emulsion was infused at 10 to 60 mg, and Lipiodol (iodine addition products of ethyl esters of fatty acids obtained from pop-

**Table 1** Baseline characteristics of study groups (mean  $\pm$  SD)

	BCAA group (n = 40)	Control group (n = 59)	P value
Gender			
Male	27	32	0.215
Female	13	27	
Age (yr)	69.9 $\pm$ 8.8	73.2 $\pm$ 10.1	0.092
Etiology of liver disease			
Chronic hepatitis C	28	43	0.287
Chronic hepatitis B	2	8	
Non B non C	10	10	
Child-Pugh classification			
Chronic hepatitis	0	9	0.006
Child-Pugh A	22	39	
Child-Pugh B	15	10	
Child-Pugh C	3	1	
WBC ( $\times 10^3/\mu\text{L}$ )	38.2 $\pm$ 10.8	44.7 $\pm$ 16.0	0.082
Hb (g/dL)	11.9 $\pm$ 1.8	12.5 $\pm$ 1.7	0.091
Platelet ( $\times 10^4/\text{mm}^3$ )	10.2 $\pm$ 9.4	11.4 $\pm$ 4.9	0.431
Alb (g/dL)	3.32 $\pm$ 0.50	3.74 $\pm$ 0.51	< 0.001
T-Bil (mg/dL)	1.28 $\pm$ 0.81	1.05 $\pm$ 0.63	0.123
PT (%)	77.5 $\pm$ 14.1	85.9 $\pm$ 17.3	0.012
AST (IU/L)	65.8 $\pm$ 39.6	73.8 $\pm$ 56.4	0.445
ALT (IU/L)	48.0 $\pm$ 38.8	54.2 $\pm$ 39.0	0.438
AFP (ng/mL)	626.1 $\pm$ 2009.8	1109.2 $\pm$ 2652.5	0.331
PIVKAII (mAU/mL)	1471.7 $\pm$ 5033.5	3421.5 $\pm$ 8211.2	0.183
HCC Stage			
Stage I	0	1	0.412
Stage II	12	11	
Stage III	23	35	
Stage IVa	5	12	
Max tumor size (cm)	3.34 $\pm$ 1.67	3.59 $\pm$ 1.47	0.422
Epirubicin dose (mg)	34.8 $\pm$ 10.4	39.5 $\pm$ 9.2	0.024

WBC: White blood cell; Hb: Hemoglobin; Alb: Albumin; T-Bil: Total bilirubin; PT: Prothrombin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; PIVKA II: Protein induced vitamin K absence or antagonist II; HCC: Hepatocellular carcinoma; BCAA: Branched-chain amino acids.

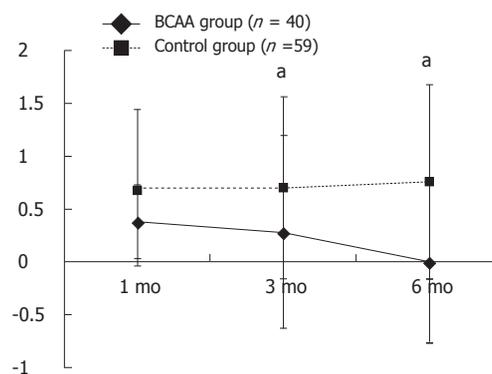
py seed oil; Mitsui, Japan) was also injected at 2 to 10 mL according to the tumor size and tumor number. This was followed by embolization with gelatin (Spongel; Yamanouchi, Japan), which was injected slowly to prevent reflux into untreated segments. The sites of injection of the embolizing agents were segmental or subsegmental in all patients.

#### Follow-up after transcatheter arterial chemoembolization

At 1 wk and 1, 3 and 6 mo after TACE, patients underwent hematological and blood biochemical tests and were assessed for their hepatic functional reserve and development of any adverse events. Dynamic CT was carried out to assess for any ascites or recurrence of HCC at 1, 3 and 6 mo after TACE.

#### Statistical analysis

Student *t* test,  $\chi^2$  test and Fisher's exact test were used to compare data between BCAA patients and the control. Serum albumin level and Child-Pugh score constituted parameters for assessment of hepatic functional reserve. Absolute changes in serum albumin level observed at 1 wk and 1, 3 and 6 mo after TACE were compared between the two groups and evaluated using Student *t* test,



**Figure 1** Overall comparison of changes in Child-Pugh score between the branched-chain amino acids group and the control group over time. There was a significant difference in changes in Child-Pugh score 3 and 6 mo after transcatheter arterial chemoembolization. <sup>a</sup>*P* < 0.05 vs control group. BCAA: Branched-chain amino acid.

and the absolute change was defined as the difference found at each assessment time point from the baseline (pre-TACE level). Changes in Child-Pugh score were also evaluated similarly using Student *t* test at 1, 3 and 6 mo after TACE.

Data were analyzed using SPSS software, version 9.0 (SPSS Inc., Chicago, IL, United States) for Microsoft Windows. Data are expressed as mean  $\pm$  SD. Values of *P* < 0.05 were considered to be statistically significant.

## RESULTS

Patient demographic characteristics are summarized in Table 1. Significant differences were noted for the following parameters: Child-Pugh score, serum albumin level, prothrombin time, and dose of epirubicin at the time of TACE. A patient in the control group had stage I HCC, for which percutaneous therapy is indicated, but TACE alone was performed because the patient refused percutaneous therapy.

#### Overall comparison of hepatic functional reserve between the branched-chain amino acid group and the control group over time

A significant difference in serum albumin level was observed at all assessment time points (*P* < 0.05). Also, there was a significant difference in Child-Pugh score 3 and 6 mo after TACE (*P* < 0.05) (Table 1, and Figure 1).

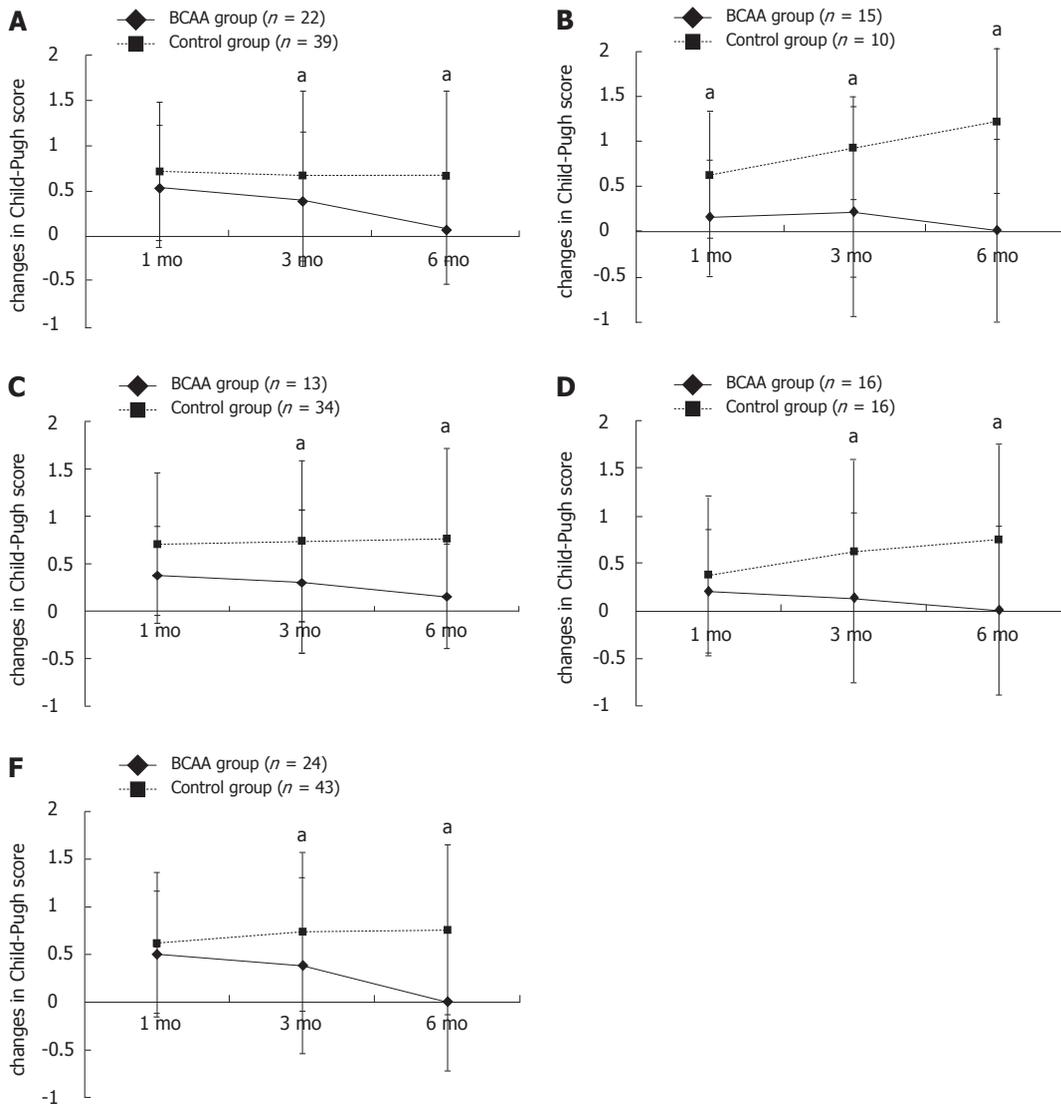
The categorized analysis results are presented below.

#### Comparison of hepatic functional reserve in Child A patients

There were 22 Child A patients in the BCAA group and 39 in the control group. A significant difference was noted in serum albumin level 1, 3 and 6 mo after TACE and in Child-Pugh score 3 and 6 mo after TACE (*P* < 0.05) (Table 1, and Figure 2A).

#### Comparison of hepatic functional reserve in Child B patients

There were 15 Child B patients in the BCAA group and 10 in the control group. A significant difference was



**Figure 2 Comparison of changes in Child-Pugh score in Child A and Child B patients: patients with a serum albumin level of 3.5 g/dL or more, low-dose epirubicin subgroups, high-dose epirubicin subgroups.** A: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after transcatheter arterial chemoembolization; B: A significant difference was noted in changes in Child-Pugh score 1, 3 and 6 mo after transcatheter arterial chemoembolization; C: A significant difference was observed in changes in Child-Pugh score 3 and 6 mo after TACE; D: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after TACE; E: A significant difference was noted in changes in Child-Pugh score 3 and 6 mo after TACE. BCAA: Branched-chain amino acids; TACE: Transcatheter arterial chemoembolization. <sup>a</sup> $P < 0.05$  vs control group.

noted in serum albumin level 3 and 6 mo after TACE and in Child-Pugh score 1, 3 and 6 mo after TACE ( $P < 0.05$ ) (Table 1 and Figure 2B).

### Comparison in patients with a serum albumin level of 3.5 g/dL or more

There were 13 and 34 patients who fell in this category in the BCAA group and the control group, respectively. A significant difference was observed in both serum albumin level and Child-Pugh score 3 and 6 mo after TACE ( $P < 0.05$ ) (Table 1, and Figure 2C).

As it is thought that antineoplastic agents used during TACE may cause hepatic impairment in a dose-dependent fashion, the data were further evaluated in patients classified into two subgroups: those treated with low-dose epirubicin (less than 40 mg) or a high-dose epirubi-

cin (40 mg or more).

### Comparison in low-dose epirubicin subgroups

Sixteen patients each received low-dose epirubicin in the BCAA group and the control group. Serum albumin level was significantly different 1, 3 and 6 mo after TACE and Child-Pugh score 3 and 6 mo after TACE ( $P < 0.05$ ) (Table 1 and Figure 2D).

### Comparison in high-dose epirubicin subgroups

Twenty-four and 43 patients received high-dose epirubicin in the BCAA group and the control group, respectively. A significant difference was noted in serum albumin level at all assessment time points and in Child-Pugh score 3 and 6 mo after TACE ( $P < 0.05$ ) (Table 1 and Figure 2E).

## DISCUSSION

PEM occurs frequently in patients with liver cirrhosis and represents an important predictive factor for the prognosis of liver cirrhosis patients with HCC<sup>[18,21]</sup>. Supplementation with BCAA formula is reportedly useful for improving PEM and QoL in these patients. However, few studies have assessed the importance of such nutritional intervention in patients with HCC who underwent nonsurgical therapies such as TACE. The purpose of the present study was to investigate to what extent BCAA treatment can contribute to maintaining hepatic functional reserve in HCC patients after TACE.

A significant difference was observed in the overall patient population in terms of change in serum albumin level at all assessment time points. As seen in Table 1, hepatic functional reserve was relatively well maintained in the control group; therefore, anticancer chemotherapy was given at relatively high doses (60% of patients treated with BCAA received epirubicin at 40 mg or more whereas the corresponding percentage for the control group was 72.9%). Patients receiving high-dose anticancer chemotherapy are often unable to sufficiently ingest food over several weeks after TACE. This may account for lower serum albumin levels observed in the control group compared with the BCAA group. Other possible causes of decreased serum albumin levels after TACE include (1) impaired ability of the liver to synthesize serum albumin due to decreased hepatocyte count; (2) inhibition of the synthesis of albumin by inflammatory cytokines; and (3) leakage of albumin due to inflammation of the cauterized areas<sup>[22,23]</sup>.

The assessments in Child-Pugh A patients revealed a significant difference in serum albumin level 1, 3 and 6 mo after TACE and in Child-Pugh score 3 and 6 mo after TACE. TACE is best indicated for Child-Pugh A HCC. In patients undergoing TACE, caution should be exercised to minimize depression of hepatic functional reserve in preparation for the next treatment session. The above results thus suggest the usefulness of BCAA treatment in this regard.

The assessments in the Child-Pugh B subgroup showed a significant difference in Child-Pugh score 1, 3 and 6 mo after TACE. Once hepatic functional reserve has worsened from Child-Pugh B to Child-Pugh C following TACE, the next TACE cannot be performed according to the Barcelona Clinic Liver Cancer guidelines<sup>[24]</sup>. Therefore, particular caution should be exercised in maintaining hepatic functional reserve at the time of TACE in patients with Child-Pugh B HCC, indicating the indispensability of BCAA therapy.

In Japan, BCAA granules are indicated for the treatment of liver cirrhosis in patients with a serum albumin level of 3.5 g/dL or less. However, conversely, the present study demonstrated similar results between patients with a serum albumin level of more than 3.5 g/dL and those in other categories of serum albumin level. Therefore, treatment with BCAA proved to improve hepatic functional reserve even in cirrhotic patients with HCC

whose serum albumin level exceeds 3.5 g/dL. It is thus recommended to actively provide BCAA treatment in such patients.

There was a conspicuous difference between the BCAA and control groups in respect of response to BCAA therapy when assessed in patients receiving high-dose epirubicin compared to those treated with low-dose epirubicin. TACE may cause a marked damage to the liver in HCC patients, eventually leading to a considerable impact on their hepatic functional reserve<sup>[9]</sup>. BCAA treatment is thus recommended at sufficient doses prior to TACE in patients with advanced HCC in whom high-dose anticancer chemotherapy is anticipated.

TACE is often repeated because a single session of therapy seldom provides complete necrosis of a tumor. The procedure is commonly repeated once every 2 to 3 mo<sup>[25-27]</sup>. In the present study, however, many patients failed to attain recovery of hepatic functional reserve to a pre-TACE level, particularly in the control group, within 2 to 3 mo of TACE. It is thus estimated that every repeated session of TACE may worsen hepatic functional reserve and thereby shorten the prognosis for survival. Treatment with BCAA would therefore be essential in order to allow for providing TACE periodically while securely maintaining hepatic functional reserve.

One of the findings commonly noted in regard to all the variables assessed in this study is that a significantly greater improvement was noted in both serum albumin level and Child-Pugh score for the BCAA group 6 mo after TACE in comparison to the control group. What is suggested by this fact is simply the usefulness of long-term BCAA treatment prior to TACE. It is also important that patients should be fully instructed on the use of BCAA granules to maintain their treatment compliance.

The present study has several limitations. Firstly, it is a retrospective study. Furthermore, there was a bias in patient demographic characteristics between the BCAA and control groups since BCAA is usually used for patients showing low serum albumin levels. Therefore, pertinent data were evaluated for improvement or exacerbation using absolute serum albumin change as a parameter. The present study did not include assessment for the prognosis for survival, which should be addressed by a prospective study using comparable demographic characteristics among patients.

In conclusion, treatment with BCAAs before TACE in HCC patients is extremely useful in maintaining their hepatic functional reserve.

## COMMENTS

### Background

Patients with hepatocellular carcinoma (HCC) due to liver cirrhosis are known to have decreased plasma branched-chain amino acid (BCAA) levels, which can lead to protein-energy malnutrition (PEM). BCAA treatment can correct malnutrition associated with liver cirrhosis.

### Research frontiers

Studies dealing with the effect of treatment with BCAA granules before transcatheter arterial chemoembolization (TACE) in patients with HCC are few as

yet. In this study, the authors analyzed the effect of BCAA treatment before TACE for HCC patients.

### Innovations and breakthroughs

Recent studies imply that by BCAA supplementation, malnutrition associated with liver cirrhosis is corrected and liver function improves. The present study shows that in HCC patients who underwent TACE, liver function was maintained by BCAA supplementation.

### Applications

This study emphasizes the importance of BCAA treatment before TACE for HCC patients with regard to maintaining liver function.

### Peer review

This is a very good and novel study in which authors analyze the effect of BCAA treatment before TACE for HCC patients. The results are interesting and suggest the usefulness of BCAA treatment before TACE in HCC patients in maintaining their hepatic functional reserve.

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## Assessment of disease progression in patients with transfusion-associated chronic hepatitis C using transient elastography

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### Abstract

**AIM:** To evaluate the relationship between liver stiffness and duration of infection in blood transfusion-associated hepatitis C virus (HCV) patients with or without hepatocellular carcinoma (HCC).

**METHODS:** Between December 2006 and June 2008, a total of 524 transfusion-associated HCV-RNA positive patients with or without HCC were enrolled. Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France). The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood

transfusion and the date of liver stiffness measurement (LSM). Patients were stratified into four groups based on the duration of infection (17-29 years; 30-39 years; 40-49 years; and 50-70 years). The difference in liver stiffness between patients with and without HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness.

**RESULTS:** A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements. Thus 516 patients were included in the current analysis (225 with HCC and 291 without). The patients were 244 men and 272 women, with a mean age of  $67.8 \pm 9.5$  years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). The patients who developed HCC in short duration of infection were male dominant, having lower platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an old age. Liver stiffness was positively correlated with duration of infection in patients without HCC ( $r = 0.132$ ,  $P = 0.024$ ) but not in patients with HCC ( $r = -0.103$ ,  $P = 0.123$ ). Liver stiffness was significantly higher in patients with HCC than in those without in each duration group ( $P < 0.0001$ ). The factors significantly associated with high liver stiffness in multiple regression were age at blood transfusion ( $P < 0.0001$ ), duration of infection ( $P = 0.0015$ ), and heavy alcohol consumption ( $P = 0.043$ ).

**CONCLUSION:** Although liver stiffness gradually increases over time, HCC develops in patients with high stiffness value regardless of the duration of infection.

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**Key words:** Transfusion-associated hepatitis C; Transient elastography; Hepatocellular carcinoma; Liver stiffness; Ultrasonography; Liver fibrosis

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## INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, presenting serious public health problems worldwide<sup>[1,2]</sup>. HCV infection generally shows an asymptomatic onset and slow fibrosis progression, with cirrhosis developing after 20-50 years<sup>[3-7]</sup>. Progression of disease is known to depend on patients' characteristics at the onset of infection<sup>[8-12]</sup>. Infection at old age, male gender and heavy alcohol consumption are reported to be independently associated with rapid disease progression.

The onset of HCV infection can be reliably estimated in transfusion-associated chronic hepatitis C patients, in contrast to repeating injecting-drug users. In Japan, about 40% of chronic hepatitis C patients and HCV-related HCC patients have a history of blood transfusion typically in 1950s and 1960s<sup>[13]</sup>, when blood supply depended on paid blood donors. Not a few of the blood donors were also injecting-drug users, mainly methamphetamine, among whom HCV spread first after the end of World War II. Viral spread started to decline in Japan after commercial blood banks were entirely abolished in 1969<sup>[14]</sup>.

Chronic hepatitis C with cirrhosis is a strong risk factor for hepatocellular carcinoma (HCC)<sup>[15,16]</sup>. It has been shown that the risk of HCC increases with the degree of liver fibrosis<sup>[17]</sup>. Until recently, however, the degree of liver fibrosis could be reliably assessed only with liver biopsy, an invasive procedure with the possibility of serious complications<sup>[18,19]</sup>.

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage<sup>[20]</sup>. We previously reported that liver stiffness is strongly associated with the risk of HCC<sup>[21,22]</sup>. The calculated stratum-specific likelihood ratio indicated that the post-test odds for the presence of HCC increase five-fold when liver stiffness is higher than 25 kPa and decrease to one-fifth when lower than 10 kPa. Furthermore, we have confirmed in a prospective study that liver stiffness is a significant risk factor for HCC development, together with male gender, clinical cirrhosis and serum albumin level. However, in those studies we did not fully consider the duration of HCV infection and the age at the onset of infection, which are indicated in several studies to be

associated with disease progression.

The aim of this study is to evaluate the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without HCC, focusing on the risk of HCC development.

## MATERIALS AND METHODS

### Patients

This study conformed to the ethical guideline of the 1975 Helsinki Declaration and the ethical guidelines for epidemiologic research designed by Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labor and Welfare. The study design was approved by the ethics committee of the authors' institution. Between December 2006 and June 2008, a total of 1562 patients positive for HCV RNA visited the liver clinic of authors' institution. Among these patients, those with a history of receiving blood transfusion were consecutively enrolled (229 with HCC and 295 without). We excluded from the present study those patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon therapy, and patients who received multiple blood transfusions.

### Transient elastography

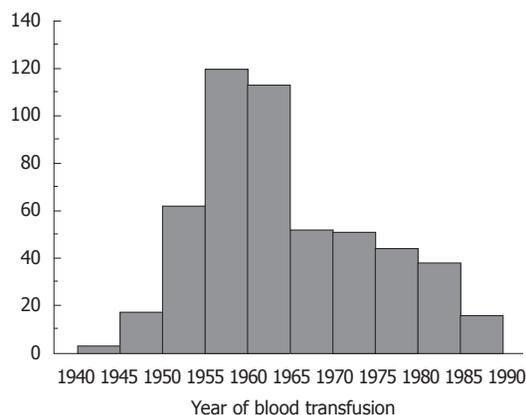
Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France), a newly developed medical device based on elastometry. Liver stiffness measurement (LSM) was considered valid only when at least eight acquisitions were successful with a success rate of at least 60% and the ratio of inter-quartile range (IQR) to the median value was larger than 30%.

### Diagnosis of hepatocellular carcinoma

In patients with HCC, the cancer was diagnosed by dynamic computed tomography (CT), where intrahepatic nodules with hyperattenuation in the arterial phase with washout in the late phase were considered as definite HCC<sup>[23,24]</sup>. Histopathological diagnosis, using ultrasound-guided biopsy samples, was also performed when required. In patients without HCC, the cancer was ruled out by ultrasonography. No HCC was detected in the subsequent six-month follow-up period among these patients.

### Laboratory tests

We determined the following parameters on the day of LSM: serum albumin and total bilirubin concentrations, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, prothrombin activity and platelet count. Serogrouping of HCV was assessed by enzyme-linked immunosorbent assay (ELISA) (Immucheck F-HCV Gr Kokusai; Sysmex, Kobe, Japan)<sup>[25]</sup>. Based on the prevalence of each HCV genotype in Japan, serogroup 1 was assumed to represent genotype 1b and serogroup 2 to represent genotype 2a or 2b.



**Figure 1** Frequency distribution of the year of receiving blood transfusion among the subjects. There is a peak around the year 1960.

**Table 1** Characteristics of patients according to presence of hepatocellular carcinoma *n* (%)

Characteristics	HCC	Non-HCC	<i>P</i> value
Overall patients	<i>n</i> = 225	<i>n</i> = 291	
Gender (M/F)	126/99	118/173	0.0005
Age (yr) <sup>1</sup>	71.2 (66.1-75.7)	68.1 (58.7-72.4)	< 0.0001
Platelet count (10 <sup>9</sup> /L) <sup>1</sup>	95 (74-133)	161 (111-200)	< 0.0001
ALT (IU/L) <sup>1</sup>	48 (34-68)	42 (25-69)	0.006
Alcohol consumption > 50 g/d	51 (22.7)	28 (9.6)	< 0.0001
Liver stiffness (kPa) <sup>1</sup>	25.8 (17.3-37.4)	7.6 (5.6-13.9)	< 0.0001
IQR (kPa) <sup>1</sup>	4.0 (2.5-6.0)	1.6 (1.2-2.6)	< 0.0001
Duration (17-29 yr)	<i>n</i> = 34	<i>n</i> = 64	
Gender (M/F)	25/9	38/26	0.0028
Age (yr) <sup>1</sup>	73.1 (65.7-77.1)	59.7 (47.2-69.2)	0.033
Platelet count (10 <sup>9</sup> /L) <sup>1</sup>	95 (76-154)	180 (116-229)	< 0.0001
ALT (IU/L) <sup>1</sup>	51 (34-89)	42 (22-77)	0.2071
Alcohol consumption > 50 g/d	12 (35.3)	9 (14.1)	0.023
Liver stiffness (kPa) <sup>1</sup>	26.1 (16.8-53.3)	5.9 (4.9-12.1)	< 0.0001
Duration (30-39 yr)	<i>n</i> = 40	<i>n</i> = 59	
Gender (M/F)	16/24	23/36	0.9191
Age (yr) <sup>1</sup>	72.0 (65.4-76.7)	62.3 (55.7-68.6)	< 0.0001
Platelet count (10 <sup>9</sup> /L) <sup>1</sup>	93 (68-120)	151 (97-215)	< 0.0001
ALT (IU/L) <sup>1</sup>	42 (33-65)	48 (27-80)	0.7591
Alcohol consumption > 50 g/d	6 (15)	7 (11.9)	0.7641
Liver stiffness (kPa) <sup>1</sup>	28.7 (20.1-37.8)	7.4 (5.7-13.8)	< 0.0001
Duration (40-49 yr)	<i>n</i> = 101	<i>n</i> = 127	
Gender (M/F)	58/43	51/76	0.0113
Age (yr) <sup>1</sup>	69.2 (65.8-73.6)	69.9 (65.7-72.7)	0.8107
Platelet count (10 <sup>9</sup> /L) <sup>1</sup>	97 (67-136)	163 (112-195)	< 0.0001
ALT (IU/L) <sup>1</sup>	48 (34-69)	38 (23-64)	0.0080
Alcohol consumption > 50 g/d	25 (24.8)	8 (6.3)	0.0001
Liver stiffness (kPa) <sup>1</sup>	25.1 (17.5-37.4)	8.2 (5.8-14.1)	< 0.0001
Duration (50-70 yr)	<i>n</i> = 50	<i>n</i> = 41	
Gender (M/F)	27/23	18/23	0.4016
Age (yr) <sup>1</sup>	74.4 (70.0-78.1)	73.7 (66.3-79.2)	0.5658
Platelet count (10 <sup>9</sup> /L) <sup>1</sup>	97 (81-141)	147 (117-189)	0.0001
ALT (IU/L) <sup>1</sup>	52 (36-69)	46 (32-63)	0.1700
Alcohol consumption > 50 g/d	8 (16)	4 (9.8)	0.5363
Liver stiffness (kPa) <sup>1</sup>	16.0 (8.0-36.3)	7.9 (6.5-15.8)	< 0.0001

<sup>1</sup>Data are expressed as median (25th-75th percentiles). ALT: Alanine aminotransferase; IQR: Inter-quartile range; HCC: Hepatocellular carcinoma; M: Male; F: Female.

### Duration of infection and liver stiffness progression

The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood transfusion and the date of LSM. The rate of liver stiffness progression was calculated as follows: present liver stiffness-minimal stiffness value in the cohort (kPa)/interval after blood transfusion (years).

### Statistical analysis

Data were expressed as median and 25th-75th percentiles in parenthesis unless otherwise indicated. The categorical variables were compared by  $\chi^2$  tests, whereas continuous variables were compared with unpaired Student's *t* test (parametric) or Mann-Whitney *U* test (non-parametric). A *P* value < 0.05 on two-tailed test was considered significant.

The correlation between liver stiffness and the interval from blood transfusion was assessed by Spearman's rank correlation. The duration of infection was arbitrarily stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years. The difference in liver stiffness according to the presence of HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness. Processing and analysis were performed by using the S-plus Version 7 (TIBCO Software Inc., Palo Alto, CA, United States).

## RESULTS

### Patients' profile

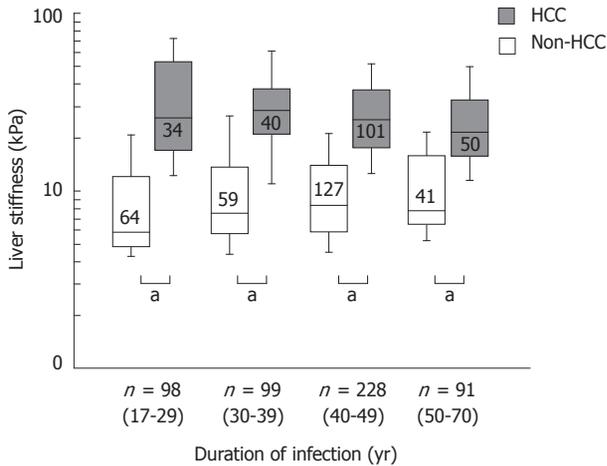
A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements, mostly due to obesity (four patients had IQR/median > 30% and four had a success rate lower than 60%). Thus 516 patients were included in the current analysis (225 with HCC and 291 without). Their characteristics at the time of LSM are summarized in Table 1. The patients were 244 men and 272 women, with a mean age of 67.8 ± 9.5 years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). Figure 1 shows the frequency distribution of the year of receiving blood transfusion among the subjects. A peak is noted around the year 1960.

### Characteristics of patients according to the duration of infection

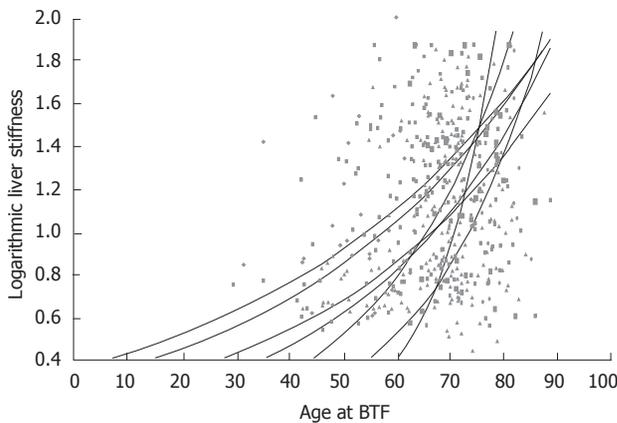
Characteristics of patients were compared between patients with and without HCC in each duration of infection group (Table 1). The patients who developed HCC in short duration of infection were male dominant, having low platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an older age.

### Correlation between liver stiffness and duration of infection

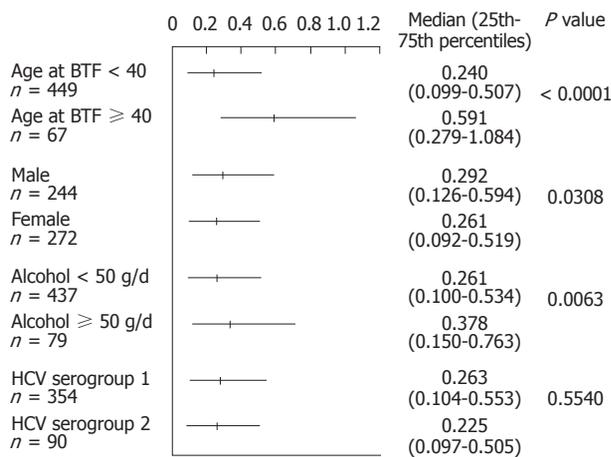
The correlation between liver stiffness and duration of



**Figure 2 Duration of infection and liver stiffness.** Liver stiffness was higher in patients with HCC than in patients without in each infection duration group ( $P < 0.0001$  by Mann-Whitney *U* test).



**Figure 3 Age at blood transfusion and liver stiffness.** Stiffness at present (each dot) and stiffness at BTF (assumed to normal value) were connected approximate logarithmic curve. Stiffness progressions become rapid in older age at BTF.



**Figure 4 Liver stiffness progression rate.** The progression rate is significantly higher in patients who were older than 40 at the time of blood transfusion, whose alcohol consumption is more than 50 g/d, and who are male. There is no significant difference according to hepatitis C virus (HCV) serotypes. Horizontal bar represents median value and 25th-75th percentiles.

infection was significant in patients without HCC ( $r = 0.132$ ,  $Z = 2.256$ ,  $P = 0.024$ ) but not in patients with HCC ( $r = -0.103$ ,  $Z = -1.54$ ,  $P = 0.123$ ). When the duration of infection was stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years, liver stiffness was higher in patients with HCC than in patients without in each group ( $P < 0.0001$ , Figure 2).

### Multiple regression analysis

The relationship between present liver stiffness and patients' characteristics, i.e., the age at blood transfusion, duration of infection, gender, and alcohol consumption (alcohol > 50 g/d) was analyzed with multiple linear regression analysis. The results showed that the age at blood transfusion was positively correlated with liver stiffness, with a coefficient of +0.336 per year for kPa,  $P < 0.0001$ , independently of the duration of infection (coefficient +0.272 per year for kPa,  $P = 0.0015$ ). This suggests that fibrosis progression is more rapid when infection is acquired at older ages. Alcohol consumption was also significantly correlated with a positive coefficient (coefficient +4.183 for kPa,  $P = 0.043$ ).

### Stiffness progression and the age at blood transfusion

The progression of liver fibrosis, as represented by the increase in liver stiffness, must have been rapid in patients who have high liver stiffness in spite of short duration of HCV infection. We assumed that the liver stiffness was normal, that is, 2.9 kPa, when patients received blood transfusion. In Figure 3, the slopes represent the estimated increase rates of liver stiffness. In accordance with the results of multiple regression, the estimated increase rate was higher when patients received blood transfusion at older ages.

The progression rate of liver stiffness was assessed in subgroups according to three parameters (Figure 4). The progression rate was significantly higher in patients who were older than 40 at the time of blood transfusion ( $P < 0.0001$ ), which is in accordance with the results of multiple regression. Heavy alcohol consumption (more than 50 g ethanol/d,  $P = 0.0308$ ) and male gender ( $P = 0.0063$ ) also showed significant difference by Mann-Whitney *U* test. There was no significant difference among HCV genotypes.

## DISCUSSION

The natural history of chronic hepatitis C concerning liver fibrosis progression has been vigorously studied using liver biopsy specimens. The extent of liver fibrosis is usually evaluated as categorical stages. For example, METAVIR Score uses five stages, F0-F4, for fibrosis evaluation<sup>[26]</sup>. The fibrosis progression in hepatitis C patients, calculated by using paired liver biopsy, was reported to be 0.1-0.133 Unit per year<sup>[12,13]</sup>. Liver stiffness measured by transient elastography is now widely accepted as a surrogate marker of liver fibrosis<sup>[27]</sup>. Liver stiffness is expressed as a continuous variable in kPa unit. The cut-

off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus LSM has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

In the present study, the increase rate of liver stiffness was positively correlated with the age at blood transfusion, as shown by the steeper slopes of approximation curves when patients received BTF at older ages. The cause of this phenomenon is not clear but age-related changes in immunity may be involved. If this is the case, the increase rate is likely to become higher in the same patient with age. Indeed, each approximation curve in the figure apparently becomes steeper with age, suggesting age-related acceleration. This is to be confirmed in future longitudinal studies.

LSM is useful not only as a surrogate of liver biopsy but also as a risk indicator of HCC development. Indeed, in the present study, liver stiffness is high in patients with HCC regardless of duration of infection. The patients who developed HCC with short duration of infection received blood transfusion at an older age and were older at the time of LSM, male dominant, and showed higher liver stiffness than patients without HCC with similar duration of infection. The difference between patients with and without HCC became smaller with longer duration of infection, as the average liver stiffness in patients with HCC became lower and that in patients without HCC became higher. We speculated that patients with high liver stiffness who received blood transfusion in the early period have already died of HCC or liver failure and were eliminated from the study population. Another possibility is that HCC may develop in patients with relatively low liver stiffness when infection has lasted a long time.

In the present study, the median increase in liver stiffness was calculated as 0.275 kPa per year. Using 13.01 kPa as a cut-off for cirrhosis<sup>[28]</sup>, it will take around 40 years on average to develop cirrhosis, which is consistent with previous reports based on liver biopsy<sup>[29]</sup>. Admittedly, the present study is basically cross-sectional, and prospective longitudinal LSM will be obviously superior in understanding the natural course of liver fibrosis progression. However, the estimated average increase rate of liver stiffness indicates that such studies will require repeated LSM at an interval of at least five years.

Age at viral infection, alcohol consumption, and male gender were reported to be associated with accelerated fibrosis progression<sup>[8-11]</sup>. In the present study, we performed subgroup analysis and indeed found that blood transfusion at an age older than 40, male gender, and alcohol consumption more than 50 g ethanol/d were significantly associated with rapid increase in liver stiffness. There is consensus that heavy alcohol consumption is associated with fibrosis progression<sup>[30]</sup>. Alcohol, which by itself can cause liver disease and fibrosis, may affect liver stiffness and worsen fibrosis in hepatitis C<sup>[31]</sup>. We did not find a difference in liver stiffness increase rate between HCV genotypes 1 (mostly 1b) and 2 (2a/2b), although we could not evaluate genotypes 1a, 3 or 4.

This study has some limitations. First, since this is a cross-sectional study performed after LSM became available, patients with more rapid disease progression may have died and been excluded from the study. Second, because transfusion-associated HCV infection has been virtually eliminated in Japan since 1992, we could not include patients with shorter duration of infection. Lastly, we did not perform paired LSM but assumed that liver stiffness was normal at the time of infection. Longitudinal observation is now on-going but will take several years to obtain results.

In conclusion, although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

## COMMENTS

### Background

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage.

### Research frontiers

This study evaluated the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without hepatocellular carcinoma (HCC), focusing on the risk of HCC development.

### Innovations and breakthroughs

Liver stiffness is expressed as a continuous variable in kPa unit. The cut-off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus liver stiffness measurement (LSM) has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

### Applications

Although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired hepatitis C virus (HCV) infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

### Terminology

Transient elastography (Fibro-Scan<sup>®</sup>; EchoSens, Paris, France) is a rapid, reliable and well-tolerated imaging technique for the assessment of liver fibrosis by measuring liver stiffness.

### Peer review

This is an interesting and timely study on liver stiffness in patients with transfusion associated HCV. The authors show that HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness. Co-exposure to alcohol is critical. The methodology is sound and the paper is well and clearly written.

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## Opiate-induced constipation related to activation of small intestine opioid $\mu$ 2-receptors

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### Abstract

**AIM:** To investigate the role of opioid  $\mu$ -receptor subtype in opiate-induced constipation (OIC).

**METHODS:** The effect of loperamide on intestinal transit was investigated in mice. Ileum strips were isolated from 12-wk-old male BALB/c mice for identification of isometric tension. The ileum strips were precontracted with 1  $\mu$ mol/L acetylcholine (ACh). Then, decrease in muscle tone (relaxation) was characterized after cumulative administration of 0.1-10  $\mu$ mol/L loperamide into the organ bath, for a concentration-dependent study. Specific blockers or antagonists were used for pretreatment to compare the changes in loperamide-induced relaxation.

**RESULTS:** In addition to the delay in intestinal transit, loperamide produced a marked relaxation in isolated ileum precontracted with ACh, in a dose-dependent manner. This relaxation was abolished by cyprodime,

a selective opioid  $\mu$ -receptor antagonist, but not modified by naloxonazine at a dose sufficient to block opioid  $\mu$ -1 receptors. Also, treatment with opioid  $\mu$ -1 receptor agonist failed to modify the muscle tone. Moreover, the relaxation by loperamide was attenuated by glibenclamide at a dose sufficient to block ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels, and by protein kinase A (PKA) inhibitor, but was enhanced by an inhibitor of phosphodiesterase for cyclic adenosine monophosphate (cAMP).

**CONCLUSION:** Loperamide induces intestinal relaxation by activation of opioid  $\mu$ -2 receptors via the cAMP-PKA pathway to open  $K_{ATP}$  channels, relates to OIC.

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**Key words:** ATP-sensitive  $K^+$  channels; Isometric tension; Loperamide; Opioid  $\mu$ -receptors; Small intestine

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### INTRODUCTION

Opiate-induced constipation (OIC) is widely observed among patients receiving chemotherapy<sup>[1,2]</sup>. In the gastrointestinal system, the opioid peptides are released and activate opioid receptors, which regulate the enteric circuitry by controlling motility and secretion, resulting in an increase in sphincter tone, inhibition of gastric emptying, and induction of stationary motor patterns. Together with the inhibition of ion and fluid secretion, these effects result in constipation, one of the most troublesome

side effects of opiate analgesic treatment<sup>[3]</sup>. The development of a better therapy for treating OIC is urgent and necessary.

Loperamide is widely used clinically to treat a variety of diarrheal syndromes, including acute and nonspecific (infectious) diarrhea<sup>[4,5]</sup>. Loperamide is a peripheral agonist of opioid  $\mu$ -receptors with poor ability to penetrate the blood-brain barrier<sup>[6,7]</sup>. Some analgesic agents have been shown to have relaxant effects on smooth muscle<sup>[8,9]</sup>. (+)-Tramadol activates peripheral opioid  $\mu$ -receptors, inducing concentration-dependent relaxation of the aorta<sup>[10]</sup>. Opioid  $\mu$ -receptors are divided into three subtypes:  $\mu$ -1,  $\mu$ -2 and  $\mu$ -3<sup>[11]</sup>. The activation of opioid  $\mu$ -1 receptors has been reported to be associated primarily with the phospholipase C (PLC)-protein kinase C (PKC) pathway<sup>[12]</sup>. PLC-PKC signals can increase the intracellular calcium concentration, inducing gastrointestinal or bladder contraction<sup>[13,14]</sup>. Therefore, it is unlikely that intestinal relaxation is induced by the activation of opioid  $\mu$ -1 receptors.

ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels are involved in the regulation of intestinal smooth muscle<sup>[15]</sup>. In addition, the opening of  $K_{ATP}$  channels has been reported to reduce intracellular  $Ca^{2+}$  concentration<sup>[16-18]</sup>. The  $K_{ATP}$  channel opener diazoxide has been shown to have the ability to attenuate indomethacin-induced small intestinal damage in rats<sup>[19]</sup>. However, the role of  $K_{ATP}$  channels in loperamide-induced gastrointestinal transit remains obscure.

In an attempt to determine the subtype of opioid  $\mu$ -receptors involved in the regulation of intestinal tone, we used loperamide as an agonist to induce intestinal relaxation in the present study. In addition, specific blockers or antagonists were applied to investigate the potential mechanisms of action of loperamide.

## MATERIALS AND METHODS

### Experimental animals

We obtained 12-wk-old male BALB/c mice from the Animal Center of National Cheng Kung University Medical College. Mice were maintained in a temperature-controlled room ( $25 \pm 1$  °C) under a 12-h light-dark cycle (lights on at 06:00 h). All mice were given water and fed standard chow (Purina Mills, LLC, St Louis, MO, United States) *ad libitum*. All animal-handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the Animal Welfare Act.

### Gastrointestinal transit assay

Gastrointestinal tract (GIT) in mice was measured according to the method used in a previous study<sup>[20]</sup>. Briefly, 18 h before the experiment, food was withheld from the animals but free access to water was allowed. The mice received 0.25 mL of a suspension of charcoal consisting of 10% vegetable charcoal in 5% gum acacia (Sigma-Aldrich, St Louis, MO, United States) that was administered by an intragastric cannula. In subsequent experiments, the effects of loperamide or other compounds on GIT were evaluated 20 min after administration of the marker. At

that time, the animals were sacrificed, the stomach and small intestine removed and the omentum was separated, avoiding stretching. The length of the intestine from the pyloric sphincter to the ileocecal junction and the distance travelled by the charcoal front were measured and recorded. We also recorded the time at which the mice started to drain stool after the administration of the charcoal meal.

We evaluated the effects of loperamide, stevioside (an agonist of opioid  $\mu$ -1 receptor) or vehicle on GIT using subcutaneous injection into mice. Loperamide, stevioside and vehicle were given 30 min before the charcoal meal. The opioid antagonists cyprodime and naloxonazine were intraperitoneally injected at 60 min before the charcoal meal.

### Preparation of isolated ileum

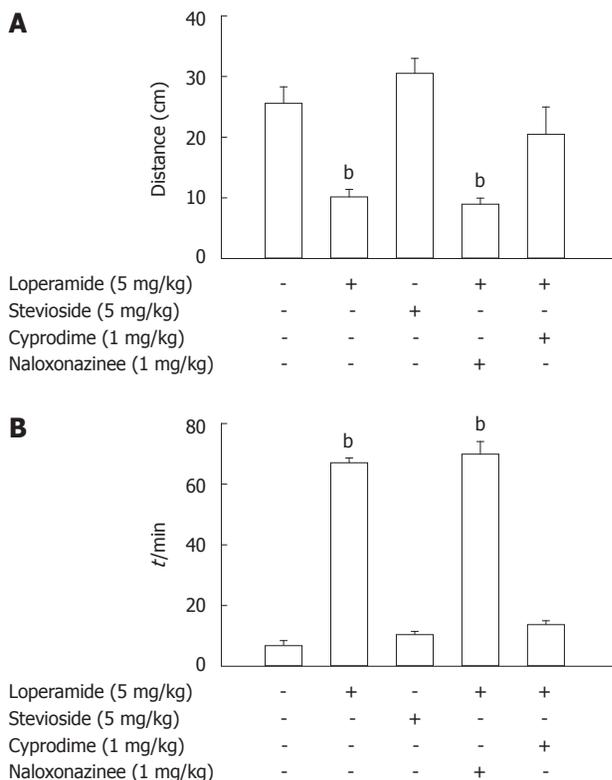
In the *in vitro* experiments, isolated ileum from BALB/c mice was used. Each mouse was killed by decapitation under anesthesia with pentobarbital (50 mg/kg). After the ileum strips had been carefully freed from the fat and connective tissue, the strips were mounted in organ baths filled with 10 mL oxygenated Krebs' buffer (95%  $O_2$ , 5%  $CO_2$ ) at 37 °C containing: 135 mmol/L NaCl; 5 mmol/L KCl; 2.5 mmol/L  $CaCl_2$ ; 1.3 mmol/L  $MgSO_4$ ; 1.2 mmol/L  $KH_2PO_4$ ; 20 mmol/L  $NaHCO_3$ ; and 10 mmol/L D-glucose (pH 7.4). Each preparation was connected to strain gauges (FT03; Grass Instruments, Quincy, MA, United States). The isometric tension was recorded using chart software (MLS023, Powerlab; ADInstruments, Bella Vista, NSW, Australia). Strips were mounted and allowed to stabilize for 2 h. Each preparation was then gradually stretched to achieve an optimal resting tension of 0.5 g.

### Intestinal relaxation induced by loperamide

After the resting tension had stabilized, a solution of acetylcholine (ACh; Sigma-Aldrich) prepared in distilled water was added to the bathing buffer to induce a rapid increase in ileum tone followed by stable constriction (tonic contraction). The final ACh concentration in the organ bath was 1  $\mu$ mol/L. Ileum strips in the treatment group were exposed to loperamide (0.1-10  $\mu$ mol/L) to observe the decrease in the tonic contraction (ileum relaxation). In addition, stevioside, an opioid  $\mu$ -1 receptor agonist<sup>[21]</sup>, was also used to investigate the effect on tonic contraction. Relaxation was expressed as the percentage decrease in the maximum tonic contraction. Concentration-relaxation curves were generated in a cumulative fashion.

### Effects of antagonists on loperamide-induced intestinal relaxation

Ileum strips were exposed to glibenclamide (Research Biochemicals, Wayland, MA, United States), a specific opioid  $\mu$ -1 receptor antagonist (naloxonazine) or a general opioid  $\mu$  receptor antagonist (cyprodime) (Tocris Cookson, Bristol, United Kingdom), for 15 min before addition of loperamide to the organ bath. The strips were treated with an inhibitor of cyclic adenosine monophosphate (cAMP) phosphodiesterase (3-isobutyl-1-methylxanthine; IBMX) or an inhibitor of protein kinase A (PKA) (H-89)



**Figure 1** Role of opioid  $\mu$ -receptors in gastrointestinal tract using charcoal as an indicator. The data represent the distance (A) and time (B) for the transit of charcoal. Data represent the mean  $\pm$  SEM of eight animals. <sup>b</sup> $P < 0.01$  vs the distilled water (vehicle)-treated control.

in the same manner. Forskolin (Sigma-Aldrich) was used as a control. The changes in the relaxation caused by antagonists or blockers were compared with those of the vehicle-treated control.

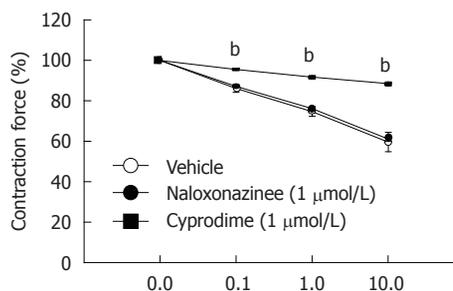
### Statistical analysis

All values are presented as the mean  $\pm$  SEM for a given number of animals or samples. Analysis of variance and Dunnett's post hoc test were used to evaluate the significance between groups.  $P < 0.05$  was considered significant.

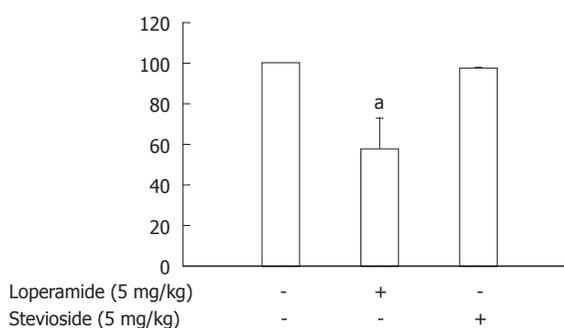
## RESULTS

### Role of opioid receptor in loperamide-induced gastrointestinal transit

As shown in Figure 1A, the distance travelled by charcoal in the loperamide-treated group (5 mg/kg) was shorter than that in the vehicle-treated group. However, the distance travelled in the stevioside-treated (5 mg/kg) group was similar to that in the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) significantly abolished the effect of loperamide on GIT, but naloxonazine (1 mg/kg) failed to produce the same effect. Moreover, the time for transit of charcoal from the stomach to the anus stool drain in the loperamide-treated group (5 mg/kg) was longer than that in the vehicle-treated group. The transit time of the stevioside-treated (5 mg/kg) group was the same as that of the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) at-



**Figure 2** Inhibitory effect of cyprodime or naloxonazine on relaxation induced by loperamide (10  $\mu$ mol/L) in isolated ileum contracted with 1  $\mu$ mol/L acetylcholine. Data represent the mean  $\pm$  SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. <sup>b</sup> $P < 0.01$  vs the distilled water (vehicle)-treated control.



**Figure 3** Effect of stevioside on the tone of isolated ileum strips contracted with 1  $\mu$ mol/L acetylcholine. Data represent the mean  $\pm$  SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. <sup>a</sup> $P < 0.05$  vs the distilled water (vehicle)-treated control, shown in the first column.

tenuated the loperamide-induced delay in charcoal transit, but naloxonazine (1 mg/kg) failed to exhibit the same action (Figure 1B).

### Effect of opioid receptor blockade on loperamide-induced intestinal relaxation

Ileum strips strongly contracted in response to the application of ACh at 1  $\mu$ mol/L. As shown in Figure 2, loperamide relaxed the ACh-contracted ileum strips in a concentration-dependent manner. At the maximum concentration tested (10  $\mu$ mol/L), loperamide significantly attenuated the tonic contraction of ileum strips to 63.59%  $\pm$  5.60% of the contraction induced by ACh. Cyprodime (1  $\mu$ mol/L) produced a marked attenuation of the relaxation induced by loperamide. However, naloxonazine failed to modify the action of loperamide, even at a higher concentration (1  $\mu$ mol/L). In addition, treatment with stevioside at a dose sufficient to activate the opioid  $\mu$ -1 receptor<sup>[21]</sup> failed to modify the intestinal tone in ACh-contracted ileum (Figure 3).

### Role of cyclic adenosine monophosphate and protein kinase A in loperamide-induced intestinal relaxation

In the present study, forskolin (5  $\mu$ mol/L), a direct activator of adenylate cyclase, was used as a positive control to increase the activity of cAMP, based on the findings of a previous study<sup>[22]</sup>. In ileum strips contracted with

**Table 1** Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation

	ACh (%)
Loperamide (10 $\mu\text{mol/L}$ )	
+ Vehicle	63.59 $\pm$ 5.60
+ H-89 (1 $\mu\text{mol/L}$ )	80.49 $\pm$ 3.07 <sup>a</sup>
+ IBMX (10 $\mu\text{mol/L}$ )	41.02 $\pm$ 2.57 <sup>b</sup>
+ Glibenclamide (1 $\mu\text{mol/L}$ )	83.52 $\pm$ 0.89 <sup>a</sup>
Forskolin (5 $\mu\text{mol/L}$ )	
+ Vehicle	31.64 $\pm$ 7.39
+ H-89 (1 $\mu\text{mol/L}$ )	79.29 $\pm$ 2.76 <sup>b</sup>
+ IBMX (10 $\mu\text{mol/L}$ )	27.77 $\pm$ 1.40 <sup>b</sup>
+ Glibenclamide (1 $\mu\text{mol/L}$ )	80.98 $\pm$ 2.75 <sup>b</sup>
IBMX (10 $\mu\text{mol/L}$ )	93.41 $\pm$ 2.15 <sup>b</sup>
H-89 (1 $\mu\text{mol/L}$ )	91.31 $\pm$ 3.47 <sup>b</sup>
Glibenclamide (1 $\mu\text{mol/L}$ )	92.45 $\pm$ 3.29 <sup>b</sup>

Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation induced by loperamide (10  $\mu\text{mol/L}$ ) or forskolin (5  $\mu\text{mol/L}$ ) in isolated ileum contracted with 1  $\mu\text{mol/L}$  acetylcholine (ACh). IBMX: 3-isobutyl-1-methylxanthine. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs vehicle treated control.

ACh (1  $\mu\text{mol/L}$ ), forskolin-induced relaxation was also abolished by pretreatment with glibenclamide (1  $\mu\text{mol/L}$ ). Moreover, intestinal relaxation induced by forskolin was increased by the addition of IBMX at a concentration (10  $\mu\text{mol/L}$ ) sufficient to inhibit cAMP-phosphodiesterase<sup>[23]</sup>, and was decreased by addition of H-89 at a concentration (1  $\mu\text{mol/L}$ ), which was sufficient to inhibit the activity of PKA<sup>[24]</sup>. The loperamide-induced intestinal relaxation was also modified by these agents in the same manner. Our results showed that intestinal relaxation induced by loperamide was increased by IBMX and attenuated by H-89 (Table 1).

## DISCUSSION

In the present study, we found that loperamide caused a dose-dependent delay in GIT using charcoal as an indicator in mice. In addition, loperamide induced relaxation in the ileum strips contracted with stimulant. This action of loperamide seems to be related primarily to the activation of opioid receptors in peripheral tissue, because loperamide does not cross into the central nervous system<sup>[7]</sup>. The loperamide-induced action was effectively abolished by cyprodime, suggesting that opioid  $\mu$  receptors were involved. However, this action of loperamide was not reversed by naloxonazine even at a dose sufficient to block opioid  $\mu$ -1 receptors. In addition, as shown in Figure 2, relaxation was not induced by stevioside, which is an agonist specific for opioid  $\mu$ -1 receptors<sup>[21]</sup>. The involvement of opioid  $\mu$ -1 receptors in the intestinal relaxation mechanism of loperamide seems unlikely.

Thus, another opioid  $\mu$  receptor must be involved in this action of loperamide. There is no doubt that loperamide is an agonist of peripheral opioid  $\mu$  receptors<sup>[7,25]</sup>. Opioid  $\mu$  receptors have been divided into three subtypes<sup>[11]</sup>:  $\mu$ -1,  $\mu$ -2 and  $\mu$ -3<sup>[26-28]</sup>. The analgesic action mediated by the activation of opioid  $\mu$ -1 receptors has been report-

ed to exert spinal antinociception<sup>[29,30]</sup>. In addition, the activation of opioid  $\mu$ -1 receptors seems to be related to smooth muscle contraction *via* the PLC-PKC pathway<sup>[14,31]</sup>. Moreover, opioid  $\mu$ -3 receptors are present predominantly in endothelial cells associated with the production of nitric oxide to induce vasodilatation<sup>[32]</sup>. Therefore, the involvement of opioid  $\mu$ -1 or  $\mu$ -3 receptors in intestinal relaxation seems unlikely. Taken together, our results suggest that the activation of opioid  $\mu$ -2 receptors is more likely to participate in the action of loperamide with respect to intestinal relaxation. The activation of opioid  $\mu$ -2 receptors has been reported to be involved in the relaxation of guinea pig ileum and in the inhibition of GIT<sup>[33,34]</sup>. In addition, opioid  $\mu$ -receptor-expressing myenteric neurons are distributed primarily in the small intestine, followed by the stomach and the proximal colon<sup>[35]</sup>. Although constipation is predominantly a large bowel disorder, the presence of opioid  $\mu$  receptors in the colon and the longer GIT time of charcoal to the anus stool drain in mice that received loperamide support the role of opioid  $\mu$ -2 receptors in opiate-induced constipation. Unfortunately, there is no suitable tool or agent that can be used to provide further evidence supporting this hypothesis. Therefore, we focused on the subcellular signals as an alternative experimental approach.

Potassium channels play an important role in the regulation of intestinal smooth muscle cells<sup>[36,37]</sup>. ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels are composed of four inwardly rectifying  $\text{K}^+$  channel subunits and four regulatory sulfonylurea receptors<sup>[38]</sup>. The activation of  $\text{K}_{\text{ATP}}$  channels induces hyperpolarization of the cell membrane and consequently relaxes the smooth muscle. Thus, we focused on the involvement of  $\text{K}_{\text{ATP}}$  channels in the intestinal relaxation induced by loperamide. We used forskolin as a positive control because forskolin is a direct activator of adenylate cyclase that can increase intracellular cAMP concentration to activate cAMP-dependent PKA, resulting in the opening of  $\text{K}_{\text{ATP}}$  channels<sup>[24]</sup>. As shown in Table 1, we observed that forskolin-induced intestinal relaxation was also blocked by glibenclamide. The intestinal relaxation induced by forskolin was abolished by H-89 at a concentration sufficient to block the activity of PKA<sup>[24]</sup> and was enhanced by IBMX at a concentration sufficient to inhibit the activity of cAMP-phosphodiesterase<sup>[23]</sup>. Similar changes were also observed in ileum strips relaxed by loperamide (Table 1). These data suggest that the potential mechanism responsible for loperamide-induced intestinal relaxation is mediated *via* the cAMP-PKA pathway to open  $\text{K}_{\text{ATP}}$  channels. Therefore, the results provide a novel insight into the mechanism of action of loperamide and increase our understanding of intestinal relaxation. It is reasonable to consider that similar results will be obtained for the parts of the colon that have opioid  $\mu$  receptors. Further investigations are required in the future.

In conclusion, we suggest that the activation of opioid  $\mu$ -2 receptors, which induce the opening of  $\text{K}_{\text{ATP}}$  channels, is responsible for loperamide-induced intestinal relaxation. Therefore, peripheral opioid  $\mu$ -2 receptors will

be a new target in the development of agents for treating OIC.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Opioid-induced constipation (OIC) is a frequent disorder in tumor patients receiving morphine-like compounds. Thus, it is important to prevent this disorder. To date, it is still unclear which subtype of opioid receptors should be used for development of suitable agents. Loperamide is a well-known agonist of opioid receptors, without the ability to enter the brain. Many studies have reported that loperamide can be used to treat diarrhea, but the receptor site has not been established.

### Research frontiers

Loperamide is a widely used agent in clinics and its effectiveness is believed to arise from peripheral action. In the area of prevention of constipation with loperamide, the research hotspot is how to distinguish the receptor subtype to improve its adverse reactions. Then, it will be useful for prevention of OIC.

### Innovations and breakthroughs

In previous studies of loperamide for treatment of diarrhea, it was found that intestinal motility was significantly decreased. In order to decrease the side effects of loperamide, the authors investigated the receptor subtype that is selectively activated, and the results will be useful for the development of new agents with similar side effects. Thus, the authors compared loperamide with stevioside, which is mainly effective against opioid  $\mu$ -1 receptors. We found that opioid  $\mu$ -2 receptors linked with ATP-sensitive K<sup>+</sup> channels are responsible for intestinal relaxation. Therefore, agents with less effect on opioid  $\mu$ -2 receptors will be useful to decrease the side effects of constipation.

### Applications

The results suggest that opioid  $\mu$ -2 receptors are mainly responsible for intestinal relaxation. Clinical application of agents showing less affinity than loperamide to opioid  $\mu$ -2 receptors could be useful for prevention of constipation.

### Terminology

OIC is a frequent side effect in cancer patients who received morphine-like compounds to reduce pain. Loperamide is a widely used agent for treatment of diarrhea in clinics, and it has no effect in the brain. Opioid receptors are the action site of opioids and related agents. Receptors are generally expressed in various tissues and located on the cell membrane. Subtypes of opioid receptors have been established.

### Peer review

This is a good descriptive study in which the authors analyzed the subtype of opioid  $\mu$  receptors in the intestine of mice. The results are interesting and suggest that opioid  $\mu$ -2 receptors are responsible for intestinal relaxation, which could be useful in preventing constipation by agents with less affinity for this receptor site.

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## Notch3 regulates the activation of hepatic stellate cells

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### Abstract

**AIM:** To investigate whether Notch signaling is involved in liver fibrosis by regulating the activation of hepatic stellate cells (HSCs).

**METHODS:** Immunohistochemistry was used to detect the expression of Notch3 in fibrotic liver tissues of patients with chronic active hepatitis. The expression of Notch3 in HSC-T6 cells treated or not with transforming growth factor (TGF)- $\beta$ 1 was analyzed by immunofluorescence staining. The expression of Notch3 and myofibroblastic marker  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I in HSC-T6 cells transfected with pcDNA3.1-N3ICD or control vector were detected by Western blotting and immunofluorescence staining. Moreover, effects of Notch3 knockdown in HSC-T6 by Notch3 siRNA were investigated by Western blotting and immunofluorescence staining.

**RESULTS:** The expression of Notch3 was significantly up-regulated in fibrotic liver tissues of patients with

chronic active hepatitis, but not detected in normal liver tissues. Active Notch signaling was found in HSC-T6 cells. TGF- $\beta$ 1 treatment led to up-regulation of Notch3 expression in HSC-T6 cells, and over-expression of Notch3 increased the expression of  $\alpha$ -SMA and collagen I in HSC-T6 without TGF- $\beta$ 1 treatment. Interestingly, transient knockdown of Notch3 decreased the expression of myofibroblastic marker and antagonized TGF- $\beta$ 1-induced expression of  $\alpha$ -SMA and collagen I in HSC-T6.

**CONCLUSION:** Notch3 may regulate the activation of HSCs, and the selective interruption of Notch3 may provide an anti-fibrotic strategy in hepatic fibrosis.

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**Key words:** Notch signaling; Myofibroblast; Liver fibrosis; Hepatic stellate cells; siRNA

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Chen YX, Weng ZH, Zhang SL. Notch3 regulates the activation of hepatic stellate cells. *World J Gastroenterol* 2012; 18(12): 1397-1403 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i12/1397.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i12.1397>

### INTRODUCTION

Hepatic fibrosis is a reversible wound-healing response characterized by the accumulation of extracellular matrix (ECM) to liver injury<sup>[1]</sup>. In the process of hepatic fibrosis, activated hepatic stellate cells (HSCs) synthesize a large amount of ECM and then change into myofibroblasts<sup>[2]</sup>, which is characterized by the expression of  $\alpha$ -smooth

muscle actin ( $\alpha$ -SMA) and ECM, particularly collagen I. Myofibroblast is one of the key cellular components involved in liver fibrosis, therefore, the majority of anti-fibrotic therapies are designed to inhibit the activation, proliferation, or synthetic products of HSCs<sup>[3]</sup>.

Notch signaling is an ancient cell signaling that regulates cell fate specification, stem cell maintenance, and initiation of differentiation in embryonic and postnatal tissues<sup>[4,5]</sup>. More recently, some researches reported that Notch signaling was implicated in human fibrosis diseases, such as pulmonary, renal and peritoneal fibrosis<sup>[6-8]</sup>. Several researches suggested that the Jagged/Notch pathway may selectively mediate fibrogenic properties of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) which was essential to promote the production and deposition of ECM<sup>[9-11]</sup>.

Notch receptors (Notch1, Notch2 and Notch4) were present at the mRNA level in freshly isolated HSCs, and the synthesis of Notch1 decreased during culture and development of HSCs into myofibroblast-like cells<sup>[12]</sup>. However, the expression of Notch3 in phenotype activated HSCs remains unknown. Ono *et al.*<sup>[13]</sup> reported that Notch3 was required for TGF- $\beta$ 1-induced myofibroblastic differentiation of myoblasts. Based on the studies above, the present study was undertaken to investigate whether Notch3 was expressed in fibrotic liver tissues of patients with chronic active hepatitis and in activated HSCs, and sequentially contributed to liver fibrosis by regulating the activation of HSCs.

## MATERIALS AND METHODS

### Patients and liver biopsy samples

Liver tissue samples were obtained by biopsy from 11 patients with chronic active hepatitis (5 women and 6 men; median age 43 years, range 31-55 years). Control liver biopsy specimens were obtained from healthy volunteers ( $n = 6$ ). All patients and controls signed consent forms approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Tissue samples were fixed in 10% formalin and paraffin-embedded for immunohistochemical analysis.

### Antibodies and reagents

Rabbit polyclonal to  $\alpha$ -SMA and Notch3 were obtained from Abcam (Cambridge, United States). Rabbit polyclonal anti-collagen I antibody was purchased from Bioss Corporation (Beijing, China). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, United States). Recombinant human transforming growth factor (TGF)- $\beta$ 1 was purchased from PeproTech EC Ltd (London, United Kingdom). Lipofectamine™ 2000 transfection reagent was obtained from Invitrogen (Carlsbad, United States).

### Cell line and culture conditions

HSC-T6 cells, an immortalized rat HSC line, purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (China), were cultured in Dulbecco's

-modified Eagle's medium (DMEM; Gibco, United States) supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% fetal bovine serum (FBS) (Gibco, United States). TGF- $\beta$ 1 (2 ng/mL) was incubated in growth medium for 24 h.

### Immunohistochemistry and immunocytofluorescence analysis

Liver tissue sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> followed by serum blocking with 10% goat serum in 5% bovine serum albumin (BSA). The Notch3 was detected by staining with polyclonal rabbit anti-Notch3 (1:250) overnight at 4 °C. Irrelevant isotype antibodies (Santa Cruz Biotechnology, United States) at the same concentration were used as control. The staining was carried out using SABC kit (Boster, China).

Cells were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde at room temperature for 30 min and were penetrated in blocking solution (Amresco, United States) containing 0.3% Triton X-100, and incubated overnight with either anti-Notch3 (1:250), anti- $\alpha$ -SMA (1:100) or anti-collagen I (1:100) antibody in 1% BSA solution. Then, cells were incubated with secondary antibodies for 1 h at 37 °C. After incubation, cells were stained with nuclear stain marker 4',6-diamidino-2-phenylindole. The reaction was examined under confocal microscope (Nikon, Japan).

### Transfection of siRNA and plasmid

HSC-T6 was seeded into 6-well plates at a density of  $1 \times 10^5$  cells 12 h before transfection. siRNA was mixed with 5  $\mu$ L lipofectamine 2000 in 250  $\mu$ L Opti-MEM I medium for 20 min. The transfection mixture was then added to each well with 1.5 mL FBS free DMEM at a concentration of 100 nmol/L. After 6-h incubation, liquid mixture containing siRNA was disposed. Two mL DMEM containing 10% FBS was added and incubated for another 72 h. The following siRNA sequences were used: Notch3 siRNA: 5' ACAAGAUCAAUACAGGAGCTT 3'; the control siRNA sequence: 5' UUGUAC UACACAAAAGUACUG 3'. These siRNA were synthesized by Shanghai Genepharma Co. Ltd. (Shanghai, China).

HSC-T6 was transfected with Notch3 intracellular domain (Notch3-ICD) cDNA cloned into pcDNA3.1 (pcDNA3.1-N3ICD) vector, which was a gift from Dr. Tao Wan. The control (pcDNA3.1-empty) vector was purchased from Shanghai Genepharma Co. Ltd. (Shanghai, China). All transfections were performed using Lipofectamine 2000 following the manufacturer's instructions.

### TaqMan quantitative reverse transcription polymerase chain reaction

Total RNA from each sample was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed using a StepOne/StepOne-Plus (ABI) and the TaqMan PCR Reagent (Genepharma). Primer sequences are summarized in Table 1. Comparative threshold (Ct)

**Table 1** Primer sequences for TaqMan real-time reverse transcription polymerase chain reaction

Notch3	
Forward	5'-CCTGCCTGCCTCTATGACAAC-3'
Reverse	5'-ACACTCCTCGGTGTTACAGCC-3'
Probe	5'-ACTGCTACTCTGGTGGCCCGAC-3'
Jagged1	
Forward	5'-GTGGAAGAGGATGATATGGATAAGC-3'
Reverse	5'-CTCCTCTCTGTCTACCAGCGTGTAC-3'
Probe	5'-CCAGCAGAAAGTCCGGTTTGCCA-3'
Hes1	
Forward	5'-TGCTACCCAGCCAGTGTC-3'
Reverse	5'-GCTTTGATGACTTTCTGTGCTCA-3'
Probe	5'-CIGTCTTTGGTTTGTCGGGTGTCGT-3'
GAPDH	
Forward	5'-GATGACATCAAGAAGGTGGTGAAG-3'
Reverse	5'-ACCTGTGCTGTAGCCATATTC-3'
Probe	5'-ACTCAACAGCAACTCCCACTCTCCACC-3'

method was used for calculating the relative amount of mRNA of treated sample compared with control samples.

### Western blotting assay

Cells were washed with PBS and lysed. The extracts were cleared by centrifugation at  $12\,000 \times g$  for 15 min. After blocking with 5% non-fat milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with either anti-Notch3 (1:1000), anti- $\alpha$ -SMA (1:300) or anti-collagen I (1:200) antibody in tris-buffered saline (TBS) containing 0.05% Tween 20 at 4 °C overnight. Then membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (1:2000) antibody in TBS and Tween 20 for 1 h at room temperature, and visualized by chemiluminescence using an electrochemiluminescence immunoblotting kit (Cell Signaling Technology) with a digital luminescent image analyzer Bio-Spectrum600 (UVP, United States). Band intensity was assessed using Gel-Pro analyzer.

### Statistical analysis

All experiments were repeated three times, and data recorded as mean  $\pm$  SD and analyzed by Student's *t* test using SPSS12.0 software.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Expression of Notch3 in fibrotic liver tissues of patients with chronic active hepatitis

All patients were positive for the Notch3 in fibrotic liver tissues (Figure 1A). In contrast, Notch3 was not detected by immunohistochemistry in normal liver tissues (Figure 1C).

### Expression of Notch3 in hepatic stellate cell-T6 cells

To detect expression of Notch3 in HSCs, immunofluorescence staining analysis was performed to examine the expression of Notch3 in HSC-T6 cells. The result showed that Notch3 protein was localized in the cytoplasm and nucleus of HSC-T6 cells (Figure 2).

### Up-regulation of Notch3 expression by transforming growth factor- $\beta$ 1 in hepatic stellate cell-T6 cells

We investigated the effect of TGF- $\beta$ 1 on Notch signaling in HSC-T6 treated with TGF- $\beta$ 1 (0.5, 1, 2 and 4 ng/mL) for 24 h. RT-PCR analysis showed that the expression of Notch signaling components including Notch3, Jagged1 and Hes-1 were obviously increased in HSC-T6 treated with 2 ng/mL TGF- $\beta$ 1 as compared with the control group without TGF- $\beta$ 1 treatment ( $P < 0.05$ , Figure 3).

### Over-expression of Notch3 increased the expression of myofibroblastic marker in hepatic stellate cell-T6

To investigate the effect of Notch3 in activation of HSCs, we examined if overexpression of Notch3 in HSC-T6 would enhance the activation. The results showed that the increased expression of Notch3 in pcDNA3.1-N3ICD introduced HSC-T6 cells as compared with cells transfected with pcDNA3.1-empty vector ( $P < 0.05$ , Figure 4A). Western blotting and immunofluorescence staining analyses demonstrated that over-expression of Notch3 led to increased expression of  $\alpha$ -SMA and collagen I compared with control group ( $P < 0.05$ , Figure 4A and B).

### Knockdown of Notch3 by siRNA downregulated the expression of myofibroblastic marker in hepatic stellate cell-T6

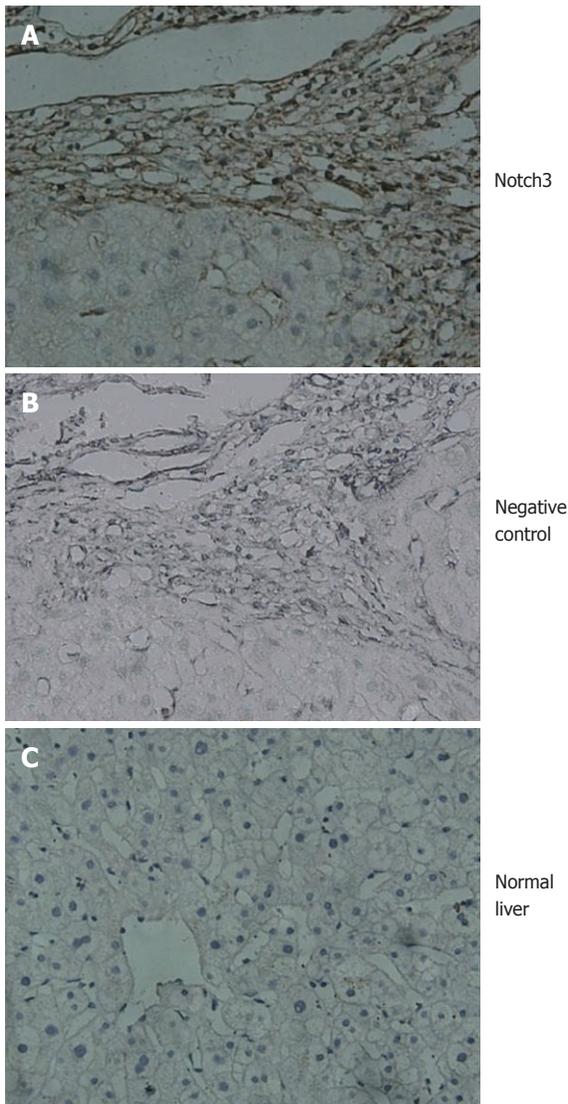
To further confirm the role of Notch3 in regulating activation of HSCs, siRNA was employed to specifically knockdown Notch3. Western blotting analysis showed that siRNAs targeting Notch3 reduced Notch3 protein levels by approximately 80%. Seventy and two hour after transfection in HSC-T6 ( $P < 0.05$ , Figure 5A). We also observed that knockdown of Notch3 in HSC-T6 down-regulated the expression of  $\alpha$ -SMA and collagen I detected by Western blotting and immunofluorescence staining 72 h after siRNA transfection ( $P < 0.05$ , Figure 5A and B).

To investigate the relationship between Notch3 and TGF- $\beta$ 1, TGF- $\beta$ 1 (2 ng/mL) was added into HSC-T6 24 h before transfection with siRNAs targeting Notch3 or control siRNAs. Western blotting and immunofluorescence staining analyses demonstrated that knockdown of Notch3 antagonized TGF- $\beta$ 1-induced expression of  $\alpha$ -SMA and collagen I in HSC-T6 ( $P < 0.05$ , Figure 5A and B).

## DISCUSSION

Liver fibrosis is the result of the wound-healing response to repeated injury in liver. It is well known that HSCs activation plays an important role in fibrosis because these cells become the primary source of extracellular matrix in liver upon injury. TGF- $\beta$ 1 is known to promote fibrogenesis *in vivo* and *in vitro*, however, development of anti-fibrotic strategies targeting the TGF- $\beta$  axis is problematic owing to the pleiotropic nature of TGF- $\beta$  action<sup>[1]</sup>.

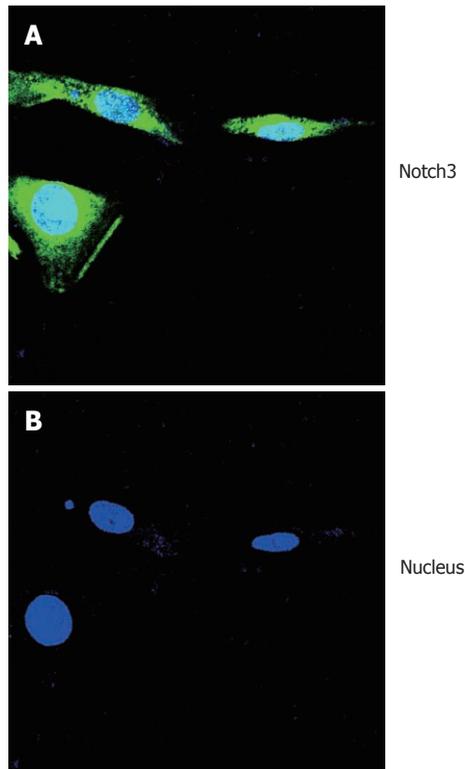
Notch signaling is an evolutionarily conserved local cell-signaling that functions in the determination of cellular identity during developmental stages<sup>[14]</sup>. Four Notch proteins (Notch1, Notch2, Notch3 and Notch4) have



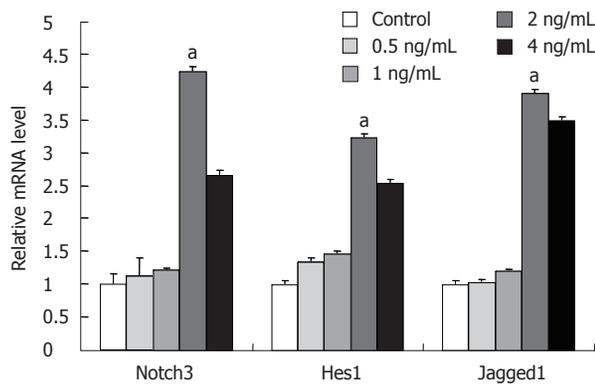
**Figure 1** Immunohistochemical staining of Notch3 in liver tissues of patients with chronic active hepatitis and normal livers. A: Intense staining of Notch3 in fibrotic tissues of livers from patients with chronic active hepatitis; B: Negative control; C: Notch3 was not detected in normal liver tissues (x 400).

been identified in vertebrates, while membrane-bound proteins (Delta and Jagged) have been recognized as Notch ligands<sup>[15]</sup>. Activation of the pathway usually occurs via expression of the ligand in signal-giving cells. Upon interaction with the ligand, Notch undergoes a series of proteolytic cleavages in the signal-receiving cells. Finally, the cytoplasmic domain, referred to as the Notch intracellular domain (NICD), translocates to the nucleus, where it binds with the transcription factor CSL [CBF-1/RBP-Jk, Su (H) and Lag-1] and co-activator Mastermind-like to trigger downstream target genes expression, such as HES1 and HEY which act as transcription factors<sup>[16-20]</sup>.

Notch signaling is essential for the regulation of cell differentiation, and its aberrant activation was implicated in human fibrosis diseases. Notch1 signaling in response to inflammatory zone 1 may play a significant role in myofibroblast differentiation during lung fibrosis<sup>[6]</sup>. Active Notch pathway in tubular epithelial cells was demonstrat-



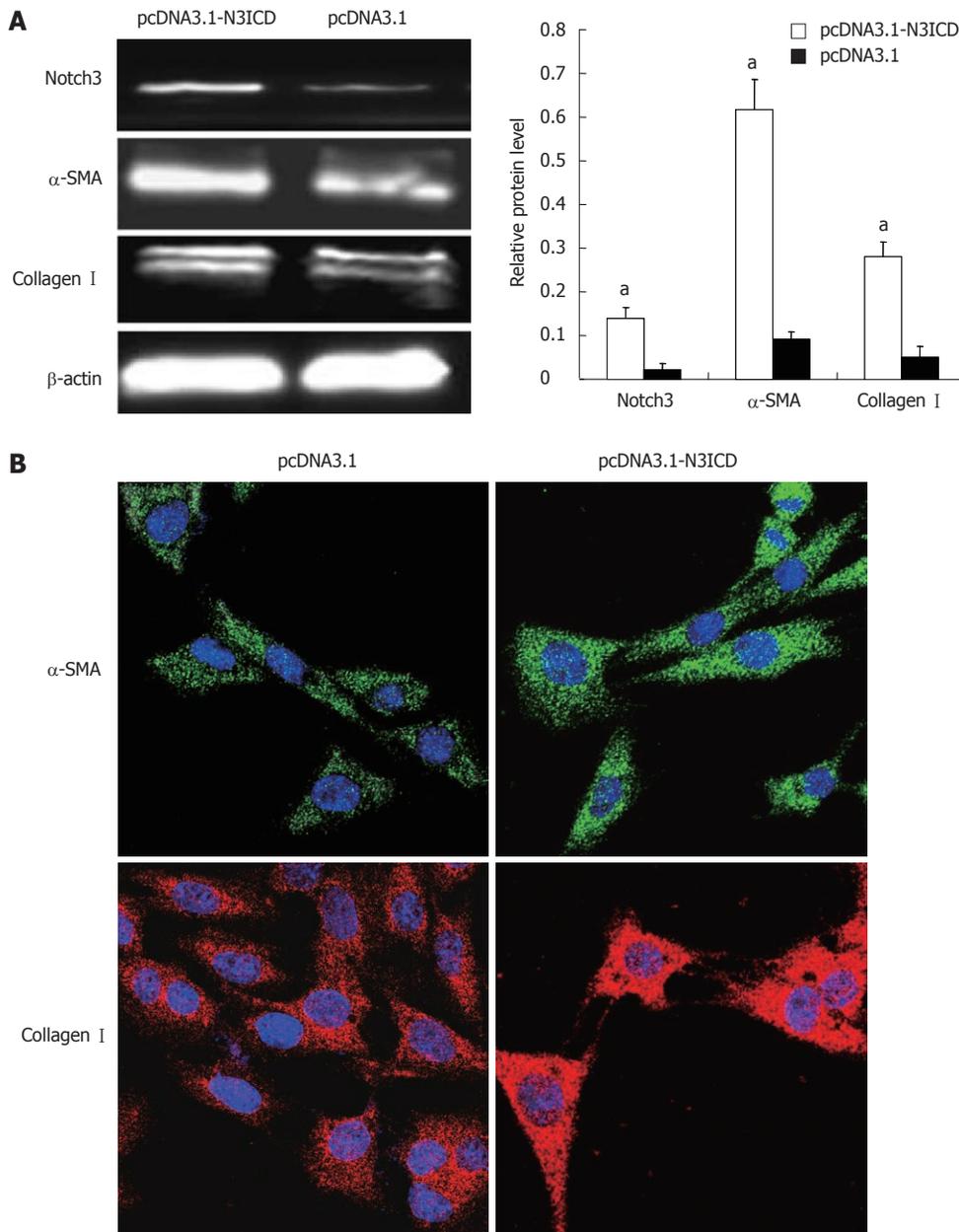
**Figure 2** Immunofluorescence staining analysis was performed to examine expression of Notch3 in hepatic stellate cell-T6 cells. A: The green fluorescence represents the expression of Notch3; B: Blue one represents nucleus of hepatic stellate cell-T6 cells (x 400).



**Figure 3** TaqMan reverse transcription polymerase chain reaction analysis was performed to detect expression of Notch3, Jagged1 and Hes1 in hepatic stellate cell-T6 cells treated or not with transforming growth factor- $\beta$ 1 (0.5, 1, 2 and 4 ng/mL) for 24 h. <sup>a</sup>*P* < 0.05 vs control group.

ed as a critical regulator of tubulointerstitial fibrosis<sup>[7]</sup>. It was reported that Notch signaling was highly activated in rats with fibrotic peritoneum induced by peritoneal dialysis fluid, as indicated by increased expression of Jagged1, Notch1, and HES1. Blocking Notch signaling activation by intraperitoneal injection of a  $\gamma$ -secretase inhibitor significantly attenuated peritoneal fibrosis as indicated by the decreased expression of  $\alpha$ -smooth muscle actin and collagen I<sup>[8]</sup>.

In this study, the Notch3 was not detected in normal liver tissues. In contrast, intense staining of the Notch3

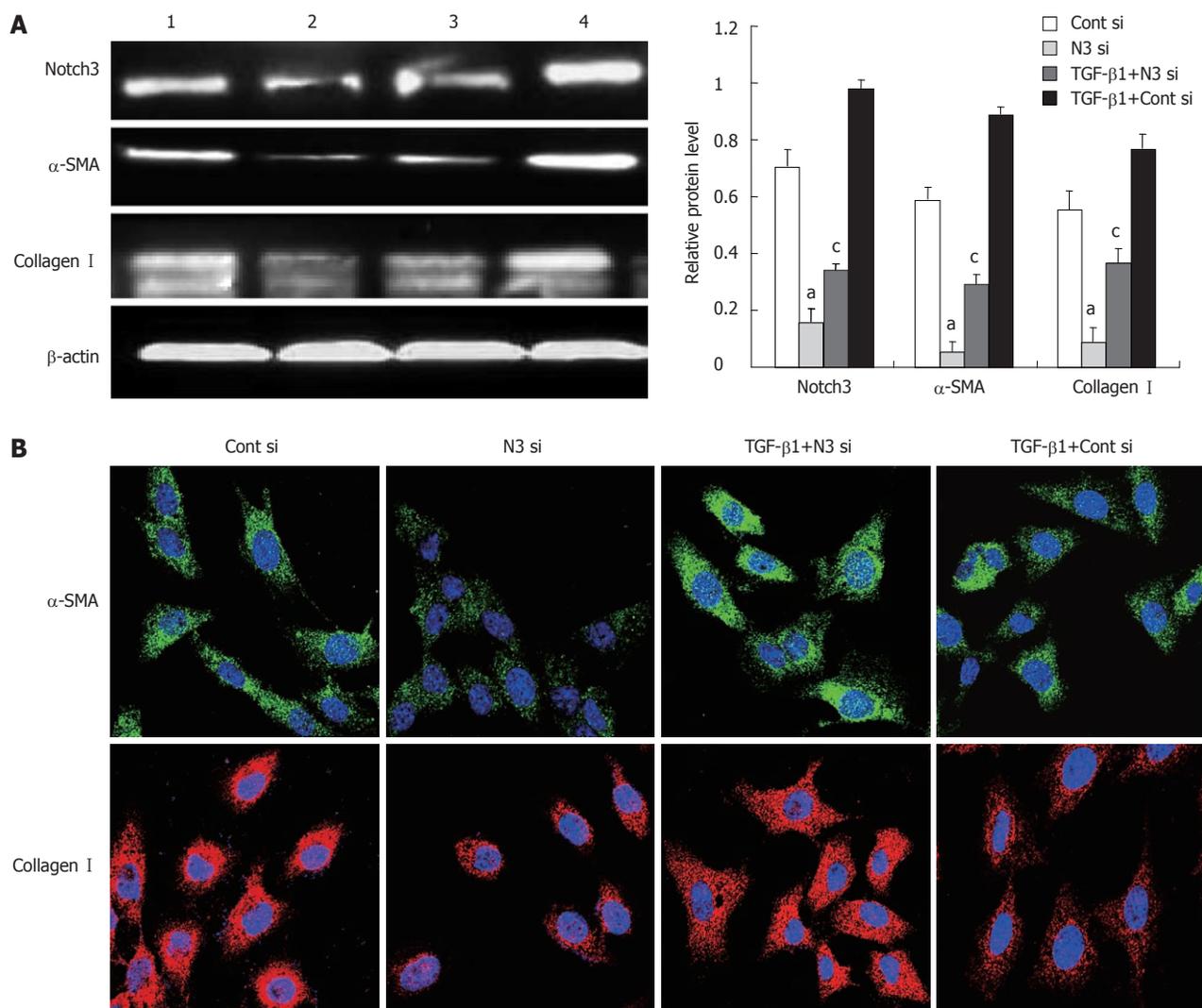


**Figure 4** Over-expression of Notch3 intracellular domain increased the expression of myofibroblastic marker in hepatic stellate cell-T6. A: The expression of Notch3,  $\alpha$ -smooth muscle actin (SMA) and collagen I were detected by Western blotting; B: The expression of  $\alpha$ -SMA and collagen I was also detected by immunofluorescence staining (x 400), <sup>a</sup> $P < 0.05$  vs control group.

was observed in fibrotic liver tissues of patients with chronic active hepatitis, which suggested that Notch3 was involved in liver fibrosis. Furthermore, several lines of evidence clearly confirmed that Notch3 contributed to liver fibrosis by regulating activation of HSCs. First, the expression of active Notch3 was found in the nucleus of HSC-T6. Second, the expression level of Notch signaling components was elevated including Notch3, Jagged1 and Hes1 in HSC-T6 after TGF- $\beta$ 1 treatment. More importantly, we also found that specific knockdown of Notch3 by siRNA antagonized TGF- $\beta$ 1-induced expression of myofibroblastic marker  $\alpha$ -SMA and collagen I in HSC-T6, and on the contrary, over-expression of Notch3 increased the expression of myofibroblastic marker in HSC-T6.

Inhibition of the  $\gamma$ -secretase complex required for release of the active NICD is the most common method for targeting Notch signaling<sup>[21,22]</sup>. Two different inhibitors are being evaluated in clinical trials for the treatment of resistant T cell acute leukemia and advanced breast cancer (www.clinicaltrials.gov). However, targeting of  $\gamma$ -secretase would result in several adverse events when Notch receptors from Notch1 to Notch4 are affected<sup>[23,24]</sup>. In this study, we found that the selective interruption of Notch3 by siRNA decreased the expression of myofibroblastic marker in HSC-T6 cells, which provided a potential novel therapeutic target for liver fibrosis.

However, further studies are required to elucidate how do other members of Notch family, such as Notch1, Notch2 and Notch4, play a part in activated HSCs as



**Figure 5** Knockdown of Notch3 decreased the secretion of myofibroblastic marker in hepatic stellate cell-T6. A: Effects of Notch3 knockdown in HSC-T6 using Notch3 siRNA (N3 si), and control siRNA (Cont si) were investigated. Expression of Notch3,  $\alpha$ -smooth muscle actin (SMA) and collagen I was detected by Western blotting; B: The expression of  $\alpha$ -SMA and collagen I was also detected by immunofluorescence staining (x 400). <sup>a</sup> $P < 0.05$  vs control siRNA group, <sup>c</sup> $P < 0.05$  vs transforming growth factor (TGF)- $\beta$ 1+control siRNA group. 1: Cont si; 2: N3 si; 3: TGF- $\beta$ 1+N3 si; 4: TGF- $\beta$ 1+Cont si.

well as the mechanism underlying the Notch signaling in liver fibrosis. In addition, the contribution of Notch and TGF- $\beta$ 1 signaling to the liver fibrosis should also be further investigated.

In conclusion, we demonstrated for the first time that Notch3 plays a role in regulating the activation of HSCs (HSC-T6). Therefore, the selective interruption of Notch3 may have a potential anti-fibrogenic effect in liver fibrosis.

## COMMENTS

### Background

It is well known that hepatic stellate cell (HSC) activation plays an important role in fibrosis because these cells are the primary source of extracellular matrix in liver upon injury. Notch signaling regulates many aspects of morphogenesis through diverse effects on differentiation, proliferation, and cell survival. Recently, some researches reported that Notch signaling was implicated in human fibrosis diseases, such as pulmonary, renal and peritoneal fibrosis.

### Research frontiers

This study was undertaken to investigate whether Notch signaling is activated in HSCs and sequentially contributed to liver fibrosis by regulating the activation of HSCs.

### Innovations and breakthroughs

This is the first study to characterize the role of Notch signaling in liver fibrosis. This finding indicated that Notch3 may contribute to liver fibrosis by regulating the activation of HSCs.

### Applications

This study showed that the selective interruption of Notch3 by siRNA decreased the expression of myofibroblastic marker in hepatic stellate cell-T6 cells, which provided a potential novel therapeutic target for liver fibrosis.

### Peer review

This is a very interesting study aimed at investigating the role of the Notch signaling pathway in liver fibrosis. The text is generally well written, with structured abstract and organized sections. The manuscript has scientific value since it includes original information about a relevant topic.

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## Endoscopic stenting and concurrent chemoradiotherapy for advanced esophageal cancer: A case-control study

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### Abstract

**AIM:** To evaluate the role of endoscopic stenting with or without concurrent 3-dimensional conformal chemoradiotherapy (3D-CRT) in patients with inoperable esophageal cancer.

**METHODS:** Advanced esophageal cancer patients indicated for esophagectomy received esophageal stents. A part of patients completed 3D-CRT after stenting. Efficacy was assessed by endoscopy and computed tomographic scan before and 4 wk after completion of the treatment. The median survival, 3D-CRT toxicity and complications were compared between 3D-CRT and control groups.

**RESULTS:** From 1999 to 2008, 99 consecutive patients with T3/T4 disease and unsuitable for esophagectomy were placed with esophageal stents. Sixty-seven patients received 3D-CRT, while 36 patients treated with

endoscopic stents alone were recruited as controls. After 3D-CRT treatment, the median tumor volume of 3D-CRT patients were reduced significantly from  $43.7 \pm 10.2 \text{ cm}^3$  to  $28.8 \pm 8.5 \text{ cm}^3$  ( $P < 0.05$ ). The complete and partial response rate was 85.1%, and no response was 14.9%. After 3D-CRT, the incidence rate of T2 and T3 disease evident on CT scan increased to 78.4% while T4 decreased from 66.7% to 21.6% ( $P < 0.05$ ). 3D-CRT Karnofsky Performance Status improved in 3D-CRT patients compared with the control group ( $P = 0.031$ ). 3D-CRT patients had a longer survival than the control group (251.7 d vs 91.1 d,  $P < 0.05$ ). And the median half-year survival rate in 3D-CRT group (91%) was higher than in the control group (50%,  $P < 0.05$ ). The most common toxicity was leukocytopenia in the 3D-CRT group (46.7% vs 18.8%,  $P = 0.008$ ). The control group had a higher rate of restenosis than the 3D-CRT group (81.3% vs 9.0%,  $P < 0.05$ ). The rate of nephrotoxicity was increased in 3D-CRT as compared with the control group (31.3% vs 15.6%,  $P < 0.05$ ).

**CONCLUSION:** 3D-CRT can improve dysphagia in patients with inoperable esophageal carcinoma. 3D-CRT combined with stenting results in better survival as compared with endoscopic stents used alone.

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**Key words:** Esophageal Cancer; Stents; Chemoradiotherapy; Three-Dimensional Imaging; Case Control Study

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## INTRODUCTION

Esophageal cancer is one of the most common malignant tumors with a high mortality rate in almost a half of the cases, and is the fourth leading cause of cancer-related deaths in China<sup>[1,2]</sup>. Most patients have been already in an advanced stage at the diagnosis of this aggressive malignancy. At least 60% of patients are unsuitable for surgical resection either due to the advanced stage or their comorbidity<sup>[3]</sup>. At this stage, esophageal carcinoma has infiltrated surrounding tissues and caused esophageal stenosis, and even esophagotracheal fistula in some cases. These patients could only be treated with palliative procedures which play an important role in improving the patient's life quality. The current palliative means include radiotherapy, chemotherapy and esophageal stent placement. However, esophagitis is usually unavoidable after radiotherapy, and once esophagitis occurred, dysphagia would be exacerbated<sup>[4]</sup>. Some patients even can not swallow liquid diet, some can not endure the chemotherapy and radiotherapy for their poor nutritional status. Stents placement has become a safe and effective palliation for dysphagia due to malignant esophageal obstruction or strictures after radiotherapy<sup>[5]</sup>. A randomized trial demonstrated that the combination of endoscopic stenting with additional radiation and chemotherapy could improve the survival of advanced esophageal cancer patients<sup>[6,7]</sup>. We evaluated the feasibility and efficacy of esophageal stenting combined with simultaneous radiotherapy and chemotherapy in the treatment of advanced esophageal cancer.

## MATERIALS AND METHODS

### Patients

All esophageal cancer patients treated in Qingdao Municipal Hospital from January 1999 to September 2008 were identified. The care of esophageal cancer patients was planned under the auspices of a multidisciplinary gastrointestinal disease management team which includes surgical oncologists, medical oncologists, gastroenterologists, pathologists, interventional radiologists, and radiation oncologists. This study was approved by the Ethics Committee of our institution and informed consent was obtained from all the patients before enrollment.

Clinical staging was performed with endoscopy and endoscopic ultrasound and computed tomography (CT). The tumor, node, metastasis and staging classification used for this analysis were defined according to the American Joint Committee on Cancer staging system version 6.0<sup>[8]</sup>.

Inclusion criteria for the study were: (1) esophageal cancer at stage III or IV unsuitable for esophagectomy; (2) symptoms of dysphagia  $\geq$  grade 3 (Table 1); (3) tumors were mainly located in the esophagus; (4) adequate bone marrow (white blood cell  $> 3.5 \times 10^9/L$ , hemoglobin  $> 90$  g/L, platelet count  $> 100 \times 10^9/L$ ), and hepatic (bilirubin 1.5 times the upper limit of the normal value) and renal functions (calculated creatinine clearance  $> 50$  mL/

Table 1 Dysphagia assessment

Grade
Asymptomatic
Difficulty in swallowing solid food but able to swallow semisolid food
Difficulty in swallowing solid food, but able to swallow liquid one
Difficulty in swallowing liquid
Inability to swallow anything, including saliva

min or creatinine  $< 2$  mg/dL) were examined before administration of chemotherapy; (5) a minimum life expectancy of 5 mo; and (6) stay in hospital during the entire chemoradiotherapy treatment course.

Patients were excluded because: (1) with tumors predominantly located in the stomach; (2) with prior treatment, including surgery, chemotherapy, or radiotherapy; and (3) with tumors infiltrating the tracheobronchial tree found on CT.

Data collected included patient clinical demographics, Karnofsky Performance Status, dysphagia grade, 76% meglumine diatrizoate compound swallow esophagogram (esophageal strictures) findings, endoscopic findings (tumor length at initial endoscopy, primary tumor, and lymphadenopathy location), tumor histology, and results of CT scan of the chest and abdomen with intravenous contrast.

### Procedure of stenting

The procedure was performed under local anesthetic spray, with intravenous sedation when required. Endoscopy was done to determine precisely the site and length of stenosis. All patients with strictures underwent dilation with 12-14 mm flexible rubber Savary-Guillard dilators before stent placement. Once this stricture was successfully dilated, a distal hemoclip (resolution clip, Boston Scientific, United States) was placed 2 cm below the area of stricture. Then endoscope was advanced farther and a flexible guidewire was placed into the second portion of the duodenum. The covered self-expanding Titanium Nickel alloy mesh stent (MTN, Nanjing Microinvasive Medical Inco., Nanjing, China) has a polyester at its mid-section and its proximal end is flared to a diameter of 25 mm. The stent was loaded onto a dedicated applicator (12-14 mm depending on the diameter of stent) with an atraumatic dilator tip and was placed under continuous fluoroscopic guidance using the distal hemoclip as a mark. The length chosen was at least 2 cm longer than the stenosis. An 18 mm-diameter stent was used for severe strictures; and a 21 mm-diameter stent was used for moderate strictures.

### Three-dimensional conformal chemoradiotherapy

Three-dimensional conformal chemoradiotherapy (3D-CRT) protocol was used. CT scans displayed isodose distributions and directly obtain dose-volume histograms. The primary tumors as well as the loco-regional lymph nodes were irradiated with an International Commission on Radiation Units and Measurements reference dose of 45.0 Gy in 25 fractions with 1.8 Gy/d using a LINAC

**Table 2** Clinical and demographic characteristics of 3-dimensional conformal chemoradiotherapy-treated patients and controls

	3D-CRT (n = 67)	Control (n = 32)	P value
Gender			
Male	53	24	0.796
Female	14	8	
Age (mean ± SD, yr)	56.3 ± 12.7	58.6 ± 12.1	0.435
Karnofsky performance status			
50-70	37	21	0.386
10-49	30	11	
Dysphagia grade			
3	6	4	0.673
4	46	23	
5	15	5	
Stage at diagnosis			
III	56	29	0.539
IV	11	3	
Tumor type			
SCC	53	23	0.579
Adenocarcinoma	6	4	
Unspecified	8	6	
Location			
Thoracic - middle	42	20	1.000
Thoracic - lower	25	12	
Gross tumor volume (mean ± SD, m <sup>3</sup> )	44.6 ± 10.2	48.5 ± 11.1	0.077
Length (mean ± SD, cm)	7.3 ± 1.6	7.1 ± 1.8	0.299

SCC: Squamous cell carcinoma; 3D-CRT: 3-dimensional conformal chemoradiotherapy.

6-MV X-ray unit (Varian, CA, United States), and the total dose was adjusted according to patients' tolerance. Chemotherapy regimens consisted of cisplatin 60 mg/m<sup>2</sup> infusion on day 1 and day 22, plus continuous infusion of 5-fluorouracil at 200 mg/m<sup>2</sup> per day from day 1 to day 42.

### Clinical response criteria

The clinical response to treatment was categorized as a clinically complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). CR was defined as the disappearance of all clinically detectable lesions; CR of primary tumors was defined as the disappearance of all visible lesions, including ulceration, with no microscopic evidence of tumor in randomly obtained biopsy specimens from the previous lesion sites. PR was defined as either a reduction exceeding 50% of the initial sizes (products of dimensions) of all measurable tumors according to CT or esophagography. A new lesion or more than a 25% increase of the original tumor size was defined as PD. All other conditions were categorized as SD. Chest CT was repeated every 4 wk to assess the response of the tumor. Endoscopy or abdominal CT was repeated if necessary.

### Statistical analysis

The data was analyzed using SPSS 13.0, mean ± SD were calculated. Continuous variables were analyzed using Student's *t* test. Categorical variables were compared using  $\chi^2$  test or Fisher's exact test, whichever applicable. Repeated measure analysis (two-way ANOVA) was also used. Sur-

vival curves were estimated by the Kaplan-Meier method. A *P* value of 0.05 or less was considered significant.

## RESULTS

### Patient characteristics

Among the 146 patients who were diagnosed as having inoperable esophageal cancer, 111 (76.0%) were eligible for treatment with palliative 3D-CRT. Nine patients did not complete 3D-CRT because of disease progression. One patient died of severe bleeding 12 d after 3D-CRT. Two patients developed febrile neutropenia, and stopped the chemotherapy. Thirty-two patients did not undergo 3D-CRT after stents placement because their preference, these patients were regarded as the control group. Sixty-seven patients completed the 3D-CRT segment with a total radiation dose over 40Gy. Among these patients, 38 received 40Gy, and 29 received 50-55Gy. These patients were enrolled as the 3D-CRT treatment group. The clinical and demographic characteristics of the patients in 3D-CRT and control groups are shown in Table 2. There was no obvious difference in the dysphagia grade, tumor type, tumor stage and tumor size between the two groups.

### Response after 3-dimensional conformal chemoradiotherapy

After 3D-CRT treatment, the median tumor volume of the 67 patients measured by CT scan reduced significantly from 43.7 ± 10.2 cm<sup>3</sup> to 28.8 ± 8.5 cm<sup>3</sup> (*P* < 0.05). The complete or partial response (> 50% reduction of tumor volume) was observed in 57 patients (85.1%), and no response in 10 patients (14.9%). The cases of T2 and T3 disease evident on CT scan had increased from 0 to 40 (78.4%) after 3D-CRT, and that of T4 decreased from 34 (66.7%) to 11 (21.6%) (*P* < 0.05). After 3D-CRT, the number of cases of dysphagia at grade ≤ 3 among the 67 patients increased significantly from 6 (9.0%) to 55 (82.1%), while the number of dysphagia at grade ≥ 4 reduced from 61 (91.0%) to 12 (17.9%), (*P* < 0.05). Karnofsky Performance Status improved in the 3D-CRT patients compared with the control group (*P* = 0.031). The result of response assessed by CT scan and endoscopy is listed in Table 3.

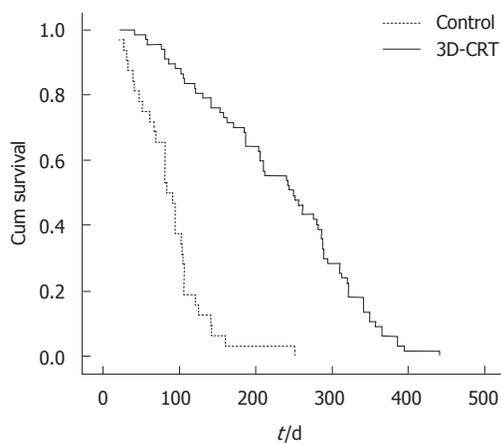
### Survival comparison

Patients treated with 3D-CRT seemed to have a better prognosis, with a longer survival duration (251.7 d *vs* 91.1 d, *P* < 0.05; Figure 1). The median six-month survival rate in 3D-CRT group (91%) was higher than in the control group (50%, *P* < 0.05, Table 4). The overall 1-year survival rate after 3D-CRT was 25%; none of the control patients survived more than 1 year. Although the mean number of hospital admissions was significantly higher in patients who received 3D-CRT (146 ± 48 d *vs* 87 ± 29 d, *P* < 0.05), the overall survival of the 3D-CRT group without hospitalization was statistically significantly higher than that of the control group (186 ± 33 d *vs* 59 ± 18 d, *P* < 0.05).

**Table 3 Radiological and clinical evaluation after 3-dimensional conformal chemoradiotherapy**

	Before 3D-CRT	After 3D-CRT	P value
Tumor staging			
T2 and T3	0	40 (78.4%)	< 0.05
T4	34 (66.7%)	11 (21.6%)	
Tumor volume (mean ± SD, cm <sup>3</sup> )	43.7 ± 10.2	28.8 ± 8.5	< 0.05
Assessment of tumor response			
Complete and partial response		57 (85.1%)	
No response		10 (14.9%)	
Dysphagia grade			
≤ 3	6 (9.0%)	55 (82.1%)	< 0.05
≥ 4	61 (91.0%)	12 (17.9%)	
Karnofsky performance status			
50-70	37	49	0.031
10-49	30	18	

3D-CRT: 3-dimensional conformal chemoradiotherapy.



**Figure 1 Comparison of overall survival between 3D-CRT and control groups.** 3D-CRT: 3-dimensional conformal chemoradiotherapy.

**Toxicity and complications**

The toxicity profile is presented in Table 5. After 3D-CRT, the most common toxicity was leukocytopenia as compared with the control group without 3D-CRT treatment (46.7% *vs* 18.8%, *P* = 0.008). However, in the control group, the most common non-hematologic toxicities included a higher rate of restenosis compared with 3D-CRT group (81.3% *vs* 9.0%, *P* < 0.05). The incidence of nephrotoxicity in 3D-CRT was higher than in control group (31.3% *vs* 15.6%, *P* < 0.05).

**DISCUSSION**

About more than 90% of esophageal cancer patients are not clinically identified until at an advanced stage, their prognosis is the poorest among the patients with digestive carcinomas, with a 5-year survival rate below 10%<sup>[1]</sup>. Because of malnourishment due to comorbid conditions in advanced carcinoma, more than 50% of esophageal cancer cases are unresectable<sup>[9]</sup>. A case-control study showed that the median survival time for patients with advanced esophageal cancer was only 3-5 mo<sup>[10]</sup>. However, once

**Table 4 Comparison of clinical and survival outcomes between 3-dimensional conformal and control groups**

Outcome	3D-CRT (n = 67)	Control (n = 32)
Median survival (d)	251.7	91.1
Overall survival at a half year	91%	50%
Over survival at 1 year	25%	0
Total hospital stay (mean ± SD, d)	146 ± 48	87 ± 29
Median hospitalization-free survival (mean ± SD, d)	186 ± 33	59 ± 18

3D-CRT: 3-dimensional conformal chemoradiotherapy.

**Table 5 Toxicity and complications in patients after 4-wk 3-dimensional conformal and control group after treatment n (%)**

	3D-CRT (n = 67)	Control (n = 32)	P value
Hematologic			
Anemia	33 (49.3)	11 (34.4)	0.198
Thrombocytopenia	21 (31.3)	7 (21.9)	0.475
Leukocytopenia	31 (46.7)	6 (18.8)	0.008
Non-hematologic			
Reflux esophagitis	28 (41.8)	15 (46.9)	0.669
Pectoralgia	29 (43.3)	14 (43.8)	1.000
Perforation	16 (23.9)	12 (37.5)	0.232
Restenosis	6 (9.0)	26 (81.3)	< 0.05
Nephrotoxicity	21 (31.3)	5 (15.6)	< 0.05
Shift and brush off of stent	8 (11.9)	5 (15.6)	0.751

3D-CRT: 3-dimensional conformal chemoradiotherapy.

these inoperable patients' physical condition was permitted, effective therapies should be performed. No matter which therapy is used, the primary purpose is to prolong the patients' survival time and improve their quality of life. In this study, we relieved the dysphagia with minimal morbidity and mortality, and then choose the suitable management to prolong the patients' life span and improve their quality of life.

Recently, 3D-CRT appeared to be a promising treatment even for esophageal carcinoma patients with distant metastasis<sup>[11]</sup>. Because CDDP and 5-FU have synergistic effects and also act as radiosensitizers, these agents were considered to be particularly effective in combination with radiotherapy<sup>[12]</sup>.

In our study, we focused on the 3D-CRT effectiveness in the inoperable esophageal cancer patients. Ten years ago, we could only place stents to palliate dysphagia among these patients. And in recent years after stents placement, 3D-CRT could be performed as long as patient's physical condition allowed, and whenever their dysphagia was relieved, the malnutrition of the patients was ameliorated. For these inoperable patients, esophageal stents placement not only improved their quality of life, but made the 3D-CRT completion possible as well. In our study, compared with the patients treated with stenting only, at least 25% of the inoperable patients after 3D-CRT survived 1-year, and their six-month survival rate was also higher. 3D-CRT could reduce the tumor volume

and improve Karnofsky Performance Status in the inoperable patients. Combined with 3D-CRT, it seemed that the esophageal stent placement is the adjuvant therapy with CRT. We found that stenting combined with 3D-CRT could improve the inoperable patients' quality of life, and prolong their survival. Fietkau<sup>[4]</sup> thought that simultaneous CRT should be considered as the standard treatment for inoperable carcinoma of the esophagus with the median survival time between 13 and 18 mo. The reason why Fietkau's result<sup>[4]</sup> was better than ours may be that there were more inoperable stage III/IV patients in our study as compared with the stage II/III patients in Fietkau's study.

3D provides the superior dose distributions in the target volume with markedly reduced morbidity to the surrounding normal tissues. Minsky found that increasing the percutaneous radiation dose from 50.4 to 64.8 Gy did not result in better survival<sup>[13]</sup>. Part of the patients in our study received radiation dose of less 40Gy due to the complications such as perforation or esophagitis. Although there is still no consensus of the optimal dose of radiation within the framework of simultaneous RCT, there has been a common opinion that the higher dose of radiation the more therapy-related deaths. In our study, our therapeutic purposes were not to remit or downstage the tumor, but to alleviate the pain and improve the quality of life of the patients. Moreover, 3D-CRT with covered metal stenting would cause a relatively higher rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse. The results from some studies about the safety of self-expandable metallic stents for patients who have undergone chemoradiotherapy were controversial<sup>[14-16]</sup>. Therefore, more studies should be conducted.

In our study, there was no difference of complications such as perforation, shift and brush off of stent and pectoralgia between the 3D-CRT group and the control group with only stenting. Because we did not determine whether lymph node metastasis occurred in these patients without biopsies from esophagectomy, we did not compare the patients with and without lymph node metastasis. Those who could not be treated by 3D-CRT due to poor physical conditions served as controls, and there might be selection bias in this study.

Fietkau<sup>[4]</sup> pointed out that CRT could increase the treatment-related toxicity, in particular, the hematological side effects. This result was in agreement with ours, which was caused by 3D-CRT such as leukocytopenia and nephrotoxicity. After 3D-CRT, more leukocytopenia and nephrotoxicity occurred, but less restenosis was found as compared with the control group. Therefore, among the patients with leukocytopenia and liver and renal dysfunctions, caution should be exercised in the application of 3D-CRT.

Furthermore, more patients were relieved from dysphagia during the later stages of life after 3D-CRT treatment. 3D-CRT is beneficial for the majority of patients with advanced esophageal cancer in the improvement of quality of life and survival. In our study, endoscopic stent-

ing with 3D-CRT for advanced esophageal cancer patients could relieve the dysphagia and prolong the survival, and decrease the incidence of restenosis as well. Therefore, more efficient and combined management should be explored and studied to improve the survival and quality of life of the patients with advanced esophageal cancer.

## COMMENTS

### Background

Placement of esophageal stents in patients with advanced esophageal cancer can improve the symptoms of dysphagia. However, the safety of esophageal stents for patients receiving chemoradiotherapy is controversial. The authors evaluated the morbidity and mortality after self-expandable metallic stent placement with and without 3-dimensional conformal chemoradiotherapy (3D-CRT) in advanced esophageal cancer patients unsuitable for surgery.

### Research frontiers

In this study, once the dysphagia in advanced esophageal cancer patients was alleviated, concurrent 3D-CRT was carried out. 3D-CRT combined with esophageal stenting could improve the symptoms of dysphagia in patients with inoperable esophageal carcinoma and could prolong their survival as well.

### Innovations and breakthroughs

This study showed that self-expandable metallic stent placement with concurrent 3D-CRT could improve the symptoms of dysphagia and result in better survival as compared with endoscopic stenting used alone in the patients with inoperable esophageal carcinoma.

### Applications

The combined treatment of endoscopic stenting and 3D-CR could be applied in patients with inoperable advanced esophageal cancer.

### Terminology

Three-dimensional conformal chemoradiotherapy (3D-CRT) is a mode of high precision radiotherapy. It is a complex process that begins with the creation of individualized, 3D digital data sets of patient tumors and normal adjacent anatomy. These data sets are then used to generate 3D computer images and to develop complex plans to deliver highly "conformed" (focused) radiation while sparing normal adjacent tissues. The radiation beam could focus on a higher radiation dose to the tumor while minimizing radiation exposure to healthy cells.

### Peer review

In this study, the authors examined the efficacy and toxicity/complication of esophageal stenting thereafter 3D-CRT for patients with inoperable esophageal cancer. The results of this study revealed that esophageal stenting after 3D-CRT induced no severe complication, prolonged their survival and effectively improved their symptoms of dysphagia than stenting alone in patients with inoperable advanced esophageal cancer.

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## A rare case of langerhans cell histiocytosis of the gastrointestinal tract

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### Abstract

Langerhans cell histiocytosis (LCH) is a group of idiopathic disorders characterized by the proliferation of specialized, bone marrow-derived langerhans cells and mature eosinophils. The clinical spectrum ranges from an acute, fulminant, disseminated disease called Letterer-Siwe disease to solitary or few, indolent and chronic lesions of the bone or other organs called eosinophilic granuloma. Involvement of the gastrointestinal tract is very rare in LCH. We present the case of a 53-year-old woman referred by her primary care physician for a screening colonoscopy. A single sessile polyp, measuring 4 mm in size, was found in the rectum. Histopathological examination revealed that the lesion was relatively well circumscribed and comprised mainly a mixture of polygonal cells with moderate-to-abundant pink slightly granular cytoplasm. The nuclei within these cells had frequent grooves and were occasionally folded. Immunohistochemical staining was positive for CD-1a which confirmed the diagnosis of LCH. On further workup, there was no evidence of involvement of any other organ. On follow up colonoscopy one year later, there was no evidence of disease recurrence. Review of the published literature revealed that LCH presenting as solitary colonic polyp is rare. However, with the increas-

ing rates of screening colonoscopy, more colonic polyps may be identified as LCH on histopathology. This underscores the importance of recognizing this rare condition and ensuring proper follow-up to rule out systemic disease.

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**Key words:** Langerhans cells; Histiocytosis; Colonic polyp; CD-1a; Eosinophilic granuloma; Screening; Colonoscopy

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### INTRODUCTION

Langerhans cell histiocytosis (LCH) is a group of idiopathic disorders characterized by the proliferation of specialized, bone marrow-derived langerhans cells (LCs) and mature eosinophils. The clinical spectrum of LCH ranges from an acute, fulminant, disseminated disease (called Letterer-Siwe disease) to solitary or few indolent and chronic lesions of the bone or other organs called (eosinophilic granuloma). The intermediate clinical form, called Hand-Schüller-Christian disease, is characterized by multifocal, chronic involvement and classically presents as a triad of diabetes insipidus, proptosis and lytic bone lesions.

LCH is a rare disease of the pediatric population,

with an estimated annual incidence of 4-5 per million. More than two-thirds of cases have single system disease with bones and skin as the most commonly involved sites<sup>[1,2]</sup>. Other organs involved are the lung, liver, spleen, bone marrow, lymph nodes and the hypothalamic-pituitary region<sup>[3]</sup>. Involvement of the gastrointestinal (GI) tract is very rare in LCH, especially in adults, with only a few isolated case reports available in English-language literature<sup>[4]</sup>.

The pathogenesis of LCH is unknown. An ongoing debate exists over whether this is a reactive or neoplastic process. From the histopathological viewpoint, the demonstration of LC (Birbeck) granules by electron microscopy remains the “gold standard” for diagnosis of the phenotype, but expression of the CD1a antigen on lesional cells also provides the basis for a definitive diagnosis<sup>[5]</sup>.

## CASE REPORT

We present a case of a 53-year-old woman with a history of hyperlipidemia who was referred by her primary care physician for a screening colonoscopy. She was essentially asymptomatic and did not report any abdominal pain, blood in stool, change in bowel habits, dysphagia, nausea, or vomiting. Findings of physical examination were unremarkable.

On colonoscopy, quality of bowel preparation was excellent. A single sessile polyp measuring 4 mm in size was found in the rectum (Figure 1). It was removed by cold snare polypectomy. The procedure was performed without complications.

Histopathological examination of the polyp revealed that the lesion was relatively well-circumscribed and comprised mainly a mixture of polygonal cells. These cells had moderate-to-abundant pink slightly granular cytoplasm. The lesion also contained inflammatory cells. The inflammatory populations included eosinophils, lymphocytes and neutrophils. The nuclei within the larger cells had frequent grooves and were occasionally folded, suggesting the presence of LCs (Figure 2). Immunohistochemical staining showed histiocytes with cytoplasmic and membranous staining for CD-1a (Figure 3). Histiocyte cytoplasm was positive for S-100 and negative for prekeratin on immunohistochemistry. Electron microscopy was not performed to document presence of Birbeck granules as staining for CD-1a was strongly positive and provided confirmation for the diagnosis<sup>[5]</sup>.

The patient was referred for medical oncology evaluation for this unusual pathologic finding with malignant potential. A computerized tomography (CT) scan of the chest, abdomen, and pelvis was performed to rule out any metastasis through staging evaluation. The CT scan showed no evidence of metastasis, although there were fibroids in the uterus and few cystic masses in the ovaries. Some prominent lymph nodes, which appeared to be benign, were observed in the right and left axillae and sub carina. Further evaluation by an oncologist and a gynecologist revealed that these lesions were benign.



Figure 1 Endoscopic view of the rectal polyp.

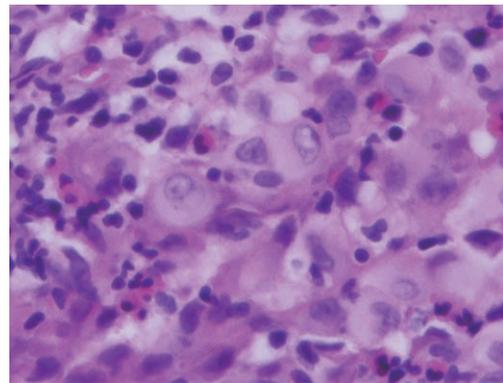


Figure 2 Rectal biopsy with histiocytic infiltrate in the lamina propria. Note eosinophils ( $\times 400$ ).

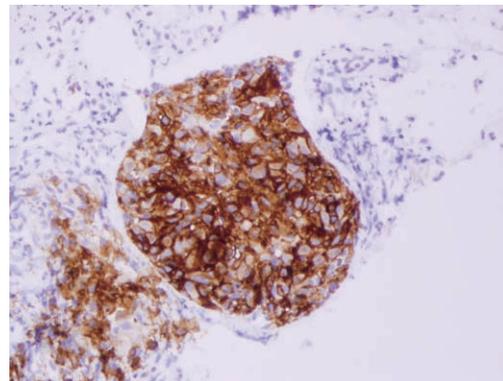


Figure 3 CD-1a immunostain. Histiocytes show positive cytoplasmic and membranous staining ( $\times 200$ ).

Therefore, we found no evidence of disseminated LCH.

The patient remained asymptomatic, and a follow-up colonoscopy was performed 1 year later. A single sessile polyp, measuring 4 mm, was found in the rectum. Another single sessile polyp, measuring 5 mm, was found in the proximal ascending colon. Histopathological examination showed that the polyps were hyperplastic and tubular adenomatous polyps, respectively.

## DISCUSSION

In the GI tract, LCH involvement of the stomach<sup>[6-8]</sup>,

small intestine<sup>[9-11]</sup>, colon<sup>[4,12,13]</sup> and perianal skin<sup>[14,15]</sup> has been reported. Rectal LCH, proven by histopathology, has been described in infants presenting with bloody diarrhea. In most cases, rectal LCH in infants is indicative of widespread, multisystem disease<sup>[16]</sup>.

Among the 6 reported cases of LCH of stomach, all patients were in the fourth and fifth decade of life, and the male-to-female ratio was 1:1; five patients presented with abdominal pain, and one was asymptomatic. The gastric lesion was described as a large, flat raised area in 1 case and a single polyp in 1 case. In other 2 cases, the gastric lesion was described as an ulcerating mass and multiple polyposis, respectively. Histopathological examination of lesions from 2 cases revealed malignant features<sup>[8,17]</sup>. In the case reported by Terracciano *et al.*<sup>[8]</sup>, the patient presented with abdominal pain and weight loss; the tumor was located on the lesser curvature of the stomach. During laparotomy, the tumor was found to have invaded the distal pancreas. Some areas of the tumor had high mitotic indexes and cytologic atypia. Most neoplastic cells were intensely positive for vimentin, S-100 and CD 1a on immunohistochemistry. Two months after surgical resection, the patient died at home. The cause of death was unclear. The remainder of the cases did not show histopathological or clinical malignant features.

Small bowel LCH is rare, with only 3 cases being reported in infants<sup>[10,18]</sup> and 1 in an adult<sup>[11]</sup>. All infants with small bowel LCH presented with diarrhea, weight loss, and failure to thrive. Laboratory findings were significant for anemia and hypoalbuminemia, and the duodenum was always involved. All patients simultaneously or subsequently developed widespread, multisystem involvement, requiring chemotherapy. Two patients responded well to chemotherapy with complete remission, but 1 succumbed to the disease after poor response to treatment and finally refusing chemotherapy<sup>[18]</sup>. In adults, only 1 proven case of small bowel LCH has been reported. LCH occurred in a patient who had undergone right hemicolectomy for steroid-refractory Crohn's disease; this patient presented with worsening diarrhea, abdominal pain, and weight loss. Barium follow-through showed extensive mucosal infiltration of the small bowel wall, and the duodenal biopsy result was consistent with a diagnosis of LCH. Results of bone marrow examination were consistent with chronic myelomonocytic leukemia. The authors concluded that in this case, LCH was a complication of Crohn's disease because of the increased incidence of myeloproliferative disorders in inflammatory bowel disease<sup>[11,19]</sup>.

In children, colonic involvement in LCH is extremely rare. Only 1 case of LCH has been reported in an infant who died of widespread multisystem disease consistent with Litterer-Siwe disease; LCH was diagnosed post-mortem in this case<sup>[13]</sup>. In adults, there have been only 2 reported cases of colonic LCH presenting as isolated polyps in English-language literature<sup>[4,12]</sup>. Both patients were essentially asymptomatic, and polyps were detected during screening colonoscopy. In both cases, diagnosis was confirmed by positive immunochemical staining for CD1a

antigen. Extensive workup in both cases did not reveal involvement of any other organ. In the case presented herein, a polyp was located in the rectum, and diagnosis was supported by the presence of an eosinophilic infiltrate and LCs. The diagnosis was confirmed by strong immunochemical staining for CD1a antigen. The workup to rule out involvement of other organs was negative, and repeat colon examination did not reveal recurrence.

In conclusion, LCH of the GI tract is extremely rare. Colonic involvement in adults usually presents as a solitary polyp without multisystem disease. With the increasing rates of screening colonoscopy, more colonic polyps may be identified as LCH on histopathology. This underscores the importance of recognizing this rare condition and ensuring proper follow-up to rule out systemic disease.

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## Pseudomelanosis duodeni associated with chronic renal failure

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### Abstract

Pseudomelanosis duodeni (PD) is a rare dark speckled appearance of the duodenum associated with gastrointestinal bleeding, hypertension, chronic heart failure, chronic renal failure and consumption of different drugs. We report four cases of PD associated with chronic renal failure admitted to the gastroenterology outpatient unit due to epigastric pain, nausea, melena and progressive reduction of hemoglobin index. Gastroduodenal endoscopy revealed erosions in the esophagus and stomach, with no active bleeding at the moment. In addition, the duodenal mucosa presented marked signs of melanosis; later confirmed by histopathological study. Even though PD is usually regarded as a benign condition, its pathogenesis and clinical significance is yet to be defined.

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**Key words:** Pseudomelanosis duodeni; Chronic renal failure; Diabetes mellitus; Anti hypertensive drugs; Gastro-duodenal endoscopy

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### INTRODUCTION

Melanosis duodeni was first described in 1976 and refers to a rare endoscopic appearance of discrete speckled black pigmentation of duodenal mucosa, which was initially postulated to represent a form of stored iron<sup>[1]</sup>. The term melanosis gives a false idea that the pigment is produced by melanocytes, justifying renaming this endoscopic finding as pseudomelanosis duodeni (PD)<sup>[2]</sup>. Nevertheless, the clinical significance of this condition is yet to be established. PD is more common in the sixth and seventh decade of life with a female predominance. It has been postulated to be associated with gastric hemorrhage, certain chronic illnesses, such as diabetes mellitus, hypertension, as well as various medications, such as sulfur-containing antihypertensive agents and ferrous sulfate<sup>[3-5]</sup>.

### CASE REPORT

#### Case 1

A 66-year-old woman was admitted with a history of melena and a progressive decrease of hemoglobin index. She presented with a history of diabetes mellitus and

systemic arterial hypertension for > 30 years, with a diagnosis of chronic renal failure in the last 6 mo, treated with hemodialysis. She was on long-term treatment with angiotensin-converting enzyme inhibitors, furosemide, ferrous sulfate, folic acid and insulin. Gastroduodenal endoscopy was performed and revealed multiple superficial erosions in the stomach without signs of recent bleeding. In addition, in the duodenal mucosa, pigmented lesions with a speckled pattern were evident (Figure 1). Biopsies revealed numerous macrophages containing brown pigment granules in their cytoplasm within the lamina propria (Figure 2). Staining of the specimen with Masson Fontana stain revealed iron-containing deposits inside the macrophages (Figure 3).

### Case 2

A 37-year-old woman with a history of renal transplantation (hypertensive nephropathy) had been previously treated with furosemide, propranolol, hydralazine and ferrous sulfate. She was referred to the gastroenterology section due to classical gastroesophageal reflux disease symptoms. She was submitted to gastroduodenal endoscopy that revealed reflux esophagitis and diffuse, multiple, dark brown spots in the duodenum. Biopsies were taken and light microscopy confirmed the presence of brown pigment granules in the lamina propria.

### Case 3

A 70-year-old woman with diabetes and long-term systemic arterial hypertension developed chronic renal failure that was initially treated conservatively with calcium channel blockers, propranolol,  $\alpha$ -methyl dopa, furosemide and glibenclamide. She underwent a left nephrectomy due to nephrolithiasis. Progressive reduction of hemoglobin index was observed during follow-up, therefore, gastroduodenal endoscopy was performed, revealing multiple non-actively bleeding superficial gastric erosions and pigmented lesions in the duodenum. Biopsies were collected and histopathological examination showed features of PD.

### Case 4

A 30-year-old woman with diabetes started complaining of epigastric pain, nausea and vomiting 1 mo after renal and pancreatic transplantation (diabetic nephropathy). She had been previously taking insulin, and after the transplant, she started tracolumis, mycophenolate and corticosteroid therapy. Gastroduodenal endoscopy revealed cytomegalovirus (CMV) esophagitis and multiple small pigmented duodenal spots. Biopsies were taken and histopathological examination confirmed the diagnosis of PD. Another gastroduodenal endoscopy was performed after CMV treatment, and demonstrated total regression of the esophageal lesions but no changes in the aspect of the duodenal mucosa.

## DISCUSSION

PD represents a fine granular brown material inside the

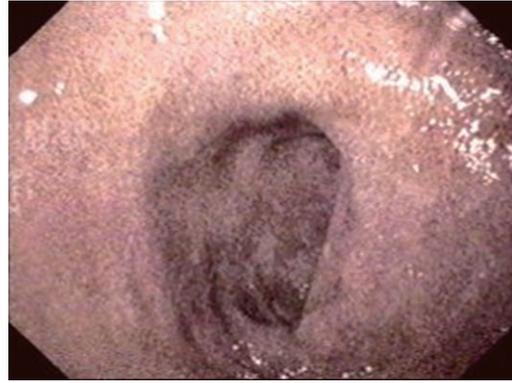


Figure 1 Endoscopic picture showing multiple dark brown spots in the duodenum.

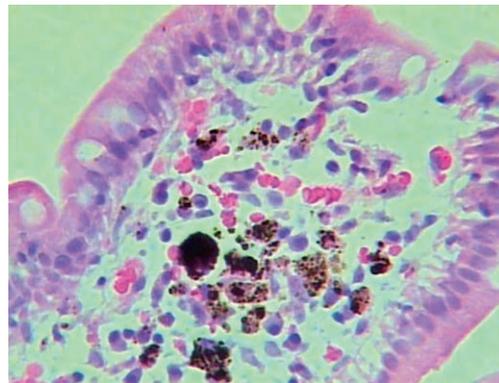


Figure 2 Many macrophages containing brown pigmented granules within the lamina propria (hematoxylin and eosin stain, x 200).

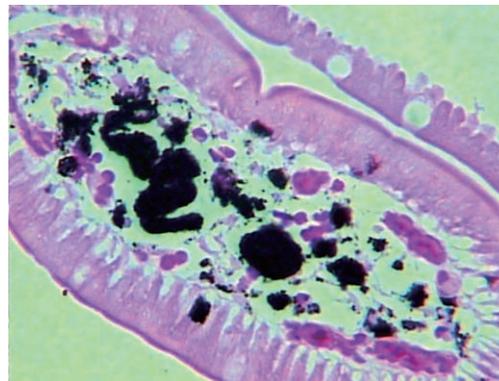


Figure 3 Iron deposits inside macrophage cytoplasm (Masson-Fontana stain, x 200).

macrophage lysosomes in the lamina propria around the tips of the duodenal villi, detected by histochemical staining and electron microscopy. It has been postulated that this heterogeneous pigment may represent a deposit of melanin-like substances, hemosiderin, lipomelanin and lipofuscin<sup>[6,7]</sup>. Even though iron (ferrous sulfide) is the main pigment compound, varying amounts of sulfur, calcium, potassium, aluminum, magnesium and silver can also be detected<sup>[5,8]</sup>. The color of the pigment could represent various degrees of auto-oxidation of ferrous sulfide<sup>[2,6]</sup>.

The pathogenesis still remains unclear. It could be related to iron deposition secondary to intramucosal hemorrhage or impaired intramucosal iron transport after oral ferrous sulfate supplementation<sup>[6,9]</sup>. Iron sulfide storage can also be a product of an acquired inherent defect in macrophage metabolism. In that regard, the pigment present in the duodenal mucosa has also been shown to be partially associated with impaired macrophage metabolism of drugs containing cyclic compounds such as phenols, indoles and skatoles<sup>[8]</sup>.

All four patients had undergone previous gastroduodenal endoscopy without any pathological findings, suggesting that this condition might be acquired rather than congenital, which is in keeping with previous reports<sup>[5]</sup>. Importantly, it must be stressed that this entity might be identified histologically even before it becomes endoscopically visible, making it difficult to establish a temporal association between disease onset and endoscopic manifestation<sup>[10]</sup>.

In this report, all patients were female, with chronic renal failure, and taking antihypertensive drugs. Of note, only two patients were taking oral iron supplements. The biopsy specimens were positive for hematoxylin and eosin and Masson-Fontana stains but not reactive for Pearl's stain, suggesting a melanin-like compound. Although Pearl's stain is a classic method for demonstrating iron in tissues, there is a possibility of a false-negative reaction if an iron oxide compound is present instead of iron sulfide<sup>[11,12]</sup>.

In conclusion, these findings suggest that the duodenal involvement can occur in the absence of a history of oral iron supplementation. Importantly, although the

long-term clinical impact of these depositions remains unclear, these endoscopic findings still do not require any specific treatment or recommended follow-up.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

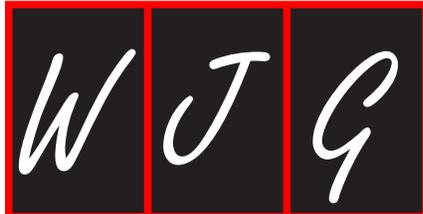
October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States



## GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the “priority” and “copyright” of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers’ names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid

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### Name of journal

*World Journal of Gastroenterology*

## Instructions to authors

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

### Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read “Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest” from International Committee of Medical Journal Editors (ICMJE), which is available at: [http://www.icmje.org/ethical\\_4conflicts.html](http://www.icmje.org/ethical_4conflicts.html).

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is <http://www.clinicaltrials.gov> sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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**Title:** Title should be less than 12 words.

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**Authorship:** Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically

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**Institution:** Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

**Author contributions:** The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

**Supportive foundations:** The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

**Correspondence to:** Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. [montgomery.bissell@ucsf.edu](mailto:montgomery.bissell@ucsf.edu)

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### Abstract

There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections.

## Instructions to authors

AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g.  $6.92 \pm 3.86$  vs  $3.61 \pm 1.67$ ,  $P < 0.001$ ; CONCLUSION (no more than 26 words).

### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

### Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: [http://www.wjgnet.com/1007-9327/g\\_info\\_20100315215714.htm](http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm).

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: ...*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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beled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

### Acknowledgments

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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*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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*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 dis-

eases. *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/index.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

## Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

## Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 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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## Pancreatic cancer: Translational research aspects and clinical implications

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### Abstract

Despite improvements in surgical techniques and adjuvant chemotherapy, the overall mortality rates in pancreatic cancer have generally remained relatively unchanged and the 5-year survival rate is actually below 2%. This paper will address the importance of achieving an early diagnosis and identifying markers for prognosis and response to therapy such as genes, proteins, microRNAs or epigenetic modifications. However, there are still major hurdles when translating investigational biomarkers into routine clinical practice. Furthermore, novel ways of secondary screening in high-risk individuals, such as artificial neural networks and modern imaging, will be discussed. Drug resistance is ubiquitous in pancreatic cancer. Several mechanisms of drug resistance have already been revealed, including human equilibrative nucleoside transporter-1 status, multidrug resistance proteins, aberrant signaling pathways, mi-

croRNAs, stromal influence, epithelial-mesenchymal transition-type cells and recently the presence of cancer stem cells/cancer-initiating cells. These factors must be considered when developing more customized types of intervention ("personalized medicine"). In the future, multifunctional nanoparticles that combine a specific targeting agent, an imaging probe, a cell-penetrating agent, a biocompatible polymer and an anti-cancer drug may become valuable for the management of patients with pancreatic cancer.

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### INTRODUCTION

Pancreatic cancer has an approximate incidence of 11.4/100 000 inhabitants per year, and is recognized as the fourth cause of cancer-related death, with an overall 5-year survival of less than 1%-2%<sup>[1-3]</sup>. Total costs, including care-related costs and loss of production (due especially to premature death) related to pancreatic cancer in Sweden in the year 2009 were 86-93 million euros (population 9.1 million), corresponding to a society cost in the West of up to 10 million euros per 1 million inhabitants per year<sup>[4]</sup>. Smoking and also family history (in about 5%-10% of cases) are established risk factors for the development of pancreatic cancer<sup>[5]</sup>. There is a weaker positive associa-

tion for other factors including obesity, diabetes mellitus, chronic pancreatitis, ABO genotype, race, periodontal disease, occupational exposures, dietary factors, *Helicobacter pylori* and gallstones<sup>[5,6]</sup>. It is to be stated that the median age at diagnosis is in general 66-68 years<sup>[7]</sup>, though early onset pancreatic cancer, i.e., occurring prior to 50 years of age, accounts for less than 6% of patients and is associated with more advanced disease at presentation and a tendency for shorter overall survival<sup>[8]</sup>. Gender-specific differences in the incidence of pancreatic cancer have been observed, including higher rates in males<sup>[9]</sup>.

Chemotherapy and to a lesser extent, radiotherapy, have emerged as valuable adjuncts to the management of pancreatic cancer. A few studies reported that “marginally resectable” pancreatic tumors shrink after radiochemotherapy and may become resectable<sup>[10-12]</sup>. Neoadjuvant treatment of resectable pancreatic cancer is associated with fewer positive lymph nodes and increased survival (median 34 mo *vs* 19 mo,  $P = 0.03$ )<sup>[13]</sup>. In the ESPAC-1 study, 6 mo of postoperative 5-fluorouracil (5-FU) and folinic acid (FA) increased median survival from 14 mo to 19.7 mo, but there was no effect provided by radiochemotherapy<sup>[14]</sup>. Long-term follow-up after adjuvant chemotherapy demonstrated even better results with a median 21-23 mo survival following adjuvant chemotherapy *vs* 8-16 mo for observation<sup>[15,16]</sup>. The validity of gemcitabine as an adjuvant agent has been confirmed<sup>[17]</sup>. The ESPAC-3 study reported similar outcomes between 5-FU and FA *vs* gemcitabine ( $n = 1088$ )<sup>[18]</sup>. In unresectable pancreatic cancer, most regimens are also gemcitabine-based. The use of gemcitabine has increased median survival from 3-4 mo to 5.5-7 mo<sup>[19-21]</sup>. Recently, FOLFIRINOX (oxaliplatin, irinotecan, leucovorin, fluorouracil) surpassed the effectiveness of gemcitabine by showing longer survival (11.1 mo *vs* 6.8 mo;  $P < 0.001$ )<sup>[22]</sup>. The utilization of molecular targeted treatment in pancreatic cancer outside of clinical trials has been limited. Erlotinib provided a modest survival benefit in advanced pancreatic cancer when used in combination with gemcitabine (6.2 mo *vs* 5.9 mo)<sup>[23]</sup>, but due to increased side-effects and increased costs it has not received wide clinical acceptance.

This paper will focus on clinical and molecular aspects of pancreatic cancer, discussing novel ways to improve early detection and prognostic prediction, as well as the design of future targeted therapy, which is imperative in this era of personalized medicine.

## MOLECULAR PATHOGENESIS

Pancreatic ductal adenocarcinoma (PDAC) is believed to arise from precursor lesions that develop into invasive carcinoma through a multistep carcinogenic process. Pancreatic intraepithelial neoplasia (PanIN) is the most common preneoplastic lesion in patients with pancreatic cancer, being observed in approximately 80% of cases<sup>[24]</sup>. Other precursor lesions of PDAC are intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN). The *KRAS* oncogene is the most commonly altered gene in pancreatic cancer. Inactivation

of the tumor-suppressor genes *CDKN2A*, *TP53*, *DPC4* and *BRC42*, as well as chromosomal losses, gene amplifications and telomere shortening have also been observed<sup>[6]</sup>. Reactivation of developmental pathways, such as hedgehog, notch and wnt/ $\beta$ -catenin, may be crucial for the development of PDAC<sup>[25]</sup>. In addition to genetic alterations, many lines of evidence indicate that epigenetic changes play a role in pancreatic carcinogenesis. DNA methylation and histone modification frequently alter gene function without changing the DNA sequence, and have the potential to be used as diagnostic markers in pancreatic cancer<sup>[26]</sup>. MicroRNAs are non-coding segments of RNA that can regulate gene expression. Aberrant expression of microRNAs contributes to tumor progression and has been associated with drug resistance<sup>[27]</sup>.

Because all three precursor lesions of PDAC possess ductal characteristics, it has been suggested that the lesions develop from ductal cells. However, the study of mouse models of pancreatic cancer has broadened the current understanding of pancreatic carcinogenesis by showing other cells to be the cancer-initiating cells. Differentiated acinar cells have been shown to cause PanIN and pancreatic cancer following activation of *KRAS in vivo*<sup>[28-30]</sup>. Moreover, insulin-positive endocrine cells and PDX1-expressing cells have been demonstrated to induce PDAC<sup>[31]</sup>. It should be noted that the cell of origin, in which tumorigenesis is initiated, could be different from the cancer stem cell, which propagates the tumor<sup>[32]</sup>. Identification of cells of origin in PDAC may allow earlier detection of malignancy and better preventive and treatment tools.

## Crosstalk tumor-stroma

Desmoplasia is a characteristic feature of pancreatic cancer and the stromal compartment has been considered to be a physical barrier for drug delivery<sup>[33]</sup>. The pancreatic stellate cell (PSC) has a key role in stroma formation. In addition to endogenous quiescent PSCs, bone marrow may also contribute to the population of activated PSCs<sup>[34]</sup>. PSCs are involved in tumor growth, invasion, metastasis and resistance to radiochemotherapy<sup>[35-37]</sup>. Furthermore, PSCs accompany cancer cells to distant metastatic sites, stimulate angiogenesis and have the capacity to migrate over the endothelial barrier to and from blood vessels<sup>[38]</sup>. A limited number of studies have attempted to block PSC activity in the setting of pancreatic cancer. For example, halofuginine, a smad3-phosphorylation-inhibitor, reduces PSC activation and prevents pancreatic xenograft tumor development<sup>[39]</sup>. Retinoic acid can also inhibit PSC activity and reduces wnt- $\beta$ -catenin signaling in cancer cells and their invasive ability<sup>[40]</sup>. Key signaling pathways between PSCs and cancer cells have been identified and involve e.g., sonic hedgehog, galectins, endothelins and platelet-derived growth factor<sup>[35]</sup>, which thereby represent potential therapeutic targets.

## Cancer stem cells and epithelial-mesenchymal transition

Pancreatic cancer stem cells constitute a minority of cancer cells (1%-5%) and have the ability to self-renew, and

are resistant to chemotherapy and radiation<sup>[41]</sup>. They are characterized by several surface markers including CD44, CD24, epithelial specific antigen, aldehyde dehydrogenase, CD133 and CXCR4<sup>[42]</sup>. Furthermore, it has been observed that pancreatic cancer cells that were cultured in gemcitabine demonstrated characteristics of epithelial-mesenchymal transition (EMT)<sup>[43]</sup>. They also showed increased expression of cell surface proteins associated with cancer stem cells. In pancreatic cancer xenografts, radiation or gemcitabine therapy leads to enrichment of the EMT cells<sup>[44]</sup>. Wnt, notch and hedgehog are important signaling events in cancer stem cells, and can become novel therapeutic targets<sup>[41]</sup>. Ongoing clinical trials are currently investigating PRI-724 (inhibitor of wnt), MK-0752 (inhibitor of notch) and GDC-0449 (inhibitor of hedgehog) in patients with advanced pancreatic cancer ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). Future therapeutic strategies may need to combine targeting of cancer stem cells and EMT cells with the targeting of other cells in the microenvironment, e.g., stromal cells, in order to achieve maximal benefit.

### Pro-inflammatory response

Inflammation is closely related to the development and progression of pancreatic cancer, and molecular factors such as STAT3 have been suggested to play a key role in creating a pro-inflammatory tumor microenvironment<sup>[45]</sup>. Clinical studies have shown that a pro-inflammatory response is both prognostically negative and promotes tumor proliferation<sup>[46]</sup>. Inflammatory factors may also contribute to the profound weight loss and cancer cachexia frequently seen in pancreatic cancer<sup>[47]</sup>. Elucidation of the mechanisms underlying the crosstalk between inflammation, cancer and stroma may improve the management of pancreatic cancer, as a frequent desmoplastic reaction is noted.

### Chemoresistance

Gemcitabine has represented the first-line of chemotherapeutic agents in pancreatic cancer. A frequent problem, though, is drug resistance and lack of response to therapy given. Nucleoside transporters, such as human equilibrative nucleoside transporter-1 (hENT-1), appear to regulate the intracellular uptake of gemcitabine<sup>[48]</sup>. One of the proposed mechanisms of chemoresistance is a reduction in hENT-1 expression. Determination of hENT-1 status at the time of cancer diagnosis, and also modifications of gemcitabine in order to bypass the nucleoside receptor, may represent novel types of targeted approaches in the management of patients with pancreatic cancer<sup>[48]</sup>. Multidrug resistance (MDR) proteins including ABC-transporters have also been implicated in drug resistance in pancreatic cancer and limit the efficacy of gemcitabine<sup>[49]</sup>. Another mechanism that contributes to chemoresistance is the tumor microenvironment surrounding the cancer cells, including cancer stem cells, EMT cells and stellate cells. Furthermore, the hypoxic stroma could be a physical barrier preventing chemotherapeutic drugs from

reaching pancreatic cancer cells, and depletion of the stroma could enhance cancer drug delivery<sup>[33]</sup>. Aberrant signaling pathways also have a role in drug resistance. The PI3K/Akt signaling pathway is commonly overactive in pancreatic cancer. PI3K stimulates proliferation and confers chemoresistance<sup>[50]</sup>. MicroRNAs have received increased attention in recent years. Targeting of microRNAs may help overcome drug resistance in pancreatic cancer and improve clinical outcome<sup>[27]</sup>.

## BIOMARKERS

Biomarkers can be applied in several areas of disease management including diagnosis, prognosis, staging and prediction and monitoring of therapeutic response. The different types of biomarkers include genes, proteins, metabolites, microRNAs and epigenetic modifications. CA 19-9 has some value for detection of recurrent disease<sup>[51]</sup>, but so far no other biomarker is recommended for routine clinical use in pancreatic cancer. Recently, a seven-gene panel was identified as being differentially expressed between pancreatic cancer ( $n = 36$ ) and normal samples ( $n = 19$ )<sup>[52]</sup>. Validation using two blood-based biomarkers from this panel, tenascin C and tissue factor pathway inhibitor, yielded a combined area under the curve (AUC) of 0.88 and, with addition of CA19-9, a combined AUC for the three-gene panel of 0.99 with 100% specificity at 90% sensitivity and 97% sensitivity at 90% specificity.

Proteomic profiling of pancreatic cancer serum has been promising. Most studies have used surface enhanced laser desorption (SELDI) or matrix assisted laser desorption/ionization (MALDI) yielding a sensitivity in the range of 78% to 100% and a specificity between 74% and 100%<sup>[53]</sup>. Immunohistochemistry (IHC) is the most practical method for evaluating protein expression changes in histopathology. It can be combined with tissue microarray technology to allow rapid testing of immunohistochemical markers on many tumors in a single experiment. During the past decade, a multitude of immunohistochemical biomarkers that are potentially involved in pancreatic carcinogenesis and drug responsiveness have been studied for their prognostic and predictive value, but none of them have yet proved to be sufficiently useful for use in routine clinical practice<sup>[54]</sup>. Apart from the tumor compartment, stromal tissue may also be analyzed and it has been discovered that stromal secreted protein acidic and rich in cysteine has been associated with outcome in pancreatic cancer<sup>[55]</sup>. A panel of IHC markers may prove clinically valuable in the future. Furthermore, metabolomic studies of pancreatic cancer are promising and may be useful in identifying benign from malignant conditions<sup>[56-58]</sup>. MicroRNA is a new class of biomarkers. Aberrant expression of miRNA-21 and miRNA-34a has been associated with survival in resectable pancreatic cancer<sup>[59]</sup>. Epigenetic changes, such as histone modification, may be used as novel biomarkers in pancreatic cancer<sup>[60]</sup>.

Although a multitude of investigational biomarkers have been identified, translation into routine clinical

practice has been difficult. To improve methodological reporting several guidelines have been developed. For diagnostic biomarkers, the STAndards for Reporting Diagnostic accuracy (STARD) guidelines are available<sup>[61]</sup>. For prognostic studies, the REporting recommendations for tumor MARKer prognostic studies (REMARK) guidelines are available<sup>[62]</sup>. The process of translating biomarkers is complex and the path from discovery to clinical application may be long and arduous. The effective demonstration of clinical utility of the biomarker will remain the key to its gaining widespread acceptance, but regulatory issues and budgetary constraints of the biomarker industry remain major challenges<sup>[63]</sup>.

## IMAGING

The detection of precursor lesions of pancreatic cancer would be a key factor in improving the prognosis. Non-invasive imaging techniques such as ultrasound, computed tomography and magnetic resonance imaging do not accurately identify PanINs. Positron emission tomography (PET) is a functional imaging modality that utilizes the principle that metabolic alterations in tumors occur prior to notable morphological alterations. The radioactive tracer <sup>18</sup>F-fluorodeoxyglucose (FDG) has been used extensively for PET imaging of malignant tumors. Malignant tissue has increased glucose metabolism as compared to its surrounding tissue, which leads to focal FDG-uptake visualized by PET. PET/CT has come to play an increasing role in pancreatic cancer, due to the ability to accurately detect small primary pancreatic lesions and distant metastases, as well as recurrences following surgery<sup>[64-67]</sup>. It has been shown that an elevated glucose metabolism occurs already in precursor lesions of pancreatic cancer, with the opportunity of detecting these changes with PET/CT, and thus improving diagnosis and outcome<sup>[68]</sup>. Eser *et al*<sup>[69]</sup> recently described a technique that could improve diagnosis and also grading of PanINs using *in vivo* molecular imaging based on catepsins.

## PANCREATIC CANCER AND DIABETES MELLITUS

Up to 80% of patients with pancreatic cancer have diabetes mellitus or pathologic glucose tolerance test at diagnosis<sup>[70]</sup>. Long-standing type II diabetes is a predisposing factor for pancreatic cancer, while new-onset diabetes may indicate subclinical cancer<sup>[71]</sup>. The molecular mechanisms linking long-standing diabetes to pancreatic cancer are incompletely understood. Diabetes may promote the neoplastic process by several mechanisms including hyperinsulinemia (endogenous or exogenous), hyperglycemia and chronic inflammation<sup>[72]</sup>. The insulin and insulin-like growth factor (IGF) receptors are frequently expressed in pancreatic cancer and contribute to neoplastic growth and progression<sup>[73]</sup>. The administration of the anti-diabetic agent metformin may reduce the incidence

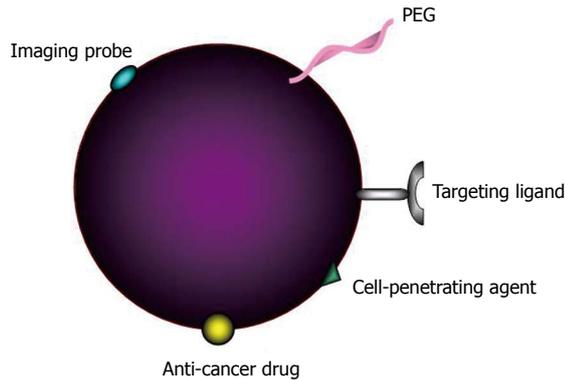
of pancreatic cancer in patients with type II diabetes<sup>[74,75]</sup>. In xenograft models, metformin inhibits the growth of pancreatic cancer cells by interfering with insulin/IGF-1 receptor and G-protein-coupled receptor signaling<sup>[76]</sup>. In addition, metformin can inhibit tumor growth by inactivating cancer stem cell-like cells<sup>[77]</sup>. Studies have sought to elucidate molecular alterations that link diabetes and cancer, and one such molecular connection could be TCF7L2 (T-cell factor 7-like 2) and p53<sup>[78]</sup>. In a recently published case-control study, rs780094 was selected as one of 10 diabetes-associated single-nucleotide polymorphisms related to increased pancreatic cancer risk<sup>[79]</sup>. However, diabetes in pancreatic cancer is mostly new-onset, i.e., occurring 24 mo prior to cancer diagnosis, and is likely related to secondary effects from the tumor, which is supported by the observation that glucose metabolism is improved following tumor resection<sup>[71,80]</sup>. Although the exact mechanisms behind pancreatic cancer-induced diabetes are yet to be disclosed, there is ample evidence for a tumor-derived influence on glucose metabolism, leading to disturbed  $\beta$ -cell function, peripheral insulin resistance, hyperglycemia and finally diabetes mellitus<sup>[70]</sup>.

## SCREENING

Pancreatic cancer develops over a long time span, providing a strong rationale for developing techniques for early detection. It may take ten years or more between the initial mutation and first non-metastatic tumor cell, and another five years for the development of metastatic capacity and death after an additional two years<sup>[81]</sup>. This implies a therapeutic window of opportunity for both early diagnosis and treatment. Chromothripsis is a new concept that involves the simultaneous acquisition of multiple mutations in a single catastrophic event. This phenomenon may be present in 2%-3% of all human cancers, but the incidence may be higher in certain tumors, such as osteosarcomas and chordomas<sup>[82]</sup>.

Patients with pancreatic cancer usually have generic symptoms and are often difficult to diagnose at an early stage. There are several risk groups where secondary screening for pancreatic cancer may be appropriate, e.g., patients with heredity, IPMN, or new-onset diabetes mellitus<sup>[70,83-85]</sup>. Distinguishing pancreatic cancer-associated diabetes from the more common general type 2 diabetes may identify individuals with a potentially resectable pancreatic cancer<sup>[70-71]</sup>. Huang *et al*<sup>[86]</sup> identified vanin-1 and matrix metalloproteinase 9 as useful biomarkers for the discrimination of pancreatic cancer-associated diabetes from type II diabetes.

Artificial neural networks represent non-linear pattern recognition techniques that simulate the analytic processes of the human brain. They have been utilized in complex medical decision-making, including diagnosis, prognosis and risk stratification<sup>[87]</sup>. A major benefit of these networks is the ability to recognize complex relationships between input and output data that may be hidden to conventional statistical methods. Initial reports on the use of artificial neural networks combined with proteomic



**Figure 1 Multifunctional nanoparticle.** PEG: Polyethylene glycol.

data have provided promising results concerning the detection of pancreatic cancer<sup>[88]</sup>. The future application of artificial neural networks based on parameters including age, smoking, heredity, chronic pancreatitis, new-onset diabetes mellitus, biomarkers and imaging findings imply promise for early detection of pancreatic cancer, and may be used as screening tools.

## NANOMEDICINE

Nanomedicine is defined as the application of nanotechnology to medicine. Nanoparticles are in the range of 1-100 nm. Examples of nanoparticles include liposomes (phospholipid vesicles), dendrimers (synthetic polymers), carbon nanotubes (fullerene), quantum dots (colloidal fluorescent semiconductor nanocrystals), magnetic nanoparticles (spherical nanocrystals with a  $Fe^{2+}$  and  $Fe^{3+}$  core) and gold nanoparticles (metallic nanoparticles). The application of nanoparticles in medicine include e.g., diagnostics, imaging and drug delivery.

Nanoparticles enable refined diagnostics at the level of single cells and molecules. For example, magnetic nanoparticles have been coupled with molecular targeting ligands to improve imaging of early pancreatic tumors *in vivo*<sup>[89]</sup>. Quantum dots conjugated with RGD peptides have been reported for *in vivo* imaging of pancreatic tumor vasculature<sup>[90]</sup>. Drug resistance is a recognized challenge in pancreatic cancer. Gemcitabine-squalene obtained by covalently coupling gemcitabine at the 4-amino group with squalene, a natural lipid, has been shown to make tumor cells more sensitive to gemcitabine<sup>[91]</sup>. Recently, polymeric nanoparticles encapsulating hedgehog-inhibitors or curcumin have been produced that inhibit the growth of orthotopic pancreatic cancer xenografts<sup>[92,93]</sup>. Gold nanoparticles have been utilized to induce intracellular hyperthermia in a murine model of pancreatic cancer after radiofrequency field exposure<sup>[94]</sup>. An ongoing phase I study (NCT00968604) of advanced pancreatic cancer is currently investigating the effects of intravenous injection of the liposome nanoparticle BikDD, which contains a pro-apoptotic agent. Several nanoparticle-based anticancer drugs are already on the market, e.g., Abraxane<sup>®</sup> (albumin-bound paclitaxel), Myocet<sup>®</sup> (liposomal doxorubicin) and

Oncaspar<sup>®</sup> (PEG-L-asparaginase)<sup>[95]</sup>.

While monofunctional nanoparticles only carry out one function, multifunctional nanoparticles have the ability to perform several tasks. Multifunctional nanocarriers using a specific targeting agent, an imaging probe, a cell-penetrating agent such as TAT peptide, a biocompatible polymer such as polyethylene glycol (PEG) and an anti-cancer drug, may result in effective tumor destruction with minimal toxicity (Figure 1).

## CONCLUSION

Pancreatic cancer is a condition with an almost total lethal outcome. Despite advancement in surgical techniques and adjuvant treatment, the prognosis has only marginally improved. Novel therapeutic interventions have been tested but with limited effect. Research should continue to focus on biomarkers for early diagnosis, prognosis and prediction and monitoring of therapeutic response. Screening of high-risk individuals using novel approaches such as artificial neural networks could be considered. Mechanisms of chemoresistance have been elucidated, including hENT-1 status, MDR proteins, aberrant signaling pathways, microRNAs and micro-environmental factors, which should underlie future development of targeted therapy. The identification of cancer-initiating cells represents a fundamental shift in our understanding of the intrinsic drug resistance of pancreatic cancer. Multifunctional nanoparticles have the potential to combine imaging, diagnosis and therapy in a single vehicle. It is expected that nanomedicine will have a prominent role in the quest for a successful therapy for this recalcitrant disease.

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## Worldwide epidemiology of liver hydatidosis including the Mediterranean area

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### Abstract

The worldwide incidence and prevalence of cystic echinococcosis have fallen dramatically over the past several decades. Nonetheless, infection with *Echinococcus granulosus* (*E. granulosus*) remains a major public health issue in several countries and regions, even in places where it was previously at low levels, as a result of a reduction of control programmes due to economic problems and lack of resources. Geographic distribution differs by country and region depending on the presence in that country of large numbers of nomadic or semi-nomadic sheep and goat flocks that represent the intermediate host of the parasite, and their close contact with the final host, the dog, which mostly provides the transmission of infection to humans. The greatest prevalence of cystic echinococcosis in human and animal hosts is found in countries of the temperate zones, including several parts of Eurasia (the Mediterranean

regions, southern and central parts of Russia, central Asia, China), Australia, some parts of America (especially South America) and north and east Africa. Echinococcosis is currently considered an endemic zoonotic disease in the Mediterranean region. The most frequent strain associated with human cystic echinococcosis appears to be the common sheep strain (G1). This strain appears to be widely distributed in all continents. The purpose of this review is to examine the distribution of *E. granulosus* and the epidemiology of a re-emerging disease such as cystic echinococcosis.

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**Key words:** Epidemiology; *Echinococcus granulosus*; Cystic echinococcosis

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### INTRODUCTION

Cystic echinococcosis (CE) is a near-cosmopolitan zoonosis caused by adult or larval stages of tapeworms (cestodes) belonging to the genus *Echinococcus* (family Taeniidae). Actually, six species of *Echinococcus* have been recognized, but the most important members of the genus in respect of their public health importance and their geographical distribution are *Echinococcus granulosus* (*E. granulosus*) (which causes cystic echinococcosis) and *Echinococcus multilocularis*

(which causes alveolar echinococcosis). Infection with *E. granulosus* results in the development of one or several unilocular hydatid cysts that in humans develop mainly in the liver (70%), but also lungs (20%) and 10% of cysts can occur almost anywhere in the body (e.g., brain, body musculature, wall of the heart, kidneys, orbit of the eye, marrow cavity of bones). *E. multilocularis* metacestodes develop as a series of small, interconnected cysts, growing as a metastasising lesion almost exclusively in the liver (98%-100%), but in the later phase of infection distant metastases in other organs may occur.

*E. multilocularis* is a cestode whose life cycle involves a tapeworm stage during which it lives in the small intestine of carnivores (definitive hosts, usually wild or domestic canids), and a tissue-invading metacestode (larval) stage during which echinococcal cysts develop in internal organs (mainly liver and lungs) of humans and other intermediate hosts as unilocular fluid-filled bladders surrounded by a host-produced layer of granulomatous adventitial reaction. Small vesicles called brood capsules bud internally from the germinal layer and produce multiple protoscolices by asexual division. In humans, the slowly growing echinococcal cysts may reach a volume of several litres and contain many thousands of protoscolices. Moreover, internal septations and daughter cysts may appear over time, disrupting the unilocular pattern typical of the young echinococcal cysts.

Infection of an intermediate host is due to accidental ingestion of tapeworm eggs passed into the environment with faeces from definitive hosts. Transmission of *E. granulosus* could be due to domestic and wildlife reservoirs, and is influenced by human activities, behaviour, and politics.

CE represents an increasing public health and socio-economic concern in many areas of the world<sup>[1-3]</sup> and is currently considered an endemic zoonosis in the Mediterranean region (MR), in addition to brucellosis, rabies, leishmaniasis and food-borne zoonotic infections<sup>[4]</sup>. Given a geographic distribution and extent greater than previously believed, several studies have shown that hydatidosis is currently considered an emerging or re-emerging disease<sup>[5,6]</sup>. The distribution and prevalence of CE depends on the presence in that country of large numbers of nomadic or semi-nomadic sheep and goat flocks that represent the intermediate host of the parasite, and their close contact with the final host, the dog, which mostly provides the transmission of infection to humans.

Molecular studies conducted on mitochondrial DNA (mtDNA) sequences, have shown that *E. granulosus* complex consists of three species and comprise ten defined strains (genotype G1-10), based on morphology, host specificity and molecular characteristics<sup>[7,8]</sup>. The intraspecific variants have substantial variation at the genetic level and DNA sequence<sup>[9]</sup>, conferring several characteristics such as life-cycle patterns, host specificity, development rate, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents, and pathology<sup>[10,11]</sup>. These characteristics may have important implications for the

design and development of vaccines, diagnostic reagents and drugs impacting on the epidemiology and control of echinococcosis<sup>[12,13]</sup>. Indeed, each *Echinococcus* species maintains a specific host-adapted genetic identity that only rarely overlaps in some geographical areas<sup>[5,11,14]</sup>.

In this review we discuss aspects of the current epidemiology of *E. granulosus* complex and highlight worldwide and specific distribution in recognised endemic areas.

## SPECIES AND DISTRIBUTION OF

### *E. GRANULOSUS* COMPLEX

*E. granulosus* has a worldwide geographical distribution with endemic foci present on every inhabited continent (Figure 1). The greatest prevalence of CE in human and animal hosts is found in countries of the temperate zones, including several parts of Eurasia (the Mediterranean regions, southern and central parts of Russia, central Asia, China), Australia, some parts of America (especially South America) and north and east Africa<sup>[2,15]</sup>.

The distinct genetic types of *E. granulosus* include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camelid strain (G6), a pig strain (G7), and a cervid strain (G8). A ninth genotype (G9) has been described in swine in Poland<sup>[8,16]</sup> and a tenth strain (G10) in reindeer in Eurasia. Among these strains, we have available data for preliminary epidemiological analyses only for some strains. In fact, some of them are still poorly characterised and further research is needed to determine with higher detail their host and geographic ranges and whether their genetic characteristics are conserved between different endemic regions.

The most frequent strain associated with human CE appears to be the common sheep strain (G1). This strain appears to be widely distributed in all continents. Highest rates of infection are recorded in communities involved in extensive sheep farming and epidemiological studies suggest that this genetic variant is the principal strain infecting humans<sup>[2,5,9,17]</sup>. Consequently, its presence coincides with areas which have high prevalence of human CE such as in Morocco, Tunisia, Kenya, Kazakhstan, western China and Argentina.

The G2 strain is known to be transmitted among sheep and infect humans also, but genetic differences biologically distinguish it from the G1 strain, conferring a different life cycle<sup>[18]</sup>. It has been found in Australia and previously also documented in Tasmania.

The G3 strain which is diffused among buffalos and transmitted by water, has been recorded in South Asia<sup>[19]</sup>, but no susceptibility among humans has been found.

The G4 strain, formerly known as *Echinococcus equinus*, appears to infect exclusively equines as intermediate hosts and no human cases have been documented<sup>[9,20]</sup>. It is known to be diffused in the Mediterranean regions of Spain, Italy, Lebanon, and Syria, as well as in South Africa.

The former cattle strain (G5), known as *Echinococcus ortleppi*, is transmitted by cattle in Europe, Asia, parts of

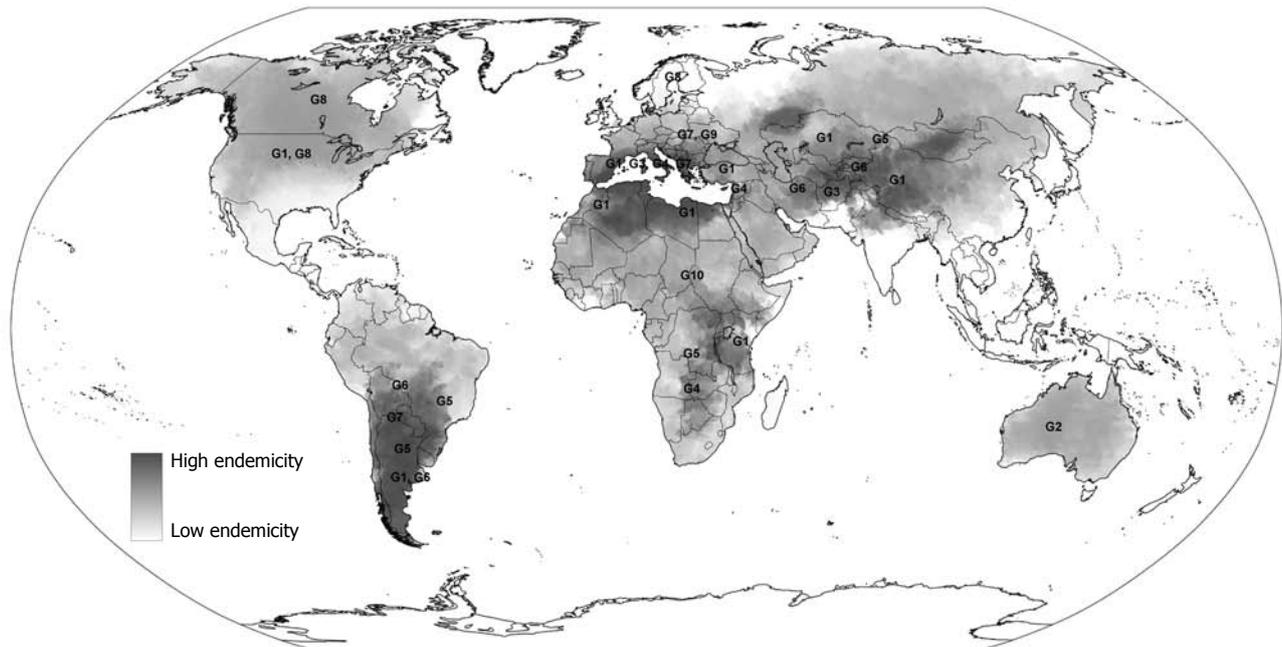


Figure 1 Worldwide distribution of the zoonotic strains of *Echinococcus granulosus* and geographical endemicity.

Africa and South America and only one case in humans has been isolated in past years<sup>[21]</sup>, suggesting a less pathogenic risk for humans than the sheep strain of *E. granulosus*.

G6-10 strains are poorly distinguished from each other but they are clearly distinct from the common sheep strain<sup>[5]</sup>. The G6 strain is known to principally affect camels and goats. Animal infection is diffused in the Middle East, Africa, southern Asia and South America<sup>[9]</sup> and cases of human infection have been found in Nepal, Iran, Mauritania, Kenya and Argentina<sup>[5,17]</sup>.

The G7 strain is transmitted by domestic pigs in Europe (Spain and Italy), Asia and South America, as well as the closely related genetic variant G9 that has been documented to affect Polish patients<sup>[16]</sup> although the animal reservoir is unknown.

The G8 strains are known to be transmitted between wolves and wild cervids in the northern regions of Europe, Asia and North America. Few cases of human infection have been documented with a lower severity of the disease than CE caused by other forms of *E. granulosus*<sup>[22]</sup>. However, transmission between humans of this genetic variant seems to be low and further data is needed to better assess its pathogenicity.

Finally, some other genetic variants which are poorly characterized have been found in several countries. For example, the wildlife “lion” strain transmitted among lions and wild ungulates has been documented in Africa but no human infection has been found.

## EPIDEMIOLOGY OF *E. GRANULOSUS* COMPLEX: WORLDWIDE DISTRIBUTION

### America

The most ubiquitous taxa of *E. granulosus* that occur in North America are the cervid strain (G8) and the sheep

strain (G1). The former is diffused in wildlife mainly in Canada, Alaska and Minnesota<sup>[23]</sup>. The wildlife reservoir was found to be largely diffused among cervids and wolves, coyotes and domestic dogs<sup>[24]</sup>. EC started to be diagnosed in Canada after the 1950s following the introduction of routine chest X-rays for tuberculosis in some tribes of native Americans (such as Indians and Eskimo) who were identified with pulmonary hydatidosis<sup>[24]</sup>. In the same period, a review of 101 autochthonous cases of *E. granulosus* infection in Alaska were documented<sup>[22]</sup>. It has been estimated that 50% of moose in Ontario and British Columbia are infected with the parasite<sup>[25]</sup> and that 28%-50% of dogs in the Canadian Northwest Territories are infected with *E. granulosus*<sup>[26]</sup>. In humans, pulmonary localization is quite diffuse. Indeed, a recent chart review performed in Alberta documented 22 definite and probable cases, of which 77% were female and 41% aboriginal; 40% had pulmonary involvement and 50% hepatic involvement<sup>[27]</sup>.

Sporadic autochthonous transmission among humans of the sheep strain in the western States of North America such as Arizona, California, New Mexico and Utah has been documented in reports from the 1960s<sup>[28]</sup>. The source of these *E. granulosus* infections was Australian sheep dogs imported into Utah in 1938 when the parasite diffused among sheep of this area as well as adjoining states through trading of live sheep<sup>[29]</sup>. Moreover, another source of infection were immigrants from countries in which echinococcosis disease is highly endemic, historically Icelanders, Italians and Greeks, but in more recent years, mostly persons of Middle Eastern and Asian origin. After the Second World War, foci of transmission involving swine and dogs were reported in several areas such the Mississippi valley due to the close relationship between humans and dogs<sup>[30]</sup> but transmission of infection ap-

peared to have ended by mid-century<sup>[31]</sup>. Then, an epidemic focus of sheep and human infection in western states including California, Utah, New Mexico and Arizona in the mid-1960s was traced<sup>[28]</sup>. Most infections occurred in high-risk groups such as sheep farmers and those involved in home slaughter including Basque-Americans in California<sup>[32]</sup>, Mormons in central Utah<sup>[33]</sup>, and Navajo and Zuni Indians in New Mexico and Arizona<sup>[33-35]</sup>.

As well as in the United States, all genetic variants of the *E. granulosus* complex have been introduced into South America with domestic animals imported from other regions, such as Europe. The principal strain of *E. granulosus* is the sheep strain (G1), widely diffused in Peru, Chile, Argentina and Brazil<sup>[2]</sup>.

In the central Peruvian Andes, the prevalence of hydatidosis in livestock has been noted to be 89% in sheep and 80% in cattle in a livestock raising community<sup>[36]</sup>. Among definitive hosts, the prevalence of infection in dogs in endemic areas has been reported to range from 32% to 46%<sup>[36-38]</sup> and from 46% to 88%<sup>[38-40]</sup>. The recorded surgical incidence of CE in the central and southern Peruvian Andes has been noted to be 1-2 cases per 100 000 inhabitants<sup>[36]</sup> and the prevalence of asymptomatic CE between 3% and 9.3% in rural villages in the central Peruvian highlands<sup>[38]</sup>. However, a study in a coastal city of Peru reported an annual surgical incidence of 32 per 10 000 for 1998<sup>[37]</sup> leading to the conclusion that incidence of CE is significantly under-reported. Recently, a re-emergence of transmission has been documented after the failure of previous control activities<sup>[41]</sup>.

Chile is an endemic area for *E. granulosus* infection. During 2000, the prevalence of bovine, sheep and canine hydatidosis for the entire country decreased to 22.3%, 6.3% and 11%, respectively<sup>[42]</sup>, after a control program<sup>[2,43,44]</sup>. With regard to human infection, although the overall incidence of diagnosed disease has been assessed as 2-2.5 cases per 100 000 inhabitants between 1992 and 2004, taking under-notification into account, the incidence has been estimated at 10 per 100 000. A major endemic area for EC is the southern part of Chile where annual surgical incidence ranged from 6 to 20 cases per 100 000 in August 2005 but reaching 162 per 100 000 in some regions<sup>[45]</sup>.

In Argentina, several strains of *E. granulosus* and *E. ortleppi* have been found in different host animals and humans such as the sheep strain (G1) (mostly infecting humans), the Tasmanian sheep strain (G2), the cattle strain (G5) and the camel strain (G6)<sup>[14,46]</sup>, while the pig strain (G7) has been detected in pigs and dogs but not humans<sup>[46]</sup>. The prevalence of EC affecting livestock has been documented as reaching 7% of cattle, 12.5% of sheep, 9.8% of pigs and 6.0% of goats<sup>[2]</sup>. In humans, prevalence rates depend on the endemicity of the area, ranging from 1.4 per 100 000 to 404, 260 and 30 cases per 100 000 in Neuquen, Chubut and Rio Negro (regions of Patagonia), respectively<sup>[42]</sup>.

In southern Brazil only sheep strain and *E. ortleppi* have been recorded<sup>[47,48]</sup>, although the most endemic area is the southern part of Brazil. Indeed, a recent analysis of

hydatidosis prevalence in animals in this area reported a prevalence of infection to be 25.5% of cattle, 30.2% of sheep<sup>[42]</sup> and from 11.4% to 38% of dogs<sup>[49]</sup>. Data about human hydatidosis documented a seroprevalence of 6% in the rural population and 3.5% in the urban population of Sena Madureira<sup>[50]</sup>. However, the few data available to allow conclusions on epidemiology of different taxa often depend on control activities that are inconsistent in their consideration of the economic and public health impact of echinococcosis in these areas.

## Australia

The most common strain currently found in Australia is the G1, while the G2 strain was previously also found in Tasmania<sup>[7,8,51]</sup>. This G2 strain probably evolved as a genetically modified variant after a Tasmanian hydatid control campaign aimed to strictly control helminthic diffusion among dogs. Thus, this genetic variant became dominant because of the limited gene pool on an island<sup>[18]</sup>. However, the absence of diffusion of the hydatid infection in wildlife and the intense hydatid control programmes allowed the eradication of *E. granulosus* from Tasmania in the middle 1990s<sup>[52]</sup>.

In Australia several areas have been documented at high risk of transmission of *E. granulosus*, especially in wildlife. The definitive hosts most commonly involved in transmission in south eastern Australia are represented by the wild dog<sup>[53,54]</sup>, while the most common intermediate hosts are grey kangaroos and wallabies<sup>[54]</sup>. Western Australia, south of Perth, is another active area of transmission of *E. granulosus*<sup>[55]</sup>. In this region, similar intermediate hosts have been found<sup>[10]</sup> while in northern Western Australia the source of infection has yet to be confirmed<sup>[56]</sup>.

However, wildlife reservoirs play the main role in maintaining a constant source of transmission for domestic livestock, domestic dogs and humans<sup>[53,54,57-60]</sup>. Recent analyses assessed infection in wild dogs caught in the outer suburbs of Townsville, Queensland<sup>[61]</sup>, and in those examined from the Maroochy Shire, eastern Queensland<sup>[62]</sup>. Sheep infection is still common in farms with a high number of poorly managed domestic dogs; additionally livestock are often hunted by wild dogs contaminating the pasture with eggs of *E. granulosus*<sup>[53]</sup>. However, dog and sheep infection prevalence seems to be decreasing over the last years<sup>[60]</sup>, although recent surveys reported a re-emergence of domestic transmission of *E. granulosus* in some rural areas of south eastern regions where it was found that 29% of 344 rural dogs in New South Wales and 18% of 218 Victorian dogs tested positive<sup>[63]</sup>.

Annually, new cases of human hydatidosis appear stable, numbering between 80 and 100 among the entire country<sup>[60,64]</sup>. Human transmission has traditionally been a public health problem of rural people due to *E. granulosus* infected domestic animals, but there is increasing potential for accidental exposure of urban residents due to the infiltration in urban centres by infected wildlife definitive hosts such as foxes and wild dogs. In fact, these animals are attracted to public recreation areas commonly fre-

quented by urban residents to scavenge food scraps<sup>[61,65]</sup>. Thus, urban residents could accidentally have direct contact with *E. granulosus* eggs through wild dog or fox faeces or *via* coprophagous flies when visiting parks and forests for recreational purposes. Furthermore, it has been documented that there has been a potential infection of the dogs of recreational pig hunters living in urban centres<sup>[66]</sup>.

The reporting of hydatidosis or echinococcosis does not depend on any monitoring system but only on individual case reports. Thus, assessing accurate prevalence and incidence, as well as trend changes over time, is still difficult to achieve.

### Western and Central Asia

The G1 strain, infecting sheep, goats, cattle and camels, is the most common genetic variant documented in Iran<sup>[67]</sup>. On the other hand, the G6 strain has also been found in camels, sheep and cattle in the same area<sup>[67]</sup>. Both of these were diagnosed in human hydatid infection confirming the pathogenicity of G6 for humans<sup>[67]</sup>.

In Kazakstan, it has been assessed that the prevalence of infection in sheep ranges between 20%-25% in 1-year-old sheep and 74%-80% in sheep 6 years old and over. Among wild and village dogs, the prevalence of infection is 23% and 6%, respectively<sup>[68]</sup>. Although the highest worm burdens have been recorded in rural dogs, only those closer to human habitation are responsible for transmitting disease to humans<sup>[68]</sup>. Human infection has increased since the middle 1990s till present time from 200 surgical cases annually to the current level of nearly 1000 cases per year<sup>[69,70]</sup>. Similar trends in human cases have been assessed in all other Central Asian countries. However, no detailed data is available about transmission and diffusion of *E. granulosus* infection in Central Asian countries.

Hydatidosis is a serious public health problem in Turkey where *E. granulosus* infection in dogs ranges between 0.32% and 40%<sup>[71]</sup>. The predominant genotype of *E. granulosus* in Turkey is the G1 strain with a prevalence infection rate in farm animals ranging from 26.6% to 50.9% in sheep, from 13.3% to 35.68% in cattle, and reaching 22.1% in goats, 44.31% in cows and 24.39% in bulls in the most endemic areas such the Budur region<sup>[72]</sup>, the Kirikkal region<sup>[73]</sup>, the Afyonkarahisar district<sup>[74]</sup>, and the Sivas region<sup>[75]</sup>. Lower rates in sheep (3.5%) and cattle (11.6%) have been found in less endemic areas such as Thrace region<sup>[76]</sup>. Surgical cases of human hydatidosis have been estimated to range from 0.87 to 6.6 per 100 000 inhabitants between 1987 and 1994<sup>[71]</sup>. A more recent survey based on hospital, regional and ministerial documents showed that, from 2001 to 2005, a total of 14 789 CE surgical cases were recorded with a higher incidence in the Middle Anatolian region (38.57%) and lower in the Black Sea region<sup>[77]</sup>.

Several regions of the Arab peninsula such as Syria, Israel and Palestine are considered endemic for *E. granulosus*. In fact, hydatidosis is mostly associated with main risk factors such as livestock production, raising of sheep

and nomadic tribal life that characterize northern Syria, northern Israel and western Palestine. Epidemiological evidence in Syria showed a prevalence of *E. granulosus* infection ranging between 9% and 15% in dogs and between 5% and 17% in livestock<sup>[78]</sup>; in Israel, ranging between 5.4% to 14.2% in dogs and between 4.56% and 10% in sheep<sup>[79,80]</sup>; and in Palestine, ranging between 7.9% and 14.3% in dogs<sup>[81]</sup>. Human infection rates have been assessed in individual studies. Annual surgical prevalence recorded from the Al-Maqased Hospital in Jerusalem was documented to be 1.76 per 100 000 inhabitants in the middle 1990s<sup>[81]</sup>, while in hospitals of the Palestinian West Bank this value was 3.1 per 100 000 inhabitants, with the highest rates of 4.9, 5.0 and 5.1 per 100 000 inhabitants found in Hebron, Jericho and Bethlehem, respectively<sup>[82]</sup>. In an epidemiological study conducted in northern Israel a cumulative infection rate of 1.5 per 100 000 inhabitants was found<sup>[83]</sup>, while in another study conducted in a Bedouin group from southern Israel this rate was 0.68%<sup>[83]</sup>.

### China

China is one of the most important endemic regions of CE<sup>[2]</sup>. The sheep strain (G1) and the camel strain (G6) are the only two *E. granulosus* strains found in China<sup>[84]</sup>, both of them infectious to humans<sup>[85]</sup>. The most endemic areas for *Echinococcus spp.* have been recognized as the provinces and autonomous regions stretching from western Xinjiang<sup>[86]</sup>, Ningxia and Inner Mongolia, with the highest prevalence rates occurring in pastoral communities of the eastern Tibetan plateau<sup>[87-89]</sup> (south western Qinghai and north western Sichuan) and the Tibetan autonomous area of south Gansu<sup>[90]</sup>, located in western and northwestern China<sup>[85,91-95]</sup>. Infection by cysts of *E. granulosus* can be found in organs of ungulate intermediate hosts<sup>[96-99]</sup>. High prevalence of hydatid infection has been reported in sheep and yaks (99%), cattle (88%) and pigs (70%)<sup>[90]</sup>. In fact, in the western and northwestern pastoral areas of China, livestock pastoralism is a major industry with a total of 350 million sheep and other domesticated large herbivores including horses, camels, and red deer<sup>[90]</sup>. On the other hand, the definitive host is mainly represented by canids, predominantly the domestic dog. Indeed, they are kept in large populations in northwestern China for pastoralism and cultural reasons<sup>[87]</sup>. Given the close contact with local people, dogs are considered the most important definitive host transmitting *E. granulosus* to humans<sup>[2,87]</sup>. However, in certain rural regions, wild canids such as wolves and foxes are involved in the sylvatic cycle<sup>[2]</sup>.

The first human CE was reported in China in 1905<sup>[86]</sup>. Over the last century, about 35 000 cases of human cystic echinococcosis have been treated surgically in China. However, given the documented 21 560 cases in Xinjiang alone with a prevalence of 80 cases/100 000 inhabitants<sup>[86]</sup>, it has been assessed that an underestimation occurred in past years. Now, it has been estimated that about one million existing cases of human echinococcosis occur in China<sup>[100]</sup>. Of these, about 70% present with chronic cystic lesions of the liver as well as in other organs includ-

ing the brain<sup>[101]</sup>. The infection rate of females has been assessed to be considerably higher than that of males because of their role in the home activities including feeding dogs, collecting yak dung for fuel, and milking livestock<sup>[87,102]</sup>. Thus, nomadic or seminomadic pastoral lifestyle is one of the most important risk factors for CE in China, especially in western and northwestern areas where livestock pastoralism is a major industry<sup>[90]</sup>, and women are more frequently exposed to the definitive hosts of CE. Consequently, adults have much higher infection rates than children<sup>[87]</sup>, and the infection rate increases with age<sup>[102]</sup>.

The increasing number of diagnosed cases may reflect improved diagnostic methods and improved outreach programs. In fact, China is now recognized as a new focus for echinococcosis research.

### Africa

Although most regions of Africa are poorly researched and limited information is available, several taxa have been found in the African countries<sup>[19,103,104]</sup>. The most common strain is the G1, highly diffused in the North and East African sheep raising areas. Moreover, the exclusive presence of the camel strain (G6) has been documented. In addition, wild strains such as the *E. equinus* (the "horse strain")<sup>[105]</sup> and the "lion strain"<sup>[106]</sup> have been found in South Africa. However, the nature of *Echinococcus* in African wildlife is poorly documented.

In a recent study carried out in Libya, 25.8% of stray dogs and 21% of owned dogs have been assessed to be positive for EC<sup>[107]</sup> while another study found a prevalence of 58% of hydatidosis in the same area<sup>[108]</sup>. Nevertheless, several surveys assessed that other animals also, especially camels, are frequently infected by *E. granulosus*<sup>[109,110]</sup>, while infection rates in livestock varied from 1.7% to 33.4% in sheep, 1.0% to 13.9% in cattle, 1.4% to 40.0% in camels and 0% to 18% in goats<sup>[111-113]</sup>, often associated with human cases<sup>[114]</sup>. The sheep strain has been considered the most common genetic variant diffused among humans in another survey, reporting a prevalence rate of 1.7% of 20 200 patients screened by ultrasound for hydatid cysts in 36 villages along the northern coast of Libya<sup>[115]</sup> and an incidence rate of 4.2 cases per 100 000 inhabitants in Eastern Libya<sup>[112]</sup>. Indeed, in a genetic survey conducted on 179 isolates from humans collected in the border area of northwestern Kenya and south-eastern Sudan, only one was associated with the camel strain (G6) while the remaining were the common sheep strain (G1)<sup>[117]</sup>. On the other hand, other surveys conducted in central Sudan<sup>[110,116]</sup> and Egypt<sup>[117]</sup> documented the presence of human echinococcosis cases diagnosed as G6 and at least two other distinct strains (camel and equine)<sup>[118]</sup>.

CE is currently of low endemicity in Egypt with a mean prevalence in dogs ranging between 3.2% in urban areas and 6% in rural areas<sup>[119]</sup>. Higher prevalence has been documented in Cairo with about 15% of dogs infected<sup>[120]</sup>. Among ruminants, confirming earlier re-

sults<sup>[121]</sup>, recent data demonstrated an overall prevalence infection rate of 0.3% in sheep and goats, 0.68% in pigs, 6.4% in cows and buffaloes, 2.53% in camels<sup>[121]</sup> and 10.62% in donkeys<sup>[122]</sup>. In humans, a retrospective hospital study showed an annual surgical incidence ranging between 1.34 and 2.60 per 100 000 inhabitants<sup>[123]</sup>.

In Tunisia, echinococcosis is a major public health problem due to its high prevalence and morbidity. Molecular analysis has demonstrated that the most common genetic variants of *E. granulosus* circulating in Tunisia are the G1 sheep strain and the G6 camel strain<sup>[124,125]</sup>. Sheep breeding is a significant risk factor, being practised by 94.7% of patients *vs* 58.3% of the farming population<sup>[126]</sup>. A series of studies carried out between 1999 and 2007 assessed that the prevalence of *E. granulosus* infection reached 10.41% in lambs (6-12 mo), 75.42% in sheep aged 1-2 years and 83.83 to 100% in sheep over 2 years old<sup>[127]</sup>; and 10.1% of camels<sup>[124]</sup> and 40% of sheep in a further analysis conducted in North-East Tunisia<sup>[128]</sup>. Despite the lack of recent published data, the last report of EC in humans reported an annual surgical incidence of hydatidosis of about 15 per 100 000 inhabitants<sup>[129]</sup>.

In Algeria similar strain distribution has been found, identifying the sheep strain G1 infecting sheep, cattle and humans and the camel strain G6 infecting camels<sup>[130]</sup>. Dogs likely represent the main source of infection for farm animals and humans<sup>[19]</sup> with a prevalence rate of 24.8% in camels, 13.9% in cattle and 6.0% in horses<sup>[131]</sup>. Despite poor data regarding recently reported human infection, it is documented that more than 700 surgical cases are notified each year to the Ministry of Health. Last published work assessed that the annual incidence of human EC reached 3.6-4.6 per 100 000 inhabitants<sup>[132]</sup>.

Morocco is considered an endemic area for echinococcosis. A genotype almost similar to the common G1 sheep strain with some nucleotide variations was found in camels and horses. Infection rate in dogs ranges from 22.0% to 62.8%, depending on the region<sup>[133]</sup>. In a more recent analysis, CE infection prevalence rates have been documented to be 10.58% in sheep, 1.88% in goats, 22.98% in cattle, 12.03% in camels and 17.80% in equines, mostly in Middle Atlas (48.72% in cattle) and in North West (37.61% in cattle and 31.65% in sheep)<sup>[134]</sup>. In humans, an annual rate of 4.55 surgical cases per 100 000 inhabitants has been documented in 2006, with a higher prevalence in the middle Atlas mountainous region<sup>[135]</sup>.

### Europe and the Mediterranean Basin

With the exception of Malta and the area controlled by the Government in southern Cyprus, where the disease has been practically eliminated, all the Mediterranean regions including the Arab peninsula countries are facing problems due to CE. Indeed, in Cyprus CE had an annual surgical incidence rate of 12.9 per 100 000 inhabitants before the first eradication program implemented in the 1970s and, subsequently, a second program in the 1990s<sup>[136]</sup>. In the northern part of Cyprus, disease rates

decreased from 1.95% in dogs examined in 1998-1999 to 0.012% in 2000-2003, from 23.58% to 6.61% in cattle, from 5.31% to 1.53% in sheep, while in goats rates were consistently below 0.5% and remained at 0.13%. On the other hand, the south part of Cyprus that maintained its control programme was able to keep positive testing levels at virtually 0%<sup>[137]</sup>.

In Europe, *E. granulosus* is present in most countries with the exception of Ireland, Iceland and Denmark. EC of animals is rare in northern and central Europe with the exception of cervid-transmitted echinococcosis in Finland and pig-transmitted echinococcosis in regions further east. The cervid strain in Finland was found to differ genetically from the previously described North American cervid strain G8, and was identified as a new strain, G10<sup>[138]</sup>. Transmission has been documented to occur mostly between wolves, reindeer and elks<sup>[139]</sup>.

The most endemic areas have been documented to be the Mediterranean regions where annual incidence rates for human CE of 4-8 per 100 000 have been reported, and parts of Eastern Mediterranean countries such as Bulgaria<sup>[2]</sup>. In some other eastern regions such as Poland, Slovakia and Ukraine, the pig strains (G6-G10) often occur as animal and sometimes human CE<sup>[140,141]</sup>. In Serbia and Montenegro the most frequent intermediate hosts for *E. granulosus* are pigs, with a percentage of infected animals ranging between 4.6% and 57.6%<sup>[142]</sup> but no information is available about human infection. Although several other countries such as Albania<sup>[78]</sup>, Bosnia and Herzegovina<sup>[143,144]</sup> are recognized as endemic for CE, none of them have available published data on the exact incidence of CE in livestock, carnivores or humans.

In Greece, investigation of the prevalence and the genotype of *E. granulosus* in sheep and goats in Peloponnese (southern Greece) revealed that sheep were infected by the G1 (sheep) strain and the G3 (buffalo) strain, while the 20 goats examined harboured the G7 (pig) strain<sup>[145]</sup>. The prevalence of CE in farm animals ranged from the mid 1980s to the mid 1990s between 82% and 56.6% in cattle, 80% and 100% in sheep, 24% and 15.4% in goats and 5% and 9.3% in pigs, while surgical human cases reached 12.9 per 100 000 inhabitants in 1984 and up to 29% in 1999<sup>[146]</sup>. Furthermore, surveillance in livestock species since 1998 has documented a prevalence of 31.3% in sheep, 10.3% in goats, 0.6% in pigs and 0% in cattle<sup>[146]</sup>. Finally, a more recent survey conducted on sheep in central Greece from 2002 to 2006, revealed an incidence rate of 39.3%<sup>[147]</sup>. In humans, the overall incidence rate was estimated to have increased from 9.77 per 100 000 in 1967<sup>[148]</sup> to 10.59 per 100 000 inhabitants in 1983<sup>[149]</sup>; results which were confirmed in another survey where an incidence of 12.7 per 100 000 inhabitants (varying from 11.6 to 13.35) has been reported<sup>[150]</sup>. Incidence rates steadily declined in the most recent survey carried out in 2007 where they have been documented to be 0.122 per 100 000 inhabitants<sup>[151]</sup>. Published data for the entire country are not available but according to personal communications with surgeons it is estimated that approxi-

mately 800 cases of cystic echinococcosis are diagnosed each year, of which between 300 and 400 of them were undergoing surgical treatment.

In Western Europe, the sheep strain (G1) is the principal cause of human CE. In the past, the cattle-based transmission cycle of *E. ortlepp* in Germany and Switzerland has been documented<sup>[2,152]</sup>, but now cases are reduced to sporadic occurrence and only a single case from a human patient in the Netherlands has been reported<sup>[21]</sup>.

In the United Kingdom, the parasite has a restricted distribution, being found mainly in mid and southern Wales<sup>[2,152]</sup>. Recently, a re-emergence of *E. granulosus* in Wales has been reported, noting a rise in prevalence in rural dogs between 1989 and 2002 of 3.4% to 8.1%<sup>[153]</sup>.

In Spain, CE is an endemic disease in north-eastern, central and western parts of the country, with prevalence rates rising in the last few years. The most common strains found in these areas were the sheep strain (G1) infecting sheep, cattle, goats, pigs, wild boars and humans, the pig strain (G7) infecting pigs, goats and wild boars, and *E. equinus* (old G4 strain) infecting horses<sup>[154]</sup>. In the province of Alava, two recent surveys documented prevalence of *E. granulosus* infection of 8% in the dog definitive hosts<sup>[155]</sup> and 15% in Iberian wolves<sup>[156]</sup>. In the municipality of Madrid, it has been assessed that hydatidosis affected 2.88% of sheep<sup>[157]</sup>. In Laroja region, the overall prevalence has been calculated to reach 20.3% in adult sheep and up to 23% in sheep and cows in the north-eastern, central and western parts of the country<sup>[158]</sup>.

With regard to human hydatidosis, a higher incidence of surgical cases occurs in Salamanca, with 10.8/100 000 inhabitants affected between the end of the 1980s and 2000<sup>[159]</sup>. On the other hand, in the Laroja region, prevalence of CE decreased from 19 to 4 cases per 100 000 inhabitants until 2000<sup>[158]</sup> and in the rest of the country it ranges between 1.1 and 3.4 cases per 100 000 inhabitants<sup>[159]</sup>.

In France, a surveillance system in the mid 1990s revealed a prevalence of hydatidosis of 2.5% in livestock and less than 0.28 per 100 000 in humans<sup>[160]</sup>. A higher annual incidence has been documented in Corsica (10/100 000) and eastern regions (4.5/100 000 inhabitants)<sup>[78]</sup>. In recent years, the European Centre for Disease Prevention and Control reported 17 human cases in 2005.

Italy is considered a medium to high risk country for echinococcosis. The G1 (sheep), G2 (Tasmanian sheep), G3 (buffalo), G4 (horse), and G7 (pig) genotypes of *E. granulosus* are commonly found in livestock of several regions of Italy, especially in the southern part (such as in the Campania region), in Sardinia and in Sicily<sup>[3]</sup>. Indeed, the prevalence rate of *E. granulosus* in sheep has been reported to be 5%-28% in Basilicata, 22% in Abruzzo and 47% in Tuscany<sup>[3]</sup>. In Sicily, CE was found in 67.1% of cattle, 11.13%-57.6% of sheep and 5.6%-19% of shepherd dogs<sup>[161,162]</sup>. CE prevalence of infection in Sardinia has been assessed to be 70%-92.8% of sheep, 9.4% of cattle, 9.4%-11.1% of pigs, 1% of horses and 3%-19% of dogs<sup>[3,163-166]</sup>. In Campania, the prevalence rate in cattle has been reported to range from 10.4%<sup>[167]</sup> to 14.8%<sup>[163]</sup> while in

buffalos this ranges from 10%<sup>[168]</sup> to 18.6%<sup>[169]</sup>.

Infection of *E. granulosus* in animals seems to occur also in several regions of the centre of Italy while north regions could be considered of low endemicity. Indeed, in Central Italy medium prevalence values usually range from 20.2% to 47%-81.18% in sheep, from 7.34% to 15.3% in cattle, and reach 71.97% in goats, and 0.82% in pigs<sup>[170-172]</sup>. In Abruzzo, prevalence infection rates in sheep and cattle are 20.2% and 15.3%, respectively<sup>[163]</sup>. On the other hand, in Emilia Romagna the prevalences were low for several animals: 0.39%-0.54% in cattle, 0.30% in sheep, 0.39% in goats, 0.34% in horses and 0.95 per million in pigs<sup>[173]</sup>. In dogs and wolves retrieved along the whole Apennines the prevalence of *E. granulosus* infection has been noted to be 31% and 15%, respectively<sup>[172,174]</sup>.

Despite these findings, the overall national occurrence of CE in farm animals can be considered low with prevalence rates of 0.52% of cattle, 1.30% of sheep, 0.6% of goats, 3.86% of sheep and goats, 0.0013% of pigs and 0.019% of horses<sup>[175]</sup>. On the other hand, human hydatidosis represents a serious public health problem, with an incidence of 1.3 cases per 100 000 inhabitants, a maximum of 4-8 cases per 100 000 inhabitants in Sardinia<sup>[176]</sup>, and the occurrence of over 1000 cases requiring surgery each year<sup>[177]</sup>. Endemic zones reflect animal infection, with higher incidence rates in Sardinia and Sicily, medium in the Central-South regions, and a sporadic diffusion in the northern part of the country where this disease plays a minor role (prevalence < 1%). Annual mean incidence rates of surgical cases have been reported to be 6.6-10.6 per 100 000 inhabitants in Sardinia<sup>[178,179]</sup>, 1.57-5.6 in Emilia Romagna<sup>[180,181]</sup>, 1.22 in Lombardia, 2.30 in Sicily<sup>[182]</sup>, 1.76 in Basilicata, 0.46 in Campania and 2.33 in Apulia<sup>[178]</sup>.

Risk factors for infection are now considered to be widespread use of extensive or semi-extensive sheep farming (echinococcosis being a work-related disease), illegal slaughtering, and high numbers of sheepdogs and other types of dogs<sup>[183]</sup>.

## CONCLUSION

Given the wide geographic distribution, CE caused by *E. granulosus* is a re-emerging disease in several countries and regions, even in places where it was previously at low levels. Evidence suggests this is a result of a reduction of control programmes due to economic problems and lack of resources, leading to severe disease, considerable economic loss and, definitely, a public health problem of increasing concern.

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## Multidisciplinary imaging of liver hydatidosis

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### Abstract

Liver hydatidosis is a parasitic endemic disease affecting extensive areas in our planet, a significant stigma within medicine to manage because of its incidence, possible complications, and diagnostic involvements. The diagnosis of liver hydatidosis should be as fast as possible because of the relevant complications that may arise with disease progression, involving multiple organs and neighboring structures causing disruption, migration, contamination. The aim of this essay is to illustrate the role of imaging as ultrasonography (US), multi detector row computed tomography, and magnetic resonance imaging (MRI) in the evaluation of liver hydatidosis: the diagnosis, the assessment of extension, the identification of possible complications and the monitoring the response to therapy. US is the screening method of choice. Computed tomography (CT) is indi-

cated in cases in which US is inadequate and has high sensitivity and specificity for calcified hydatid cysts. Magnetic resonance is the best imaging procedure to demonstrate a cystic component and to show a biliary tree involvement. Diagnostic tests such as CT and MRI are mandatory in liver hydatidosis because they allow thorough knowledge regarding lesion size, location, and relations to intrahepatic vascular and biliary structures, providing useful information for effective treatment and decrease in post-operative morbidity. Hydatid disease is classified into four types on the basis of their radiologic appearance.

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**Key words:** Liver hydatidosis; Hepatic cyst; Daughter cysts; Calcified cyst; Pericyst

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### INTRODUCTION

Hydatid disease is a worldwide zoonosis caused by the larval stage of the echinococcus tapeworm, that is endemic in many parts of the world (in European, Middle Eastern, Mediterranean, South American and African countries)<sup>[1-4]</sup>. There are two types of Echinococcus infections: Echinococcus granulosus, the more common type, and Echinococcus multilocularis, the less common but more invasive. Hydatid disease is a relevant health problem in underdeveloped areas where veterinary control does not exist. The

most frequent location of hydatid cystic lesions is in the liver (up to 80% of cases), followed by the lung (about 20% of cases), and with a lower reported incidence in any other organ or tissue in the body<sup>[1-4]</sup>.

Dogs or other carnivores are definitive hosts, whereas sheep or other ruminants are intermediate hosts. Humans are secondarily infected by the ingestion of food or water contaminated by dog feces containing the eggs of the parasite. After the ingestion of the eggs, the freed embryo enters a branch of the portal vein by passing through the duodenal mucosa; most of these embryos become lodged in the hepatic capillaries where they either die or grow into hydatid cysts. Some embryos pass through the hepatic capillaries and become lodged in the lungs and other organs.

The definitive diagnosis of liver echinococcosis requires a combination of imaging, serologic, and immunologic studies<sup>[4]</sup>.

At biochemical analysis, there is usually eosinophilia, and a serologic test is positive in 25% of patients<sup>[5]</sup>. At histopathologic analysis, a hydatid cyst is composed of three layers: the outer pericyst, which corresponds to compressed liver tissue; the endocyst, an inner germinal layer; and the ectocyst, a translucent thin interleaved membrane<sup>[5]</sup>.

Imaging procedures are essential in diagnosis and evaluation of the extent of liver hydatidosis; ultrasound (US), computed tomography (CT), and magnetic resonance (MR) can depict hydatid disease<sup>[2-4,6]</sup>.

The imaging method used depends on the involved organ, and the radiologic findings range from purely cystic lesions to a completely solid appearance<sup>[3]</sup>. US is the screening method of choice and is also used to monitor efficacy of medical therapy<sup>[2-4,6]</sup>. CT is always performed because it has a high sensitivity (94%)<sup>[7]</sup>. It is an important preoperative diagnostic tool to determine vascular, biliary or extrahepatic extension, to recognize complications, such as rupture and infections, and therefore to assess respectability<sup>[8-10]</sup>. MR is the best imaging procedure to demonstrate a cystic component. It helps to determine vascular or biliary tree involvement, as well as extrahepatic extension<sup>[10,11]</sup>.

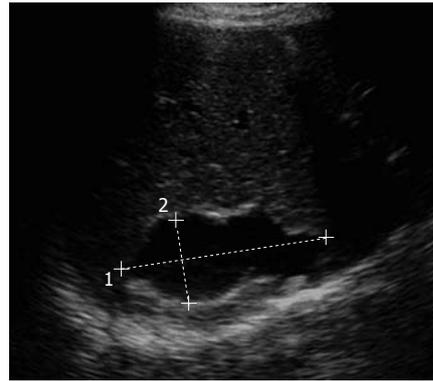
There are many potential complications such as exophytic growth, transdiaphragmatic thoracic involvement, peritoneal seeding, biliary communication, portal vein involvement, abdominal wall invasion and hematogenous dissemination in any anatomic location (lung, kidney, spleen, bone, brain)<sup>[6,10,11]</sup>.

## RADIOLOGIC FINDINGS

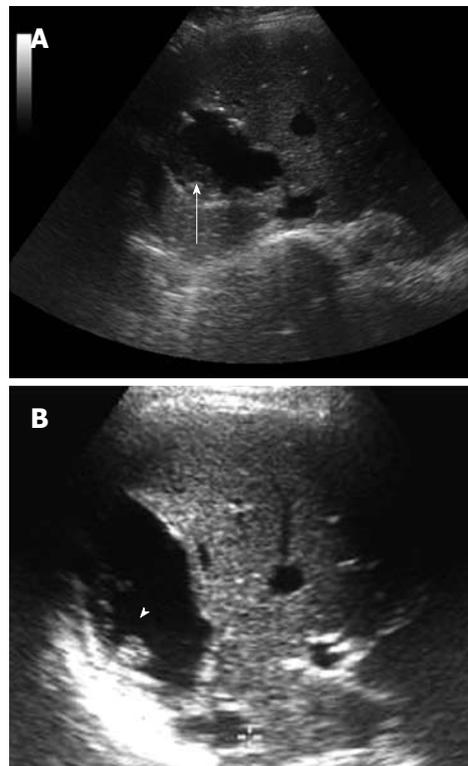
### Ultrasonographic findings

The ultrasonographic appearance of hydatid cysts may vary, from a simple aspect to a more complex one, in relation to the stage of evolution and maturity<sup>[5-7,10]</sup>. US can categorize cysts as solitary univesicular, solitary multivesicular, solid echogenic mass, multiple, either uni- or multivesicular, or collapsed, flattened and calcified<sup>[8]</sup>.

In the first stage, the hydatid cyst may manifest as a



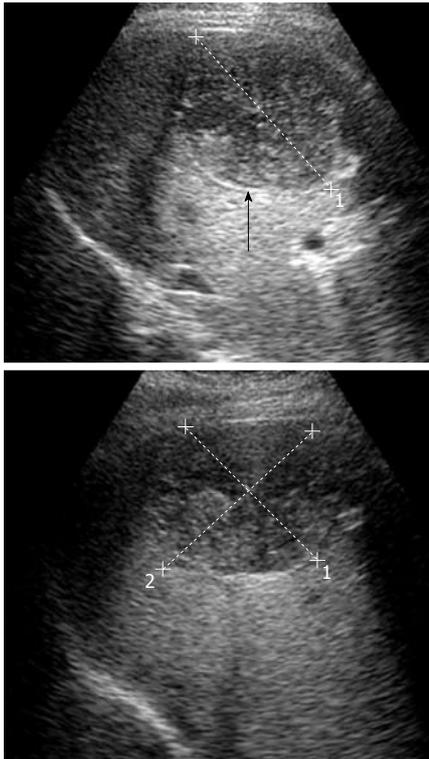
**Figure 1** Liver hydatid disease in a 50-year-old man appears as a well-defined anechoic mass without hydatid sand and septa (type I).



**Figure 2** Liver hydatidosis in a 27-year-old female. Ultrasonography images (A, B) show a lesion with mixed echogenicity, with hydatid sand (the arrow) and multiple echogenic foci (the arrowhead).

well-defined anechoic cyst (Figure 1), an anechoic cyst except for hydatid “sand”<sup>[2,6,7]</sup>. The more complex aspect is typical of the advanced stages and is related to the presence of multiple internal septa, daughter cysts, multiple echogenic foci and floating membranes inside the cavity (Figures 2 and 3)<sup>[7-9]</sup>. Membranes may appear as serpentine linear structures, a finding that is highly specific for hydatid disease<sup>[7,12,13]</sup>. The detachment of the membrane inside the cyst is considered the US “water lily sign”<sup>[13-16]</sup>. The cyst wall is visible as double echogenic lines separated by a hypoechoic layer (Figure 3)<sup>[16]</sup>.

Multivesicular cysts manifest as well-defined fluid collections in a honeycomb pattern with multiple septa representing the wall of the daughter cysts. Daughter cysts



**Figure 3** Ultrasonography images of hydatid disease show multiple internal septa and floating membranes inside the cyst. Note the cyst wall is visible as double echogenic lines (see the black arrow).

appear as cysts within a cyst<sup>[7-9]</sup>. Altering patient's position may change the position of daughter cysts.

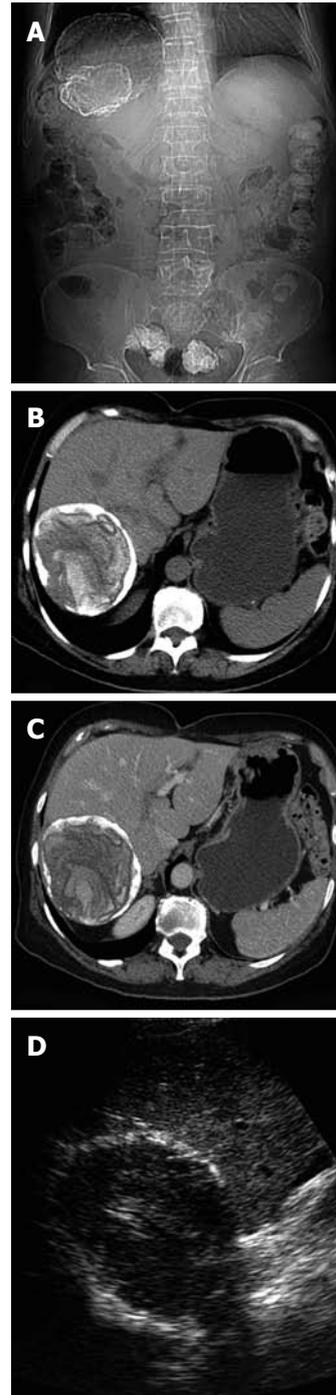
The more complex aspects of hydatid cyst may also mimic solid hepatic masses, and differential diagnosis becomes difficult but fundamental; it is important to look for daughter vesicles or membranes within the lesion that may help in differential diagnosis<sup>[7,8]</sup>. Cyst calcification is seen in dead hydatid cysts; US shows a hyperechogenic contour with a cone-shaped acoustic shadow<sup>[13,15,16]</sup>.

When the cyst wall is heavily calcified, only the anterior portion of the wall is visualized and appears as a thick arch with a posterior concavity. Partial calcification of the cyst does not indicate the death of the parasite, on the contrary densely calcified cysts may be assumed to be inactive<sup>[8,9]</sup>.

US is considered the preferred investigatory test to monitor efficacy of medical antihydatid therapy because of its low cost<sup>[12,14]</sup>. Positive response findings include reduction in cyst size, membrane detachment, progressive increase in cyst echogenicity and mural calcification<sup>[12]</sup>.

### Computed tomography findings

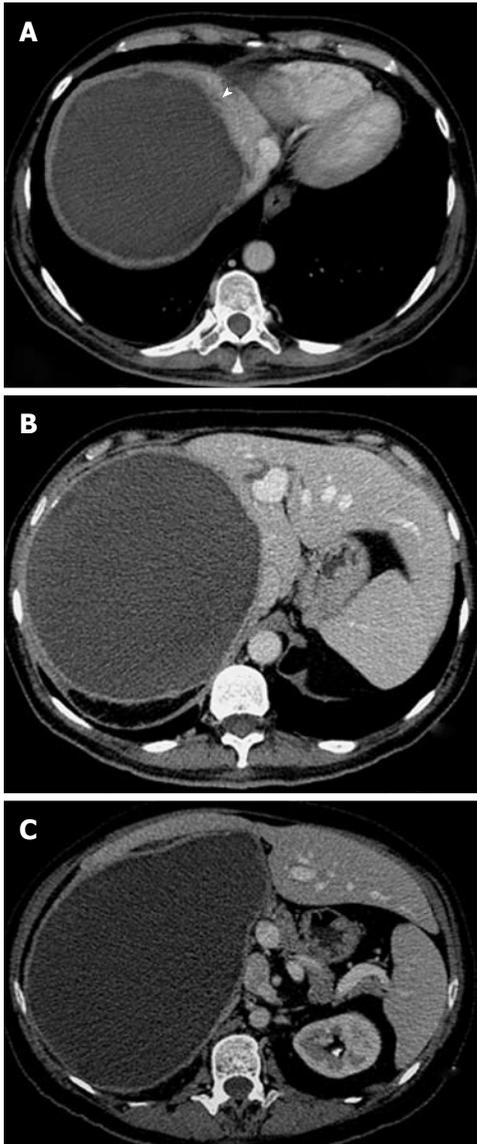
CT is indicated in cases in which US is inadequate due to patient-related difficulties (obesity, excessive intestinal gas, previous surgery<sup>[3,5-7]</sup>). CT has high sensitivity and specificity for hepatic hydatid disease<sup>[7]</sup>. Intravenous administration of contrast medium is useful to give a vascular map to the surgeon, and when complications (especially infection and communication with the biliary tree) and extra-hepatic diffusion are suspected.



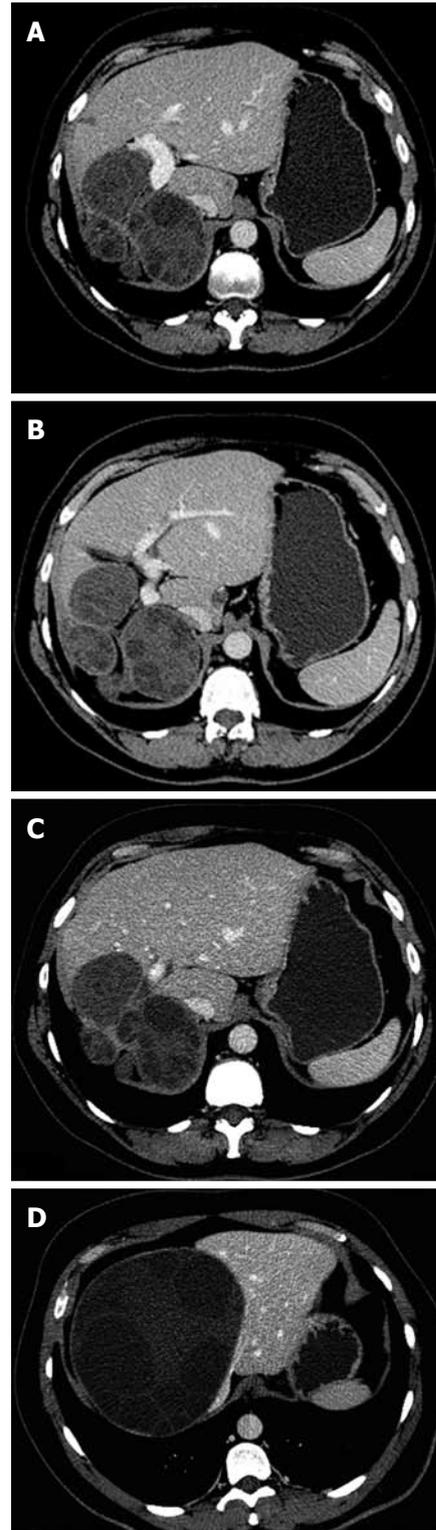
**Figure 4** Calcified unilocular hydatid cyst. Digital scout image (A) shows a round, densely calcified lesion supra-elevating the right diaphragm. Computed tomography basal (B) and contrast-enhanced (C) images reveal a hypoattenuating lesion with peripheral wall calcification in the right lobe. Membranes appear as serpentine linear structures. Note the complex ultrasonography aspect of the cyst and the hyperechoic wall (D).

CT may show the same findings as US<sup>[6,7]</sup>. Calcification of the cyst wall, internal septa, floating membranes and daughter vesicles are easily detected at CT<sup>[3,5]</sup>.

A hydatid cyst typically is seen as a round lesion with water attenuation density, surrounded by a calcified ring-like (Figure 4) or highly attenuated wall, representing the pericyst (Figure 5)<sup>[17]</sup>. Detachment of the laminated mem-



**Figure 5** Computed tomography scan shows a huge nonenhancing mass with regular contours and thickened wall representing the pericyst (the white arrowhead) occupying all the right lobe of the liver (type I); either the right portal vein or the right hepatic vein is completely replaced.



**Figure 6** Computed tomography scan (A, B, C, D) shows some unenhanced hypoattenuating masses with well defined borders occupying the right lobe of the liver; multiple round daughter cysts are seen peripherally inside the lesion (type II). Note the "rosette appearance".

branes from the pericyst are visualized as linear areas of increased attenuation within the cyst<sup>[17]</sup>.

At CT daughter vesicles are visible as round structures located peripherally within the mother cyst; they usually contain fluid with a lower attenuation than that of the fluid of the mother cyst (Figure 6)<sup>[5,7]</sup>.

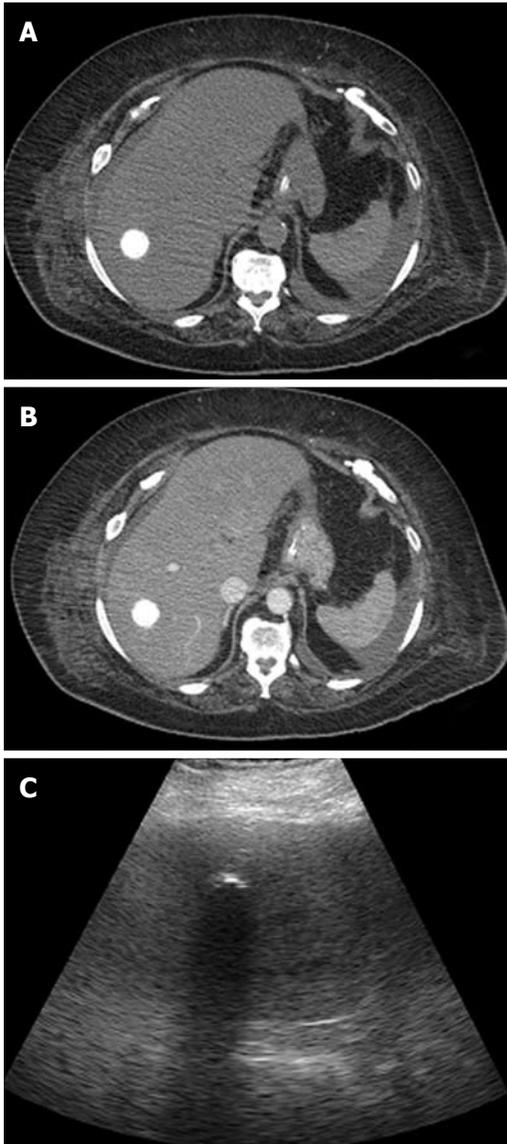
Contrast-enhanced CT may show the typical high-attenuation rim representing abscesses surrounding the lesion. Sometimes, patchy areas of contrast-enhanced liver parenchyma are seen in the vicinity of the lesion, representing inflammatory changes<sup>[18]</sup>.

The dead cysts are totally calcified and at CT they appear as round hyperattenuating areas (Figure 7)<sup>[5,7]</sup>.

CT also may depict gas or air-fluid levels or fat inside the hydatid cyst, indirect signs of infection and/or communication with the biliary tree (Figures 8 and 9)<sup>[6,10,11]</sup>.

CT is the modality of choice to study extra-hepatic diffusion because it allows imaging of the entire abdomen, pelvis and thorax.

Extra-hepatic diffusion may regard peritoneum, the



**Figure 7** Seventy-three year-old-woman with a dead hydatid cyst. Computed tomography scan basal and enhanced images (A, B) show a totally calcified cyst (type III). At ultrasonography (C) calcified cyst shows strong posterior shadowing.

diaphragm and the thorax cavity, the abdominal wall, the portal system, and the hematogenous dissemination<sup>[4,6,7]</sup>.

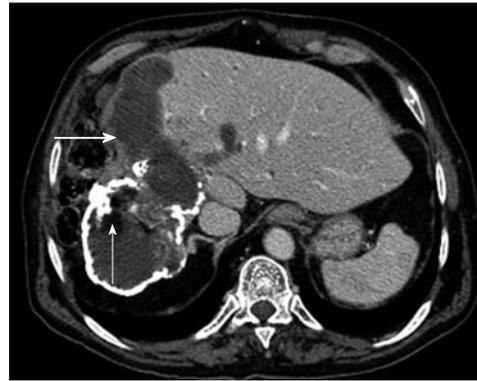
**Magnetic resonance findings**

MR may be performed to confirm the hypothesis of hepatic hydatidosis and visualize the lesion in different planes. It is the best diagnostic investigation to differentiate the cystic component from the others and to demonstrate a biliary tree involvement<sup>[13]</sup>.

The hydatid cysts may show variable signal intensities on T1- and T2-weighted images, according to the different components inside the lesion<sup>[13,17,18]</sup>.

The necrotic and the fluid components are hypointense on T1-weighted images and markedly hyperintense on T2-weighted images<sup>[13,18,19]</sup>.

When present the daughter cysts are seen as cystic structures attached to the germinal layer that are hypointense relative



**Figure 8** Contrast-enhanced upper abdominal computed tomography scan demonstrates a partially calcified hydatid cyst in direct communication with the biliary tree markedly dilated (the bold white arrow). The presence of fat components within the cyst derives from the lipid elements in bile (the thin white arrow) (type IV).



**Figure 9** Fifty-five-year-old male with acute abdominal pain, fever and leucocytosis. Contrast-enhanced computed tomography shows intracystic gas in the anterior part of a unilocular partially calcified hydatid cyst, suggesting super-infection; finding confirmed at surgery (type IV).

to the intracystic fluid on T1-weighted images (Figure 10)<sup>[13]</sup>.

The characteristic sign of hydatid disease is represented by the pericyst that usually appears as a low-signal-intensity rim on T2-weighted images (Figure 10)<sup>[17,19]</sup>.

In addition, there may be an intermediate-signal-intensity inner ring representing the detachment of the membranes<sup>[19]</sup>.

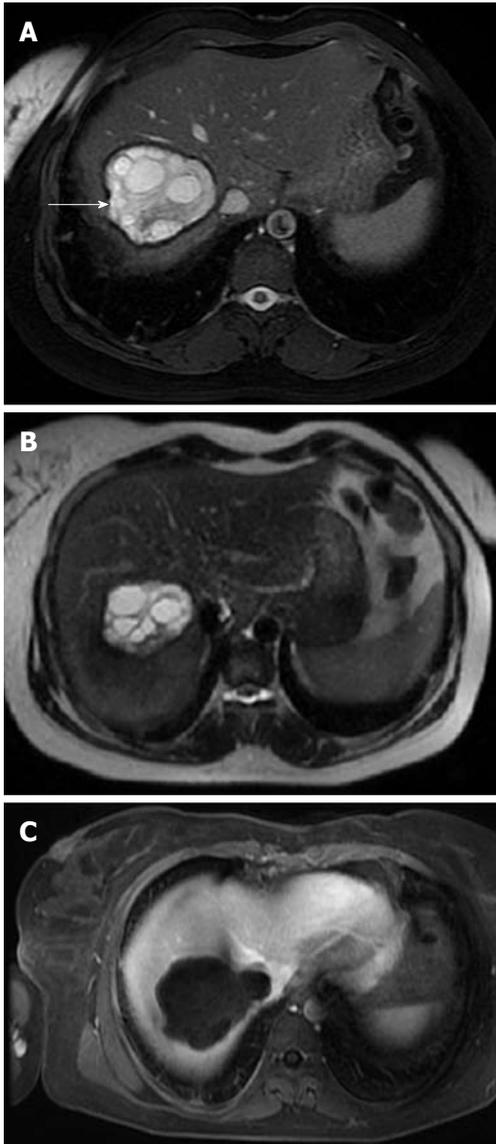
After the i.v. injection of gadolinium contrast agent the pericyst may show slight enhancement (Figure 11).

MR is the best diagnostic tool in demonstrating the floating membranes (Figure 12) and irregularities of the rim representing incipient detachment of the membranes (Figure 13)<sup>[17,19,20]</sup>. On the other hand MR is less sensitive than CT scan in showing cyst wall calcification.

The “snake sign” is another typical MR imaging feature: it represents collapsed parasitic membranes, secondary to damage or degeneration of the hydatid cyst: these membranes have low signal intensity with all sequences (Figure 13).

Intracystic air-fluid level may be visible on MR, as a possible sign of super-infection (Figure 14)<sup>[16,21]</sup>.

MR cholangiopancreatography (MRCP) is useful to



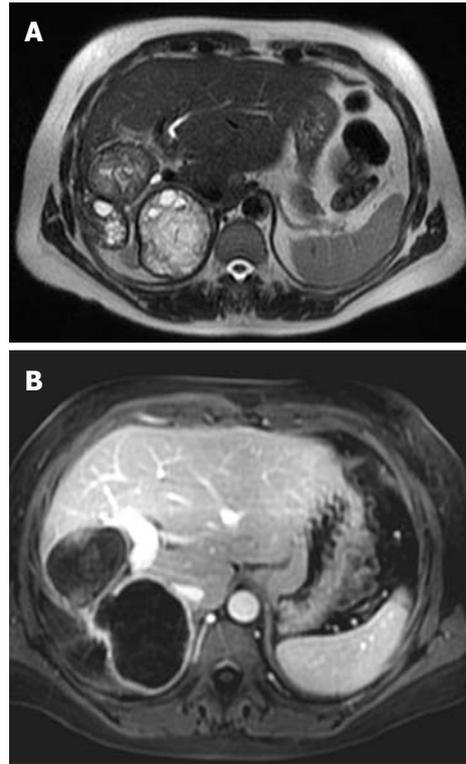
**Figure 10** Axial T1-weighted (A) and T2-weighted (B) images show a well defined cystic lesion and the typical peripheral location of the daughter cysts within the mother cyst. Note the marked hypointensity of the pericyst (the white arrow). After contrast administration (C) the cystic lesion does not show contrast enhancement (type II).

study potential involvement of the biliary tree: communication between the cysts and the biliary tree; dilatation of the biliary system secondary to compression of the hydatid cyst<sup>[20,21]</sup> (Figure 15).

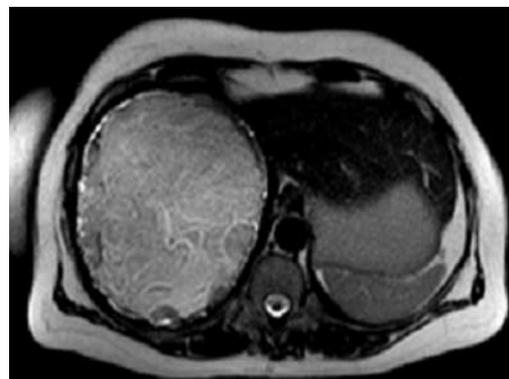
It is known that routine MRI does not adequately differentiate completely liquid hydatid cysts (type I, see following paragraph) from simple cysts: Inan *et al*<sup>[22,23]</sup> have demonstrated in their study that diffusion-weighted (DW-MRI), a recent MRI technique, can be helpful in the differential diagnosis.

DW-MRI has long been used exclusively in brain imaging due to technical problems and sensitivity to motion artifacts (caused by cardiac motion and respiration); with the advent of faster sequences, DW-MRI has been applied to abdominal imaging<sup>[22,23]</sup>.

Using DW MRI with a high b factor (1000 s/mm<sup>3</sup>) the



**Figure 11** Axial T2-weighted and post-contrast images show the exophytic growth of hydatid cyst (type II) (A). After the injection of contrast media the septa and cyst wall enhance (B). Note the proximity of the cyst to the diaphragm which facilitates transdiaphragmatic thoracic involvement and to main portal vein.



**Figure 12** 35-year-old woman living in an endemic region. Axial T2-weighted magnetic resonance image show the hydatid cyst that occupies almost the entire right lobe of the liver with thickened pericyst and multiple floating membranes inside the lesion (type II).

hydatid cysts are hyperintense, whereas none of the simple cysts show significant hyperintensity (Figure 16)<sup>[22-24]</sup>.

In addition, using DW MRI it is possible to calculate a parameter, called the apparent diffusion coefficients (ADCs), that measures the difference in cellular density of hepatic lesions<sup>[22-24]</sup>.

This quantitative parameter can be used to differentiate hydatid cysts from simple cysts. The difference between the ADCs of the hydatid cysts and those of simple cysts can be attributed to the difference in cyst contents<sup>[22-24]</sup>.

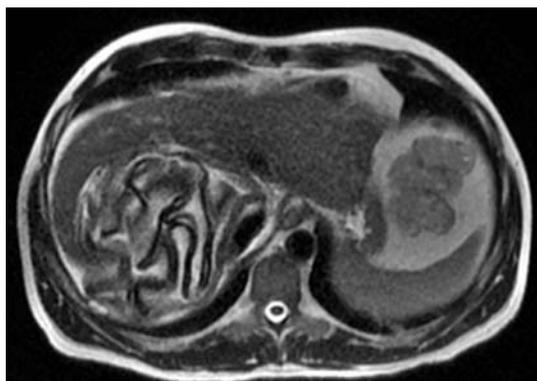


Figure 13 The detachment of the pericyst and the collapsed membranes inside the cyst due to damage or degeneration may give the hydatid cyst a serpentine linear aspect; this is the “snake sign”.

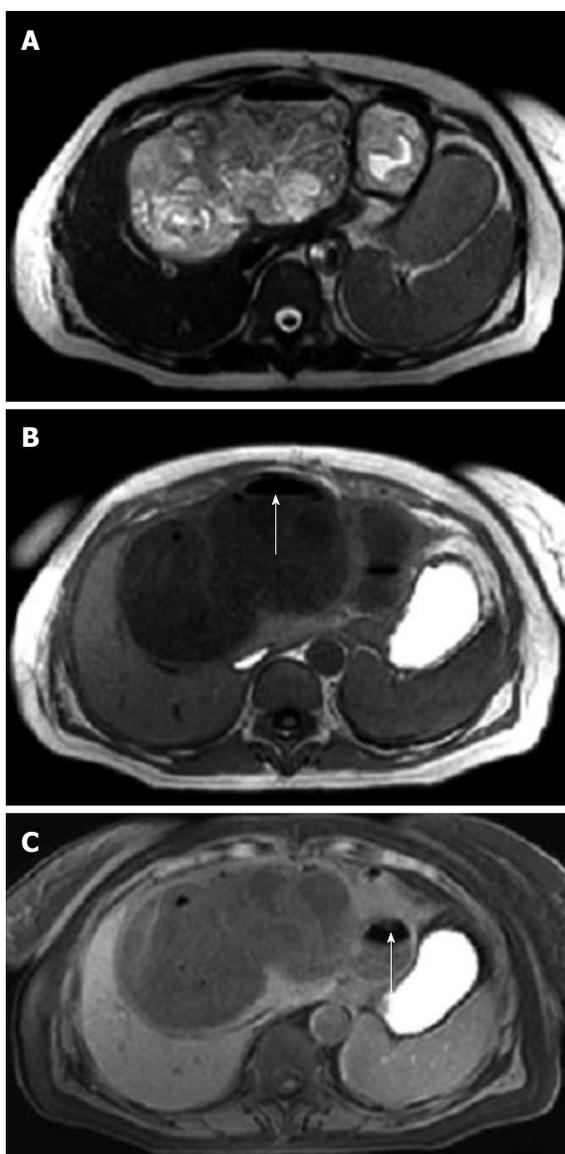


Figure 14 Axial T2-weighted (A) and T1-weighted (B, C) magnetic resonance images demonstrate a round, cystic lesion in the left hepatic lobe, with thickened pericyst, small daughter cysts, floating membranes and an air-fluid level within the cyst (white arrow). The diagnosis is an infected hydatid cyst (type IV).

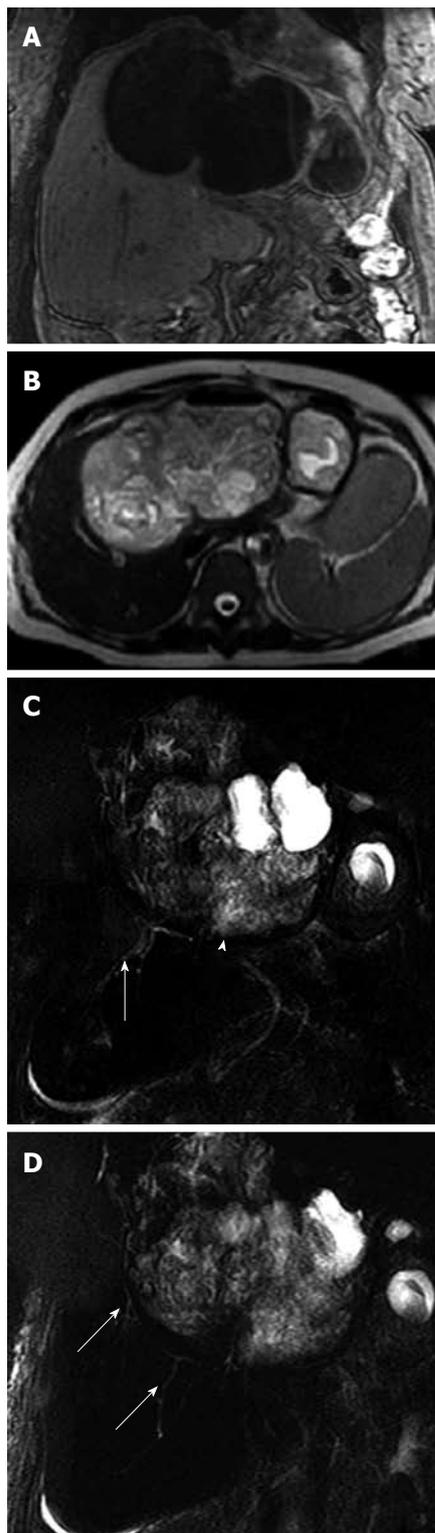
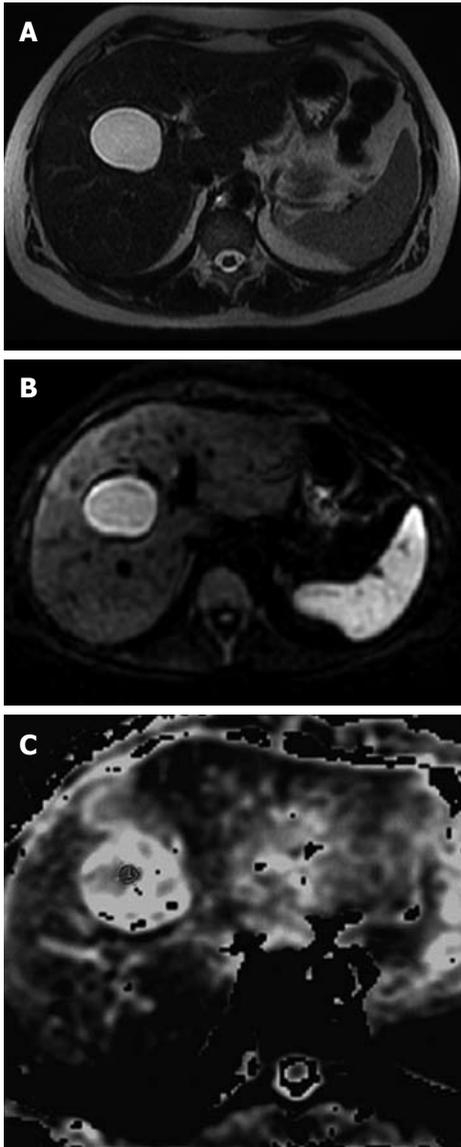


Figure 15 T1 coronal image and T2 axial image show a huge and multi-locular hydatid cyst occupying the entire left lobe, partially the right lobe (A, B) and protruding into the hepatic hilum. Magnetic resonance cholangiopancreatography (MRCP) sequences show the compression of the common bile duct at the confluence and of the right hepatic duct (the white arrowhead) and the consequent intrahepatic biliary tree dilatation (the white arrows) (C, D).

Because the hydatid cyst contains viscous hydatid sand that consists of scolices, sodium chloride, proteins, glucose, ions, lipids, and polysaccharides, the ADC of the hydatid



**Figure 16** A type I hydatid cyst. A: Axial T2 weighted magnetic resonance image depicts a round cystic mass in the anterior segment of the right lobe, with no septa or solid portions; B: On the diffusion-weighted image the lesion exhibits high signal intensity ( $b = 1000$ ); C: On apparent diffusion coefficient map, apparent diffusion coefficient value is  $2.4 \times 10^{-3}$ .

cyst is decreased; on the contrary the simple cyst has lower viscosity, hence the higher viscosity. In Inan's series the mean ADCs of the hydatid cysts was significantly lower ( $2.5 \times 10^{-3} \pm 0.9$ ) than that of the simple cysts ( $3.5 \times 10^{-3} \pm 0.5$ ) (Figure 16)<sup>[22-24]</sup>.

In patients affected by hepatic hydatidosis, contrast enhanced magnetic resonance angiography may be useful in detecting hepatic venous outflow obstruction or thrombosis or invasion<sup>[25]</sup>; in these patients, pulmonary embolism may be a possible complication (Figure 17)<sup>[26]</sup>.

## CLASSIFICATION OF HYDATID DISEASE ON THE BASIS OF IMAGING

Hydatid disease is classified into four types on the basis

of their radiologic appearance<sup>[27]</sup>:

### **Type I : Simple cyst with non internal architecture**

Hydatidosis appear at US as a well-defined anechoic mass with or without hydatid sand and septa. Unilocular cysts are considered to be an initial stage in the development of the parasite<sup>[28]</sup>. A solitary type I cyst may be difficult to distinguish from a simple epithelial cyst<sup>[8]</sup>.

At CT, a type I appears as a well-defined water-attenuation mass; after injection of contrast material the septa and cyst wall enhance, a finding that helps differentiate type I from a simple liver cyst<sup>[13,19]</sup>. MR images are also similar to those of a simple liver cyst, including hypointensity on T1-weighted images and marked hyperintensity on T2-weighted images; a low signal intensity rim (“rim sign”)<sup>[19,20]</sup>, which is more evident on T2-weighted images, has been described as typical of hydatidosis, and it can be used to differentiate hydatid cysts from simple cysts; this finding represents the pericyst.

Recently the emerging role of DW MRI may play a decisive role in the differential diagnosis of hydatid liver disease and simple cysts<sup>[22-24]</sup> (Figures 1, 5 and 16).

### **Type II : Cyst with daughter cysts and matrix**

Daughter cysts are inside the mother cyst, usually arranged at the periphery<sup>[15,18]</sup>. Floating membranes or vesicles can be also seen in the cyst. Multiple daughter cysts are enclosed together looking like an echogenic solid lesion. (Figures 6, 10, 11 and 12)

Type II may manifest as a well-defined fluid collection in a honeycomb pattern with multiple septa representing the walls of the daughter cysts, creating a “rosette” appearance<sup>[15]</sup>. Peripheral calcification may occur and involves the pericyst; it is easily detected in CT images as a curvilinear or ring-like structure. CT can distinguish the mother cyst: the average density attenuation of the mother cyst is higher than that of daughter cysts. At MR imaging, daughter cysts may appear hypointense or isointense relative to the maternal matrix on T1 and T2-weighted images<sup>[19,20]</sup>.

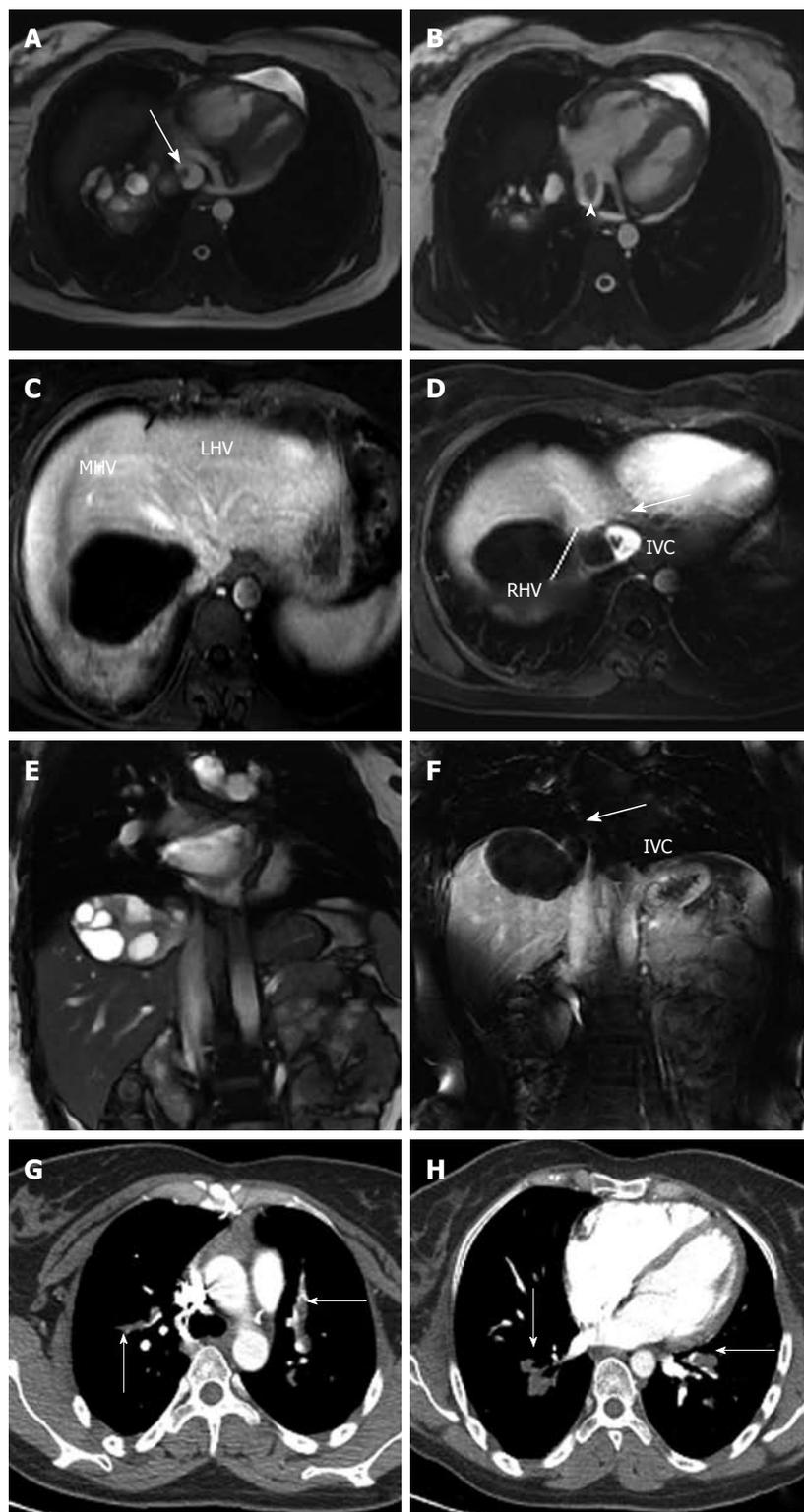
### **Type III : Calcified cyst**

Type III lesions are dead cysts with total calcification. At US calcified cysts show strong posterior shadowing, at CT they appear as round hyperattenuating areas, at MR they appear as hypointense areas (Figure 7).

### **Type IV**

Hydatid complications include rupture and superinfection and may be seen in both type I and type II. CT and MRI play a key role in recognizing the complications such as rupture and infection of cysts associated with hydatid disease.

Ruptures may occur in 50% of cases<sup>[6,9,11]</sup>; cyst rupture is mainly due to the degeneration of parasitic membranes. Cyst rupture is usually due to the degeneration of parasitic membranes, as a result of age, or a host defense mechanism<sup>[6,9,11]</sup>. The rupture may be contained, commu-



**Figure 17** Hepatic hydatidosis in a 30-year-old woman who presented with short of breath, fatigue and edema to the lower limbs. Magnetic resonance (MR) steady-state-free-precession sequences (A, B, E) and MR angiography (C, D, F) images showed the hydatid cyst invading the right hepatic vein (RHV), protruding in the inferior vein cava (IVC) (the bold white arrow) and in the right atrium (the white arrowhead). The mid hepatic vein (MHV) and the left hepatic vein (LHV) were normally patent (C). The multidetector computed tomography-angiography revealed diffuse pulmonary parasitic embolism (the thin white arrows) (G, H).

nicating or direct.

Fissures in the cyst wall can be visualized at both CT and MR imaging. Perforation to the biliary tree has been reported in up to 90% of hydatid cysts<sup>[7,10,11]</sup>. Hydatid cysts

may also rupture into pleural and peritoneal cavities. Up to 25% of ruptured cysts may become infected<sup>[10,11]</sup>. Signs of cyst infection include air-fluid or fluid-fluid levels<sup>[9,11]</sup> (Figures 8, 9 and 14).

## CONCLUSION

Imaging plays a primary role in liver hydatidosis. It is used for diagnosis, for assessment of extension, for identification of possible complications, for classification and for monitoring the response to therapy. US, MDCT and MR have different roles depending on accuracy in depicting the different goals.

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## Hepatic echinococcosis: Clinical and therapeutic aspects

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alveolar and cystic forms, associated with *Echinococcus multilocularis* (*E. multilocularis*) and *Echinococcus granulosus* (*E. granulosus*) infection, respectively. Cystic echinococcosis (CE) has a worldwide distribution, while hepatic alveolar echinococcosis (AE) is endemic in the Northern hemisphere, including North America and several Asian and European countries, like France, Germany and Austria. *E. granulosus* young cysts are spherical, unilocular vesicles, consisting of an internal germinal layer and an outer acellular layer. Cyst expansion is associated with a host immune reaction and the subsequent development of a fibrous layer, called the pericyst; old cysts typically present internal septations and daughter cysts. *E. multilocularis* has a tumor-like, infiltrative behavior, which is responsible for tissue destruction and finally for liver failure. The liver is the main site of HD involvement, for both alveolar and cystic hydatidosis. HD is usually asymptomatic for a long period of time, because cyst growth is commonly slow; the most frequent symptoms are fatigue and abdominal pain. Patients may also present jaundice, hepatomegaly or anaphylaxis, due to cyst leakage or rupture. HD diagnosis is usually accomplished with the combined use of ultrasonography and immunodiagnosis; furthermore, the improvement of surgical techniques, the introduction of minimally invasive treatments [such as puncture, aspiration, injection, re-aspiration (PAIR)] and more effective drugs (such as benzimidazoles) have deeply changed life expectancy and quality of life of patients with HD. The aim of this article is to provide an up-to-date review of biological, diagnostic, clinical and therapeutic aspects of hepatic echinococcosis.

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### Abstract

Echinococcosis or hydatid disease (HD) is a zoonosis caused by the larval stages of taeniid cestodes belonging to the genus *Echinococcus*. Hepatic echinococcosis is a life-threatening disease, mainly differentiated into

**Key words:** Hydatidosis; Cystic echinococcosis; Alveolar echinococcosis; Liver; PAIR; Albendazole; Treatment; Diagnosis

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## INTRODUCTION

Echinococcosis or hydatid disease (HD) is a zoonosis caused by the larval stages of taeniid cestodes belonging to the genus *Echinococcus*. Six species of *Echinococcus* are known, but only four of them are responsible for human disease: *Echinococcus granulosus* (*E. granulosus*) (which causes cystic echinococcosis), *Echinococcus multilocularis* (*E. multilocularis*) (which causes alveolar echinococcosis), *E. vogeli* and *E. oligartbrus* (which cause polycystic echinococcosis). Recent studies have identified two new species, *E. felidis* and *E. shiquicus*, even if no data are available about their pathogenicity to humans.

Hepatic alveolar and cystic echinococcosis are both life-threatening diseases because of their medical and economical impact and their wide geographical distribution. Polycystic echinococcosis is, on the contrary, confined to Central and South America and only few cases of this condition have been reported in man<sup>[1,2]</sup>.

The liver is the major site of HD involvement (about 75% of cases) both in the alveolar and in the cystic form<sup>[3]</sup>. This review is focused on the biological, epidemiological, clinical and therapeutic aspects of hepatic echinococcosis, with particular reference to *E. granulosus* cystic and *E. multilocularis* alveolar hydatidosis (Table 1).

## HEPATIC ECHINOCOCCOSIS CAUSED BY *E. GRANULOSUS*

Cystic echinococcosis (CE) occurs as the result of infection by the larval stages of *E. granulosus*. CE is the most common form of HD, with a worldwide distribution, and it can be regarded as an emerging or re-emerging disease in several countries of the world.

### *E. granulosus*: The parasite biology and life cycle

*E. granulosus* is a small tapeworm (length of 2-7 mm), whose body is made up by a mean number of three proglottids. There are ten distinct genetic types (G1-10) within *E. granulosus*, with a different geographical distribution, and these have been identified by molecular studies based on mitochondrial DNA sequences<sup>[4,5]</sup>: 2 sheep strains (G1-G2), 2 bovid strains (G3 and G5), a horse strain (G4), a camelid strain (G6), a pig strain (G7), a cervid strain (G8), a swine strain (G9) and a reindeer strain (G10).

The parasite life cycle involves dogs and other canids (coyotes, dingoes, red foxes) as definitive hosts and un-

gulates (sheeps, pigs, goats, horses) as intermediate hosts. Definitive hosts are infected by ingestion of offal containing hydatid cysts; the adult worms reside in the canine small bowel and their eggs or gravid proglottids are shed in the feces. After oral uptake of eggs by intermediate hosts, an oncosphere larva is released from the egg and penetrates the intestinal lamina propria, reaching the blood and lymph vessels which transport it to liver, lungs and other organs, where oncosphere larvae can develop into metacestodes (also known as hydatid cysts). Humans can accidentally become "aberrant" intermediate hosts, after ingestion of *Echinococcus* eggs excreted by infected carnivores.

Hydatid cysts are spherical, fluid-filled, unilocular vesicles, consisting of an internal cellular layer (germinal layer) and an outer acellular, laminated layer. The parasite cysts gradually expand and cause a granulomatous host reaction, followed by the development of a fibrous tissue layer (pericyst). Brood capsules and protoscolices bud from the germinal membrane; with time, internal septations and daughter cysts usually develop, modifying the unilocular morphology that is typical of young hydatid cysts. When definitive hosts ingest the cyst-containing organs of intermediate herbivore hosts, the *Echinococcus* life cycle can restart, as the protoscolices evaginate, attach to the intestinal mucosa and develop to adult stage in 30-80 d<sup>[6,7]</sup>.

### Molecular crosstalk between human host and parasite

Several studies have focused on the mechanisms of host-parasite interplay in CE.

The immune response to *E. granulosus* infection has been investigated through both clinical studies on patients with hydatidosis and sheep and mouse experimental models<sup>[8]</sup>. In the early stage of hydatid cyst development, a cell-mediated response involving macrophages, neutrophils and eosinophils is established<sup>[9-11]</sup>; antibody response is usually undetectable during the first weeks after infection, but IgE, IgG2 and IgG4 levels subsequently significantly increase<sup>[8]</sup>. Elevated levels of IgE for echinococcal antigens are responsible for allergic reactions, such as itching, urticaria and anaphylactic shock<sup>[12]</sup>.

*E. granulosus* induces both TH1 and TH2 response: elevated levels of TH1 cytokines, especially interferon- $\gamma$  (IFN- $\gamma$ )<sup>[13]</sup>, but also TH2 cytokines, such as IL-4, IL-5 and IL-6, have been recorded in patients with HD<sup>[8,11]</sup>. The reason for this duplex cytokine secretion pattern is not known: TH1 and TH2 responses usually down-regulate each other, with a cross-inhibitory mechanism; it is assumed that the complex antigenic organization of *Echinococcus* may stimulate both T-cell subsets<sup>[14]</sup>. After chemotherapy treatment, surgical removal or natural death of a cyst, TH2 response quickly drops and TH1 response becomes predominant<sup>[15]</sup>.

The metacestode attempt to escape from the host protective response involves complex and intriguing strategies aimed at modulating host response and protecting itself from elimination. *Echinococcus* tries, in fact, to minimize host reaction by exposing several immunomodulatory molecules to its host<sup>[16]</sup>, interfering with complement

Table 1 Hydatid disease epidemiology and characteristics<sup>16,71</sup>

	Cystic echinococcosis	Alveolar echinococcosis
Causative agent	<i>E. granulosus</i>	<i>E. multilocularis</i>
Definitive hosts	Dogs and other canids (coyotes, dingoes, red foxes)	Red foxes, arctic foxes, coyotes, dogs and cats
Intermediate hosts	Ungulates	Rodents
Geographic distribution	Worldwide	North America, northern and central Eurasia
Worldwide incidence	1-200/100 000	0.03-1.2/100 000
Organ localization	Mainly liver and lungs	Mainly liver
Characteristics of hydatid lesions	Young cysts: spherical, fluid-filled, unilocular vesicles (diameter: 1-15 cm) Old cysts: internal septations, daughter cysts Three-layered structure: germinal layer, laminated layer, pericyst	Alveolar-like pattern, with numerous vesicles (< 1 mm up to 15 cm in diameter) and surrounding dense connective tissue, no cyst fluid, sometimes central necrosis
Type of growth in human organs	Concentric expansion	Tumor-like, infiltrative behaviour
Therapeutic options	Surgery, PT (especially PAIR), chemotherapy	Surgery, chemotherapy, EPIs

PTs: Percutaneous treatments; PAIR: Puncture, aspiration, injection, re-aspiration; EPIs : Endoscopic percutaneous interventions.

activity<sup>[17]</sup>, altering leukocyte function<sup>[18]</sup> or using molecular mimicry<sup>[19]</sup>.

### Epidemiology and infection risk

*E. granulosus* has a worldwide distribution; the highest prevalence is recorded in the Mediterranean countries, Russia and China (in Sichuan Province human CE had a prevalence of 2.1% in 1997-1998<sup>[20]</sup>). Other hyperendemic areas are North and East Africa (prevalence > 3%), South America and Australia<sup>[21]</sup>. CE infection has re-emerged in certain parts of the world where it was once believed to be controlled, including Israel, Central Asia and Eastern Europe<sup>[21,22]</sup>. In Bulgaria the annual incidence of CE in children has increased from 0.7 per 100 000 in 1971-1982 to 5.4 in 1995<sup>[23]</sup>; in Kazakhstan the annual surgical incidence of CE over the whole country was below 1.4 per 100 000 inhabitants from 1988 until 1995 but has increased to 5.9 in 2000<sup>[24,25]</sup>.

CE is typically a rural and occupational disease, since certain human activities, such as feeding dogs with the viscera of slaughtered livestock, increase the risk of infection. Humans acquire the parasite through fecal-oral contact, generally by handling infected domestic dogs or egg-containing feces. *Echinococcus* eggs adhere to the coat of animals, especially to hairs around the anus and on the muzzle and paws<sup>[26]</sup>. Eggs can also be ingested with contaminated water or vegetables; it is also possible that the contamination of surfaces and foodstuffs with *Echinococcus* eggs occurs *via* wind, flies, birds or beetles.

Some studies have evaluated several risk factors for infection: Campos-Bueno *et al.*<sup>[27]</sup> studied a Spanish cohort of 127 CE infected patients, matched with 127 healthy controls, associating an increased risk for CE with having a higher number of dogs in the family and with dogs' ease of access to raw viscera of slaughtered animals. In Tibet a rise of infective risk was associated with nomadic life, age, playing with dogs, not protecting food from flies and raising yaks or sheep. Water wells were suspected to be a source of infection in African arid lands, where animals and humans often share the same water points<sup>[28]</sup>.

### Clinical aspects

After infection, humans are usually asymptomatic for a long period of time, since cyst growth is usually slow; in the liver the growth rate is variable, ranging from 1 mm to 5 mm in diameter per year. Most primary infections consist of a single cyst, but up to 20%-40% of infected people have multiple cysts. Presenting symptoms depend not only on the size and number of cysts, but also on the mass effect within the organ and upon surrounding structures. The signs and symptoms of liver hydatidosis include hepatomegaly, right/epigastric pain, nausea and vomiting. Cyst leakage or rupture may be responsible for systemic immunological responses, causing anaphylaxis; in one series, anaphylaxis complicated 10% of all intra-peritoneal ruptures.

Cyst rupture in the peritoneal cavity may cause secondary CE, with the release of protoscolices and/or small cysts, which can grow to larger cysts.

Portal vein or bile duct obstruction, caused by the expanding cysts, may be responsible for segmental or lobar liver atrophy in the cyst-bearing lobes<sup>[29]</sup>.

Other complications are rupture in the biliary tree with secondary cholangitis<sup>[30]</sup>, biliary obstruction by daughter cysts, portal hypertension, ascites, intracystic or subphrenic abscess formation, development of a bronchobiliary fistula<sup>[31,32]</sup>. Hydatid cyst suppuration has been reported as occurring in 5% to 40% of patients<sup>[33]</sup>. Perforation in the biliary tree has been described in up to 90% of HD<sup>[34]</sup>.

### Diagnosis

Considering that the early stages of infection are usually asymptomatic, the diagnosis of liver CE may often be incidental, associated with an abdominal ultrasonography performed for other clinical reasons. In endemic areas, the presence of symptoms suggestive of CE in a person with a history of exposure to sheepdogs supports the suspicion of hydatidosis.

A non-invasive diagnosis of hepatic CE is usually accomplished with the combined use of radiologic imaging

and immunodiagnostic techniques. Abdominal ultrasonography is considered the gold standard for defining the number, site, dimensions and vitality of cysts<sup>[32,35,36]</sup> and it is also important to evaluate treatment options. A standardized ultrasonographic classification system for hepatic cysts has been developed by the World Health Organization (WHO)<sup>[37]</sup>, in order to update the older Gharbi classification<sup>[38]</sup>.

Ultrasonography is not always able to differentiate hydatid cysts from other space-occupying lesions, like tumors or liver abscesses, so that additional imaging techniques, such as magnetic resonance imaging (MRI) and CT scans, may be required. MRI should be preferred to CT, due to better visualization of liquid areas within the matrix<sup>[39]</sup>. MRI is also important for pre-surgical evaluation of CE.

Immunodiagnosis is useful to confirm a radiologic diagnosis and can also be an important tool for the follow-up after surgical or pharmacological treatment, even if not all patients with CE have a detectable immune response<sup>[40-42]</sup>. Serological test sensitivity is indeed inversely related to the degree of sequestration of the echinococcal antigens inside cysts; for instance, healthy, intact cysts can elicit a minimally detectable response, whereas previously ruptured or leaking cysts are associated with stronger immune responses.

Almost all traditional immunodiagnostic methods (e.g., Casoni intradermal test, complement fixation test, indirect hemagglutination test, indirect immunofluorescence antibody test, immunoelectrophoresis and latex agglutination test) have now been replaced by the enzyme-linked immunosorbent assay (ELISA) and/or immunoblotting<sup>[43]</sup>. In order to detect antibody response to parasite, several hydatid antigens have been extracted and used for serological diagnosis. Hydatid cyst fluid antigen B (AgB) and antigen 5 (Ag5) from *E. granulosus* are considered the most specific native antigens for the immunodiagnosis of CE<sup>[40,41]</sup>, even though lack of sensitivity and specificity, technique standardization and cross-reactivity with antigens of other parasites<sup>[44-46]</sup> are major problems associated with immunodiagnosis of CE.

In doubtful cases, for example undetectable anti-*Echinococcus* antibodies in patients with small lesions resembling hydatid cysts or in patients whose hepatic cysts cannot be differentiated from liver abscess or neoplasms, ultrasonography-guided fine needle puncture may represent an additional diagnostic option. The demonstration of protoscolices or hydatid membranes or echinococcal antigens/DNA in the aspirated cyst fluid can confirm, in fact, the diagnosis of CE. Anthelmintic coverage is important to minimize the risk of secondary CE: albendazole should be recommended for 4 d before the procedure and should be continued for at least 1 mo after having punctured a lesion recognized as an *E. granulosus* cyst<sup>[32,47]</sup>. Detection of parasite-specific IgE has no significant diagnostic advantages, even if eosinophilia is often present after rupture/leakage of the cyst<sup>[48]</sup>.

## Treatment

The goals of hepatic hydatid cyst treatment are a com-

plete elimination of the parasite and prevention of recurrence, minimizing mortality and morbidity risk. In order to achieve these aims, it is essential to choose the most appropriate treatment with regard to disease-specific characteristics (cyst number, size, site, presence of cystobiliary communication), to patient clinical conditions, availability of an experienced surgeon or an interventional radiologist.

Three therapeutic modalities are available to treat hepatic CE: chemotherapy, surgery (with open or laparoscopic approach) and percutaneous treatments (PTs). A stage-specific approach is recommended<sup>[49]</sup>.

**Surgery:** Until the 1980s, surgery was the only therapeutic option for patients with CE. Surgery is still the first choice for large CE2-CE3b cysts with multiple daughter cysts or for single superficial cysts, considering the likelihood of spontaneous or traumatic rupture, when PT is not available. Presence of complicated cysts, e.g., infected cysts or cysts communicating with the biliary tree, and cysts exerting pressure on other vital organs, are other indications for surgical approach. Surgery is contraindicated in patients whose preexisting medical conditions put them at risk or in patients having inactive asymptomatic cysts or multiple cysts which are difficult to access. If feasible, surgical removal of hydatid cysts has the best chance to completely remove cysts and to immediately cure CE.

Surgical options can be divided into radical (pericystectomy) and conservative approaches (for instance unroofing or capitonnage)<sup>[50-53]</sup>. Radical procedures are associated with a lower risk of recurrence, but also with a higher operative risk; conservative procedures, on the contrary, are easier to perform but have a higher likelihood of recurrence. Recurrence is usually due to either inadequate cyst removal or to previously undetected cysts; reported recurrence rates range from 2% to 25%<sup>[54]</sup>.

Whichever technique is used, a benzimidazole (BMZ) agent is usually used to reduce the risk of anaphylaxis and secondary CE<sup>[55]</sup>. BMZ is administered from 1 d before surgery to 1 mo after surgery but, again, no conclusive data about the best timing are available. Major complications of surgery are postoperative hemorrhage, cholangitis, sepsis and fistulae formation. Operative mortality varies from 0.5% to 4%<sup>[55]</sup>.

**Percutaneous treatments:** PTs of hepatic CE can aim at the destruction of the germinal layer [puncture, aspiration, injection, re-aspiration (PAIR)] or the evacuation of the entire endocyst ("modified catheterization technique").

PAIR is an acronym that stands for "puncture, aspiration, injection, re-aspiration". PAIR consists of four steps: (1) percutaneous puncture of the cyst using ultrasound guidance; (2) aspiration of the cyst fluid; (3) injection of a protoscolicidal agent (e.g., 95% ethanol or 20% NaCl) for at least 15 min; and (4) re-aspiration of the fluid<sup>[37,56]</sup>.

PAIR is indicated for CE1 and CE3a cysts > 5 cm<sup>[49,56]</sup>; CE2 and CE3b cysts treated by PAIR tend to relapse. PAIR has also been used for patients who refused surgery

or relapsed after surgical treatment. It is contraindicated for inaccessible or superficially located liver cysts and for inactive or calcified cystic lesions. The possibility of secondary echinococcosis can be minimized by concurrent treatment with benzimidazoles; indeed, combined treatment (PAIR plus albendazole) may yield better results than those of either chemotherapy or PAIR alone<sup>[57,58]</sup>. The length of administration of chemotherapy with albendazole usually ranges between 4 h before and 1 mo after PAIR, in order to reduce the risk of disease recurrence and intraperitoneal seeding of infection. PAIR must be avoided in patients with cystobiliary communications, to prevent the risk of sclerosing cholangitis.

**Chemotherapy:** Mebendazole (MBZ) and albendazole (ABZ) are the BMZ agents used for the treatment of hepatic CE. They interfere with the absorption of glucose through the wall of the parasite, causing glycogen depletion and degenerative changes in echinococcal mitochondria and endoplasmic reticulum. BMZ may be favorably used alone for the treatment of small (< 5 cm) CE1-CE3a liver cysts<sup>[59]</sup> or for inoperable patients; BMZs are also usually associated with PAIR or surgery to prevent secondary CE<sup>[55]</sup>. BMZs are not indicated for the treatment of inactive or calcified asymptomatic cysts, unless they are complicated lesions<sup>[49]</sup>.

Both ABZ and MBZ are effective, but ABZ is considered the drug of choice, because it is more active *in vitro* and it has a better gastrointestinal absorption and bioavailability<sup>[60,61]</sup>. The usual dose of orally-administered ABZ is 10-15 mg/kg per day in two divided doses; if MBZ, the daily dose is 40-50 mg/kg in three divided doses. Treatment with BMZ should be administered continuously, for 3-6 mo<sup>[49]</sup>.

Clinical and radiographic improvement (in most studies defined as > 25% reduction in cyst size, membrane separation, or cyst calcification<sup>[62]</sup>) is quite frequent and is favorably influenced by the duration of treatment. Unfortunately, complete cure (i.e., cyst disappearance) only occurs in approximately a third of patients treated with BMZ alone and, interestingly, the number of patients with cure does not significantly increase by extending the duration of treatment<sup>[60]</sup>. A recent systematic review<sup>[63]</sup> has confirmed that the size and stage of cysts are the key factors to evaluate the likelihood of response to chemotherapy.

Usual adverse effects include nausea, hepatotoxicity, neutropenia and occasionally alopecia. Thus, all patients should have regular monitoring of leukocyte counts and liver function tests. Contraindications to chemotherapy include pregnancy, chronic hepatic diseases and bone marrow depression.

Praziquantel has been used (40 mg/kg once a week) with ABZ for combined treatment of CE; this therapeutic association seems to be more effective than ABZ alone<sup>[64]</sup>.

For uncomplicated CE4 and CE5 cysts a “watch and wait” strategy is currently advised<sup>[49]</sup>.

## HEPATIC ALVEOLAR ECHINOCOCCOSIS CAUSED BY *E. MULTILOCULARIS*

Hepatic alveolar echinococcosis (AE) results from infection by the larval forms of *E. multilocularis*. The echinococcal metacestode develops in the liver and is characterized by an alveolar structure, made up by several vesicles surrounded by large granulomas. Human AE is a severe and emerging disease, whose prognosis is bleak in absence of treatment or if it is not diagnosed at an early stage of disease.

### *E. multilocularis*: The parasite life cycle

*E. multilocularis* is a small cestode (1.2-4.5 mm), whose definitive hosts are wild carnivores such as red fox and arctic fox (sylvatic cycle) or domestic dogs and cats (synanthropic cycle). The adult tapeworms, whose bodies are characterized by a mean number of five proglottids, reside in the small bowel of their definitive hosts, where gravid proglottids release eggs which are passed in the feces. Intermediate hosts, usually small rodents, or aberrant hosts such as humans, become infected by ingestion of embryonated eggs. Human infection can happen through direct contact with the definitive host or it can be indirect, through contamination of food or water with parasite eggs<sup>[7,65]</sup>. The echinococcal metacestode develops in the liver and is characterized by an alveolar structure, made up by several vesicles whose diameter varies from < 1 mm up to 15-20 cm<sup>[47,65]</sup>. Each vesicle has a wall structure similar to that of the *E. granulosus* cyst, consisting of a germinal and a laminated layer<sup>[66]</sup>. Brood capsules or protoscolices are only occasionally seen and lesions may be complicated by central necrosis, producing a cavity or pseudocyst after liquidization. Small cysts are surrounded by a dense connective tissue and they usually do not contain fluid but instead a semisolid matrix<sup>[6]</sup>.

### Host-parasite interaction

*E. multilocularis* is able to elicit a strong cellular immune response: in the liver, parasitic lesions appear to be surrounded by large granulomas made up by macrophages, T-lymphocytes and myofibroblasts<sup>[67-69]</sup>. Observations in humans and experiments with rodents have shown that cellular immunity, related to TH1 cytokine profile, has a crucial role in host defense against the parasite<sup>[70]</sup>. IL-12, a key factor in the induction of TH1 profile, has been shown to inhibit, in mice, the development of alveolar lesions, leading to the formation of abortive parasitic vesicles surrounded by fully efficient periparasitic immune cell infiltration and fibrosis<sup>[71]</sup>. In mice treated with IFN- $\gamma$ , a typical TH1 cytokine, a partial reduction in larval growth has been observed<sup>[72]</sup>. In contrast, a TH2 cytokine profile has been associated with disease progression: high levels of IL-5 and IL-10 have been detected in serum of patients with progressive disease, compared with individuals with abortive forms<sup>[73-76]</sup>. As in the case of *E. granulosus*, several mechanisms have been proposed

to explain *E. multilocularis* avoidance from host-protective responses, including antigenic disguise<sup>[77]</sup>, immunomodulation<sup>[78-80]</sup>, molecular mimicry<sup>[81]</sup>, antigen and DNA polymorphism<sup>[82,83]</sup>.

### Epidemiology and infection risk

Data on human AE are difficult to be evaluated due to its low prevalence<sup>[21]</sup>, which does not allow a reliable recognition of temporal developments or differences in spatial distribution. The long asymptomatic period also makes it difficult to determine time and place of infection<sup>[84]</sup>. *E. multilocularis* is endemic in the Northern hemisphere, including North America (Alaska, Canada), Asia (some of the newly independent states of the former Soviet Union<sup>[85]</sup>, China<sup>[86]</sup> and Japan) and some European countries<sup>[87]</sup> (mainly France, Switzerland, Austria, Germany)<sup>[21,22]</sup>. In endemic areas, annual incidence of AE ranges from 0.03 to 1.2/100 000 inhabitants<sup>[88,89]</sup>. Increasing fox population, increased fox encroachment into urban areas and *E. multilocularis* spillover from wild carnivores to domestic hosts, are all factors that may explain *E. multilocularis* spreading from endemic areas to previously non-endemic European countries<sup>[21,90]</sup>.

Considering the parasite life cycle, exposure of humans to echinococcal eggs may be influenced by occupational and behavioral factors. Hunters, trappers and persons who work with fox fur should be more frequently exposed to *E. multilocularis* eggs, but there is no evidence that these groups are at increased risk<sup>[91,92]</sup>.

### Clinical aspects

Slow larval growth results in an asymptomatic phase of several years (5-15 years). Initially, the liver, usually the right lobe, is the organ where the metacestodes establish themselves; then, later in the infection, it is possible to find blood metastasis to lung, brain, bones and local extension of the lesion (abdomen, retroperitoneum, diaphragm)<sup>[66]</sup>. First symptoms are usually vague: patients may complain of fatigue, weight loss or may have hepatomegaly. One third of them have cholestatic jaundice; one third present with abdominal pain<sup>[54,66,93]</sup>. In advanced stages, liver failure usually occurs and it is frequently associated with portal hypertension, ascites and splenomegaly. The prognosis in untreated or inadequately treated patients with AE is poor. Treatment has radically changed average life expectancy at diagnosis from 3 years in the 1970s to 20 years in 2005<sup>[94]</sup>.

### Diagnosis

As for CE, AE diagnosis is based on clinical and epidemiologic findings, imaging techniques, nucleic acid detection and serology.

Among the imaging techniques, ultrasonography is the method of choice to identify hydatid lesions: ultrasound (US) typical aspect shows a pseudotumoral mass, with irregular limits and scattered calcification, where hypoechogenic and hyperechogenic areas are juxtaposed; central necrosis may give to the mass the appearance of a cystic-

like structure, surrounded by a hyperechogenic ring<sup>[95,96]</sup>. Color doppler may be useful to evaluate biliary and vascular infiltration. Abdominal CT gives further anatomical details and information about the lesion pattern of calcification<sup>[65]</sup>. MR imaging is the best standard to study the invasion of adjacent structures and may help in unclear cases<sup>[97]</sup>. Pre-surgical percutaneous cholangiography is important to assess the presence of communication between the biliary tree and the alveolar lesions<sup>[96]</sup>; it is also fundamental to exclude extra-hepatic involvement, through pulmonary and cerebral radiological examination.<sup>[18f]</sup> Fluorodeoxyglucose positron emission tomography (FDG-PET) scanning gives indirect information on the parasite metabolic activity, especially if combined with MRI or CT scan; if negative, this finding does not mean that the parasite is not viable but that there is a suppressed periparasitic inflammatory activity<sup>[98]</sup>.

WHO classification of AE is based on imaging findings and it is useful to have an internationally recognized, uniform standard for disease diagnosis and treatment strategies. The WHO-IWGE PNM classification system<sup>[65,99]</sup> is similar to tumor TNM classification: "P" refers to the extent of parasite localization inside the liver, "N" establishes the involvement of neighboring organs, "M" evaluates the absence (M0) or presence (M1) of distant metastasis, after having performed a chest X-ray and a cerebral CT.

As in CE, immunodiagnosis has a complementary role to other procedures, not only in primary diagnosis but also for follow-up of patients after surgical treatment or chemotherapy<sup>[100,101]</sup> and for the specific differential diagnosis between AE and CE in those regions where the diseases are co-endemic<sup>[102,103]</sup>. Immunodiagnosis (with indirect hemoagglutination test or ELISA) is more reliable for the diagnosis of AE than for CE, because more specific antigens are available. For example, the Em2plus-ELISA, which is a mixture of affinity purified *E. multilocularis* metacestode antigens (Em2-antigen) and a recombinant antigen (Em II /3-10), has shown a great sensitivity and specificity<sup>[104]</sup>, but it is not able to discriminate between active and inactive lesions; in fact, Em2-ELISA may be positive for years after spontaneous or pharmacological-induced dying out of the metacestode in patients with calcified lesions, because the Em2 antigen main source is the laminated layer of the parasite which obviously persists in these inactive lesions. Surgical removal of the dried-out lesion results in an immediate seroconversion to negative anti-Em2 antibodies<sup>[105,106]</sup>. Considering that the protoscolex is the most active component of echinococcal tissues, protoscolex antigens Em16 and Em18 have been isolated and used for immunoblot tests, in order to discriminate between active and inactive lesions<sup>[107]</sup>; recombinant (r) Em18 appears to be a promising immunodiagnostic tool for serological differentiation between AE and CE<sup>[107,108]</sup>. Combining US and serological data, it is possible to classify seropositive patients into three groups: patients with active hepatic lesions, patients with calcified lesions and patients with no

evidence of hepatic lesions<sup>[49]</sup>. The latter cases are a consequence of immune system pressure, which can cause larval degeneration and death, so that the only radiological sign of the host-parasite interaction may be the US finding of calcifications<sup>[109]</sup>.

Some studies have shown that patients with AE have high levels of IgG1 and IgG4 antibodies and that after treatment they usually become seronegative for IgG4 antibodies<sup>[110-113]</sup>; IgG4 antibody reappearance can be considered a warning sign of disease reactivation.

Liver needle biopsy can be performed in uncertain cases and it can confirm AE diagnosis if histopathological examination identifies the presence of alveolar vesicles. RT-PCR on liver specimens, obtained by biopsy or surgery, has been used to assess parasite viability, while PCR can detect *E. multilocularis* DNA. These tests have a good positive predictive value, but a negative result does not exclude parasite activity and parasite presence in the liver, respectively<sup>[114]</sup>.

### Treatment

The key concept of AE treatment is to adopt a multidisciplinary approach to disease. Surgery and chemotherapy are the cornerstones of AE treatment and, as for CE, a stage-specific approach is recommended<sup>[49]</sup>.

**Surgery:** Surgery is the first-choice option in all operable patients. Radical resection of the entire hepatic parasitic lesions is the only curative procedure, even though it is often difficult to achieve because of echinococcal dissemination into host tissues. Palliative liver surgery is almost always contraindicated, because it does not offer advantages when compared with conservative treatment<sup>[115,116]</sup>. Pre-operative evaluation is important to establish lesions full resectability; WHO-IWGE PNM classification estimates quite well the likelihood to achieve radical resection<sup>[99]</sup>.

Liver transplant (LT) has been employed in otherwise terminal cases<sup>[117]</sup>. Indications for LT are the presence of severe liver failure or recurrent life-threatening cholangitis and the inability to perform a radical liver resection. The absence of extra-hepatic AE localizations is mandatory for LT<sup>[49]</sup>.

BMZ chemotherapy should be carried out for at least 2 years after surgery and patients should be monitored for at least 10 years, because of the risk of recurrence: in fact, unrecognized or invisible parasites can re-grow, even after some years, especially in post-LT immunosuppressed patients<sup>[118]</sup>.

**Chemotherapy:** Inoperable AE patients should receive continuous BMZ treatment for life; moreover, long-term BMZ administration (at least 2 years) is mandatory after surgical treatment. Pre-surgical BMZ therapy is advised only in the case of LT. ABZ is given orally at a dosage of 10-15 mg/kg per day, in two divided doses; if it is not tolerated, MBZ may be given at daily doses of 40-50 mg/kg per day, split into three divided doses with fat-rich meals<sup>[49]</sup>. Conventional and liposomal amphotericin B has

been used in patients who did not tolerate BMZ<sup>[119]</sup>. In a recent study nitazoxanide has not shown any efficacy for AE treatment<sup>[120]</sup>.

Therapy with BMZ has resulted in an increased 10-year survival rate of approximately 80% (6%-25% in untreated historical controls)<sup>[121]</sup>. BMZs are parasitostatic, not parasitocidal: after several years of BMZ treatment, in the absence of progression of AE lesions, it is possible to discuss whether treatment should be continued or not. Decision-making should be supported by the evaluation of parasite viability, usually by PET-CT<sup>[98]</sup>, and serum specific antibodies<sup>[101,102]</sup>. These tools may also be useful for the follow-up after BMZ withdrawal.

All AE patients should be monitored by US at frequent intervals and CT and/or MRI at intervals of 2-3 years, to evaluate disease recurrence or progression<sup>[49]</sup>.

**Endoscopic percutaneous interventions:** Interventional procedures may be considered in inoperable patients in the presence of complications such as liver abscesses, jaundice due to biliary duct obstruction, portal vein thrombosis or bleeding esophageal varices associated with portal hypertension<sup>[96]</sup>. EPIs with BMZ avoid palliative surgery and may improve the patient life expectancy and quality of life.

## CONCLUSION

Liver echinococcosis is a severe, neglected, often misdiagnosed disease; both AE and CE may be considered emerging public health problems, since CE is endemic in several countries in the world and AE is one of the most lethal helminthic diseases.

The last years have been characterized by significant advances in the knowledge of *Echinococcus* biology and interaction with the immune system; the development of more specific and sensitive immunological tests and the introduction of PCR for detection of parasite nucleic acid have increased the range of diagnostic tools. Furthermore, the improvement in surgical techniques, the introduction of effective drugs (e.g., BMZ) and minimally invasive treatments (e.g., PAIR) have deeply changed the life expectancy and quality of life of patients with HD.

Despite diagnostic and therapeutic progress, many unresolved problems are still waiting for a solution; for instance, there is a need for prevention programs able to monitor and control parasite spreading. Additionally, randomized, controlled trials comparing different therapeutic options, especially for CE, are urgently required, in order to provide new evidence to guide treatment decision-making.

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## Increased presence of effector lymphocytes during *Helicobacter hepaticus*-induced colitis

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### Abstract

**AIM:** To identify and characterize drosophila mothers against decapentaplegic (SMAD)3-dependent changes in immune cell populations following infection with *Helicobacter hepaticus* (*H. hepaticus*).

**METHODS:** SMAD3<sup>-/-</sup> (*n* = 19) and colitis-resistant SMAD3<sup>+/-</sup> (*n* = 24) mice (8-10 wk of age) were infected with *H. hepaticus* and changes in immune cell populations [T lymphocytes, natural killer (NK) cells, T regulatory cells] were measured in the spleen and mesenteric lymph nodes (MsLNs) at 0 d, 3 d, 7 d and 28 d post-infection using flow cytometry. Genotype-

dependent changes in T lymphocytes and granzyme B<sup>+</sup> cells were also assessed after 28 d in proximal colon tissue using immunohistochemistry.

**RESULTS:** As previously observed, SMAD3<sup>-/-</sup>, but not SMAD3<sup>+/-</sup> mice, developed colitis, peaking at 4 wk post-infection. No significant changes in T cell subsets were observed in the spleen or in the MsLNs between genotypes at any time point. However, CD4<sup>+</sup> and CD8<sup>+</sup>/CD62L<sup>lo</sup> cells, an effector T lymphocyte population, as well as NK cells (NKp46/DX5<sup>+</sup>) were significantly higher in the MsLNs of SMAD3<sup>-/-</sup> mice at 7 d and 28 d post-infection. In the colon, a higher number of CD3<sup>+</sup> cells were present in SMAD3<sup>-/-</sup> compared to SMAD3<sup>+/-</sup> mice at baseline, which did not significantly change during infection. However, the number of granzyme B<sup>+</sup> cells, a marker of cytolytic lymphocytes, significantly increased in SMAD3<sup>-/-</sup> mice 28 d post-infection compared to both SMAD3<sup>+/-</sup> mice and to baseline values. This was consistent with more severe colitis development in these animals.

**CONCLUSION:** Data suggest that defects in SMAD3 signaling increase susceptibility to *H. hepaticus*-induced colitis through aberrant activation and/or dysregulation of effector lymphocytes.

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**Key words:** Transforming growth factor- $\beta$ ; Colitis; Drosophila mothers against decapentaplegic; Colon cancer; T lymphocytes

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## INTRODUCTION

Individuals with inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), are at a higher risk of developing colon cancer than the general population<sup>[1]</sup>. A meta-analysis of 116 studies indicated that the prevalence of colon cancer in patients with UC is approximately 3.7% (95% CI: 3.2-4.2), with the cumulative probability reaching 18% by 30 years regardless of disease severity<sup>[2]</sup>. Although the etiology of UC is poorly understood, there are indications that the immune system of individuals with UC reacts abnormally to bacteria in the digestive tract. This altered immune response leads to the inflammation-associated pathology of IBD<sup>[3-5]</sup>.

Imbalances in both innate and adaptive immune cells, such as natural killer (NK) cells and T cell subsets, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD4/CD25/Foxp3<sup>+</sup> T regulatory (Treg) cells, are associated with the pathogenesis of IBD<sup>[2]</sup>. The inflammation and damage caused by increased secretion of inflammatory cytokines during an active disease state is thought to be triggered by cytotoxicity against the commensal bacteria<sup>[6]</sup>. For example, levels of NK cytotoxicity in UC are related to the clinical stage of the disease<sup>[7]</sup>. In active disease states, NK cells are present in normal numbers, but are functionally defective, whereas NK cells exhibit normal cytotoxic activity in an inactive disease state<sup>[7]</sup>. Induction of inflammatory cytokines can also result from the disruption of the homeostatic balance between Treg and effector T helper (Th) cells. Elevated levels of pro-inflammatory CD4<sup>+</sup> T cells lead to excess cytokine/chemokine production, thereby recruiting additional leukocytes and influencing the severity of the inflammatory response<sup>[2]</sup>. CD8<sup>+</sup> T cells are also important in the pathogenesis of UC in humans, as demonstrated by extensive CD8<sup>+</sup> T cell infiltration within intestinal lesions contributing to mucosal damage<sup>[8,9]</sup>.

Transforming growth factor (TGF)- $\beta$  is a multifunctional cytokine that plays an important role in epithelial and immune cell homeostasis<sup>[10,11]</sup>. TGF- $\beta$  mediates many diverse biological functions on different cell types through receptor-mediated phosphorylation and activation of the drosophila mothers against decapentaplegic homolog (SMAD) family proteins, notably SMAD2 and SMAD3, which migrate to the nucleus and induce transcription of a targeted set of genes<sup>[12,13]</sup>. Dysfunctions in one or more components of TGF- $\beta$  signaling are commonly observed in human IBD and during colon cancer development. For example, loss of expression of the TGF receptor type II is observed in 90% of microsatellite instable colon

cancers, leading to loss of growth regulation in epithelial cells<sup>[14]</sup>. Additionally, although the TGF- $\beta$  isoform is overexpressed in the colon of individuals with IBD<sup>[15]</sup>, nuclear signaling is impaired due to increased levels of SMAD7<sup>[16]</sup>. SMAD7 normally inhibits TGF- $\beta$  signaling by blocking activation of SMAD2/3 in response to receptor-ligand binding. Normalizing SMAD7 expression restores TGF- $\beta$  signaling through SMAD3 and inhibits proinflammatory cytokine production by lamina propria mononuclear cells<sup>[16]</sup>.

Impairments in one or more components of the TGF- $\beta$  signaling pathway are implicated in intestinal inflammation in rodent models. For example, homologous knockout of the *TGF- $\beta$ 1* gene in mice causes an excessive inflammatory response in multiple organs, including the heart, lungs, and intestinal tract leading to premature death<sup>[17,18]</sup>. Additionally, Maggio-Price *et al.*<sup>[19]</sup> have demonstrated that disruption of the transcription factor SMAD3 modulates colitis susceptibility following infection with certain *Helicobacter spp.* Among these, *Helicobacter hepaticus* (*H. hepaticus*) is a Gram-negative spiral bacterium that colonizes the lower intestine and the hepatobiliary tract of mice<sup>[20]</sup>. Although generally asymptomatic, infection can lead to hepatic and intestinal inflammation in certain strains of immunodeficient mice<sup>[21-24]</sup>. In the complete absence of SMAD3 signaling, *H. hepaticus* induces a moderate inflammatory response in the cecum and colon, eventually leading to mucinous adenocarcinoma formation after 15-30 wk<sup>[19]</sup>. It is generally accepted that chronic low levels of inflammation lead to cancer promotion and progression<sup>[25-28]</sup>, therefore, the SMAD3 mouse model is very similar to the development of specific human cancers where pathogen-induced inflammation is necessary (but not sufficient) to cause dysplasia and tumor formation.

Using this model, the focus of the current study was to investigate the effect of SMAD3 deficiency on changes in local and systemic immune cell populations following infection with *H. hepaticus*. We hypothesized that colitis susceptibility in SMAD3<sup>-/-</sup> mice induced by *H. hepaticus* is associated with altered immune cell populations compared to colitis resistant SMAD3<sup>+/-</sup> mice. The aims of this study were to: (1) characterize the colitis induced by *H. hepaticus* in colitis-sensitive SMAD3<sup>-/-</sup> vs resistant SMAD3<sup>+/-</sup> mice; (2) compare the immune cell population changes in the spleen and mesenteric lymph nodes (MsLNs); and (3) compare local immune cell changes by immunohistochemistry in the colon.

## MATERIALS AND METHODS

### Murine model

SMAD3<sup>+/-</sup> and SMAD3<sup>-/-</sup> (129-Smad3<sup>tm1Par</sup>/J) mice were bred in-house. Homozygous males and heterozygous females were mated to obtain both SMAD3<sup>+/-</sup> and SMAD3<sup>-/-</sup> pups. Genotypes were confirmed by polymerase chain reaction (PCR). Animals were housed under specific pathogen-free (SPF) conditions in 60 square

inch plastic cages (maximum of five adult mice per cage) with microisolator lids in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at Michigan State University. SPF conditions were assured through quarterly serology testing by Charles Rivers (Wilmington, MA, United States) and in-house testing for ectoparasites, endoparasites and fecal *Helicobacter* species (PCR). Full necropsies (including culture and sensitivity) were performed at least yearly on rodent breeding colonies. Animal rooms were maintained at  $23.3 \pm 2.2$  °C with a 12-h light/dark cycle. Mice were fed Harlan Teklad 7913 rodent chow and sterile water *ad libitum*. Animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

### Bacterial culture and infection

The wild-type *H. hepaticus* strain 3B1 (ATCC 51488) was utilized for these experiments. Isolates were aseptically streaked onto sheep blood agar and incubated at 37 °C for 24–48 h inside GasPak™ gas generating pouch systems (BD Diagnostic Systems, Sparks, MD, United States). Mice were infected as previously described<sup>[19]</sup>. Briefly, bacteria were collected and resuspended in tryptic soy broth at  $A_{600\text{ nm}} \geq 1.8$ . Animals were then gavaged with 0.3 mL doses of fresh bacterial suspension on two consecutive days. Previously, Maggio-Price *et al.*<sup>[19]</sup> have shown that *Helicobacter* infection is localized primarily in the cecum and proximal colon, and that bacterial DNA is still present in the tissue and luminal contents of the cecum at 12 wk post-infection. Bacterial presence was confirmed in the current study *via* DNA isolation at 3 d post-infection using a commercial kit (QIAGEN tissue kit; Valencia, CA, United States) as previously described<sup>[24]</sup>.

### Experimental design

In study 1, SMAD3<sup>-/-</sup> mice ( $n = 30$ ) at 8–10 wk of age were infected with *H. hepaticus* to determine onset and duration of colitis. At the time of necropsy, mice were asphyxiated with CO<sub>2</sub> and exsanguinated *via* cardiac puncture. Intestinal tissue was collected and processed for histopathology at 2–8 wk post-infection. In study 2, SMAD3<sup>+/-</sup> ( $n = 24$ ) and SMAD3<sup>-/-</sup> mice ( $n = 19$ ) at 8–10 wk of age were infected with *H. hepaticus* once per day for two consecutive days. At select time points after infection (0, 3, 7 and 28 d), the spleen and MsLNs were collected and processed for lymphocyte isolation as described below. Colon and cecum tissue was collected, fixed, and processed for immunohistochemistry.

### Histopathology

The colon and cecum were removed and flushed with phosphate-buffered saline (PBS). Tissues were fixed in 10% formalin overnight, embedded in paraffin, then sectioned and stained with hematoxylin and eosin (HE). Longitudinal sections were graded for inflammation and epithelial dysplasia/neoplasia by a pathologist using a blinded scoring system adapted from Maggio-Price *et al.*<sup>[29]</sup>. Cecum and colons were scored on a 1 to 4

scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low-grade dysplasia; 3, high-grade dysplasia; 4, high-grade dysplasia with invasion/adenocarcinoma). The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and neoplasia.

Immunohistochemistry was performed on paraffin-embedded colon sections. Antibodies specific for CD3 and granzyme B were purchased from Abcam (Cambridge, MA, United States). Colons were sectioned at 5 µm, mounted on coated slides, deparaffinized in xylene, and rehydrated through graded ethanol-water baths. Antigen retrieval was performed using citrate buffer (10 mmol/L, pH 6.0) and a vegetable steamer. Tissues were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated overnight at 4 °C in primary antibody. On the following day, tissues were washed in Tris-buffered saline containing Tween-20 (0.05%), then incubated with biotinylated secondary antibodies followed by streptavidin horseradish peroxidase for 45 min each at room temperature (Dako, Carpinteria, CA, United States). After extensive washing, antigen-bound horseradish peroxidase was detected using the chromagen 3,3'-diaminobenzidine (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) dissolved in PBS (10 mmol/L, pH 7.2). Identification of cellular infiltrate in the colons of mice was performed by a pathologist. CD3<sup>+</sup> and granzyme B<sup>+</sup> cells were identified under a light microscope using a 20 × objective. The occurrence of positively stained cells was scored in proximal colons of mice in five fields using a 1-cm<sup>2</sup> grid reticle as follows: 0 = average of 0 cells/grid, 1 = average of ≤ 1 cell/grid, 2 = average of 2–10 cells/grid, 3 = average of 11–20 cells/grid, 4 = average of > 21 cells/grid. Final values represent mean ± SE per group ( $n = 3$ –5/group).

### Lymphocyte isolation

Spleens and MsLNs were removed and placed in ice-cold RPMI medium at the time of necropsy. Spleens were processed with a dounce homogenizer, pelleted, and washed in RPMI. Cells were resuspended in ACK lysing buffer (Invitrogen, Carlsbad, CA, United States) and washed twice in RPMI. MsLNs were treated with 5 mL enzymatic digest [5% fetal bovine serum (FBS), 0.5 mg/mL collagenase, 0.05 mg/mL DNase I] for 30 min at 37 °C. Cells were passed through 70-µm filters and washed with RPMI. Cell counts were performed with a hemocytometer using trypan blue exclusion and resuspended to a concentration of one million cells per milliliter of medium.

### Flow cytometry

Lymphocytes were resuspended in fluorescence-activated cell sorting (FACS) buffer (0.1% sodium azide, 1% FBS, in dPBS) blocked with anti-Fc receptor R II / III [CD16/

CD32 (purified from clone 2.4G2 hybridoma; ATCC, Manassas, VA, United States)] for 10 min on ice, and subsequently incubated with combinations of the following fluorochrome-conjugated antibodies (E-bioscience, San Diego, CA, United States; or BD Bioscience, San José, CA, United States) at concentrations ranging from 1:100 to 1:300 in FACS buffer: CD3 (PerCP-Cy5.5), CD4 (eFluor450), CD8 (PE-Cy7), CD25 (PE), FoxP3 (FITC or Alexa Fluor488), CD62 (APC), Nkp46 (FITC) and DX5 (APC). Cells were incubated in staining cocktails (one million cells per cocktail) on ice in the dark for 30 min. Intracellular staining was performed using FoxP3 staining buffer set as per the manufacturer's instructions (E-bioscience). Briefly, after surface staining, cells were washed twice in FACS buffer, fixed in 4% paraformaldehyde for 25 min, and permeabilized for 30 min. Permeabilization was followed by incubation for 30 min with the appropriate antibodies diluted in permeabilization diluent. Samples were then acquired on a LSR II (BD Bioscience) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, United States). The number of cells in each population of interest was determined by multiplying cell percentages by the total cell number.

### Statistical analysis

Data for the colitis and immunohistochemistry scores were analyzed using the nonparametric Kruskal-Wallis test and Dunn's post-test for specific comparisons. Flow cytometric data was analyzed using a two-way analysis of variance in GraphPad Prism (GraphPad Software, La Jolla, CA, United States). When statistical differences were detected, Tukey's multiple comparison test was used to determine differences between the two groups.  $P < 0.05$  was considered significant. All data are represented as mean  $\pm$  SE.

## RESULTS

### SMAD3-deficient mice are susceptible to colitis 4 wk post-infection

Colitis severity in SMAD3<sup>-/-</sup> mice (Figure 1A) peaked at 4 wk post-infection, with an average colitis score of  $7.8 \pm 0.4$ . This value was significant compared to samples taken at all other time points ( $P < 0.05$ ). Colitis resolved to baseline levels in SMAD3<sup>-/-</sup> mice by 8 wk post-infection. In comparison, SMAD3<sup>+/-</sup> mice were resistant to colitis development at all time points (data not shown). There was no statistically significant change in colitis scores in SMAD3<sup>+/-</sup> mice compared to baseline at any time point post-infection. Representative HE images from SMAD3<sup>+/-</sup> and SMAD3<sup>-/-</sup> mice prior to and 4 wk following infection are presented in Figure 1B.

### SMAD3-dependent changes in lymphocyte populations following *H. hepaticus* infection

We next evaluated genotype- and time-dependent changes in lymphocyte populations in the spleen and MsLNs using flow cytometry. There were no significant changes

in total CD3<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes in the spleen at baseline or at any time point following infection (Figure 2A-C). Tregs (FoxP3<sup>+</sup>/CD25<sup>+</sup>/CD4<sup>+</sup>) and NK cells (Nkp46<sup>+</sup>/DX5<sup>+</sup>) increased in both genotypes following infection but returned to baseline by 28 d (Figure 2D and E).

In the MsLNs, CD3<sup>+</sup>, CD4<sup>+</sup> and Treg cells were significantly higher in both genotypes at 7 d and 28 d post-infection (Figure 3A, C, and D), whereas there were no significant changes in CD8<sup>+</sup> cells at any time point examined (Figure 3B). NK cells increased in SMAD3-deficient mice by 7 d post-infection, and were significantly different from baseline values at 28 d (Figure 3E). Comparably, NK cells were not significantly altered at any time point in SMAD3<sup>+/-</sup> mice (Figure 3E).

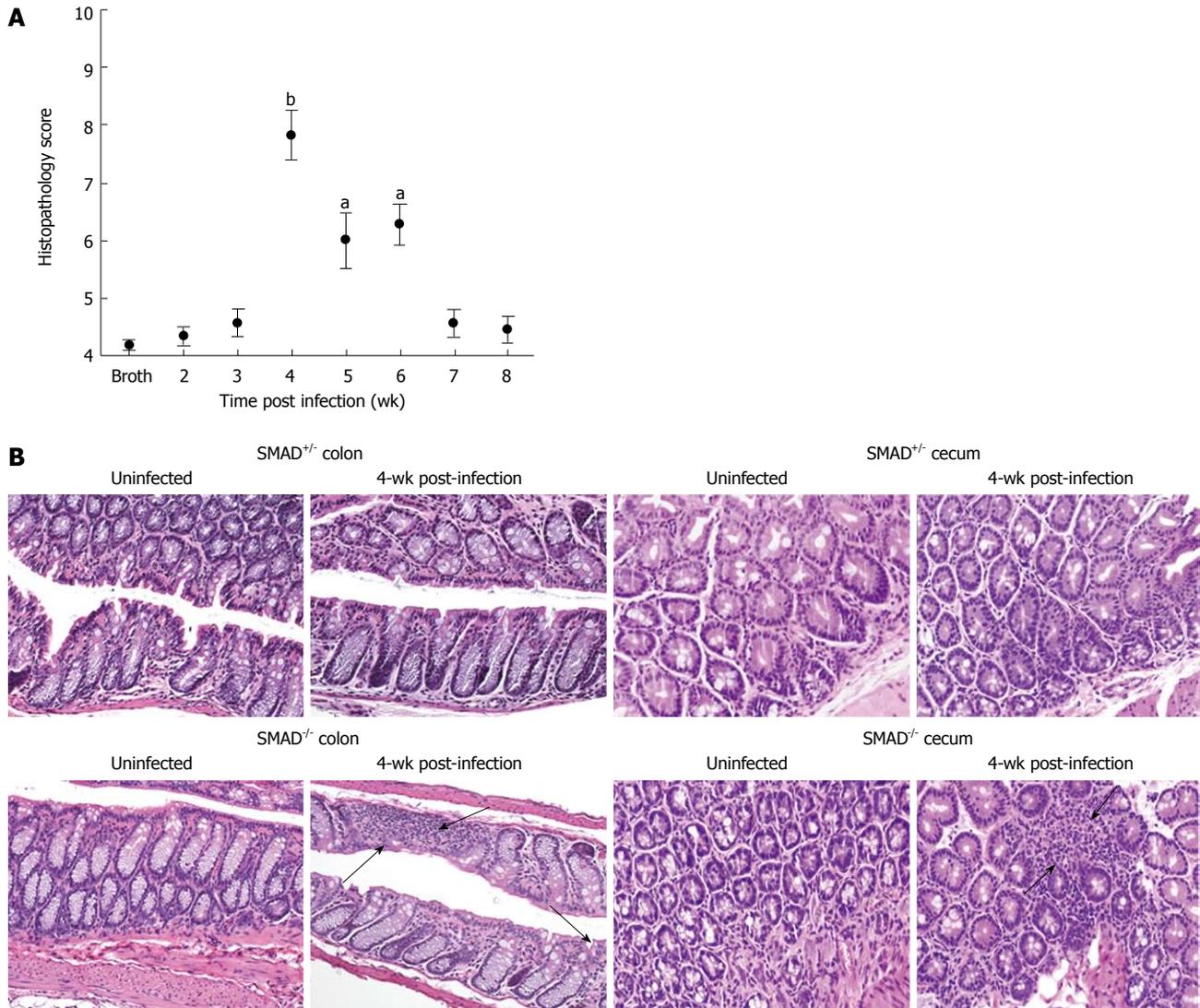
To determine activation status of the different T lymphocyte populations, we next evaluated surface expression of CD62L. L-Selectin (CD62L) is an adhesion marker expressed at high levels in naïve T cells and is cleaved from the surface (CD62L<sup>lo</sup>) in activated and/or in memory T cells. There were no statistically significant changes or observable trends in the proportion or total number of activated T cells in the spleen at any time point after infection (data not shown). However, the proportion of CD3<sup>+</sup>, CD8<sup>+</sup>, CD62L<sup>lo</sup> and CD3<sup>+</sup>, CD4<sup>+</sup>, CD62L<sup>lo</sup> cells was significantly higher in SMAD3<sup>-/-</sup> mice at 7 d and 28 d compared to baseline values and to SMAD3<sup>+/-</sup> mice (Figure 4A and D). Effector Treg cells increased in both strains at 7 d and 28 d compared to baseline values (Figure 4G). CD62L expression became dimmer at later time points in the SMAD3<sup>-/-</sup> mice for both CD8<sup>+</sup> and CD4<sup>+</sup> populations (Figure 4C and F) in the MsLNs, however, the intensity of CD62L expression was maintained consistently in SMAD3<sup>+/-</sup> mice through all time points (Figure 4B and E). No significant differences were observed in the percentage of Treg cells expressing reduced levels of CD62L between genotypes at any time point (Figure 4H and I).

### Immunohistochemical analysis of colon sections 28 d post-infection

We next evaluated local changes in CD3<sup>+</sup> cells and the serine protease, granzyme B, in the proximal colons of SMAD3<sup>+/-</sup> and SMAD3<sup>-/-</sup> mice 4 wk post-infection. The lamina propria in SMAD3<sup>-/-</sup> mice was moderately expanded by lymphocytic cells. Based on morphology and immunohistochemistry, these cells consisted primarily of CD3<sup>+</sup> lymphocytes (Figure 5A). Additionally, numerous granzyme B<sup>+</sup> cells were noted in the intestine of SMAD3<sup>-/-</sup> infected mice, primarily within the villous epithelium, but sometimes also within the lamina propria (Figure 5B).

## DISCUSSION

Functional TGF- $\beta$  signaling is crucial for maintaining immune cell homeostasis<sup>[30]</sup>. In the present study, we evaluated changes in local and systemic immune cell populations in colitis resistant SMAD3<sup>+/-</sup> and sensitive

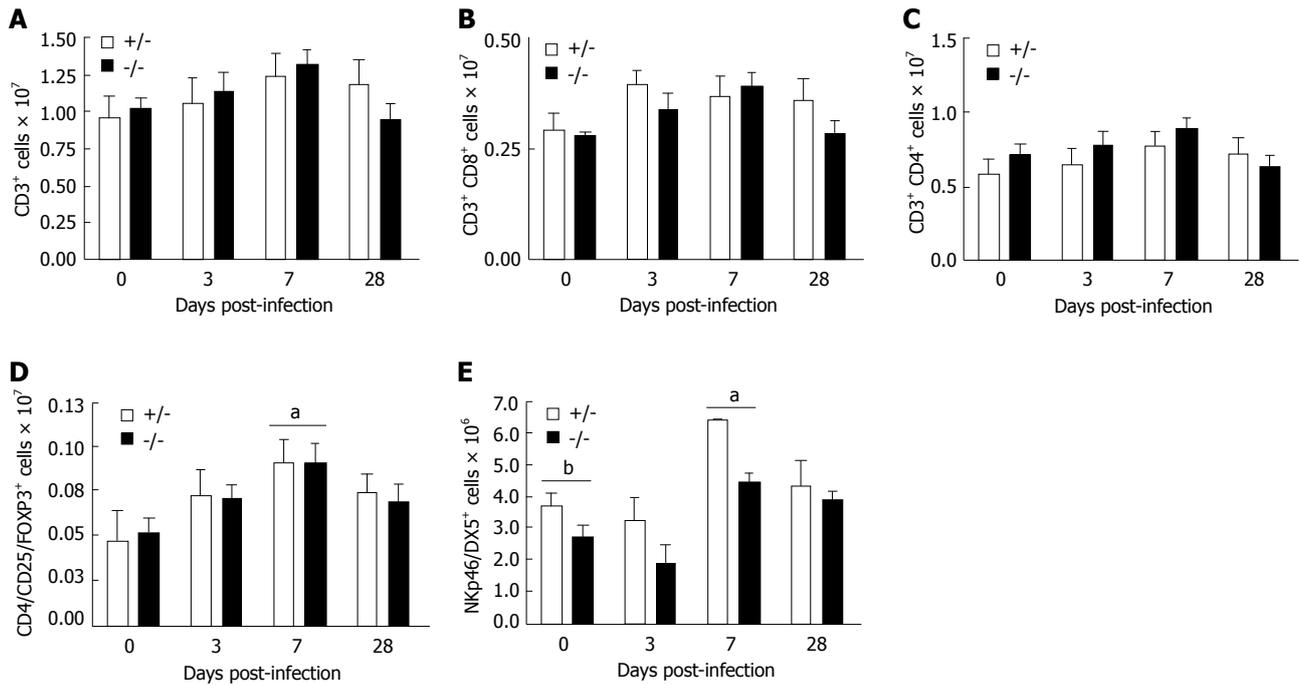


**Figure 1** *Drosophila* mothers against decapentaplegic 3<sup>+</sup> mice are more susceptible to colitis following infection with *Helicobacter hepaticus*. **A**: Inflammation and dysplasia scores in *drosophila* mothers against decapentaplegic (SMAD3)<sup>+/+</sup> mice post-infection (wk). The colon and cecum from each animal were given a separate score for inflammation and dysplasia (*n* = 30 animals/trt). Each animal received a total of four numerical scores for each of these criteria. The figure displays the average total of these scores with a lowest possible score of 4 and a highest possible of 16. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.001 vs control animals. There was no change in colitis scores among SMAD3<sup>-/-</sup> mice throughout the course of infection (data not shown); **B**: Hematoxylin and eosin-stained sections from the cecum and colon of SMAD3<sup>+/+</sup> (upper panel) and SMAD3<sup>-/-</sup> mice (lower panel) comparing uninfected and 4 wk after infection with *Helicobacter hepaticus* (*H. hepaticus*). Four weeks following infection, the number of inflammatory cells and primarily lymphocytes in the lamina propria was slightly increased in both tissues, consistent with mild inflammation (arrows denote infiltrate).

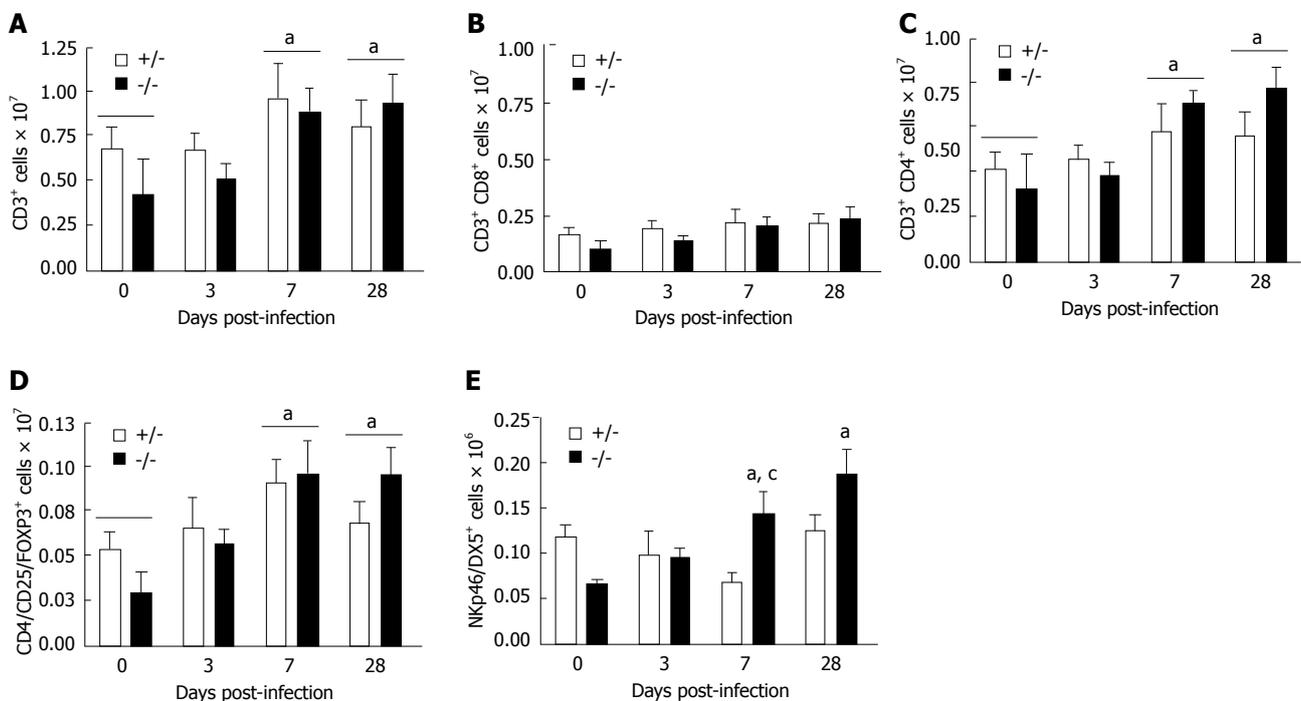
SMAD3<sup>-/-</sup> mice during the course of infection with the enteric pathogen, *H. hepaticus*. A major finding of this study was a significantly higher number of CD4 and CD8 effector cell populations in the mesenteric lymph nodes of SMAD3<sup>-/-</sup> mice at 7 d and 28 d post-infection compared to both baseline values and to SMAD3<sup>+/+</sup> mice. The number of granzyme B<sup>+</sup> cells, a marker of cytolytic lymphocytes, was also higher in proximal colon tissue at 28 d post-infection, consistent with colitis development in these animals. Our findings suggest loss of TGF-β signaling through SMAD3 leads to aberrant activation of colitogenic T cell subsets in response to *H. hepaticus*, whereas changes in specific T cell numbers were unaffected by genotype. These data are consistent with Maggio-Price *et al*<sup>[19]</sup>, who reported no significant T cell response to infection with *Helicobacter* *in vitro*, although

it is important to note that in that study only splenic lymphocytes were assessed, and that both *H. hepaticus* and *Helicobacter bilis* were used for infection. Additionally, Yang *et al*<sup>[31]</sup> reported no differences between SMAD3<sup>-/-</sup> and wild-type controls on development of T and B lymphocytes and NK cells, but found increased activated phenotype of T lymphocytes in SMAD3<sup>-/-</sup> mice that were resistant to TGF-β1 inhibition *in vitro*.

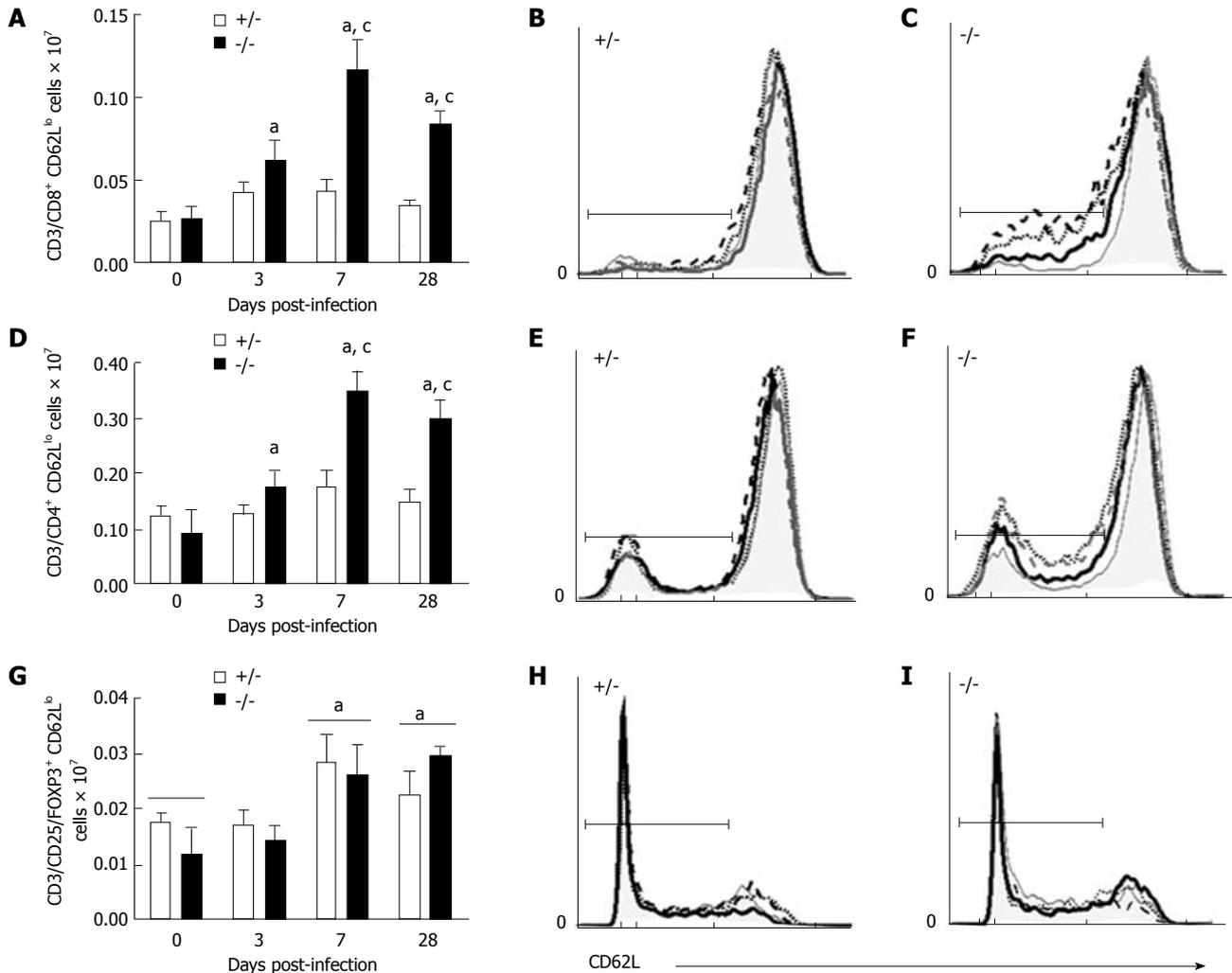
The inflammation associated with *H. hepaticus* infection in susceptible strains leads to a dysregulated Th1-type immune response, characterized by increased expression of interleukin (IL)-12 and interferon (IFN)-γ<sup>[19,32,33]</sup> as well as the proinflammatory cytokines IL-1α, IL-1β, IL-6 and tumor necrosis factor-α<sup>[19]</sup>. Treg cells normally function to control the inflammatory response by suppressing proliferation and activation of CD4<sup>+</sup> and CD8<sup>+</sup>



**Figure 2** Changes in T lymphocyte populations and natural killer cells in the spleen of drosophila mothers against decapentaplegic 3<sup>+/+</sup> and drosophila mothers against decapentaplegic 3<sup>-/-</sup> mice following infection with *Helicobacter hepaticus*. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. A: Total CD3<sup>+</sup> lymphocytes gated on forward scatter vs CD3; B: Total CD8<sup>+</sup> lymphocytes gated on CD3<sup>+</sup> lymphocytes; C: Total CD4<sup>+</sup> lymphocytes gated on CD3<sup>+</sup> lymphocytes; D: Total CD25<sup>+</sup>/FOXP3<sup>+</sup> Treg cells gated on CD3<sup>+</sup>/CD4<sup>+</sup> lymphocytes; E: Total natural killer (NK)p46<sup>+</sup>/DX5<sup>+</sup> NK cells in spleen tissue (n = 4-6 animals per time point). <sup>a</sup>P < 0.05 vs baseline values (7 d vs 0 d); <sup>b</sup>P < 0.05 denotes significance between genotypes [drosophila mothers against decapentaplegic (SMAD)3<sup>+/+</sup> vs SMAD3<sup>-/-</sup>].



**Figure 3** Changes in T lymphocyte populations and natural killer cells in the mesenteric lymph nodes of drosophila mothers against decapentaplegic 3<sup>+/+</sup> and drosophila mothers against decapentaplegic 3<sup>-/-</sup> mice following infection with *Helicobacter hepaticus*. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. A: Total CD3<sup>+</sup> lymphocytes gated on forward scatter vs CD3; B: Total CD8<sup>+</sup> lymphocytes gated on CD3<sup>+</sup> lymphocytes; C: Total CD4<sup>+</sup> lymphocytes gated on CD3<sup>+</sup> lymphocytes; D: Total CD25<sup>+</sup>/FOXP3<sup>+</sup> Treg cells gated on CD3<sup>+</sup>/CD4<sup>+</sup> lymphocytes; E: Total natural killer (NK)p46<sup>+</sup>/DX5<sup>+</sup> NK cells in mesenteric lymph nodes (n = 4-6 animals per time point). <sup>a</sup>P < 0.05 vs baseline values; <sup>c</sup>P < 0.05 denotes significant interaction between genotypes [drosophila mothers against decapentaplegic (SMAD)3<sup>+/+</sup> vs SMAD3<sup>-/-</sup>].

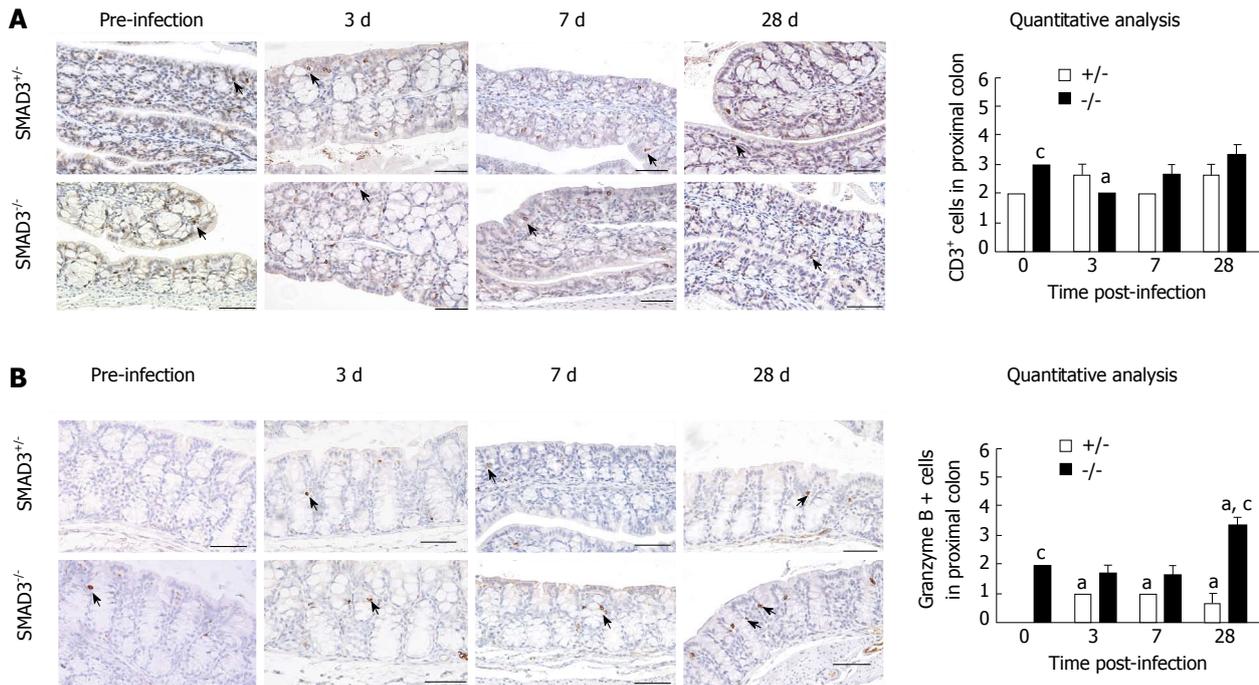


**Figure 4** Changes in effector T lymphocyte populations in the mesenteric lymph nodes of drosophila mothers against decapentaplegic 3<sup>-/-</sup> and drosophila mothers against decapentaplegic<sup>+/-</sup> mice following infection with *Helicobacter hepaticus*. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. Histograms represent CD62L expression at 0 d (solid grey), 3 d (dotted black), 7 d (dashed black) and 28 d (solid black) post-infection. Y-axis represents relative cell frequency. X-axis is CD62L expression. Brackets indicate CD62L<sup>lo</sup> gate used to determine cell percentages. A: Total CD8<sup>+</sup> CD62L<sup>lo</sup> lymphocytes gated on CD3<sup>+</sup>/CD8<sup>+</sup> lymphocytes; B: CD62L expression in drosophila mothers against decapentaplegic (SMAD3)<sup>3<sup>-/-</sup></sup> CD8<sup>+</sup> lymphocytes; C: CD62L expression in SMAD3<sup>3<sup>-/-</sup></sup> CD8<sup>+</sup> lymphocytes; D: Total CD4<sup>+</sup> CD62L<sup>lo</sup> lymphocytes gated on CD3<sup>+</sup>/CD4<sup>+</sup> lymphocytes; E: CD62L expression in SMAD3<sup>3<sup>-/-</sup></sup> CD4<sup>+</sup> lymphocytes; F: CD62L expression in SMAD3<sup>3<sup>-/-</sup></sup> CD4<sup>+</sup> lymphocytes; G: Total CD25<sup>+</sup>/FOXP3<sup>+</sup> CD62L<sup>lo</sup> Treg cells gated on CD3<sup>+</sup>/CD4<sup>+</sup> lymphocytes (n = 4-6 animals per time point); H: CD62L expression in SMAD3<sup>3<sup>-/-</sup></sup> CD25<sup>+</sup>/FOXP3<sup>+</sup> lymphocytes; I: CD62L expression in SMAD3<sup>3<sup>-/-</sup></sup> CD25<sup>+</sup>/FOXP3<sup>+</sup> lymphocytes. <sup>a</sup>P < 0.05 vs baseline values; <sup>c</sup>P < 0.01 denotes significant interaction between genotypes (SMAD3<sup>3<sup>-/-</sup></sup> vs SMAD3<sup>3<sup>-/-</sup></sup>).

lymphocytes, inhibiting production of the cytokines IL-2 and IFN- $\gamma$ , as well as producing the anti-inflammatory cytokine IL-10<sup>[34,35]</sup>. Transgenic mice lacking T and B lymphocytes, including *scid* and *rag-2*-deficient mice exhibit a more severe colitis that can be partially alleviated by adoptive transfer of IL-10-producing Treg cells<sup>[36-40]</sup>. Additionally, adoptive transfer of wild-type Treg cells into *rag2*-deficient mice inhibits *H. hepaticus*-induced colon cancer development<sup>[38,40]</sup>, further establishing an important role for this cell type in suppressing inflammatory signaling.

Importantly, Treg cell development is intricately dependent on TGF- $\beta$  signaling, whereas Treg cells themselves are a major source of this cytokine, deriving much of their suppressive function from TGF- $\beta$  production as well as IL-10. Given the importance of this cell type

in suppressing colitis in other models, we next evaluated whether SMAD3-deficiency impaired the development and/or activation of CD4<sup>+</sup>/CD25<sup>+</sup>/Foxp3<sup>+</sup> T regulatory cells. We found no significant difference between genotypes at baseline, suggesting normal development of this cell type in the absence of SMAD3 signaling. Following infection, Treg cells increased proportionally in both genotypes in both the spleen and MsLNs at 7 d and remained elevated in the latter at 28 d. To assess further whether activation of Treg cells may be impaired, we evaluated L-selectin (CD62L) expression, which is required for migration to sites of inflammation and is cleaved from the surface upon activation<sup>[41,42]</sup>. Although the number of activated Tregs (CD4<sup>+</sup>/CD25<sup>+</sup>/FOXP3<sup>+</sup>CD62L<sup>lo</sup>) increased at 7 d and 28 d post-infection in the MsLNs, there was no further difference between genotypes. Thus,



**Figure 5** Immunohistochemical staining for (A) CD3 lymphocytes and (B) granzyme B in proximal colon tissue of drosophila mothers against decapentaplegic 3<sup>+/+</sup> and drosophila mothers against decapentaplegic 3<sup>-/-</sup> mice following infection with *Helicobacter hepaticus*. <sup>a</sup>P < 0.05 vs baseline values; <sup>b</sup>P < 0.01 denotes significant interaction between genotypes [drosophila mothers against decapentaplegic (SMAD3)<sup>+/+</sup> vs SMAD3<sup>-/-</sup>] (n = 4-5 animals per stain). Arrows denote areas of positive staining. Scale bars represent 100 μm.

our findings suggest that SMAD3 deficiency does not influence Treg cell numbers in peripheral lymphoid tissue; however, this does not rule out the possibility that the suppressive effect of this cell type is influenced in a SMAD3-dependent manner. This is further supported by recent findings of Fantini *et al*<sup>[43]</sup> who have reported that overexpression of SMAD7 in CD4 T lymphocytes, which blocks TGF-β-mediated activation of SMAD2/3, impairs the ability of Treg cells to suppress T cell proliferation and proinflammatory cytokine expression both *in vitro* and *in vivo*.

NK cells are generally acknowledged to be important for cell-mediated immunity, and play an important role in the control of cellular infections as well as in antitumor immunity<sup>[44]</sup>. For example, NK cells can directly lyse infected/dysplastic cells through perforin-granzyme-dependent mechanisms and induce apoptosis<sup>[45-51]</sup>. Additionally, NK cells activate other effector immune cells through local production of cytokines<sup>[52]</sup>. Fort *et al*<sup>[53]</sup> have demonstrated that NK cells exert a protective effect on colitis by controlling the responses of effector CD4 T cells through perforin-dependent mechanisms<sup>[53]</sup>. Other studies have provided evidence that NK cells are in fact an innate source of IL-22 in the colon; a cytokine that has proinflammatory properties but is also proposed to protect tissues during inflammation<sup>[54-56]</sup>.

Yang *et al*<sup>[31]</sup> previously have found no effect of SMAD3 deficiency on development of NK cells in the spleen or MsLNs. In the current study, we determined whether SMAD3 deficiency would influence NK cells in peripheral lymphoid tissue in response to infection with *H. hepaticus*. Surprisingly, we found higher numbers of NK cells

(NKp46<sup>+</sup>/DX5<sup>+</sup>) in SMAD3<sup>-/-</sup> mice both at 7 d and 28 d post-infection in the MsLNs, whereas no corresponding changes in population numbers were observed in SMAD3<sup>+/-</sup> mice. Our findings of increased NK cell populations are somewhat inconsistent with the previously established protective role of this cell type<sup>[53]</sup>; however, it is possible that SMAD3 signaling mediates the balance of NK cell subsets in response to infection and/or cytotoxicity of NK cells. For example, significant enrichment of lamina propria NK cells of the CD56<sup>+</sup>CD16<sup>+</sup> cytotoxic subset in individuals with IBD has been reported<sup>[57]</sup>. Additionally, individuals with Crohn's disease have been reported to have a higher proportion of NKp46<sup>+</sup> compared to NKp44<sup>+</sup> NK cells in the intestinal mucosa, which is suggested to mediate pathogenesis through increased production of IFN-γ<sup>[58]</sup>. Importantly, TGF-β inhibits INF-γ production by NK cells<sup>[30,59]</sup>, suggesting the possibility of altered balance of NK cell subsets in our model.

Our findings highlight that mice infected with *H. hepaticus* and deficient in SMAD3 signaling have elevated levels of effector lymphocyte subsets in the MsLNs and in the colon likely contributing to increased colitis severity. Although no genotype differences in numbers of natural Treg cells were found following infection, it is possible that defective TGF-β signaling through SMAD3 may impair suppressive function. Alternatively, the latent presence of effector T cells may indicate continuous antigen presenting cell stimulation which was not addressed in these studies. Given the pleiotropic role of TGF-β signaling in immune cell homeostasis, further evaluation of cytokine production by activated T cells

derived from infected SMAD3-deficient mice would lead to a more thorough understanding of SMAD3 in colitis susceptibility. Additionally, very little is known about the role of SMAD3 in NK cell function, however, the higher presence of NKp46<sup>+</sup>/DX5<sup>+</sup> NK cells in the MsLNs of SMAD3-deficient mice might indicate altered NK subsets present in the MsLNs due to different chemokines being released throughout the course of the infection. The signaling pathways involved in initiating the inflammatory response to *H. hepaticus* in susceptible mouse strains has also not been well characterized. *H. hepaticus* activates nuclear factor- $\kappa$ B and extracellular signal-regulated kinase signaling in bone-marrow-derived macrophages<sup>[60]</sup>, which can induce both pro- and anti-inflammatory pathways<sup>[33,36,60,61]</sup>. Given the importance of TGF- $\beta$  signaling in both IBD and colon cancer development in humans, further identifying innate targets involved in initiating the SMAD3-dependent inflammatory response to pathogenic stimuli would prove highly useful in understanding the pathogenesis of IBD as well as for designing interventions that may alter immune cell populations and/or activation. Future studies addressing some of these possibilities are currently under investigation.

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## COMMENTS

### Background

Individuals with inflammatory bowel disease (IBD) are at an increased risk of developing colon cancer. Imbalances in immune cells, such as natural killer (NK) cells and many T cell subsets, are important in the pathogenesis of IBD. These imbalances, in addition to an abnormal reaction to natural gut bacteria, lead to increased inflammation and create an environment favorable for tumor formation in the colon.

### Research frontiers

Previous studies using the drosophila mothers against decapentaplegic 3 (SMAD3) mouse model, in which SMAD3<sup>-/-</sup> but not SMAD3<sup>+/-</sup> mice develop colitis and colon cancer after infection with *Helicobacter* bacteria, highlight similarities to the development of specific human cancers in which pathogen-induced inflammation is necessary (but not sufficient) to cause dysplasia and tumor formation. This study used this model to investigate the effect of a SMAD3 deficiency on changes in both tissue-specific and systemic immune cell populations after bacterial infection.

### Innovations and breakthroughs

Novel findings from this study illustrate that changes in immune response, due to genetic alteration and/or specific susceptibility, can affect the severity of colitis and potentially contribute to the development of colon tumors.

### Applications

These data also suggest potential targets for prevention and treatment of chronic IBD-related inflammation. Furthermore, the SMAD3 model may also prove useful in identifying dietary and/or other interventions that alter immune cell functionality, thereby reducing inflammation and cancer.

### Terminology

SMAD3 is an intracellular protein that functions as a signal transducer and tran-

scription factor for the transforming growth factor  $\beta$  superfamily.

### Peer review

This is an interesting manuscript. Overall, the topic is complicated and the authors present it well. There of course was a great deal of interest at one time in the treatment of ulcerative colitis with anti-*Helicobacter* antibiotics.

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## Immunological milieu in the peritoneal cavity at laparotomy for gastric cancer

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### Abstract

**AIM:** To investigate the immunological repertoire in the peritoneal cavity of gastric cancer patients.

**METHODS:** The peritoneal cavity is a compartment in which immunological host-tumor interactions can occur. However, the role of lymphocytes in the peritoneal cavity of gastric cancer patients is unclear. We observed 64 patients who underwent gastrectomy for gastric cancer and 11 patients who underwent laparoscopic cholecystectomy for gallstones and acted as controls. Lymphocytes isolated from both peripheral blood and peritoneal lavage were analyzed for surface markers of lymphocytes and their cytokine production by flow cytometry. CD4<sup>+</sup>CD25<sup>high</sup> T cells isolated from

the patient's peripheral blood were co-cultivated for 4 d with the intra-peritoneal lymphocytes, and a cytokine assay was performed.

**RESULTS:** At gastrectomy, CCR7<sup>+</sup>CD45RA<sup>-</sup>CD8<sup>+</sup> effector memory T cells were observed in the peritoneal cavity. The frequency of CD4<sup>+</sup>CD25<sup>high</sup> T cells in both the peripheral blood and peritoneal cavity was elevated in patients at advanced stage [control *vs* stage IV in the peripheral blood: 6.89 (3.39-10.4) *vs* 15.34 (11.37-19.31), *P* < 0.05, control *vs* stage IV in the peritoneal cavity: 8.65 (5.28-12.0) *vs* 19.56 (14.81-24.32), *P* < 0.05]. On the other hand, the suppression was restored with CD4<sup>+</sup>CD25<sup>high</sup> T cells from their own peripheral blood. This study is the first to analyze lymphocyte and cytokine production in the peritoneal cavity in patients with gastric cancer. Immune regulation at advanced stage is reversible at the point of gastrectomy.

**CONCLUSION:** The immunological milieu in the peritoneal cavity of patients with advanced gastric cancer elicited a Th2 response even at gastrectomy, but this response was reversible.

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**Key words:** Cytokines; Gastric cancer; Lymphocytes; Peritoneal cavity; Regulatory T cell

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## INTRODUCTION

Tumor progression is governed not only by the genetic changes intrinsic to cancer cells, but also by epigenetic and environmental factors. Therefore, neoplastic cell factors and biophylactic side factors such as immune reactions are interacting in the survival and development of micrometastasis. Increasing evidence gleaned from studies in immune-compromised hosts suggests that the cellular mechanisms of immunosurveillance influence tumor development. There are several lines of research which indicate the critical role of the immune system in controlling the growth of malignant cells<sup>[1-5]</sup>. Thus, impairment of anti-tumor immunity, which leads to immunologic toleration of malignant cells, contributes to the development and progression of peritoneal metastasis<sup>[6]</sup>. The elimination phase of the cancer immunosurveillance mechanism is thought to be a continuous process, and local control of metastatic invasion by the immune system may be critical for survival. However, the role of lymphocytes in the peritoneal cavity for anti-tumor immunity in gastric cancer patients is unknown<sup>[7]</sup>.

Studies in rodents have demonstrated that adoptive immunotherapy with antigen-specific CD8<sup>+</sup> T cells is effective for cancer, and there is evidence that this approach has therapeutic activity in humans<sup>[8-10]</sup>. Memory T cells circulate throughout all tissues of the body and are primed to rapidly produce secondary immune responses upon antigen challenge<sup>[11]</sup>. The nature of the cells that mediate the different facts of immunological memory remains unresolved. Natural killer T cells are a specialized subset of T cells. They express T-cell and natural killer-lineage cell surface markers and key cytokines, which regulate the course of the immune response. There are many mechanisms that regulate and dampen the immune response to cancers<sup>[12-15]</sup>. Regulatory T cells protect the host from autoimmune disease by suppressing self-reactive immune cells. As such, regulatory T cells may also block antitumor immune responses. Regulatory T cells have been an active research area in basic as well as in clinical immunology<sup>[16-18]</sup>. Th1 immune responses are considered to be essential for eradicating malignant cells. Based on the cytokine profile, interferon-gamma is a Th1 cytokine with an antitumor effect. Interleukin-10, a Th2 cytokine, inhibits Th1 immune responses and enhances the production of other Th2 cytokines<sup>[19-22]</sup>.

In order to clarify the clinical significance of the host immune response within the peritoneal cavity in patients with gastric cancer, we conducted an immunological analysis of the peritoneal lavage obtained from patients at the time of gastrectomy.

## MATERIALS AND METHODS

### Patients

A total of 75 patients (50 males and 25 females; mean age: 64.3 years) were included in this study. Sixty-four patients were histologically diagnosed as having gastric cancer. Among these, 56 had gastrectomy, 2 underwent bypass op-

**Table 1** Clinicopathological features in the examined gastric cancer patients

Variables	No. of patients
Total cases	64
Age (yr)	67.5 ± 2.8
Sex (male/female)	42/22
Depth of tumor invasion	
T1	32
T2	20
T3	9
T4	3
Lymphnode metastasis	
N0	34
N1	12
N2	14
N3	4
Peritoneal metastasis	
Absent	56
Present	8
Cytology	
Negative	57
Positive	7
Stage	
Stage I A	25
Stage I B	13
Stage II	7
Stage III	7
Stage IV	12

eration, and 6 had exploratory laparotomy. Eleven patients who underwent laparoscopic cholecystectomy for benign disease acted as controls. The resected specimens were histologically examined by hematoxylin and eosin staining according to the general rules of the Japanese Classification of Gastric Carcinoma<sup>[23]</sup>. The investigation protocol was approved by the Institutional Review Board of the Nagasaki University School of Medicine (#14122694). Written informed consent was obtained from all patients. The stages of gastric cancer patients were as follows: stage I A, *n* = 25 patients; stage I B, *n* = 13; stage II, *n* = 7; stage III, *n* = 7; and stage IV, *n* = 12. The clinicopathological features of the patients are shown in Table 1.

### Isolation of mononuclear cells from peripheral blood and peritoneal lavage

Endotracheal general anesthesia was induced and 10 mL of peripheral blood was taken from all patients. Four hundred milliliter of physiological saline was poured into the peritoneal cavity prior to manipulation of the tumor, and was recovered after being gently stirred. Half of the peritoneal lavage was allocated for conventional cytology and carcinoembryonic antigen (CEA) analysis by an enzyme-linked immunosorbent assay. The other half of the peritoneal lavage was immediately centrifuged at 2000 rpm for 10 min, and the supernatants were assayed for CEA values. The peritoneal CEA levels were then measured using an enzyme immunoassay kit (IMx-SERECT CEA, Dainabot, Tokyo) and the protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA, United States). The cell component was used for lymphocyte analysis. Lymphocytes from peripheral

**Table 2** Carcinoembryonic antigen values in sera and peritoneal lavage

Source	Control	Stage I A	Stage I B	Stage II	Stage III	Stage IV
CEA						
PB (ng/mL)	Not tested	2.09 (1.39-2.78)	2.03 (0.96-3.1)	3.06 (2.04-4.07)	2.54 (0.38-4.69)	7.98 (1.18-15.82)
PL (ng/g protein)	56.53 (21.82-91.24)	44.17 (27.37-60.96)	61.95 (11.98-111.91)	83.14 (7.31-187.54)	262.63 (7.26-517.26)	1234.00 (87.77-2380.22)
CD4/CD8						
PB (ratio)	5.379 (2.705-8.052)	5.595 (3.224-7.967)	4.571 (2.057-7.086)	5.277 (1.369-9.184)	7.999 (3.366-12.632)	4.156 (2.228-6.083)
PL (ratio)	0.494 (0.338-0.649)	0.553 (0.421-0.685)	0.697 (0.511-0.883)	0.638 (0.395-0.881)	1.242 (0.961-1.522)	1.158 (0.907-1.408)
CD45RA <sup>-</sup> /CCR7 <sup>-</sup>						
PB (%)	60.43 (46.42-74.44)	58.29 (48.93-67.64)	53.92 (32.65-75.2)	57.36 (42.01-72.71)	49.01 (29.31-68.71)	45.73 (32.79-58.67)
PL (%)	81.17 (81.12-93.22)	81.67 (76.35-87.01)	76.2 (59.43-92.96)	72.3 (61.01-83.58)	68.36 (58.70-78.02)	51.92 (38.34-65.50)
NKT						
PB (%)	9.19 (5.83-12.54)	7.59 (5.63-9.56)	9.47 (4.41-14.53)	10.71 (1.55-19.87)	5.43 (0.54-10.33)	7.16 (3.95-10.3)
PL (%)	18.1 (9.83-26.37)	17.25 (13.54-20.97)	15.74 (9.23-22.25)	15.38 (7.71-23.04)	9.66 (1.2-18.11)	9.91 (6.94-12.88)

PB: Peripheral blood; PL: Peritoneal lavage; CI: Confidence interval. The data are presented as the median and 95% CI. The statistical analysis of the differences revealed higher CEA and CD4/CD8, lower CD8<sup>+</sup> effector memory T cells and NKT cells in the peritoneal cavity in patients with advanced stage than in controls.

blood were isolated by density centrifugation over Ficoll-Paque<sup>TM</sup> gradients (Amersham, Uppsala, Sweden).

### Flow cytometry

The following monoclonal antibodies were used in the present study: fluorescein isothiocyanate (FITC)-conjugated anti-CD8, FITC-CD25, FITC-CD45RA, phycoerythrin (PE)-conjugated anti-CD4, PE-CD56, PE-CCR7, PE-IFN- $\gamma$ , PE-IL-10, PE-Foxp3, cychrome (Cy)-conjugated anti-CD3, and Cy-CD8 (BD Pharmingen, San Diego, CA, United States). Single-cell suspensions were stained in phosphate-buffered saline-1% fetal calf serum at saturating concentrations according to standard procedures. Flow cytometry was performed on the BD Biosystems-FACSCanto II system (BD Biosciences, San Diego, CA, United States), and FACSDiva software (BD Biosciences, San Diego, CA, United States) was used for analysis. All analyses of T cells were carried out after gating by CD3. The ratio of the percentage of CD4 and CD8 cells was represented as the CD4/CD8 ratio.

### Intracellular staining for Foxp3

Intracellular staining for Foxp3 was performed using the Human Foxp3 Buffer set (BD Pharmingen, San Diego, CA, United States) according to the manufacturer's protocol.

### Cytokine assays

Anti-IFN- $\gamma$ -PE and anti-IL-10-PE mAbs were used for the intracellular analysis of cytokine production. Peripheral and intra-peritoneal lymphocytes were activated with 10 ng/mL phorbol 12-myristate-13-acetate (PMA), 0.5  $\mu$ g/mL Ionomycin, and 1  $\mu$ L/mL GolgiPlug (BD Pharmingen, San Diego, CA, United States) for 4 h. Cells were washed, fixed and permeabilized by Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA, United States), and stained with titrated amounts of cytokine-specific antibodies.

Next, the CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from peripheral blood by magnetic beads (Miltenyi Biotech, BergischGladbach, Germany). These CD4<sup>+</sup> CD25<sup>+</sup> T cells

were mixed with intraperitoneal lymphocytes at a ratio of 1:10 and co-cultivated for 4 d in RPMI with 10% FBS. The CD4<sup>+</sup> CD25<sup>-</sup> T cells were co-cultivated with intraperitoneal lymphocytes as controls. The cytokine assay was performed by the intracellular cytokine method after 4 d of co-cultivation.

### Statistical analysis

The statistical analysis was performed using the Kruskal-Wallis test (non-parametric ANOVA) using a personal computer and the StatViewV.5.0 software package (SAS Institute, Cary, NC, United States). *P* values less than 0.05 were considered to indicate statistical significance.

## RESULTS

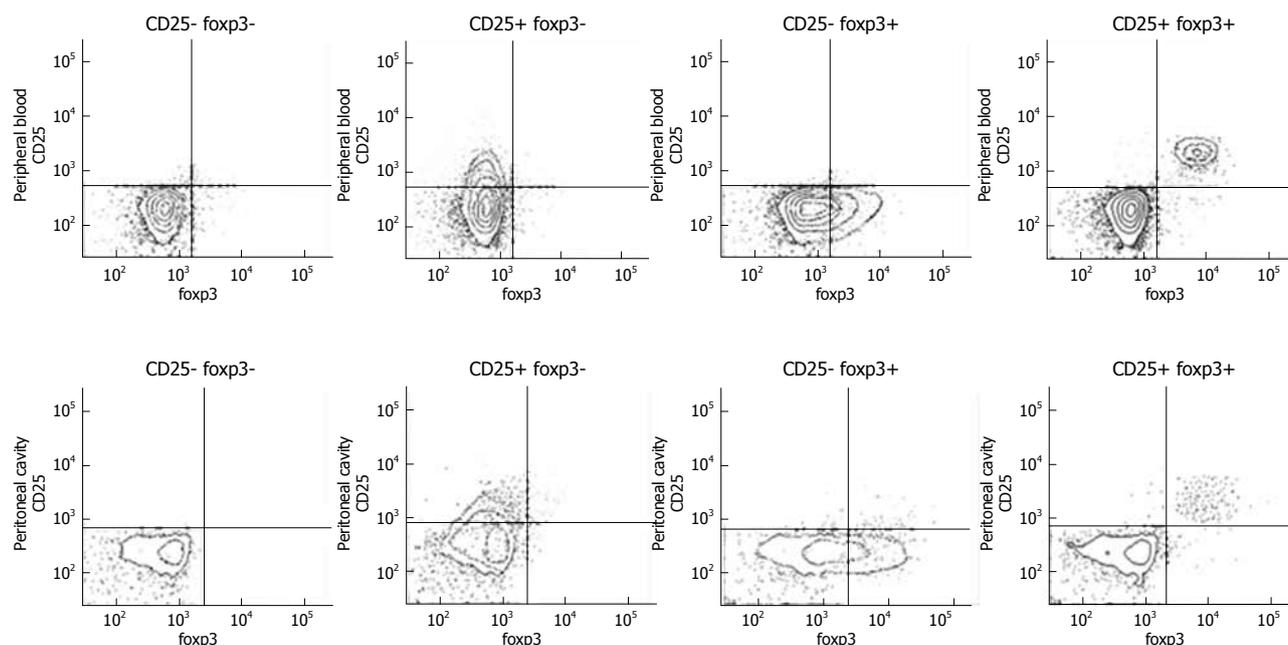
### Carcinoembryonic antigen values in sera and peritoneal lavage

For the interaction between peripheral blood and the peritoneal cavity, we investigated the CEA values in both serum and peritoneal lavage at the time of surgery. The serum CEA values were elevated only in patients with stage IV disease. On the other hand, the values in peritoneal lavage were found to be elevated even at stage III, and they were also related to the clinical stage (Table 2).

### Analysis of lymphocyte populations in peripheral blood and the peritoneal cavity

After purification of lymphocytes from peritoneal lavage, we investigated the phenotypes of lymphocytes in both peripheral blood and the peritoneal cavity. The mean value of the CD4/CD8 ratio for all patients was 2.17 in peripheral blood. The CD8<sup>+</sup> T cells were dominant in the peritoneal cavity and the CD4/CD8 ratio was reversed. The ratio in patients with stage III or IV was significantly higher than in stage I or control patients (Table 2).

The CCR7<sup>+</sup> CD45RA<sup>-</sup> CD8<sup>+</sup> T cells were counted as effector memory T cell subsets. The percentage of effector memory T cells in the peritoneal cavity was higher than that in peripheral blood. However, the percentage



**Figure 1** Co-staining with foxp3 and CD25 for CD4<sup>+</sup> T cells. High correlation was shown between both populations.

decreased in association with the clinical stage (Table 2). The CD3<sup>+</sup>CD56<sup>+</sup> cells were measured as natural killer T cells. The percentage of these cells in the peritoneal lavage was also low in patients with stage III or stage IV (Table 2). As the co-staining of foxp3 and CD25 revealed a high correlation between both populations, CD25<sup>high</sup> was used following cytokine producing assays (Figure 1). The frequency of CD4<sup>+</sup> CD25<sup>high</sup> T cells in patients with advanced stage cancer was higher than that in control patients in both peripheral blood and the peritoneal cavity (Figure 2A and B).

### Cytokine production by lymphocytes

The cytokine production from CD3<sup>+</sup> T cells after stimulation with PMA + ionomycin was evaluated by a cytokine production assay. The lymphocytes in the peritoneal cavity were more sensitive for the production of IFN- $\gamma$  than those in the peripheral blood. The ratio of IFN- $\gamma$  producing cells in the peritoneal cavity was significantly lower in patients with advanced stage disease in comparison to the controls (Figure 3A and B). The ratio of IL-10 producing cells in the peritoneal cavity in patients with advanced stages was higher in comparison to the controls (Figure 3C and D).

### Cytokine assays of intra-peritoneal lymphocytes after co-cultivation with self- CD4<sup>+</sup> CD25<sup>high</sup> T cells

In order to investigate whether the suppression of IFN- $\gamma$  production from T cells in the peritoneal cavity at advanced stages was caused by CD4<sup>+</sup> CD25<sup>high</sup> T cells, further assays were performed. The IFN- $\gamma$  production of CD8<sup>+</sup> T cells was suppressed in intra-peritoneal lymphocytes co-cultivated with isolated CD4<sup>+</sup> CD25<sup>high</sup> T cells from self-peripheral blood (Figure 4A). No inhibition was seen when the lymphocytes were co-cultivated with CD4<sup>+</sup>

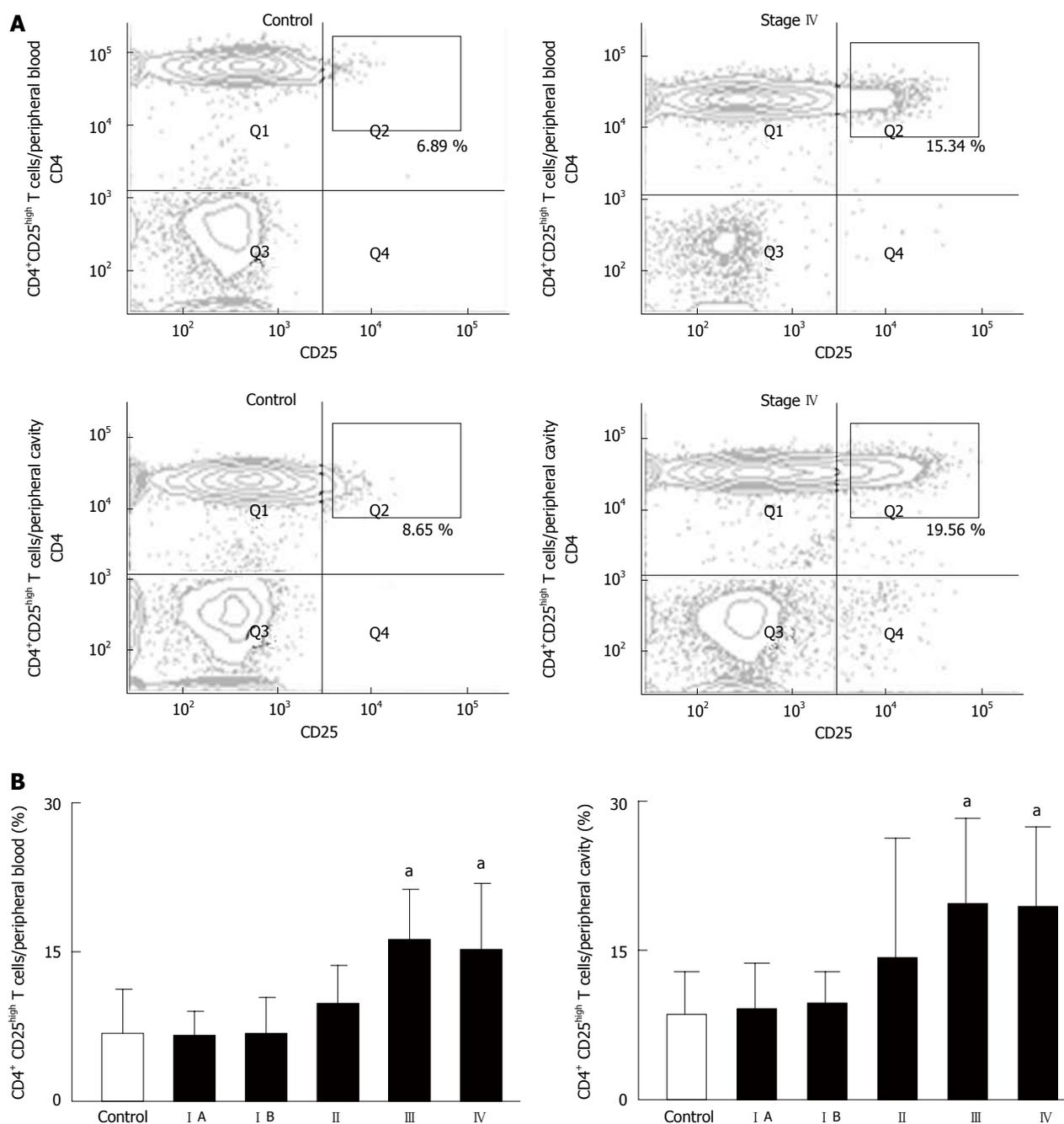
CD25<sup>-</sup> cells (Figure 4B).

## DISCUSSION

The peritoneal cavity is a compartment in which the immunological host-tumor interaction can occur<sup>[24]</sup>. This study investigated lymphocytes in the peritoneal cavity of patients with gastric cancer in relation to anti-tumor immunity. Some tumors can acquire the ability to down-regulate immune responses and exploit this action to promote tumor cell proliferation, survival, and invasion<sup>[10,25]</sup>. Therefore, the presence of leukocytes in the peritoneal cavity may be a consequence of an immune response that favors either dissemination of tumor cells or a protective host response. Malignant ascites has been used as a common source of immunological analysis in previous reports<sup>[11,26]</sup>. To the best of our knowledge, there are no reports describing the lymphocyte and cytokine production ability in peritoneal lavage from patients with gastric cancer at the time of gastrectomy.

In our initial experiments, the CEA values in peritoneal lavage were found to correlate with the clinical stages. Interestingly, the CEA values were elevated even in cases without serosal invasion. This result suggests that some fragments of cancer cells may spread throughout the peritoneal cavity and induce an immune reaction between the tumor and host<sup>[26,27]</sup>.

The frequency of CD4<sup>+</sup> T cells in all patients was higher than that of CD8<sup>+</sup> T cells in peripheral blood, but this pattern was reversed in peritoneal lavage fluid. CD8<sup>+</sup> T cells were dominant in the peritoneal cavity. Our data suggested that the immunological environment in the peripheral blood is different from that in the peritoneal cavity. There were significant differences in the CD4/CD8 ratio in the peritoneal cavity between gastric cancer



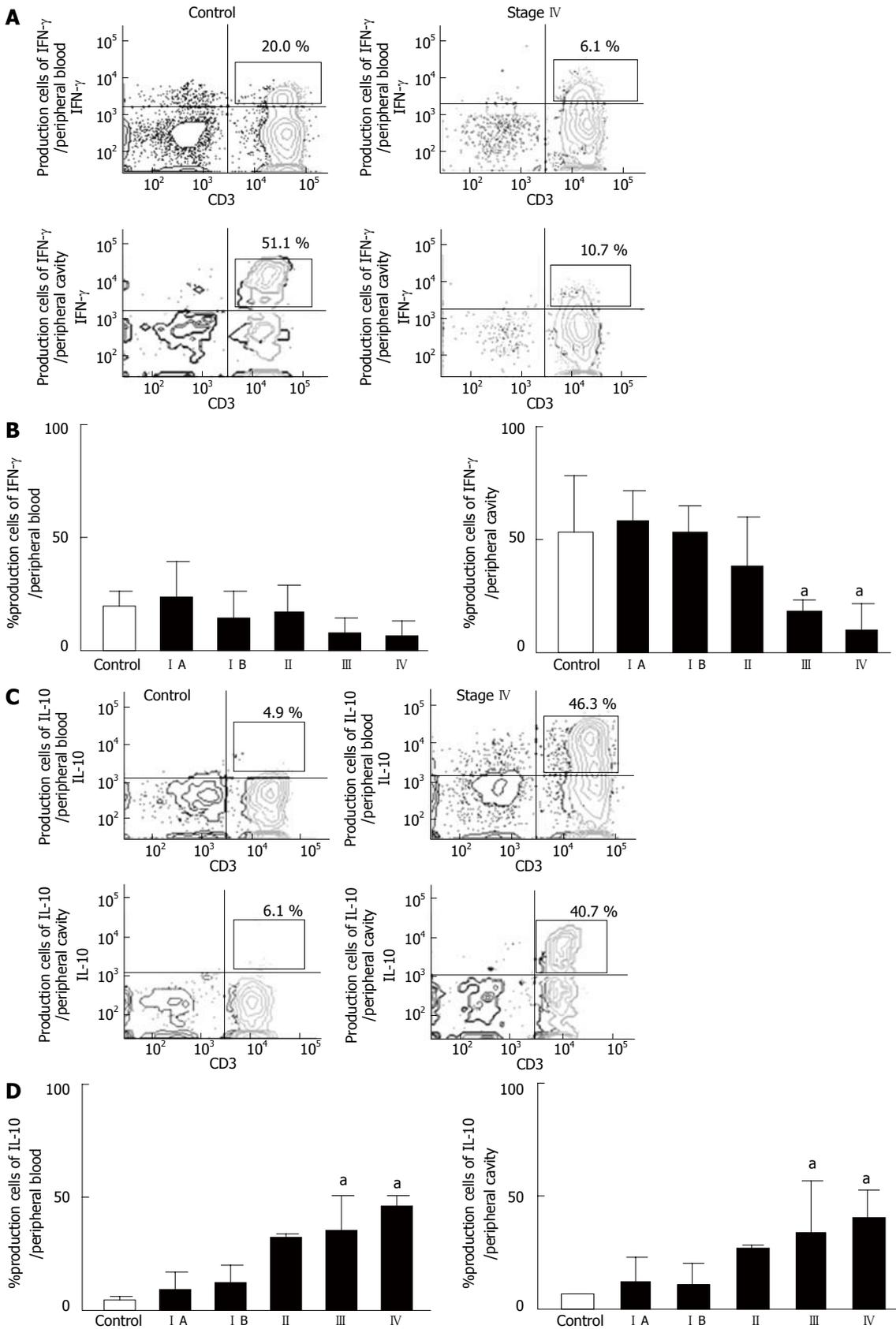
**Figure 2 Analysis of lymphocyte populations in peripheral blood and the peritoneal cavity.** A: The gating and counting of CD4<sup>+</sup> CD25<sup>high</sup> T cell population by flow cytometry; B: The percentage of CD4<sup>+</sup> CD25<sup>high</sup> T cells in the CD4<sup>+</sup> T cell population in peripheral blood and peritoneal lavage of patients at each stage of gastric cancer and control patients. Data are presented as the mean ± SD.

patients at advanced stage and control patients. Cancer progression may have an effect on the balance of the T cell population in the peritoneal cavity.

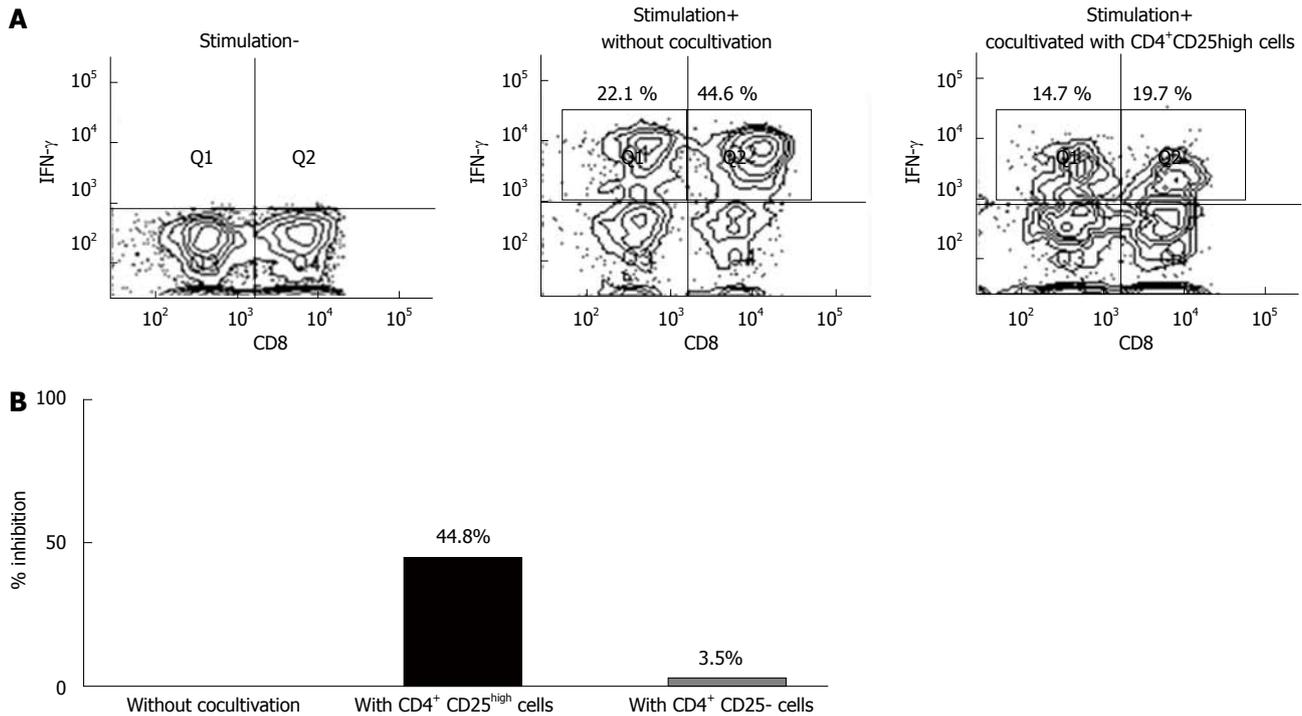
Immunological memory is demonstrated by following T cell subsets: lymph-node-homing cells lacking inflammatory and cytotoxic function (defined as central memory T cells, CCR7<sup>+</sup> CD45RA<sup>-</sup>) and tissue-homing cells endowed with various effector functions (defined as effector memory T cells, CCR7<sup>-</sup> CD45RA<sup>+</sup>). These two subsets allow for the division of labor among memory cells. Effector memory T cells represent a readily available pool of antigen-primed cells that can enter peripheral tissues

to mediate inflammatory reactions or cytotoxicity, thus rapidly containing invasive pathogens and cancer antigens<sup>[11,28-31]</sup>. Our data show that CD8<sup>+</sup> effector memory T cells were rich in the peritoneal cavity. This indicates the migration of effector memory cells from the peripheral blood to local sites. However, in advanced cases, the frequency of CD8<sup>+</sup> effector memory cells in the peritoneal lavage was low. These results suggest that the peritoneal cavity exerts the local immune response, more than peripheral blood.

Natural killer T cells, a unique lymphocyte subpopulation, are characterized by the expression of invariant an-



**Figure 3 Cytokine production by lymphocytes.** A: The gating and counting of the IFN- $\gamma$  producing cell population by flow cytometry; B: The percentage of IFN- $\gamma$  producing cells in the CD3<sup>+</sup> cell population stimulated with PMA + ionomycin in peripheral blood and peritoneal lavage of patients at each stage of gastric cancer and control patients. Data are presented as the mean  $\pm$  SD. The statistical analysis was performed by the Kruskal-Wallis test. After gating of CD3<sup>+</sup> T cells, 10 000 events were analyzed. The production of IFN- $\gamma$  in the peritoneal cavity was higher than that in the peripheral blood. The ratio of IFN- $\gamma$  production cells in the peritoneal lavage was significantly lower in patients with advanced-stage than in controls [control vs stage IV: 51.1 (35.1-67.1) vs 10.7 (2.6-22.1), <sup>a</sup> $P < 0.05$ ]; C: The gating and counting of the IL-10 producing cell population by flow cytometry; D: The percentage of IL-10 producing cells in the CD3<sup>+</sup> cells stimulated with PMA + ionomycin in peripheral blood and peritoneal lavage of patients at each stage of gastric cancer and control patients. Data are presented as the mean  $\pm$  SD. The ratio of IL-10 producing cells in peripheral blood and intra-peritoneal lymphocytes was significantly higher in patients at advanced stage than in controls [control vs stage IV: 6.1 (3.94-8.25) vs 40.7 (18.35-63.0), <sup>a</sup> $P < 0.05$ ].



**Figure 4** Cytokine assays of intra-peritoneal lymphocytes after co-cultivation with self-CD4<sup>+</sup>CD25<sup>high</sup>T cells. A: IFN- $\gamma$  production in intra-peritoneal lymphocytes co-cultivated with self- CD4<sup>+</sup> CD25<sup>high</sup> T cells; B: Either CD4<sup>+</sup> CD25<sup>high</sup> T cells or CD4<sup>+</sup> CD25<sup>-</sup> T cells.

tigen receptors<sup>[12,13]</sup>. Natural killer T cells have been suggested to serve as a bridge between innate and acquired immunity<sup>[14,15]</sup>. However, the mechanisms underlying the anti-tumor effect of human natural killer T cell-mediated immunotherapy remain unclear so far. The frequency of natural killer T cells was lower in patients with stages III and IV than in control patients. Therefore, a decrease in the number of natural killer T cells in the peritoneal cavity may be one aspect of the interaction between host-immunity and cancer progression.

Recent studies have shown that CD4<sup>+</sup> CD25<sup>high</sup> foxp3<sup>+</sup> T cells exhibiting regulatory/suppressive properties are naturally present in humans<sup>[16-18]</sup>. The roles of regulatory T cells have been active topics of research in both basic and clinical immunology. Naturally-occurring regulatory T cells represent a small fraction (5%-6%) of the overall CD4<sup>+</sup> T cell population, and play an important role in down-regulation of the response of T cells to foreign and self antigens<sup>[31]</sup>. The depletion of this subset of regulatory T cells in normal hosts results in various autoimmune diseases because the host immune system is unchecked and attacks the body's own tissues<sup>[28]</sup>. Despite the importance of these cells in preventing autoimmune disease, their presence in the tumor microenvironment diminishes anti-tumor immune responses<sup>[32-36]</sup>.

Within the CD4<sup>+</sup> T cell subset, there is a population of naturally occurring foxp3<sup>+</sup> T cells that are defined as regulatory T cells. These cells can be identified as CD4<sup>+</sup>foxp3<sup>+</sup> T cells by flow cytometry. However, because foxp3 is intracellular and requires permeabilization of cells for detection by flow cytometry, regulatory T cells are isolated as CD4<sup>+</sup>CD25<sup>high</sup> T cells, which were shown to

have functional suppressive abilities in our co-culture experiments<sup>[37]</sup>. In the present study, the mean percentage of CD4<sup>+</sup> CD25<sup>high</sup> T cells in the peritoneal cavity in advanced gastric cancer patients was higher than that of control patients. After the co-cultivation of the self-CD4<sup>+</sup> CD25<sup>high</sup> T cell population of intra-peritoneal lymphocytes, the production of IFN- $\gamma$  was inhibited.

IFN- $\gamma$ , a Th1 cytokine, not only exerts an anti-tumor effect, but also inhibits the proliferation of Th2 clones<sup>[19-20]</sup>. IL-10, a Th2 cytokine, suppresses the synthesis of Th1 cytokines such as IFN- $\gamma$ <sup>[21-22]</sup>. This study showed that the production of intracellular cytokines in the peritoneal cavity was higher than that in the peripheral blood after appropriate stimulation. IFN- $\gamma$  production was down-regulated in advanced cases, but not in the controls and stage I patients. On the other hand, IL-10 production was up-regulated, which revealed the switch of Th1 and Th2 responses in the peritoneal cavity of these patients. IFN- $\gamma$  production in intra-peritoneal lymphocytes was suppressed after co-cultivation with self-CD4<sup>+</sup> CD25<sup>high</sup> T cells, but not CD4<sup>+</sup> CD25<sup>-</sup> T cells. Interestingly, the replacement of CD4<sup>+</sup> CD25<sup>-</sup> T cells for CD4<sup>+</sup> CD25<sup>high</sup> T cells could recover the production of IFN- $\gamma$  in intra-peritoneal lymphocytes.

## COMMENTS

### Background

The peritoneal cavity is a compartment in which immunological host-tumor interactions can occur. Neoplastic cell factors and biophylactic side factors such as immune reactions are interacting in the survival and development of micro-metastasis. However, the role of lymphocytes in the peritoneal cavity of gastric cancer patients is unclear.

## Research frontiers

Clinical and experimental studies have established that leukocyte infiltrations around tumors promote the development or regression of solid tumors, but whether the organ-specific cellular and molecular programs promote tumor growth or exhibit anti-tumor immunity by leukocytes are incompletely understood. Recent studies have shown that CD4<sup>+</sup> CD25<sup>high</sup> foxp3<sup>+</sup> T cells exhibiting regulatory/suppressive properties are naturally present in humans. The roles of regulatory T cells have been active topics of research in both basic and clinical immunology.

## Innovations and breakthroughs

In most previous studies, malignant ascites have been a common source of immunological analysis. However, there are no reports describing the lymphocyte and cytokine production ability in peritoneal lavage from patients with gastric cancer at the time of gastrectomy. In the present study, CD4<sup>+</sup> CD25<sup>high</sup> T cells were found to be increased in the peritoneal cavity of advanced gastric cancer patients, but in the co-cultivation of the self- CD4<sup>+</sup> CD25<sup>high</sup> T cell population of intra-peritoneal lymphocytes, the production of IFN- $\gamma$  was inhibited.

## Applications

Peritoneal lavage samples from patients with gastric cancer are more susceptible than peripheral blood for monitoring the interaction between the host's immune system and tumor cells.

## Terminology

Regulatory T cells: Regulatory T cells contribute to the maintenance of immunologic self-tolerance. Recent reports underscore that regulatory T cells not only play a role in the maintenance of immunotolerance but are also potent inhibitors of antitumor immune responses.

## Peer review

The authors have investigated T-cells isolated from peripheral blood and peritoneal lavage in patients with gastric cancer and controls. Main findings are that in stage III and IV gastric cancers the lavage fluid contains less CD8 memory cells, NKT cells and more CD25<sup>high</sup> regulatory T cells.

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## Curcumin prevents indomethacin-induced gastropathy in rats

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### Abstract

**AIM:** To investigate the effects of curcumin on gastric microcirculation and inflammation in rats with indomethacin-induced gastric damage.

**METHODS:** Male Sprague-Dawley rats were randomly divided into three groups. Group 1 (control group,  $n = 5$ ) was fed with olive oil and 5%  $\text{NaHCO}_3^-$  (vehicle). Group 2 [indomethacin (IMN) group,  $n = 5$ ] was fed with olive oil 30 min prior to indomethacin 150 mg/kg body weight (BW) dissolved in 5%  $\text{NaHCO}_3^-$  at time 0th and 4th h. Group 3 (IMN + Cur group,  $n = 4$ ) was fed with curcumin 200 mg/kg BW dissolved in olive oil 0.5 mL, 30 min prior to indomethacin at 0th and 4th h. Leukocyte-endothelium interactions at postcapillary

venules were recorded after acridine orange injection. Blood samples were determined for intercellular adhesion molecule (ICAM)-1 and tumor necrosis factor (TNF)- $\alpha$  levels using enzyme linked immunosorbent assay method. Finally, the stomach was removed for histopathological examination for gastric lesions and grading for neutrophil infiltration.

**RESULTS:** In group 2, the leukocyte adherence in postcapillary venules was significantly increased compared to the control group ( $6.40 \pm 2.30$  cells/frame vs  $1.20 \pm 0.83$  cells/frame,  $P = 0.001$ ). Pretreatment with curcumin caused leukocyte adherence to postcapillary venule to decline ( $3.00 \pm 0.81$  cells/frame vs  $6.40 \pm 2.30$  cells/frame,  $P = 0.027$ ). The levels of ICAM-1 and TNF- $\alpha$  increased significantly in the indomethacin-treated group compared with the control group ( $1106.50 \pm 504.22$  pg/mL vs  $336.93 \pm 224.82$  pg/mL,  $P = 0.011$  and  $230.92 \pm 114.47$  pg/mL vs  $47.13 \pm 65.59$  pg/mL,  $P = 0.009$  respectively). Pretreatment with curcumin significantly decreased the elevation of ICAM-1 and TNF- $\alpha$  levels compared to treatment with indomethacin alone ( $413.66 \pm 147.74$  pg/mL vs  $1106.50 \pm 504.22$  pg/mL,  $P = 0.019$  and  $58.27 \pm 67.74$  pg/mL vs  $230.92 \pm 114.47$  pg/mL,  $P = 0.013$  respectively). The histological appearance of the stomach in the control group was normal. In the indomethacin-treated group, the stomachs showed a mild to moderate neutrophil infiltration score. Gastric lesions were erosive and ulcerative. In rats treated with indomethacin and curcumin, stomach histopathology improved and showed only a mild neutrophil infiltration score and fewer erosive lesions in the gastric mucosa.

**CONCLUSION:** The results indicate that curcumin prevents indomethacin-induced gastropathy through the improvement of gastric microcirculation by attenuating the level of ICAM-1 and TNF- $\alpha$ .

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**Key words:** Curcumin; Nonsteroidal anti-inflammatory drugs; Gastric damage; Gastric microcirculation; Inter-cellular adhesion molecule-1; Tumor necrosis factor- $\alpha$

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## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed medications worldwide. However, NSAIDs have adverse effects on the gastric mucosa, resulting in various clinical presentations, ranging from nonspecific dyspepsia to ulceration, upper gastrointestinal bleeding, and death, summarized by the term "NSAID gastropathy"<sup>[1]</sup>. NSAIDs-induced gastric damage is the major side effect of this kind of drug<sup>[2]</sup>.

The main action of NSAIDs is to inhibit prostaglandin synthesis. There is substantial evidence supporting the view that the ulcerogenic effect of this medication correlates with its ability to suppress prostaglandin synthesis<sup>[3-5]</sup>. Endogenous prostaglandins normally regulate mucosal blood flow, epithelial cell proliferation, epithelial restitution, mucosal immunocyte function, mucus and bicarbonate secretion, and basal acid secretion<sup>[6]</sup>. Therefore, decreases in prostaglandins, protective factors for ulcer formation, lead to gastric mucosal injury.

Animal studies have shown that neutrophil adherence to the endothelium of the gastric microcirculation is critical in NSAIDs injury<sup>[7]</sup>. Neutrophil adherence damages the mucosa by producing oxygen-free radicals, releasing proteases, and obstructing capillary blood flow. NSAIDs might induce the synthesis of tumor necrosis factor (TNF)- $\alpha$  and leukotrienes<sup>[8,9]</sup>. These inflammatory mediators subsequently stimulate neutrophil adherence by the upregulation of adhesion molecules<sup>[10]</sup>.

NSAID administration in rats caused a rapid and significant increase in adhesion between neutrophils and vascular endothelial cells in both gastric and mesenteric venules<sup>[11-13]</sup>. This was dependent on intercellular adhesion molecule (ICAM)-1 expression on the endothelium and CD11/CD18 expression on the leukocyte<sup>[14,15]</sup>. Interestingly, Andrews *et al.*<sup>[10]</sup> recently reported that administration of aspirin or indomethacin to rats resulted in a significant increase in ICAM-1 expression in the gastric microcirculation.

Curcuma, a genus in the plant family of Zingiberaceae, is the biological source for curcuminoids, including curcumin. *Curcuma longa*, the yellow tuberous root re-

ferred to as turmeric, was taken from India to Southeast Asia<sup>[16]</sup>. The yellow pigmented fraction of *Curcuma longa* contains curcuminoids, which are chemically related to its principal ingredient, curcumin<sup>[16]</sup>. It possesses a broad range of pharmacological activities, including antioxidant, anti-carcinogenic, wound-healing, and anti-inflammatory effects<sup>[17-19]</sup>. There are currently limited studies investigating the effect of curcumin on NSAIDs-induced gastric damage. The aim of this study was to investigate the anti-inflammation effect of curcumin on indomethacin-induced gastric damage in rats.

## MATERIALS AND METHODS

### Animal preparation and curcumin preparation

Male Sprague-Dawley rats weighing 180-220 g, purchased from the National Laboratory Animal Center, Mahidol University, Salaya ( $n = 18$ ), Nakorn pathom, were used in this study. All rats were kept in a controlled temperature room at  $25 \pm 1$  °C under standard conditions (12 h day-night rhythm). They were cared for in accordance with the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Thailand. Curcumin powder (Cayman Chemical Company, United States) was suspended in olive oil.

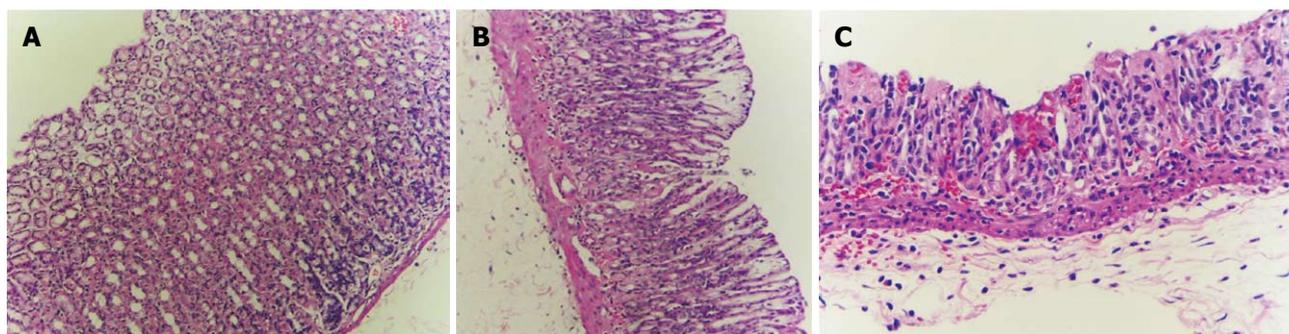
### Experimental protocol

All rats were fasted, with free access to water ad libitum, for 22-24 h before the experiment. They were randomly divided into three experimental groups. Group 1 (control,  $n = 6$ ): Rats were fed with olive oil 30 min prior to 5% sodium bicarbonate 1 mL orally *via* an intragastric tube at time 0th and 4th h. Group 2 [indomethacin (IMN),  $n = 6$ ]: Rats were fed with olive oil 30 min prior to indomethacin (150 mg/kg body weight in 5% sodium bicarbonate 1 mL orally *via* an intragastric tube) at time 0th and 4th h. Group 3 (IMN + Cur,  $n = 6$ ): Rats were fed with curcumin (200 mg/kg body weight dissolved in olive oil 0.5 mL) 30 min prior to indomethacin [150 mg/kg body weight (BW) dissolved in 5% sodium bicarbonate 1 mL orally *via* an intragastric tube] at time 0th and 4th h.

After 8 h 30 min, animals were anesthetized with intraperitoneal injection of thiopental (50 mg/kg body weight). After tracheostomy, the carotid artery and jugular vein were cannulated for blood pressure measurement using a polygraph and for the administration of a fluorescent marker; acridine orange was infused intravenously (Sigma chemical Co., United States, 0.5 mg/kg BW/min). The abdominal wall was incised and the stomach was extended and fixed. Leukocyte adherence in the stomach was observed by intravital fluorescence microscopy. At the end of the experiment, blood samples were collected for ICAM-1 and TNF- $\alpha$  determination using enzyme linked immunosorbent assay (ELISA) methods. The stomach was cut and fixed in 10% formalin solution to inspect the histopathology.

### Study of the interaction between leukocytes and endothelial cells in postcapillary venule

It has been stated that NSAIDs-induced leukocyte adherence could contribute to the pathogenesis of gastric



**Figure 1** Hematoxylin-eosin stained stomach sections ( $\times 200$ ). A: The control group showed normal stomach histopathology; B: The indomethacin treated group showed gastric ulcer formation and infiltration of inflammatory cells; C: The curcumin treated group showed a reduced degree of gastric ulcer formation and inflammation.

mucosal injury. To visualize leukocytes, acridine orange was infused intravenously (0.3 mg/kg body weight). The number of leukocyte adhesions was recorded using a video recorder. Videotape of each experiment was replayed and leukocyte adherence was monitored. Most leukocytes were adhered to the postcapillary venule (about 15-30  $\mu\text{m}$  in diameter). Leukocytes were considered adherent to the vessel endothelium if they remained stationary for 30 s or longer. Adherent leukocytes were expressed as the number of leukocyte adhesions per frame of view, as previously described<sup>[20]</sup>.

#### Determination of serum cytokine levels

After the experiment, blood samples were taken by cardiac puncture, and allowed to clot overnight before centrifuging at approximately 2000  $\times g$ . Serum was stored at -80  $^{\circ}\text{C}$  for determining ICAM-1 and TNF- $\alpha$  levels by ELISA kit (R and D systems).

#### Histopathological examination

Samples of the stomach were excised and transferred to formalin. The samples were subsequently processed by routine techniques before embedding in paraffin. Sections were cut at the thickness of 5  $\mu\text{m}$  and stained with hematoxylin and eosin (HE), as previously described<sup>[20]</sup>. One pathologist performed all the histopathological examinations. All histopathological changes were observed under a light microscope. The neutrophil infiltration score in each section was graded according previously determined criteria<sup>[21]</sup>.

#### Statistical analysis

All data were presented as mean  $\pm$  SD. To compare data among all groups of animals, one-way analysis of variance (one-way ANOVA) and Duncan comparisons were employed. All statistical tests were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, United States). Differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

#### Histopathological examination

The histological appearance of the stomach in the con-

**Table 1** Summary of the infiltration of inflammatory cells and erosion in all groups ( $n = 6$ )

Experimental group	Neutrophil infiltration <sup>1</sup>				Pathology		
	0	1	2	3	No erosion	Erosion	Ulcer
Control	6	-	-	-	6	-	-
IMN	-	3	3	-	-	2	4
IMN + Cur	4	2	-	-	4	2	-

<sup>1</sup>The severity of neutrophil infiltration was graded as: 0: No neutrophil infiltration; 1: Neutrophil infiltration found in 1/3 of gastric mucosal layer; 2: Neutrophil infiltration found in 2/3 of gastric mucosal layer; 3: Neutrophil infiltration found in the muscularis mucosae of gasm. IMN: Indomethacin.

trol group (Figure 1) was normal. In the indomethacin treated group, the stomachs showed mild to moderate gastric mucosal injury. Gastric lesions were erosive and ulcerative. In rats treated with indomethacin and curcumin, the stomach histopathology improved and showed only mild gastric mucosal injury and reduced amounts of erosive lesions in the gastric mucosa. The summary of infiltration of inflammatory cells and gastric lesions are shown in Table 1.

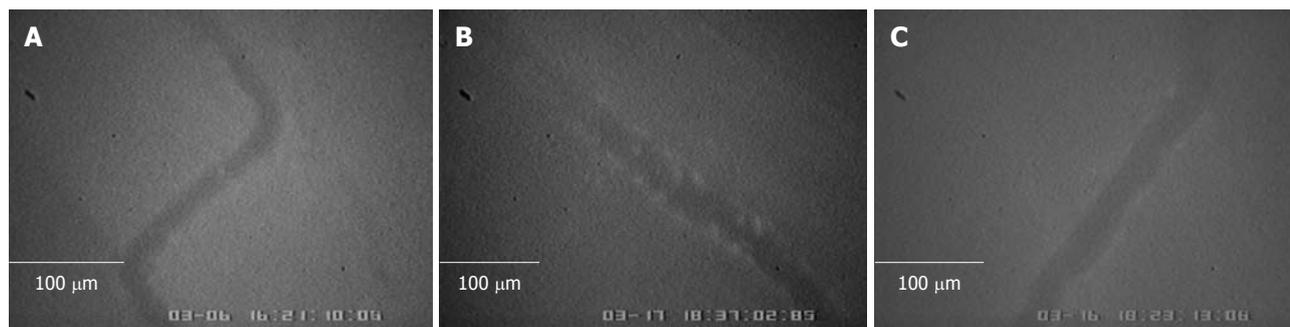
#### Interaction between leukocytes and endothelial cells

After gastric injury was induced by the administration of indomethacin, leukocyte adherence to endothelial cells of postcapillary venules (15-30  $\mu\text{m}$  in diameter) was observed under intravital fluorescence microscopy, 10-15 min after acridine orange injection (Figure 2). The number of leukocytes adhering to postcapillary venules for 30 s or longer was counted. The mean number of leukocyte adhesions in the IMN group without curcumin treatment was significantly higher than in the control group ( $6.40 \pm 2.30$  cells/frame *vs*  $1.20 \pm 0.83$  cells/frame,  $P = 0.001$ ).

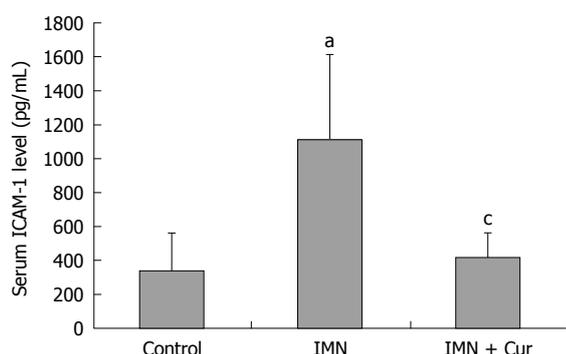
The number of leukocyte adhesions significantly decreased after pretreatment with curcumin as compared to the IMN group ( $3.00 \pm 0.81$  cells/frame *vs*  $6.40 \pm 2.30$  cells/frame,  $P = 0.027$ ).

#### Changes in ICAM-1 levels

The levels of ICAM-1 increased significantly in the indomethacin treated group compared with the control group



**Figure 2** Intravital microscopic ( $\times 40$ ) images of leukocyte adherence on vascular endothelium of postcapillary venules 10-15 min after acridine orange injection in the control group (A), IMN group (B), and IMN + Cur group (C). IMN: Indomethacin.



**Figure 3** Serum intercellular adhesion molecule-1 levels in all groups. All data are expressed as mean  $\pm$  SD. The mean intercellular adhesion molecule (ICAM)-1 levels were significantly higher in the indomethacin treated group (IMN) when compared with the control group ( $^aP < 0.05$ ). Pretreatment with curcumin significantly decreased ICAM-1 level when compared with indomethacin ( $^cP < 0.05$ ). IMN: Indomethacin.

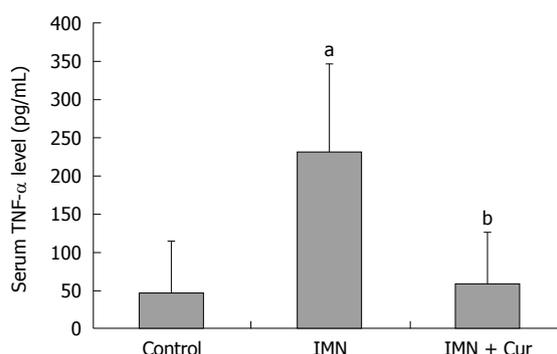
( $1106.50 \pm 504.22$  pg/mL *vs*  $336.93 \pm 224.82$  pg/mL,  $P = 0.011$ ). Pretreatment with curcumin markedly decreased the elevation of the ICAM-1 level compared with the indomethacin treated group ( $413.66 \pm 147.74$  pg/mL *vs*  $1106.50 \pm 504.22$  pg/mL,  $P = 0.019$ ) (Figure 3).

### Changes in TNF- $\alpha$ levels

The level of TNF- $\alpha$  markedly increased in the indomethacin treated group compared with the control group ( $230.92 \pm 114.47$  pg/mL *vs*  $47.13 \pm 65.59$  pg/mL,  $P = 0.009$ ). Pretreatment with curcumin decreased the elevation of TNF- $\alpha$  levels compared with the indomethacin treated group ( $58.27 \pm 67.74$  pg/mL *vs*  $230.92 \pm 114.47$  pg/mL,  $P = 0.013$ ) (Figure 4).

## DISCUSSION

In the present study, we investigated the effects of curcumin on indomethacin-induced gastric damage in rats. The results clearly demonstrated that curcumin administration prevented the ulcerogenic effect of indomethacin, possibly through its anti-inflammatory action. Evidence suggests that NSAIDs-induced gastric ulceration is a neutrophil-dependent process. NSAIDs administration to rats caused a rapid and significant increase in adhesion



**Figure 4** Serum tumor necrosis factor-alpha level in all groups. All data are expressed as mean  $\pm$  SD. The mean of tumor necrosis factor (TNF)- $\alpha$  levels increased in the indomethacin treated group when compared with the control group ( $^aP = 0.009$ ). Pretreatment with curcumin decreased serum of TNF- $\alpha$  when compared with indomethacin alone ( $^bP = 0.013$ ). IMN: Indomethacin.

between neutrophils and vascular endothelial cells in both the gastric and mesenteric venules<sup>[11,12]</sup>. Indeed, monoclonal antibodies that blocked NSAID-induced neutrophil adherence to vascular endothelium could significantly alleviate NSAID-induced gastric mucosal injury<sup>[7,15,22]</sup>. Neutrophils play an important role in the development of inflammation and tissue injury by releasing a variety of inflammatory mediators<sup>[23,24]</sup>. These inflammatory mediators are capable of producing tissue injury; therefore, they may be involved in the pathogenesis of indomethacin-induced gastric mucosal injury<sup>[25]</sup>. Furthermore, adhesion molecules expressed on activated neutrophils, such as CD11b and CD18, have been shown to play an important role in neutrophil-induced tissue injury<sup>[14]</sup>.

Moreover, NSAIDs are believed to have the effect on nuclear translocation of nuclear factor (NF)- $\kappa$ B, which modulates the expression of several adhesion molecules, including ICAM-1<sup>[26]</sup>. ICAM-1, one of the major adhesion molecules, plays a pivotal role in the inflammatory reaction by increasing leukocyte adhesion to endothelium and promoting transendothelial migration of leukocytes to inflammatory sites<sup>[27]</sup>. Another important mechanism that induces ICAM-1 expression is the increment of TNF- $\alpha$  levels<sup>[27,28]</sup>. The inhibitory effect of NSAIDs on COX-2 leads reduced prostaglandin E2 (PGE2) levels. Thus, TNF- $\alpha$  production, which is normally inhibited

by PGE<sub>2</sub>, increases<sup>[28]</sup>. TNF- $\alpha$  is an important mediator causing NSAIDs induced gastropathy. Apart from its effect on adhesion molecules, TNF- $\alpha$  may have the ability to activate pro-apoptosis caspases, which regulate gastric epithelial cells apoptosis in NSAID-treated rats<sup>[27]</sup>.

A previous study demonstrated that indomethacin administration caused significantly elevated TNF- $\alpha$  levels in rats<sup>[8]</sup>. Moreover, pretreatment with anti-TNF- $\alpha$ , dexamethasone, and PGE<sub>2</sub> could prevent the increases in gastric mucosal injuries and TNF- $\alpha$  levels. Similarly, we found significant rises in TNF- $\alpha$  and ICAM-1 levels in the serum of the indomethacin-treated group compared to the control. Other formulations of NSAIDs could also increase TNF- $\alpha$  production. A study by Jainu *et al*<sup>[29]</sup> noted a significant increase in TNF- $\alpha$ , IL-1 $\beta$  and NOS-2 activity in aspirin-administered rats. The elevations of inflammatory mediators and adhesion molecules in serum correlated with the pathological findings of gastric mucosa and the numbers of adhered leukocytes in the gastric microcirculation. These were also true for curcumin-treated group. Pretreatment with curcumin could significantly reduce TNF- $\alpha$  and ICAM-1 levels in the serum, accompanied by an improvement in gastric mucosal inflammation and leukocyte adhesion.

Curcumin, a substance rich in phenolics, is known to possess antioxidant properties. Curcumin reduces gastric injury induced by NSAIDs<sup>[30]</sup>. It has been reported that curcumin can decrease gastric injury by preventing the peroxidase inactivation effect of indomethacin and scavenging reactive oxygen produced by this enzyme<sup>[30]</sup>. Several studies showed that curcumin is also an anti-inflammatory substance, with an inhibitory effect on transcription factor NF- $\kappa$ B activation. NF- $\kappa$ B is required for the expression of many genes linked with the host immune response, such as ICAM-1, TNF- $\alpha$ , IL-1 $\beta$ , and iNOS<sup>[31]</sup>. Cytoplasmic NF- $\kappa$ B is complexed with its inhibitor I $\kappa$ B and is, therefore, inactive. The cytokine-mediated activation of NF- $\kappa$ B requires activation of various kinases, which ultimately leads to the phosphorylation and degradation of I $\kappa$ B. Several beneficial effects of curcumin are consistent with its ability to inhibit the activity of NF- $\kappa$ B<sup>[32-34]</sup>. Singh *et al*<sup>[31]</sup> observed that curcumin inhibited NF- $\kappa$ B activation pathway after the convergence of various stimuli mediated by protein tyrosine kinase, protein kinase, and ubiquitin conjugation enzymes, but before the phosphorylation and subsequent release of I $\kappa$ B complexed to NF- $\kappa$ B. Jobin *et al*<sup>[35]</sup> and Plummer *et al*<sup>[36]</sup> examined the modulatory potential of curcumin on NF- $\kappa$ B signaling pathways and found that curcumin prevented phosphorylation of I $\kappa$ B by inhibiting the activation of I $\kappa$ B-kinase (IKKs). Our previous study demonstrated that *Helicobacter pylori*-induced gastric inflammation in rats is associated with increased NF- $\kappa$ B activation and macromolecular leakage, which can be reduced by curcumin<sup>[37]</sup>. In this study, 200 mg/kg curcumin was a sufficient dose for reducing gastric epithelial NF- $\kappa$ B p65 expression and mucosal macromolecular leakage. Despite its inconclusive mechanism of action, we clearly demonstrated that curcumin has a protective and beneficial

effect on NSAIDs-induced gastropathy in rats. Further studies on the expression of inflammatory mediators and adhesion molecules in the gastric mucosa are necessary to demonstrate the exact curative effect of curcumin on NSAID-induced gastric pathology. Clinical studies might also be needed to verify the protective effect of curcumin in humans.

In conclusion, NSAIDs could induce gastric injury through increases in inflammatory cytokines and leukocyte adhesions. Curcumin, an anti-oxidant herbal substance, could prevent these adverse events and might be used as a preventive method for NSAIDs-induced gastropathy.

## COMMENTS

### Background

Nonsteroidal anti-inflammatory drugs (NSAIDs)-induced gastric damage is the major side effect of this kind of medication. Although the underlying pathogenesis of NSAIDs-induced gastric damage is unclear, neutrophils are believed to play an important role in the development of gastric inflammation and injury following NSAID administration. Curcumin possesses several biological activities, including an anti-inflammatory effect. Authors postulated that curcumin, acting through nuclear factor (NF)- $\kappa$ B inhibition, could reduce the production of adhesion molecules and inflammatory cytokines, resulting in the amelioration of gastric injury in NSAIDs-induced gastropathy in rats.

### Research frontiers

Curcumin (diferuloylmethane) is an active ingredient of *Curcuma longa* (turmeric), which exerts many biological activities by the inhibition of NF- $\kappa$ B-mediated reactions. NSAIDs can cause gastric mucosal damage through an increase in leukocyte-endothelial adhesions and the release of inflammatory mediators, leading to the free radical production. This study demonstrated an improvement in gastric mucosal damage and decreases in leukocyte adhesions, and intercellular adhesion molecule 1 and tumor necrosis factor (TNF)- $\alpha$  production after curcumin administration in the indomethacin-treated group.

### Innovations and breakthroughs

The previous study showed that curcumin is an anti-inflammatory agent and can inhibit NF- $\kappa$ B activation in an *in vitro* study. However, it is not known whether curcumin's anti-inflammatory effects will help prevent NSAIDs-induced gastropathy *in vivo*. In this study, authors investigated the protective effect of curcumin in indomethacin-induced gastric damage in rats. Authors found that curcumin could alleviate indomethacin-induced gastric injury *via* a decrease in leukocyte adhesions and TNF- $\alpha$  production.

### Applications

Curcumin might be used as a new protective agent for NSAIDs-induced gastric damage in clinical use.

### Terminology

NSAIDs gastropathy: NSAIDs are well-known for their adverse effects on gastric mucosa, resulting in various clinical presentations, ranging from nonspecific dyspepsia to ulceration, upper gastrointestinal bleeding and death, summarized by the term "NSAID gastropathy".

### Peer review

This is an interesting study of the effects of curcumin on indomethacin-induced gastric damage in rats. The results clearly demonstrated that curcumin administration prevented the ulcerogenic effect of indomethacin, possibly through its anti-inflammatory action.

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## HBx activates FasL and mediates HepG2 cell apoptosis through MLK3-MKK7-JNKs signal module

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### Abstract

**AIM:** To investigate the possible mechanism by which hepatitis B virus X protein (HBx) mediates apoptosis of HepG2 cells.

**METHODS:** HBx expression vector pcDNA3.1-X was transfected into HepG2 cells to establish an HBx high-expression cellular model as pcDNA3.1-X transfected group. The pcDNA3.1-X and pSilencer3.1-shHBX (HBx antagonist) were cotransfected into HepG2 cells to establish an HBx low-expression model as RNAi group. Untransfected HepG2 cells and HepG2 cells transfected with negative control plasmid were used as controls. Apoptosis rate, the expression of Fas/FasL signaling pathway-related proteins and the phosphorylation levels of MLK3, MKK7 and JNKs, which are upstream molecules of death receptor pathways and belong to the family of mitogen-activated protein kinases (MAPKs),

were measured in each group.

**RESULTS:** Compared with HepG2 cell group and RNAi group, apoptosis rate, the expression of Fas and FasL proteins, and the activation of MLK3, MKK7 and JNKs were increased in the pcDNA3.1-X transfected group. The activation of JNKs and expression of FasL protein were inhibited in the pcDNA3.1-X transfected group when treated with a known JNK inhibitor, SP600125. When authors treated pcDNA3.1-X transfected group with K252a, a known MLK3 inhibitor, the activation of MLK3, MKK7 and JNKs as well as expression of FasL protein was inhibited. Furthermore, cell apoptosis rate was also significantly declined in the presence of K252a in the pcDNA3.1-X transfected group.

**CONCLUSION:** HBx can induce HepG2 cell apoptosis *via* a novel active MLK3-MKK7-JNKs signaling module to upregulate FasL protein expression.

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**Key words:** Hepatitis B virus X protein; MLK3; FasL; HepG2 cell; Apoptosis

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Tang RX, Kong FY, Fan BF, Liu XM, You HJ, Zhang P, Zheng KY. HBx activates FasL and mediates HepG2 cell apoptosis through MLK3-MKK7-JNKs signal module. *World J Gastroenterol* 2012; 18(13): 1485-1495 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i13/1485.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i13.1485>

### INTRODUCTION

Hepatitis B virus (HBV) is one of the major pathogenic causes of primary hepatocellular carcinoma (HCC),

among which, HBV X gene is considered as a key gene that plays a critical role in the occurrence and progression of HBV-related HCC<sup>[1]</sup>. It has been proposed that HBx is a cellular transactivator that may indirectly stimulate a variety of viral and host gene promoters by interacting with transcription factors, including AP-1, ATF/CREB, ERCC, and is involved in several signal transduction pathways, including mitogen-activated protein kinase, Ras-Raf-mitogen-activated protein kinase, and JAK/STAT signaling pathways, therefore HBx affects several cellular processes, such as proliferation and differentiation<sup>[2-4]</sup>. In contrast to its proliferative effects, HBx also participates in the apoptotic destruction of liver cells during the virus infection. Several mechanisms might be involved in this process: (1) HBx induces cell apoptosis on its own or sensitizes cells to apoptotic stimuli such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) or UV irradiation<sup>[5-8]</sup>; (2) HBx increases expression of IL-18 and enhances transcription activity of Egr-2 and Egr-3, which up-regulates FasL expression and induces the apoptosis of hepatic cells by the death receptor pathway<sup>[9,10]</sup>; (3) HBx may directly not only target to mitochondria to enhance translocation of Bax to mitochondria but also interact with the mitochondrial protein voltage-dependent anion channel (HVDAC3) to induce cell death by causing loss of mitochondrial membrane potential; and (4) HBx also interacts with heat shock protein 60 which is also localized in mitochondria to enhance HBx-mediated apoptosis<sup>[11-13]</sup>.

Cell death signals from the extracellular environment or internal sensors for the cellular response are major constituents of apoptotic machinery. Cell surface death receptors that transmit cell death signals are activated by specific death ligands. It is demonstrated that Fas is one of the best-characterized death receptors. Upon binding of FasL onto Fas, apoptotic signals are subsequently transmitted *via* death adaptor molecule FADD which can mediate the activation of caspase 8, and active caspase 8 can proteolytically activate downstream effector caspases, such as caspase 3, to trigger apoptosis<sup>[14,15]</sup>. It is reported that liver cell apoptosis is mediated by Fas<sup>[16-18]</sup>. Understanding the molecular mechanism responsible for the regulation of Fas and FasL may aid in developing novel therapeutic strategies for HCC.

The mixed lineage kinases (MLKs) are a family of serine/threonine protein kinases that function in a phospho-relay module to control the activity of specific mitogen-activated protein kinases (MAPKs). The family includes three subgroups: MLKs (mixed lineage kinases, including MLK1-4), dual leucine zipper-bearing kinases (DLKs), and Zipper Sterile-a-Motif Kinases (ZAKs). MLKs as mitogen activated protein kinase kinase kinases (MAP-KKKs) could activate MKKs, such as MKK4 and/or MKK7, which in turn, activate c-Jun N-terminal kinases (JNKs)<sup>[19-21]</sup>. Ischemic brain injury studies provide the evidence that the ischemia-stimulating factor can activate MLK3-MKK7-JNKs signaling module to activate death receptor pathway, leading to the neural cell apoptosis<sup>[22,23]</sup>. It is noteworthy that hepatic cells also express MLK3, MKK7, JNK proteins<sup>[24,25]</sup>. Therefore, it is of significance

to clarify whether the HBx can also activate MLK3-MKK7-JNKs signaling module and induce apoptosis of hepatic cells.

In this study, we demonstrated that HBx induces the apoptosis of hepatic cells depending on activating MLK3-MKK7-JNKs signaling module to upregulate FasL protein expression. On this basis, this study gives a new insight into a better understanding of how HBx mediates apoptosis in hepatocytes, and lays the foundation for further revealing the role of HBx in HBV-related liver oncogenesis and development.

## MATERIALS AND METHODS

### Reagents

The RPMI 1640 medium, liposome Lipofectamine 2000, and Trizol reagent were obtained from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-HBx antibody was from Chemicon (Temecula, CA). Rabbit polyclonal anti-phospho-MLK3 and rabbit polyclonal anti-phospho-MKK7 were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-phospho-JNKs, rabbit polyclonal anti-Fas, and rabbit polyclonal anti-FasL were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-GAPDH antibody, goat anti-mouse IgG-AP, goat anti-rabbit IgG-AP, BCA Protein Assay Kit, BCIP/NBT Alkaline Phosphatase Color Development Kit, the BeyoECL Plus Western blotting detection System, Caspase3 activity assay kit, Caspase8 activity assay kit, and Hoechst33258 staining solution were from Beyotime Institute of Biotechnology (Jiangsu, China). *In situ* cell death detection kit was from Roche (Mannheim, Germany). Annexin V/PI apoptosis kit was from Biovision (Mountain View, CA). Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). TIANScript RT Kit was from TIANGEN Biotech (Beijing, China). SP600125 and K252a were obtained from Sigma (St. Louis, MO). Twenty mmol/L stock solution of SP600125 and 20  $\mu$ mol/L stock solution of K252a were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C in the dark. SP600125 and K252a were prepared freshly for each experiment by serial dilution into 0.01% DMSO in RPMI 1640 medium. All other chemicals and reagents were of analytical grade.

### Plasmids construction

Plasmid pcDNA3.1-X containing the full length HBx sequence, was constructed in mammalian expression vector pcDNA3.1 (Invitrogen) as described previously<sup>[26]</sup>.

To construct the expression vector for shRNA targeting HBx, pSilencer3.1-shHBX, two chemically synthesized oligonucleotides encoding HBx specific shRNA with the following sense sequences: 5'-GATCCGGTCTTACATAAGAGGACTTTCAAGAG AAGTCCTCTTATGTAAGACCTTTTTTGAAA-3' and antisense sequences: 5'-AGCTTTTCCAAAAAAGGTCTTACATAAGAGGACTTCTCTTGAAAGTCCTCTTAT GTAAGACCG-3' were annealed and cloned into *Bam*H I -

Table 1 Primers for the reverse transcription polymerase chain reaction amplification

Gene	Sense	Antisense	Product length (bp)
HBX	5'-TGTAAGCTTATGGCTGCTAGGC-3'	5'-TGTTGAATTCCTTAGGCAGAGGTG-3'	465
Fas	5'-GTGAACACTGTGACCCCTT-3'	5'-TCATTGACACCATTCTTTCCG-3'	349
FasL	5'-CTGGGGATGTTTCAGCTCITC-3'	5'-CTTCACTCCAGAAAGCAGGAC-3'	304
Bax	5'-TTTGCTTCAGGGTTTCATCC-3'	5'-CAGTTGAAGTTGCCGTCAGA-3'	246
Bcl-2	5'-GTGGAGGAGCTCTTCAGGGA-3'	5'-AGGCACCCAGGGTGATGCAA-3	304
$\beta$ -actin	5'-GGCATCGTGATGGACTCCG-3'	5'-GCTGGAAGGTGGACAGCGA-3	607

HBx: Hepatitis B virus X protein.

*Hind*III sites of the linearized pSilencer3.1-H1-nero vector (Ambion, Austin, TX).

### Cell culture and transfection

Human hepatocarcinoma cell line, HepG2 cell, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) was cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum, 2 mmol/mL L-glutamine, 100  $\mu$ g/mL streptomycin and 100 units/mL penicillin at 37 °C in 5% CO<sub>2</sub>. When the cell fusion rate reached 80%, HepG2 cells were transfected with negative control plasmid pcDNA3.1, pcDNA3.1-X, cotransfected pcDNA3.1-X with either pSilencer3.1-shHBX or pSilencer3.1-H1 in a ratio of 1:3, in the presence of the liposome Lipofectamine 2000 according to the manufacturer's instructions.

### Reverse transcription polymerase chain reaction analysis

The total RNA of HepG2 cells transfected with various plasmids was prepared with Trizol reagent according to the manufacturer's instructions. The reverse transcription was performed with TIANScript RT Kit. The specific primers used are shown in Table 1, the amplification condition was 94 °C for 45 s, 58 °C (55 °C-60 °C) for 35s, 72 °C for 1 min for 35 cycles and a final extension at 72 °C for 5 min each. The PCR products were subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide staining.

### Western blotting analysis

For protein extracts, cells were lysed using cell lysis buffer [20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% TritonX-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, 1% Na<sub>2</sub>VO<sub>4</sub>, 0.5  $\mu$ g/mL leupeptin and other phosphatase inhibitors, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF)]. The lysates were collected, and centrifuged at 10 000  $\times$  g at 4 °C for 5 min. The bicinchoninic acid (BCA) Protein Assay Kit was used to measure the protein concentrations. Total protein of 100  $\mu$ g of each above lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 3% bovine serum albumin (BSA) in tris buffered saline (TBS) containing 0.01% Tween-20 for 3 h at room temperature, and then incubated with specific primary antibodies: mouse mono-

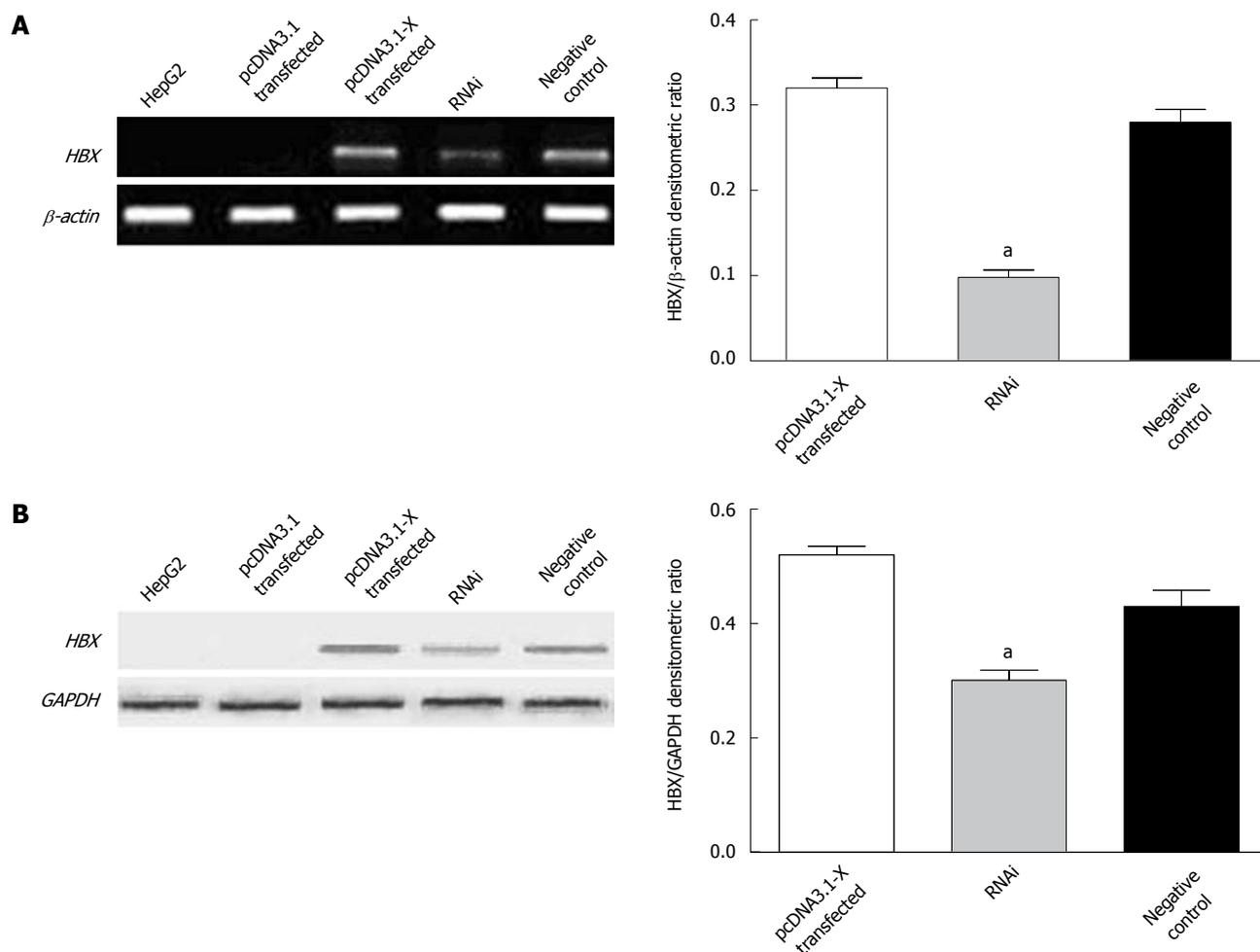
clonal anti-HBx antibody (1:250), rabbit polyclonal anti-phospho-MLK3 (1:500), rabbit polyclonal anti-phospho-MKK7(1:500), mouse monoclonal anti-phospho-JNKs (1:400), rabbit polyclonal anti-Fas (1:500), rabbit polyclonal anti-FasL (1:500), and mouse monoclonal anti-GAPDH antibody (1:500), respectively, overnight at 4 °C. The membranes were then incubated with goat anti-mouse IgG-AP (1:500), goat anti-rabbit IgG-AP (1:500), goat anti-mouse IgG-HRP (1:2000) and goat anti-rabbit IgG-HRP (1:2000) for 2 h at room temperature separately. The labeled bands were detected with NBT/BCIP Alkaline Phosphatase Color Development Kit or the BeyoECL Plus Western blotting detection system.

### Caspase activity assay

The enzyme activities of caspase3, caspase8 and caspase9 were quantified using caspase3, caspase8 and caspase9 activity assay kit, respectively. Adherent and floating cells were collected and lysed in caspase lysis buffer. Caspase3 enzyme activity in 30  $\mu$ g cell lysate was measured by cleavage of Ac-DEVD-pNA colorimetric substrate. Caspase8 enzyme activity in 30  $\mu$ g cell lysate was measured by cleavage of Ac-IETD-pNA colorimetric substrate. Caspase9 enzyme activity in 30  $\mu$ g cell lysate was measured by cleavage of Ac-LEHD-pNA colorimetric substrate. The absorbance at 405 nm was quantified in a microtiter plate reader after incubated at 37 °C for 2 h.

### Apoptosis analysis

Cells were adjusted to a density of 2  $\times$  10<sup>5</sup> cells/mL, added to 24-well plates in 0.5 mL each well. After transfection and incubation for 72 h, cell apoptosis was analyzed by three methods: (1) terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining: cell apoptosis was analyzed using the *in situ* cell death detection kit, according to the manufacturer's protocol. The number of TUNEL-positive cells was divided by the total number of cells to determine the ratio of TUNEL-positive cells. Five optical fields, about 200 cells were selected randomly and analyzed; (2) Hoechst 33258 staining: cells were fixed with 4% paraformaldehyde, washed twice with phosphate-buffered saline (PBS) and stained with Hoechst 33258 staining solution according to the manufacturer's instructions. The morphologic changes of apoptotic cells, including reduction in volume and nuclear chromatin condensation, were observed under a fluorescence microscope. Five optical fields containing about 200 cells were selected randomly and analyzed; and (3) flow



**Figure 1** Detection of hepatitis B virus X protein expression in transfected HepG2 cells. HepG2 cells were transfected with pcDNA3.1-X plasmids or cotransfected with pcDNA3.1-X and pSilencer3.1-shHBX plasmids. Forty-eight hours later, the expression of hepatitis B virus X protein (HBx) in HepG2 cells was determined by reverse transcription polymerase chain reaction (A) and Western blotting analysis (B). HepG2 group was not transfected with any plasmids. pcDNA3.1 transfected group was transfected with plasmid pcDNA3.1; pcDNA3.1-X transfected group was transfected with pcDNA3.1-X; RNAi group was cotransfected with pcDNA3.1-X and pSilencer3.1-shHBX in a ratio of 1:3; negative control group was cotransfected with pcDNA3.1-X and negative control plasmid pSilencer3.1-H1 in a ratio of 1:3. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), <sup>a</sup> $P < 0.05$  vs pcDNA3.1-X transfected group.

cytometry: cell apoptosis was evaluated by double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) using annexin V/PI apoptosis kit. Cells were washed with PBS twice and stained with Annexin V and PI for 5 min at room temperature in the dark. The level of apoptosis was determined by measuring the fluorescence of the cells with a flow cytometer (Becton-Dickinson, San Diego, CA).

### Statistical analysis

All experiments were performed three times. Semiquantitative analysis of the bands was performed with the Image J analysis software (Version 1.30v, Wayne Rasband, NIH, United States). The data were presented in mean  $\pm$  SD and analyzed by one-way ANOVA (SPSS version 13.0).  $P < 0.05$  was considered statistically significant.

## RESULTS

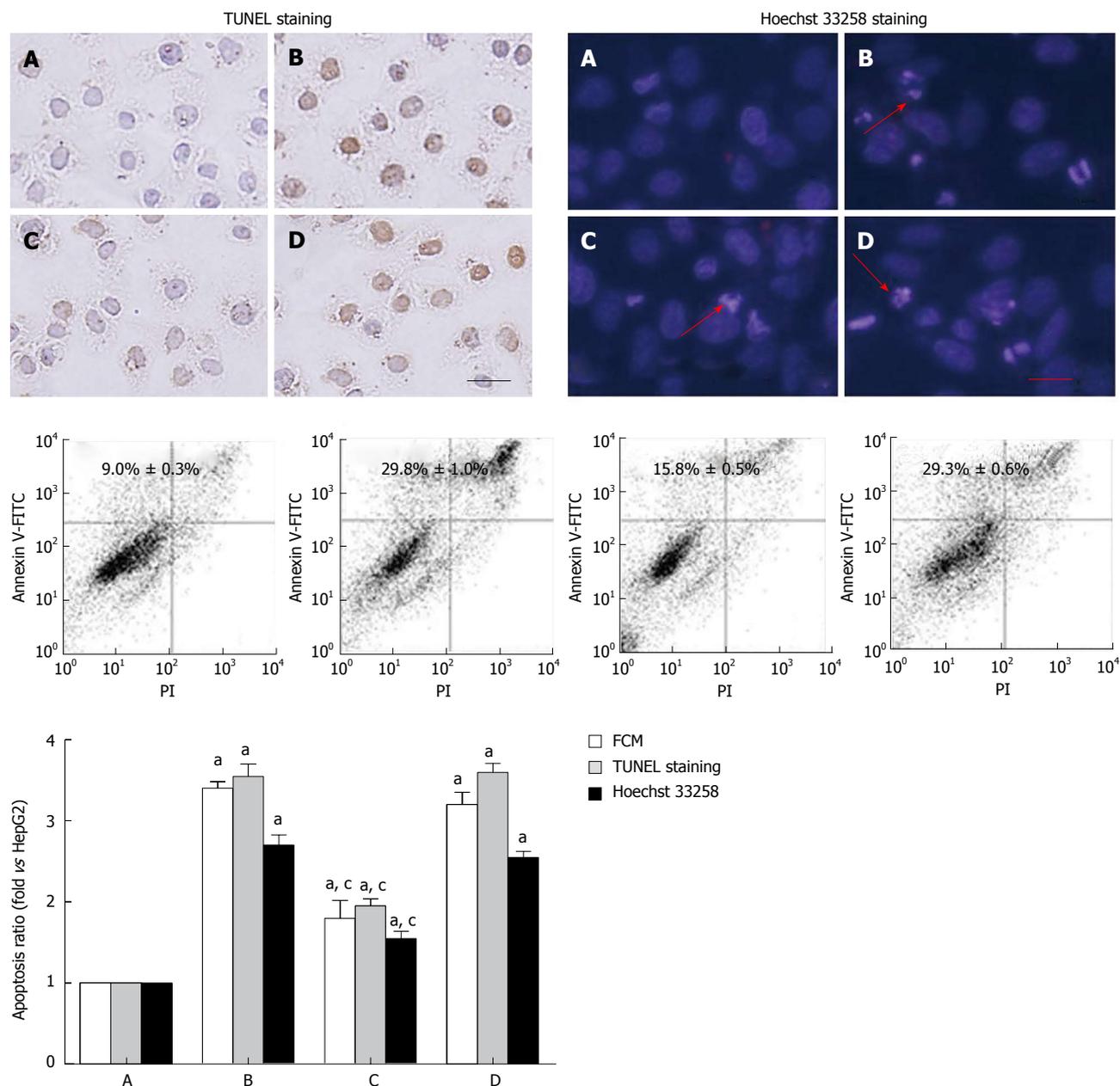
### Expression of hepatitis B virus X protein in HepG2 cells

To investigate the potential apoptotic ability of HBx,

HBx expressing plasmid pcDNA3.1-X was transiently transfected into a human HCC cell line, HepG2. RT-PCR and Western blotting analysis demonstrated that HepG2 cells transfected with pcDNA3.1-X could steadily express HBx. To further identify the function of HBx upon apoptosis of HepG2, the expression vector for shRNA targeting HBx named pSilencer3.1-shHBX was constructed. When the shRNA expression plasmid was transfected to HepG2 cells in combination with pcDNA3.1-X, the expression of HBx was specifically inhibited by shRNA against HBx, while universal negative control plasmid pSilencer3.1-H1 did not display any effect on HBx expression (Figure 1).

### Effects of hepatitis B virus X protein on induction of apoptosis in HepG2 cells

To investigate the roles of HBx in cell apoptosis, TUNEL assay was used to detect the presence of DNA strand breaks. The *in situ* TUNEL staining showed that the TUNEL-negative cells appeared with blue nucleus and TUNEL-positive cells with yellow nucleus. To fur-



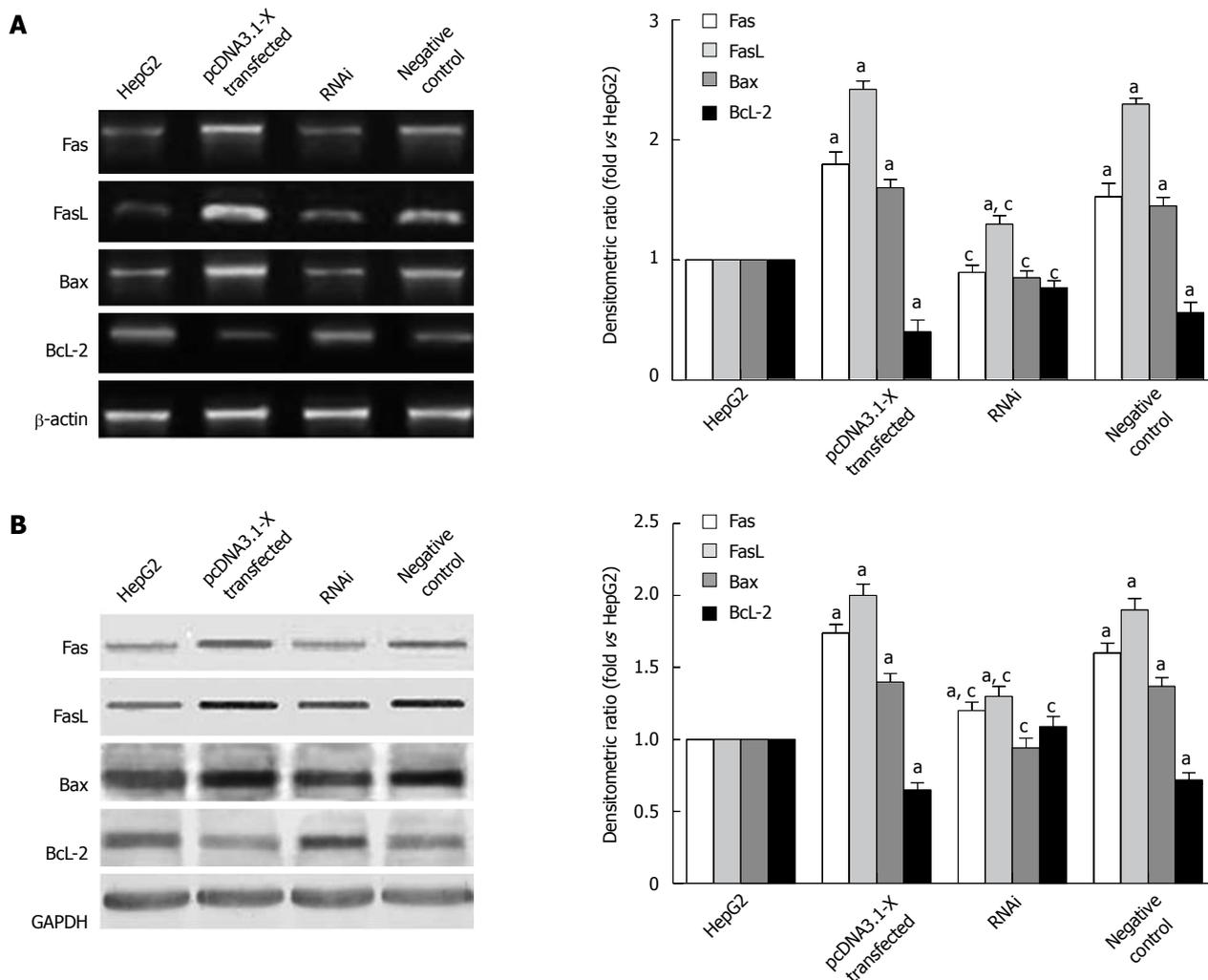
**Figure 2** Apoptosis of HepG2 cells induced by hepatitis B virus X protein. HepG2 cells were cotransfected with pcDNA3.1-X and pSilencer3.1-shHBX as RNAi group, and HepG2 cells were cotransfected with pcDNA3.1-X and pSilencer3.1-H1 plasmids as negative control. Cells were examined by TUNEL, Hoechst 33258 staining and flow cytometry as described in Materials and Methods. A: HepG2 group; B: pcDNA3.1-X transfected group; C: RNAi group; D: Negative control group. Data was expressed as mean ± SD ( $n = 3$ ),  $^*P < 0.05$  vs the HepG2 group;  $^*P < 0.05$  vs pcDNA3.1-X transfected group and negative control group. Scale bar value: 5  $\mu$ m. Red arrows indicate apoptotic nuclei. Apoptosis ratio = (apoptotic cells/total cells) × 100%. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

ther confirm the occurrence of apoptosis, the apoptosis of HepG2 cells and HepG2 transfected with HBx were detected by Hoechst 33258 staining and flow cytometry (FCM). In Hoechst 33258 staining, reduced cell sizes and increased nuclear chromatin condensation were detected in the apoptotic cells. As it is shown in Figure 2, when compared with HepG2 cells group, the apoptosis rate of pcDNA3.1-X transfected group and negative control group was increased, while, in contrast, that of RNAi group was decreased. It indicated that the high expression of HBx could promote the apoptosis of HepG2 cells, and inhibiting the expression of HBx protein could

reduce the apoptosis rate of HepG2 cells.

**Hepatitis B virus X protein-induced apoptosis was attributed to the upregulation of Fas/FasL signaling pathway-related proteins**

To determine which signaling pathway was involved in cell apoptosis by HBx, expression of Fas, FasL and apoptotic regulators Bax, Bcl-2 in HBx-transfected HepG2 cells were examined by RT-PCR and Western blotting analysis. The results showed that the Fas, FasL, Bax mRNA and protein expression were increased induced by HBx, while the Bcl-2 mRNA and protein were decreased



**Figure 3** Expression of Fas, FasL, Bax, Bcl-2 mRNA and protein in HepG2 cells induced by hepatitis B virus X protein. Transfection of HepG2 cells is described in Figure 2. Forty-eight hours after transfection, the mRNA (A) and protein (B) expression levels of Bax, Bcl-2, Fas and FasL were determined by RT-PCR and Western blotting analysis. Data are expressed as mean ± SD (n = 3), <sup>a</sup>P < 0.05 vs the HepG2 group; <sup>b</sup>P < 0.05 vs pcDNA3.1-X transfected group and negative control group.

after transfection with HBx in HepG2 cells for 48 h (Figure 3). When HepG2 cells were cotransfected with pcDNA3.1-X and pSilencer3.1-shHBX for 48 h, the levels of Fas, FasL, Bax mRNA and protein were decreased. However, Bcl-2 mRNA and protein were increased after cotransfection with pcDNA3.1-X and pSilencer3.1-shHBX in HepG2 cells.

In order to determine the dynamic effect of HBx on the Fas/FasL signaling pathway, we detected the gene and protein expression of Fas and FasL at 24 h, 48 h, and 72 h after transfection with HBx, respectively, and found that the Fas and FasL mRNA expression was increased by HBx in a time-dependent manner (Figure 4A). In parallel, the levels of Fas and FasL proteins in pcDNA3.1-X transfected cells were increased by HBx in a similar manner (Figure 4B).

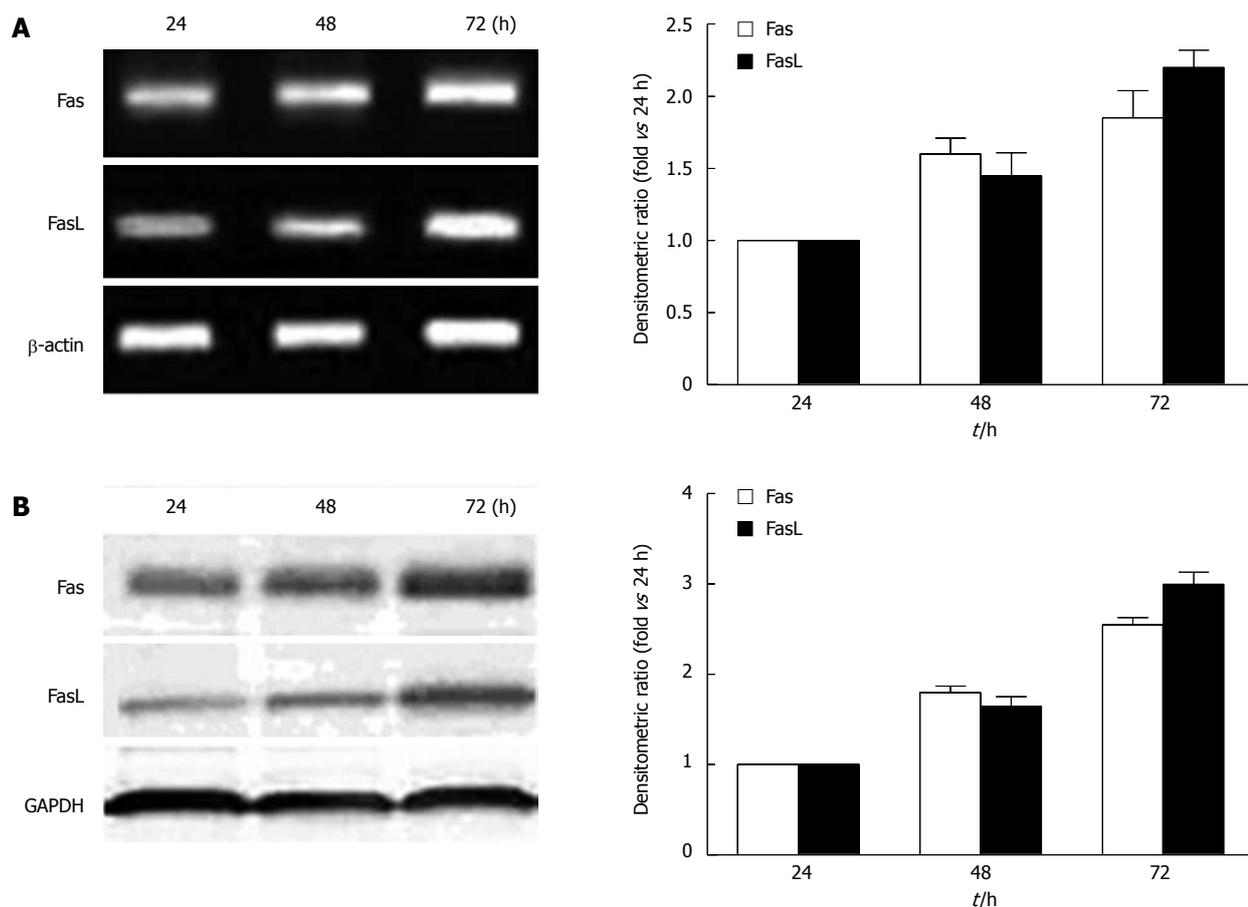
To further determine the effect of HBx on the Fas/FasL signaling pathway, enzyme activity of caspase8, caspase9 and caspase3 were also detected by spectrophotometric test. The results showed that the activation of caspase8, caspase9 and caspase3 was increased in HBx-

transfected HepG2 cells (Figure 5).

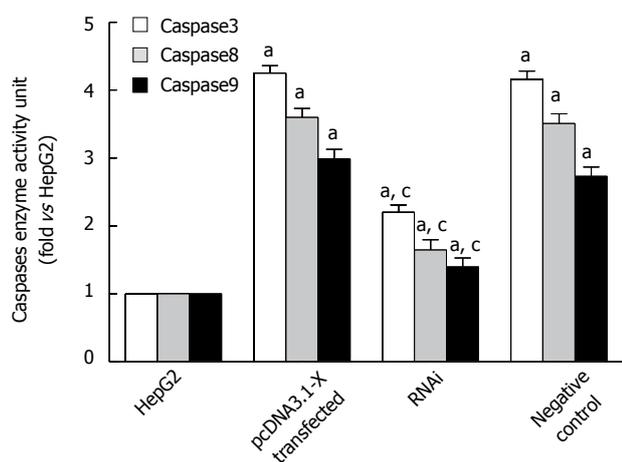
It could be concluded that HBx could mediate cell apoptosis by upregulating Fas/FasL signaling pathway-related protein expression to activate the Fas/FasL signaling pathway.

**Hepatitis B virus X protein upregulates FasL protein expression by activating MLK3-MKK7-JNKs signal module and induces apoptosis**

The upstream mechanisms of HBx on expression of Fas/FasL signaling pathway-related proteins were further investigated. Previous studies showed that MLK3, MKK7 and JNKs which belong to MAPK signaling pathways, could form MLK3-MKK7-JNKs signaling module and the activation of the signaling module could upregulate the expression of Fas/FasL signaling pathway-related protein, leading to cell apoptosis<sup>[22,23]</sup>. To find out whether the upregulation of Fas/FasL signaling pathway-related proteins induced by HBx was dependent on activation of MLK3-MKK7-JNKs signaling module, the phosphorylation levels of MLK3, MKK7 and JNKs



**Figure 4** Upregulation of Fas/FasL signaling pathway-related protein expression by hepatitis B virus X protein in a time-dependent manner. HepG2 cells were transfected with pcDNA3.1-X and incubated for various time periods as indicated. The levels of Fas, FasL mRNA (A) and proteins (B) were determined by reverse transcription polymerase chain reaction and Western blotting analysis. Data are expressed as mean  $\pm$  SD ( $n = 3$ ).

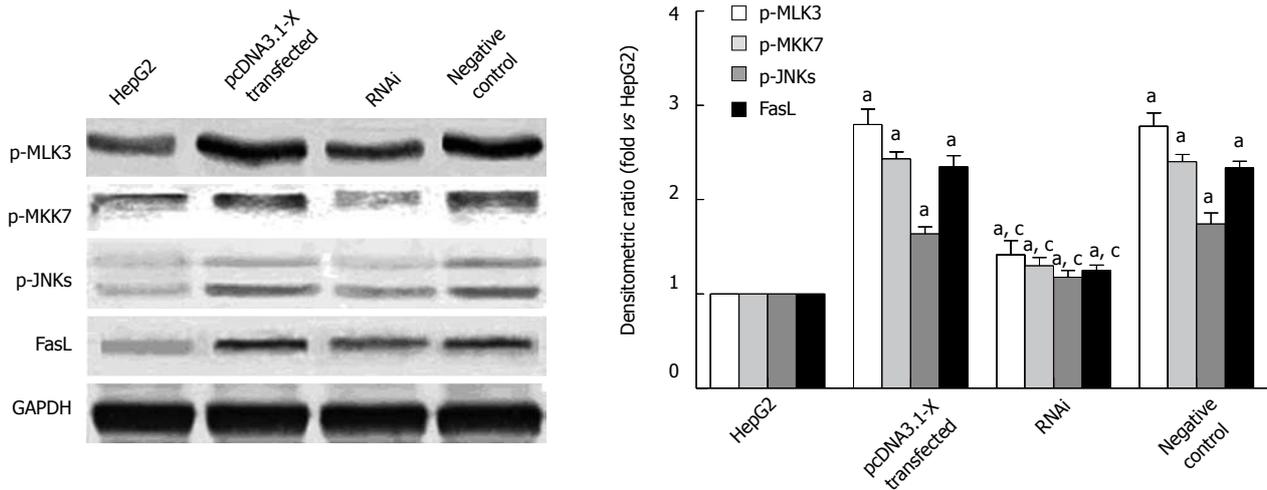


**Figure 5** Detection of activated caspases in HepG2 cells transfected with hepatitis B virus X protein. Transfection of HepG2 cells is described in Figure 2. Forty-eight hours after transfection, the enzyme activity of caspase3, caspase8 and caspase9 was analyzed by spectrophotometric test. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), <sup>a</sup> $P < 0.05$  vs the HepG2 group; <sup>c</sup> $P < 0.05$  vs pcDNA3.1-X transfected group and negative control group.

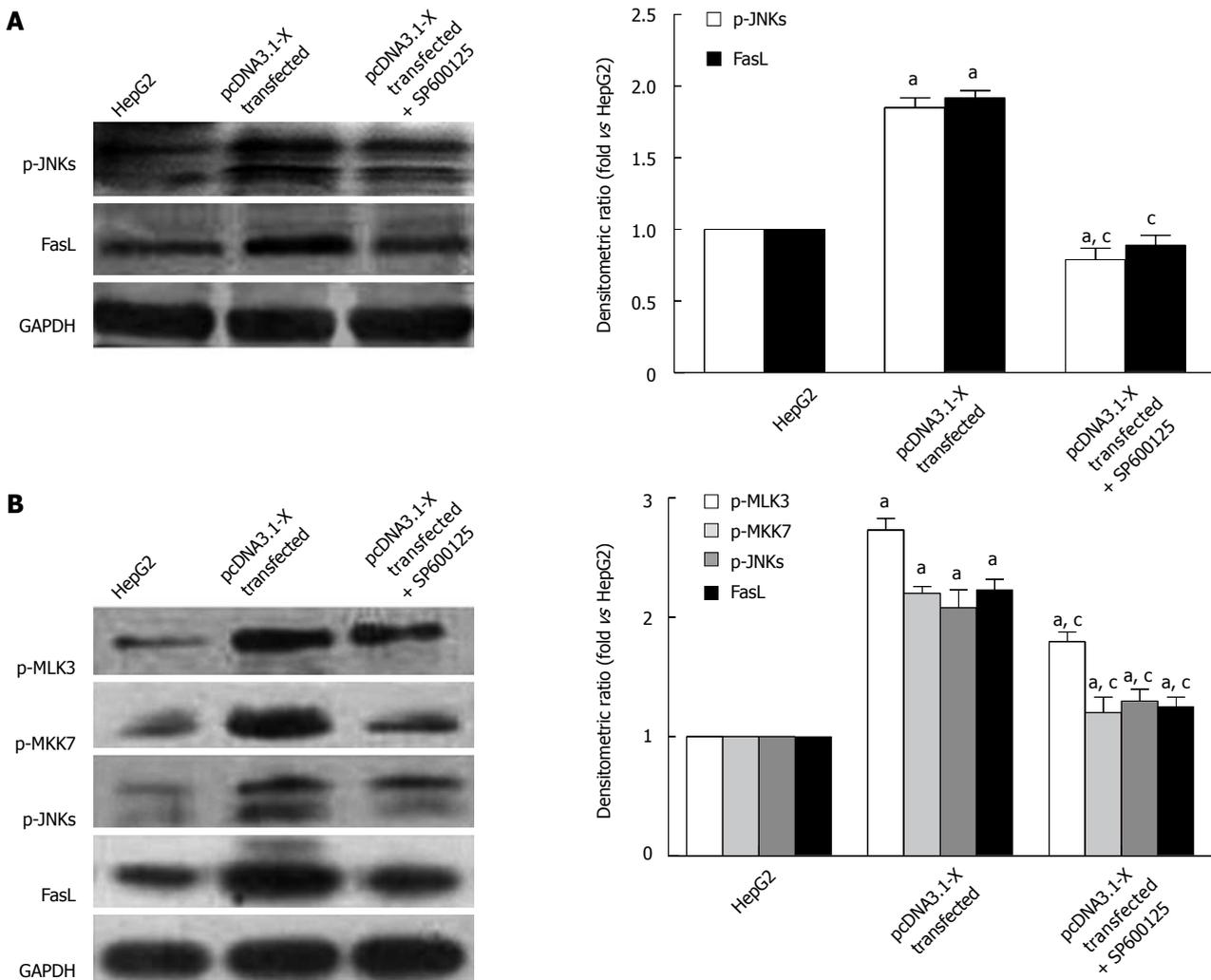
were detected. The phosphorylation levels of MLK3, MKK7 and JNKs proteins in pcDNA3.1-X transfected group were increased remarkably compared with the

HepG2 cells. When silencing HBx protein expressed by pSilencer3.1-shHBX, phosphorylation of MLK3, MKK7 and JNKs was inhibited (Figure 6). The changing tendency of expression level of FasL protein was consistent with phosphorylation levels of MLK3, MKK7 and JNKs proteins. When we treated HepG2 cells transfected with HBx with a known JNK inhibitor, SP600125, the activation of JNKs and expression of FasL protein were inhibited (Figure 7A). Furthermore, the phosphorylation levels of MLK3, MKK7 and JNKs and the protein expression of FasL also concomitantly decreased in pcDNA3.1-X transfected group when treated with K252a, a known MLK3 inhibitor<sup>[27,28]</sup> (Figure 7B). The results demonstrated that HBx could activate MLK3-MKK7-JNKs signal module and upregulate Fas/FasL death receptor pathway-related protein expression.

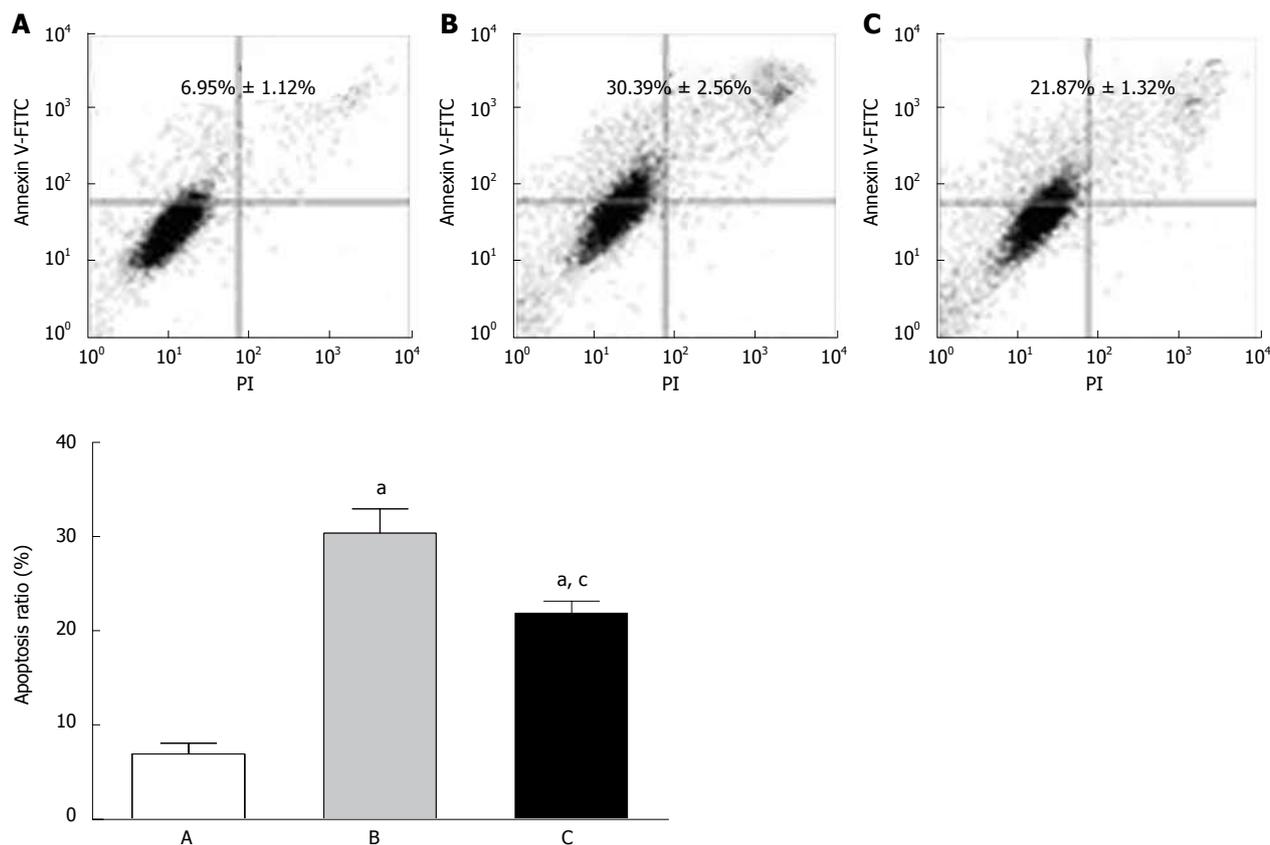
To determine the relationship between activation of MLK3-MKK7-JNKs signal module and HBx-stimulated cell apoptosis, the apoptosis rate of the pcDNA3.1-X transfected group treated with K252a or not was determined by flow cytometry. As expected, apoptosis rate of the HBx-transfected HepG2 cells was suppressed in the presence of the MLK3 inhibitor (Figure 8), indicating that HBx could activate the MLK3-MKK7-JNKs signaling modular and its downstream death receptor pathway, and



**Figure 6** Activation of MLK3-MKK7-JNKs signaling module in hepatitis B virus X protein-transfected HepG2 cells. Transfection of HepG2 cells is described in Figure 2. Forty-eight hours after transfection, the cells lysates were detected by Western blotting analysis. Data are expressed as mean  $\pm$  SD ( $n = 3$ ),  $^aP < 0.05$  vs the HepG2 group;  $^cP < 0.05$  vs pcDNA3.1-X transfected group and negative control group.



**Figure 7** Activation of MLK3-MKK7-JNKs signaling module on FasL expression mediated by hepatitis B virus X protein. A: HepG2 cells were cultured in 0.01% dimethyl sulfoxide (DMSO) in the absence or presence of 20  $\mu$ mol/L SP600125 after transfection with pcDNA3.1-X and incubated for 24 h; B: HepG2 cells were cultured in 0.01% DMSO in the absence or presence of 300 nmol/L K252a after transfection with pcDNA3.1-X and incubated for 24 h. Cell lysates were prepared and electrophoresed in SDS-PAGE and subsequently performed by Western blotting analysis. Data are expressed as mean  $\pm$  SD ( $n = 3$ ),  $^aP < 0.05$  vs the HepG2 group;  $^cP < 0.05$  vs pcDNA3.1-X transfected group.



**Figure 8** Inhibition of HBx-induced cell apoptosis by K252a. HepG2 cells were cultured in 0.01% dimethyl sulfoxide in the absence or presence of 300 nmol/L K252a after transfection with pcDNA3.1-X and incubated for 24 h, and the cell apoptosis was examined by flow cytometry. A: HepG2 group; B: pcDNA3.1-X transfected group; C: pcDNA3.1-X transfected + K252a group. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), <sup>a</sup> $P < 0.05$  vs the HepG2 group; <sup>c</sup> $P < 0.05$  vs pcDNA3.1-X transfected group.

induce the apoptosis of the hepatocarcinoma cell line.

## DISCUSSION

HBx protein has been reported to be either a promoter or an inhibitor of cell apoptosis<sup>[11,29-31]</sup>. The dual activity of HBx protein on cell apoptosis suggests that the expression of HBx gene and its physiological role depend on cellular environments and infection stage<sup>[30,32-33]</sup>. Naturally, HBx shows extremely low levels of expression during the early stage of HBV infection, which may contribute to the activation of transcription and virus replication<sup>[33,34]</sup>. With the development of chronic HBV infection, expression of HBx protein increased and activated apoptosis to contribute to virus spread and the progression of chronic hepatitis and HCC<sup>[32,35,36]</sup>. It was reported that HBx could induce the apoptosis of hepatic cells by the death receptor pathway and the mitochondrial pathway. However, the question how HBx activates the death receptor pathway to induce the apoptosis of hepatic cells remains unclear. The further elucidation of signaling pathways that regulate apoptosis by HBx would help us understand the effectiveness of HBX in the development of HCC.

In this study, the regulation mechanism of apoptosis by HBx in HepG2 cells was investigated. First, the ability

of HBx to induce apoptosis using transient transfection of HBx was tested. As a result, after transfected with HBx, HepG2 cells exhibited more conspicuous apoptotic nuclear condensation and a higher level of cell death, which is in agreement with the previous reports that HBx could induce cell apoptosis on its own<sup>[5-6,37,38]</sup>. Second, the expression level of the Fas/FasL signaling pathway-related proteins was examined in HepG2 cells. The expression of Fas/FasL signaling pathway-related proteins were remarkably upregulated in pcDNA3.1-X transfected group. RNA interference targeting HBx in HepG2 cells could reduce the cell apoptosis and down-upregulate the Fas/FasL signaling pathway-related proteins at the same time. Based on the above results, it was reasonable to conclude that HBx is able to induce apoptosis by upregulating Fas/FasL signaling pathway-related protein expression in HepG2 cells, which was similar to the previous report that HBx played a role in inducing apoptosis of hepatocyte *via* Fas/FasL system<sup>[39,40]</sup>.

Recent studies have found that the MAPKs were involved in the signal transduction for apoptosis. The animal model experiments for cerebral ischemia demonstrated that MLK3-MKK7-JNKs signaling modules, a member of the MAPK family, could activate its downstream Fas/FasL signaling pathway and induce the apoptosis of nerve cells<sup>[22-23]</sup>. In addition, it was reported that hepatic cells

could also express MLK3, MKK7 and JNKs<sup>[24-25]</sup>. We supposed HBx protein could act as the stressor to activate the signal module of MLK3-MKK7-JNKs, and to induce the apoptosis of the hepatic cells.

On the basis of this hypothesis, the phosphorylation levels of MLK3, MKK7 and JNKs were detected in all groups. It was found that the phosphorylation levels of MLK3, MKK7 and JNKs were increased obviously in the pcDNA3.1-X group, indicating that the HBx might increase the phosphorylation levels of these proteins. When we treated pcDNA3.1-X group with SP600125, the activation of JNKs and expression of FasL were significantly inhibited. Moreover, when pcDNA3.1-X group was treated with K252a, the expression levels of p-MLK3, p-MKK7, p-JNKs and FasL were also significantly decreased, and then cell apoptosis rate obviously declined, suggesting that the HBx protein could act as a stressor and activate the MLK3-MKK7-JNKs signaling module and its downstream Fas/FasL death receptor pathway to induce the apoptosis of HepG2 cells. This would provide us a new research target and a novel concept for blocking the HBx-induced apoptosis process.

The ratio of Bax and Bcl-2 could change the mitochondrial membrane potential and result in the release of cytochrome C, the activation of caspase9, and then further activate caspase3 to induce apoptosis<sup>[41,42]</sup>. As shown in Figures 3 and 5, HBx up-regulated the expression of Bax, down-regulated the expression of Bcl-2, and increased the activation of caspase9 at the same time. This data indicated that the apoptosis induced by HBx also referred to the mitochondrial pathway. Nevertheless, effects of activating the mitochondrial pathway on apoptosis needs to be further investigated.

In conclusion, this study demonstrated that the high expression level of HBx protein could activate MLK3-MKK7-JNKs module and upregulate FasL protein. It has provided a novel insight into the mechanism of HBx-induced hepatocarcinoma cell apoptosis.

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## COMMENTS

### Background

Hepatitis B virus (HBV) infection is one of the most widely spread viral diseases and strongly associated with the development of hepatocellular carcinoma (HCC). However, the mechanism of HBV-mediated HCC development is not clearly elucidated. Although it has been shown that HBx, the protein encoded by the X gene of the HBV genome, could induce cell apoptosis on its own or sensitize cells to apoptotic stimuli, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) or UV irradiation, the mechanism of HBx-mediated apoptosis remains controversial.

### Research frontiers

HBx, the protein encoded by the X gene of the HBV genome, is a multifunctional regulatory protein and has been implicated in HBV-mediated hepatocarcinogenesis. In this study, the authors further investigated the regulation mechanism of apoptosis mediated by HBx in HepG2 cells.

### Innovations and breakthroughs

Previous researches showed that HBx participated in the apoptotic destruction

of liver cells during the virus infection. Although some studies found that HBx could mediate apoptosis by the death receptor pathway or affecting mitochondrial physiology, the mechanism of HBx-mediated apoptosis is still not clear. In the present study, the authors showed that HBx could induce HepG2 cells apoptosis via a novel active MLK3-MKK7-JNKs signaling module and upregulate Fas/FasL signaling pathway-related protein expression.

### Applications

This study provided a new insight into a better understanding of how HBx mediated apoptosis, and lay the foundation for further clarifying the role of HBx in HBV-related liver oncogenesis and development.

### Terminology

Apoptosis is a common form of cell death and often referred to as programmed death. The characteristics of apoptosis include activation of cysteine proteases (caspases) and endonucleases, condensation of the nuclear chromatin and cytoplasm, cleavage of the DNA into oligonucleosomal fragments, and segmentation of the dying cell into membrane-bound apoptotic bodies. Fas is one of the best-characterized death receptor. Upon binding of FasL (Fas ligand) onto Fas, apoptotic signals are subsequently transmitted in cytoplasm to trigger apoptosis. The mixed lineage kinase 3 (MLK3) is a member of the mixed lineage kinases (MLKs), a family of serine/threonine protein kinases functioning in a phosphorelay module to control the activity of specific mitogen-activated protein kinases (MAPKs).

### Peer review

In this manuscript, the authors demonstrate that hepatitis B virus X protein induced apoptosis through Fas/FasL signaling via MLK3-MKK7-JNKs signaling module in HepG2 cells. A series of experiments are well-planned and well-performed and this manuscript is well written. Although interesting, this manuscript could be strengthened if several points were addressed.

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## Magnetic resonance imaging: A new tool for diagnosis of acute ischemic colitis?

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### Abstract

**AIM:** To define the evolution of ischemic lesions with 7T magnetic resonance imaging (7T-MRI) in an animal model of acute colonic ischemia.

**METHODS:** Adult Sprague-Dawley rats were divided into two groups. Group I underwent inferior mesenteric artery (IMA) ligation followed by macroscopic observations and histological analysis. In group II, 7T-MRI was performed before and after IMA ligation and followed by histological analysis.

**RESULTS:** Morphological alterations started to develop 1 h after IMA ligation, when pale areas became evident in the splenic flexure mesentery and progressively wors-

ened up to 8 h thereafter, when the mesentery was less pale, and the splenic flexure loop appeared very dark. The 7T-MRI results reflected these alterations, showing a hyperintense signal in both the intraperitoneal space and the colonic loop wall 1 h after IMA ligation; the latter progressively increased to demonstrate a reduction in the colonic loop lumen at 6 h. Eight hours after IMA ligation, MRI showed a persistent colonic mural hyperintensity associated with a reduction in peritoneal free fluid. The 7T-MRI findings were correlated with histological alterations, varying from an attenuated epithelium with glandular apex lesions at 1 h to coagulative necrosis and loss of the surface epithelium detected 8 h after IMA ligation.

**CONCLUSION:** MRI may be used as a substitute for invasive procedures in diagnosing and grading acute ischemic colitis, allowing for the early identification of pathological findings.

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**Key words:** Ischemic colitis; Animal models; Sprague-Dawley rats; Magnetic resonance imaging; Histopathology

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### INTRODUCTION

Ischemic colitis (IC) was first described by Boley *et al*<sup>[1]</sup>

and Theodoropoulou *et al.*<sup>[2]</sup>, and is a relatively common disease, being the most frequent form of intestinal ischemia<sup>[3]</sup> and the second-most frequent cause of lower gastrointestinal bleeding<sup>[4]</sup>. IC is the consequence of an acute interruption or chronic decrease in the colonic blood supply<sup>[5]</sup>, which may be either occlusive or non-occlusive in origin<sup>[2]</sup>. This disease results in ischemic necrosis of variable severity that can range from superficial mucosal involvement to full-thickness transmural necrosis<sup>[5,6]</sup>.

Clinically, IC presents in either a gangrenous (acute fulminant) or non-gangrenous form (acute transient; chronic)<sup>[2]</sup>, with a mortality rate ranging from 10% for the non-occlusive disease to 90% for occlusive mesenteric infarction due to embolus or thrombosis<sup>[5]</sup>. The left colon is involved in 75% of cases and the right colon in the remaining 25%; the splenic flexure and the sigmoid colon are the areas most frequently affected<sup>[3,7]</sup>.

The presentation of IC is not specific and is highly variable, therefore, its diagnosis largely depends on clinical suspicion<sup>[7]</sup>. In this context, the role of imaging techniques is controversial. Standard radiology and computed tomography (CT), the latter considered the best diagnostic modality in acute settings<sup>[8,9]</sup>, yield non-specific and late findings, whereas the advantages of magnetic resonance imaging (MRI) are still a matter of debate<sup>[5]</sup>.

The functional and morphological responses to ischemia produced by inferior mesenteric artery (IMA) ligation have been studied in the canine, porcine and rat colon<sup>[10-13]</sup>. However, to date, nothing is known about the histological evolution of the colonic ischemic injury or the relationship between these lesions and MRI findings. Therefore, the aim of our study was to define the evolution of histological ischemic lesions and to compare anatomopathological features with T2-weighted MRI in an animal model of colonic ischemia.

## MATERIALS AND METHODS

### Animal preparation

All procedures performed on animals were approved by our Institutional Animal Care and Use Committee. Nine adult male Sprague-Dawley rats (250-340 g; Harlan Laboratories, Indianapolis, IN, United States) were used in this study. The rats were maintained on a 12/12 h light/dark cycle and allowed free access to food and water. They were anesthetized with ketamine (100 mg/kg i.m.) and medetomidine (0.25 mg/kg i.m.) injections. Butorphanol (0.1 mg/kg s.c.) was used immediately before the intervention to ensure intraoperative analgesia. Further injections of these drugs were provided throughout the intervention to maintain a sufficient state of anesthesia. Each rat was allowed to breathe spontaneously. Body temperature was monitored with a rectal probe and maintained at  $37.0 \pm 0.5$  °C with a heating blanket regulated by a homeothermic blanket control unit (Harvard Apparatus Ltd., Holliston, MA, United States).

After drug injection, eight rats were prepared for surgery through thoracic and abdominal trichotomy. These areas were then washed with povidone iodine and

alcohol. These animals were divided at random into two groups of four rats each. Group I rats underwent IMA ligation followed by macroscopic observation and histological analysis. Before and after IMA ligation, group II rats underwent 7-tesla (7T) MRI followed by histological analysis of a colon specimen after euthanasia. A healthy rat underwent an MRI bowel enema without IMA ligation to enable us to study its bowel anatomy.

### Surgical procedures

After a midline laparotomy, the small and large bowel were exposed from the abdominal cavity and displaced to the left. A photograph of the physiological appearance of the bowel and mesentery was taken with a digital camera (Panasonic Lumix DMC-TZ8, 12.0 megapixels, ISO 2000; Osaka, Japan). The IMA was identified and ligated at the origin with Prolene 7/0 and sectioned.

### Macroscopic analysis

After IMA ligation, the anesthetized rats of group I were observed for 8 h. The bowel was exposed on gauze moistened with saline to prevent excessive evaporative loss. Photographs of the exposed bowel and mesentery were taken every 10 min for the first hour and every 30 min thereafter. At the end of the observation period, all rats were euthanized with an intrapulmonary injection of Tanax (0.5 mL), and the large bowel was excised for histological analysis.

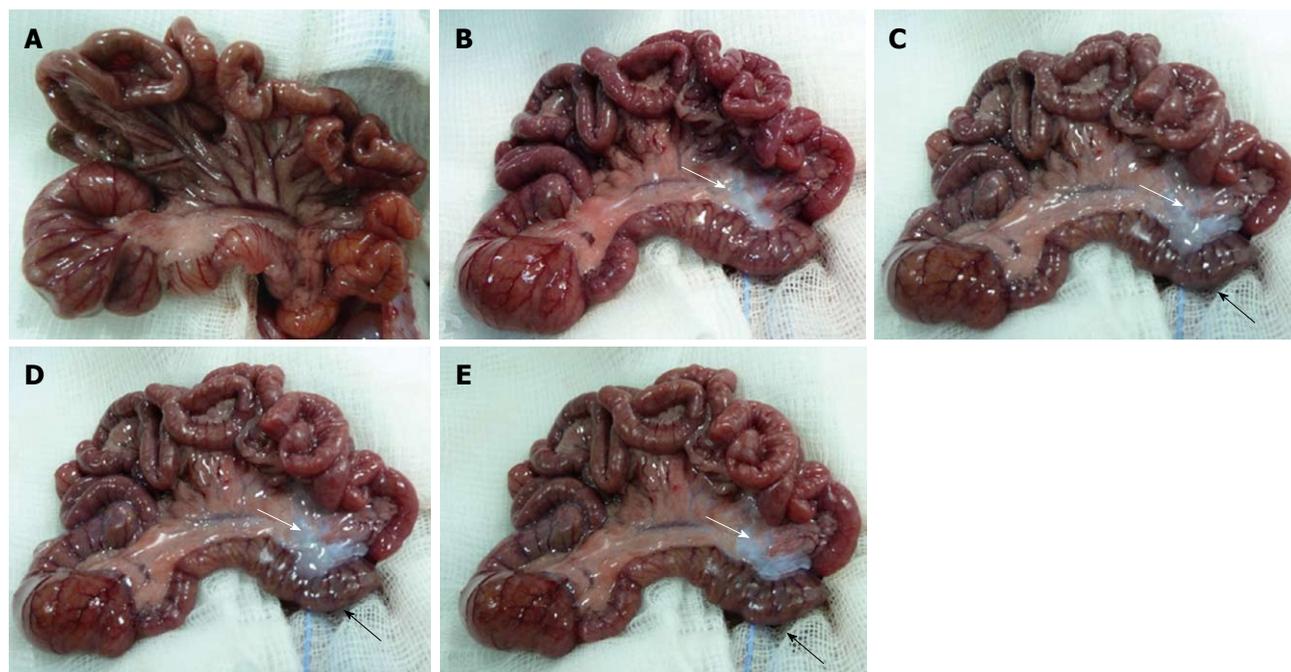
### MRI

Abdominal 7T-MRI scans were acquired in group II animals (Bruker BioSpec 70/16US; Bruker Medical Systems, Ettlingen, Germany) before IMA ligation to record the physiological appearance of the abdomen. After IMA ligation, the entire intestine was replaced in the abdomen, and the abdominal wall was closed with Vicryl 2/0 thread.

Each rat underwent 7T-MRI at 1 h, 4 h, 6 h and 8 h after IMA ligation as follows: Tripilot sequence, parameters: TR 100.0 ms; TE 6.0 ms; FOV 8.00 cm; IS 2.00 mm; N slice 3 and RareT2 sequence in axial section, parameters: TR 6060.3 ms; TE 36.0 ms; FOV 7.00 cm; matrix 256 × 256; IS 1.00/1.00 mm; N slice 52; acquisition time: 14 min, 32 s, 688 ms. To obtain bowels for histological analysis, selected rats were sacrificed at 1 h, 4 h, 6 h and 8 h after 7T-MRI.

### Histological analysis

For light microscopy, the large bowels in both groups of rats were excised from the cecum to the rectum, including the mesentery, and stored in 10% buffered formalin acetate for at least 2 d. The samples were divided into three segments: the cecum and proximal colon (first segment), the splenic flexure (second segment), and the distal colon and rectum (third segment). Sections 3 mm in size were obtained at 10-mm intervals from each segment and embedded in paraffin. Transverse sections 3 µm thick were cut and stained with hematoxylin-eosin. The sections were mounted on chrome alum/gelatin-coated slides,



**Figure 1 Macroscopic monitoring.** A: Physiological appearance of the rat bowel; B: Rat bowel 1 h after inferior mesenteric artery (IMA) ligation; C: At 4 h after IMA ligation; D: At 6 h after IMA ligation; E: At 8 h after IMA ligation.

dehydrated and coverslipped. Slides were imaged with a Zeiss Axio Skope microscope equipped with a high-resolution digital camera (ORCA-HR C4742-95-12HR, 10 MP; Hamamatsu Photonics, Hamamatsu, Japan)

### MRI bowel enema

This procedure was performed on one healthy rat to determine the large bowel anatomy and topography. Under anesthesia, a 16 G angiocatheter was inserted in the rectum and fixed to the anal skin by a purse-string suture. After the injection of 8-10 mL warm water, 7T-MRI was performed using the following protocol: Tripilot sequence, parameters: TR 100.0 ms; TE 6.0 ms; FOV 8.00 cm; IS 2.00 mm; N slice 3 and RareT2 sequence in axial section, parameters: TR 6060.3 ms; TE 36.0 ms; FOV 6.00 cm; matrix 256 × 256; IS 1.00/1.00 mm; N slice 44; acquisition time: 14 min, 32 s, 688 ms. These scans served as anatomic images of reference for comparison purposes to identify ischemic damage in group II rats.

## RESULTS

### Macroscopic evaluation

Figure 1A shows the appearance of a rat intestine immediately before IMA ligation; the colon and the ileum were of normal size and presented a uniform serous membrane and rose-colored mesentery. One hour after IMA ligation, pale areas appeared in the splenic flexure mesentery (indicated by a white arrow in Figure 1B); these areas progressively increased (white arrow) and were associated, four hours after IMA ligation, with a change in the color of the splenic flexure loop (black arrow), which appeared dark reddish blue (Figure 1C). Six

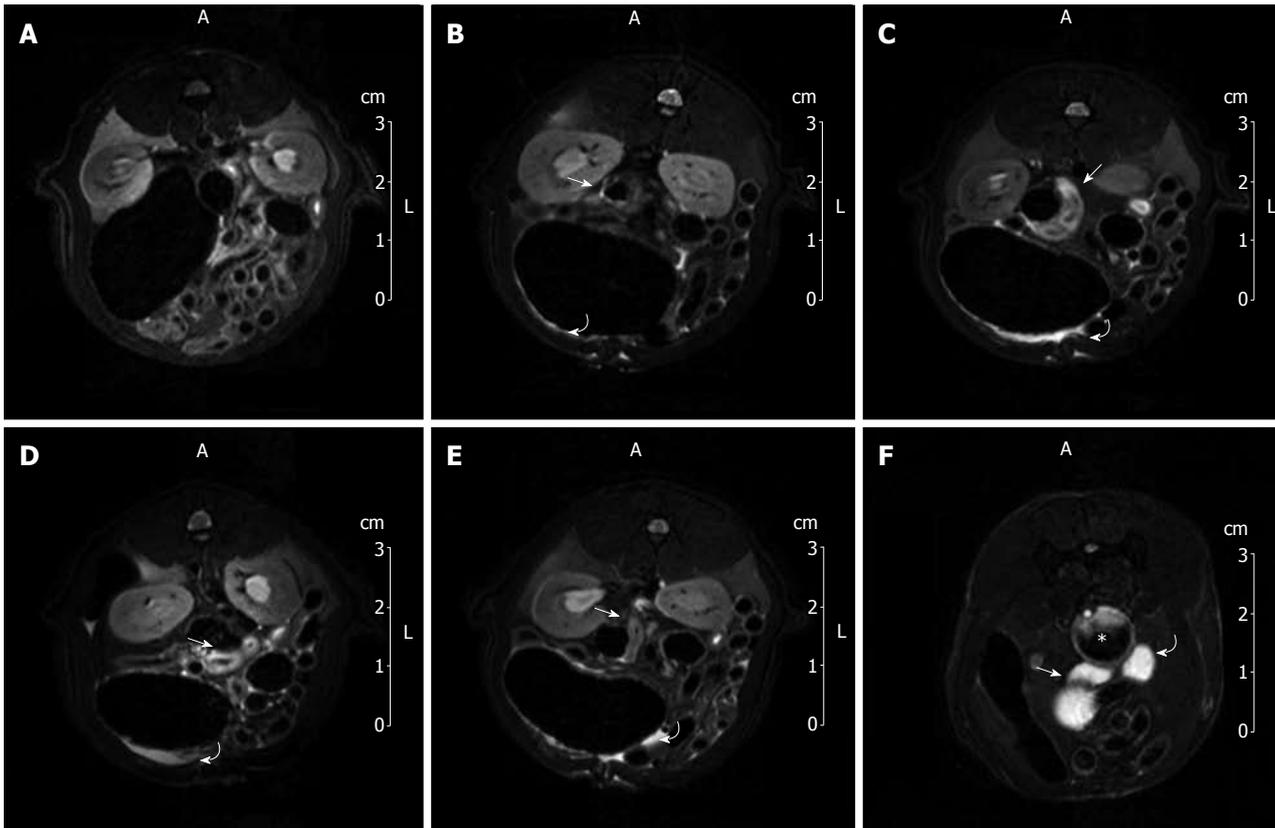
hours after IMA ligation, the splenic flexure mesentery was very pale (white arrow), and the loop was very dark (black arrow, Figure 1D). Eight hours after IMA ligation, although the loop was even darker (black arrow), the mesentery was less pale (white arrow, Figure 1E). No other macroscopic alterations were noted during the 8 h observation period, and the chronological sequence of the macroscopic morphological changes was the same in all group I animals.

### MRI

Figure 2A shows a 7T-MRI abdominal scan of a group II rat immediately before IMA ligation. No pathological findings related to ischemic damage were detected. One hour after IMA ligation, the T2 sequences showed minimal findings, namely, hyperintense signals in both the colonic loop wall (arrow) and the intraperitoneal space (curved arrow, Figure 2B). These MRI findings were more pronounced four hours after IMA ligation, suggesting colonic wall edematous thickening and a small amount of peritoneal free fluid (Figure 2C). Six hours after ligation, the amount of peritoneal free fluid increased (curved arrow), as did the colonic wall hyperintensity (arrow, Figure 2D). Eight hours after IMA ligation, MRI showed persistent colonic mural hyperintensity (arrow) associated with a reduction in the peritoneal free fluid (curved arrow), probably related to compensatory intraperitoneal drainage (Figure 2E).

### MRI colon enema

Figure 2F shows the hyperintense signals of the splenic flexure (arrow), descending colon (curved arrow) and rectum (star) distended by instilled water. Comparison



**Figure 2** 7T magnetic resonance imaging investigation. A: Image of a 7T magnetic resonance imaging (MRI) abdominal scan before inferior mesenteric artery (IMA) ligation; B: A 7T MRI abdominal scan 1 h after IMA ligation; C: At 4 h after IMA ligation; D: At 6 h after IMA ligation; E: At 8 h after IMA ligation; F: Image of 7T MRI colon enema.

of the 7T-MRI and MRI colon enema scans reveals that the splenic flexure was the colonic area most affected by ischemia.

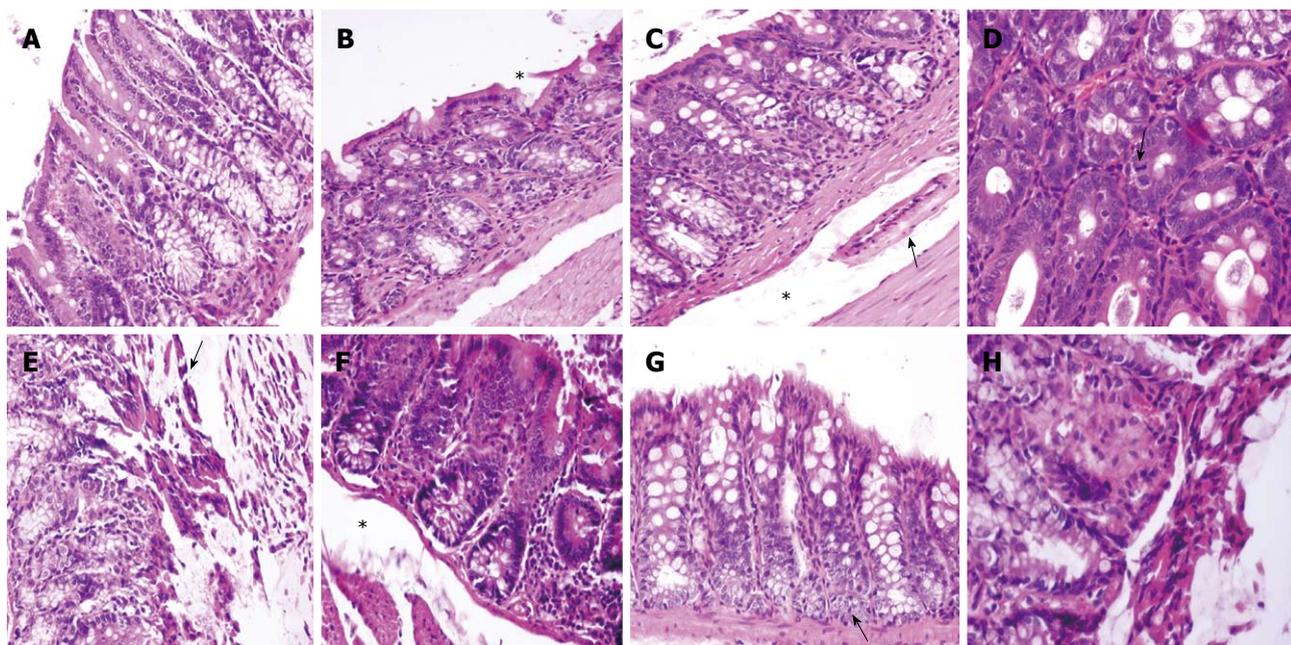
**Histological analysis**

Histology revealed anatomopathological alterations in the second segments (colonic splenic flexure), whereas the first and third segments were unaffected. The histology of the colonic sections obtained one hour after IMA ligation from the proximal colon and the splenic flexure are shown in Figure 3A and B, respectively. In contrast to the normal histological pattern of the proximal colon, the splenic flexure section showed an attenuated epithelium with glandular apex lesions (star). Four hours after IMA ligation, large ischemic lesions appeared at the splenic flexure level: superficial epithelium nuclear pyknosis and epithelium mucin depleted with submucosal edema (star) leading to vessel collapse (arrow, Figure 3C). Six hours after ligation, in the same colonic area, we found a further reduction in crypt goblet cells, clear signs of apoptosis such as nuclear pyknosis and hyper eosinophilia, and epithelial regeneration attempts, as shown by the presence of mitotic figures (arrow, Figure 3D). At the end of the observation (8 h), ischemic injury of the splenic flexure was more pronounced; necrosis and a loss of the surface epithelium, crypt drop-out (arrow) with a depletion of goblet cells (Figure 3E) and a

markedly edematous submucosa (star, Figure 3F) were observed. Signs of regeneration in response to the injury appeared at the bottom of the crypts (arrow, Figure 3G) near the images of coagulative necrosis (Figure 3H). Ischemic changes were related to vascular anatomy, and a sharp demarcation line often separated the involved from the uninvolved mucosa.

**DISCUSSION**

The availability of animal models in which to reproduce human diseases has increased our knowledge of human physiopathology and led to new diagnostic and therapeutic approaches<sup>[14]</sup>. This study, based on an animal model of colonic ischemic damage, was designed to define the evolution of histological ischemic lesions and to compare anatomopathological features with corresponding 7T-MRI findings. Thus far, the chronological sequence of early colonic ischemic damage has not been described, and the role of MRI is still widely debated. To the best of our knowledge, this is the first study using 7T-MRI for the instrumental evaluation of a human disease reproduced in a rat animal model. We decided to use T2-weighted MRI sequences because, as shown in a previous study<sup>[15]</sup>, they allow us to identify, without using contrast media, the hyperintensity of parietal edema, which is an early sign of intestinal ischemia.



**Figure 3 Histological analysis.** A: Normal histological pattern of the rat proximal colon; B: Histological pattern of the rat proximal colon 1 h after inferior mesenteric artery (IMA) ligation; C: At 4 h after IMA ligation; D: At 6 h after IMA ligation; E: At 8 h after IMA ligation; note the necrosis and loss of the surface epithelium; F: Markedly edematous submucosa (star); G: Active regeneration signs at the bottom of the crypts; H: Coagulative necrosis.

We identified anatomopathological alterations as early as one hour after IMA ligation, namely, a pale mesentery and mild surface epithelium damage at the colonic splenic flexure level. Additionally at one hour after ligation, the MRI study showed a small amount of peritoneal free fluid associated with mild edematous thickening of the colonic splenic flexure wall. Moreover, there was a relevant correlation between the MRI changes observed during the course of bowel ischemia and the pathological damage. Indeed, the morphological and MRI findings showed a progressive and parallel worsening with time up to eight hours when, despite the change in the color of the colonic loop, we observed a mild improvement in mesenteric color associated with a reduction in peritoneal free fluid. Therefore, MRI detected very early signs of colonic ischemia and allowed us to monitor the evolution of the ischemic damage. Consequently, although CT remains a valid diagnostic tool for the visualization of early signs of bowel ischemia and evaluation of colonic ischemic damage<sup>[8,9,16]</sup>, MRI, which does not require the use of a contrast medium or ionizing radiation, can play an important role in the diagnostic and therapeutic management of patients with acute colitis ischemia.

We performed MRI colon enema in a surgically untreated rat to define the large bowel anatomy and topography. We were thus able to identify the specific colonic hyperintensity area detected by MRI after IMA ligation as the colonic splenic flexure. In agreement with a previous study, although the superior mesenteric artery is more important than the IMA in maintaining the cecum and transverse colon perfusion, ligation of the IMA alone produced significant injury in a single, specific colonic area<sup>[10]</sup>. The major vulnerability of the splenic flexure to

ischemic damage may be related to the presence of more limited collateral networks in this region, apparently representing a “watershed” area where the two circulations meet<sup>[2]</sup>. In this context, the amount of ischemic injury depends on the high variability of the vascular anatomy<sup>[7]</sup>.

The rat model used in this study had several limitations: vascular occlusion was sudden and total, whereas partial occlusion is possible in humans; IMA ligation was performed at the emergence of the vessel, whereas in clinical practice, distal occlusions are also observed; the time window analysis was limited to 8 h, but these patients often reach the hospital a few days after the original occlusive event. However, despite the limitations of the animal model, this study shows that MRI is useful in the diagnosis of acute IC. Moreover, although our MRI scans were performed with a 7T-MRI machine, which is not yet available in clinical practice, both our results and those of previous studies suggest that 7T MRI machines are appropriate for clinical research on humans<sup>[17,18]</sup>.

In conclusion, the assumption of parallels between the experimental colonic ischemic damage in this animal model and humans is reasonable. Our results indicate that MRI allows for the identification of pathological findings of acute IC and their correlation with histopathological features. Therefore, MRI can play a relevant role in the diagnostic management of acute IC and may be substituted for other invasive surgical and endoscopic procedures in diagnosing and grading IC when ischemic injury is suggested. The possibility of assessing the evolution of IC over time and correlating the histological alterations with imaging patterns could result in a more accurate and earlier identification of imaging diagnostic features and thus facilitate more effective treatment.

**COMMENTS**

**Background**

Ischemic colitis (IC) is a relatively common disease, being the most frequent form of intestinal ischemia and the second-most frequent cause of lower gastrointestinal bleeding. Presentation of IC is not specific and is highly variable, therefore, the diagnosis largely depends on clinical suspicion. In this context, the role of imaging techniques remains controversial.

**Research frontiers**

The role of magnetic resonance imaging (MRI) in the diagnostic management of acute IC is still a matter of debate, and nothing is known about the histological evolution of the acute colonic ischemic injury or the relationship between these lesions and MRI findings.

**Innovations and breakthroughs**

To be known, this is the first study using 7T-MRI for the instrumental evaluation of a human disease reproduced in a rat animal model. In this study, authors established the chronological evolution of the early functional and morphological responses to ischemia produced by inferior mesenteric artery (IMA) ligation and compared their anatomopathological features with the corresponding 7T-MRI findings.

**Applications**

The possibility of detecting the early signs of colonic ischemic injury with MRI suggests that this technique can play an important role in diagnosing and grading acute IC.

**Terminology**

7T-MRI: A non-invasive method of demonstrating internal anatomy based on the principle that atomic nuclei in a strong magnetic field (7 Tesla) absorb pulses of radiofrequency energy and emit them as radio waves that can be reconstructed into computerized images. The concept includes proton-spin tomographic techniques; IC: Inflammation of the colon due to colonic ischemia resulting from alterations in systemic circulation or local vasculature.

**Peer review**

This is a good demonstration of the potential use of T2-weighted MRI for detecting edema in IC. In an experimental model, results of MRI are compared with physiological appearance and histology of the bowel at various times after IMA ligation.

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## Diagnostic yield of small bowel capsule endoscopy depends on the small bowel transit time

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### Abstract

**AIM:** To investigate whether the small bowel transit time (SBTT) influences the diagnostic yield of capsule endoscopy (CE).

**METHODS:** Six hundred and ninety-one consecutive CE procedures collected in a database were analyzed. SBTT and CE findings were recorded. A running mean for the SBTT was calculated and correlated to the diagnostic yield with a Spearman's correlation test. Subgroup analyses were performed for the various indications for the procedure.

**RESULTS:** There was a positive correlation between the diagnostic yield and SBTT (Spearman's rho 0.58,  $P < 0.01$ ). Positive correlations between diagnostic yield and SBTT were found for the indication obscure gastrointestinal bleeding ( $r = 0.54$ ,  $P < 0.01$ ), for polyposis and carcinoid combined ( $r = 0.56$ ,  $P < 0.01$ ) and for the other indications ( $r = 0.90$ ,  $P < 0.01$ ), but not for

suspected Crohn's disease ( $r = -0.40$ ).

**CONCLUSION:** The diagnostic yield in small bowel capsule endoscopy is positively correlated with the small bowel transit time. This is true for all indications except for suspected Crohn's disease.

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**Key words:** Capsule endoscopy; Small bowel transit time; Diagnostic yield

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Westerhof J, Koornstra JJ, Hoedemaker RA, Sluiter WJ, Kleibeuker JH, Weersma RK. Diagnostic yield of small bowel capsule endoscopy depends on the small bowel transit time. *World J Gastroenterol* 2012; 18(13): 1502-1507 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i13/1502.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i13.1502>

### INTRODUCTION

Capsule endoscopy (CE) is a very sensitive diagnostic technique to detect small bowel pathology. It has a higher diagnostic yield than conventional diagnostic methods, i.e., push enteroscopy, small-bowel follow-through, conventional CT and angiography<sup>[1]</sup>. The reported diagnostic yield of CE varies between 38% and 83%<sup>[2-11]</sup>. In 15%-20% of all CE's the capsule does not reach the cecum within recording time. Risk factors for incomplete CE procedures include previous small-bowel surgery, hospitalization, moderate or poor bowel cleansing, and a gastric transit time longer than 45 min<sup>[12]</sup>.

For a good and complete evaluation of the small

bowel, the capsule should reach the cecum within recording time, which is eight to eleven hours depending on the manufacturer. Therefore, some investigators use a prokinetic agent to speed up the gastric and small bowel transit. However, the short bowel transit time (SBTT) may influence the diagnostic yield of CE. With colonoscopy, the detection rate of neoplastic lesions is higher when the time to withdraw the colonoscope is longer<sup>[13-15]</sup>. It is conceivable that a similar principle also applies for small bowel CE. We therefore hypothesize that the diagnostic yield of CE depends on the small bowel transit time. To study this, we analyzed the influence of small bowel transit time on the diagnostic yield of CE in 691 consecutive procedures performed in our department.

## MATERIALS AND METHODS

Data from all consecutive CE studies performed at the University Medical Center Groningen, the Netherlands, between September 2003 and January 2009 were collected. Data that were collected included patient demographics, indications for the procedure, procedural data, including gastric transit time (GTT) and SBTT, and findings of the procedure. The GTT was defined as the time, in minutes, from the first image of the stomach until the first image of the duodenum. The SBTT was defined as the passage time, in minutes, from the first image of the duodenum until the first image of the cecum. If the capsule did not reach the cecum within recording time, the SBTT was recorded as the time during which small bowel images were captured. CE was considered complete when the cecum was reached within recording time.

### CE procedure

During the study period, all patients received bowel preparation. Patients were given standardized instructions before the procedure, and informed consent was obtained. The patients were asked to stop iron supplements seven days before CE and to use a low-fiber diet three days before CE. The patients started a fasting period at midnight before the procedure. Bowel preparation consisted of the ingestion of four liters of a polyethylene glycol solution (Colofort<sup>®</sup>), 3 L the evening before the procedure and 1 L in the morning. The capsule (Pillcam; Given Imaging Ltd, Yoqneam, Israel) was swallowed in the morning. The patients were allowed to drink fluids after three hours and to consume a light meal after five hours. Before capsule ingestion, 10 mL of antifoam and a prokinetic agent was given, 10 mg of domperidone (before July 1st 2008,  $n = 641$ ) or 250 mg of erythromycin (after July 1st 2008,  $n = 50$ ). All CE recordings were reviewed by two gastroenterologists, experienced with CE (RKW and JJK). Controversial findings were discussed, and consensus was reached upon the final diagnosis. The most relevant findings obtained from CE were documented and categorized according to standard terminology<sup>[16]</sup> as angiectasia(s); ulcer(s); bleeding of unknown origin; erosion (s); polyp(s)/tumor(s); incidental abnormality of

esophagus, stomach, or colon; no abnormality; or unable to make a diagnosis.

### Statistical analysis

The SBTT was not normally distributed (tested with a Kolmogorov-Smirnov test) in the study population. To demonstrate the correlation between average diagnostic yield and average SBTT, the average yield was calculated of 50 consecutive transit times and plotted. Diagnostic yield was expressed as 0 for absence of abnormalities and 1 for presence of abnormalities. In this way, a running mean for the SBTT was calculated for 50 consecutive patients and correlated to the diagnostic yield with a Spearman's correlation test. A rho's correlation coefficient was calculated. Comparison of SBTT between groups was performed using a Mann-Whitney *U* test.

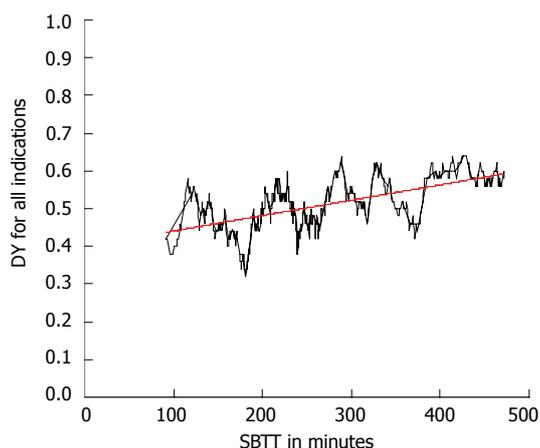
Subgroup analyses were performed for the various indications for the procedure. *P*-values below 0.05 were considered significant. SPSS 14.0 for Windows software (SPSS Inc., Chicago, IL, United States) and Microsoft Office Excel 2003 were used for statistical analyses.

## RESULTS

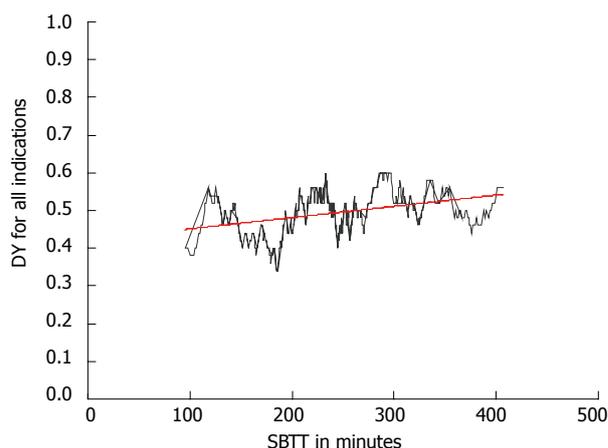
Six hundred and ninety-one consecutive CE procedures were analyzed. The mean age of the patients was 54 years (range 9-93, SD 18 years). 55% of the patients were male. Indications for CE were obscure gastrointestinal bleeding (OGIB) (67%), suspected Crohn's disease (22%), polyposis (4%), carcinoid (3%) and other (4%). CE findings were as follows in the investigated patients: angiectasia(s) in 121 cases (18%), ulcer(s) in 42 (6%), erosion(s) in 83 (12%), bleeding of unknown origin in 30 (4%), polyp(s)/tumor(s) in 56 (8%), abnormality of esophagus, stomach, or colon in 15 (2%), stenosis in 2 (0.3 %), unable to make a diagnosis in 6 (1%) and no abnormalities in 336 cases (48%). Overall, the diagnostic yield was 51%.

The cecum was reached in 82% of all procedures. The overall median small bowel transit time was 246 min (25 and 75 percentiles: 190 and 342). In CE cases with positive findings, the median SBTT was 254 min (25 and 75 percentiles: 200 and 361), in negative CE procedures, the median SBTT was 239 (25 and 75 percentiles 178 and 320), this difference was significant ( $P = 0.012$ ). There was a positive correlation between the diagnostic yield and SBTT (Figure 1) indicating that the longer the SBTT, the higher the diagnostic yield (Spearman's rho 0.58,  $P < 0.01$ ).

Next, patients were excluded in whom the cecum was not reached ( $n = 125$ ) within recording time, leaving 566 procedures with complete visualization of the small intestine. The overall median SBTT was 233 min (25 and 75 percentiles: 178 and 295). In cases with positive findings, the median SBTT was 236 (25 and 75 percentiles: 186 and 300), in negative CE procedures the median SBTT was 229 min (25 and 75 percentiles: 121 and 281), this difference was not significant ( $P = 0.078$ ). A positive correlation was again observed between the diagnostic yield and SBTT (Spearman's rho 0.40,  $P < 0.01$ , Figure 2).



**Figure 1** Correlation between the small bowel transit time in minutes and the diagnostic yield for all patients. Spearman's rho coefficient 0.58 ( $P < 0.01$ ) shown by the black line. The trend of this correlation is shown by the red line. DY: Diagnostic yield; SBTT: Small bowel transit time.



**Figure 2** Correlation between the small bowel transit time in minutes and the diagnostic yield in patient with complete capsule endoscopy. Spearman's rho coefficient 0.40 ( $P < 0.01$ ) shown by the black line. The trend of this correlation is shown by the red line. DY: Diagnostic yield; SBTT: Small bowel transit time.

Subgroup analysis for the different indications was performed for OGIB, suspected Crohn's disease, polyposis and carcinoid combined and other indications. The indications polyposis and carcinoid were taken together because both groups were too small for separate subgroup analysis. For these indications, positive correlations between diagnostic yield and SBTT were found for OGIB ( $r = 0.54$ ,  $P < 0.01$ ), for polyposis plus carcinoid ( $r = 0.56$ ,  $P < 0.01$ ) and for the other indications ( $r = 0.90$ ,  $P < 0.01$ ). However this was not observed for Crohn's disease ( $r = -0.40$ ). These results are depicted in Figure 3.

## DISCUSSION

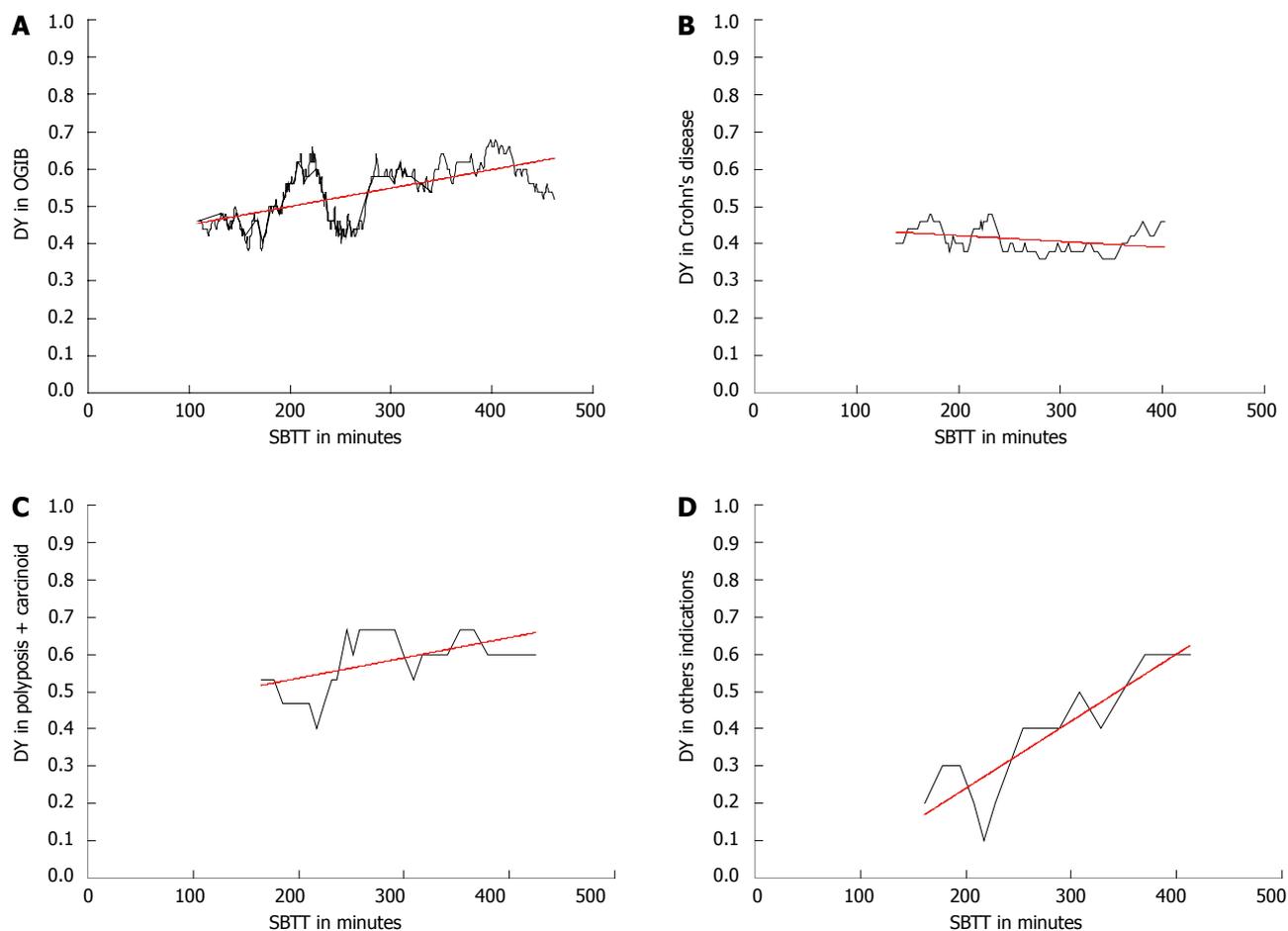
In this study, we found a positive correlation between the diagnostic yield of CE and small bowel transit time, irrespective of whether the capsule had reached the cecum within recording time. These findings are in accordance with those from colonoscopy studies, which show higher diagnostic yields for detecting neoplastic lesions when the withdrawal time during colonoscopy is longer<sup>[10-13]</sup> and from one previous study on the effect of SBTT on the diagnostic yield of CE<sup>[17]</sup>. Most of these colonoscopy studies divided the withdrawal time into more or less than a chosen number of minutes. In CE there are no known standard SBTT times, so we judged that it would not be correct to randomly divide the SBTT in two or more randomly chosen groups. Therefore we calculated a running mean to determine whether the diagnostic yield correlated with the SBTT. We found a positive correlation between the two, meaning that a longer transit time, implicating more images of the small bowel, resulted in a higher diagnostic yield.

For colonoscopy, the correlation between diagnostic yield and withdrawal time was only investigated in subjects undergoing screening for neoplastic lesions. In this study we looked at all indications for CE. We found a positive correlation between the diagnostic yield and

SBTT for the indications OGIB and polyposis/carcinoid and for other indications, but not for the indication suspected Crohn's disease. The latter is probably due to the multiple and widespread small bowel lesions usually seen in Crohn's disease. Therefore the endoscopist may be less dependent upon the mucosal inspection time to make the diagnosis. Furthermore, a previous study showed reduced capsule transit times in Crohn's disease<sup>[18]</sup>.

What does this positive correlation between diagnostic yield and SBTT mean for clinical practice? CE is less valuable when the cecum is not reached within recording time, but on the other hand our study indicates that the diagnostic yield is lower when the SBTT is shorter. So, ideally, the SBTT should be as long as possible, yet the capsule should reach the cecum within recording time. The development of capsule systems with longer battery times may be helpful. One important issue in this matter is whether there is a role for prokinetic agents in CE. In this way, one could influence the small bowel transit time. There is no consensus on this subject<sup>[1]</sup>. In most of the available studies, there are no data on the influence of prokinetics on the diagnostic yield of CE. Taking our data into account, it may not be wise to use prokinetics that speed up the SBTT. However this must be weighed against the fact that a prolonged GTT is a risk factor for incomplete CE<sup>[12]</sup>. It may therefore be useful to use an agent which shortens GTT without influencing SBTT.

A well known prokinetic agent is erythromycin. It induces high amplitude gastric propulsive contractions by activating gastric interdigestive migrating motor complexes, thereby accelerating gastric emptying<sup>[19-22]</sup>. The effects of erythromycin on SBTT are unclear. In the most recent publication on this subject, erythromycin reduced the GTT but had no significant effect on SBTT, total bowel transit time and CE completing rates<sup>[22]</sup>. Previous studies found similar results<sup>[23,24]</sup>, but in one at the cost of visibility<sup>[24]</sup>. Others found no effect of erythromycin on either GTT or SBTT<sup>[25]</sup>. Overall, the data are not very ro-



**Figure 3 Correlations between small bowel transit time and diagnostic yield.** A: Obscure gastrointestinal bleeding (OGIB;  $r = 0.54$ ,  $P < 0.01$ ); B: Suspected Crohn's disease ( $r = -0.40$ ); C: Polyposis plus carcinoid ( $r = 0.56$ ,  $P < 0.01$ ); D: Other indications ( $r = 0.90$ ,  $P < 0.01$ ), shown by the black line. The trend of this correlation is shown by the red line. DY: Diagnostic yield; SBTT: Small bowel transit time.

bust. If erythromycin mainly influences GTT, it might be an interesting prokinetic agent to use prior to small bowel CE.

Other prokinetics that have been studied in CE are metoclopramide and mosapride. Both prokinetic agents accelerated GTT and increased capsule completion rates, but had no influence on SBTT<sup>[26,27]</sup>. In our study, we used domperidone as a prokinetic agent in the majority of patients. Domperidone has shown to be effective in treating diabetic gastroparesis<sup>[28]</sup>, but there are no data on the use of this agent in CE.

Another way to use prokinetics may be with the aid of a real-time viewer system. In this way, prokinetics (or water or additional PEG) can be administered when the real-time viewer shows delayed gastric emptying. There are three studies that show a higher diagnostic yield of CE when a real-time viewer is used with on-demand administration of prokinetics, water, PEG or endoscopic-assisted duodenal placement<sup>[29-31]</sup>.

The strength of this study is that this is the first study that investigated the relation between diagnostic yield of CE and SBTT in a large study population. This allowed for a subgroup analysis for the different indications of CE. Another strong point of our study in our view is the

use of an appropriate statistical method for determining the relation between diagnostic yield and SBTT by using a running mean.

A limitation of this study is that all patients in our population received a prokinetic agent, which might have changed the SBTT and with that also the diagnostic yield. The diagnostic yield might have been higher in this study population when we would not have used a prokinetic agent. Another limitation of this study is that during this study period we switched our prokinetic agent from domperidone to erythromycin. One should realize that this is a retrospective study. Prospective studies are necessary to establish the effect of the use of prokinetics on SBTT and diagnostic yield.

There may be many factors that influence SBTT and thereby diagnostic yield in small bowel CE. In this study we did not analyze such other factors, mainly because the main goal of this study was to determine whether there was a relation between diagnostic yield and SBTT at all. A previous study on the effect of SBTT on diagnostic yield found an independent association between diagnostic yield and SBTT<sup>[17]</sup>. In that study, no relation was found between diagnostic yield and other potential risk factors such as age, gender, study indication, hospital status, and

quality of bowel preparation<sup>[17]</sup>. Since we also found a positive correlation between diagnostic yield and SBTT it might be very interesting to look further into factors influencing SBTT and thereby diagnostic yield in future studies.

In conclusion, in this study with a large group of patients, we found a positive correlation between the diagnostic yield of small bowel CE and small bowel transit time for all indications except for suspected Crohn's disease. For clinical practice, these data implicate that it may not be advisable to use prokinetic agents which accelerate small bowel transit although this remains to be proven in future studies.

## COMMENTS

### Background

Capsule endoscopy (CE) is a very sensitive diagnostic technique to detect small bowel pathology. For a good and complete evaluation of the small bowel, the capsule should reach the cecum within recording time which is eight to eleven hours depending on the manufacturer. Some investigators advocate the use of a prokinetic agent to speed up the gastric and small bowel transit. However, a short bowel transit time (SBTT) may influence the diagnostic yield of CE. With colonoscopy, the detection rate of neoplastic lesions is higher when the time to withdraw the colonoscope is longer. It is conceivable that a similar principle also applies for small bowel CE. Therefore the question is whether the diagnostic yield of CE depends on the small bowel transit time.

### Research frontiers

This the first study that investigated the relation between diagnostic yield of CE and SBTT in a large study population. This possible relation was also investigated for different indications for CE.

### Innovations and breakthroughs

Six hundred and ninety-one consecutive CE procedures were analyzed. This study found a positive correlation between the diagnostic yield of CE and small bowel transit time, irrespective of whether the capsule had reached the cecum within recording time. These findings are in accordance with those from colonoscopy studies. This means that a longer transit time, implicating more images of the small bowel, resulted in a higher diagnostic yield. We found a positive correlation between the diagnostic yield and SBTT for all indications except for suspected Crohn's disease. The latter is probably due to the multiple and widespread small bowel lesions usually seen in Crohn's disease.

### Applications

What does this positive correlation between diagnostic yield and SBTT mean for clinical practice? CE is less valuable when the cecum is not reached within recording time, but on the other hand our study indicates that the diagnostic yield is lower when the SBTT is shorter. So, ideally, the SBTT is long, but not so long that the capsule does not reach the cecum within recording time. One important issue in this matter is whether there is a role for prokinetic agents in CE to influence small bowel transit time. There is no consensus in the literature on this subject. For clinical practice, these data implicate that it may not be advisable to use prokinetic agents which accelerate small bowel transit, although this remains to be proven in future studies.

### Peer review

It is a nice and interesting work. It values an important aspect of the capsule endoscopy not well studied until now: the relation between the diagnostic yield of capsule endoscopy and small bowel transit time.

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## Identification of individuals with non-alcoholic fatty liver disease by the diagnostic criteria for the metabolic syndrome

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### Abstract

**AIM:** To clarify the efficiency of the criterion of metabolic syndrome to detecting non-alcoholic fatty liver disease (NAFLD).

**METHODS:** Authors performed a cross-sectional study involving participants of a medical health checkup pro-

gram including abdominal ultrasonography. This study involved 11 714 apparently healthy Japanese men and women, 18 to 83 years of age. NAFLD was defined by abdominal ultrasonography without an alcohol intake of more than 20 g/d, known liver disease, or current use of medication. The revised criteria of the National Cholesterol Education Program Adult Treatment Panel III were used to characterize the metabolic syndrome.

**RESULTS:** NAFLD was detected in 32.2% (95% CI: 31.0%-33.5%) of men ( $n = 1874$  of 5811) and in 8.7% (95% CI: 8.0%-9.5%) of women ( $n = 514$  of 5903). Among obese people, the prevalence of NAFLD was as high as 67.3% (95% CI: 64.8%-69.7%) in men and 45.8% (95% CI: 41.7%-50.0%) in women. Although NAFLD was thought of as being the liver phenotype of metabolic syndrome, the prevalence of the metabolic syndrome among subjects with NAFLD was low both in men and women. 66.8% of men and 70.4% of women with NAFLD were not diagnosed with the metabolic syndrome. 48.2% of men with NAFLD and 49.8% of women with NAFLD weren't overweight [body mass index (BMI)  $\geq 25$  kg/m<sup>2</sup>]. In the same way, 68.6% of men with NAFLD and 37.9% of women with NAFLD weren't satisfied with abdominal classification ( $\geq 90$  cm for men and  $\geq 80$  cm for women). Next, authors defined it as positive at screening for NAFLD when participants satisfied at least one criterion of metabolic syndrome. The sensitivity of the definition "at least 1 criterion" was as good as 84.8% in men and 86.6% in women. Separating subjects by BMI, the sensitivity was higher in obese men and women than in non-obese men and women (92.3% vs 76.8% in men, 96.1% vs 77.0% in women, respectively).

**CONCLUSION:** Authors could determine NAFLD effectively in epidemiological study by modifying the usage of the criteria for metabolic syndrome.

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**Key words:** Nonalcoholic fatty liver; Metabolic syndrome; Population based study; Methodology

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common clinical condition with histological features that resemble those of alcohol-induced liver injury, but occurs in patients who do not drink an excessive amount of alcohol (ethanol > 20 g/d)<sup>[1,2]</sup>. This disease is often associated with obesity<sup>[3]</sup>, type 2 diabetes mellitus<sup>[4,5]</sup>, dyslipidemia<sup>[6]</sup>, and hypertension<sup>[7]</sup>. Each of these abnormalities carries a cardiovascular disease risk, and together they are often categorized as the insulin resistance syndrome or the metabolic syndrome<sup>[8-15]</sup>.

NAFLD is now considered to be the hepatic representation of the metabolic syndrome<sup>[10-15]</sup>.

Conventional radiology studies used in the diagnosis of fatty liver include ultrasound (US), computed tomography, and magnetic resonance (MR) imaging. Other than these radiological studies, we have no sensitive and low invasive screening method for NAFLD. Alanine aminotransferase (ALT) > 30 IU/L was usually used as the cut off level of screening NAFLD<sup>[16,17]</sup>. This threshold had a sensitivity of 0.92 for detecting the fatty-fibrotic pattern proven by ultrasound among obese children<sup>[18]</sup>. However, ALT was within normal levels in 69% of those who had increased liver fat<sup>[19]</sup>. Similarly, in the Dallas Heart Study, 79% of the subjects with a fatty liver (liver fat content > 5.6%) had normal serum ALT<sup>[20]</sup>. This implies that a normal ALT does not exclude steatosis. Aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT) also correlate with liver fat content independent of obesity<sup>[21]</sup>, but are even less sensitive than serum ALT.

It was well known that NAFLD was associated with the metabolic syndrome and patients with NAFLD tend to be accompanied with the abnormal component of the metabolic syndrome. However, the efficiency of the criterion of metabolic syndrome for detecting NAFLD has not yet been clarified. We aimed to clarify the efficiency and perform a cross sectional study among apparent healthy Japanese.

## MATERIALS AND METHODS

### Study design

We performed a cross-sectional study involving partici-

pants of a medical health checkup program including abdominal ultrasonography. The program was conducted in the Medical Health Checkup Center at Murakami Memorial Hospital, Gifu, Japan. The purpose of the medical health checkup program is to promote public health through early detection of chronic diseases and the evaluation of their underlying risk factors. Known as a “human dock”, medical services of this kind are very popular in Japan.

### Study population

All the subjects participating in such health checkup programs at Murakami Memorial Hospital between January 2004 and December 2008 were invited to join this study. The study was approved by the ethics committee of Murakami Memorial Hospital.

Data collection and exclusion criteria were described previously<sup>[8]</sup>. In short, we collected the data from urinalysis, blood cell counts, blood chemistry and abdominal ultrasonography. The medical history and lifestyle factors were collected by using a self-administered questionnaire. Exclusion criteria were an alcohol intake of more than 20 g/d, known liver disease, or current use of medication which could influence the metabolic syndrome such as anti-diabetic drugs, anti-hypertensive drugs, anti-dyslipidemic drugs, anti-gout drugs, and/or anti-obesity drugs<sup>[8,10]</sup>.

According to the revised National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III)<sup>[22]</sup> or the new International Diabetes Federation (IDF) definition<sup>[23]</sup>, subjects who had three or more of the following criteria were diagnosed as having the metabolic syndrome. Fatty liver was defined on the basis of ultrasonographic findings<sup>[24]</sup>. Of 4 known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring), the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of fatty liver<sup>[24]</sup>.

During study period, we invited 20 012 participants in the health checkup program to enroll in the study. Of those, a total of 17 262 Japanese participants (10 329 men and 6933 women) were enrolled after giving informed consent to be included in the study. We excluded 621 participants (420 men and 201 women) who had known liver disease. In addition, 3330 participants (3042 men and 288 women) who consumed more than 20 g of ethanol per day and 1579 participants (1056 men and 541 women) who were currently receiving medication were excluded. As a result, this study ultimately consisted of 11 714 participants (5811 men and 5903 women). The mean  $\pm$  SD age was  $45.5 \pm 9.4$  years (range: 18 years to 83 years) for men and  $44.3 \pm 9.3$  years (range: 18 years to 79 years) for women, respectively. The mean body mass index (BMI) was  $23.2 \pm 3.1$  kg/m<sup>2</sup> (range: 14.3 to 41.0 kg/m<sup>2</sup>) in men and  $21.1 \pm 3.0$  kg/m<sup>2</sup> (range: 14.0 to 58.3 kg/m<sup>2</sup>) in women, respectively. The mean abdominal circumference was  $81.2 \pm 8.1$  cm (range: 57.3 cm to 127.5 cm) in men and  $71.4 \pm 8.2$  cm (range: 49.0 cm to 145.0 cm) in women, respectively.

**Table 1** The basic characteristics of the study population and the association of nonalcoholic fatty liver disease with gender difference

Men	Total <i>n</i> (%)	Obese <i>n</i> (%)	Non-obese <i>n</i> (%)
Number	5811	1441	4370
NAFLD	1874 (32.2)	970 (67.3)	904 (20.7%)
5 criteria of the metabolic syndrome			
Increased abdominal circumference	791 (13.6)	703 (48.8)	88 (2)
Elevated fasting glucose level	1967 (33.8)	704 (48.9)	1263 (28.9)
Elevated blood pressure	1294 (22.3)	575 (39.9)	719 (16.5)
Decreased HDL cholesterol level	1736 (29.9)	654 (45.4)	1082 (24.8)
Elevated triglyceride level	1063 (18.3)	484 (33.6)	579 (13.2)
ALT > 30	1269 (21.8)	670 (46.5)	599 (13.7)
MS defined by rNCEP-ATP III	873 (15)	578 (40.1)	295 (6.8)
MS defined by IDF	479 (8.2)	443 (30.7)	36 (0.8)
At least 1 criterion	3680 (63.3)	1291 (89.6)	2389 (54.7)
At least 2 criteria	1955 (33.6)	957 (66.4)	998 (22.8)
At least 1 criterion or ALT > 30 IU/L	3885 (66.9)	1337 (92.8)	2548 (58.3)
Women			
Number	5903	563	5340
NAFLD	514 (8.7)	258 (45.8)	256 (4.8)
5 criteria of the metabolic syndrome			
Increased abdominal circumference	878 (14.9)	430 (76.4)	448 (8.4)
Elevated fasting glucose level	679 (11.5)	176 (31.3)	503 (9.4)
Elevated blood pressure	578 (9.8)	185 (32.9)	393 (7.4)
Decreased HDL cholesterol level	1320 (22.4)	265 (47.1)	1055 (19.8)
Elevated triglyceride level	195 (3.3)	73 (13)	122 (2.3)
Elevated ALT (ALT > 30 IU/L)	200 (3.4)	78 (13.9)	122 (2.3)
MS defined by rNCEP-ATP III	300 (5.1)	174 (30.9)	126 (2.4)
MS defined by IDF	254 (4.3)	162 (28.8)	92 (1.7)
At least 1 criterion	2374 (40.2)	511 (90.8)	1863 (34.9)
At least 2 criteria	853 (14.5)	355 (63.1)	498 (9.3)
At least 1 criterion or elevated ALT	2430 (41.2)	515 (91.5)	1915 (35.9)

NAFLD: Nonalcoholic fatty liver disease; US: Abdominal ultrasonography; BMI: Body mass index; HDL: High density lipoprotein; MS: Metabolic syndrome; rNCEP-ATP III: Revised National Cholesterol Education Program Adult Treatment Panel III definition; IDF: International diabetes federation definition; ALT: Alanine aminotransferase.

### Statistical analysis

The R version 2.9.0 (available from <http://www.r-project.org/>) was used for statistical analyses. Two groups of subjects were compared by using the unpaired *t*-test and the chi-square test, and a *P* < 0.05 was accepted as a significant level.

## RESULTS

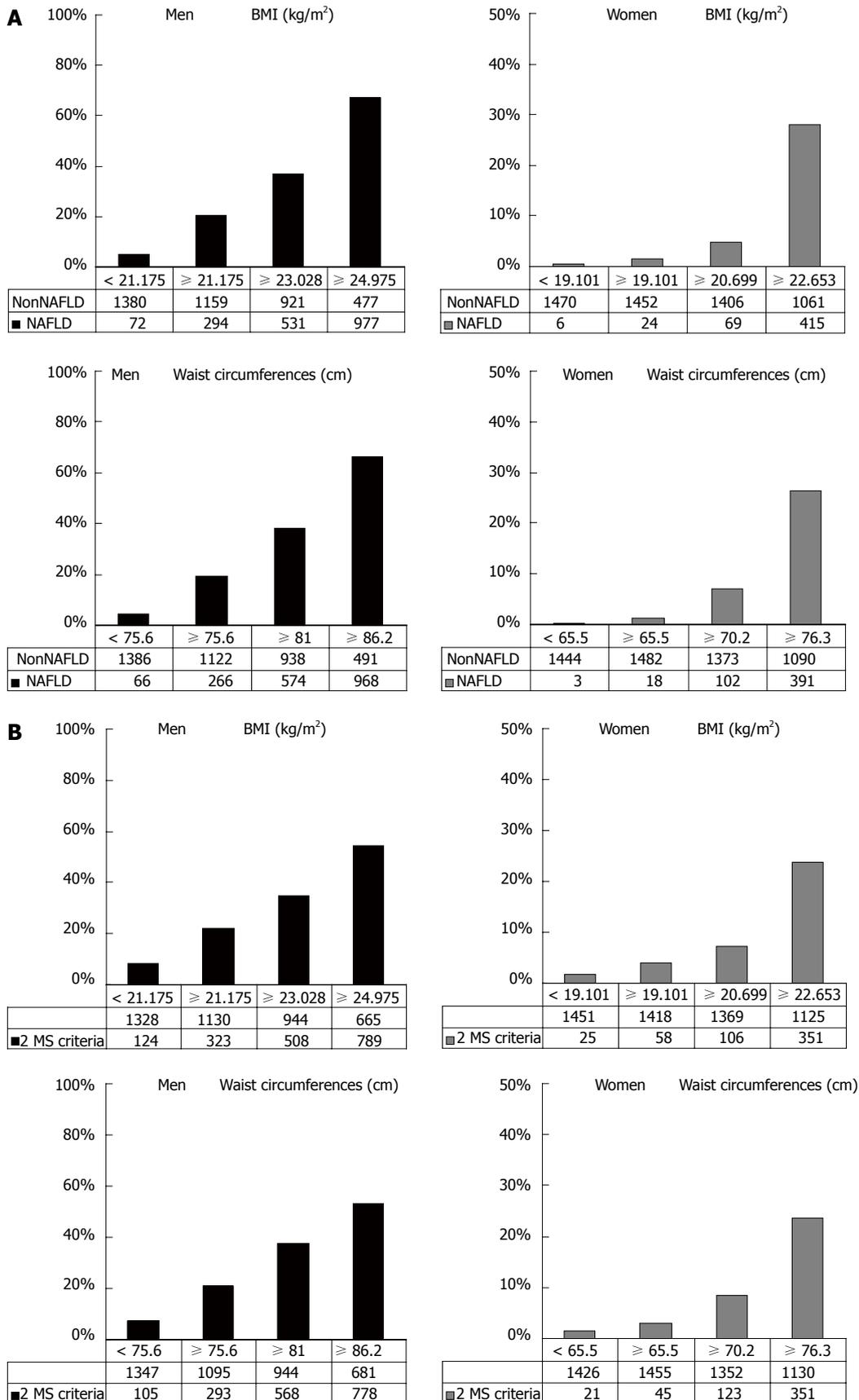
### Basic characteristics of study population

The metabolic syndrome defined by revised NCEP-ATP III definition was detected in 15.0% (95% CI: 14.1%-16.0%) of men (*n* = 873 of 5811) and in 5.1% (95% CI: 4.5%-5.7%) of women (*n* = 300 of 5903). The metabolic syndrome defined by IDF definition was detected in 8.2% (95% CI: 7.5%-9.0%) of men (*n* = 479 of 5811) and in 4.3% (95% CI: 3.8%-4.8%) of women (*n* = 254 of 5903) (Table 1). Among obese people, the metabolic syndrome defined by revised NCEP-ATP III definition was detected in 40.1% (95% CI: 37.6%-42.7%) of men and in 30.9% (95% CI: 27.1%-34.9%) of women, and the metabolic syndrome defined by IDF definition was detected in 30.7% (95% CI: 28.4%-33.2%) of men and in 28.8% (95% CI: 25.1%-32.7%) of women, respectively (Table 1).

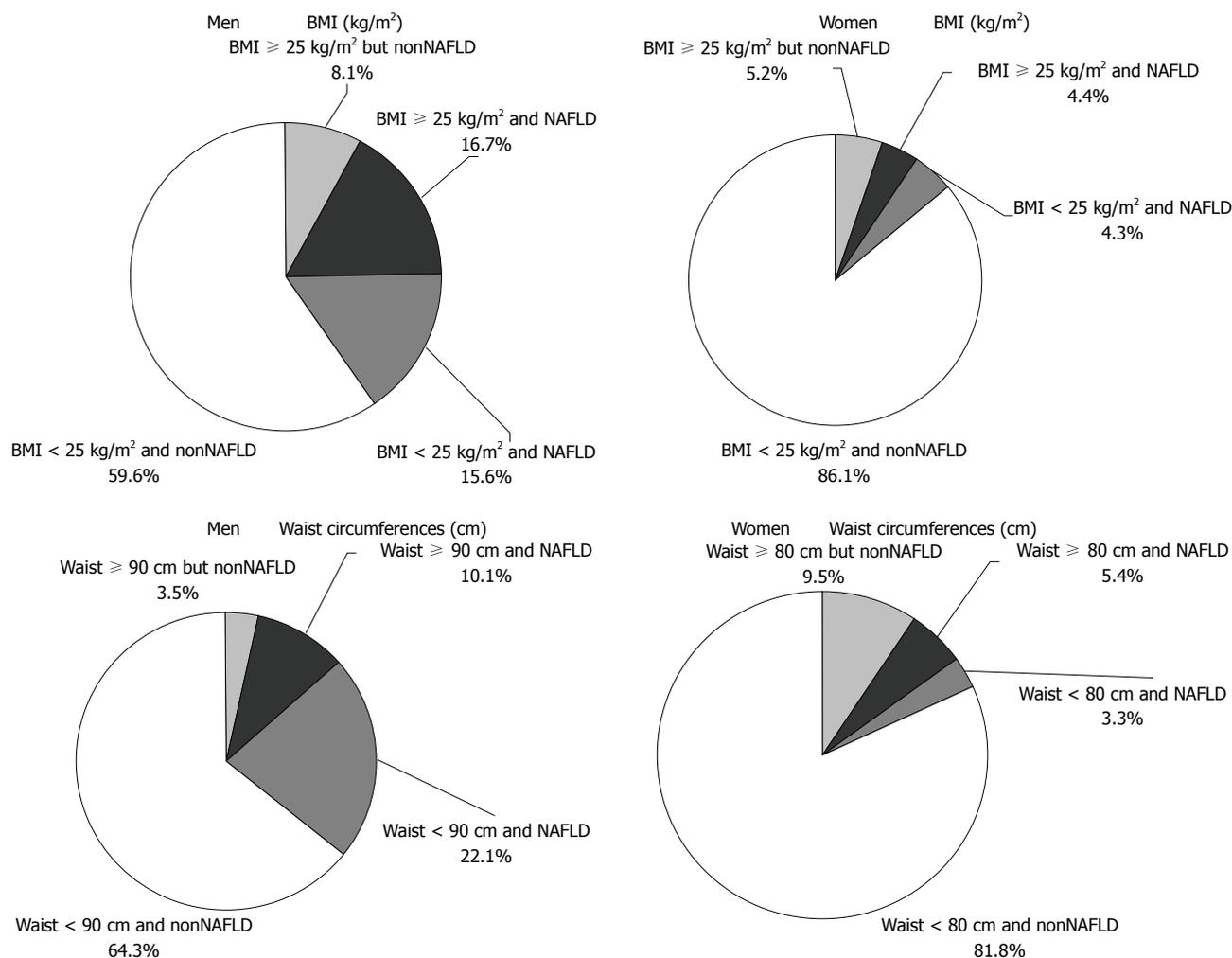
### Association of NAFLD with gender difference, or body fat accumulation

NAFLD was detected in 32.2% (95% CI: 31.0%-33.5%) of men (*n* = 1874 of 5811) and in 8.7% (95% CI: 8.0%-9.5%) of women (*n* = 514 of 5903). The prevalence of NAFLD in men was four times higher than those in women (Table 1). Among obese people, the prevalence of NAFLD was as high as 67.3% (95% CI: 64.8%-69.7%) in men and 45.8% (95% CI: 41.7%-50.0%) in women (Table 1). NAFLD was associated with body fat accumulation strongly both in men and women.

When we separated by quartile the subjects according to their BMI or abdominal circumference, half of NAFLD men and three quarters of NAFLD women were classified in the superior quartile. The prevalence of NAFLD was increased according to the increase of BMI or abdominal circumference (Figure 1A). The role of BMI for NAFLD was equal to that of abdominal circumference both in men and women. The ratio of NAFLD in the superior quartile/total NAFLD was higher in women than in men. The prevalence of individuals who met two or more of the MS criteria other than waist circumference was increased according to the increase of BMI or abdominal circumference (Figure 1B).



**Figure 1** We separated the subjects by quartile according to their body mass index or abdominal circumference. A: The bar indicated the prevalence (%) of individuals with NAFLD; B: Individuals who meet two or more of the MS criteria other than waist circumference according to BMI or waist circumference quartiles. 2 MS criteria means individuals who meets two or more of the MS criteria other than waist circumference. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; MS: Metabolic syndrome.



**Figure 2** This figure indicates the prevalence of non-alcoholic fatty liver disease and alcoholic fatty liver disease with or without patients being overweight (BMI  $\geq 25$  kg/m<sup>2</sup>) or having elevated abdominal circumferences ( $\geq 90$  cm for men and  $\geq 80$  cm for women). Data was expressed as prevalence (%). NAFLD accompanied with being overweight occurred in 51.8% of NAFLD men (970/1874) and 50.2% of NAFLD women (258/514). NAFLD accompanied by elevated abdominal circumference occurred in 31.4% of NAFLD men (588/1874) and 62.1% of women (319/514). NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

**Role of the criteria of the metabolic syndrome in detecting or diagnosing NAFLD in obese or non-obese population**

Although NAFLD was associated with obesity or body fat accumulation strongly, the population that was neither overweight (BMI  $\geq 25$  kg/m<sup>2</sup>) nor had elevated abdominal circumference was not small (Figure 2). Actually, 48.2% of men with NAFLD and 49.8% of women with NAFLD were not overweight (BMI  $\geq 25$  kg/m<sup>2</sup>). Similarly, 68.6% of men with NAFLD and 37.9% of women with NAFLD did not satisfy increased abdominal circumference classification. Half of the NAFLD group was classified as non-obese, but the prevalence of NAFLD among the non-obese population was lower. These facts means an effective method is needed to detect NAFLD among the non-obese population. Then, we separated the subjects into two groups, obese group or non-obese group, and investigated the efficacy of the criteria of metabolic syndrome for detecting NAFLD in each group.

Among the criteria for metabolic syndrome, the cri-

terion of abdominal circumferences ( $\geq 80$  cm) had high sensitivity (87.6%) for detecting NAFLD in women who were overweight (BMI  $\geq 25$  kg/m<sup>2</sup>) (Table 2). In other words, abdominal circumference was effective for detecting NAFLD in obese women. However, the criterion of abdominal circumference had low sensitivity (36.3%) in non-obese women. The sensitivity of abdominal circumference ( $\geq 90$  cm) was very low (5.8%) in non-obese men. Even in obese men the sensitivity was not high (55.3%). Other criteria for metabolic syndrome had higher sensitivity in obese men and women than in the non-obese population but sensitivity never exceeded 60%.

As a screening tool for NAFLD, the sensitivity of elevated ALT (ALT > 30 IU/L) was 49.7% in men, which exceeded the sensitivity of the criteria of metabolic syndrome, but it was 17.7% in women, which was lower than all metabolic syndrome criteria were. On the other hand, the specificity of elevated ALT was as high as 90.6% in men and 98.0% in women, but the criteria of metabolic syndrome had equally high specificity.

Next, we defined it as positive at screening for NAFLD

**Table 2** The role of the criteria of the metabolic syndrome in detecting or diagnosing nonalcoholic fatty liver disease in obese or non-obese population

	Men				Women			
	Total %	Obese %	Non-obese %	P value	Total %	Obese %	Non-obese %	P value
<b>Sensitivity</b>								
5 criteria of the metabolic syndrome								
Increased abdominal circumference	31.40	55.30	5.80	< 0.001	62.10	87.60	36.30	< 0.001
Elevated fasting glucose level	49.10	52.10	45.90	0.008	36.80	42.20	31.30	0.013
Elevated blood pressure	34.70	44.10	24.60	< 0.001	31.90	41.50	22.30	< 0.001
Decreased HDL cholesterol level	44.10	49.10	38.80	< 0.001	50.40	56.60	44.10	0.006
Elevated triglyceride level	35.20	41.00	28.90	< 0.001	17.90	20.50	15.20	0.15
Elevated ALT (ALT > 30 IU/L)	47.90	59.20	35.80	< 0.001	17.70	24.00	11.30	< 0.001
MS defined by rNCEP-ATP III	33.20	48.60	16.80	< 0.001	32.50	45.00	19.90	< 0.001
MS defined by IDF	21.00	38.10	2.70	< 0.001	29.60	43.40	15.60	< 0.001
At least 1 criterion	84.80	92.30	76.80	< 0.001	86.60	96.10	77.00	< 0.001
At least 2 criteria	61.00	74.20	46.90	< 0.001	61.10	77.50	44.50	< 0.001
At least 1 criterion or elevated ALT	90.40	96.20	84.20	< 0.001	87.40	96.90	79.70	< 0.001
<b>Specificity</b>								
5 criteria of the metabolic syndrome								
Increased abdominal circumference	94.80	64.50	99.00	< 0.001	89.60	33.10	93.00	< 0.001
Elevated fasting glucose level	73.40	57.70	75.50	< 0.001	90.90	78.00	91.70	< 0.001
Elevated blood pressure	83.60	68.80	85.70	< 0.001	92.30	74.40	93.40	< 0.001
Decreased HDL cholesterol level	76.90	62.20	78.90	< 0.001	80.30	61.00	81.50	< 0.001
Elevated triglyceride level	89.70	81.70	90.80	< 0.001	98.10	93.40	98.40	< 0.001
Elevated ALT (ALT > 30 IU/L)	90.60	79.60	92.10	< 0.001	98.00	94.80	98.20	< 0.001
MS defined by rNCEP-ATP III	93.60	77.30	95.90	< 0.001	97.50	81.00	98.50	< 0.001
MS defined by IDF	97.80	84.50	99.70	< 0.001	98.10	83.60	99.00	< 0.001
At least 1 criterion	46.90	15.90	51.10	< 0.001	64.20	13.80	67.20	< 0.001
At least 2 criteria	79.40	49.70	83.40	< 0.001	90.00	49.20	92.40	< 0.001
At least 1 criterion or elevated ALT	44.30	14.20	48.40	< 0.001	63.20	13.10	65.40	< 0.001

NAFLD: Nonalcoholic fatty liver disease; US: Abdominal ultrasonography; BMI: Body mass index; HDL: High dense lipoprotein; MS: Metabolic syndrome; rNCEP-ATP III: Revised National Cholesterol Education Program Adult Treatment Panel III definition; IDF: International Diabetes Federation definition; ALT: Alanine aminotransferase.

when participants satisfied at least one or two components of metabolic syndrome. The sensitivity of the definition “at least 1 criterion” was 84.8% in men and 86.6% in women. Separating subjects with BMI, the sensitivity was higher in obese men and women than in non-obese men and women (92.3% *vs* 76.8% in men, 96.1% *vs* 77.0% in women, respectively).

The prevalence of subjects with NAFLD who also had the metabolic syndrome is indicated in Figure 3. Although NAFLD was thought of as being the liver phenotype of metabolic syndrome, the prevalence of the metabolic syndrome among subjects with NAFLD was low both in men and women. Among men with NAFLD, 66.8% were not diagnosed with the metabolic syndrome defined by revised NCEP-ATP III definition, and 79.0% were not diagnosed with the metabolic syndrome as defined by revised IDF definition. Even in women, 70.4% and 67.5%, respectively, were not diagnosed with metabolic syndrome by revised NCEP-ATP III definition and revised IDF definition. These results mean that a large number of participants diagnosed with the metabolic syndrome have NAFLD, but a large number of participants with NAFLD were not diagnosed with the metabolic syndrome, whether we used revised NCEP-ATP III criteria or IDF criteria.

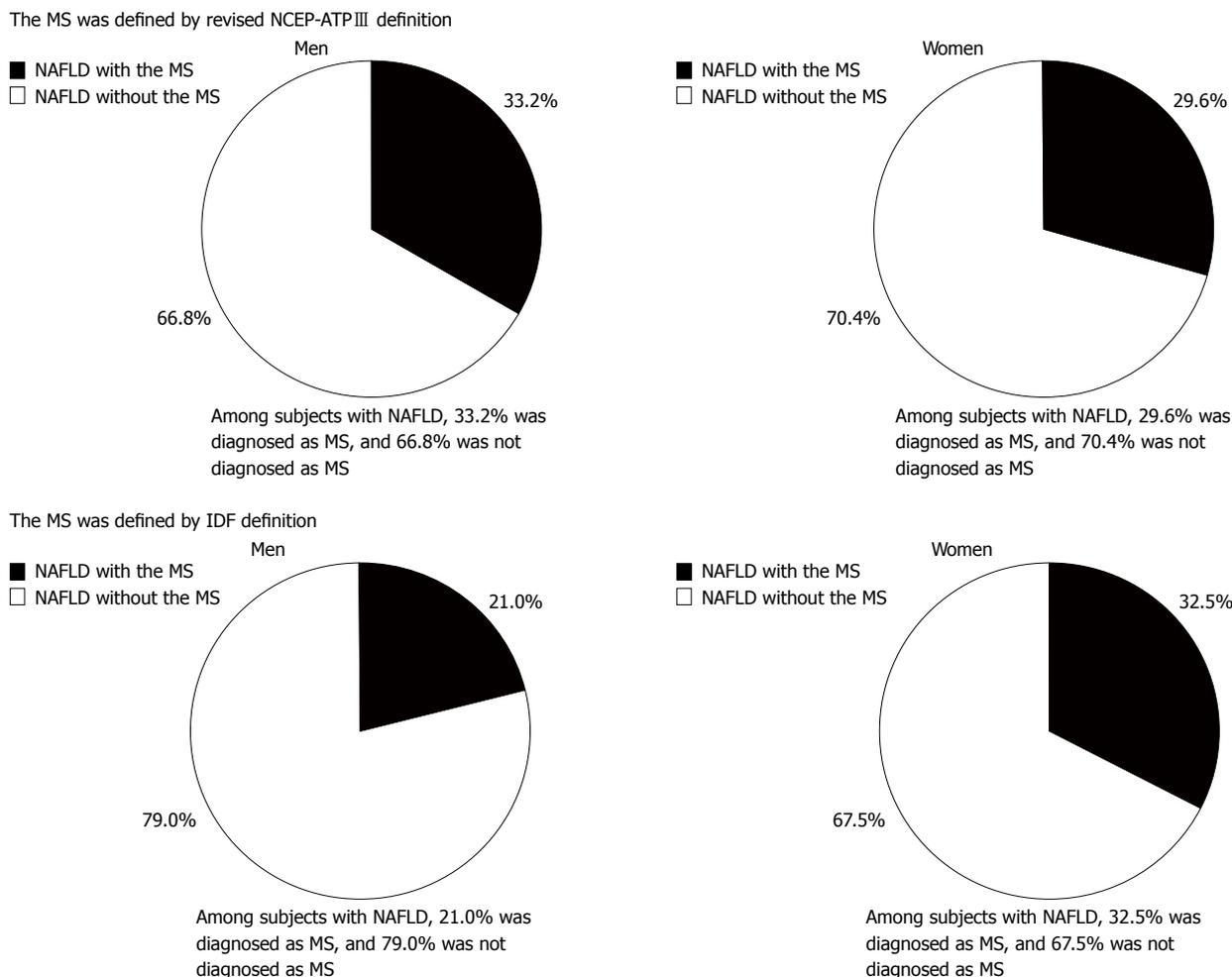
## DISCUSSION

In this study, we clarified the impact of the criteria of the

metabolic syndrome for diagnosing NAFLD in a healthy population. The metabolic syndrome was associated with abdominal obesity and its criteria include waist circumference<sup>[22,23,25,26]</sup>, and NAFLD was reported to be associated with abdominal obesity. However, our results indicated there was no significant difference between BMI and waist circumferences as the strength of association with NAFLD or the accumulation of metabolic syndrome criteria.

The presence of multiple metabolic disorders such as diabetes mellitus, obesity, dyslipidaemia and hypertension is associated with a potentially progressive, severe liver disease<sup>[15,27]</sup>. Previous reports demonstrated that prevalence of NAFLD increased to 10%-80% in individuals with obesity, 35%-90% in individuals with type 2 diabetes mellitus, 30%-56% in individuals with hypertension, and 26%-58% in individuals with dyslipidemia<sup>[9,28-30]</sup>. Another study in a Japanese population showed that prevalence of NAFLD increased to 43% in individuals with impaired fasting glucose and 62% in individuals with type 2 diabetes mellitus<sup>[28]</sup>. Some studies estimate the prevalence of NAFLD be up to 15%-30% of the general population<sup>[8,31,32]</sup>, and the prevalence of metabolic syndrome was estimated to be up to 25% of the general population<sup>[33]</sup>. In those patients with the metabolic syndrome, liver fat content is significantly increased up to 4-fold higher than those without the metabolic syndrome<sup>[34]</sup>, and the incidence of NAFLD has been shown to be increased 4-fold in men and 11-fold in women with the metabolic syndrome<sup>[8]</sup>.

Our data clearly indicated that 21% to 33% of sub-



**Figure 3** The prevalence of subjects with or without the metabolic syndrome among 1874 men and 514 women with non-alcoholic fatty liver disease. Data was expressed as prevalence (%). The metabolic syndrome (MS) was diagnosed using revised IDF. Among men with NAFLD, 66.8% and 79.0% were not diagnosed with the MS defined by revised NCEP-ATP III definition and revised IDF definition, respectively. In women, 70.4% and 67.5%, respectively, were not diagnosed with the MS by revised NCEP-ATP III definition and revised IDF definition. IDF: International Diabetes Federation; NCEP-ATP III: National Cholesterol Education Program Adult Treatment Panel III; NAFLD: Non-alcoholic fatty liver disease.

jects with NAFLD, depending on gender and the criteria used, were diagnosed with the metabolic syndrome. Several previous studies reported how many subjects with NAFLD were diagnosed with the metabolic syndrome, but almost all previous studies were hospital studies. Three population based studies mentioned the prevalence of subjects with NAFLD who were diagnosed with the metabolic syndrome among the general population<sup>[8,35,36]</sup>. In these studies, the prevalence of the metabolic syndrome among subjects with NAFLD was 17% to 36% depending on gender and the criteria used. The reported prevalence was similar to ours.

There has been no report regarding the sensitivity and specificity of the metabolic syndrome for detecting NAFLD. Among the criteria for metabolic syndrome, the criterion of abdominal circumference had high sensitivity in obese women. However, it had low sensitivity (36.3%) in non-obese women and was very low (5.8%) in non-obese men and low (55.3% in obese men. Other than the criterion of abdominal circumference, none of the sensitivities exceeded 60%. In our study, the specificity

of elevated ALT (ALT > 30 IU/L) was 90.6% in men and 98.0% in women. However, the sensitivity was as low as 47.9% in men and 17.7% in women. The specificity of elevated ALT was significantly higher among obese subjects than among non-obese subjects, and sensitivity was higher among obese subjects than among non-obese subjects.

When we investigated the predictability of each component of metabolic syndrome such as abdominal circumference, fasting blood sugar, serum lipid, and blood pressure, each component had high specificity but low sensitivity, similar to elevated ALT. Therefore, we defined it as screening positive for NAFLD, when subjects satisfied at least one criterion of metabolic syndrome; the sensitivity was 84.8% in men and 86.6% in women. Additionally, we defined it as positive when subjects satisfied at least one criterion of metabolic syndrome or elevated ALT. The sensitivity of “at least 1 criterion or elevated ALT” was 90.4% in men and 87.4% in women. However, the specificity of “at least 1 criterion or elevated ALT” was lower -44%-63%.

The result of our study means that we could identify NAFLD effectively in epidemiological study by modifying the usage of the criteria for metabolic syndrome. It is clinically critical evidence that a large part of patients with NAFLD were not diagnosed with the metabolic syndrome, when we used today's definition for the metabolic syndrome. However, our subject population consisted only of Japanese, thus, the generalizability of our study to non-Japanese populations is uncertain. It is one of our study limitations that we used abdominal ultrasonography for diagnosing NAFLD, although the validation ultrasonography had a sensitivity of 91.7% and a specificity of 100%<sup>[24]</sup>.

## COMMENTS

### Background

It is well known that non-alcoholic fatty liver disease (NAFLD) is associated with the metabolic syndrome and patients with NAFLD tend to also have the metabolic syndrome.

### Research frontiers

The impact of overlap between NAFLD and the metabolic syndrome has not been evaluated yet.

### Innovations and breakthroughs

It is clinically critical evidence that a large number of patients with NAFLD were not diagnosed with the metabolic syndrome in a healthy Japanese population.

### Applications

The authors could identify NAFLD effectively by modifying the usage of the criteria for metabolic syndrome.

### Peer review

It is a relatively large population study. The conclusion is consistent with recent observations showing the dissociation between NAFLD and other parameters of metabolic syndrome. The readers of this journal will be interested in the findings of this study.

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## Comparison of PPIs and H<sub>2</sub>-receptor antagonists plus prokinetics for dysmotility-like dyspepsia

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(PPIs) with H<sub>2</sub>-receptor antagonists (H<sub>2</sub>RAs) plus prokinetics (Proks) for dysmotility-like symptoms in functional dyspepsia (FD).

**METHODS:** Subjects were randomized to receive open-label treatment with either rabeprazole 10 mg od ( $n = 57$ ) or famotidine 10 mg bid plus mosapride 5 mg tid ( $n = 57$ ) for 4 wk. The primary efficacy endpoint was change (%) from baseline in total dysmotility-like dyspepsia symptom score. The secondary efficacy endpoint was patient satisfaction with treatment.

**RESULTS:** The improvement in dysmotility-like dyspepsia symptom score on day 28 was significantly greater in the rabeprazole group ( $22.5\% \pm 29.2\%$  of baseline) than the famotidine + mosapride group ( $53.2\% \pm 58.6\%$  of baseline,  $P < 0.0001$ ). The superior benefit of rabeprazole treatment after 28 d was consistent regardless of *Helicobacter pylori* status. Significantly more subjects in the rabeprazole group were satisfied or very satisfied with treatment on day 28 than in the famotidine + mosapride group ( $87.7\%$  vs  $59.6\%$ ,  $P = 0.0012$ ). Rabeprazole therapy was the only significant predictor of treatment response ( $P < 0.0001$ ), defined as a total symptom score improvement  $\geq 50\%$ .

**CONCLUSION:** PPI monotherapy improves dysmotility-like symptoms significantly better than H<sub>2</sub>RAs plus Proks, and should be the treatment of first choice for Japanese FD.

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**Key words:** Dysmotility; Functional dyspepsia; H<sub>2</sub>-receptor antagonist; Prokinetics; Proton pump inhibitor

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### Abstract

**AIM:** To compare efficacy of proton pump inhibitors

Sakaguchi M, Takao M, Ohyama Y, Oka H, Yamashita H, Fukuchi T, Ashida K, Murotani M, Murotani M, Majima K, Morikawa H, Hashimoto T, Kiyota K, Esaki H, Amemoto K, Isowa G, Takao F. Comparison of PPIs and H<sub>2</sub>-receptor antagonists plus prokinetics for dysmotility-like dyspepsia. *World J Gastroenterol* 2012; 18(13): 1517-1524 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v18/i13/1517.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i13.1517>

## INTRODUCTION

Functional dyspepsia (FD) is a condition characterized by persistent upper abdominal symptoms in the absence of any causative organic disease<sup>[1]</sup>. It is thought to be caused by a combination of different factors, including dysmotility or hypersensitivity of the gastrointestinal (GI) tract, gastric acid secretion, inflammation of the gastric mucosa, altered sympathetic or parasympathetic activity, altered secretion of GI hormones, and psychological factors<sup>[2,3]</sup>. Treatments vary according to the symptoms, and include gastroprokinetic agents, suppressors of gastric acid secretion, antidepressants, anxiolytics and Chinese herbal medicines. Although it has been shown that gastric acid secretion is normal in patients with FD<sup>[4]</sup>, a subset of these patients will benefit from strong acid suppression by a proton pump inhibitor (PPI)<sup>[5]</sup>. Inhibitors of acid secretion are therefore widely prescribed to patients with FD worldwide. Although treatment with acid suppression produces symptom relief in a proportion of patients with FD, this effect has not been reported consistently in all studies<sup>[6-8]</sup>. A Japanese study surveyed the prescribing habits of primary care physicians for functional GI symptoms and evaluated the efficacy and indications of the medications prescribed<sup>[9]</sup>. It was found that H<sub>2</sub>-receptor antagonists (H<sub>2</sub>RAs) are the treatment of first choice for ulcer-like symptoms such as epigastric pain, and prokinetics (Proks) for dysmotility-like symptoms such as epigastric discomfort, heaviness, and bloating. In other words, Japanese primary care physicians prefer H<sub>2</sub>RA + prokinetic combination therapy for FD symptoms.

For FD patients with at least mild heartburn and/or regurgitation at baseline, omeprazole is associated with higher treatment success rates at 4 wk than ranitidine, cisapride and placebo<sup>[10]</sup>. In those patients who have either no or minimal heartburn and/or regurgitation at baseline, omeprazole and ranitidine are superior to placebo, although no significant difference is seen between omeprazole and ranitidine<sup>[10]</sup>. The question of whether more effective acid suppression is efficacious in Japanese patients with FD has yet to be adequately tested.

It is reasonable to think that H<sub>2</sub>RAs provide adequate relief for FD symptoms in Japanese patients, who have a higher rate of *Helicobacter pylori* (*H. pylori*) infection than their western counterparts, as well as lower levels of gastric acid secretion<sup>[11]</sup>.

Complete relief of symptoms is significantly more common with omeprazole than with placebo in subgroups of

patients with ulcer-like and reflux-like dyspepsia, whereas, as might be expected, there is no indication of benefit with omeprazole in patients with dysmotility-like dyspepsia<sup>[5]</sup>. In addition, meta-analyses suggest that H<sub>2</sub>RA and Proks are superior to placebo in non-ulcer dyspepsia (NUD)<sup>[12,13]</sup>.

In this study, we concentrated on dysmotility-like symptoms in patients with FD, and compared the efficacy of PPI monotherapy and combination therapy with H<sub>2</sub>RAs and Proks, which is widely prescribed by Japanese primary care physicians for FD symptoms.

## MATERIALS AND METHODS

### Selection of patients

This study was a randomized open-label trial conducted in three hospitals (Moriguchi Keijinkai Hospital, Osaka Saiseikai Nakatsu Hospital, and Arisawa General Hospital) and nine general medical clinics (Murotani Clinic, Majima Clinic, Morikawa Clinic, Hashimoto Clinic, Kiyota Clinic, Arisawa General Hospital, Amemoto Clinic, Isowa Clinic, and Mii Clinic) in Japan from January 2009 until April 2010.

The subjects were patients of at least 18 years of age with at least 1 mo of dyspepsia symptoms and no clinically significant findings at endoscopy. The main exclusion criteria were: (1) history of erosive esophagitis, peptic ulcer disease, GI malignancy, primary esophageal motility disorder, previous upper GI surgery; (2) maintenance treatment with a PPI or H<sub>2</sub>RA within 2 wk of enrollment; and (3) severe concurrent disease. PPI, H<sub>2</sub>RA and Prok use were not permitted during the 14 d prior to endoscopy, nor during the study. Nonsteroidal anti-inflammatory drugs, acetylsalicylic acid or steroids were not permitted at any time during the study. The study protocol was approved by the relevant Institutional Review Board and/or an Independent Ethics Committee, and informed written consent was obtained from each participating subject.

### Study design

The investigators referred each enrolled subject for esophagogastroduodenoscopy. After endoscopy, eligible patients underwent a validated <sup>13</sup>C urea breath test to determine their *H. pylori* status. Subjects were randomly allocated to receive one of the following treatments for 4 wk: (1) rabeprazole 10 mg *od* (PPI); or (2) famotidine 10 mg *bid* plus mosapride 5 mg *tid* (H<sub>2</sub>RA + Prok). Group allocations were assigned in equal numbers using a central computer-generated randomization list stratified for each participating institution. Subject compliance was assessed by counting the returned medication. Subjects were considered to have complied with treatment if they took at least 75% of the dispensed medication. Subjects attended their clinic at randomization and after 4 wk of treatment.

### Symptom assessment

Subjects were asked to assess their dyspepsia symptoms at baseline and after 3 d, 7 d, 14 d and 28 d of treatment using a self-completed questionnaire for dyspepsia symp-

toms. Dysmotility-like dyspepsia symptoms were assessed using five questions (upper abdominal bloating, postprandial fullness, early satiation, belching, vomiting/nausea), and each response was graded on a five-point frequency scale as follows: 0, never; 1, occasionally; 2, sometimes; 3, often; 4, always. The scores for each question were totaled to give the total symptom score for dysmotility-like dyspepsia symptoms. The total symptom scores at each assessment time point were then expressed as a percentage of the baseline total symptom score.

### Subject satisfaction

After 14 d and 28 d of treatment, subject satisfaction was evaluated using a four-grade scale as follows: very satisfied (symptoms disappeared); satisfied (symptoms improved considerably); somewhat satisfied (symptoms improved somewhat); unsatisfied (no improvement or symptoms worse).

### Endpoints

The primary efficacy endpoint was the change (%) from baseline in total dysmotility-like dyspepsia symptom score. The secondary efficacy endpoint was subject satisfaction.

### Sample size

The sample size calculation was based on the anticipated difference in symptom improvement rates between the PPI and H<sub>2</sub>RA + Prok groups. Due to the lack of clinical trials of H<sub>2</sub>RA + Prok combination therapy, we based our calculations of the sample size on the results of comparative trials of PPIs *vs* Proks.

The estimated success rate after 4 wk treatment was 23.7% for omeprazole, and 7.5% for cisapride<sup>[10]</sup>. Assuming a two-tailed  $\alpha$  error rate of 0.05 and a power of 80%, with a 30% dropout rate during screening, 77.5 patients were required for each treatment arm.

### Statistical analysis

Data are presented as mean  $\pm$  SD. The intention-to-treat analysis included all randomized subjects. A subject who withdrew at any time was considered a dropout. We used the Wilcoxon single rank test for paired intra-individual comparisons, the Mann-Whitney *U* test for comparisons of continuous variables, and the  $\chi^2$  test for comparisons of categorized variables between the two treatment groups. In addition, we stratified primary endpoint results for differences between treatment groups according to *H. pylori* status. We performed multiple logistic regression analysis to determine factors (age, sex, *H. pylori* status, and baseline dysmotility-like dyspepsia symptom score) associated with treatment response (defined as change in total dysmotility-like dyspepsia symptom score of  $\geq$  50% after 28 d of treatment).  $P < 0.05$  was considered to signify statistical significance for all analyses.

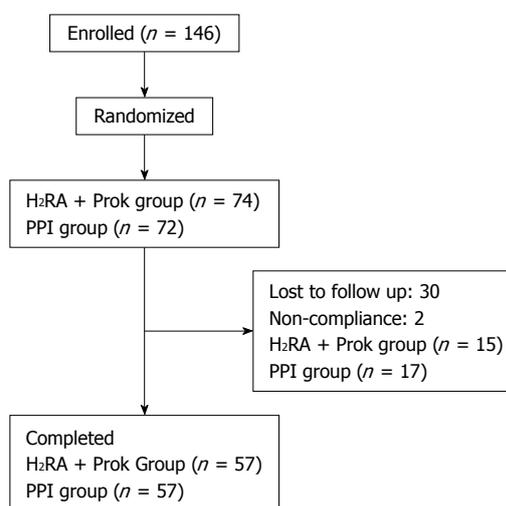
## RESULTS

A total of 146 patients were randomized. Thirty-two patients were excluded in the follow-up period (30 lost to follow-up, two for non-compliance), leaving 114 patients

**Table 1** Baseline demographic characteristics and total symptom scores for study completers

	H <sub>2</sub> RA + Prok ( <i>n</i> = 57)	PPI ( <i>n</i> = 57)	<i>P</i> value
Age (mean $\pm$ SD, yr)	51.5 $\pm$ 14.8	52.9 $\pm$ 13.8	0.6120
Sex (male/female)	17/40	12/45	0.3899
<i>Helicobacter pylori</i> infection (%)	43.8	43.8	> 0.9999
Symptom scores			
Upper abdominal bloating	1.6 $\pm$ 1.2	1.8 $\pm$ 1.3	0.6160
Postprandial fullness	2.1 $\pm$ 1.2	2.0 $\pm$ 1.2	0.7747
Early satiation	1.7 $\pm$ 1.3	1.3 $\pm$ 1.2	0.1934
Belching	1.7 $\pm$ 1.4	1.6 $\pm$ 1.4	0.7211
Vomiting/nausea	1.5 $\pm$ 1.3	1.4 $\pm$ 1.3	0.8361
Total symptom score (dysmotility-like dyspepsia symptoms)	8.6 $\pm$ 3.9	8.2 $\pm$ 4.7	0.5330

PPI: Proton pump inhibitor. H<sub>2</sub>RA: H<sub>2</sub>-receptor antagonist; Prok: Plus prokinetic.

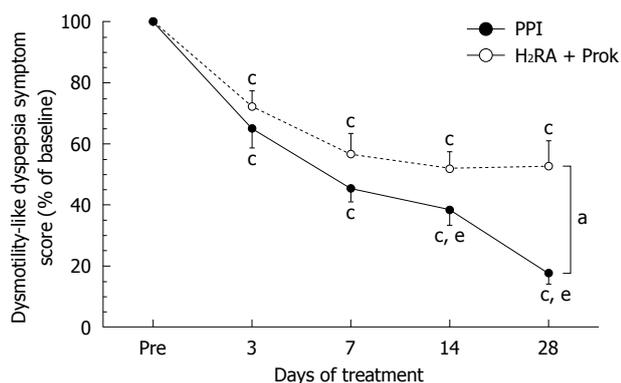


**Figure 1** Study flow chart. This study enrolled 146 subjects with functional dyspepsia and, after excluding 30 subjects for non-attendance and two for non-compliance, 57 subjects in each treatment group completed the study.

for inclusion in the analysis. Fifty-seven patients were randomized to receive PPI treatment, and 57 to receive H<sub>2</sub>RA + Prok treatment (Figure 1). Baseline demographic characteristics and symptom scores of the patients who completed the treatment period are given in Table 1. There were no significant differences between the characteristics of the two treatment groups at baseline.

### Change in dysmotility-like dyspepsia symptom score

No significant differences were seen between groups in the change in dysmotility-like dyspepsia symptom score from baseline to 3 d or 7 d of treatment. After 28 d of treatment, the change in symptom score was significantly greater in the PPI group (22.5%  $\pm$  29.2% of baseline) than in the H<sub>2</sub>RA + Prok group (53.2%  $\pm$  58.6% of baseline) ( $P < 0.0001$ ), indicating greater improvement in symptoms with PPI treatment (Figure 2). A significant improvement in total symptom score was seen over time in both groups, but in the H<sub>2</sub>RA + Prok group, the im-



**Figure 2** Change in dysmotility-like dyspepsia symptom score over time. Significantly greater improvement was seen in dysmotility-like dyspepsia symptom score on day 28 of treatment in the proton pump inhibitor (PPI) group than in the H<sub>2</sub>-receptor antagonist (H<sub>2</sub>RA) + prokinetic (Prok) group. No significant additional symptomatic improvement was seen after day 7 of H<sub>2</sub>RA + Prok treatment, whereas further symptomatic improvements were seen over time in the PPI group. <sup>a</sup>*P* < 0.05, PPI vs H<sub>2</sub>RA + Prok; <sup>c</sup>*P* < 0.05 vs pre-Rx in each group; <sup>e</sup>*P* < 0.05 vs 7 d Rx in each group.

Improvements in total symptom score on days 14 and 28 were not significantly greater than at day 7, whereas in the PPI group, the improvements in total symptom score at day 14 (38.4% ± 37.8% of baseline, *P* = 0.0034) and day 28 (*P* < 0.0001) were both significantly greater than on day 7 (45.1% ± 33.8% of baseline) (Figure 2).

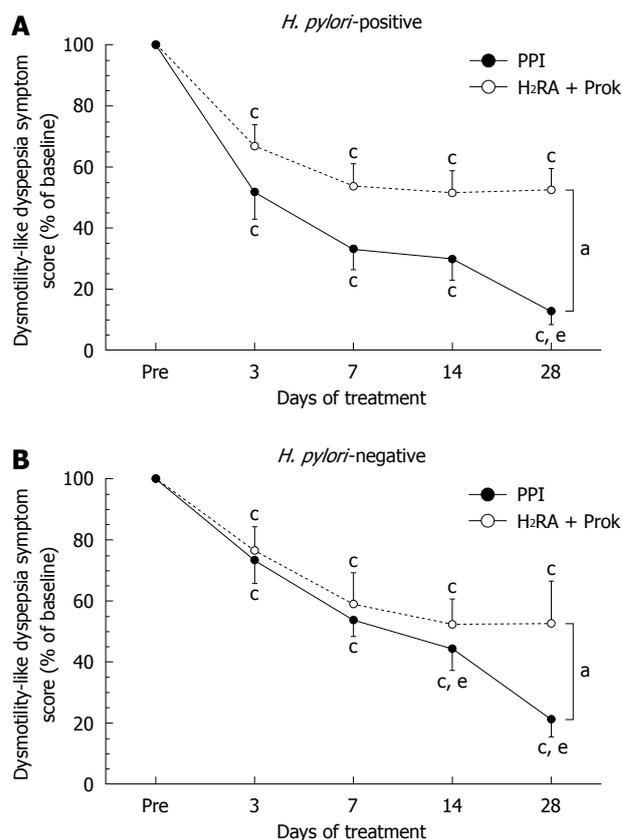
### Change in dysmotility-like dyspepsia symptom score according to *H. pylori* status

Among *H. pylori*-positive subjects, a significantly greater improvement in total symptom score was seen in the PPI group (13.4% ± 26.2% of baseline) than in the H<sub>2</sub>RA + Prok group (53.4% ± 34.7% of baseline) (*P* < 0.007) by the end of the treatment period. Significant symptomatic improvement was seen over time in both treatment groups, although in the H<sub>2</sub>RA + Prok group, no further improvement was observed after day 7 (35.7% ± 31.6% of baseline). In the PPI group, there was no significant difference between the changes in total symptom score for days 7 and 14, although there was a statistically significant difference between days 7 and 28 (*P* = 0.0277) (Figure 3A).

Among *H. pylori*-negative subjects, a significantly greater improvement in total symptom score was seen in the PPI group (24.1% ± 31.7% of baseline) than in the H<sub>2</sub>RA + Prok group (53.1% ± 72.2% of baseline, *P* < 0.0001) on day 28. Significant symptomatic improvement was seen over time in both treatment groups, although the improvements seen in the H<sub>2</sub>RA + Prok group on days 14 and 28 were not significantly superior to those observed on day 7. In the PPI group, the reductions in total symptom score on days 14 (44.2% ± 42.0% of baseline, *P* = 0.0177) and 28 (*P* = 0.0002) were both significantly greater than on day 7 (52.4% ± 34.1% of baseline) (Figure 3B).

### Subject satisfaction on days 14 and 28 of treatment

Although no significant difference was seen between



**Figure 3** Change in dysmotility-like dyspepsia symptom score over time according to *Helicobacter pylori* status. Significantly greater symptomatic improvement was seen in the proton pump inhibitor group than in the H<sub>2</sub>-receptor antagonist (H<sub>2</sub>RA) + prokinetic (Prok) group after 28 d of treatment, regardless of *Helicobacter pylori* (*H. pylori*) status. No additional significant symptomatic improvement was seen after day 7 of H<sub>2</sub>RA + Prok treatment, regardless of *H. pylori* status. <sup>a</sup>*P* < 0.05, PPI vs H<sub>2</sub>RA + Prok; <sup>c</sup>*P* < 0.05 vs pre-Rx in each group; <sup>e</sup>*P* < 0.05 vs 7 d Rx in each group.

the groups on day 14, the proportion of subjects who were satisfied or very satisfied with their treatment was significantly higher in the PPI group than in the H<sub>2</sub>RA + Prok group on day 28 (87.7% vs 59.6%, *P* = 0.0012). No significant increase was seen in subject satisfaction in the H<sub>2</sub>RA + Prok group between days 14 and 28, whereas a significant increase was seen in the proportion of subjects in the PPI group answering satisfied or very satisfied between day 14 (63.2%) and 28 (*P* = 0.0042) (Figure 4).

### Factors associated with treatment response

In this study, treatment response was defined as an improvement in dysmotility-like dyspepsia symptom score of ≥ 50% after 28 d of treatment. The only factor identified by logistic regression analysis as a positive predictor of treatment response was PPI therapy (Table 2).

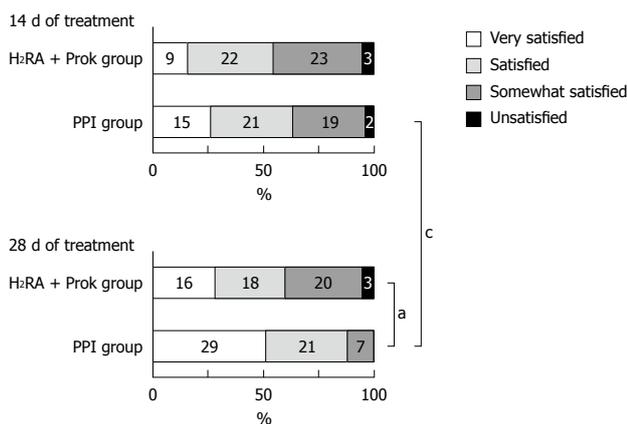
## DISCUSSION

The Rome III criteria define FD as “the chronic presence of one or more dyspepsia symptoms (bothersome postprandial fullness, early satiation, epigastric pain, epigastric burning) that are considered to originate from the

**Table 2** Multiple logistic regression analysis of treatment response

Variable	Estimated OR	95% CI	P value
Age (yr)	0.999	0.962-1.036	0.9493
Sex: male	0.853	0.312-2.332	0.7563
<i>Helicobacter pylori</i> infection	0.521	0.191-1.419	0.2019
Treatment: PPI	9.055	3.231-25.376	< 0.0001
Dysmotility-like symptom score	1.062	0.940-1.200	0.3368

OR: Odds ratio; PPI: Proton pump inhibitor.



**Figure 4** Subject satisfaction after 14 d and 28 d of treatment. Subject satisfaction was significantly higher in the proton pump inhibitor (PPI) group than in the H<sub>2</sub>-receptor antagonist (H<sub>2</sub>RA) + prokinetic (Prok) group on day 28 of treatment. No significant increase in subject satisfaction was seen in the H<sub>2</sub>RA + Prok group between days 14 and 28, whereas a significant increase was seen in the PPI group between days 14 and 28. <sup>a</sup>*P* < 0.05, PPI vs H<sub>2</sub>RA + Prok; <sup>c</sup>*P* < 0.05 vs 14 d Rx.

gastroduodenal region, with no evidence of structural disease (including at upper endoscopy) that is likely to explain the symptoms<sup>[14,15]</sup>. FD is a common condition<sup>[14,15]</sup>, with considerable adverse impact on quality of life<sup>[16]</sup>, and represents a serious problem in everyday clinical practice. Patients with FD present with a variety of symptoms<sup>[17,18]</sup>, so based on the symptom profile, they may be prescribed gastroprokinetic agents, suppressors of gastric acid secretion, antidepressants, anxiolytics or Chinese herbal medicines<sup>[19]</sup>. A Japanese survey of the prescribing habits of primary care physicians for upper GI symptoms has found that H<sub>2</sub>RAs are prescribed for epigastric pain and heartburn, and Proks for epigastric discomfort, nausea and loss of appetite<sup>[9]</sup>. In other words, H<sub>2</sub>RAs rather than PPIs are prescribed for epigastric pain syndrome<sup>[1]</sup>, characterized by the two symptoms of epigastric pain and epigastric burning, unrelated to meals and considered mainly related to gastric acid. Proks are widely prescribed for postprandial distress syndrome<sup>[1]</sup>, characterized by the two symptoms of postprandial fullness and early satiation, and considered to be strongly related to dysmotility of the GI tract; in particular, gastric accommodation of adaptive relaxation. H<sub>2</sub>RA + Prok combination therapy is widely prescribed in Japan, where dyspepsia is not a

recognized diagnosis for insurance purposes.

It has recently become clear that gastric acid secretion is strongly associated with the onset of dysmotility-like symptoms. Lee *et al*<sup>[20]</sup> have examined the influence of acid on gastric hypersensitivity and motility in healthy subjects, and have found that duodenal acidification significantly induces gastric hypersensitivity and impairs gastric motility. Compared with saline, infusion of acid into the duodenum causes not only ulcer reflux symptoms such as heartburn, but also dysmotility-like symptoms such as epigastric discomfort, belching and abdominal bloating<sup>[20]</sup>. When Miwa *et al*<sup>[21]</sup> infused acid into the stomach of Japanese subjects, they induced a variety of dyspeptic symptoms, with no significant change in acid-reflux-related symptoms such as heartburn and epigastric pain, but a significant increase in dysmotility-like symptoms such as epigastric discomfort, stomach fullness, nausea and belching. Samsom *et al*<sup>[22]</sup> have reported that decreased acid clearance and increased sensitivity to acid in patients with dyspepsia may lead to dyspeptic symptoms. There have in fact been a number of recent studies reporting the effectiveness of PPIs<sup>[7]</sup> and H<sub>2</sub>RAs<sup>[23]</sup> in the treatment of FD. Empirical omeprazole therapy induces symptom improvement in a higher proportion of patients with uninvestigated dyspepsia (defined as epigastric pain or discomfort with or without heartburn or regurgitation) than ranitidine or cisapride does, but this effect is more marked in patients with gastroesophageal reflux disease<sup>[10]</sup>. The prevalence of pathological pH monitoring (4%-6% of time at pH < 4) is significantly higher in FD patients with heartburn than those without<sup>[17]</sup>. In other words, PPIs are more effective than H<sub>2</sub>RAs in the treatment of FD associated with heartburn and regurgitation.

It is unclear, however, how important suppression of acid secretion is in Japanese patients, who have a high prevalence of *H. pylori* infection and low levels of gastric acid secretion<sup>[11]</sup>. A Japanese clinical trial found that H<sub>2</sub>RA therapy was more effective than mosapride for treatment of FD<sup>[24]</sup>, suggesting that suppression of gastric acid secretion by an H<sub>2</sub>RA may be sufficient to treat FD in Japanese patients, with no need for a PPI. In this study, we compared PPI monotherapy with H<sub>2</sub>RA + Prok combination therapy; the therapy most commonly prescribed by Japanese clinicians<sup>[9]</sup>. In consideration of the superior acid secretion suppression of PPIs over H<sub>2</sub>RAs, we combined an H<sub>2</sub>RA with a Prok, and examined only dysmotility-like symptoms, considered less responsive to PPI therapy.

Our results showed no differences between treatment groups for changes in dysmotility-like dyspepsia symptom score on days 3 and 7 of treatment, but significantly greater symptom improvement was seen in the PPI group than the H<sub>2</sub>RA + Prok group on days 14 and 28 of treatment. The proportion of subjects reporting that they were satisfied or very satisfied was significantly higher in the PPI group than in the H<sub>2</sub>RA + Prok group on day 28. PPI therapy was the only significant predictor

of treatment response, defined as a total symptom score improvement of  $\geq 50\%$ .

In a recent meta-analysis of PPI treatment for FD, the dysmotility-like subgroup did not respond to PPI therapy, unlike the reflux and ulcer-like subgroup<sup>[25]</sup>. The severity of heartburn at baseline in FD patients influences treatment response to PPI or H<sub>2</sub>RA therapy<sup>[10]</sup>. We assessed heartburn symptoms at baseline in a separate study, finding no difference between groups in the pretreatment heartburn symptom score (data not shown). Our results suggest that powerful suppression of acid secretion by PPIs is also important in the treatment of dysmotility-like symptoms in Japanese patients with FD. We saw similar symptom score improvements in both groups until day 7 of treatment. This may represent a placebo response, because it is well known that the placebo response can be substantial in trials of GI disorders, including FD<sup>[10]</sup>. There have been reports of a placebo effect until day 7 of treatment with a PPI<sup>[26]</sup>, and of the need for a 7-d run-in period to minimize the placebo effect in FD clinical trials<sup>[27-29]</sup>, so a similar placebo response lasting until day 7 of treatment was also possible in this study. The absence of any further improvement in dysmotility-like dyspepsia symptoms between day 7 and days 14 or 28 in the H<sub>2</sub>RA + Prok group, and the lack of significant change in subject satisfaction rates from day 14 to 28, indicates the development of H<sub>2</sub>RA tolerance<sup>[30]</sup>.

Stratifying the study sample by *H. pylori* status showed that significantly better symptom improvement was seen in the PPI group than the H<sub>2</sub>RA + Prok group in both *H. pylori*-positive and -negative subjects. No influence of *H. pylori* status was seen in either treatment group in our study. In the studies by Talley *et al.*<sup>[5]</sup>, no significant difference was seen in the rate of complete symptom resolution after 28 d of treatment between *H. pylori*-positive and -negative subjects in the ulcer-like, reflux-like, or dysmotility-like symptom subgroups in either treatment arm, although these were only exploratory analyses. Although *H. pylori* status did not significantly influence the response to omeprazole, this does not exclude the possibility of a small true difference between *H. pylori*-positive and -negative patients in the effect of acid inhibition on FD. Intra-gastric pH is higher in patients taking a PPI who are infected with *H. pylori*<sup>[31]</sup>. PPI therapy may therefore be more effective in *H. pylori*-positive patients with NUD, as one study has suggested<sup>[28]</sup>. Pooling all trial data suggests that symptom responses are similar in *H. pylori*-positive and -negative patients with NUD. *H. pylori* status is unlikely to have a clinically important impact on the efficacy of treatments in this patient group.

In this study, symptoms improved over time regardless of *H. pylori* status in the PPI group, whereas no further symptom improvement was seen in the H<sub>2</sub>RA + Prok group after day 7 of treatment in both *H. pylori*-positive and -negative subjects, indicating tolerance to treatment. Loss of the suppressive effect on acid secretion by H<sub>2</sub>RAs has been reported in *H. pylori*-negative patients as the duration of treatment increases<sup>[32]</sup>, whereas in this

study tolerance was seen from day 7 of treatment in the H<sub>2</sub>RA + Prok group.

Limitations of this study include the small number of subjects and the fact that it was not a placebo-controlled trial. Consideration of the Japanese medical system tells us that clinical trials of treatments for FD, a condition not covered by medical insurance, conducted with the participation of general medical clinics will be limited in scope. Nevertheless, this is the first published report of a randomized comparative trial of PPI monotherapy and H<sub>2</sub>RA + Prok combination therapy in the treatment of FD in Japanese subjects.

We demonstrated that, even in Japan with its high proportion of *H. pylori*-positive patients, PPI monotherapy significantly improves not only ulcer and reflux-like symptoms, but also dysmotility-like symptoms, better than H<sub>2</sub>RA + Prok combination therapy. In particular, tolerance to H<sub>2</sub>RA + Prok combination therapy was seen regardless of *H. pylori* status. The prevalence of *H. pylori* infection is expected to decline in Japan in the future<sup>[33]</sup>, leading to increased gastric acid secretion, so suppression of acid secretion will likely become even more important. The American College of Gastroenterology guidelines for the management of dyspepsia recommend a PPI as the treatment of first choice in regions with a low prevalence of *H. pylori* infection, with investigations and other therapies for those who fail to respond<sup>[29]</sup>. In Japan, where the prevalence of *H. pylori* infection remains high, upper GI endoscopy is considered mandatory for the exclusion of malignancy. Powerful suppression of acid secretion, such as a PPI provides, is the most effective therapy for treatment of all dyspeptic symptoms in Japanese patients, both ulcer- and reflux-like and dysmotility-like symptoms. Of particular interest is our finding that PPI therapy is useful in the treatment of dysmotility-like symptoms, usually considered less responsive to PPIs. In other words, dysmotility-like symptoms of FD are also an acid-related disorder, for which suppression of acid secretion is the most effective therapy. PPIs are extremely effective in the treatment of all symptoms of FD, and should be the treatment of first choice in Japanese patients with FD.

## COMMENTS

### Background

Many people suffer from functional dyspepsia (FD). The disease has a substantial negative effect on quality of life, therefore, daily care is of the utmost importance. However, because there are many causes of FD, including gastrointestinal motility disorders and hypersensitivity of the digestive tract, gastric acid secretion, inflammation of the mucous membrane of the stomach, nervous system and digestive tract hormone disorders, and psychological factors, no method of treatment has been established.

### Research frontiers

It has recently been clarified that gastric acid secretion plays a major role in the onset of FD symptoms. In Japan, however, where there are many *Helicobacter pylori* (*H. pylori*)-positive patients, epigastric pain, epigastric burning and other acid-related symptoms are treated with acid-secretion blockers. In contrast, dysmotility-like symptoms such as painful stomach heaviness after eating and soon feeling full are mainly treated with prokinetic agents and/or antiulcer agents, and the significance of acid secretion suppression is not clear. For this reason, we conducted a prospective randomized treatment study to examine

the importance of acid-suppressing drugs for the treatment of various FD symptoms.

### Innovations and breakthroughs

Regardless of whether cases were *H. pylori*-positive or -negative, the improvement rate for dysmotility-like symptoms as well as ulcer and acid-reflux-related symptoms in a proton pump inhibitor (PPI) single therapy group was significantly better compared to a group concomitantly administered histamine H<sub>2</sub> receptor antagonists (H<sub>2</sub>RAs) and prokinetic drugs (Proks). Logistic analysis (multivariable analysis) of factors related to improvements in dysmotility-like symptoms also showed that PPI therapy was the only significant factor. Patient satisfaction was also significantly higher in the PPI single therapy group than in the H<sub>2</sub>RA and Prok groups. In other words, in Japan, as well as other countries, regardless of whether a patient is *H. pylori*-positive or -negative, the importance of acid secretion suppression for the treatment of not only ulcer and acid-reflux-related symptoms but also digestive motility disorder symptoms has been established.

### Applications

It is thought that the *H. pylori* infection rate in Japan will gradually decrease. Assuming this will lead to more patients with increased acid secretion, acid secretion suppression will become an increasingly important issue. It has been concluded that, regardless of whether a patient is *H. pylori*-positive or -negative, PPI therapy is extremely effective as a first-choice treatment for not only ulcer and acid-reflux-related symptoms but also dysmotility-like symptoms, in other words, for all FD symptoms.

### Terminology

In the Rome III classification, FD is defined as "one or more of the chronic symptoms of painful postprandial fullness, early feeling of satiety, epigastric pain and epigastric burning for which no causal organic disease is observed during endoscopic and other examinations". This definition is divided into the subcategories of epigastric pain syndrome, indicated by the two symptoms of epigastric pain related to dietary intake and epigastric burning related to gastric acid, and postprandial distress syndrome (PDS), indicated by the two so-called dysmotility-like symptoms of early satiation and fullness. Many cases of FD are thought to be due to PDS. In Japan, Proks, drugs that improve gastrointestinal motility, are the first-choice treatment for dysmotility-like symptoms. *H. pylori*, a bacterium that exists in the mucous membrane of the stomach, is thought to cause stomach and duodenal ulcers, and stomach cancer. In *H. pylori*-positive patients, the bacterium causes chronic atrophic gastritis, progressing to gastric cancer, and low gastric acid output. The *H. pylori*-positive rate in Japan is tending to decrease, which could result in more Japanese patients with excessive gastric acid output.

### Peer review

A good paper and can be accepted for publication. Only problem is the number of patients recruited for the study was too low.

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## Noninvasive Parameters and hepatic fibrosis scores in children with nonalcoholic fatty liver disease

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### Abstract

**AIM:** To evaluate the noninvasive parameters and hepatic fibrosis scores in obese children with nonalcoholic fatty liver disease (NAFLD).

**METHODS:** A total of 77 children diagnosed with NAFLD *via* liver biopsy were included and divided into 2 subgroups according to the histopathologic staging of hepatic fibrosis: mild (stage 0-1) *vs* significant fibrosis (stage 2-4). Clinical and laboratory parameters were evaluated in each patient. The aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, AST/platelet ratio index (APRI), PGA index, Forns index, FIB-4, NAFLD fibrosis score, and pediatric NAFLD fibrosis index (PNFI) were calculated.

**RESULTS:** No clinical or biochemical parameter exhib-

ited a significant difference between patients with mild and significant fibrosis. Among noninvasive hepatic fibrosis scores, only APRI and FIB4 revealed a significant difference between patients with mild and significant fibrosis (APRI:  $0.67 \pm 0.54$  *vs*  $0.78 \pm 0.38$ ,  $P = 0.032$  and FIB4:  $0.24 \pm 0.12$  *vs*  $0.31 \pm 0.21$ ,  $P = 0.010$ ). The area under the receiving operating characteristic curve of FIB4 was 0.81, followed by Forns index (0.73), APRI (0.70), NAFLD fibrosis score (0.58), AST/ALT ratio (0.53), PGA score (0.45), and PNFI (0.41).

**CONCLUSION:** APRI and FIB4 might be useful noninvasive hepatic fibrosis scores for predicting hepatic fibrosis in children with NAFLD.

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**Key words:** Fatty liver; Hepatic fibrosis; Noninvasive; Obesity; Child

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### INTRODUCTION

The disease spectrum of nonalcoholic fatty liver disease (NAFLD) ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis; NASH progresses towards cirrhosis, even in children<sup>[1,2]</sup>. Therefore, accurate diagnosis and early detection of hepatic fibrosis are required in obese children suspicious of NAFLD.

Percutaneous liver biopsy is the gold standard for the diagnosis of NASH<sup>[3]</sup>. However, histopathologic investigation has some limitations because of its invasiveness and high costs, especially in children; moreover, the histopathologic findings of NAFLD in children are somewhat different from those in adults, revealing portal inflammation and portal fibrosis mainly in pediatric NASH in contrast to lobular inflammation, perisinusoidal fibrosis, ballooning, and Mallory's hyaline in adult NASH<sup>[4,5]</sup>.

Noninvasive markers of hepatic fibrosis and noninvasive hepatic fibrosis scores have been evaluated in previous studies, mostly in adult patients with NAFLD<sup>[6]</sup>. Although pediatric NAFLD shows peculiar histopathologic features as described above<sup>[4,5]</sup>, there have been only limited studies of noninvasive biochemical markers of hepatic fibrosis related to NAFLD in pediatric populations<sup>[7-10]</sup>, and no validation studies in children on the previously suggested noninvasive hepatic fibrosis scores excluding 1 study on the enhanced liver fibrosis panel<sup>[11]</sup>. Only 1 pediatric noninvasive score has been developed up to date<sup>[12]</sup>.

Therefore, the present study aimed to evaluate the noninvasive clinical and laboratory parameters and noninvasive hepatic fibrosis scores indicating the presence of hepatic fibrosis and its severity in obese children with NAFLD.

## MATERIALS AND METHODS

A total of 77 obese children with NAFLD under the age of 18 years who visited the Pediatric Obesity Clinic were included and divided into 2 subgroups according to the grading and staging of NAFLD. Obesity was defined as a body mass index (BMI) value higher than the 95th percentile for the child's age and sex, and overweight as a BMI between the 85th and 95th percentiles. NAFLD was diagnosed on the basis of histopathologic findings compatible with NAFLD on liver biopsy<sup>[13]</sup>. This study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital.

### Laboratory tests

Serum levels of fasting glucose, insulin, total cholesterol, TGs, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and apoprotein A1 and B levels were measured after a 12-h fast at the same time of liver biopsy. Insulin resistance was determined by the homeostatic model assessment of insulin resistance. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin,  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT), alkaline phosphatase (ALP) levels, prothrombin time (PT), and tumor necrosis factor- $\alpha$  were also measured.

For the differential diagnosis of chronic hepatitis, creatine phosphokinase, lactate dehydrogenase, ammonia, lactate, pyruvate, anti-HAV IgM antibody, HBs antigen and anti-HBs antibody, anti-HCV antibody, EBV VCA IgM antibody, CMV IgM antibody, serum ceruloplasmin, and anti-nuclear antibody were evaluated.

### Radiologic investigations

The presence of fatty liver was evaluated in each patient

using either abdominal sonography or noncontrast abdominal computed tomography (CT). Using abdominal sonography, fatty liver was detected and the degree of fatty liver was defined as mild, moderate, and severe fatty liver<sup>[14]</sup>. Regarding abdominal CT, fatty liver was diagnosed when the difference between CT numbers of the liver and spleen was greater than 10<sup>[15]</sup>.

### Histopathologic examination

Percutaneous needle liver biopsy was performed in all patients. Histopathologic grades of steatosis, lobular and portal inflammation, and hepatocyte ballooning and the histopathologic stages of fibrosis were evaluated in each patient to diagnose NAFLD and to assess the stages of hepatic fibrosis<sup>[16]</sup>. The histological scoring system of Kleiner *et al.*<sup>[13]</sup> was also applied to the liver biopsy specimens to assess the histologic stages of hepatic fibrosis and the NAFLD activity score (NAS). Fibrosis was staged as follows: stage 0: none; stage 1: perisinusoidal or periportal fibrosis (stage 1a: mild perisinusoidal; stage 1b: moderate perisinusoidal; stage 1c: portal/periportal); stage 2: perisinusoidal and portal/periportal fibrosis; stage 3: bridging fibrosis; stage 4: cirrhosis<sup>[14]</sup>.

### Noninvasive hepatic fibrosis scores

The AST/ALT ratio was calculated as the ratio of AST to ALT<sup>[10]</sup>; AST/platelet ratio index (APRI) as follows: (AST level/AST upper level of normal/platelet counts)  $\times$  100<sup>[17]</sup>; PGA index as the sum of 3 scores based on the test results of PT,  $\gamma$ GT activity, and apoprotein A1 and ranged from 0 to 12<sup>[18]</sup>; Forns index as follows:  $[7.811 - 3.131 \times \ln(\text{platelet count}) + 0.781 \times \ln(\gamma\text{GT}) + 3.467 \times \ln(\text{age}) - 0.014 \times \text{cholesterol}]$ <sup>[19]</sup>; FIB-4 as  $(\text{age} \times \text{AST level}/\text{platelet count} \times \sqrt{\text{ALT}})$ <sup>[20]</sup>; NAFLD fibrosis score as follows:  $[-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{impaired fasting glucose/diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet count} - 0.66 \times \text{albumin}]$ <sup>[21]</sup>. Pediatric NAFLD fibrosis index (PNFI) was calculated using a formula suggested by Nobili *et al.*<sup>[12]</sup>.

### Statistical analysis

The results are expressed as mean  $\pm$  SD. The data were analyzed using the SPSS 18.0 software program (SPSS Inc., Chicago, IL, United States). Frequency data were compared using Fisher's exact test for nonparametric analysis. The Mann-Whitney *U* test was used for comparisons of means between 2 groups. Multivariate logistic regression analysis was performed to determine potential variables predicting significant hepatic fibrosis. *P*-values less than 0.05 were considered statistically significant.

Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUROC) were applied to assess and compare the diagnostic accuracy of each noninvasive hepatic fibrosis scores.

## RESULTS

### Patient characteristics

A total of 77 children (M:F = 66:11; mean age 12.2  $\pm$

**Table 1** Patient characteristics and comparisons of noninvasive biochemical parameters between mild fibrosis and significant fibrosis regarding the hepatic histology of children with non-alcoholic fatty liver disease (mean  $\pm$  SD)

Parameters	Total ( <i>n</i> = 77)	Mild fibrosis (stage 0-1) ( <i>n</i> = 51)	Significant fibrosis (stage 2-3) ( <i>n</i> = 26)	<i>P</i> value
<b>Clinical characteristics</b>				
Gender (M:F)	66:11	42:9	24:2	0.316
Age (yr)	12.2 $\pm$ 2.3	12.0 $\pm$ 2.4	12.7 $\pm$ 1.9	0.132
AC (cm)	91.1 $\pm$ 10.1	92.1 $\pm$ 11.6	89.7 $\pm$ 7.8	0.637
Weight (kg)	68.9 $\pm$ 19.3	68.3 $\pm$ 21.3	70.2 $\pm$ 15.1	0.448
Height (cm)	155.5 $\pm$ 12.2	155.1 $\pm$ 12.1	156.3 $\pm$ 12.6	0.407
BMI (kg/m <sup>2</sup> )	28.1 $\pm$ 5.1	27.8 $\pm$ 5.2	28.7 $\pm$ 5.1	0.425
<b>Biochemical parameters</b>				
AST (IU/L)	82.1 $\pm$ 46.6	77.4 $\pm$ 46.3	91.3 $\pm$ 46.8	0.063
ALT (IU/L)	167.4 $\pm$ 99.1	158.7 $\pm$ 95.4	184.6 $\pm$ 105.6	0.206
ALP (IU/L)	283.7 $\pm$ 110.0	273.0 $\pm$ 113.8	304.7 $\pm$ 101.1	0.185
$\gamma$ GT (IU/L)	53.9 $\pm$ 29.1	51.1 $\pm$ 25.9	59.4 $\pm$ 34.4	0.410
Total bilirubin (mg/dL)	0.76 $\pm$ 0.37	0.75 $\pm$ 0.30	0.77 $\pm$ 0.50	0.438
Albumin (g/dL)	4.54 $\pm$ 0.31	4.54 $\pm$ 0.32	4.55 $\pm$ 0.30	1.000
PT INR	1.02 $\pm$ 0.07	1.01 $\pm$ 0.06	1.03 $\pm$ 0.08	0.090
Total cholesterol (mg/dL)	180.2 $\pm$ 32.6	176.8 $\pm$ 31.2	186.9 $\pm$ 34.7	0.139
Triglyceride (mg/dL)	132.8 $\pm$ 53.6	126.6 $\pm$ 52.4	142.7 $\pm$ 55.0	0.235
LDL cholesterol (mg/dL)	97.2 $\pm$ 24.9	93.5 $\pm$ 20.6	103.1 $\pm$ 30.2	0.198
HDL cholesterol (mg/dL)	48.1 $\pm$ 11.4	48.9 $\pm$ 12.2	46.9 $\pm$ 10.2	0.995
Apoprotein A1 (mg/dL)	120.2 $\pm$ 15.9	121.6 $\pm$ 15.7	118.1 $\pm$ 16.8	0.543
Apoprotein B (mg/dL)	83.5 $\pm$ 17.7	85.6 $\pm$ 17.5	80.0 $\pm$ 18.3	0.418
Fasting glucose (mg/dL)	98.1 $\pm$ 34.2	95.4 $\pm$ 11.9	103.4 $\pm$ 56.7	0.311
Insulin ( $\mu$ IU/mL)	24.6 $\pm$ 14.6	23.2 $\pm$ 15.5	26.2 $\pm$ 13.5	0.244
HOMA-IR	5.8 $\pm$ 4.3	6.1 $\pm$ 4.8	5.3 $\pm$ 3.2	0.586
TNF- $\alpha$ (pg/mL)	18.2 $\pm$ 15.0	15.7 $\pm$ 12.3	21.7 $\pm$ 18.0	0.400
HbA1c (%)	5.7 $\pm$ 1.1	5.5 $\pm$ 0.3	5.9 $\pm$ 1.6	0.806
Platelet ( $\times 10^9$ /L)	307.9 $\pm$ 69.2	313.2 $\pm$ 71.1	297.3 $\pm$ 65.4	0.416

AC: Abdominal circumference; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index;  $\gamma$ GT:  $\gamma$ -glutamyl transpeptidase; DBP: Diastolic blood pressure; HDL: High density lipoprotein; HOMA-IR: Insulin resistance determined by homeostasis model assessment; LDL: Low density lipoprotein; NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD activity score; Ob: Obesity; Ow: Overweight; PT: Prothrombin time; SBP: Systolic blood pressure; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

2.2 years, range 8-18 years) diagnosed as NAFLD were included. The clinical characteristics of children with NAFLD are shown in Table 1.

### Histopathologic findings and hepatic fibrosis in NAFLD

Histopathologic findings of the liver in children with NAFLD are listed in Table 2. Staging for hepatic fibrosis revealed the prevalence of stage 0 (*n* = 12), stage 1A (*n* = 12), stage 1B (*n* = 7), stage 1C (*n* = 20), stage 2 (*n* = 21), and stage 3 (*n* = 5). There was no patient with stage 4 of hepatic histology. Fibrosis stages were grouped into 2 subgroups: mild hepatic fibrosis (stage 0 to 1) (*n* = 51); and significant hepatic fibrosis (stage 2 to 3) (*n* = 26). The histopathologic findings for these 2 groups are compared in Table 2. There were no differences in steatosis, lobular inflammation, and NAS between 2 fibrosis groups (Table 2). Only hepatocyte ballooning was significantly different between the 2 groups (*P* = 0.018) (Table 2).

### Noninvasive clinical and biochemical parameters in children

Clinical data of the patients and the results of laboratory tests for obesity and obesity-related complications are listed in Table 1. None of these noninvasive clinical and

biochemical parameters exhibited a significant difference between the 2 fibrosis groups based on histopathologic findings (Table 1).

### Multivariate logistic regression analysis for hepatic fibrosis in NAFLD

The result of multivariate logistic regression analysis for clinical or biochemical factors to predict significant hepatic fibrosis in children with NAFLD is shown in Table 3.

### Comparisons of noninvasive hepatic fibrosis scores and hepatic fibrosis in children with NAFLD

The results of hepatic fibrosis scores including the AST/ALT ratio, APRI, PGA index, Forns index, FIB4, NAFLD fibrosis score, and PNFI were compared between the 2 groups based on the stage of hepatic fibrosis (Table 2).

Among the hepatic fibrosis scores, APRI and FIB4 revealed statistically significant differences between patients with mild fibrosis and significant fibrosis (APRI, *P* = 0.032; FIB4, *P* = 0.010) (Table 2). The other hepatic fibrosis scores were not significantly different between the 2 groups (AST/ALT ratio, *P* = 0.808; PGA index, *P* = 0.710; Forns index, *P* = 0.097; NAFLD fibrosis score, *P* = 0.532; PNFI, *P* = 0.314) (Table 2).

**Table 2** Comparison of hepatic fibrosis scoring systems between mild fibrosis and significant fibrosis regarding the hepatic histology of children with non-alcoholic fatty liver disease (mean ± SD)

Parameters and hepatic fibrosis scores	Total (n = 77)	Mild fibrosis (stage 0-1) (n = 51)	Significant fibrosis (stage 2-3) (n = 26)	P value
Invasive histopathologic NAFLD scores <sup>1</sup>				
Steatosis (grade 0/1/2/3)	0/13/36/28	0/7/22/22	0/6/14/6	0.085
Inflammation (grade 0/1/2/3)	9/43/25/0	7/29/15/0	2/14/10/0	0.325
Ballooning (grade 0/1/2)	27/33/17	22/21/8	5/12/9	0.018
NAS	4.3 ± 1.4	4.18 ± 1.48	4.46 ± 1.14	0.470
Noninvasive hepatic fibrosis scoring systems				
AST/ALT ratio	0.53 ± 0.22	0.52 ± 0.16	0.57 ± 0.31	0.802
AST/platelet ratio index	0.71 ± 0.49	0.67 ± 0.54	0.78 ± 0.38	0.032
PGA index	3.78 ± 1.80	3.85 ± 1.89	3.68 ± 1.70	0.710
Forns index	-0.94 ± 1.18	-1.06 ± 0.21	-0.69 ± 1.09	0.097
FIB4 score	0.27 ± 0.16	0.24 ± 0.12	0.31 ± 0.21	0.010
NAFLD fibrosis score	-4.95 ± 1.32	-5.07 ± 1.27	-4.73 ± 1.41	0.532
Pediatric NAFLD fibrosis index	7.67 ± 2.48	7.71 ± 2.79	7.61 ± 2.08	0.314

<sup>1</sup>Histopathologic non-alcoholic fatty liver disease (NAFLD) scoring on liver biopsy specimens was based on the definition by Kleiner *et al*<sup>[13]</sup>. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAS: Non-alcoholic fatty liver disease activity score.

**Table 3** Multivariate logistic regression analysis for significant hepatic fibrosis in children with non-alcoholic fatty liver disease

Variables	OR	95% CI	P value
Age	1.357	1.027-1.793	0.032
ALP	1.006	1.000-1.011	0.035

ALP: Alkaline phosphatase; OR: Odds ratio.

**Comparison of the diagnostic accuracy of the hepatic fibrosis scoring systems**

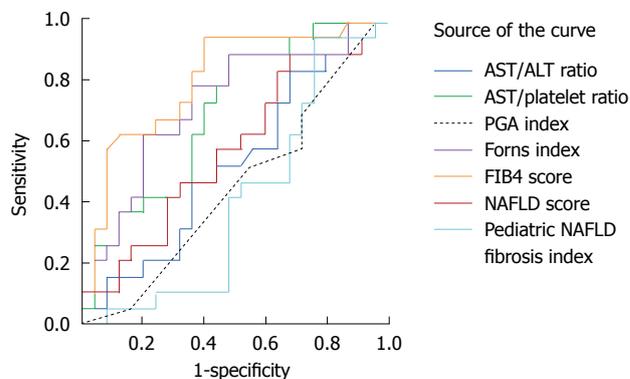
ROC curves of the hepatic fibrosis scoring systems are shown in Figure 1.

When the AUROC of each scoring system was compared, the AUROC of FIB4 was 0.81 (95% CI: 0.68-0.94), followed by the Forns index (AUROC = 0.73, 95% CI: 0.58-0.88), APRI (AUROC = 0.70, 95% CI: 0.55-0.86), NAFLD fibrosis score (AUROC = 0.58, 95% CI: 0.41-0.75), AST/ALT ratio (AUROC = 0.53, 95% CI: 0.35-0.70), PGA score (AUROC = 0.45, 95% CI: 0.28-0.62), and PNFI (AUROC = 0.41, 95% CI: 0.24-0.58).

**DISCUSSION**

Due to the invasiveness of histopathologic diagnosis based on liver biopsy, noninvasive methods including the measurement of various clinical parameters or laboratory markers have been applied to clinical practice and research, mostly in adults, to facilitate the diagnosis of NAFLD and to predict hepatic fibrosis in obese patients.

In our study performed in children with biopsy-proven NAFLD, both clinical and biochemical parameters were evaluated on the basis of the histopathologic findings of liver biopsy specimens, particularly focused on biochemical parameters. However, all of these clinical and biochemical parameters failed to distinguish significant fibrosis from no/mild fibrosis in children with NAFLD



**Figure 1** Receiver operating characteristic curves for the noninvasive hepatic fibrosis scoring systems used to diagnose clinically significant fibrosis (stage 2-3) in children with non-alcoholic fatty liver disease. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease.

by univariate analysis, although the result of multivariate analysis suggested age and ALP as possible prognostic factors that predict significant hepatic fibrosis.

Regarding the clinical markers in children with NAFLD, 1 multicenter study reached the same conclusion as our study, revealing no significantly different clinical parameters for significant fibrosis (stage 2 or more), and the serum AST level was the only biochemical parameter associated with the severity of hepatic fibrosis<sup>[9]</sup>. In contrast, another pediatric study reported that BMI was the only clinical parameter that significantly differentiated NAFLD with hepatic fibrosis from NAFLD without fibrosis, and there were no biochemical parameters predictive of hepatic fibrosis<sup>[10]</sup>. From these results, it appears that more developed noninvasive tools beyond simple parameters are needed to detect hepatic fibrosis in children suspicious of NAFLD.

To this point, a number of noninvasive hepatic fibrosis scores have been developed and applied to chronic liver diseases, such as hepatitis B, hepatitis C, alcoholic fatty

liver disease, and NAFLD<sup>[22,23]</sup>. These scores include indices based on indirect biochemical markers and/or clinical parameters developed for adults, such as the AST/ALT ratio<sup>[10]</sup>, APRI<sup>[17]</sup>, PGA index<sup>[18]</sup>, Forns index<sup>[19]</sup>, FIB-4<sup>[20]</sup>, and NAFLD fibrosis score<sup>[21]</sup>. These scores have been validated in a number of previously published studies in adults<sup>[22]</sup>, but no validation studies have been performed in children with NAFLD, excluding the AST/ALT ratio, which did not differentiate hepatic fibrosis from no fibrosis in children<sup>[10]</sup>.

In our study, only APRI and FIB4 exhibited statistically significant differences between patients with mild fibrosis and those with significant fibrosis among these noninvasive scores. Regarding APRI, no studies have been reported in children with NAFLD. However, a study on APRI in children with chronic viral hepatitis revealed that the AUROC of APRI was 0.71 for hepatic fibrosis, and the sensitivity and the specificity of APRI were 47% and 90%, respectively, at the cutoff of 0.5<sup>[24]</sup>. In addition, another study comparing APRI with FibroScan and FibroTest in children with chronic liver disease reported the AUROC of APRI to be 0.73<sup>[25]</sup>. In our study on children with NAFLD, the AUROC of APRI was 0.70, which was similar to those of previous studies on children with chronic liver diseases.

FIB4, comprising age in addition to AST, ALT, and platelet counts, also differentiated significant hepatic fibrosis from mild fibrosis in our study, with the highest AUROC of 0.81. In previous studies on FIB4 in adult patients with NAFLD, the AUROCs of FIB4 were 0.802 and 0.86, respectively, which were higher than those of the AST/ALT ratio, APRI, NAFLD fibrosis score, and the BMI, AST/ALT ratio, diabetes score in both studies<sup>[26,27]</sup>. FIB4 was significant in distinguishing advanced fibrosis (stage 3 to 4) from mild fibrosis (stage 0 to 2) in these 2 studies<sup>[26,27]</sup>. These results were similar to those of our study, indicating that FIB4 had the highest AUROC among a variety of hepatic fibrosis scores using standard laboratory tests. Thus, FIB4 also appears to be a desirable noninvasive hepatic fibrosis score, even in children, with the application of age-appropriate cutoffs.

Up to date, PNFI was the only noninvasive hepatic fibrosis scoring system developed for children with NAFLD, which revealed the AUROC for significant fibrosis (stage 2 to 3) was 0.663<sup>[28]</sup>, and according to previous studies, PNFI was significant in differentiating children with hepatic fibrosis from children without fibrosis<sup>[12,28]</sup>. However, in our study, PNFI failed to distinguish significant hepatic fibrosis from no/mild fibrosis in children with the lowest AUROC of 0.41.

Because histopathologic findings of pediatric NASH are to some extent distinct from adult NASH<sup>[4,5]</sup>, the application of noninvasive hepatic fibrosis scores to children with NAFLD should be considered from a different point of view. More validation studies may be required in the future to apply noninvasive hepatic fibrosis scoring systems to clinical fields in pediatric population.

In conclusion, it appears that no single clinical or labo-

ratory parameter can reflect the presence of hepatic fibrosis or the severity of fibrosis in children with NAFLD. Therefore, APRI and FIB4 might be regarded as useful noninvasive methods to evaluate hepatic fibrosis even in children with NAFLD.

## COMMENTS

### Background

Although liver biopsy is the gold standard for the diagnosis of nonalcoholic fatty liver disease (NAFLD), it has some limitations in children because of its invasiveness and high costs, and moreover, the histopathologic findings of pediatric nonalcoholic steatohepatitis (NASH) are somewhat different from adult NASH. Thus, noninvasive methods to predict hepatic fibrosis are required in children suspicious of NAFLD.

### Research frontiers

The application of noninvasive hepatic fibrosis scores to detect liver fibrosis may be useful in detecting and predicting significant hepatic fibrosis in pediatric NAFLD.

### Innovations and breakthroughs

There have been only limited studies of noninvasive markers or hepatic fibrosis scores of hepatic fibrosis related to NAFLD in pediatric populations. This is the first study applying noninvasive clinical or biochemical markers and various noninvasive hepatic fibrosis scores together to the detection of hepatic fibrosis. Our study suggests that AST/platelet ratio index and FIB4, among hepatic fibrosis scores, might be useful to detect hepatic fibrosis even in children with NAFLD.

### Applications

The study may represent a future diagnostics for NAFLD in pediatric population, suggesting APRI and FIB4 as noninvasive tools to evaluate hepatic fibrosis in children possibly instead of invasive liver biopsy.

### Terminology

NAFLD is a form of chronic liver disease with histopathologic features of alcohol-induced liver disease occurs in persons who do not consume a significant amount of alcohol. Because the main etiology of NAFLD is obesity, the prevalence of NAFLD is increasing in children as the prevalence of pediatric obesity is increasing.

### Peer review

In this study, authors investigate several non-invasive scores for liver fibrosis in children with established and histology-proven NAFLD. This study is of interest, as today only view reports about the diagnostic accuracy of these scores in children are available.

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## Clinical implication of 14-3-3 epsilon expression in gastric cancer

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examined by real-time quantitative RT-PCR in gastric tumors and their matched non-neoplastic gastric tissue samples.

**RESULTS:** Authors observed a significant reduction of 14-3-3 $\epsilon$  protein expression in gastric cancer (GC) samples compared to their matched non-neoplastic tissue. Reduced levels of 14-3-3 $\epsilon$  were also associated with diffuse-type GC and early-onset of this pathology. Our data suggest that reduced 14-3-3 $\epsilon$  may have a role in gastric carcinogenesis process.

**CONCLUSION:** Our results reveal that the reduced 14-3-3 $\epsilon$  expression in GC and investigation of 14-3-3 $\epsilon$  interaction partners may help to elucidate the carcinogenesis process.

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**Key words:** Gastric cancer; 14-3-3 epsilon; YWHAE; Gene expression; Protein expression

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### Abstract

**AIM:** To evaluate for the first time the protein and mRNA expression of 14-3-3 $\epsilon$  in gastric carcinogenesis.

**METHODS:** 14-3-3 $\epsilon$  protein expression was determined by western blotting, and mRNA expression was

### INTRODUCTION

Although gastric cancer (GC) rates have decreased substantially in most parts of the world, it is still the fourth most frequent cancer type and the second highest cause of cancer mortality worldwide. A total of 989 600 new

stomach cancer cases and 738 000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths by cancer<sup>[1]</sup>.

About 90% of stomach tumors are adenocarcinomas<sup>[2]</sup>. However, the etiology and disease evolution may vary among populations, primary tumor location, histological subtypes of adenocarcinoma, and other variables<sup>[3]</sup>.

GC, as with other neoplasms, is a multifactorial disease that results from a combination of environmental factors and accumulation of generalized and specific genetic and epigenetic alterations. Chromosomal instability is characterized by changes in chromosome copy number (aneuploidy) and alterations in chromosomal regions, which may induce oncogene activation, tumor suppressor gene inactivation, or both<sup>[4]</sup>. The chromosomal aberrations that are constantly found in GC include gains of 3q, 7p, 7q, 8q, 13q, 17q, 20p and 20q and losses of 4q, 9p, 17p and 18q (for a review, see<sup>[5]</sup>). Our research group previously reported that the loss of one copy of *TP53* locus (17p13) is commonly found in gastric tumors of individuals from a Brazilian population<sup>[6]</sup>, as well as in GC cell lines<sup>[7-9]</sup>. Although *TP53* is a key tumor suppressor gene in the carcinogenesis process<sup>[10]</sup>, additional genes at 17p13 may play a role in gastric carcinogenesis.

The *YWHAE* gene is located at 17p13.3 and encodes the 14-3-3 $\epsilon$  protein, one of the mammalian 14-3-3 protein family members that are highly conserved in eukaryotes. There are at least seven distinct *14-3-3* genes in vertebrates, giving rise to nine isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\sigma$  and  $\tau/\upsilon$ , with  $\alpha$  and  $\delta$  being phosphorylated forms of  $\beta$  and  $\zeta$ , respectively)<sup>[11,12]</sup>. The 14-3-3 proteins are predominantly dimeric within the cell and bind either to multiple sites within single proteins or act as a bridge between two targets<sup>[13-15]</sup>. Up to now, > 300 proteins have been reported to interact with 14-3-3 proteins, including key signaling components, such as p53, Raf-1 kinase, Bcl-2 antagonist of cell death, protein kinase C, phosphatidylinositol 3-kinase, and cdc25 phosphatase (*RASGRF1*)<sup>[12,13,15,16]</sup>. Although the exact 14-3-3 protein functions are not fully known, these proteins may act as a molecular scaffold, bringing together proteins that interact functionally and effecting phosphorylation-dependent cell regulation<sup>[12]</sup>. This protein family is involved in several biological processes and plays a regulatory role in processes such as apoptotic cell death, mitogenic signal transduction, and cell cycle control<sup>[13,17,18]</sup>.

The isoform 14-3-3 $\epsilon$  is the most highly conserved member of the 14-3-3 family, with conserved sequence in plants, yeast, and mammals<sup>[19,20]</sup>. Abnormal expression of 14-3-3 $\epsilon$  has been found in some types of cancers. However, the role of 14-3-3 $\epsilon$  in the carcinogenesis process is ambiguous and contradictory. Low expression of 14-3-3 $\epsilon$  occurs in small cell lung cancer<sup>[21]</sup>, laryngeal squamous cell carcinoma<sup>[22]</sup>, and medulloblastoma<sup>[23]</sup>, which suggest its role as a tumor suppressor gene. On the other hand, high expression of 14-3-3 $\epsilon$  has been detected in renal carcinoma<sup>[24]</sup>, astrocytoma<sup>[25]</sup>, meningioma<sup>[26]</sup> and subependymomas<sup>[27]</sup>, and, thus, probably it acts as an oncogene.

To the best of our knowledge, no study has evaluated

the role of 14-3-3 $\epsilon$  in gastric carcinogenesis until now. In the present study, we analyzed the *14-3-3 $\epsilon$*  gene and protein expression in GC and matched non-neoplastic gastric samples. We also evaluated the possible associations between 14-3-3 $\epsilon$  and clinicopathological characteristics.

## MATERIALS AND METHODS

### Tissue samples

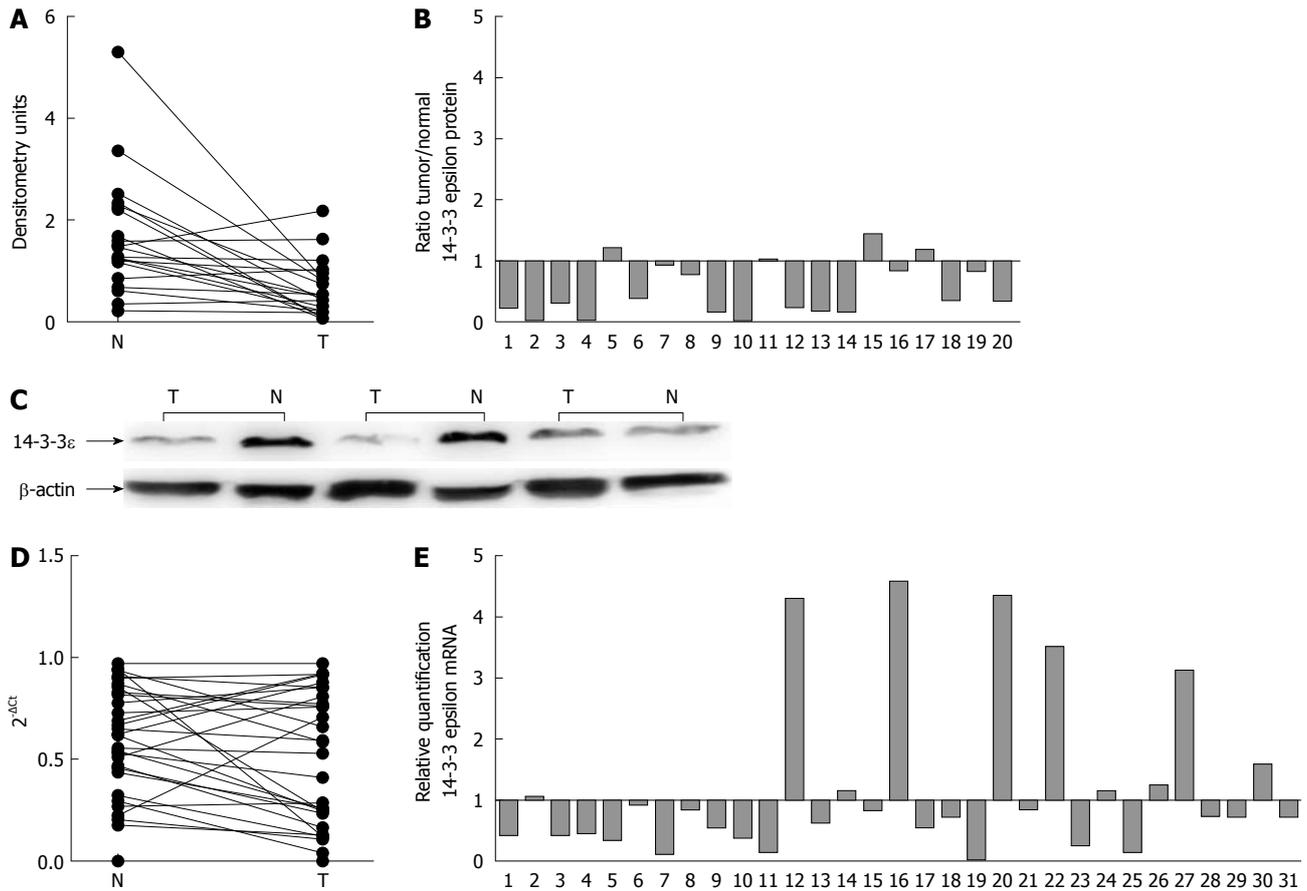
14-3-3 $\epsilon$  protein expression was evaluated in 20 pairs of GC samples and corresponding non-neoplastic gastric tissues (distant location of primary tumor). The mRNA expression was evaluated in 31 pairs of GC samples and corresponding non-neoplastic gastric tissues. Dissected tumor and paired non-neoplastic tissue specimens were immediately cut from stomach samples, frozen in liquid nitrogen, and stored at -80 °C until use for protein and RNA extraction. All the gastric samples were obtained surgically from João de Barros Barreto University Hospital (HUIBB) in Pará State, Northern Brazil. Informed consent with approval of the ethics committee of HUIBB was obtained. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there was no other co-occurrence of diagnosed cancers. All samples were classified according to Laurén<sup>[28]</sup> and tumors were staged using standard criteria by TNM staging<sup>[29]</sup>.

### Protein and mRNA purification

Total protein and total mRNA were simultaneously isolated from gastric tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To allow greatest solubilization, the protein pellet was dissolved in buffer containing 7 mol urea, 2 mol thiourea, 4% CHAPS, 50 mmol dithiothreitol, 1% Protease Inhibitor Cocktail (Sigma, St Louis, MO, United States), and 0.5% of each Phosphatase Inhibitor Cocktail 1 and 2 (Sigma-Aldrich, St Louis, MO, United States). Protein concentration was determined by the method of Bradford (Sigma-Aldrich). RNA concentration and quality were determined using a NanoDrop spectrophotometer (Kisker, Germany) and 1% agarose gels. Samples were stored at -80 °C until use.

### 14-3-3 $\epsilon$ expression by western blotting

Reduced protein (30  $\mu$ g) of each sample was separated on 12.5% homogeneous SDS-PAGE gel and electroblotted to a polyvinylidene fluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The PVDF membrane was blocked with PBS containing 0.1% Tween 20, 5% low-fat milk, and incubated overnight at 4 °C with corresponding primary antibodies to anti-14-3-3 $\epsilon$  (sc-31962, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, United States) and anti- $\beta$ -actin (Ac-74, 1:3000; Sigma-Aldrich). After extensive washing, a peroxidase-conjugated secondary antibody was incubated for 1 h at room temperature. Immunoreactive bands were visualized using western blotting Luminol reagent and the images were acquired using an ImageQuant 350 digital image system (GE Healthcare, Uppsala, Sweden). The  $\beta$ -actin was used as a loading reference control.



**Figure 1** Expression of 14-3-3ε in tumor and non-neoplastic gastric tissue. A: Protein expression normalized by β-actin (ACTB); B: Ratio of protein expression between tumor and matched non-neoplastic gastric tissues; C: Western blotting using anti-14-3-3ε and anti-β-actin antibodies; D: mRNA expression normalized by the internal controls ACTB and glyceraldehyde-3-phosphate dehydrogenase; E: Relative mRNA quantification-gastric tumor samples normalized by matched non-neoplastic gastric tissues. T: Tumor samples; N: Non-neoplastic samples.

### 14-3-3ε mRNA expression by real-time quantitative RT-PCR

First, cDNA was synthesized using High-Capacity cDNA Archive kit (Applied Biosystems, Warsaw, Poland) according to the manufacturer's protocol. All real-time qRT-PCR reactions were performed in triplicate for both target gene (YWHAE: Hs00356749\_g1, Applied Biosystems, United States) and internal controls (β-actin: Hs03023943\_g1; glyceraldehyde-3-phosphate dehydrogenase: Hs99999905\_m1; Applied Biosystems, United States). To compare 14-3-3ε mRNA expression between GC and non-neoplastic gastric samples, we converted  $\Delta\text{Ct}$  ( $\Delta\text{Ct} = \text{Ct of YWHAE} - \text{Ct of internal controls}$ ) to linear form ( $2^{-\Delta\text{Ct}}$ ). Relative quantification of the gene expression was calculated according to Pfaffl method<sup>[30]</sup>. Non-neoplastic gastric samples were designated as a calibrator of each paired tumor sample.

### Statistical analysis

We first evaluated the normal distribution of all data using the Shapiro-Wilk normality test to determine subsequent use of appropriate tests for statistical comparison. Since 14-3-3ε mRNA and protein data did not present with a normal distribution, we performed parametric tests with bootstrapping, a re-sampling method. The re-sampling methods are relatively powerful and can control

a type I error (false positive), reducing over-fit bias and internally validating the accuracy estimates. Bootstrapping methods also produce confidence intervals (CIs) around the observed effects. Paired *t* test was performed to compare the mean of 14-3-3ε expression between neoplastic and matched non-neoplastic samples. The associations between clinicopathological parameters and the mean of 14-3-3ε expression were assessed using a *t* test for independent samples. The correlation among the 14-3-3ε mRNA and protein expression was analyzed by Pearson test. All the analyses performed in this article were based on 1000 bootstrap samples. In all analyses, the CI was 95% and  $P < 0.05$  was considered significant.

## RESULTS

14-3-3ε protein expression was significantly reduced in GC samples (densitometry units:  $0.656 \pm 0.552$ ) compared to matched non-neoplastic gastric samples ( $1.656 \pm 1.158$ ) ( $P = 0.005$ , 95% CI: -1.59 to -0.53). 14-3-3ε protein was 1.5-fold lower in 60% of GC samples compared to their paired non-neoplastic gastric tissues. However, 14-3-3ε mRNA expression did not differ between GC ( $2^{-\Delta\text{Ct}}$ :  $0.492 \pm 0.325$ ) and corresponding non-neoplastic gastric tissue ( $0.579 \pm 0.272$ ) ( $P = 0.075$ , 95% CI: -0.18 to 0.003) (Figure 1). Although mRNA and protein were simultaneously purified,

Table 1 Clinicopathological characteristics and 14-3-3 $\epsilon$  expression in gastric cancer samples (mean  $\pm$  SD)

Variable	14-3-3 $\epsilon$ protein expression				14-3-3 $\epsilon$ mRNA expression			
	Total	Ratio T/N	Adjusted <i>P</i> -value	95% CI	Total	Relative quantification	Adjusted <i>P</i> -value	95% CI
Gender								
Male	10	0.418 $\pm$ 0.41	0.210	-0.13 to 0.63	18	1.404 $\pm$ 1.43	0.279	-1.38 to 0.40
Female	10	0.667 $\pm$ 0.47			13	0.898 $\pm$ 1.09		
Onset (yr)								
< 45	6	0.303 $\pm$ 0.26	0.052	-0.68 to -0.05	8	0.583 $\pm$ 0.31	0.028 <sup>a</sup>	-1.49 to -0.25
$\geq$ 45	14	0.646 $\pm$ 0.48			23	1.403 $\pm$ 1.45		
Tumor location								
Cardia	3	0.293 $\pm$ 0.11	0.067	0.03 to 0.56	4	0.796 $\pm$ 0.35	0.177	-0.12 to 1.23
Non-cardia	17	0.587 $\pm$ 0.47			27	1.250 $\pm$ 1.39		
Laurén classification								
Diffuse-type	4	0.244 $\pm$ 0.14	0.024 <sup>a</sup>	0.12 to 0.65	13	1.119 $\pm$ 1.04	0.785	-0.69 to 1.07
Intestinal-type	16	0.618 $\pm$ 0.46			18	1.245 $\pm$ 1.49		
Stage								
Early	4	0.517 $\pm$ 0.64	0.921	-0.55 to 0.81	4	0.549 $\pm$ 0.38	0.046 <sup>a</sup>	-1.41 to -0.16
Advanced	16	0.549 $\pm$ 0.41			27	1.287 $\pm$ 1.37		
Tumor invasion								
T1/T2	8	0.491 $\pm$ 0.49	0.674	-0.48 to 0.34	9	0.642 $\pm$ 0.33	0.052	-1.40 to -0.13
T3/T4	12	0.578 $\pm$ 0.43			22	1.417 $\pm$ 1.49		
Lymph node metastasis								
Absent	6	0.672 $\pm$ 0.57	0.510	-0.29 to 0.72	8	0.687 $\pm$ 0.53	0.069	-1.31 to -0.01
Present	14	0.488 $\pm$ 0.39			23	1.367 $\pm$ 1.45		
Distant metastasis								
Unknown/absent	16	0.557 $\pm$ 0.47	0.754	-0.42 to 0.47	23	0.978 $\pm$ 0.99	0.242	-2.25 to 0.51
Present	4	0.486 $\pm$ 0.37			8	1.807 $\pm$ 1.89		

Differentially expressed between groups, <sup>a</sup>*P* < 0.05. T: Tumor gastric samples; N: Non-neoplastic gastric samples.

no correlation was observed between 14-3-3 $\epsilon$  mRNA and protein expression (*R* = 0.236, *P* = 0.345).

The associations between clinicopathological characteristics and 14-3-3 $\epsilon$  expression are summarized in Table 1. A significant decrease in 14-3-3 $\epsilon$  mRNA level was observed in early-onset GC (at age  $\leq$  45 years<sup>[31]</sup>) compared to late-onset GC (*P* = 0.028, 95% CI: -1.49 to -0.25). Moreover, a tendency towards 14-3-3 $\epsilon$  protein down-expression was also observed in early-onset compared to late-onset GC (*P* = 0.052, 95% CI: -0.69 to -0.05).

The 14-3-3 $\epsilon$  mRNA level was reduced in early GC compared to advanced GC (*P* = 0.046, 95% CI: -1.41 to -0.16) and tended to present reduced levels with less invasive tumors (*P* = 0.052, 95% CI: -1.40 to -0.13). In contrast, these observations were not observed for protein level.

Concerning the 14-3-3 $\epsilon$  protein, we observed that the diffuse-type GC presented reduced expression compared to intestinal-type GC (*P* = 0.024, 95% CI: 0.12-0.65). However, a significant difference was observed between non-neoplastic gastric tissues and intestinal-type GC (*P* = 0.045, 95% CI: -1.55 to -0.29) and diffuse-type GC (*P* = 0.024, 95% CI: -2.22 to -1.14).

## DISCUSSION

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis<sup>[32]</sup>. The 14-3-3 proteins continue to generate intense interest due to their roles in signal transduction pathways that control

cell cycle checkpoints, mitogen-activate protein kinase activation, apoptosis, and regulation of gene expression. 14-3-3 stabilizes non-native conformations of bound ligands to promote their interactions with downstream targets, or facilitates their subsequent modification by kinases and phosphatases<sup>[18]</sup>.

In the present study, we evaluated the 14-3-3 $\epsilon$  mRNA and protein expression in gastric carcinogenesis. To the best of our knowledge, no study has evaluated 14-3-3 $\epsilon$  expression in this neoplasm. Here, we observed that 14-3-3 $\epsilon$  protein expression was reduced in GC samples compared to matched non-neoplastic gastric tissue. Low expression of 14-3-3 $\epsilon$  has also been reported in small cell lung cancer<sup>[21]</sup>, laryngeal squamous cell carcinoma<sup>[22]</sup>, and medulloblastoma<sup>[23]</sup>, suggesting a tumor suppressor function. The reduced 14-3-3 $\epsilon$  expression may be in part due to the loss of its locus (17p13), which is a common finding in gastric tumors of individuals from the studied population<sup>[6]</sup>.

Little is known about the role of 14-3-3 $\epsilon$  in carcinogenesis. One of the major partners of 14-3-3 $\epsilon$  is the CDC25 protein. CDC25 protein is virtually inactive during interphase, but undergoes a strong activation at mitosis due to phosphorylation of its N-terminal regulatory domain<sup>[33]</sup>. 14-3-3 $\epsilon$  acts as a negative regulator of CDC25<sup>[33,34]</sup>. The overexpression of CDC25 proteins has previously been described in GC<sup>[35-37]</sup>. Thus, the reduced expression of 14-3-3 $\epsilon$  in GC cells may enhance the ability of CDC25 to induce mitosis, contributing to the gastric carcinogenesis process.

Several classification systems have been described for

GC. According to the Laurén classification, one of the most used, gastric adenocarcinoma is classified mainly into intestinal and diffuse types<sup>[28]</sup>. Intestinal-type GC progresses through a number of sequential steps, beginning with atrophic gastritis followed by intestinal metaplasia, intraepithelial neoplasia, and carcinoma<sup>[38]</sup>. In contrast, diffuse-type GC generally does not evolve from precancerous lesions<sup>[39,40]</sup>. In the present study, we observed that the expression of 14-3-3 $\epsilon$  protein was lower in diffuse-type than in intestinal-type GC, confirming that these two histological GC subtypes follow different genetic pathways and may be two distinct entities<sup>[39]</sup>.

MYC deregulation is a frequent finding in GC<sup>[41]</sup>. Moreover, MYC immunoreactivity seems to be more frequently detected in intestinal-type than diffuse-type GC<sup>[42,43]</sup>. Gene amplification is the main mechanism of MYC deregulation in GC<sup>[41]</sup>, and we have previously described a higher frequency of *MYC* locus amplification in intestinal-type than diffuse-type GC<sup>[44,45]</sup>. Interestingly, one of the MYC target proteins is CDC25, which is negatively regulated by 14-3-3 $\epsilon$ . A correlation between MYC and CDC25 has previously been reported in GC<sup>[46]</sup>. Here, we hypothesize that the relative increase of 14-3-3 $\epsilon$  in the subset of tumors of the intestinal-type may be a compensatory mechanism to control the increase of cell proliferation due to MYC and CDC25 action. Moreover, MYC overexpression also induces the production of reactive oxygen species, which may lead to double-stranded DNA breaks and point mutations resulting in genomic instability<sup>[47]</sup>. Thus, the relative increase in 14-3-3 $\epsilon$  in the intestinal-type GC may be also a compensatory response to other oncogenic mutations accumulated in cancer cells, which lead to genomic instability and initiate a DNA damage check-point in cells that contain some functional p53 alleles, as already proposed for other 14-3-3 isoforms<sup>[18]</sup>.

Here, we also described a decrease in 14-3-3 $\epsilon$  expression in early-onset compared to late-onset GC. Early-onset GC is observed in < 10% of GC patients, and only 10% of these patients have a positive family history<sup>[31]</sup>. Most young patients present at an advanced clinical stage similar to elderly patients, so the prognosis in both age groups is poor. However, early-onset GC shows different clinicopathological and molecular profiles compared to late-onset GC, suggesting that they represent a separate entity within gastric carcinogenesis, with genetic factors probably presenting a more important role in early-onset GC patients<sup>[48-50]</sup>. 14-3-3 $\epsilon$  deregulation may have a direct or indirect function in early-onset GC because this protein interacts with several others.

In addition, no correlation was observed between 14-3-3 $\epsilon$  mRNA and protein expression, corroborating a previous study of laryngeal squamous cell carcinoma<sup>[22]</sup>. The lack of correlation between 14-3-3 $\epsilon$  protein and mRNA expression patterns indicates the post-translational regulation mechanism involved in this protein expression, and highlights the complexity of the relationship between protein and mRNA expression.

In conclusion, our data suggest that, for the first time, reduced 14-3-3 $\epsilon$  may have a role in gastric carcinogenesis, mainly in diffuse-type and early-onset GC. Moreover, further investigations are necessary to understand which proteins interact with 14-3-3 $\epsilon$  in these subtypes of GC.

## COMMENTS

### Background

Gastric cancer (GC) is the fourth most frequent cancer type and the second highest cause of cancer mortality worldwide. Although this neoplasm is a serious public health problem due to its high incidence and mortality, little is known about the molecular events involved in gastric carcinogenesis.

### Research frontiers

GC, as with other neoplasms, is a multifactorial disease that results from a combination of environmental factors and the accumulation of generalized and specific genetic and epigenetic alterations. The 14-3-3 protein family has been recently associated with carcinogenesis, but not in the stomach. In this study, the authors evaluated mRNA and protein expression of 14-3-3 $\epsilon$  in gastric neoplasms and corresponding non-neoplastic samples.

### Innovations and breakthroughs

No previous study has evaluated the gene and protein expression of 14-3-3 $\epsilon$  in gastric carcinogenesis. This study demonstrates that 14-3-3 $\epsilon$  has a role in gastric carcinogenesis, especially in diffuse-type and early-onset GC.

### Applications

14-3-3 $\epsilon$  may have a role in gastric carcinogenesis as a tumor suppressor protein.

### Terminology

The *YWHAE* gene is located at 17p13.3, a region frequently deleted in gastric neoplasms. This gene encodes the 14-3-3 $\epsilon$  protein, one of the mammalian 14-3-3 protein family members that are highly conserved in eukaryotes. This protein family is involved in several biological processes and plays a regulatory role in processes such as apoptotic cell death, mitogenic signal transduction, and cell cycle control.

### Peer review

The results report for the first time the expression of 14-3-3 $\epsilon$  in GC, both at the protein and mRNA level, and the data obtained reveal reduced 14-3-3 $\epsilon$  protein expression in GC. The data contribute to the biology of GC with possible future clinical implications to be tested in a subsequent study in a larger number of GC patients.

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## Pancreatic schwannoma: Case report and an updated 30-year review of the literature yielding 47 cases

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### Abstract

Pancreatic schwannomas are rare neoplasms. Authors briefly describe a 64-year-old female patient with cystic pancreatic schwannoma mimicking other cystic tumors and review the literature. Databases for PubMed were searched for English-language articles from 1980 to 2010 using a list of keywords, as well as references from review articles. Only 41 articles, including 47 cases, have been reported in the English literature. The mean age was 55.7 years (range 20-87 years), with 45% of patients being male. Mean tumor size was 6.2 cm (range 1-20 cm). Tumor location was the head (40%), head and body (6%), body (21%), body and tail (15%), tail (4%), and uncinate process (13%). Thirty-four percent of patients exhibited solid tumors and 60% of patients exhibited cystic tumors. Treatment included pancreaticoduodenectomy (32%), distal pancreatectomy (21%), enucleation (15%), unresectable (4%), refused operation (2%) and the detail of resection was not specified in 26% of patients. No patients died of disease with a mean follow-up of 15.7 mo (range 3-65 mo), although

5 (11%) patients had a malignancy. The tumor size was significantly related to malignant tumor ( $13.8 \pm 6.2$  cm for malignancy vs  $5.5 \pm 4.4$  cm for benign,  $P = 0.001$ ) and cystic formation ( $7.9 \pm 5.9$  cm for cystic tumor vs  $3.9 \pm 2.4$  cm for solid tumor,  $P = 0.005$ ). The preoperative diagnosis of pancreatic schwannoma remains difficult. Cystic pancreatic schwannoma should be considered in the differential diagnosis of cystic neoplasms and pseudocysts. In our case, intraoperative frozen section confirmed the diagnosis of a schwannoma. Simple enucleation may be adequate, if this is possible.

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**Key words:** Pancreatic schwannoma; Pancreas; Schwannoma; Neurinoma; Resection; Imaging; Enucleation; Prognosis; Cystic

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### INTRODUCTION

Pancreatic schwannomas are rare neoplasms that originate from Schwann cells. The Schwann cells line the nerve sheath and can generate either schwannoma or neurofibroma<sup>[1]</sup>. Schwannoma usually occur in the extremities, but can also be found in the trunk, head and neck, retroperitoneum, mediastinum, pelvis and rectum<sup>[2,3]</sup>. Pancre-

atic schwannomas are even more unusual neoplasms that affect adults with an equal gender distribution<sup>[4,5]</sup>. These tumors vary considerably in size and approximately two-thirds are reported to undergo degenerative changes including cyst formation, calcification, hemorrhage, hyalinization and xanthomatous infiltration<sup>[4,5]</sup>. As a result, they may radiographically mimic cystic pancreatic lesions (e.g., mucinous cystic neoplasms, solid and pseudopapillary neoplasms, serous cystic neoplasms, and pseudocysts).

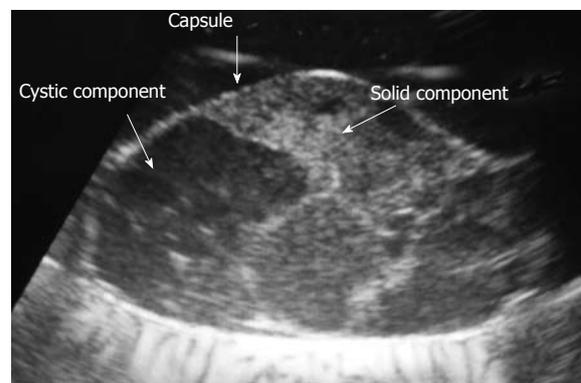
Only 47 cases have been reported in the English literature in the last three decades<sup>[2-42]</sup>. In this report, we present a case of pancreatic schwannoma and provide a pertinent review of literature with emphasis on clinical presentation, diagnosis, treatment options, and outcome.

## CASE REPORT

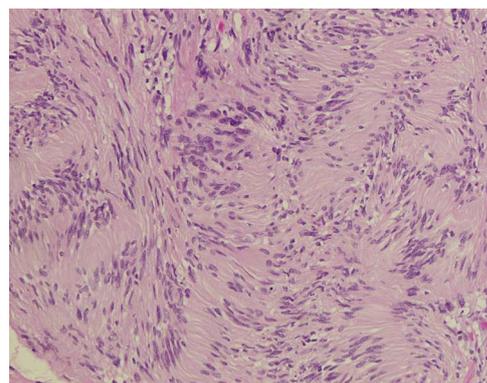
A 64-year-old previously healthy woman was incidentally discovered to have a cystic tumor in the pancreas during an ultrasound examination for a health check. She was referred to our institution for further investigation. The abdominal physical examination did not detect any marked finding and all laboratory data were normal, including tumor markers. The computed tomography (CT) scan demonstrated a well-encapsulated tumor, which was composed of solid and cystic areas (Figure 1), and neither liver mass nor peripancreatic lymph node swelling was detected. Magnetic resonance imaging (MRI) showed a mass, with hypointensity on T1-weighted images and hyperintensity on T2-weighted images. Magnetic resonance cholangiopancreatography (MRCP) showed a hyperintense mass in the pancreatic head with no dilatation of the main pancreatic duct. Endoscopic retrograde cholangiopancreatography revealed no communication between cystic tumor and pancreatic duct. Endoscopic ultrasonography showed that the tumor was composed of cystic part and solid part. We performed surgery under the diagnosis of cystic tumor of the pancreas as mucinous cystic tumor, solid pseudo papillary tumor or gastrointestinal stromal tumor. The laparotomy disclosed a well-encapsulated 4-cm mass in the uncinate process of the pancreas that had no signs of inflammation. Intraoperative ultrasound confirmed a solitary mass composed of solid and cystic components (Figure 2). The mass was enucleated and an intraoperative frozen section demonstrated a benign schwannoma. No further resection was performed based on these findings. The tumor was 4 cm × 4 cm × 3 cm in size, and was composed of a mixture of solid and hemorrhage areas. On microscopic examination, the tumor was composed of spindle cells strongly positive for S-100 proteins and foci of hemorrhage (hematoxylin and eosin, × 100) (Figure 3). The tumor cells were negative for smooth muscle actin and CD-34. The tumor was therefore histologically diagnosed as benign schwannoma. The patient was discharged uneventfully on postoperative day 17. At a 65-month follow up after resection, the patient is doing well without any recurrent disease.



**Figure 1** Contrast-enhanced computed tomography scan obtained in the arterial phase showing a multilocular cystic mass in the uncinate process of the pancreas. No pancreatic ductal dilatation or invasion into adjacent arteries or portal vein are identified.



**Figure 2** Intraoperative ultrasound showing the well-encapsulated pancreatic mass that is composed of solid and cystic components.



**Figure 3** Microscopic examination demonstrating spindle cells without nuclear atypism (HE × 100). Immunohistochemical staining for S-100 protein was positive. HE: Hematoxylin and eosin.

## DISCUSSION

A review of the patient's chart was performed along with a review of English-language articles using a PubMed search for the last three decades. We found 41 articles including 47 patients with pancreatic schwannoma. Details of the cases are summarized in Table 1 along with the current patient. Table 2 summarizes the important

Table 1 Summary of cases with pancreatic schwannoma

Author	Year	Sex	Age	Presenting symptoms	Size (cm)	Location	Solid/cystic by image	Treatment	Histology	Follow-up (mo)
Current case	2010	F	64	Asymptomatic	4.0	Uncinate	Solid and cystic	Enucleation	Benign	65
Dorsey <i>et al</i> <sup>[6]</sup>	2010	F	54	Abdominal pain, weight loss	1.4	Head	Solid	NA	Benign	NA
Stojanovic <i>et al</i> <sup>[7]</sup>	2010	F	24	Abdominal pain, dyspepsia, weight loss, palpable tumor	18.0	Body/tail	Cystic	DP + transverse colon	Malignant	28
Suzuki <i>et al</i> <sup>[8]</sup>	2010	F	66	Asymptomatic	3.0	Body	Solid and cystic	DP	Benign	24
Aggarwal <i>et al</i> <sup>[9]</sup>	2010	M	20	Upper abdominal discomfort	3.0	Head	NA	Enucleation	Benign	NA
Ohshima <i>et al</i> <sup>[10]</sup>	2010	F	32	Back pain	4.0	Head	Solid and cystic	PD	Benign	NA
Mummadi <i>et al</i> <sup>[11]</sup>	2009	M	35	Epigastric pain	7.0	Body	Solid and cystic	NA	Benign	6
Gupta <i>et al</i> <sup>[5]</sup>	2009	F	56	Asymptomatic	8.3	Head/body	Cystic	PD	Benign	NA
Li <i>et al</i> <sup>[12]</sup>	2009	M	37	Asymptomatic	16.0	Head	Solid and Cystic	PD	Benign	NA
Tafe <i>et al</i> <sup>[13]</sup>	2008	M	46	Abdominal pain	11.0	Body/tail	Cystic	DP	Benign	NA
Hirabayashi <i>et al</i> <sup>[14]</sup>	2008	M	51	Asymptomatic	6.0	Tail	Cystic	DP	Benign	NA
Okuma <i>et al</i> <sup>[15]</sup>	2008	F	71	Epigastric pain	4.0	Body	Solid and cystic	DP	Benign	NA
Tofigh <i>et al</i> <sup>[16]</sup>	2008	M	54	Epigastric pain, weight loss, nausea, intermittent jaundice	3.0	Head	Solid (by specimen)	PD	Benign	
Fasanella <i>et al</i> <sup>[17]</sup>	2007	M	36	Abdominal discomfort	3.6	Uncinate	Cystic	NA	Benign	NA
Di Benedetto <i>et al</i> <sup>[18]</sup>	2007	M	42	Asymptomatic	2.5	Body	Solid	DP	Benign	NA
Yu <i>et al</i> <sup>[19]</sup>	2006	M	72	Upper abdominal pain	1.0	Head/body	Solid	NA	Benign	NA
Wu <i>et al</i> <sup>[20]</sup>	2005	M	71	Epigastric pain, decreased appetite	1.5	Head	Cystic	Enucleation	Benign	10
Novellas <i>et al</i> <sup>[21]</sup>	2005	F	46	Asthenia, weight loss, empyema	3.0	Head	Solid	PD	Benign	24
Soumaoro <i>et al</i> <sup>[22]</sup>	2005	F	64	Asymptomatic	2.5	Head	Solid	Enucleation	Benign	24
Bui <i>et al</i> <sup>[23]</sup>	2004	F	69	Abdominal pain	5.0	Head	Solid	Unresectable	NA <sup>1</sup>	NA
Akiyoshi <i>et al</i> <sup>[24]</sup>	2004	F	67	Asymptomatic	5.0	Head	Cystic	PD	Benign	43
Von Dobschuetz <i>et al</i> <sup>[25]</sup>	2004	F	55	Asymptomatic	8.0	Head	Cystic	PD + PV reconstruction	Benign	10
Paranjape <i>et al</i> <sup>[4]</sup>	2004	F	77	Upper abdominal pain, weight loss	3.5	Body	Solid	Enucleation	Benign	3
Tan <i>et al</i> <sup>[26]</sup>	2003	F	46	Right upper quadrant pain	2.2	Head	Solid and Cystic	PD	Benign	NA
Almo <i>et al</i> <sup>[2]</sup>	2001	F	73	Abdominal pain, nausea, vomiting	3.0	Head	Cystic	PD	Benign	17
Almo <i>et al</i> <sup>[2]</sup>	2001	F	47	Abdominal pain, back pain	5.5	Head	Solid	PD	Benign	14
Lee <i>et al</i> <sup>[27]</sup>	2001	F	63	Upper abdominal pain	10.0	Tail	Cystic	DP	Benign	6
Morita <i>et al</i> <sup>[28]</sup>	1999	F	50	Upper abdominal pain	9.5	Body/tail	Cystic	DP	Benign	7
Brown <i>et al</i> <sup>[29]</sup>	1998	M	52	Asymptomatic	5.5	Body	Cystic	Resection <sup>2</sup>	Benign	NA
Brown <i>et al</i> <sup>[29]</sup>	1998	M	69	Asymptomatic	6.0	Head	Cystic	PD	Benign	NA
Hsiano <i>et al</i> <sup>[30]</sup>	1998	F	70	Palpable tumor	18.0	Body/tail	Cystic	Resection <sup>2</sup>	Benign	24
Feldman <i>et al</i> <sup>[31]</sup>	1997	M	63	Asymptomatic	2.5	Body	Solid	Enucleation	Benign	NA
Feldman <i>et al</i> <sup>[31]</sup>	1997	F	54	Abdominal pain	2.0	Uncinate	Solid	Enucleation	Benign	22
Ferrozzi <i>et al</i> <sup>[32]</sup>	1995	M	47	Right-sided abdominal pain	3.5	Body	NA	DP	Benign	48
Ferrozzi <i>et al</i> <sup>[32]</sup>	1995	M	63	Abdominal pain	NA	Body	Cystic	NA	Benign	NA
Ferrozzi <i>et al</i> <sup>[32]</sup>	1995	F	68	Upper abdominal pain	NA	Head/body	Cystic	NA	Benign	6
Sugiyama <i>et al</i> <sup>[33]</sup>	1995	M	41	Asymptomatic	1.5	Uncinate	Cystic	PD	Benign	NA
Steven <i>et al</i> <sup>[34]</sup>	1994	M	59	Asymptomatic	4.0	Uncinate	Solid	PD	Benign	10
Melato <i>et al</i> <sup>[35]</sup>	1993	M	87	Upper abdominal pain	20.0	Body/tail	Cystic	NA	Benign	NA
David <i>et al</i> <sup>[3]</sup>	1993	M	46	Right sided abdominal pain	6.0	Uncinate	Cystic	NA	Benign	NA
Urban <i>et al</i> <sup>[36]</sup>	1992	F	56	Right sided hip pain	4.0	Body	Cystic	DP	Benign	NA
Burd <i>et al</i> <sup>[37]</sup>	1992	M	73	Right upper quadrant abdominal pain	2.0	Body/tail	Solid	NA	Benign	NA
Coombs <i>et al</i> <sup>[38]</sup>	1990	F	74	Anemia, melena	7.0	Head	Solid with necrotic center	NA	Malignant	NA
Liessi <i>et al</i> <sup>[39]</sup>	1990	F	75	Abdominal pain	7.0	Head	Solid	Not resected	Benign	7
Walsh <i>et al</i> <sup>[40]</sup>	1989	F	35	Abdominal pain, melena, anemia	NA	Head	NA	PD	Malignant	24
Eggermont <i>et al</i> <sup>[41]</sup>	1987	F	40	Upper abdominal pain, jaundice, weight loss	10.0	Head	Solid with necrotic center	PD	Malignant	9
Moller-Pederson <i>et al</i> <sup>[42]</sup>	1982	M	60	Back pain, weight loss	20.0	Body/tail	Cystic	Unresectable	Malignant	4

M: Male; F: Female; NA: Not available. <sup>1</sup>Unresectable because of encasing the superior mesenteric artery and the portal vein, although the malignant finding was not confirmed by histopathology; <sup>2</sup>No specific operation documented.

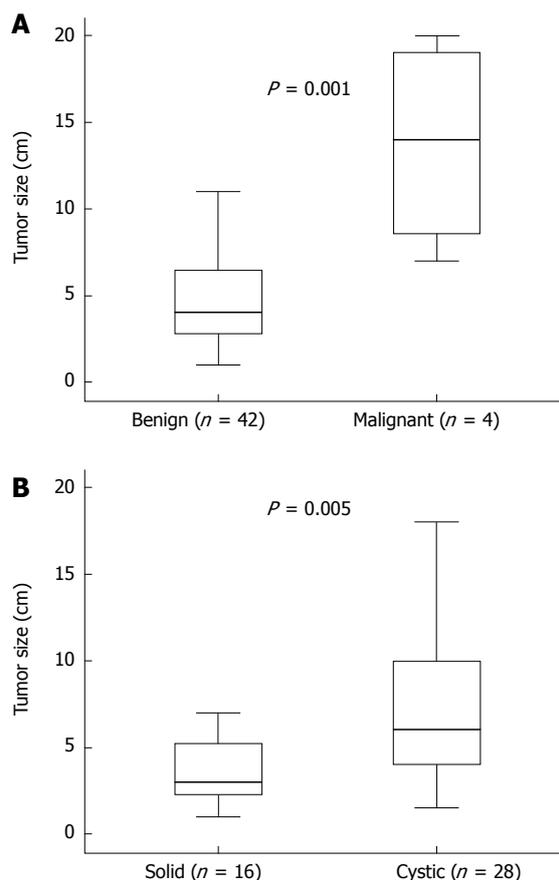
**Table 2** Summary of clinicopathological data from all 47 cases of pancreatic schwannoma

	<i>n</i> (%) or mean $\pm$ SD (range)
Age (yr)	55.7 $\pm$ 15.1 (20-87)
Sex (male/female), (male %)	21/26 (45%)
Symptoms <sup>1</sup>	
Asymptomatic	14 (30%)
Symptomatic	
Abdominal pain	27 (57%)
Weight loss	6 (13%)
Back pain	3 (6%)
Nausea/vomiting	2 (4%)
Abdominal mass	2 (4%)
Anemia	2 (4%)
Melena	2 (4%)
Jaundice	1 (4%)
Location	
Head	19 (40%)
Head/body	3 (6%)
Body	10 (21%)
Body/tail	7 (15%)
Tail	2 (4%)
Uncinate process	6 (13%)
Mean size (cm), ( <i>n</i> = 44)	6.2 $\pm$ 5.1 (1-20)
Operation	
Pancreaticoduodenectomy <sup>2</sup>	15 (32%)
Distal pancreatectomy <sup>3</sup>	10 (21%)
Enucleation	7 (15%)
Unresectable	2 (4%)
Refused	1 (2%)
Not specified	12 (26%)
Histology	
Malignant	5 (11%)
Benign	41 (87%)
Not specified	1 (2%)
Nature of tumor	
Solid	16 (34%)
Cystic	28 (60%)
Not specified	3 (6%)
Mean follow-up months ( <i>n</i> = 23)	18.9 $\pm$ 15.7 (3-65)
Died of disease	0 (0%)

<sup>1</sup>Patients had several symptoms; <sup>2</sup>One patient underwent resection of portal vein; <sup>3</sup>One patient underwent resection of transverse colon.

available facts regarding all patients. We examined the correlation between tumor size and malignancy, as well as tumor size and cystic degeneration. Continuous data are presented as mean  $\pm$  standard deviation and range. Student *t*-test was used for all comparisons among continuous variables. A *P* < 0.05 was considered statistically significant.

A PubMed search of the literature indicated 41 reports including 47 patients with pancreatic schwannoma in the English literature. Details of all the 47 cases are summarized in Table 1. Table 2 summarizes the important available clinicopathological factors. The mean age of the patients was 55.7  $\pm$  15.1 years (range 20-87 years) and the male-female ratio was 21:26. Thirty percent of patients were asymptomatic and 70% of patients were symptomatic. Symptoms included abdominal pain (57%), weight loss (13%), back pain (6%), nausea/vomiting (4%), abdominal mass (4%), melena (4%), and jaundice (4%). The symptoms did not correlate with tumor size and tu-



**Figure 4** Analysis for relation between tumor size and malignant potential and tumor nature (solid or cystic) in all 47 cases of pancreatic schwannoma. A: Relationship between tumor size and malignancy. Larger tumor size is related to malignant tumor (13.8  $\pm$  6.2 cm for malignancy vs 5.5  $\pm$  4.4 cm for benign, *P* = 0.001); B: Relationship between tumor size and nature of tumor. Larger tumor size is related to cystic degeneration (13.8  $\pm$  6.2 cm for cystic tumor vs 5.5  $\pm$  4.4 cm for solid tumor, *P* = 0.005).

mor location. The lesion was located in the pancreas head in 19 patients (40%), head and body in 3 patients (6%), body in 10 patients (21%), body and tail in 7 (15%), tail in 2 patients (4%), and uncinate process in 6 patients (13%). Mean tumor size was 6.2  $\pm$  5.1 cm (range 1-20 cm). Treatment included pancreaticoduodenectomy for 15 patients (32%) including one portal vein reconstruction, distal pancreatectomy for 10 patients (21%) including combined transverse colon resection, enucleation for 7 patients (15%), unresectable for 2 patients (4%), refused operation for 1 patient (2%) and the detail of resection was not specified in 12 patients (26%). Enucleation was performed for 7 patients, and out of these, 3 lesions were located in the head, 2 lesions were in the uncinate process and 2 lesions were in the body. The mean tumor size in the patients who underwent enucleation was 2.7 cm (range 1.5-4.0 cm). Regarding gross appearance, 34% of patients exhibited solid tumors and 60% of patients exhibited cystic tumors. No patient died of disease with a follow-up of 15.7 mo (range 3-65 mo), although 4 (9%) patients had a malignancy. The tumor size was related to malignant tumor (13.8  $\pm$  6.2 cm for malignancy vs 5.5  $\pm$  4.4 cm for benign, *P* = 0.001) (Figure 4A) and cystic for-

mation ( $7.9 \pm 5.9$  cm for cystic tumor *vs*  $3.9 \pm 2.4$  cm for solid tumor,  $P = 0.005$ ) (Figure 4B).

In 1910, Verocay reported a schwannoma as a true neoplasm which originated from Schwann cells, and which did not contain neuroganglion cells<sup>[1]</sup>. Since then, schwannomas have become well known as benign spindle cell tumors derived from Schwann cells that line the nerve sheaths. Schwannomas usually occur in the extremities, but can also be found in the trunk, head and neck, retroperitoneum, mediastinum, pelvis and rectum<sup>[2-4]</sup>. Pancreatic schwannomas are rare neoplasms that arise from either autonomic sympathetic or parasympathetic fibers, both of which course through the pancreas as branches of the vagus nerve<sup>[2-4]</sup>.

Microscopically, a typical schwannoma is composed of 2 areas, namely Antoni A and Antoni B areas. The Antoni A area is hypercellular and characterized by closely packed spindle cells with occasional nuclear palisading and Verocay bodies, whereas the Antoni B area is hypocellular and is occupied by loosely arranged tumor cells<sup>[43]</sup>. Most of the pancreatic schwannomas reported had both Antoni A and Antoni B areas in various proportions. Degenerative or cystic changes such as calcification or hemorrhage are often recognized in the Antoni B area. These changes result from vascular thrombosis and subsequent necrosis<sup>[43]</sup>. Cystic pancreatic schwannomas can mimic the whole spectrum of cystic pancreatic lesions including: intraductal mucinous-papillary neoplasms, mucinous cystic neoplasms, serous cystic neoplasms, solid and pseudo-papillary neoplasms, lymphangiomas, and pancreatic pseudocysts. Immunohistochemically, schwannomas stain strongly positive for S-100 protein, vimentin and CD 56, while negative for other tumor markers including cytokeratin AE1/AE3, desmin, smooth muscle myosin, CD 34 and CD 117<sup>[43]</sup>.

The symptoms of the reported patient cases of pancreatic schwannoma vary. Seventy percent of patients were symptomatic. Abdominal pain was the most common symptom reported (57%). Symptoms such as back pain (6%), nausea/vomiting (4%), weight loss (13%), melena (4%) and jaundice (4%) have been also reported. Thirty percent of patients were asymptomatic and the lesions were incidentally discovered on CT scans performed for other reasons.

The preoperative diagnosis of pancreatic schwannoma is very difficult, especially in cystic schwannomas. Suzuki *et al*<sup>[8]</sup> reviewed imaging features of pancreatic schwannomas. The most characteristic feature on CT scan was the presence of an area of low density and/or cystic images reflecting the Antoni B component or degenerative cystic areas of the schwannoma. Contrast-enhanced CT scan showed the difference between the Antoni A and the Antoni B areas based on their vascularity, i.e., well-enhanced areas corresponding to Antoni A, and unenhanced areas corresponding to Antoni B. The CT findings correlated well with pathological features<sup>[8,19]</sup>. The MRI findings usually showed hypointensity on T1-weighted images and hyperintensity on T2-weighted images<sup>[21]</sup>. However, other

pancreatic tumors often share those imaging features, and differential diagnoses should always be considered. Ultrasound-guided Fine Needle Aspiration (EUS-FNA) biopsy has been used increasingly commonly at many institutions. This procedure may be useful for accurate preoperative diagnosis. Cytologically, schwannomas are characteristically composed of spindle-shaped cells, which possess indistinct cytoplasmic borders and wavy nuclei embedded in a fibrillary and occasionally myxoid or collagenous matrix. The Antoni A (cohesive cellular clusters) and Antoni B (loosely cohesive or poorly cellular sheets) areas are occasionally found. Immunohistochemical staining is useful for accurate diagnosis of schwannoma<sup>[12,14]</sup>. It is diffusely and strongly positive for S-100 protein. There has only been one previous report of pancreatic schwannoma diagnosed preoperatively by EUS-FNA cytology combined with immunohistochemistry<sup>[12]</sup>.

Although malignant pancreatic schwannomas have been reported in 5 articles<sup>[7,38,40-42]</sup>, in 3 of 5 the methods of diagnosing malignancy were inconsistent, as some previous reports pointed out<sup>[4,5]</sup>. Immunohistochemical examination was not used or was not available in these 3 patients. Walsh and Bradspigel<sup>[40]</sup> described a case of pancreatic schwannoma eroding into the bowel wall and presenting with gastrointestinal bleeding that mimicked a recurrently bleeding duodenal ulcer. Another two patients reported had disease associated with von Recklinghausen's disease<sup>[41,42]</sup>. These could represent misdiagnosed neurofibromas that underwent malignant degeneration<sup>[4,5]</sup>. Stojanovic *et al*<sup>[7]</sup> reported malignant pancreatic schwannoma with node metastasis and infiltrating serosa of transverse colon. This tumor was confirmed using immunohistochemical examination. This may be the first definite report of malignant schwannoma with subsequent radical resection.

Since malignant transformation of pancreatic schwannomas is uncommon, simple enucleation is usually sufficient. A review of the treatment showed that the most common resection was pancreaticoduodenectomy (32%), followed by distal pancreatectomy (21%) and enucleation (15%). This result may account for the difficulty in accurate diagnosis of pancreatic schwannoma and relate to larger size of this tumor. Enucleation was performed for 7 patients for whom 3 lesions were located in the head, 2 lesions were in the uncinate process and 2 lesions were in the body. The mean tumor size of the patients who underwent enucleation was 2.7 cm (range 1.5-4.0 cm). Intraoperative consultation with the pathologist was carried out in most of the enucleated cases. An intraoperative frozen section should be performed, as it helps to establish the diagnosis of a benign schwannoma and avoid more radical resection. Large tumors, tumors involving portal vein, ampulla, or splenic hilum, may require a more radical resection than simple enucleation.

The present report shows the correlation between tumor size and malignant formation (Figure 4A), and tumor size and cystic degeneration (Figure 4B). Malignant schwannomas were more likely to be larger-sized compared to many other tumors. On the other hand, the par-

ticular feature of pancreatic schwannoma was that larger tumor size was related to cystic degeneration, as shown in Figure 4B. Cystic degeneration could make it difficult to diagnose pancreatic schwannoma preoperatively, because of mimicking other cystic neoplasms. Caution should be applied when diagnosing cystic neoplasm. An intraoperative frozen section may help to establish the diagnosis of a schwannoma and avoid more radical resection. To our knowledge, the present report is the first to analyze the relation among tumor size, malignant formation and cystic degeneration. Our results suggest that pancreatic schwannoma might be resected even though diagnosed preoperatively, because if schwannomas are smaller, enucleation should be oncologically adequate. However, when tumors become larger with associated bleeding risk, more invasive resection such a PD or DP might be necessary. In particular, in cases of tumors more than 10 cm in size, we should pay special attention to malignant degeneration and should perform a more extended resection. To avoid extended resection, earlier resection and accurate diagnosis are very important.

In conclusion, pancreatic schwannomas deserve attention with regard to the differential diagnosis of pancreatic lesions. Preoperative diagnosis is very difficult. Simple enucleation is adequate if this is possible to achieve. Intraoperative frozen section is useful to diagnose schwannoma.

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## Esophageal combined carcinomas: Immunohistochemical and molecular genetic studies

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### Abstract

Primary esophageal combined carcinoma is very rare. The authors herein report 2 cases. Case 1 was a combined squamous cell carcinoma and small cell carcinoma, and case 2 was a combined squamous cell carcinoma, adenocarcinoma, and small cell carcinoma. Case 1 was a 67-year-old man with complaints of dysphagia. Endoscopic examination revealed an ulcerated tumor in the middle esophagus, and 6 biopsies were obtained. All 6 biopsies revealed a mixture of squamous cell carcinoma and small cell carcinoma. Both elements were positive for cytokeratin, epithelial membrane antigen, and p53 protein, and had high Ki-67 labeling. The small cell carcinoma element was positive for synaptophysin, CD56, KIT, and platelet-derived growth factor- $\alpha$  (PDGFRA), while the squamous cell carcinoma element was not. Genetically, no mutations of *KIT* and *PDGFRA* were recognized. The patient died of systemic carcinomatosis 15 mo after presentation. Case 2 was a 74-year-old man presenting with dysplasia. Endoscopy revealed a polypoid tumor in the distal esophagus. Seven biopsies were taken, and 6 showed a mixture of squamous cell carcinoma, small cell carcinoma, and adenocarcinoma. The 3 elements were positive for cytokeratins, epithe-

lial membrane antigen, and p53 protein, and had high Ki-67 labeling. The adenocarcinoma element was positive for mucins. The small cell carcinoma element was positive for CD56, synaptophysin, KIT, and PDGFRA, but the other elements were not. Mutations of *KIT* and *PDGFRA* were not recognized. The patient died of systemic carcinomatosis 7 mo after presentation. These combined carcinomas may arise from enterochromaffin cells or totipotent stem cell in the esophagus or transdifferentiation of one element to another. A review of the literature was performed.

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**Key words:** Esophagus; Combined carcinoma; Histopathology; Immunohistochemistry; Molecular genetics

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### INTRODUCTION

Combined esophageal carcinomas are very rare and interesting tumors. A full review of the English literature revealed 24 reporting combined carcinoma of the esophagus<sup>[1-24]</sup>. Most were small cell carcinomas, and a few were non-small cell carcinomas<sup>[1-24]</sup>. The author herein reports 2 cases of combined carcinoma of the esophagus. One case is a combined squamous cell carcinoma and small cell carcinoma, and another case is a combined squamous cell carcinoma, adenocarcinoma, and small cell carcinoma

## CASE REPORT

### Case 1

A 67-year-old man was admitted to our hospital with dysphagia. An endoscopic examination revealed an ulcerated tumor (3 cm × 4 cm × 3 cm) in the middle esophagus (Figure 1A), and 6 biopsies were obtained. All 6 biopsies revealed a mixture of squamous cell carcinoma (Figure 1B) and small cell carcinoma (Figure 1C). The squamous element was composed of malignant cells arranged in a layer with focal keratinization (cancer pearls). The small cell carcinoma element consisted of malignant small cells with hyperchromatic nuclei, nuclear molding, absent nucleoli, and very scant cytoplasm. There was a gradual merging of the 2 elements.

The authors performed an immunohistochemical study using Dako Envision method, as previously described<sup>[25,26]</sup>. The immunohistochemical antibodies used were as follows: cytokeratins (AE1/3, Dako; CAM5.2 Becton-Dickinson, CA, United States), epithelial membrane antigen (E29, Dako), neuron-specific enolase (BBS/NC/VI-H14, Dako), chromogranin (DAK-A3, Dako), synaptophysin (polyclonal, Dako), CD56 (UJ13A, Dako), p53 protein (DO-7, Dako), Ki-67 (MIB-1, Dako), KIT (polyclonal, Dako), and platelet derived growth factor receptor- $\alpha$  (PDGFRA) (polyclonal, Santa Cruz, CA, United States). The squamous cell carcinoma element was positive for cytokeratin, epithelial membrane antigen, p53 protein, and Ki-67 antigen (57% labeled), but negative for other antigens examined. The small cell carcinoma element was positive for cytokeratin (Figure 1D), p53 protein, Ki-67 (96% labeled), synaptophysin (Figure 1E), CD56, and chromogranin, KIT (Figure 1F), and PDGFRA (Figure 1G).

The authors performed a molecular genetic study for *KIT* (exons 9, 11, 13 and 17) and *PDGFRA* (exons 12 and 18) genes in paraffin sections using microdissection and the polymerase chain reaction-direct sequencing method, as previously described<sup>[27-30]</sup>. There were no mutations of the *KIT* (exons 9, 11, 13 and 17) and *PDGFRA* (exons 12 and 18) genes.

The patient was diagnosed with combined carcinoma of esophagus (stage II, T2 N0 M0). Surgery was not considered because the tumor contained small cell carcinoma. The patient was treated with cisplatin-based chemotherapy and radiation, but died of systemic carcinomatosis 15 mo after presentation.

### Case 2

A 74-year-old man presented with dysplasia, and attended our hospital. An endoscopy revealed a polypoid tumor (2 cm × 2 cm × 3 cm) in the middle esophagus (Figure 2A). Seven biopsies were taken, and 6 showed a mixture of squamous cell carcinoma (Figure 2B), small cell carcinoma (Figure 2C), and adenocarcinoma (Figure 2D). The squamous cell carcinoma element showed malignant cells in a layer with focal keratinization. The small cell carcinoma element was composed of small malignant cells with hy-

perchromatic nuclei, inconspicuous nucleoli, and scant cytoplasm. The adenocarcinoma element showed sheet-like tumor cells with focal acinar formations, in which mucins were identified. The 3 elements were positive for cytokeratins, epithelial membrane antigen, p53 protein, and Ki-67 (labeling: squamous cell carcinoma element, 34%; adenocarcinoma element, 29%; small cell carcinoma element 87%). The squamous cell carcinoma and adenocarcinoma elements were negative for CD56, chromogranin, synaptophysin, neuron-specific enolase, KIT and PDGFRA. In contrast, the small cell carcinoma element was positive for CD56 (Figure 2E), synaptophysin, KIT (Figure 2F), and PDGFRA (Figure 2G). Mutations of *KIT* and *PDGFRA* were not found.

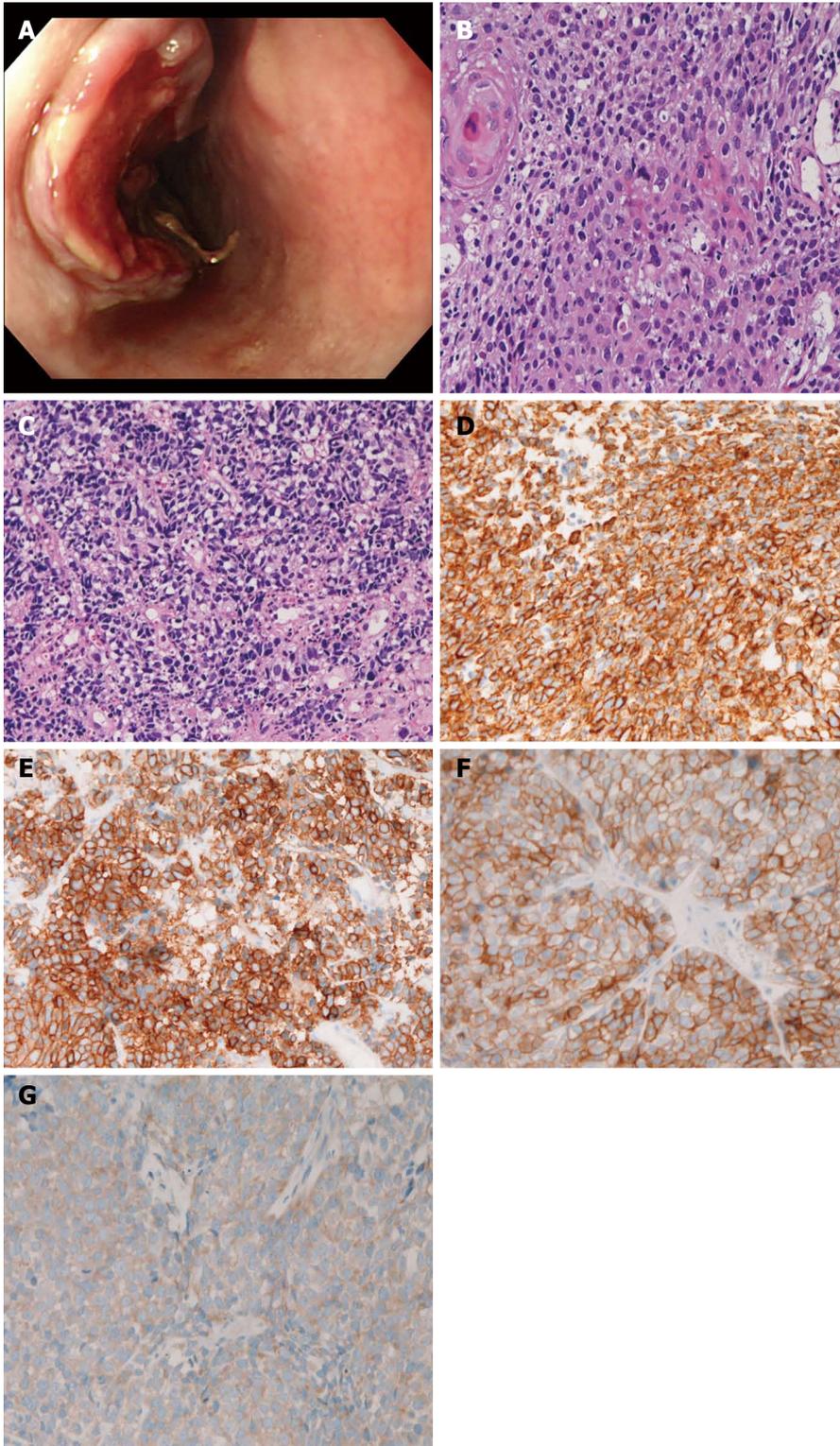
The patient was diagnosed with combined carcinoma of the esophagus (stage II, T2 N1 M0). Surgery was not considered because the tumor contained small cell carcinoma. The patient received chemoradiation, but died of systemic carcinomatosis 7 mo after presentation.

## DISCUSSION

The present 2 cases of combined carcinoma of the esophagus were associated with small cell carcinoma. Small cell carcinoma is diagnosed with hematoxylin and eosin (HE) staining and is defined as an undifferentiated carcinoma consisting of small cells with characteristic cellular and nuclear features, such as small-sized cells, scant cytoplasm, hyperchromatic, finely granular, and molded nuclei, and inconspicuous nucleoli, according to the World Health Organization Blue Book<sup>[31]</sup>. Neuroendocrine features are recognized in more than 90% of small cell carcinoma<sup>[31]</sup>. Squamous cell carcinoma is characterized by a squamoid cell arrangement and the presence of intercellular bridges and keratinization. Adenocarcinoma is characterized by tubular formations and the presence of mucins. Case 1 in the present study fulfilled these criteria, and was definitely combined small cell carcinoma and squamous cell carcinoma. Likewise, Case 2 was an apparently combined small cell carcinoma, squamous cell carcinoma, and adenocarcinoma. The presence of p53 protein and high Ki-67 labeling supports the above diagnosis.

In the present study, there was gradual merging of the 2 elements in case 1 and of the 3 elements in case 2. These findings may indicate that each element is derived from transdifferentiation of other elements. Traditionally, small cell carcinoma of the esophagus is thought to be derived from enterochromaffin cells or APUD cells present in the normal esophagus. Otherwise, this esophageal tumor arises from totipotent stem cells of the esophagus, as suggested by Ho *et al.*<sup>[2]</sup>. The present study could not determine the histogenesis of the combined carcinomas associated with small cell carcinomas.

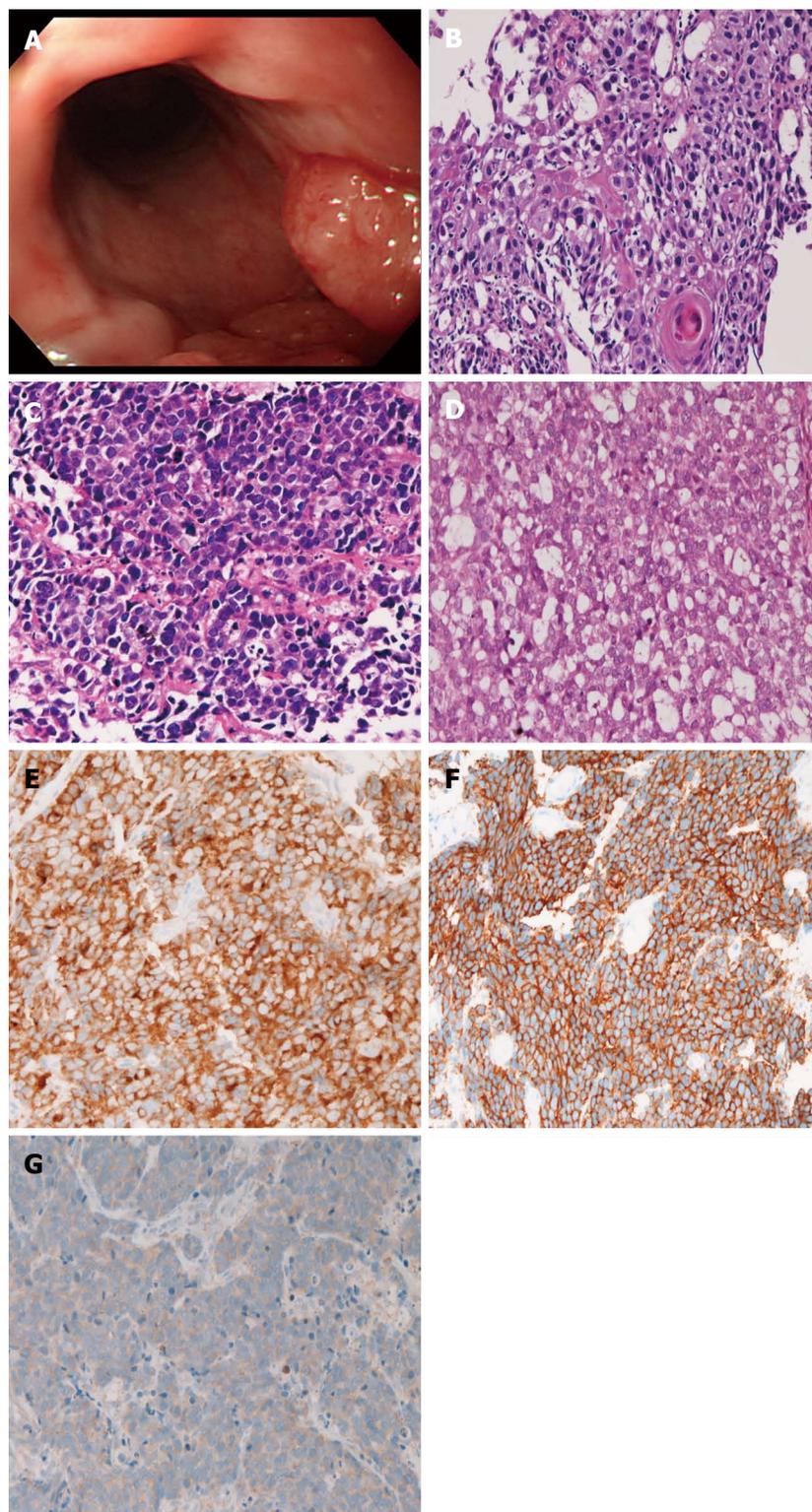
Most of esophageal tumors with multiple differentiation (combined carcinoma) are associated with small cell carcinoma<sup>[1-15,17-24]</sup>, although basaloid cell squamous cell carcinoma also shows multiple differentiation<sup>[16]</sup>. The cel-



**Figure 1 Case 1.** A: Endoscopy. An ulcerated tumor is seen in the esophagus; B: Histology of the squamous cell carcinoma element of the esophageal tumor. Keratinization is seen [hematoxylin and eosin (HE), x 200]; C: Small cell carcinoma element of the esophageal carcinoma. The tumor cells show characteristic morphologies of small cell carcinoma (HE, x 200); D: Cytokeratins are expressed in the small cell carcinoma component (x 200); E: Synaptophysin is expressed in the small cell carcinoma component (x 200); F: KIT is expressed in the small cell carcinoma component (x 200); G: Platelet-derived growth factor- $\alpha$  is expressed in the small cell carcinoma component (x 200).

ular origin of small cell carcinoma is unknown. In the full review of the English literature on combined carcinomas of esophageal cancers, Rosen *et al*<sup>[1]</sup> reported an epidermoid carcinoma simulating oat cell carcinoma. Ho *et al*<sup>[2]</sup> reported that 2 of 4 cases of esophageal small cell carcinoma contained foci of squamous cell carcinoma. Reid *et al*<sup>[3]</sup> described a case of esophageal small cell carcinoma with foci of squamous cell carcinoma. Reyes *et al*<sup>[4]</sup> reported that foci of squamous cell carcinoma were seen in 4/16

esophageal small cell carcinoma. Sarma<sup>[5]</sup> mentioned that there were oat cell carcinomas with squamous cell carcinoma foci and adenocarcinoma foci. Doherty *et al*<sup>[6]</sup> reported that there were oat cell carcinomas with squamous cell carcinoma *in situ*, with squamous cell carcinoma, with adenocarcinoma, and with carcinoid. Sato *et al*<sup>[7]</sup> reported a case of small cell carcinoma with invasive squamous cell carcinoma. Sasajima *et al*<sup>[8]</sup> demonstrated one case of esophageal carcinoma showing multiple differentiations into oat



**Figure 2 Case 2.** A: Endoscopy. An elevated tumor is seen in the esophagus; B: Histology of the squamous cell carcinoma element of the esophageal tumor. A cancer pearl is seen [hematoxylin and eosin (HE), x 200]; C: Small cell carcinoma element of the esophageal carcinoma. The tumor cells show characteristic morphologies of small cell carcinoma (HE, x 200); D: Adenocarcinomatous element shows focal tubular formations (HE, x 200); E: CD56 is expressed in the small cell carcinoma component (x 200); F: KIT is expressed in the small cell carcinoma component (x 200); G: Platelet-derived growth factor- $\alpha$  is expressed in the small cell carcinoma component (x 200).

cell carcinoma, adenoid cystic carcinoma, adenocarcinoma, and squamous cell carcinoma. Mori *et al*<sup>[9]</sup> reported that 7 squamous cell foci and 2 adenocarcinoma foci were recognized in 10 small cell carcinomas. Attar *et al*<sup>[10]</sup> showed concomitant squamous cell carcinoma in small cell carcinoma. Beyer *et al*<sup>[11]</sup> mentioned that there was considerable histological heterogeneity in small cell carcinoma. Fujiwara *et al*<sup>[13]</sup> reported a case of small cell carcinoma with concomitant squamous cell carcinoma.

Takubo *et al*<sup>[14]</sup> found a combination of small cell carcinoma and squamous cell carcinoma in 11 of 21 cases, and a combination of small cell carcinoma and mucoepidermoid carcinoma in 1 of 21 cases. Medgyesy *et al*<sup>[15]</sup> found a combination of small cell carcinoma and adenocarcinoma in 1 of 8 cases, and a combination of small cell carcinoma and squamous cell carcinoma in 1 of 8 cases. Cho *et al*<sup>[16]</sup> identified a combination of basaloid squamous cell carcinoma and squamous cell carcinoma in 8 of 18 cases, a

combination of basaloid squamous cell carcinoma and adenocarcinoma in 3 of 18 cases, a combination of basaloid squamous cell carcinoma and small cell carcinoma in 2 of 18 cases. Uğraş *et al*<sup>[18]</sup> reported a combined carcinoma composed of small cell carcinoma and squamous cell carcinoma. Ishihara *et al*<sup>[19]</sup> found an esophageal combined carcinoma consisting of Pagetoid squamous cell carcinoma, choriocarcinoma, and mucoepidermoid carcinoma. Yamamoto *et al*<sup>[20]</sup> reported *in situ* and invasive squamous cell carcinomas were present in 3 of 6 cases of small cell carcinoma. Wu *et al*<sup>[21]</sup> reported that small cell carcinoma with squamous cell carcinoma was found in 3 of 9 cases. Yun *et al*<sup>[22]</sup> identified squamous differentiation in small cell carcinoma in 2 of 21 cases. Bilbeau *et al*<sup>[23]</sup> reported a case of small cell carcinoma with adenocarcinoma in a Barrett's esophagus. Maru *et al*<sup>[24]</sup> reported that a combination of small cell carcinoma and adenocarcinoma was seen in 15 of 40 cases, and a combination of small cell carcinoma and squamous cell carcinoma in 1 of 40 cases. Therefore, this literature review showed that combined carcinoma of the esophagus is not so rare among small cell esophageal carcinomas, and that the majority of combined carcinoma is associated with small cell carcinoma. The review also confirmed that esophageal combined carcinoma composed of small cell carcinoma and squamous cell carcinoma is the most common, followed by a combination of small cell carcinoma and adenocarcinoma. The present 2 cases also are the common type of combined esophageal carcinoma.

As mentioned above, small cell carcinoma is diagnosed by HE staining<sup>[31]</sup>. About 90% of small cell carcinoma has neuroendocrine features<sup>[31]</sup>. The neuroendocrine features can be demonstrated by immunohistochemical demonstration of neuroendocrine antigens such as chromogranin, synaptophysin, CD56, and neuron-specific enolase or by ultrastructural demonstration of neuroendocrine secretory vesicles<sup>[32]</sup>. Yamamoto *et al*<sup>[20]</sup> described that CD56, neuron-specific enolase, and chromogranin were positive in a small cell carcinoma component while they were negative in the squamous cell carcinoma component in 3 cases of combined esophageal carcinoma. They also demonstrated that both components were positive for cytokeratins and epithelial membrane antigen. Wu *et al*<sup>[21]</sup> described that esophageal small cell carcinomas were positive for neuron-specific enolase, chromogranin A, and synaptophysin in all 9 cases investigated. Yun *et al*<sup>[22]</sup> described that the percentage of endocrine markers in 21 esophageal small cell carcinomas was as follows: synaptophysin, 95%; CD56, 76%; chromogranin A, 62%; neuron-specific enolase, 62%; TTF-1, 71%; epithelial membrane antigen, 62%; cytokeratins, 57%; S100 protein, 19%. Maru *et al*<sup>[24]</sup> described that chromogranin was positive in 31 of 40 and synaptophysin in all 40 esophageal neuroendocrine carcinomas. In the present case, synaptophysin, CD56 and chromogranin were positive in the small cell carcinoma component in case 1, and CD56 and synaptophysin were positive in the small cell carcinoma component in case 2.

In both cases in the present study, all the elements were positive for cytokeratin and epithelial membrane antigen. The non-small cell carcinoma components were negative for the neuroendocrine carcinoma. These findings are compatible with those of previous studies.

The present study has new findings: it showed positive expression of *KIT* and *PDGFRA* in the small cell carcinoma element of the 2 combined esophageal carcinomas. The present study also revealed that the squamous cell carcinoma and adenocarcinoma components were negative for *KIT* and *PDGFRA* protein and were negative for *KIT* and *PDGFRA* mutations in the esophageal combined carcinoma. *KIT* and *PDGFRA* are transmembrane receptor tyrosine kinase oncoproteins involved in carcinogenesis<sup>[33-35]</sup>. The vast majority of small cell carcinoma develops in the lung. In small cell lung carcinoma, *KIT* is frequently expressed, but no mutations of *KIT* gene have been recognized<sup>[36-46]</sup>. In small cell lung carcinoma, protein expression and mutations of *PDGFRA* are unknown. In extrapulmonary small cell carcinoma, *KIT* and *PDGFRA* proteins are frequently expressed, but there have been no mutations of *KIT* and *PDGFRA* genes found<sup>[46-48]</sup>. Many more studies of the *KIT* and *PDGFRA* gene status in esophageal combined carcinomas are necessary to elucidate the molecular mechanism of the carcinogenesis.

The biological behavior of these combined carcinomas of the esophagus is not known. However, it is thought that these combined carcinomas behave like small cell carcinoma, because the great majority of these combined carcinomas contain a small cell carcinoma element<sup>[1-24]</sup>. The option for treatment is not surgery but chemotherapy and radiation as in pulmonary small cell carcinoma<sup>[1-24]</sup>. The chemotherapy employed was cisplatin and etoposide<sup>[1-24]</sup>. Adjuvant radiation therapy may be effective. The combined carcinomas of the esophagus have a higher propensity for systemic metastases<sup>[1-24]</sup>. The survival rate is not clear because of a limited number of cases. However, survival was thought to be similar to that of pulmonary small cell carcinoma<sup>[1-24]</sup>.

In summary, the authors presented 2 rare cases of esophageal combined carcinoma with double (squamous cell carcinoma and small cell carcinoma) and triplicate differentiation (squamous cell carcinoma, small cell carcinoma, and adenocarcinoma). The authors speculate that the combined carcinomas are basically small cell carcinomas with squamous and/or adenocarcinomatous differentiation. The present esophageal combined carcinomas may arise from enterochromaffin or totipotent stem cell of the esophagus. It is also possible that each element of the esophageal combined carcinomas may be derived from transdifferentiation of other elements. There were expressions of *KIT* and *PDGFRA* in the small cell carcinoma component of the esophageal combined carcinomas, but were negative for mutations of *KIT* and *PDGFRA*.

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## Esophageal space-occupying lesion caused by *Ascaris lumbricoides*

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*caris lumbricoides* in the esophagus, emphasizing the importance of awareness of this parasitic infection as it often presents with different and unspecific symptoms.

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**Key words:** Ascariasis; *Ascaris lumbricoides*; Esophagus; Endoscope; Radiograph

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### Abstract

*Ascaris lumbricoides* is the largest intestinal nematode parasite of man, which can lead to various complications because of its mobility. As the esophagus is not normal habitat of *Ascaris*, the report of esophageal ascariasis is rare. An old female presented with dysphagia after an intake of several red bean buns and haw jellies. The barium meal examination revealed a spherical defect in the lower esophagus. Esophageal bezoar or esophageal carcinoma was considered at the beginning. The patient fasted, and received fluid replacement treatment as well as some oral drugs such as proton pump inhibitor and sodium bicarbonate. Then upper gastrointestinal endoscopy was done to further confirm the diagnosis and found a live *Ascaris lumbricoides* in the gastric antrum and two in the duodenal bulb. The conclusive diagnosis was ascariasis. The esophageal space-occupying lesion might be the entangled worm bolus. Anthelmintic treatment with mebendazole improved patient's clinical manifestations along with normalization of the radiological findings during a 2-wk follow-up. Authors report herein this rare case of *As-*

### INTRODUCTION

*Ascaris lumbricoides* is the giant intestinal roundworm, causing infection of the gastrointestinal tract and affects approximately one quarter of the world's population. In clinical observations, the majority of patients are infected with intestinal ascariasis. Adult roundworms can be stimulated to migrate to any orifice by stressful conditions such as gastrointestinal disease, fever, anesthesia and anthelmintic drugs, so some complications such as acute cholecystitis, acute cholangitis, and acute pancreatitis caused by ascariasis of bile or pancreatic ducts have been reported. However, demonstration of the worms in esophagus is extremely rare. A case of esophageal ascariasis in a 15-year-old boy was reported by an Indian author in 1999<sup>[1]</sup>, but it did not discuss the possible mechanism. Herein, we describe a case that showed a space-occupying lesion of the esophagus in an old woman caused by *Ascaris* and discuss the likely causes.

## CASE REPORT

A 70-year-old female presented with recurrent dysphagia for 4 years, and was admitted to our hospital with complaints of symptoms worsening in the past 3 d. The patient complained that the dysphagia often occurred when she ate too fast, which was unrelated to the position or season and relieved by inducing vomiting through physical stimulation. She also had abdominal distension and frequent belching. Three days before admission, the symptoms of dysphagia could not be relieved by inducing vomiting after an intake of several red bean buns and haw jellies. Barium meal examination in a local hospital revealed a spherical defect in the lower esophagus allowing little barium to pass through (Figure 1). She had no specific past medical history which included digestive system disease. Upon physical examination, her general condition was normal, temperature 37 °C, blood pressure 120/75 mmHg, heart rate 64 beats/min, and respiratory rate of 18 breaths/min. She was thin but no signs of anemia. The abdomen was soft with normal bowel sounds and no peritoneal signs. The laboratory results were as follows: Hb 139 g/L, white blood cell  $11.54 \times 10^9/L$ , neutrophils 77.8%, eosinophils 0.3%. Markers for hepatitis A, B, C were negative. Kidney and liver function tests and serum electrolytes were normal. Fecal examination was not available due to absence of stools in the hospital. X-ray films of the chest and abdomen were performed immediately upon admission to our hospital and showed a globular high density shadow on the right side of trachea in the mediastinum, as well as a strand-like high density shadow in the right upper quadrant of abdomen, and excess barium remained in the colon, and lumbar spondylosis. We considered the following diagnoses: hiatal hernia, esophageal bezoar or esophageal carcinoma on the basis of the radiological appearance.

The patient fasted and was given fluid replacement treatment. Other oral drugs such as proton pump inhibitor (PPI) and sodium bicarbonate were also administered considering the possible existence of hiatal hernia and esophageal bezoar, while soybean oil was taken orally to help the barium pass with the feces. To further confirm the diagnosis, the patient underwent upper gastrointestinal endoscopy on the second day of admission. Endoscopy showed the mucous membrane of esophagus to be smooth and clear except for some slight congestion in the lower part; the body of stomach was normal and a live *Ascaris lumbricoides* worm was seen in the gastric antrum, where mucosa was thinner; there was a diverticula approximately 0.6 cm × 0.6 cm in size in the posterior wall of duodenal bulb and another two live worms were present (Figure 2). The congestion of duodenal mucosa also appeared. Endoscopic diagnoses were esophagitis, atrophic antral gastritis, duodenitis with diverticulum, and ascariasis.

The patient reported a history of passage of worms in stools, and a history of eating raw vegetables. She received mebendazole for anthelmintic treatment along with PPI therapy. The patient improved symptomatically during a 2-wk follow-up, and did pass 5-6 worms with



Figure 1 Barium meal examination revealed a spherical filling defect in the lower esophagus.



Figure 2 A live *Ascaris* worm appeared in the duodenal bulb, which was extracted with a grasper.



Figure 3 The defect filling disappeared in repeated barium examinations.

feces. Repeated barium examination for upper gastrointestinal tract was unremarkable (Figure 3).

## DISCUSSION

*Ascaris lumbricoides*, the largest intestinal nematode parasite of man, are commonly seen in rural areas of China, especially among people with poor hygienic conditions and/or having a habit of eating raw food. However, the prevalence of ascariasis has declined with improvement of sanitation and the application of pesticides and chemi-

cal fertilizers. The serious harm and complications of this round worm should be stressed. *Ascaris* larvae can cause transient eosinophilic pulmonary infiltrates (Loeffler's syndrome) when they migrate through the lungs, which is characterized by pulmonary infiltrates and peripheral blood eosinophilia. Infection of adult *Ascaris lumbricoides*, which mostly inhabit the jejunum and ileum, usually presents with anorexia, nausea and vomiting, intermittent periumbilical pain, and malnutrition. More serious complications of *Ascaris* infection may occur when a large worm burden is present in the lumen of the intestine, such as intestinal obstruction, intussusception, volvulus or even gangrene. Intestinal hemorrhage<sup>[2]</sup> and perforation by *Ascaris* have also been reported, with the latter being able to lead to acute diffuse peritonitis or peritoneal granuloma<sup>[3]</sup>. In addition, *Ascaris* infection can cause allergic reactions, presenting with urticaria, skin itch, angio-neurotic edema or even eosinophilic cholecystitis<sup>[4]</sup>.

When the living environment becomes unfavorable such as gastrointestinal disease, hunger, fever, failed deworming therapy or impaction of a mass of worms in the intestinal lumen, adult *Ascaris* will try to enter into any orifice and advance into any channel leading off from it. Then various complications are encountered. The worms commonly enter the biliary or pancreatic ducts, causing cholecystitis, cholangitis, liver abscess, and pancreatitis. *Ascaris* may migrate into appendix as well, resulting in appendiceal colic and appendicitis. *Ascaris* has also been found in the lacrimal passage by being regurgitated into the nasolacrimal duct when they accidentally enter nasopharynx; in the air way causing mechanical asphyxia; and in the urethra and urinary bladder through vesico-intestinal fistulae or transanal migration causing urinary retention. Moreover, the emergence of an *Ascaris* from mouth, nostrils and external auditory meatus has been documented. Esophagus ascariasis is extremely rare, because the esophagus is not normal habitat of *Ascaris* as it prefers an alkaline environment and rarely travels from the jejunum and duodenum to the stomach (an acid environment) and then to esophagus.

The patient in our case is at high risk of ascariasis as she is from rural area and in favor of eating fresh vegetables. This old woman was admitted to the hospital due to dysphagia with an abnormal barium study of the esophagus. In Figure 1, we can find several high-density string shadows and globular shadows in the esophageal spherical filling defect, which actually are *Ascaris* worms. The most likely explanation in our case is that the worms were forced to migrate by gastroduodenal antiperistalsis, which is induced by eating too many red bean buns and haw jellies. Subsequently these worms became entangled with each other to form a small worm bolus in the alkaline esophagus, resulting in the symptom of dysphagia. The patient received treatment of soybean oil after admission, and she drank lidocaine hydrochloride mucilage before endoscopic examination, which relaxed the lower esophageal sphincter, possibly stimulating the worms. All of these measures induced the migration of the *Ascaris* bolus to the gastrointestinal tract. Therefore, the esophagus ap-

peared normal except for some slight mucosal congestion on endoscopy. After anthelmintic therapy, more ascarides were passed out with feces, and a repeated barium study of upper gastrointestinal showed that the former defect filling had disappeared, which confirmed that the abnormal radiological appearance in the esophagus was a worm bolus. In a previous case<sup>[1]</sup> with similar radiographic appearance in the esophagus, the boy later vomited out six live *Ascaris* worms, and his esophageal lumen and mucosa were normal on the barium examination done the next day. Because of lack of exact causative factors in this case, we consider that children's anatomically smaller intestine and larger worm loads caused the rapid transit of worms across the esophagus. This kind of anti-peristaltic migration of *Ascaris* is truly uncommon.

As our patient had a history of recurrent dysphagia and eating too many haw jellies before admission, we highly suspected that hiatal hernia combining with esophageal bezoar might cause this kind of space-occupying lesion at the beginning. However, bezoars rarely form in the esophagus and are often associated with structural and functional abnormalities of the esophagus, such as achalasia and hiatal hernia. Esophageal bezoar is also a complication of enteral feeding and the predisposing factors include mechanical ventilation, supine position, neurological diseases, diabetes mellitus, hypothyroidism, obesity and history of partial gastrectomy<sup>[5]</sup>. Therefore, we performed the gastrointestinal endoscopy to clarify the diagnosis. The unexpected result suggests that careful history-taking and complete examinations are necessary.

In conclusion, we reported a case of an old woman with esophageal ascariasis and discussed the possible causes. We often lack of awareness of *Ascaris* infection because of the dramatically decreasing incidence and several different and unspecific symptoms of this infection. This report also listed some other common and rare complications, and we would like to warn all medical workers that we should pay more attention to such a disease and reduce any misdiagnosis in the future work.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States

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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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- 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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- 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

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- 15 Morse SS. Factors in the emergence of infectious dis-

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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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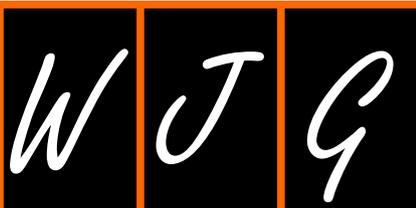
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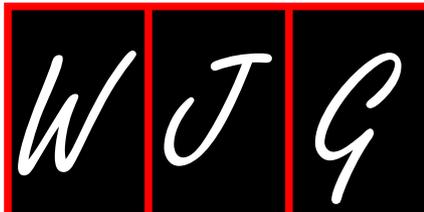
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## Consensus statement AIGO/SICCR: Diagnosis and treatment of chronic constipation and obstructed defecation (part I : Diagnosis)

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### Abstract

Chronic constipation is a common and extremely troublesome disorder that significantly reduces the quality of life, and this fact is consistent with the high rate at which health care is sought for this condition. The aim of this project was to develop a consensus for the diagnosis and treatment of chronic constipation and obstructed defecation. The commission presents its results in a "Question-Answer" format, including a set of graded recommendations based on a systematic review of the literature and evidence-based medicine. This section represents the consensus for the diagnosis. The history includes information relating to the onset and duration of symptoms and may reveal secondary causes of constipation. The presence of alarm symptoms and risk factors requires investigation. The physical examination should assess the presence of lesions in the anal and perianal region. The evidence does not support the routine use of blood testing and colonoscopy or barium enema for constipation. Various scoring systems are available to quantify the severity of constipation; the Constipation Severity Instrument for constipation and the obstructed defecation syndrome score for obstructed defecation are the most reliable. The Constipation-Related Quality of Life is an excellent tool for evaluating the patient's quality of life. No single test provides a pathophysiological basis for constipation. Colonic transit and anorectal manometry define the pathophysiologic subtypes. Balloon expulsion is a simple screening test for defecatory disorders, but it does not define the mechanisms. Defecography detects structural abnormalities and assesses functional parameters. Magnetic resonance imaging and/or pelvic floor sonography can further complement defecography by providing information on the movement of the pelvic floor and the organs that it supports. All these investigations are indicated to differentiate

between slow transit constipation and obstructed defecation because the treatments differ between these conditions.

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**Key words:** Slow transit constipation; Dyssynergic defecation; Obstructed defecation; Constipation scoring system; Quality of life; Anorectal manometry; Colon motility; Balloon expulsion test; Defecography

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## INTRODUCTION

The mission of the Italian Association of Hospital Gastroenterologists (AIGO) is to advance the knowledge of digestive pathologies, to promote progress in the prevention, diagnosis, care and rehabilitation of gastrointestinal diseases, and to promote research.

The aim of the Italian Society of Colo-Rectal Surgery (SICCR) is to ensure the highest therapeutic standards through the evaluation and introduction into medical practice of the latest advances in the areas of prevention, diagnosis and care of pathologies involving the colon, rectum and anus.

The Joint Committee AIGO/SICCR is made up of members of these two scientific societies, elected on the basis of their experience in treating functional and organic problems of the colon and rectum.

The objective of the committee was to develop a consensus statement on the most important diagnostic and therapeutic aspects of functional constipation and obstructed defecation, including a set of graded recommendations based on a review of the literature and on evidence-based medicine.

## LITERATURE SEARCH

A search of the literature was carried out using the online databases of PUBMED, MEDLINE and COCHRANE to identify articles published in English before April 2011 and reporting trials conducted on adult subjects with chronic constipation. The key words used were: Rome criteria, constipation, slow transit constipation,

Table 1 Levels of evidence<sup>[1]</sup>

Levels of evidence	
I	Randomised clinical trials with $P < 0.05$ , adequate sample size, and appropriate methodology
II	Randomised clinical trials with $P < 0.05$ , inadequate sample size, and/or inappropriate methodology
III	Non-randomised trials with simultaneous controls
IV	Non-randomised trials with historical controls
V	Case series

pelvic floor dyssynergia, dyssynergic defecation, dyschezia, colonic inertia, bowel questionnaire, constipation scoring system, quality of life, anorectal manometry, rectal compliance, colonic transit, colon motility, gastrointestinal motility, colonic manometry, balloon expulsion test, pelvic floor imaging, proctography, cystoproctography, dynamic magnetic resonance, anal ultrasound, endosonography, constipation medical therapy, alimentary fibres, laxatives, prokinetics, probiotics, biofeedback, pelvic floor rehabilitation, sacral nerve stimulation, obstructed defecation, outlet obstruction, rectocele, rectal intussusception, rectal prolapse, enterocele, Duhamel operation, Block operation, Sarles operation, stapled transanal resection, Delorme operation, Ripstein operation, colorectal surgery, colectomy, ileorectal anastomosis, segmental colonic resection, laparoscopic colectomy, antiperistaltic cecoproctostomy, cecorectal anastomosis, antegrade colonic enema, Malone's procedure, Malone antegrade continence enema, colostomy, ileostomy, colonic irrigation, pelvic organ prolapse, posterior vaginal prolapse, posterior colporrhaphy, transanal repair, transvaginal repair and mesh.

## LEVELS OF EVIDENCE AND GRADING OF RECOMMENDATIONS

The recommendations of the committee were defined and graded based on the current levels of evidence and in accordance with the criteria adopted by the American College of Gastroenterology's Chronic Constipation Task Force<sup>[1]</sup>.

Five evidence levels were defined (Table 1). The recommendations were graded A, B and C (Table 2).

The committee wishes to underline that insufficient evidence does not automatically imply "evidence against" a statement. Many decisions in daily practice are based on clinical experience. Sometimes, it is difficult to find scientific papers supporting a widespread clinical practice, but this difficulty does not mean that we need to refute or abandon therapies that clinicians have been using for years with their patients. Evidence-based medicine is a useful tool to guide clinical practice, but if applied mechanically and without the application of common sense and personal experience, it can lead to erroneous conclusions<sup>[2]</sup>.

In the development of this consensus statement, the committee identified five key areas (Table 3) and divided

Table 2 Grading of the recommendations<sup>[1]</sup>

Grading of the recommendations	
A	Recommendation supported by two or more level I trials, without conflicting evidence from other level I trials
B	Recommendation based on evidence from a single level I trial OR, evidence from two or more level I trials with conflicting, evidence from another level I trial OR, evidence from two or more level II trials
C	Recommendations based on levels of evidence III-V

OR: Odds ratio.

them into subsections. Each subsection was researched, and recommendations were prepared by one or more members of the committee in accordance with specific themes defined by the committee.

The process of drafting the consensus statement involved constant communication and evaluation conducted online and during four face-to-face working meetings held at 3-mo intervals. During these meetings, the levels of evidence and the grading of the recommendations were discussed to reach a consensus in all the areas covered in the consensus statement.

The commission presents its results here in a “Question-Answer” format, which will allow clinicians to find concise responses to their specific questions quickly and easily and to peruse the full text at their leisure.

## DEFINITION OF CONSTIPATION

Constipation can be either primary or secondary. The commission adopted the definition of primary functional constipation outlined in the Rome III criteria<sup>[3]</sup>. This set of criteria was developed by an international group of experts through a process of consensus, and it has been reviewed and revised more than once since it was first published<sup>[5-5]</sup>.

Stool form was defined using the Bristol stool form score<sup>[6]</sup>; constipation may involve slow intestinal transit and/or abnormal defecation; the definition of abnormal defecation from the Rome III criteria was adopted<sup>[7]</sup>.

## CLINICAL EVALUATION AND SCORING SYSTEMS

### Clinical evaluation

Is a patient history useful in the evaluation of chronic constipation? A thorough medical history should always be taken in patients with chronic constipation. This process constitutes the first approach to the patient and is designed to detect events that may be directly or indirectly linked to the patient's symptoms<sup>[8-11]</sup>.

The patient history can identify conditions responsible for secondary constipation<sup>[11,12]</sup>, such as the following: (1) alarm symptoms, such as weight loss, bloody stools, anaemia, or a family history of colon cancer; (2) conditions and/or diseases potentially associated with

Table 3 Areas defined by the committee for the consensus statement

Area	
1	Clinical evaluation and scoring systems
2	Diagnostic techniques
3	Medical and rehabilitative treatment
4	Surgery for slow transit constipation
5	Surgery for obstructed defecation with or without associated pelvic diseases

constipation, such as inappropriate diet<sup>[13]</sup>, low physical activity<sup>[10]</sup>, the use of constipating drugs, and metabolic, psychiatric or neurological diseases; and (3) the negative outcome of perineal-pelvic-abdominal or obstetric-gynaecological surgery<sup>[14,15]</sup>.

Can the medical history distinguish among the different subtypes of chronic constipation? No, there are as yet no specific criteria that can distinguish among the subtypes of chronic constipation based on anamnesis<sup>[7,16-18]</sup>. Level I evidence, Grade A recommendation.

Are there specific symptoms that are present only in patients with functional constipation? No, there are no specific symptoms that distinguish patients with functional constipation from normal subjects<sup>[3]</sup>. Level I evidence, Grade A recommendation.

The occurrence of two or more symptoms during at least 25% of bowel movements distinguishes patients with chronic constipation from normal subjects<sup>[3,19]</sup>.

Should a physical examination be performed in patients with chronic constipation? A physical examination is essential in the initial workup of a patient with chronic constipation<sup>[11]</sup>. The examination should include inspection of the anorectal region and exploration of the rectum. This process can detect external signs of anal disease, pelvic organ prolapse, or descending perineum syndrome. A digital rectal examination should detect any signs of organic disease or obstructed defecation. The examination is particularly important if functional alterations in defecation are suspected.

Is blood testing useful in the diagnostic algorithm of functional constipation? Blood testing does not provide useful input. Functional constipation is defined as a primitive condition and is not accompanied by any organic or biochemical alterations, being associated instead with a “functional” pathology of visceral motility. For this reason, there are no laboratory tests for the diagnosis of functional constipation<sup>[3,9]</sup>. Level I evidence, Grade A recommendation.

Blood tests can, however, be performed to exclude conditions of secondary chronic constipation<sup>[12]</sup>.

Should morphological investigations (colonoscopy, barium enema, or computerised tomographic colonography) be performed in all patients with chronic constipation? Prospective studies on this point are lacking in the literature<sup>[20,21]</sup>. There is no clear evidence to support the usefulness of colonoscopy in patients with chronic constipation. Level IV evidence, Grade C recommendation.

However, morphologic investigations should always be performed in patients with alarm symptoms, in patients > 50 years of age, and in patients with a family history of colon cancer.

## SCORING SYSTEMS IN CHRONIC CONSTIPATION

### Scoring systems to quantify disease severity

Various scoring systems have been developed to quantify the severity of constipation. These systems are particularly important in a subjective, functional disease, such as constipation, to evaluate the results of therapy.

An early scoring system, the chronic idiopathic constipation index (CICI), was published in *Techniques of Coloproctology* in 1996<sup>[22]</sup>. It is based on seven variables (scored from 0 to 3, with a maximum score of 21) and was designed to detect chronic idiopathic slow transit constipation. The CICI was the first evaluation system that took into consideration signs of autonomic neuropathy. However, it has never been validated in a prospective study.

In 1999, the Patient Assessment of Constipation Symptoms<sup>[23]</sup> was published. This 12-item, patient-administered questionnaire has been validated and found to be effective, but it is rarely used in clinical studies.

The most widely adopted instrument is the Cleveland Clinic Constipation Score<sup>[24]</sup>. It is easy to understand and administer and therefore has won broad acceptance, although it has not been formally validated. It consists of 8 items scored from 0 to 4 for a maximum score of 30. It should be noted that one of the items, "duration of symptoms", cannot be modified by therapy.

In 2002, a new, prospectively validated score, the symptom scoring system for constipation<sup>[25]</sup>, consisting of 11 items scored from 0 to 3 or 4 for a maximum possible score of 39, was published, but it is rarely used.

More recently the Constipation Severity Instrument (CSI)<sup>[26]</sup> was developed. It is a well-designed scoring system consisting of 78 items that can identify and quantify different types of constipation (IBS, slow transit and obstructed defecation).

In 2008, the first instrument specifically designed for obstructed defecation syndrome, the obstructed defecation syndrome (ODS) score, was published in *Colorectal Disease*<sup>[27]</sup>. It consists of 7 items scored from 0 to 4 with a maximum score of 27, and it has been prospectively validated.

### Measuring quality of life in constipation

Three Quality of Life (QoL) questionnaires for constipation have been published. The gastrointestinal QoL questionnaire<sup>[28]</sup> was designed to address all gastrointestinal symptoms and therefore is not specific for constipation. It includes 36 items with 5 possible answers, and it has a maximum possible score of 180.

In 2005, the first disease-specific questionnaire on constipation appeared, the Patient Assessment of Constipation Quality of Life<sup>[29]</sup>. It consists of 28 items

scored from 0 to 4 with a maximum score of 112.

Recently, a new, statistically validated QoL questionnaire, the Constipation-Related Quality of Life (CRQOL)<sup>[30]</sup>, was published. It includes 4 domains: social impact (11 items), distress (11 items), usual diet (11 items), and defecation features (4 items).

## Conclusions

Several scoring systems for constipation can be found in the medical literature. The consensus of the committee is that the most reliable instruments for scoring disease severity are the CSI for constipation in general and the ODS score for obstructed defecation. The CRQOL is an excellent tool for evaluating the effects of constipation on the patient's quality of life. The use of these instruments is recommended for clinical trials.

## DIAGNOSIS OF FUNCTIONAL CONSTIPATION

### Imaging in chronic constipation and obstructed defecation syndrome

Currently available imaging techniques for chronic constipation and ODS include the following: (1) transit time (TT) studies<sup>[31,32]</sup>; (2) X-ray videoproctography<sup>[33]</sup> and colpo-cysto-entero-defecography<sup>[34,35]</sup>; (3) magnetic resonance (MR)-defecography<sup>[36]</sup>; and (4) ultrasonography (US) of the pelvic floor<sup>[37-40]</sup>.

Can a TT study differentiate slow transit constipation from obstructed defecation? Depending on the site of accumulation of the radiopaque markers along the large bowel, an initial TT study can differentiate between patients with total or segmental colonic slow transit constipation and patients with outlet obstruction. Unfortunately, lack of standardisation in the procedure makes it difficult to compare results among centres. Level V evidence, Grade C recommendation. In the case of distal obstruction, X-ray defecography is recommended as a second-line examination. The fact that this examination has been universally adopted makes it the benchmark against which to test newer modalities.

When should defecography be performed as opposed to colpo-cysto-entero-defecography? Defecography is indicated to rule out a variety of conditions that could play a role in the aetiology of the presenting symptom(s), such as paradoxical contraction of the puborectalis muscle<sup>[41,42]</sup>, a rectocele, recto-anal intussusception and complete external rectal prolapse. Colpo-cysto-entero-defecography should be performed when multiple compartment defects are suspected, including cystocele, enterocele, or descending perineum syndrome<sup>[43]</sup>.

Because their clinical significance remains a matter of debate, there is general agreement<sup>[44-46]</sup> that the results of contrast radiography should not be relied on exclusively when making decisions regarding the treatment of a patient (including surgery).

When should MR defecography be considered as an alternative to X-ray examination? Due to the panoramic

view that they provide and the absence of ionising radiation, MR imaging of the pelvic floor and MR defecography are now frequently recommended as a valid alternative to contrast radiography, especially in young patients, female patients of reproductive age, pregnant patients, and those patients at risk for adverse reactions to the contrast medium.

Are the findings commonly observed on defecography captured equally well by MR defecography? Despite the less natural (horizontal) position of the patient during the exam, MR imaging can provide similar, and sometimes better, results than conventional X-rays, with the added advantage (especially in the case of defects affecting multiple compartments) of the superior reproducibility of the results<sup>[47,48]</sup>. Consequently, while MR defecography is widely recommended as a tool to increase diagnostic confidence in cases of evacuation dysfunctions, MR neurography of the pelvic floor can be extremely useful in detecting pudendal nerve entrapment neuropathy in patients with chronic pelvic pain<sup>[49]</sup>. Level V evidence, Grade C recommendation.

Can defecographic findings be assessed and measured by perineal, endovaginal and endoanal sonography? There has been a reappraisal of the use of perineal, introital, endoanal and endovaginal US (conventional 2-D and 3-D images recorded using a variety of probes: convex, end-fire, linear and axial 360° rotating models) in the evaluation of the pelvic floor anatomy in patients with evacuation dysfunctions<sup>[50-55]</sup>. With the exception of rectal evacuation<sup>[56]</sup>, the presence and severity of the most common ODS abnormalities visible on defecography can be equally well documented by any one of these sonography techniques. Level V evidence, Grade C recommendation.

What is the role of endovaginal sonography in chronic constipation? Currently, 2-D and 3-D endovaginal sonography are recommended as alternatives to defecography and MR imaging, respectively, when assessing the overall anatomic configuration and movement of the urogenital hiatus in patients with multiple defects affecting the muscular and fascial components of various compartments (anterior, middle and posterior), which are possibly indicative of descending perineum syndrome or pelvic organ prolapse<sup>[53,54]</sup>. Level V evidence, Grade C recommendation.

What is the role of endoanal sonography in chronic constipation? Given the inherently static nature of this examination and the presence of a foreign object in the anal canal (i.e., the endocavitary probe), endoanal sonography is of limited value in the diagnosis of chronic constipation. Recently, however, the advent of 3-D reconstruction has significantly increased the diagnostic confidence associated with this technique<sup>[55]</sup>, which can provide detailed imaging of abnormalities, such as the extent of anal sphincter defects, the anatomy of fistulous tracts in complex perianal sepsis, and submucosal invasion in early anorectal cancers.

In summary, general agreement exists among authors

that the first-line examination remains TT, followed by X-ray defecography. When the appropriate instruments and trained personnel are available, MRI and/or pelvic floor sonography can further complement defecography by providing information on the movement of the pelvic floor and the organs that it supports.

### **Anorectal manometry**

Anorectal manometry measures anal canal pressures. Perfusion catheters are generally employed, rather than solid-state microtransducers, which are more reliable but too expensive for routine use<sup>[57]</sup>. Vector volume manometry has been developed to provide a 3-D view of the anal sphincter, but its clinical utility is still under evaluation<sup>[58]</sup>. Recently, the high-resolution manometry has been shown to provide greater details than water-perfused manometry, but it is still in the experimental stage<sup>[59]</sup>.

The reproducibility of anal manometry is high<sup>[60]</sup>, but its reliability depends on the operator's experience, and its utility is limited by the absence of standardised protocols<sup>[61,62]</sup> and of data from large numbers of normal subjects<sup>[57,63]</sup>. Moreover, most of the parameters measured by anorectal manometry (anal canal pressure, sensory thresholds) are influenced by sex and age<sup>[64]</sup>.

Should anorectal manometry always be performed in patients with chronic constipation and/or obstructed defecation? The main indication for anorectal manometry is the presence of obstructed defecation<sup>[65,66]</sup>. It should also be performed in patients who do not improve with first-line treatments for chronic constipation (a defecation disorder is reported in 51% of such patients)<sup>[12,67]</sup>.

Anorectal manometry, together with other tests, can provide essential information on the rectoanal function defects involved in the physiopathology of obstructed defecation, including increased pressure in the anal canal, rectoanal inhibitory reflex defects, lower rectal sensitivity, and increased rectal compliance<sup>[7]</sup>. Level II evidence, Grade B recommendation.

Is anorectal manometry sufficient for the diagnosis of obstructed defecation? There is no gold standard for the diagnosis of obstructed defecation, and manometry alone does not provide sufficient grounds for the diagnosis. A comprehensive evaluation of anorectal function is necessary and should include tests to evaluate various aspects of defecation, including the balloon expulsion test, imaging techniques, and perhaps electromyography, in addition to manometry<sup>[7]</sup>. Defecography can evaluate the morphological and dynamic factors of defecation; anorectal manometry measures anorectal sensitivity and motility; and electromyography can provide information on electrical activity in the external anal sphincter muscle during straining. The balloon expulsion test can confirm the diagnosis of obstructed defecation<sup>[68,69]</sup>. Level II evidence, Grade B recommendation.

Anorectal manometry consists of several tests; which of them are most useful in the diagnosis of obstructed defecation? At a minimum, the following tests should be performed<sup>[70]</sup>: resting anal pressure, squeezing pressure,

Table 4 Interpretation of the manometric data

Test	Parameter evaluated	Interpretation
Resting pressure	IAS (70% of resting pressure) and EAS (30% of resting pressure)	<i>P</i> increased: Hypertonic sphincters (IAS and/or EAS). Oral nitroglycerin can identify the sphincter involved because it relaxes IAS, but not EAS
Squeeze pressure	EAS	The fatigue rate index can be calculated based on the pressure and duration of the contraction. However, the usefulness of the test in both constipated and incontinent patients is disputed <sup>[112,113]</sup>
Rectoanal inhibitory reflex	IAS relaxation during rectal inflation	Absent: Possible hirschsprung; If present with elevated volume inflation: Megarectum <sup>[57]</sup>
Rectal sensitivity	Rectal sensory function at different volumes	Elevated sensory thresholds may be linked to changes in rectal biomechanics (megarectum) or to afferent pathway dysfunction <sup>[114,115]</sup>
Rectal compliance	Mechanical rectal function	Increased compliance: megarectum <sup>[57]</sup>
Attempted defecation	Synchronisation between the increase in rectal pressure and the decrease in anal pressure during attempts to defecate	Three types of dysfunction may be detected <sup>[65]</sup> : Type 1: Adequate rectal <i>P</i> increase but associated with anal <i>P</i> increase; Type 2: Inadequate rectal <i>P</i> increase associated with anal <i>P</i> increase or inadequate anal <i>P</i> decrease; Type 3: Adequate rectal <i>P</i> increase but inadequate anal <i>P</i> decrease

IAS: Internal anal sphincter; EAS: External anal sphincter; *P*: Pressure. Modified from Azpiroz *et al*<sup>[57]</sup>.

rectoanal inhibitory reflex, rectal sensations (first sensation, maximum tolerable volume), rectal compliance, and rectal and anal pressure during attempted defecation (straining)<sup>[57,71]</sup>. The results will vary with age and sex; normal values based on a large cohort of healthy individuals are still lacking<sup>[57]</sup>. Level III evidence, Grade C recommendation.

How should I interpret the results of anorectal manometric tests for obstructed defecation? The interpretation of the manometric data in clinical and physiopathologic terms is summarised in Table 4.

Are there typical manometric abnormalities in chronic constipation and/or obstructed defecation? The main abnormality in obstructed defecation is absent or inadequate relaxation of the anal sphincter, sometimes associated, paradoxically, with contraction during straining (dyssynergia). Obstructed defecation may also be associated with absent or inadequate rectal pressure<sup>[65,67]</sup>. The “defecation index”, or the ratio of maximal rectal pressure to minimal anal residual pressure<sup>[65]</sup>, quantifies recto-anal coordination during attempted defecation. Abnormalities have also been reported in anal canal resting pressure, anal canal squeezing pressure (external anal sphincters exhaustion), rectoanal inhibitory reflex (RAIR), rectal sensitivity, and compliance. Level III evidence, Grade C recommendation.

How can anorectal manometry be used to guide choices regarding therapy? Anorectal manometry can shed light on the physiopathologic mechanisms of obstructed defecation and help to develop a pelvic floor rehabilitation program for the patient<sup>[72]</sup>. It should be included in the pre-operative evaluation when a surgical reduction in rectal capacity is planned<sup>[73,74]</sup>. If RAIR is absent, Hirschsprung disease should be suspected. Elevated sensory thresholds, increased compliance, and rectal motor dysfunction are frequent in constipated patients<sup>[75,76]</sup> and can be treated with sensory retraining biofeedback therapy, based on sensory values obtained by means of anorectal manometry<sup>[77]</sup>. The results of bio-

feedback and electrical stimulation can be measured with anorectal manometry, and in fact, a reduction in rectal sensory thresholds has been demonstrated<sup>[78,79]</sup>. Level III evidence, Grade C recommendation.

### Balloon expulsion test

The balloon expulsion test is a simple, inexpensive test that can identify patients with abnormal defecation.

What is the usefulness of the balloon expulsion test to diagnose dyssynergic defecation? The balloon expulsion test has not yet been standardised; the filling volume of the balloon, the position of the patient, and the expulsion time have differed in various studies.

Trials including healthy controls. Two trials performed the test with the patient seated and the balloon filled with 50 mL of water; 59%<sup>[67]</sup> and 25%<sup>[80]</sup> of the constipated patients and 16%<sup>[67]</sup> of the controls were unable to expel the balloon within 5 min.

In the third trial<sup>[81]</sup>, the expulsion time was not specified, and the test was performed with a balloon filled with different volumes of water; 100% of patients with idiopathic megarectum, 53% of patients with a normal colonic transit time, 36% of patients with a slow transit colonic time, and 7% of controls were unable to expel the balloon.

Other trials. Some trials<sup>[82-84]</sup> have assessed patients with pelvic floor dyssynergia and have reported positive results in 23% to 57% of patients. However, different methods were used, so the results are not comparable.

In one trial<sup>[85]</sup>, the balloon was filled to the point at which the need to defecate was triggered, and the balloon had to be expelled within one minute. The authors concluded that a negative test is useful “to identify patients who do not have dyssynergia” and resulted in a specificity of 89%, a sensibility of 88%, a positive predictive value of 67%, and a negative predictive value of 97%.

The balloon expulsion test cannot be used as a gold standard for the diagnosis of “dyssynergic defecation” and should be integrated with other anorectal tests. Level

III evidence, Grade C recommendation.

### Colonic manometry

Slow transit constipation (STC) is characterised by prolonged colonic transit, generally measured in terms of intestinal transit time using radiopaque markers<sup>[86]</sup>. Colon manometry shows the daily patterns of bowel activity, identifying high amplitude waves, which correspond to mass movement in the intestine, and low amplitude waves<sup>[87,88]</sup>. Manometric studies<sup>[89,90]</sup> in STC patients have shown that propagating activity may be altered in frequency, amplitude and duration; segmental activity can be maintained or drastically lost, but there is, above all, a subversion of the periodicity of motor activity in the colon. Recently, a new method of evaluating propagated motor activity or “propagating sequences” has been developed, but it is still in the experimental stage<sup>[91]</sup>.

What are the clinical applications of colonic manometry? In patients with serious STC symptoms, colonic manometry can be helpful in the diagnosis and in decisions regarding therapy (whether conservative or surgical)<sup>[61]</sup>. Level IV evidence, Grade C recommendation.

How should colon manometry be performed in patients with slow transit constipation? In the clinical setting, the bisacodyl test should be used. This procedure tests the stimulation of residual colonic propulsive activity, and it can be used to identify the subgroup of patients with severe slow transit constipation or “inertia coli”, one incontrovertible indication for total colectomy<sup>[92-94]</sup>. Thus, colonic manometry may help to diagnose an underlying myopathy or neuropathy and to differentiate slower transit due to neuromuscular function<sup>[95]</sup>. Level V evidence, Grade C recommendation.

### Pathologies of the colon

The pathophysiology of slow transit constipation is not known<sup>[96]</sup>, but there is evidence to indicate that certain subtypes of idiopathic constipation are secondary to visceral neuropathy<sup>[97-99]</sup>, such as aberrant regulation of the nervous enteric system or parasympathetic alterations<sup>[100]</sup>.

What STC alterations can be verified on histology? Qualitative and quantitative alterations in the enteric nervous system can be observed on histology, from alterations in the neurotransmitters to the loss of argyrophilic neurons and neurofilaments and myenteric plexus hypoganglionosis<sup>[101]</sup>. More recently, reductions in the number of cells of Cajal have been described<sup>[102,103]</sup>. Level III evidence, Grade C recommendation.

Is an endoscopic biopsy sufficient, or is a full-wall thickness biopsy necessary? Endoscopic biopsies only provide information on the mucosa and cannot detect other histological alterations; therefore, they are not useful in the pathogenetic evaluation of STC. Given the nature of the alterations, it is necessary to conduct biopsies that reach the muscle layer.

What is the role of the suction biopsy in STC? Suction biopsy is the gold standard for the diagnosis of intestinal neurodysplasia, particularly in children. In the

differential diagnosis, four biopsy samples should be taken between 2 cm and 10 cm from the pectinea line<sup>[104]</sup>. The histological findings can distinguish STC from Hirschsprung disease and contribute to the diagnosis of intestinal neurodysplasia and other degenerative diseases of the colon (i.e., amyloidosis, desmosis, elastosis)<sup>[105]</sup>. Level II evidence, Grade B recommendation.

What is the role of immunohistochemistry? Immunohistochemistry is the main tool for the histological evaluation of nerves and connective tissues. There are no clinical studies in the literature that focus on this particular examination. Pathologists recommend that immunohistochemical analysis be undertaken in suspected cases of STC<sup>[106]</sup>.

The Consensus Committee therefore recommends that immunohistochemistry be performed to document patterns of slow transit constipation.

### Gastrojejunal manometry

There is evidence that slow transit constipation subtends diffuse enteric neurological involvement, probably of the myenteric plexus and, above all, the system of interstitial cells of Cajal<sup>[107]</sup>. Various studies have highlighted different ileal dysfunctions: in two retrospective analyses, 20.6% of patients with chronic constipation showed gastrojejunal abnormalities<sup>[61,108]</sup>. Cardiovascular tests for dysautonomia, which are widely used in diabetic neuropathy, are not applicable in the diagnostic workup of slow transit constipation.

The most meaningful test for myopathic or neuro-pathic involvement (especially in the pre-surgical evaluation) in patients with chronic constipation is gastrojejunal manometry, as stated recently by the American Neurogastroenterology and Motility Society<sup>[109-111]</sup>.

What are the clinical applications of gastrojejunal manometry? Gastrojejunal manometry can be used to analyse antro-duodenal activity and fasting jejunal motility, particularly in patients with autonomic dysfunctions, such as diabetic neuropathy. In a recent study of 61 subjects undergoing gastrojejunal manometry, all STC patients and 94% of those patients with normal transit constipation exhibited alterations in small bowel motility in the postprandial and fasting phases, but there were no significant differences between the two groups<sup>[109]</sup>.

When should gastrojejunal manometry be performed in STC patients? In cases of STC, gastrojejunal manometry is recommended before surgery<sup>[93,109]</sup>. Level III evidence, Grade C recommendation.

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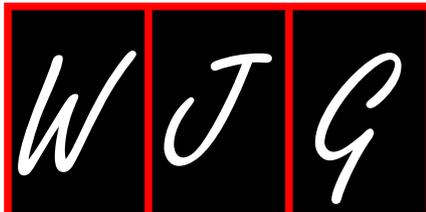
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## Adjuvant and neoadjuvant treatment in pancreatic cancer

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### Abstract

Pancreatic adenocarcinoma is one of the most aggressive human malignancies, ranking 4th among causes for cancer-related death in the Western world including the United States. Surgical resection offers the only chance of cure, but only 15 to 20 percent of cases are potentially resectable at presentation. Different studies demonstrate and confirm that advanced pancreatic cancer is among the most complex cancers to treat and that these tumors are relatively resistant to chemotherapy and radiotherapy. Currently there is no consensus around the world on what constitutes "standard" adjuvant therapy for pancreatic cancer. This controversy derives from several studies, each fraught with its own limitations. Standards of care also vary somewhat with regard to geography and economy, for instance chemo-radiotherapy followed by chemotherapy or *vice versa* is considered the optimal therapy in North America while chemotherapy alone is the current stan-

dard in Europe. Regardless of the efforts in adjuvant and neoadjuvant improved therapy, the major goal to combat pancreatic cancer is to find diagnostic markers, identifying the disease in a pre-metastatic stage and making a curative treatment accessible to more patients. In this review, authors examined the different therapy options for advanced pancreatic patients in recent years and the future directions in adjuvant and neoadjuvant treatments for these patients.

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**Key words:** Pancreatic ductal adenocarcinoma; Adjuvant; Neoadjuvant; Fluorouracil; Gemcitabine

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### INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies, ranking 4th among causes of cancer-related death in the Western world<sup>[1]</sup>.

Unlike most of the more frequent causes of cancer mortality (lung, colon, prostate and breast cancers) whose death rates are declining, the death rate for pancreatic cancer is relatively stable.

The poor prognosis is reflected by a median survival of 5-8 mo and a 5-year survival of less than 5% when all stages are combined<sup>[1-3]</sup>.

PDAC is characterized by a rapid disease progression

and absence of specific symptoms, largely precluding an early diagnosis and curative treatment<sup>[5,4]</sup>.

In most cases, PDAC is already locally advanced at time of diagnosis and only approximately 10%-20%<sup>[1,5]</sup> of patients are considered candidates for curative resection. The majority of patients (50%-60%) present with metastatic disease, and thus palliative chemotherapy remains the only option for almost all of these patients<sup>[6]</sup>. Owing to the high recurrence rate, surgical PDAC patients require adjuvant chemotherapy with or without radiotherapy providing a 5-year survival rate of 15%-25%<sup>[7]</sup> (Table 1).

Due to the described overall prognosis for all pancreatic cancer patients, systemic chemotherapy, radiation therapy or a combination of both is used following surgical resection (adjuvant therapy) and also prior to the tumor resection (neoadjuvant therapy) to improve cure rates.

Although the benefit of adjuvant and neoadjuvant therapy has been improved in recent years, the best choice of treatment modality still remains highly controversial.

The objective of this review is to examine therapies received by advanced pancreatic cancer patients in recent years and to examine the principal chemotherapeutic agents or molecular-targeted therapies useful for clinicians.

## ADJUVANT THERAPY

In an effort to improve the outcome in patients undergoing potentially curative resection, systemic chemotherapy (Table 2), radiotherapy or a combination of both have been applied following surgery.

## SYSTEMIC THERAPY

### Chemotherapy

The first randomized controlled trial of adjuvant therapy in pancreatic cancer was designed by the Gastrointestinal Tumor Study Group, which concluded that treatment with 5-fluorouracil (5-FU) plus radiation followed by two years of weekly 5-FU maintenance provided better outcomes than surgery alone<sup>[8]</sup>. Although this trial was criticized for many reasons, it served to establish 5-FU as the only standard adjuvant therapy for many years in pancreatic cancer. Different drugs and combinations have emerged and been incorporated for the best treatment of these patients (Table 2).

**5-FU:** 5-FU is a thymidylate synthase inhibitor that blocks the synthesis of pyrimidine thymidine, a nucleotide required for DNA replication.

5-FU had been considered the only chemotherapeutic option for about 20 years until the registration of gemcitabine. Several trials conducted in the late 1970s and early 1980s demonstrated that adjuvant chemotherapy using bolus 5-FU therapy conferred a survival benefit in patients with resected pancreatic cancer<sup>[8]</sup>.

Different studies in the last years have demonstrated a survival benefit from six months of postoperative leucovorin-modulated 5-FU in patients with resected pan-

Table 1 Staging of pancreatic cancer

Stage	TNM classification	Clinical classification	5-year percent survival (mo)
Stage 0	TisN0M0	Resectable	
Stage I A	T1N0M0	Initial	31.4
Stage I B	T2N0M0		27.2
Stage II A	T3N0M0		15.7
Stage II B	TXN1M0	Locally advanced	7.7
Stage III	T4NXM0		6.8
Stage IV	TXNXM1	Metastatic	2.8

Tis: Cancer *in situ*; T: Size and/or extent of invasion; N: Extent of lymph node involvement; M: Whether distant metastases are present.

creatic cancer, compared to those receiving no adjuvant chemotherapy (median overall survival 19.7 mo *vs* 14 mo respectively, statistically significant)<sup>[9-11]</sup>.

Although for locally advanced and metastatic patients this drug leads to an improved survival compared to the best supportive care<sup>[12,13]</sup>, the combination of 5-FU with other drugs such as doxorubicin or mitomycin did not prove superior to the antimetabolite alone. Similar results were obtained comparing single agent 5-FU to 5-FU plus cyclophosphamide, methotrexate and vincristine<sup>[14]</sup> as the combination did not offer a survival advantage over 5-FU alone.

Only the combination of 5-FU/irinotecan/oxaliplatin (FOLFIRINOX) has been associated with a high objective response rate based on imaging study, and this finally is the preferred regimen for patients who have good performance status and a normal serum bilirubin level<sup>[15]</sup>.

In the last years, new fluoropyrimidines that mimic the effect of a continuous infusion of 5-FU have been approved. One of the most common but not available in all countries is S-1, an orally active fluoropyrimidine, with favorable antitumor activity in gemcitabine-refractory disease<sup>[16,17]</sup>.

**Capecitabine:** Capecitabine is an orally administered fluoropyrimidine that is absorbed intact through the intestinal wall and then converted to 5-FU in three sequential enzymatic reactions: carboxylesterases, cytidine deaminase and thymidine phosphorylase. The last enzyme is present at consistently higher levels in tumor rather than normal tissue, thereby providing the basis for enhanced selectivity and better tolerability<sup>[18]</sup>. The efficacy of capecitabine in monotherapy was shown with high clinical response rate (24%) but low objective response (7%)<sup>[19]</sup>; however, no advantage using capecitabine in monotherapy over gemcitabine alone has been demonstrated.

**Gemcitabine:** The development of gemcitabine may be considered a major advance in the treatment of pancreatic cancer. This drug is a difluorinated analog of deoxycytidine. As a prodrug, gemcitabine must be phosphorylated by cytoplasmic and mitochondrial enzymes to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. The cytotoxic effect of this drug is attributed to a combination of two actions of the

Table 2 Mode of action of principal drugs used in pancreatic cancer

Agent	Mode of action
5-FU	5-FU is a folate antimetabolite that forms a ternary complex involving 5-fluoro-2-deoxyuridine-5-monophosphate, thymidylate synthase, and 5,10-methylene THF. The formation of this complex thereby inhibits thymidylate synthase activity, which subsequently depletes intracellular thymidylate levels and ultimately suppresses DNA synthesis
Gemcitabine (Gemzar®)	Also, two metabolites of 5-FU, 5-fluoro-2-deoxyuridine-5-triphosphate and 5-fluorouridine-5-triphosphate, can be incorporated into DNA and RNA, respectively, resulting in DNA instability and interfering with RNA processing and function Gemcitabine is an S-phase nucleoside analogue (difluorodeoxycytidine) that is phosphorylated to difluorodeoxycytidine triphosphate by deoxycytidine kinase. Gemcitabine also stimulates deoxycytidine kinase and inhibits both ribonucleotide reductase and deoxycytidine monophosphate deaminase. Gemcitabine triphosphate is incorporated into nascent DNA to inhibit DNA synthesis
Capecitabine (Xeloda®)	Capecitabine an oral, tumor-selective fluoropyrimidine carbamate that is sequentially converted to 5-FU by three enzymes located in the liver and in tumors. The final step is the conversion of 5'-deoxy-5-fluorouridine to 5-FU by thymidine phosphorylase in tumors
Platinum analogues	Platinum forms adducts with DNA inhibiting transcription and replication causing cell death. Oxaliplatin is a third-generation platinum analogue (a diaminocyclohexane platinum derivative) that may have activity in tumors resistant to cisplatin or carboplatin and may have an additive/synergistic activity in doublet or triplet therapy
Taxanes	The taxanes include paclitaxel and docetaxel (Taxotere®) and are semi-synthetic microtubule inhibitors with a different mechanism of action from the vinca alkaloids. Taxanes bind to $\beta$ -tubulin, promoting microtubule assembly and preventing depolymerisation thus forming stable non-functional complexes and inhibiting the function of the mitotic spindle; This results in cell cycle arrest and increased sensitivity to radiation
Irinotecan (CPT11, Camptosar®)	Irinotecan is a topoisomerase I inhibitor that impedes the DNA helix torsional stress-relieving activity of DNA topoisomerases and also prevents their release from the DNA thus prompting apoptosis

5-FU: 5-fluorouracil.

diphosphate and triphosphate nucleosides, which leads to inhibition of DNA synthesis<sup>[20,21]</sup>.

The first pivotal trial found that gemcitabine is more effective than 5-FU in alleviation of some disease-related symptoms in patients with advanced, symptomatic pancreatic cancer, conferring a modest survival advantage over treatment with 5-FU. As the treatment with gemcitabine was associated with significant clinical response and better survival, this drug was approved for first-line therapy of metastatic pancreatic cancer. This pivotal phase III trial demonstrated improvement in median overall and 1-year survival compared to 5-FU (5.7 mo *vs* 4.4 mo and 18% *vs* 2%, respectively)<sup>[22]</sup>.

Many phase II studies have demonstrated the efficacy of gemcitabine combination treatments, but not all of the phase III trials confirmed the improvement in overall survival (OS) of gemcitabine-based regimens compared to gemcitabine alone. However, an improvement in six-month survival was seen by combining gemcitabine-fluoropyrimidine analogues and gemcitabine-platinum analogues, as demonstrated in the meta-analysis of Heinemann and colleagues<sup>[2]</sup>.

Due to the results obtained in monotherapy, gemcitabine has been combined with many other active cytotoxic agents including 5-FU, cisplatin, docetaxel, oxaliplatin and irinotecan, in an attempt to improve the response in pancreatic cancer patients and each will be discussed here separately.

**Gemcitabine and 5-fluorouracil:** Based on the complementary pharmacology of their mechanisms of action, the combination of 5-FU and gemcitabine has been evaluated in phase I, II and III trials. Finally, phase III trials showed that there is no significant improvement in

median OS and median progression-free survival when evaluating the combined regimen compared to that of gemcitabine alone<sup>[23-26]</sup>.

**Gemcitabine and capecitabine:** Different phase III trials have shown that patients who received gemcitabine and capecitabine compared to gemcitabine alone have a significant improvement in survival<sup>[27,28]</sup>.

These data and the meta-analysis performed by these authors suggest that the combination of gemcitabine plus capecitabine should be considered a standard first-line option for locally advanced and metastatic pancreatic cancer.

**Gemcitabine and platinum combinations:** Since gemcitabine enhances the formation of cisplatin-DNA adducts, an effect that may be due to suppression of nuclear excision repair by gemcitabine, and the platinum may augment the incorporation of gemcitabine triphosphate into DNA<sup>[29]</sup>, the gemcitabine and platinum combination has been assessed in different trials.

Although in preclinical studies the combination of gemcitabine and cisplatin is synergistic, at least three phase III trials comparing gemcitabine to the combination of gemcitabine plus cisplatin showed no significant survival advantage for this approach<sup>[30-32]</sup>. Furthermore, the combination of gemcitabine and platins has not shown improvement in terms of response and is not a considered option for pancreatic cancer patients.

**Gemcitabine and irinotecan:** As irinotecan (a topoisomerase inhibitor) had minimal clinical activity in patients with advanced pancreatic cancer, combined therapy with gemcitabine is not recommended<sup>[33,34]</sup> and in some

cases the combination could lead to major toxicity.

**Gemcitabine and taxanes:** Antitumoral action of taxanes is due to their mechanism of microtubule stabilization and consequently to cell cycle arrest. The association of gemcitabine with paclitaxel or docetaxel in advanced pancreatic patients was studied in different trials and has shown encouraging response rates<sup>[35,36]</sup>. A phase III trial has not yet been completed. Thus, whether this regimen represents an improvement over gemcitabine alone is unclear.

The available data suggest that if there is a benefit to gemcitabine combination therapy compared to gemcitabine alone, it is modest and best documented for capecitabine plus gemcitabine. Today, only gemcitabine alone and the combination of gemcitabine plus capecitabine represent good options for initial therapy.

In summary, adjuvant fluorouracil has been shown to be of benefit for patients with resected pancreatic cancer but gemcitabine is the most effective agent in advanced disease. Compared with the use of fluorouracil, gemcitabine does not result in improved overall survival in patients with completely resected pancreatic cancer<sup>[37]</sup>.

### Combined therapies

As compared with gemcitabine, FOLFIRINOX was associated with a survival advantage and had increased toxicity. FOLFIRINOX is an option for the treatment of patients with metastatic pancreatic cancer and good performance status<sup>[38]</sup>.

### Targeted molecular therapy

Based on the biological properties of pancreatic cancer, new systemic therapies have been tried. The most common molecular targets have been epidermal growth factor receptor (EGFR)/KRAS, human epidermal growth factor receptor type 2 (HER2) and vascular endothelial growth factor (VEGF), as these genes are overexpressed or mutated in pancreatic tumors.

**Targeting EGFR:** Currently, there are two approaches targeting the EGFR system: monoclonal antibodies (i.e., cetuximab/Erbitux<sup>®</sup>) and small molecule tyrosine kinase inhibitors. In spite of promising preclinical trials, cetuximab as monotherapy, or in combination with other cytotoxic agents such as gemcitabine or with radiotherapy, has failed to improve the outcome of PDAC patients<sup>[39,40]</sup>.

Up to now, the only EGFR targeting demonstrating a clinical benefit is erlotinib (Tarceva<sup>®</sup>, OSI 774), a tyrosine kinase inhibitor that inhibits ErbB-1 phosphorylation. One phase III trial of erlotinib with gemcitabine was able to show at least a small gain in the survival of patients with advanced PDAC<sup>[41]</sup>. Although erlotinib obtained Food and Drug Administration approval and access in clinical application in 2005, the therapeutic benefit for patients with advanced PDAC remains poor.

**Targeting HER2:** In several studies, HER-2 overexpression in pancreatic cancer has been reported to vary widely (10%-82%)<sup>[42,43]</sup> and it does not correlate with poor prognosis<sup>[44]</sup>. Although studies in a mouse model have shown that combination of anti-HER2 antibodies (i.e., trastuzumab) and other chemotherapy may be effective for HER2-overexpressing pancreatic cancer patients<sup>[45]</sup>, the clinical significance is uncertain. A phase II clinical trial of trastuzumab for pancreatic cancer has been conducted and showed only 6% response to combined therapy with trastuzumab and gemcitabine in patients with metastatic pancreatic cancer, which is not superior to therapy with gemcitabine alone<sup>[46]</sup>.

Currently, anti-Her2 therapy is experimental and still under investigation for the treatment of pancreatic cancer.

**Targeting VEGF:** A phase III trial concluded that there is no benefit for the addition of bevacizumab to gemcitabine *vs* gemcitabine alone and *vs* gemcitabine and cetuximab<sup>[47]</sup>.

Also some studies have failed to demonstrate a benefit for adding axitinib (an oral inhibitor of VEGF receptors 1, 2 and 3) to gemcitabine<sup>[48,49]</sup>. Currently, the anti-VEGF approach is not recommended in pancreatic cancer.

### Hormonal therapy

Tamoxifen and octreotide are not indicated in metastatic pancreatic cancer because both of them have failed to demonstrate any survival advantage for treated patients<sup>[50,51]</sup>.

## RADIOTHERAPY

The use of adjuvant radiotherapy for pancreatic cancer is controversial and the role of radiation therapy continues to be investigated. Currently, the addition of radiotherapy depends on the country in which a patient is being treated<sup>[52]</sup>.

Chemo-radiotherapy followed by chemotherapy is considered the optimal therapy in North America (Gastrointestinal Tumor Study Group; Radiation Therapy Oncology Group) while chemotherapy alone is the current standard in Europe (European Study Group for Pancreatic Cancer; Charité Onkologie)<sup>[10,53,54]</sup>.

The rationale for adjuvant radiotherapy for pancreatic cancer is to improve loco-regional control. Modern radiation delivery techniques, such as intensity-modulated radiation therapy or image-guided and stereotactic body radiation therapy, permit dose escalation in order to reduce normal tissue toxic effects and simultaneously deliver increased doses of radiation to affected areas<sup>[55,56]</sup>. It is clear that breakthroughs in the treatment of this devastating disease will come mostly from advances in systemic therapy, so radiotherapy should not be abandoned, but rather, intensified.

Intraoperative radiotherapy has also been considered, since local recurrence rates are very high. In general, intraoperative radiotherapy can slightly increase survival rates among patients with pancreatic cancer in localized

stages. There is no clear evidence to indicate that intraoperative radiotherapy is more effective than other therapies in treating pancreatic cancer in locally advanced and metastatic stages<sup>[57]</sup>.

## CHEMORADIO THERAPY

Some studies demonstrated improved survival when radiotherapy was combined with 5-FU chemotherapy compared with radiotherapy alone, in patients with locally advanced unresectable pancreatic cancer<sup>[58]</sup>. This combined therapy has been applied to patients undergoing RO resection to improve surgical cure rate.

In locally advanced pancreatic cancer, recent evidence using modern radiotherapy techniques and dosing suggests a continued role for radiotherapy. In both resected and unresected disease, further studies are needed to define optimal radiation dose, field size, and technique, and to assess the effect of radiotherapy not only on survival, but also on local disease control and quality of life<sup>[59]</sup>.

## NEOADJUVANT THERAPY

The low rate of resectability and the poor outcomes following pancreaticoduodenectomy have led to the investigation of preoperative and postoperative therapies to identify those patients who are not candidates for surgery and who could benefit from neoadjuvant chemotherapy and/or radiotherapy.

The initial reports using radiation therapy with or without 5-FU did not demonstrate an obvious improvement in either resectability or overall survival<sup>[60,61]</sup>. Subsequent studies improved the treatment by increasing radiotherapy dose, adding intraoperative radiotherapy and using combined chemotherapy. The drugs tested were mitomycin, 5-FU, 5-FU and cisplatin, and paclitaxel, but their efficacy remains uncertain<sup>[62-64]</sup>.

Subsequent reports used gemcitabine-based chemotherapy which provided an enhanced local effect, although with potentially more toxicity than 5-FU-based regimens. Gemcitabine has also been combined with radiotherapy and cisplatin<sup>[65,66]</sup>.

Currently, neoadjuvant radiation is associated with improved survival in patients with resectable pancreatic cancer<sup>[67]</sup> but chemotherapy alone without radiotherapy is beginning to be studied and the experience is limited<sup>[68,69]</sup>.

## ADJUVANT VS NEOADJUVANT THERAPY

Although the median survival times reported from some uncontrolled trials of neoadjuvant therapy compare favorably to those reported with adjuvant therapy approaches<sup>[65,70,71]</sup>, the question as to whether preoperative therapy is better than postoperative therapy is uncertain as there are no randomized trials comparing the two approaches.

One advantage of neoadjuvant therapy is that it avoids the morbidity of pancreaticoduodenectomy in patients who have occult, micrometastatic disease that

becomes evident during therapy. A second advantage is that in patients undergoing surgery, prolonged recovery prevents the delivery of postoperative adjuvant chemotherapy in about a quarter of them<sup>[72]</sup>.

Recent studies have shown that neoadjuvant therapy is associated with a lower rate of lymph node positivity and improved overall survival and should be considered an acceptable alternative to the surgery-first paradigm in operable pancreatic cancer<sup>[73]</sup>.

## SECOND-LINE THERAPY

There are few trials of second-line therapy in patients who have failed chemotherapy, and there is no widely accepted standard of care.

For patients who retain a good performance status after failing initial gemcitabine therapy, benefit has been suggested from a second-line therapy based on oxaliplatin/fluoropyrimidine combination such as 5-FU and oxaliplatin<sup>[65,70,71]</sup>. Other oxaliplatin combinations are also acceptable with the agents gemcitabine, irinotecan or capecitabine<sup>[74-76]</sup>.

There are no data for patients who fail initial 5-FU and oxaliplatin, but a reasonable option is gemcitabine as monotherapy.

## CONCLUSION

All the treatment options examined in this review demonstrate and confirm that advanced pancreatic cancer is among the most complex cancers to treat.

Currently there is no consensus regarding the optimal management of patients after resection of an exocrine pancreatic cancer, and the approach is different in Europe and in the United States. Most European clinicians use chemotherapy alone after resection of a pancreatic neoplasm. The American approach more often includes chemoradiotherapy as well as adjuvant chemotherapy.

Although it is mainly accepted that a 6-mo course of systemic chemotherapy with gemcitabine or 5-FU should be part of any adjuvant treatment, there is no single adjuvant regimen of chemotherapy or chemoradiotherapy that can claim unequivocal superiority over others. Among these options there are no differences in outcome but fewer side effects occur with gemcitabine, and this is nowadays the preferred regimen.

Based on current data, it is clear that treatment with gemcitabine or 5-FU results in a median survival of just a few months<sup>[77,78]</sup>. The limitation of this treatment is mainly due to the profound resistance of PDAC cells towards anti-cancer drugs, emerging from the efficient protection against chemotherapeutic drugs by an altered balance of pro- and anti-apoptotic proteins which results in a markedly reduced apoptotic responsiveness<sup>[79,80]</sup>.

Currently there are around 1070 clinical trials focusing on studying new biomarkers, different drug combinations and vaccines designed for pancreatic cancer ([www.clinicaltrial.gov](http://www.clinicaltrial.gov)).

Regardless of these efforts in adjuvant and neoadjuvant therapy, the major goal to combat PDAC is to find diagnostic markers, identifying the disease in a pre-metastatic stage and making a curative treatment accessible to more patients. Given an earlier diagnosis, surgical interventions together with adjuvant radio/chemotherapy are the most promising options. Considering such evidence, the urgent need for an individualized and more effective adjuvant therapy is evident.

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## 2011 update on esophageal achalasia

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### Abstract

There have been some breakthroughs in the diagnosis and treatment of esophageal achalasia in the past few years. First, the introduction of high-resolution manometry with pressure topography plotting as a new diagnostic tool has made it possible to classify achalasia into three subtypes. The most favorable outcome is predicted for patients receiving treatment for type II achalasia (achalasia with compression). Patients with type I (classic achalasia) and type III achalasia (spastic achalasia) experience a less favorable outcome. Second, the first multicenter randomized controlled trial published by the European Achalasia Trial group reported 2-year follow-up results indicating that laparoscopic Heller myotomy was not superior to endoscopic pneumatic dilation (PD). Although the follow-up period was not long enough to reach a convincing conclusion, it merits the continued use of PD as a generally available technique in gastroenterology. Third, the novel

endoscopic technique peroral endoscopic myotomy is a promising option for treating achalasia, but it requires increased experience and cautious evaluation. Despite all this good news, the bottom line is a real breakthrough from the basic studies to identify the actual cause of achalasia that may impede treatment success is still anticipated.

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**Key words:** Esophageal achalasia; High resolution manometry; Endoscopic pneumatic dilations; Minimally invasive surgical procedures; Peroral endoscopic myotomy

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### INTRODUCTION

Achalasia is the only primary motor disorder of the esophagus with a well-understood pathophysiology. It affects both sexes and all races equally<sup>[1-3]</sup>. Achalasia involves the selective loss of inhibitory neurons in the myenteric plexus, leading to the production of vasoactive intestinal polypeptide (VIP), nitric oxide (NO), and inflammatory infiltrate responsible for abnormal lower esophageal sphincter (LES) dysfunction. An unopposed excitation of the LES causes its dysfunction or failure to relax in response to swallowing<sup>[3,4]</sup>.

Clinical presentations include dysphagia for both liquid and solid food, and food regurgitation may be severe enough to produce pulmonary complications such as cough or aspiration pneumonia. Weight loss usually

occurs as a result. The diagnosis of achalasia is made on the basis of the results of barium esophagography, esophageal manometry, and endoscopy. Typical radiological signs are classic “bird-beak” of the gastroesophageal junction, with atonia and a dilated esophageal body observed by barium ingestion and fluoroscopy. Manometry is still the standard diagnostic test for achalasia. The basic criteria required for diagnosis of achalasia include the absence of relaxation of the LES or abnormal swallowing relaxation of the LES and the absence of peristalsis in the esophageal body. However, an endoscopic examination, preferably endoscopic ultrasonography or computed tomography, is always necessary to distinguish primary achalasia from the secondary form<sup>[5]</sup>, in cases of possible malignancy<sup>[6]</sup>. A greater risk of esophageal squamous cell carcinoma among achalasia patients is well established. Male achalasia patients have substantially greater risks for both squamous cell carcinoma and adenocarcinoma of the esophagus<sup>[7]</sup>. Recent advances in the diagnosis of esophageal achalasia by using updated high-resolution manometry (HRM) with pressure topography plotting have made it possible to classify achalasia into three subtypes. Furthermore, it is now possible to predict the outcome of each type of achalasia<sup>[8-10]</sup>. In this paper, the implications of the recent findings in the diagnosis and treatment of esophageal achalasia are reviewed and discussed.

## **PATHOGENESIS**

The mechanisms responsible for the selective loss of inhibitory neurons in the myenteric plexus that produces VIP, NO and inflammatory infiltrate responsible for abnormal LES dysfunction is still not well understood<sup>[11]</sup>. However, specimens taken from autopsy or myotomy have shown the histological damage of the esophageal myenteric plexus and an inflammatory response consisting of CD3/CD8-positive cytotoxic T lymphocytes, variable numbers of eosinophils and mast cells, loss of ganglion cells, and neurofibrosis<sup>[12,13]</sup>. These events occur even at the early inflammatory stages of achalasia, and the underlying cause has not yet been identified. Previous studies have implicated hereditary, neurodegenerative, genetic, infectious, and autoimmune mechanisms. The most acceptable hypothesis suggests that achalasia may be caused by viral and autoimmune factors, leading to the inflammatory changes and damage to the myenteric plexus.

Achalasia patients with certain genetic backgrounds are reported to develop an autoimmune reaction and hence the production of autoantibodies that cause chronic inflammation and destruction of inhibitory neurons<sup>[12,13]</sup>. In addition, infiltration of the myenteric ganglia with CD3/CD8-positive lymphocytes that express activation markers, IgM antibodies, and evidence of complement activation have been observed within the myenteric ganglia<sup>[14-16]</sup>. Moreover, antibodies against myenteric neurons have been detected in the serum of patients with esophageal achalasia; especially in those with a specific

human leukocyte antigen genotype (DQA1 × 0103 and DQB1 × 0603 alleles)<sup>[17-19]</sup>. The above evidence indicates that an autoimmune mechanism likely plays an important role in achalasia. However, the trigger for the destructive autoimmune events is unknown. So far, viral infection is believed to be the main cause. Some possible causative infections are varicella zoster and measles viruses<sup>[16,20-22]</sup>. However, some may argue that antineuronal antibodies have also been reported in the serum of gastroesophageal reflux disease patients and even in healthy individuals, suggesting that these antibodies may simply result from tissue damage secondary to inflammation<sup>[19]</sup>. The other unanswered question is why only neurons in the esophagus and LES are destroyed. The results of some studies have led to the hypothesis that neurotropic viruses, especially those with a predilection for squamous epithelium, could be involved; however, these findings have been inconsistent<sup>[16,20-23]</sup>.

Although there have been many excellent basic studies, the presence of viral antibodies in the serum of patients has been an inconsistent finding. The method to verify the actual cause of achalasia that may impede treatment success is yet to be determined.

## **DIAGNOSIS**

The diagnosis of achalasia is on the basis of the results of gastroscopy, manometry, and timed barium esophagography. Pseudoachalasia is always excluded by either computed tomography or endoscopic ultrasonography. Since the emergence of HRM with pressure topography plotting, esophageal achalasia can be classified into three subtypes<sup>[8-10]</sup>. In type I achalasia (classic achalasia), impaired LES relaxation but no significant pressurization within the esophageal body is observed. In type II achalasia (with compression), swallowing of water causes rapid panesophageal pressurization. This may exceed LES pressure, causing the esophagus to empty. Type III achalasia (spastic achalasia) is also associated with rapidly propagated pressurization; however, the pressurization is attributable to an abnormal lumen, obliterating contraction. HRM can be used to predict the outcome of each type of achalasia. Patients in whom HRM shows type II achalasia (esophageal pressurization) are more likely to respond to therapies such as pneumatic dilation (PD), heller myotomy (HM), and botulinum toxin (BT) (overall, 70%-100%), compared to those with type I (overall, ≥ 50%-63.3%) and type III (overall, about 30%) achalasia<sup>[8,10]</sup>. HRM may play an increasingly important role in the diagnosis of esophageal achalasia in the future, especially when the technique becomes more affordable.

## **CURRENT TREATMENT OPTIONS**

Currently, there is no cure for esophageal achalasia. The only available therapeutic options are to loosen the LES and treat the symptoms<sup>[24]</sup>. However, the advantages of each option must be considered in the patients.

Pharmacological management such as smooth muscle relaxation usually plays a minor role in the treatment of esophageal achalasia<sup>[2,24]</sup>. Nitrates increase NO concentration in smooth muscle cells, and calcium antagonists block calcium and hence esophageal muscle contractions. By so doing, LES pressure can be reduced, but the efficacy is usually unsatisfactory and incomplete, with intolerable side effects such as headache, dizziness, and pedal edema. This is the same for other drugs such as sildenafil<sup>[25]</sup>.

## ENDOSCOPIC TREATMENT

Endoscopic treatment with BT injection at the terminal nerve endings of myoneural junctions prevents the release of acetylcholine from vesicles. This causes chemical denervation, which may last for several months<sup>[26]</sup>. As a result of its wider safety range and fewer complications, local injection of BT into the LES muscle of patients with achalasia lowers LES tone, and the patient becomes asymptomatic. This treatment yields excellent immediate responses with success rates of > 90%. However, the results last only 6-9 mo on average in most patients, and only half of all the patients benefit for > 1 year<sup>[27]</sup>. Complications of BT therapy for achalasia are minor because the dosage used is too small to induce serious adverse effects such as generalized paralysis. It is therefore used to treat elderly patients or patients with high surgical risks<sup>[28]</sup>.

The most commonly used endoscopic balloon dilator is the rigiflex dilator. The dilation procedure can be performed under fluoroscopic<sup>[29]</sup> or endoscopic guidance<sup>[30,31]</sup>. The number of dilation sessions and the inflation time needed for a successful dilation vary and are operator dependent. Immediate and short-term results have reportedly been good in most series<sup>[30-33]</sup>. However, large-scale long-term follow-up investigations<sup>[28,34,35]</sup> have reported unfavorable recurrence in patients who have undergone fluoroscopically guided PD. During a prolonged observation period (median, 13.8 years) in a prospective follow-up investigation study conducted by Eckardt *et al.*<sup>[29]</sup>, only 40% of the patients treated using a single PD procedure remained in remission at 5 years. Generally, the response to PD is still determined on the basis of subjective improvement in symptoms, such as dysphagia, regurgitation, and chest pain, by performing structured interviews with validated symptom score methods<sup>[29,36]</sup>. However, additional radiographic findings could reliably predict clinical remission and strongly suggest the need for further treatment in patients with poor esophageal clearance after each dilation. This could prevent sigmoid-type achalasia<sup>[37-39]</sup>. It is generally accepted that the predictors of risk factors for relapse after PD include young age (< 40-45 years), male sex, single dilation with a 3.0 cm balloon, post-treatment LES pressure > 10-15 mmHg, poor esophageal emptying after timed barium swallow, and type I and type III achalasia pattern on HRM<sup>[2,40]</sup>. Complications attributable to PD are uncommon. The most severe complication is perfora-

tion<sup>[41]</sup>, which was reported to be approximately 2% in a recent analysis by Katzka *et al.*<sup>[42]</sup>.

Peroral endoscopic myotomy (POEM) is a novel endoscopic esophagomyotomy for achalasia that was first reported by Pasricha *et al.*<sup>[43]</sup> in porcine models and subsequently by Inoue *et al.*<sup>[44]</sup> in humans. POEM is performed by dissection and division of the inner circular muscle layer of the esophagus through a submucosal tunnel created endoscopically by a small proximal opening in the esophageal mucosa. POEM can be used to perform deeper myotomy incisions in the thoracic esophagus than that performed in surgical myotomy, which is difficult for the surgeon, and is indicated especially for patients with advanced disease and for those with severe fibrosis. Theoretically, injury to the vagus nerve should be less than that with the surgical approach. So far, several centers are using the POEM technique and have achieved good short-term results without serious complications, but long-term follow-up results are required<sup>[45]</sup>. There is concern that POEM is a sophisticated and demanding technique, even for experienced endoscopists, and serious complications such as purulent mediastinitis may develop. Revisional surgery might be difficult and involve extensive procedures such as esophagectomy because the plane between the submucosal and muscular layers will be inflamed and scarred after the endoluminal approach<sup>[46]</sup>.

A few Chinese studies have reported the utility of self-expanding, 30-mm metallic stents for achalasia at a single center over a 10- to 13-year period, with a long-term clinical success rate > 80%<sup>[47-50]</sup>. There were no perforations or mortality associated with the treatment, but stent migration occurred in 5% of the patients, reflux in 20%, and chest pain in 38.7%. Overall, the self-expanding, 30-mm metallic stents were associated with better long-term clinical efficacy in the treatment of patients with achalasia than treatment with PD.

## SURGICAL TREATMENT

From a surgical point of view, minimally invasive HM has become the gold standard procedure for achalasia in the spectrum of current treatment options<sup>[51,52]</sup>. Myotomy of the LES is the most direct method used and by far the best treatment modality for satisfactory long-term results with very low mortality. Overall success rates of laparoscopic HM (LHM) were 47%-82% at 10 years<sup>[53,54]</sup>. Systematic reviews and meta-analysis that have compared existing treatment methods for achalasia have found that surgery is superior to PD<sup>[55,56]</sup>. However, the major adverse event after surgery is severe reflux. There is much debate on the role of fundoplication with myotomy in the reported literature<sup>[57-59]</sup>. Intraoperative endoscopy during videoscopic HM is used to guide the extent and adequacy of myotomy by utilizing a focused dissection with preservation of the natural antireflux mechanisms around the gastroesophageal junction and by limiting the extent of myotomy along the cardia. By

so doing, postoperative reflux symptoms are minimized. A concomitant endoscope examination during HM to guide myotomy and routine fundoplication is clinically necessary, despite disagreement about the fundoplication procedure<sup>[60,61]</sup>. In addition, there is a lot of debate on the choice of laparoscopic cardiomyotomy as the primary treatment for achalasia or as a second-line treatment following the failure of nonsurgical intervention<sup>[62]</sup>. Some doctors believe that laparoscopic cardiomyotomy can be more technically difficult following PD<sup>[63]</sup>. However, it has been shown that laparoscopic cardiomyotomy can be as safe and effective as first- or second-line treatment, even after the failure of PD<sup>[64]</sup>. In general, esophagectomy should be reserved only for those cases in which simpler operations have failed. In summary, as stated in the recent Kagoshima consensus, despite the variations as to the length of the myotomy and the addition of an antireflux procedure, good overall long-term results suggest that these operative variations are not critical<sup>[65]</sup>.

## WHICH IS THE BETTER CHOICE: LHM OR PD? THE ONGOING DEBATE

In general, LHM is considered to be superior to PD for treating achalasia. Many experts have regarded LHM as the first choice of treatment for achalasia, at the cost of reflux complications. However, it should be noted that the first randomized controlled multicenter trial published by the European Achalasia Trial group that compared LHM and PD reported that, after 2 years of follow-up, LHM was not associated with superior rates of therapeutic success<sup>[66]</sup>. The large sample size gathered from 15 European centers and the excellent design of the study gave adequate statistical power for obtaining convincing results for the two treatment groups. Perforation of the esophagus occurred in 4% of the patients during PD, whereas mucosal tears occurred in 12% during LHM. Abnormal exposure to esophageal acid was observed in 15% and 23% of the patients in the PD and LHM groups, respectively. In addition, when considering the cost-effectiveness of treatment strategies for achalasia, laparoscopic myotomy has a higher initial cost, and PD is the most cost-effective treatment option for adults with achalasia. It is unclear how the results of the European Achalasia Trial actually affect the ongoing debate between gastroenterologists and surgeons on the treatment of choice for esophageal achalasia. This study had the following limitations. First, this was only a 2-year cohort study, and the intermediate and long-term remission rates have yet to be proven. Second, the good results for PD may be questioned by the definition of treatment failure in redilation sessions. In fact, all patients in the PD group routinely received at least two sessions of dilation. A maximum of three redilation sessions was allowed. However, this is an ongoing study, and more information will be collected in the future. The debate on which is the better choice between LHM and PD for

esophageal achalasia is ongoing; however, it is generally accepted that myotomy is usually suggested for younger patients (age, < 40-45 years), male patients, and those with pulmonary symptoms who failed to respond to one or two initial dilations<sup>[2,67]</sup>.

In summary, despite the ongoing debate and the report of the first randomized control trial, the minimally invasive surgical treatment seems to yield better results than PD with the currently available evidence, despite being less cost-effective and resulting in more reflux symptoms. POEM is a promising technique and is associated with good short-term results without serious complications, but long-term results are not yet available. Despite these advancements, the actual cause of achalasia has not yet been identified, and this knowledge may improve treatment success in the future.

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## Dual regulatory role for phosphatase and tensin homolog in specification of intestinal endocrine cell subtypes

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Enzyme-linked immunosorbent assay was used to measure blood circulating ghrelin, somatostatin (SST) and glucose-dependent insulinotropic peptide (GIP) levels.

**RESULTS:** Results show an unexpected dual regulatory role for epithelial *Pten* signalling in the specification/differentiation of enteroendocrine cell subpopulations in the small intestine. Our data indicate that *Pten* positively regulates chromogranin A (CgA) expressing subpopulations, including cells expressing secretin, ghrelin, gastrin and cholecystokinin (CCK). In contrast, *Pten* negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells.

**CONCLUSION:** The present results demonstrate that *Pten* signalling favours the enteroendocrine progenitor to specify into cells expressing CgA including those producing CCK, gastrin and ghrelin.

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**Key words:** Phosphatase and tensin homolog; Enteroendocrine cells; Intestinal epithelial cell specification; Chromogranin A

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### Abstract

**AIM:** To investigate the impact of phosphatase and tensin homolog (*Pten*) in the specification of intestinal enteroendocrine subpopulations.

**METHODS:** Using the Cre/loxP system, a mouse with conditional intestinal epithelial *Pten* deficiency was generated. *Pten* mutant mice and controls were sacrificed and small intestines collected for immunofluorescence and quantitative real-time polymerase chain reaction. Blood was collected on 16 h fasted mice by cardiac puncture.

### INTRODUCTION

The phosphatase and tensin homolog (*PTEN*) tumour

suppressor gene is one of the most frequently mutated/deleted genes in various human cancers<sup>[1,2]</sup>. PTEN is a lipid and protein phosphatase. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate (PIP3), is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks)<sup>[3]</sup>. PTEN dephosphorylates PIP3 to produce phosphatidylinositol 4,5-bisphosphate, which inhibits PI3K-dependent effectors such as the downstream kinases Akt and pyruvate dehydrogenase kinase 1. PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism<sup>[4]</sup>. PTEN has also been shown to regulate genomic stability<sup>[5,6]</sup>, stem cell renewal<sup>[7,8]</sup>, senescence<sup>[9]</sup> and cell differentiation<sup>[10-12]</sup>.

The multiple cellular functions of PTEN suggest that this protein plays major roles in overall system homeostasis. Indeed, homozygous deletion of *Pten* in the mouse causes early embryonic lethality by embryonic day (E) 9.5, whereas *Pten* heterozygous mice (*Pten*<sup>+/-</sup>) develop, over a period of time, various dysplasia and hyperplasia in organs such as the breast, thyroid, prostate and intestine<sup>[1,2,13]</sup>. As reviewed by Knobbe *et al*<sup>[14]</sup>, *Pten* has also been conditionally deleted in many specific tissues. These models have established the tumour suppressive function of *Pten* but have also unravelled its important role in the maintenance of normal physiological functions in various tissues such as the immune system, skin, lung, liver, pancreas and hypothalamus<sup>[14]</sup>.

In a previous study, we reported that *Pten* is important for intestinal homeostasis<sup>[10]</sup>. The villin-Cre system was used to specifically inactivate *Pten* in the mouse intestinal epithelium. *Pten* mutant mice developed an intestinalomegaly associated with an increase in epithelial cell proliferation. Histological analysis also demonstrated significant perturbation of the crypt-villus architecture, a marked increase in goblet cells and a decrease in enteroendocrine cells, suggesting a role for *Pten* in the commitment of the multipotential-secretory precursor cell<sup>[10]</sup>.

Enteroendocrine cells are hormone-secreting epithelial cells that are scattered throughout the gastrointestinal epithelium and although they represent only 1% of the intestinal epithelium, taken together, they constitute the major endocrine organ of the body<sup>[15,16]</sup>. At least 10 different enteroendocrine cell types have been identified in the small intestine and are classified based on their main hormonal products<sup>[16,17]</sup>. The various hormones produced by these endocrine cells [ghrelin (GHR), gastrin-releasing peptide (GRP), glucose-dependent insulinotropic peptide (GIP), secretin (SCT), peptide YY (PYY), glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), cholecystokinin (CCK), neurotensin, serotonin, substance P, somatostatin (SST) and motilin] control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite<sup>[16,18,19]</sup>. Most enteroendocrine cell types secrete chromogranin A (CgA), a soluble glycoprotein stored with hormones and neuropeptides in secretory granules of endocrine cells.

The important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations. Using our Cre/loxP *Pten* conditional knock out mouse model<sup>[10]</sup>, we report herein an unexpected dual regulatory role for epithelial *Pten* signalling in the specification of enteroendocrine cells. Our data indicate that *Pten* positively influences the determination and specification of CgA-expressing cell subpopulations in the small intestine including those expressing secretin, ghrelin, gastrin and CCK. Conversely, *Pten* limits determination and specification of non-expressing CgA endocrine cell subpopulations, including GIP and SST.

## MATERIALS AND METHODS

### Animals

BALB/*c-Pten*<sup>fx/fx</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME, United states). The C57BL/6 12.4KbVilCre transgenic line was provided by Dr. Deborah Gumucio (University of Michigan, Ann Arbor, MI, United states)<sup>[20]</sup>. Genomic DNA was isolated using the Spin Doctor genomic DNA kit from Gerard Biotech according to the manufacturer's protocol. Both mutations were genotyped following protocols already published<sup>[20]</sup> or as directed by The Jackson Laboratory. For this study, the BALB/*c-Pten*<sup>fx/fx</sup> mice were first crossed with the C57BL/6 12.4KbVilCre to generate F1-generation heterozygous animals. F1-generation heterozygous animals were then backcrossed with BALB/*c-Pten*<sup>fx/fx</sup> mice to produce F2-generation experimental animals. All experiments were conducted in F2-generation experimental animals. All mice were maintained on regular diet in the transgenic mouse facility at the Faculty of Medicine and Health Sciences of the Université de Sherbrooke. All experiments were approved by the animal research committee of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke.

### Tissue collection, tissue preparation, RNA extraction and gene expression analysis

Digestive tracts from 120-d-old *Pten*<sup>ΔIEC</sup> mice and control littermates were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, then dehydrated and embedded in paraffin. Sections of 5 μm were applied to Probe-On Plus slides (Fisher Scientific, Ottawa, ON, Canada) and kept at room temperature until used<sup>[10,21]</sup>. Total RNA was isolated and processed using the Totally RNA extraction kit (Ambion, Grand Island, NY, United states). Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR were performed as described previously<sup>[21]</sup>. Quantitative real-time PCR conditions were as follows: one cycle of 15 min at 95 °C; 50 cycles at 95 °C for 15 s; 59 °C for 30 s and 72 °C for 30 s. The following forward and reverse primers were used: Hairy and enhancer of split 1 (NM\_008235), 5'-TTCCAAGC-TAGAGAAGGCAGA-3', 5'-GTTGATCTGGGT-

CATGCAGTT-3'; Atonal homolog 1 (NM\_007500), 5'-GCTTCCTCTGGGGGTTACTC-3', 5'-ACAACGAT-CACCACAGACCA-3'; Neurogenin 3 (NM\_009719), 5'-CGGATGACGCCAAACTTACAAAG-3' 5'-CA-CAAGAAGTCTGAGAACAACAG-3'; Growth factor independent 1 (NM\_010278), 5'-TCCGAGTTCGAG-GACTTTTG-3', 5'-CATGCATAGGGCTTGAAAGG-3'; Neurogenic differentiation 1 (NM\_010894), 5'-AGC-CACGGATCAATCTTCTCT-3', 5'-GACGTGCCCTCTA-ATCGTGAAA-3'; Pancreatic and duodenal homeobox 1 (NM\_008814), 5'-AACCCGAGGAAAACAAGAGG-3', 5'-TTCAACATCACTGCCAGCTC-3'; Forkhead box O1 (NM\_019739), 5'-CCGGAGTTTAACCAGTCCAA-3', 5'-TGCTCATAAAGTCCGGTGCTG-3'; Forkhead box a1 (NM\_008259), 5'-CAAGGATGCCTCTCCACACTT-3', 5'-TGACCATGATGGCTCTCTGAA-3'; Forkhead box a2 (NM\_010446), 5'-GAGCACCATTACGCCTTCAAC-3', 5'-GGCCTTGAGGTCCATTTTGT-3'; PDGB (NM\_013551), 5'-TGCACGATCCTGAAACTCTG-3', 5'-TGCATGCTATCTGAGCCATC-3'.

### Immunofluorescence

Immunofluorescence staining was performed as previously described<sup>[21]</sup>. The following antibodies were used at the indicated dilutions: FITC-conjugated anti-mouse IgG (1:200, Vector, Burlingame, CA, United states), FITC-conjugated anti-rabbit IgG (1:200, Vector), AlexaFluor 568 donkey anti-goat (1:400, Invitrogen, Grand Island, NY, United states), AlexaFluor 488 donkey anti-goat (1:400, Invitrogen), AlexaFluor 488 donkey anti-rabbit (1:400, Invitrogen), rabbit anti-SP-1 CgA (1:1000, ImmunoStar, Hudson, WI, United states), goat anti-CgA (1:50, SantaCruz, Santa Cruz, CA, United states), rabbit anti-gastrin (1:200, Chemicon, Billerica, MA, United states), goat anti-GIP (1:100, SantaCruz), mouse anti-serotonin (1:200, LabVision, Kalamazoo, MI, United states), rabbit anti-secretin (1:1000, Phoenix pharmaceuticals, Burlingame, CA, United states), goat anti-SST (1:100, SantaCruz), goat anti-ghrelin (1:100, SantaCruz), rabbit anti-CCK (1:100, ab92128 gift from Rehfeld JF)<sup>[22]</sup>.

### Measurement of circulating hormone levels

Blood was collected on 16 h fasted mice by cardiac puncture. Serum levels of total ghrelin and GIP were measured using Millipore ELISA kits (EZRGRT-91K, EZRMGIP-55K) (Millipore, Billerica, MA, United states) according to manufacturer's instructions. Serum levels of SST were measured using the Phoenix Pharmaceuticals ELISA kit EK-060-03, according to the manufacturer's instructions.

### Statistical analysis

All cell count analyses were performed using continuous serial sections from low-powered fields of well-oriented intestinal cross-sections in a blind manner on an average of 10 independent fields per animal. Three different intestinal sections were evaluated: duodenum, jejunum and ileum. The total number of enteroendocrine cells

was counted per crypt-villus axis. Image magnification was calibrated by comparison with a stage micrometer (graticules™ Ltd., Tonbridge, Kent, England). Statistical analyses were performed using two-way ANOVA. For qRT-PCR, data were analyzed using the Mann Whitney-test for abnormal distribution. Differences were considered significant with a *P* value of < 0.05. All statistical analyses were carried out using Graph Pad Prism 5 (Graph Pad Inc., San Diego, CA).

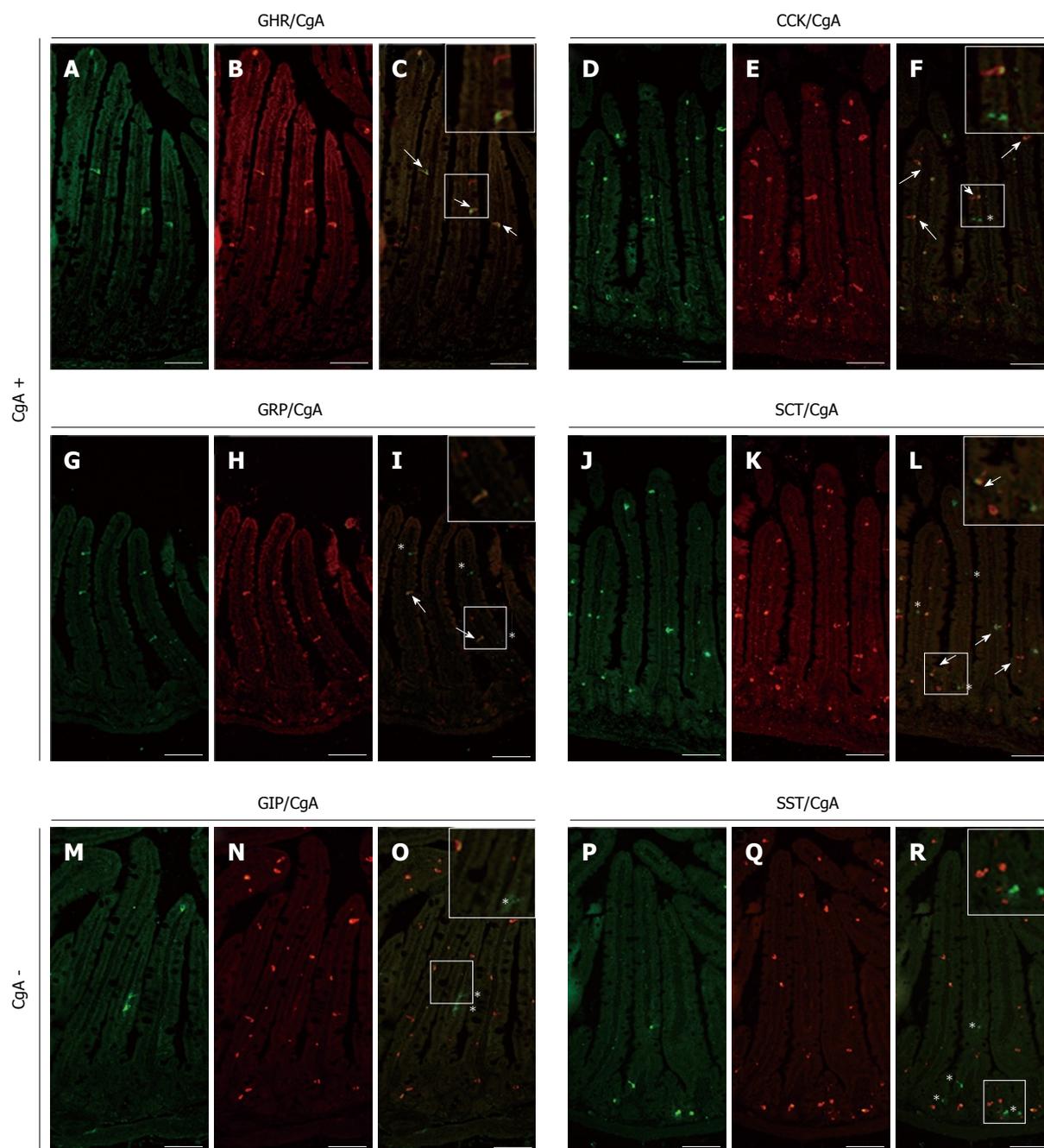
## RESULTS

### **CgA is not expressed in all enteroendocrine cell subtypes of the mouse small intestinal epithelium**

Mice homozygous for the floxed exon 5 of the *Pten* gene<sup>[23]</sup> were bred to the *villin*-Cre transgenic line, which directs expression of the transgene in all epithelial cells of the small intestine and colon, including stem cells, but not in the mesenchymal compartment<sup>[20]</sup>. Conditional knock-out mice for *Pten* (*Pten*<sup>Δ<sup>Ex5</sup></sup>) were born at the expected Mendelian ratios, survived for more than 1 year, and grew normally without obvious gross physical abnormalities<sup>[10]</sup>. In a previous study with these mice, we reported an overall decrease in the number of enteroendocrine cells using a CgA antibody<sup>[10]</sup>. Over the years, there has been a lingering controversy where a number of studies showed that all endocrine cell subpopulations express CgA<sup>[18,24,25]</sup> while others reported that some cell subpopulations do not express CgA<sup>[17,26]</sup>. Therefore, individual analysis of various intestinal endocrine subpopulations was first performed for their co-expression with CgA in the mouse small intestine. Double-labelling with CgA (Figure 1B, E, H and K) and specific antibodies directed against ghrelin (Figure 1A), CCK (Figure 1D), gastrin (Figure 1G) and secretin (Figure 1J) confirmed co-expression of CgA with ghrelin (Figure 1C) as well as with CCK- (Figure 1F), gastrin- (Figure 1I) and secretin- (Figure 1L) producing enteroendocrine cells in the mouse small intestine. On the other hand, double-labelling with CgA antibody (Figure 1N and Q) and specific antibodies directed against GIP (Figure 1M) and SST (Figure 1P) supported the exclusion of co-expression between GIP (Figure 1O), SST (Figure 1R) and CgA in the mouse small intestine. The specificity of our CgA antibodies was confirmed with the use of two different CgA antibodies from two different commercial sources, in which the exact same cells were labeled in consecutive sections from a same specimen with both antibodies.

### **Loss of intestinal epithelial *Pten* impairs the specification of CgA expressing enteroendocrine cells**

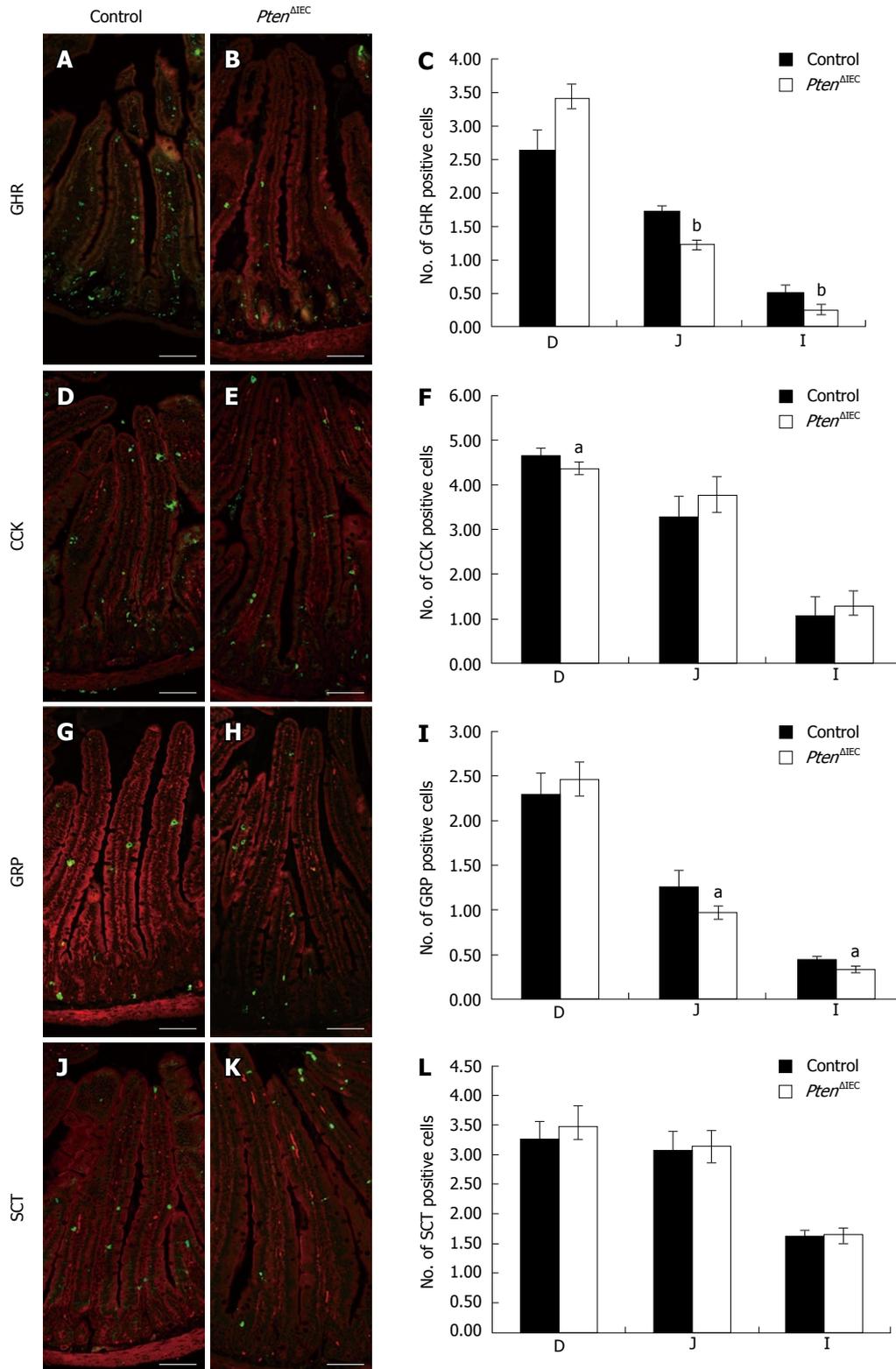
Enteroendocrine subtype specification appears to be regulated by distinct mechanisms<sup>[17,26]</sup>. Since our previous study only investigated CgA-expressing cells, the impact of *Pten* loss of expression on the specification of the various enteroendocrine cell subpopulations in the small intestine was further analyzed. We first analyzed how the loss of epithelial *Pten* alters specification of CgA-expressing en-



**Figure 1 Analysis of chromogranin A co-expression in mouse small intestinal endocrine subpopulations.** Small intestine sections of adult control mice were co-immunostained with antibodies directed against ghrelin (GHR) (A), cholecystokinin (CCK) (D), gastrin-releasing peptide (GRP) (G), secretin (SCT) (J) glucose-dependent insulinotropic peptide (GIP) (M) or somatostatin (SST) (P) and against chromogranin A (CgA) (respectively B, E, H, K, N and Q). The arrows in images C, F, I and L show co-expression of CgA respectively with GHR, CCK, GRP and SCT while asterisks in images F, I, L, O and R point to CgA-negative enteroendocrine cells. The number of arrows and asterisks within the crypt-villus axis represents the average proportion of labelled cells per units. Scale bar: 50  $\mu$ m.

teroendocrine cells along the various sections of the small intestine (duodenum, jejunum and ileum). Although some enteroendocrine cells are restricted to specific regions of the small intestine, each region was analyzed in order to verify the possible delocalization of subpopulations along the rostro-caudal axis of the gut. The intestinal mucosa of *Pten*<sup>AIEC</sup> and control mice was stained with specific markers for each enteroendocrine cell subtype and positive cells were counted (Figure 2). A significant decrease of 29% in the jejunum (1.2 positive cells per crypt-villus axis

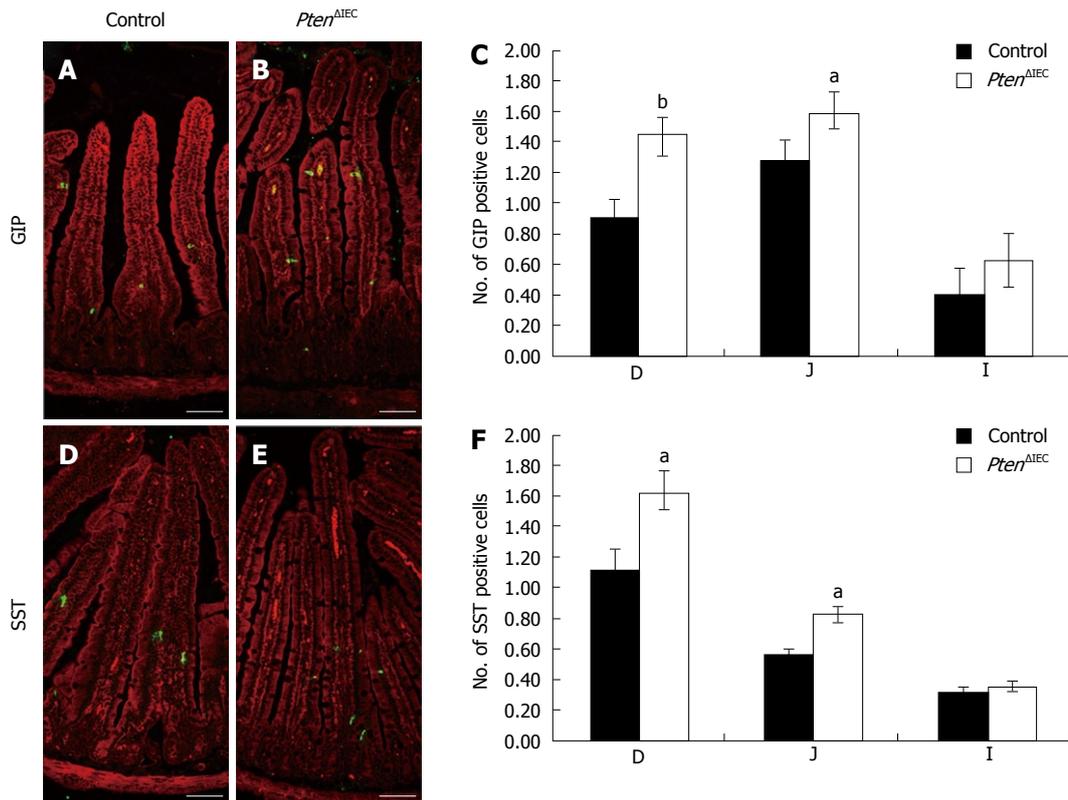
*vs* 1.7) and 51% in the ileum (0.25 cell *vs* 0.52 cell) was observed (Figure 2C) in the ratio of positive ghrelin cells in *Pten* mutant mice (Figure 2B) compared to control littermates (Figure 2A). A modest but significant decrease of 10% (Figure 2F) was also observed in the ratio of positive CCK cells in the duodenum of the mutant mice (4.4 cell *vs* 4.7 cell) (Figure 2E). There was also a significant 23% decrease in gastrin-positive cells in the jejunum (1 cell *vs* 1.3 cell) and a decrease of 29% in the ileum (0.34 cell *vs* 0.44 cell) in *Pten*<sup>AIEC</sup> (Figure 2H and I) when compared to



**Figure 2** Epithelial *Pten* positively regulates commitment of chromogranin A-positive enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*<sup>AIEC</sup> mice were immunostained with antibodies against ghrelin (GHR) (A and B), cholecystokinin (CCK) (D and E), gastrin-releasing peptide (GRP) (G and H) and secretin (SCT) (J and K). Positive cells were counted from intestinal sections of controls (*n* = 6) and mutants (*n* = 5). Statistical analysis (C, F, I, L) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50  $\mu$ m. D: Duodenum; J: Jejunum; I: Ileum. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.001.

control mice (Figure 2G and I). Finally, secretin immunostaining showed no modulation in the number of secretin-positive cells in *Pten*<sup>AIEC</sup> (Figure 2K and L) *vs* control mice

(Figure 2J and L). Taken together, these results suggest that *Pten* positively influences production of CgA-expressing enteroendocrine cell subpopulations.



**Figure 3** Epithelial *Pten* negatively regulates commitment of chromogranin A-negative enteroendocrine subpopulations in the small intestine. Duodenum, jejunum and ileum of adult control and *Pten*<sup>ΔIEC</sup> mice were immunostained with antibodies against glucose-dependent insulinotropic peptide (GIP) (A and B) and somatostatin (SST) (D and E). Positive cells were counted from intestinal sections of controls (*n* = 6) and mutants (*n* = 5). Statistical analysis (C and F) represents the average number of positive cells per crypt-villus axis in each section of the intestine. Error bars represent SE. Scale bar: 50 μm. D: Duodenum; J: Jejunum; I: Ileum. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.

### Loss of epithelial intestinal *Pten* positively influences the specification of CgA negative enteroendocrine cells

We next examined if the specification of CgA-negative cells was affected following the loss of epithelial *Pten*. As illustrated in Figure 3, there was a marked 61% (duodenum) and 25% (jejunum) increase in GIP-positive cells in *Pten*<sup>ΔIEC</sup> (Figure 3B and C) when compared to control mice (Figure 3A and C) (respectively 1.45 positive cells *vs* 0.9 in the duodenum and 1.6 cells *vs* 1.28 in the jejunum). SST immunostaining in both duodenum and jejunum revealed an increase of 45% in the number of SST-positive cells in *Pten*<sup>ΔIEC</sup> (Figure 3E and F) *vs* control mice (Figure 3D and F) (respectively 1.65 cells *vs* 1.15 cell and 0.85 cell *vs* 0.6 cell). Hence, these data suggest that *Pten* signalling negatively controls specification of CgA-negative cells in the intestinal epithelium.

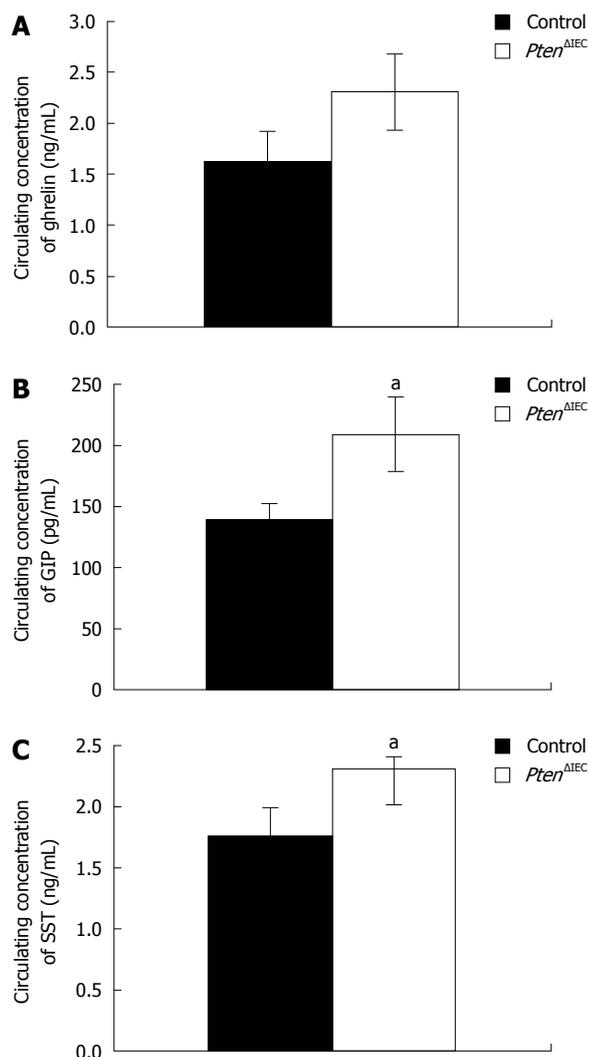
### Loss of epithelial *Pten* signalling leads to deregulation of circulating GIP and SST levels

In light of these observations, we next investigated whether deregulation in the number of enteroendocrine cells in the intestinal epithelium of the *Pten*<sup>ΔIEC</sup> mice has an impact on their circulating levels. We chose to focus on the enteroendocrine subpopulations where the deregulation was more considerable. Circulating ghrelin, GIP and SST levels were analysed by ELISA assay. A 1.5-fold and 1.3-fold increase in GIP (Figure 4B) and SST (Figure 4C)

levels, respectively, were observed in *Pten*<sup>ΔIEC</sup> mice when compared to control littermates. No significant difference in ghrelin levels was observed between *Pten*<sup>ΔIEC</sup> mice and control mice (Figure 4A).

### *Pten* expression impacts differently on various pro-enteroendocrine specification factors

Comparative analysis of secretory lineage and specific pro-enteroendocrine determination factors was next investigated by quantitative PCR to clarify the role of *Pten* during enteroendocrine subtype specification. The Notch pathway, and more specifically the transcription factors Hairy enhancer of Split (Hes-1) and Math1, is crucial in the determination of the intestinal progenitor cell to absorptive or secretory cell fate (Figure 5)<sup>[27,28]</sup>. We also investigated if loss of epithelial *Pten* could deregulate the production of secretory precursors. Quantitative PCR analysis of mutant *vs* wild-type littermates revealed no modulation of Math1 or Hes-1 mRNA levels in the mutant animals (Table 1). Modifications downstream of the Notch pathway during enteroendocrine cell determination were also subsequently assessed. The proendocrine bHLH transcription factor Ngn3 has been shown to contribute to the maintenance and specification of enteroendocrine precursors (Figure 5)<sup>[29]</sup>. Our analysis revealed that the Ngn3 mRNA expression was significantly reduced by 2.07-fold in the mutant animals (Table 1). BETA2/



**Figure 4** Loss of epithelial *Pten* signalling modulates circulating levels of glucose-dependent insulinotropic peptide and somatostatin. A: Analysis of circulating ghrelin level revealed no significant modulation between adult *Pten*<sup>AIEC</sup> mice ( $n = 10$ ) and control littermates ( $n = 10$ ); B: Analysis of circulating glucose-dependent insulinotropic peptide (GIP) level revealed a 1.5-fold increase in adult *Pten*<sup>AIEC</sup> mice ( $n = 10$ ) when compared to control littermates ( $n = 10$ ); C: Analysis of circulating somatostatin (SST) level revealed a 1.3-fold increase in adult *Pten*<sup>AIEC</sup> mice ( $n = 10$ ) when compared to control littermates ( $n = 10$ ). Error bars represent SE. <sup>a</sup> $P < 0.05$ .

NeuroD1, Pancreatic and duodenal homeobox 1 gene (*Pdx1*), the winged helix *Foxa1* and the forkhead box-containing (*FoxO1*) transcription factors have also been shown to control the determination of specific enteroendocrine subpopulations<sup>[16,30-33]</sup>. The gene transcript level of BETA2/NeuroD1, linked to specification of secretin and CCK producing cells<sup>[34]</sup>, was reduced by 1.40-fold in mutant animals (Table 1). *Pdx1*, which regulates serotonin and GIP producing cells (Figure 5)<sup>[31,32]</sup>, was found to be significantly increased by 2.15-fold at the gene transcript level in mutant mice (Table 1). *FoxO1* factors are downstream targets of the PI3K/AKT pathway<sup>[35]</sup> and affect the subcellular localization of *Pdx1* in the pancreas and, hence, its transcriptional activity<sup>[36]</sup>. *FoxO1* gene transcript level was found to be reduced by 1.95-fold in the *Pten*<sup>AIEC</sup>

**Table 1** Gene expression changes in the small intestine of *Pten*<sup>AIEC</sup> mice

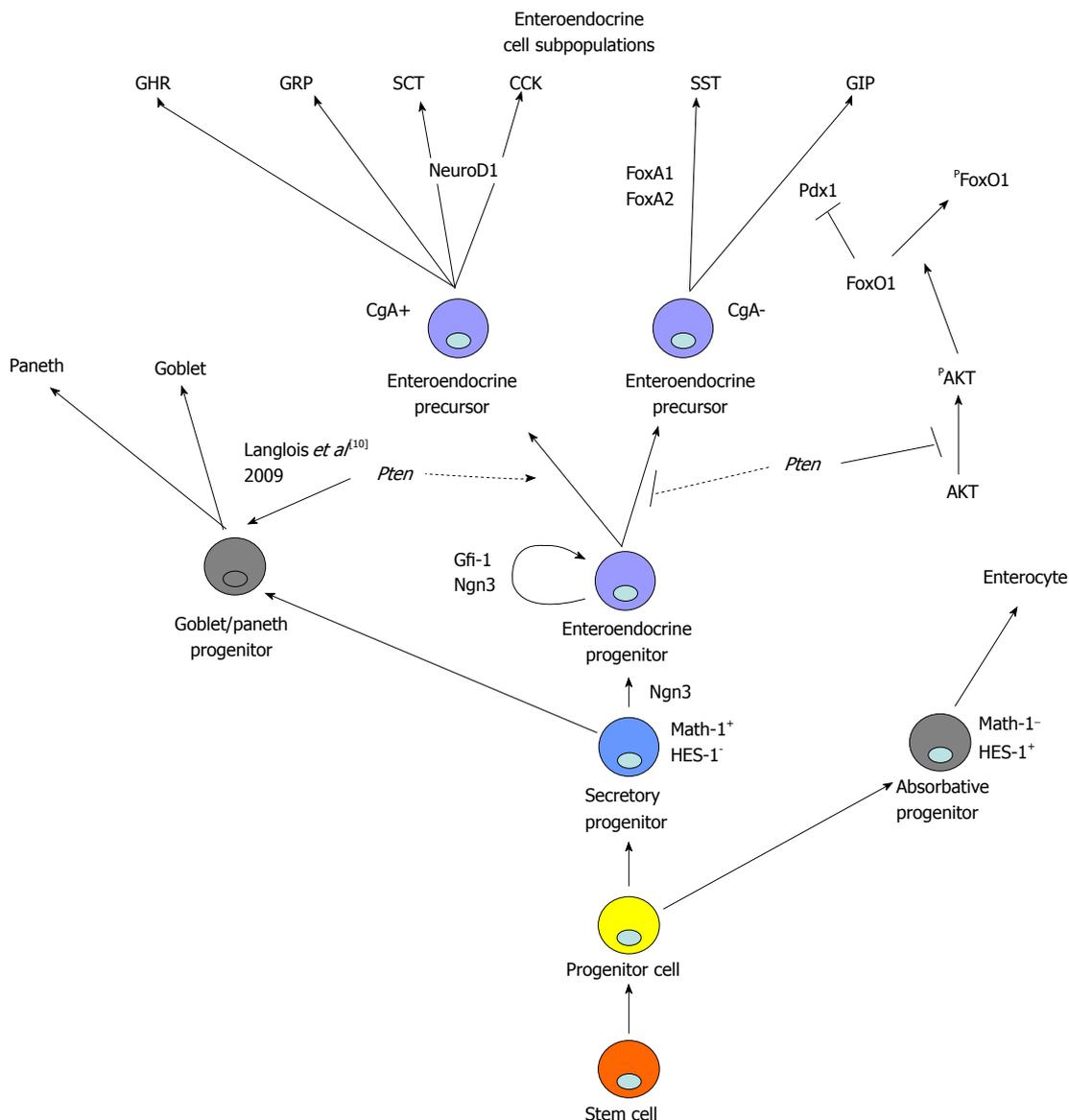
Gene description	Gene symbol	Fold	P value
Hairy and enhancer of split 1	<i>Hes1</i>	-1.08	NS
Atonal homolog 1	<i>Math 1</i>	-2.05	NS
Neurogenin 3	<i>Ngn3</i>	-2.07	0.033
Growth factor independent 1	<i>Gfi1</i>	1.07	NS
Neurogenic differentiation 1	<i>NeuroD1</i>	-1.40	0.002
Pancreatic and duodenal homeobox 1	<i>Pdx1</i>	2.15	0.048
Forkhead box O1	<i>FoxO1</i>	-1.95	0.017
Forkhead box a1	<i>Foxa1</i>	2.64	0.017
Forkhead box a2	<i>Foxa2</i>	-1.25	NS

Target expression was quantified relatively to PDGF expression. Fold changes represent the ratio of mean expression values (control/mutant). Negative values indicate reduction in *Pten*<sup>AIEC</sup> intestines. NS: Non significant fold change (Mann-Whitney test).

mice (Table 1). Finally, the winged helix transcription factors *Foxa1* is essential for the differentiation of SST-, GLP-1- and PYY-expressing endocrine cells (Figure 5)<sup>[33]</sup>. Accordingly, we found an increase of 2.64-fold in *Foxa1* gene transcript expression in *Pten*<sup>AIEC</sup> mice (Table 1).

## DISCUSSION

Endocrine cells found scattered in the gastrointestinal epithelium represent the major endocrine organ of the body<sup>[15,16]</sup>. The various hormones produced by these endocrine cells control numerous physiological functions<sup>[16,18,19]</sup>. Recently, by using conditional tissue-specific disruption of *Pten* in the epithelium of the gut, we revealed a key role for epithelial *Pten* in intestinal morphogenesis, in the maintenance of crypt-villus axis architecture, in cell proliferation and in secretory cell commitment<sup>[10]</sup>. We had also reported an overall decrease in the number of enteroendocrine cells using a CgA antibody. However, the choice of CgA as a pan marker for all enteroendocrine cells has been challenged. Commonly used as a biomarker for endocrine granules, CgA plays a role in the biogenesis of mobile secretory granules and the release of hormones through the regulated secretory pathway<sup>[37]</sup>. Over the years, there has been a lingering controversy in which some studies showed that all endocrine cell subpopulations express CgA<sup>[18,24,25]</sup> while others reported that enteroendocrine cell subpopulations producing GIP, GLP-1 or SST do not express CgA<sup>[17,26]</sup>. Fixation artefacts and different CgA antibodies may account for this controversy. Also, it has been demonstrated that CgA expression in enteroendocrine subpopulations varies from one species to another as well as in pathologies such as colorectal cancer and inflammatory bowel diseases<sup>[38-42]</sup>. Herein, our analysis of the various intestinal endocrine subpopulations with CgA antibodies confirmed the absence of co-expression between GIP and SST with CgA. Therefore, the important role of enteroendocrine cells in whole body homeostasis prompted us to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various en-



**Figure 5 Proposed model for mode of action of epithelial *Pten* signalling in intestinal epithelial determination and specification of enteroendocrine progenitor cell fate.** Epithelial *Pten* signalling is not essential for maintenance or determination of the secretory precursor. *Pten* represses specification of glucose-dependent insulinotropic peptide (GIP)-expressing cells by maintaining FoxO1 in the nucleus. GHR: Ghrelin; GRP: Gastrin-releasing peptide; SCT: Secretin; CCK: Cholecystokinin; SST: Somatostatin; GIP: Glucose-dependent insulinotropic peptide; CgA: Chromogranin A.

teroendocrine subpopulations. Since all enteroendocrine subtype cells are still detectable in the mutant mice, our results suggest that *Pten* is not a direct and indispensable regulator of enteroendocrine cell determination. Nevertheless, our data revealed a dual role for *Pten* signalling in enteroendocrine cell specification. Indeed, our results indicate that *Pten* signalling facilitates the specification of CgA-expressing enteroendocrine cell subpopulations while it negatively controls specification of CgA-negative cells in the intestinal epithelium. Furthermore, our results showed that the number of GIP and SST cells as well as their associated circulating hormone levels was increased in mutant mice. Although the number of ghrelin cells was decreased, no significant modulation in ghrelin serum level was observed in the *Pten*<sup>ΔIEC</sup> mice. This may be explained by the fact that ghrelin endocrine cells found in

the stomach epithelium are strong contributors for total circulating ghrelin levels<sup>[43,44]</sup>, and are not likely affected by the loss of epithelial *Pten* in the intestine. Nevertheless, the lack of modulation in circulating levels of ghrelin does not imply that the reduction observed in the cell number in the intestine has no local consequences in this tissue. Indeed, such a reduction could influence specific physiological intestinal functions, such as motility, digestion and epithelial renewal<sup>[16,18,19]</sup>. Finally, analyses of each enteroendocrine cell subtypes along the rostro-caudal axis of the small intestine confirmed that the loss of *Pten* does not influence normal distribution of these endocrine cell subpopulations.

Our data also indicate that *Pten* affects the expression of key regulators for cell lineages and/or proenteroendocrine determination. Since the Notch/Hes-1

path is required for the specification of progenitor cells into the absorptive lineage and since *Math1* is required for specification into the secretory lineage<sup>[27,28]</sup>, we therefore analyzed whether the loss of epithelial *Pten* could alter their expression. Lack of modulation in *Math1* and *Hes-1* gene transcripts suggest that *Pten* is not involved in the initial decision steps for lineage determination. Once the initial decision is made between secretory and absorptive cell lineages, the fate of enteroendocrine progenitor cells is defined by proendocrine bHLH transcription factors such as *Ngn3* and *BETA2/NeuroD1*. *Ngn3* acts downstream of *Math1*<sup>[27,29]</sup> and has been shown to contribute to the maintenance of the enteroendocrine precursors and to the differentiation of all enteroendocrine subpopulations in mice<sup>[29,45,46]</sup>. Unlike *Ngn3*, expression of *BETA2/NeuroD1* is restricted to a subset of enteroendocrine cells<sup>[34]</sup>. *BETA2/NeuroD1* controls terminal differentiation of secretin and CCK producing cells in the intestine as revealed by the absence of these subpopulations in *BETA2/NeuroD1* null mice<sup>[34]</sup>. In addition, *BETA2/NeuroD1* acts downstream of *Ngn3*<sup>[45]</sup>. Our analysis revealed that the expression of both bHLH transcription factors was reduced in absence of epithelial *Pten*, thereby impacting on the production of specific enteroendocrine subpopulations (Figure 5). Over the years, other factors have been shown to be important in the differentiation/specification of several enteroendocrine cell subpopulations<sup>[16,30-33]</sup>. Such is the case for the winged helix transcription factor *Foxa1*, previously shown to be essential for the differentiation of SST, GLP-1 and PYY expressing cells<sup>[33]</sup>. *Foxa1* expression was found to be increased in *Pten*<sup>ΔIEC</sup> mice, hence correlating with the increased production of SST-expressing cells in these mice (Figure 5). The same logic can be applied to *Pdx-1*. Indeed, studies from *Pdx1*-null mice revealed an increase in the number of serotonin cells and a decrease in the GIP-expressing cell population<sup>[31,32]</sup>. Herein, *Pdx-1* gene transcript was found to be significantly increased in absence of epithelial *Pten*, thereby matching the deregulation seen in GIP cell specification (Figure 5). In addition, *FoxO1* gene transcript was found to be significantly reduced in the absence of epithelial *Pten*. *FoxO1* competes with *FoxA2* for binding to the *Pdx1* promoter, resulting in inhibition of *Pdx1* transcription<sup>[36]</sup> (Figure 5). Aside from these observations, one could speculate that phosphorylation of *FoxO1* affects its subcellular localisation leading to its exclusion from the nucleus. This nuclear/cytoplasm shuttling phosphorylation of *FoxO1* ultimately decreases its transactivation potential<sup>[36,47]</sup>. Furthermore, PI3K/Akt is a major upstream signalling pathway leading to the phosphorylation of *FoxO1* and its exclusion from the nucleus<sup>[35]</sup>. In a previous study with *Pten*<sup>ΔIEC</sup> mice, we reported that loss of *Pten* resulted in increased phosphorylation levels of *Akt*<sup>[10]</sup>. Thus, it is tempting to extrapolate that following the loss of intestinal epithelial *Pten* and activation of *Akt*, targeted *FoxO1* protein would become more phosphorylated and exported to the cytoplasm allowing expression of *Pdx1* and specification of GIP-expressing cells.

In summary, our results reveal a distinctive role for *Pten* in specification/differentiation of enteroendocrine cell subpopulations. *Pten* signalling negatively regulates the enteroendocrine subtype specification of non-expressing CgA cells such as GIP and SST expressing cells. In contrast, *Pten* signalling positively affects CgA-expressing cells such as ghrelin, gastrin and CCK cells. Many of these enteroendocrine cell subtypes are known to play critical roles in whole body physiological functions. Incretin hormones such as GLP-1 and GIP have been shown to potentiate glucose-stimulated insulin secretion<sup>[48]</sup>, while double-mutant mice for GIP and GLP-1 exhibit glucose intolerance<sup>[49]</sup>. Likewise, the importance of enteroendocrine cells in lipid absorption has recently been shown with the generation of intestinal-conditional *Ngn3* null mice<sup>[46]</sup>. A study with *Gip*-receptor null mice revealed a crucial role for GIP in promoting the efficient storage of ingested fat suggesting that inhibition of the GIP signal could represent a therapeutic approach against obesity<sup>[50]</sup>. Further analysis will be needed to better evaluate the impact and possible networking of small intestinal endocrine cell deregulation following the loss of *Pten* signalling on overall metabolism in the mouse.

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## COMMENTS

### Background

The phosphatase and tensin homolog (*PTEN*) tumour suppressor gene is a lipid and protein phosphatase frequently mutated/deleted in various human cancers. Its best-known substrate, the phosphatidylinositol 3,4,5-trisphosphate, is a lipid second messenger mainly produced by class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks have been implicated in many signalling pathways that regulate cell survival, growth, proliferation, migration, phagocytosis, and metabolism. In previous study, authors reported that *Pten* is important for intestinal homeostasis as well as in the commitment of enteroendocrine cells. The important role of enteroendocrine cells in whole body homeostasis prompted people to further analyze the effect of intestinal epithelial deletion of *Pten* on the specification of the various enteroendocrine subpopulations.

### Research frontiers

Enteroendocrine cells located in the gut epithelium are the largest and least understood population of hormone-producing cells in the body. The various hormones and peptides produced by these endocrine cells control important physiological functions, such as gastrointestinal motility, glycaemia, exocrine pancreatic secretion, biliary secretion, digestion, gut epithelial renewal and appetite. In recent years, studies have placed the regulation of these gut hormones as potential targets for novel treatments of metabolic diseases such as type 2 diabetes and obesity.

### Innovations and breakthroughs

In the current study, the authors report a distinctive role for *Pten* in specification/differentiation of enteroendocrine cell subpopulations. *Pten* signalling negatively regulates the enteroendocrine subtype specification of non-expressing chromogranin A (CgA) cells such as glucose-dependent insulinotropic peptide and somatostatin expressing cells. In contrast, *Pten* signalling affects positively CgA-expressing cells such as ghrelin, gastrin and cholecystokinin cells.

## Applications

Many of these enteroendocrine cell subtypes are known to play critical roles in whole body homeostasis. These experimental data can be used in further studies to better evaluate the impact on general metabolism and possible networking of small intestinal endocrine cell deregulation following the loss of Pten signalling.

## Peer review

This is a high quality descriptive study in which authors analyze the impact of the *PTEN* intestinal knockdown in the specification of intestinal enteroendocrine subpopulations.

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## Overexpression of Dickkopf-3 induces apoptosis through mitochondrial pathway in human colon cancer

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### Abstract

**AIM:** To investigate the mechanisms of the biological roles of Dickkopf-3 (Dkk-3) in cell invasion, survival and apoptosis in colon cancer cells.

**METHODS:** Three human colon cancer cell lines, i.e., HT-29, LoVo and SW480, were used. Overexpression of Dkk-3 induced by pEGFP-N1-Dkk-3-GFP plasmid in LoVo cells was performed using Lipofectamine 2000 reagent. Reverse transcription polymerase chain reaction and Western blotting were performed to determine the mRNA and protein expression levels of Dkk-3, respectively. Cell proliferation assay, cell cycle analysis, hoechst 33258 assay and Matrigel invasion assay were performed on Dkk-3 overexpressing transfectants.

**RESULTS:** The mRNA and protein expressions of Dkk-3 in HT-29 (mRNA:  $0.06 \pm 0.02$ , protein:  $0.06 \pm 0.01$ ) and LoVo (mRNA:  $0.07 \pm 0.02$ , protein:  $0.07 \pm 0.02$ ) cells were significantly lower than that in SW480 cells (mRNA:  $0.92 \pm 0.04$ , protein:  $0.69 \pm 0.13$ ; all  $P < 0.05$ ), and the greatest levels of invasiveness was

in LoVo cells. Dkk-3 overexpression inhibited the proliferation and invasion of LoVo cells and induced cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase and subsequent apoptosis, as indicated by increased chromatin condensation and fragments, upregulated Bax and cytochrome c protein, downregulated survivin and Bcl-2 protein, and the activation of caspase-3 and caspase-9. Furthermore, Dkk-3 overexpression reduced the accumulation of cytosolic fraction of  $\beta$ -catenin.

**CONCLUSION:** Dkk-3 overexpression induced apoptosis in human colon cancer possibly through the mitochondrial pathway. Dkk-3 may be involved in the Wnt/ $\beta$ -catenin signaling pathways in colon cancer.

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**Key words:** Dickkopf-3; Overexpression; Invasion; Apoptosis; Colon cancer; Mitochondria

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### INTRODUCTION

The prevalence of colorectal cancer is increasing in Asia. Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced a 2-4 folds increase in the incidence of colorectal cancer (CRC) during the past few decades<sup>[1]</sup>. Even in the United States, colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer deaths among cancers

that affect both men and women<sup>[2,3]</sup>. However, the cellular mechanisms involved in CRC are not fully described. Recent studies have shown that the Wnt signaling pathway, which is composed of canonical Wnt signaling *via* Wnt/ $\beta$ -catenin and noncanonical Wnt signaling *via* the Wnt/ $\text{Ca}^{2+}$  pathway and Wnt/c-Jun N-terminal kinase (JNK) (planar cell polarity), regulates proliferation, fate specification, polarity and migration of cells<sup>[4,5]</sup>. The Wnt signaling pathway can be blocked by two functional classes of Wnt antagonists: the secreted frizzled-related proteins (sFRP) and the Dickkopf (Dkk)<sup>[6]</sup>.

Dkk-3, also known as reduced expression in immortalized cells, is a member of a recently identified gene family encoding secreted proteins that control cell fate during embryonic development<sup>[7-9]</sup>. Deletion at Dkk-3 locus has been found in many cancers, such as lung cancer<sup>[10]</sup>, gastric cancer<sup>[11]</sup> and ovarian cancer<sup>[12]</sup>. In acute lymphoblastic leukaemia<sup>[13]</sup>, prostate cancer<sup>[14]</sup>, bladder cancer<sup>[15,16]</sup> and renal cell carcinoma<sup>[16]</sup>, Dkk-3 expression is reduced or silenced. Interestingly, Dkk-3 is strongly expressed at the base of the crypts in human colon, which is known to contain proliferating epithelial precursor cells<sup>[17]</sup>. Therefore, Dkk-3 may be an important component of the gastrointestinal proliferative regulatory network<sup>[17]</sup>.

However, the relationship between Dkk-3 and colon cancer remains unclear. We hypothesized that: (1) Dkk-3 expression may be inhibited epigenetically in colon cancer cells; (2) Dkk-3 may be a tumor suppressor and plays an important role in mitochondria-mediated apoptosis; and (3) Dkk-3 may be involved in the Wnt/ $\beta$ -catenin signaling pathways in colon cancer cells. In the present study, we investigated the mechanisms of the biological roles of Dkk-3 in cell invasion, survival and apoptosis of human colon cancer cells.

## MATERIALS AND METHODS

### Construction of expressing plasmids

The pEGFP-N1-Dkk-3-GFP plasmid constructed to target Dkk-3 (RefSeq ID: BC007660) was obtained from Genechem Co., Ltd. (Shanghai, China). pEGFP-N1 plasmid (Genechem Co., Ltd.) was cut with *Xho*I/*Kpn*I and ligated by T4 DNA ligase with gene encoding Dkk-3, making Dkk-3-pEGFP construct. The plasmid construct was confirmed by DNA sequencing.

### Cell culture and transfection conditions

The human colon cancer cell lines HT-29, LoVo and SW480 were obtained from the Cell Collection Center of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GibcoBRL, Grand Island, NY, United States) supplemented with 10% fetal bovine serum and were maintained in a humidified incubator at 37 °C with a supply of 5% CO<sub>2</sub>/95% air atmosphere. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of transfection, cells were used

for cell cycle analysis, hoechst 33258 assay, Matrigel invasion assay, reverse transcription polymerase chain reaction (RT-PCR) analysis and Western blotting analysis. The transient expression of green fluorescent protein (GFP) was detected under a fluorescence microscope (Olympus; Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 460-490 nm.

### RT-PCR

After 48 h of transfection, total cellular RNA was isolated by Trizol (Invitrogen, Carlsbad, CA) and 2  $\mu$ g of RNA was treated with DNase and used as a template for the reverse transcription reaction following the manufacturer's instructions (Fermentas, United States). The resultant cDNA was then used in PCRs and analyzed by gel electrophoresis. The following primers were used: Dkk-3 sense 5'-GGGAGACGAAGAAGGCAGAAGG-3' and Dkk-3 antisense 5'-CCAGGTGATGAGGTCCAGAAGC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-AGGTGAAGGTCGGAGTCAAC-3', and GAPDH anti-sense, 5'-CGCTCCTGGAAGATGGT-GAT-3'. The PCR conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The final extension was at 72 °C for 5 min. PCR products were analyzed on 1.2% agarose gels containing 0.5 g/mL ethidium bromide and were visualized under ultraviolet light. Band density was analyzed and quantified using Genetools software (Syngene, Cambridge, United Kingdom).

### Western blotting analysis

Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, United States) by wet transfer system (Bio-Rad, Hercules, CA, United States). Membranes were blocked with 10% non-fat dry milk in Tris-buffered saline Tween-20 and incubated first with primary antibodies at 4 °C overnight and then with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody for 2 h at room temperature. The following antibodies were used: Dkk-3 (R and D Systems Inc., Minneapolis, MN, United States) 1.5  $\mu$ g/mL,  $\beta$ -catenin (Abcam, Cambridge, United Kingdom) 1:5000, survivin, Bax, Bcl-2 and Cyt-c (Santa Cruz Biotechnology, Santa Cruz, CA, United States) 1:1000, Caspase-9 (Abcam, United States) 1:1000, Caspase-3 (Abcam, United States) 1:250 and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, United States) 1:2000. Specific proteins were visualized using an enhanced chemiluminescence system (Millipore, Bedford, MA, United States) and then exposed with Kodak X-ray film. Protein band intensities were determined densitometrically using the video-imaging CMIASWIN system (Bio-Rad, Hercules, CA, United States).

### Cell proliferation assay

Cell proliferation was determined by the WST-8 tetrazolium salt assay (Cell Counting Kit-8, Beyotime Inst Bio-

tech, China), which quantifies the amount of formazan dye formed when tetrazolium salt is cleaved by cellular mitochondrial dehydrogenase present in viable cells. Cells were seeded in 96-well plates at a density of  $2 \times 10^3$ /well in 0.1 mL of culture medium. Viability of cells 0, 12, 24, 36, 48, 60 and 72 h after transfection was evaluated. Two hours before the end of the specified incubation period, 10  $\mu$ L WST-8 reagent was added to the cells. At the end of the incubation, cell density was estimated by measuring the absorbance of the colored formazan reaction product at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, United States).

### Cell cycle analysis

Cell cycle status was determined by measuring cellular DNA content after staining with propidium iodide by flow cytometry. After 48 h transfection, cells were centrifuged, washed twice with ice-cold phosphate buffer saline (PBS), and fixed in 70% ethanol at 4 °C for 24 h. Cells were then centrifuged at 1000 r/min for 5 min, and the supernatant was discarded. The pellets were then washed twice with 4 mL PBS and then stained with 0.5 mL RNase A (2 mg/mL) and 0.5 mL propidium iodide (0.1% in 0.6% Triton-X in PBS) for 30 min in the dark. Samples were then analyzed on a FACSCalibur flow cytometer (Beckman Coulter, Inc. Fullerton, CA).

### Hoechst 33258 assay for apoptosis

Apoptotic cells were detected by Hoechst 33258 staining following the manufacturer's protocol (Apoptosis Hoechst staining kit, Beyotime Biotechnology, Jiangsu, China) after 48 h transfection. Briefly, cells were first fixed in 0.5 mL methanol for 30 min and then rinsed with PBS twice; 1 mg/mL Hoechst 33258 reagent was used to stain the apoptotic cells in dark at room temperature for 5 min, after which the cells were again washed with PBS twice. The stained cells were examined and immediately photographed under a fluorescence microscope (Olympus; Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 330-380 nm. Apoptotic cells were identified on the basis of morphologic changes in their nuclear assembly by observing chromatin condensation and fragment staining by Hoechst 33258. In each group, ten microscopic fields were selected randomly and counted.

### Invasion assay

Transwell chambers (Corning, New York, NY, United States) were used to examine the ability of cells to invade through a Matrigel-coated filter following the manufacturer's instructions. DMEM was added to the upper chambers and allowed to hydrate for 2 h at 37 °C with 5% CO<sub>2</sub>. Next,  $1 \times 10^5$  LoVo cells transfected with various plasmids were added to the upper chamber and grown in serum-free medium on 8.0  $\mu$ m porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with DMEM medium containing 10% fe-

tal bovine serum. After 24 h incubation, the cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that migrated to the underside of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa (Sigma). Cells in 10 random fields of view at  $\times 400$  magnifications were counted and expressed as the average number of cells/field of view.

### Colony formation assay

Cells from the colon cancer cell line LoVo ( $2 \times 10^5$  cells per well) were transfected with 0.5  $\mu$ g Dkk-3-expressing or empty vector (pEGFP-N1) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Transfected cells were selected with antibiotic G-418 Sulfate (0.4 mg/mL) (Merck, Darmstadt, Germany) for 2 wk. Colonies were fixed with methanol/acetone (1:1), stained with Giemsa, and counted. All experiments were performed in triplicate.

### Statistical analysis

All continuous values were expressed as mean  $\pm$  SD. One-way analysis of variance was used for comparisons among groups. Student's *t* test was used for comparison of the values between two groups. SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Statistical significance was defined as  $P < 0.05$ .

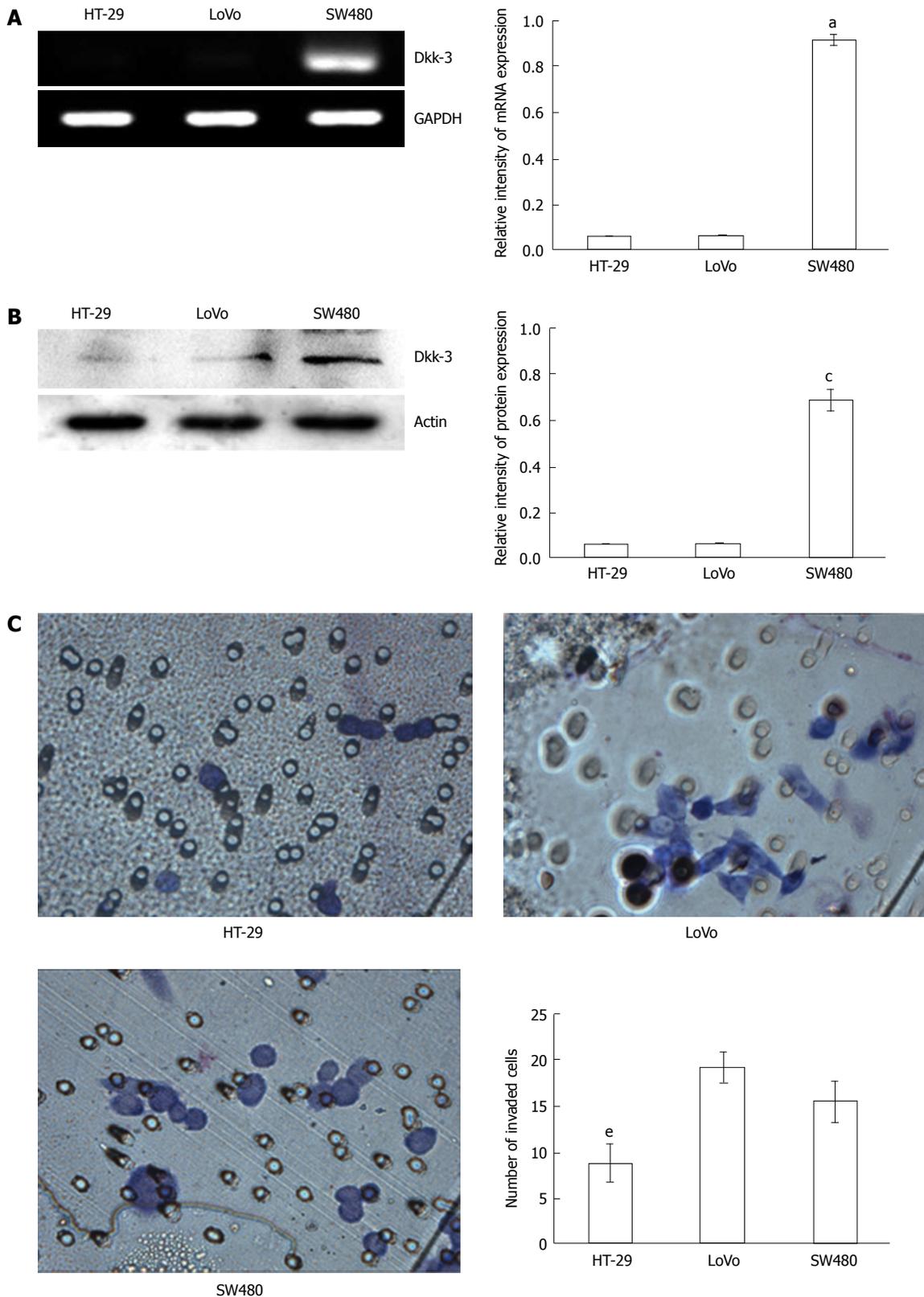
## RESULTS

### Correlation between Dickkopf-3 expression levels and invasion ability in human colon cancer cell lines

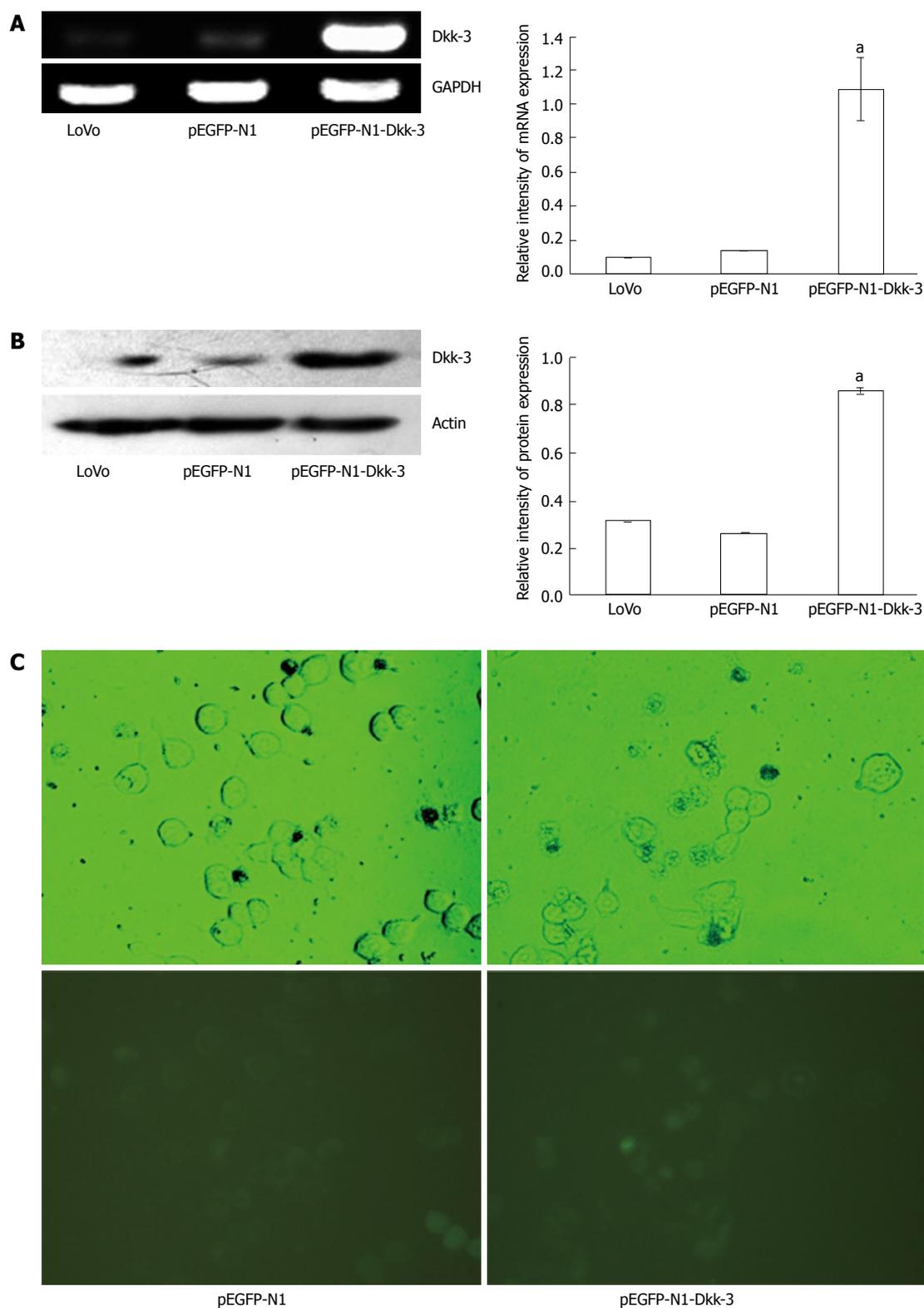
To determine the endogenous expression of Dkk-3, we compared the Dkk-3 level in three human colon cancer cell lines (HT-29, LoVo and SW480). As shown in Figure 1A and B, Dkk-3 expression was significantly higher in SW480 cells (mRNA:  $0.92 \pm 0.04$ , protein:  $0.69 \pm 0.13$ ; all  $P < 0.05$ ) as compared with HT-29 (mRNA:  $0.06 \pm 0.02$ , protein:  $0.06 \pm 0.01$ ) and LoVo cells (mRNA:  $0.07 \pm 0.02$ , protein:  $0.07 \pm 0.02$ ). We also examined the ability of these cells to invade Matrigel, which is a well-established *in vitro* model for assessing tumor invasiveness. The result showed that the greatest levels of invasiveness was in LoVo cells ( $19.25 \pm 1.65$ ), which was followed by the SW480 ( $15.50 \pm 2.12$ ) and HT-29 ( $8.75 \pm 2.10$ ,  $P < 0.05$  vs LoVo or SW480), an order consistent with their known metastatic potentials (Figure 1C). These preliminary findings provoked us to track the question of whether modulation of Dkk-3 could affect colon cancer progression.

### Overexpression of Dickkopf-3 by pEGFP-N1-Dkk-3-GFP plasmid in human colon cancer LoVo cells

To study the biological role of Dkk-3 in colon cancer progression, we used pEGFP-N1-Dkk-3-GFP plasmid coding for full-length human Dkk-3 to enhance the Dkk-3 gene expression in the human colon cancer LoVo cells. The expression of the recombinant human Dkk-3 was analyzed by RT-PCR and Western blotting



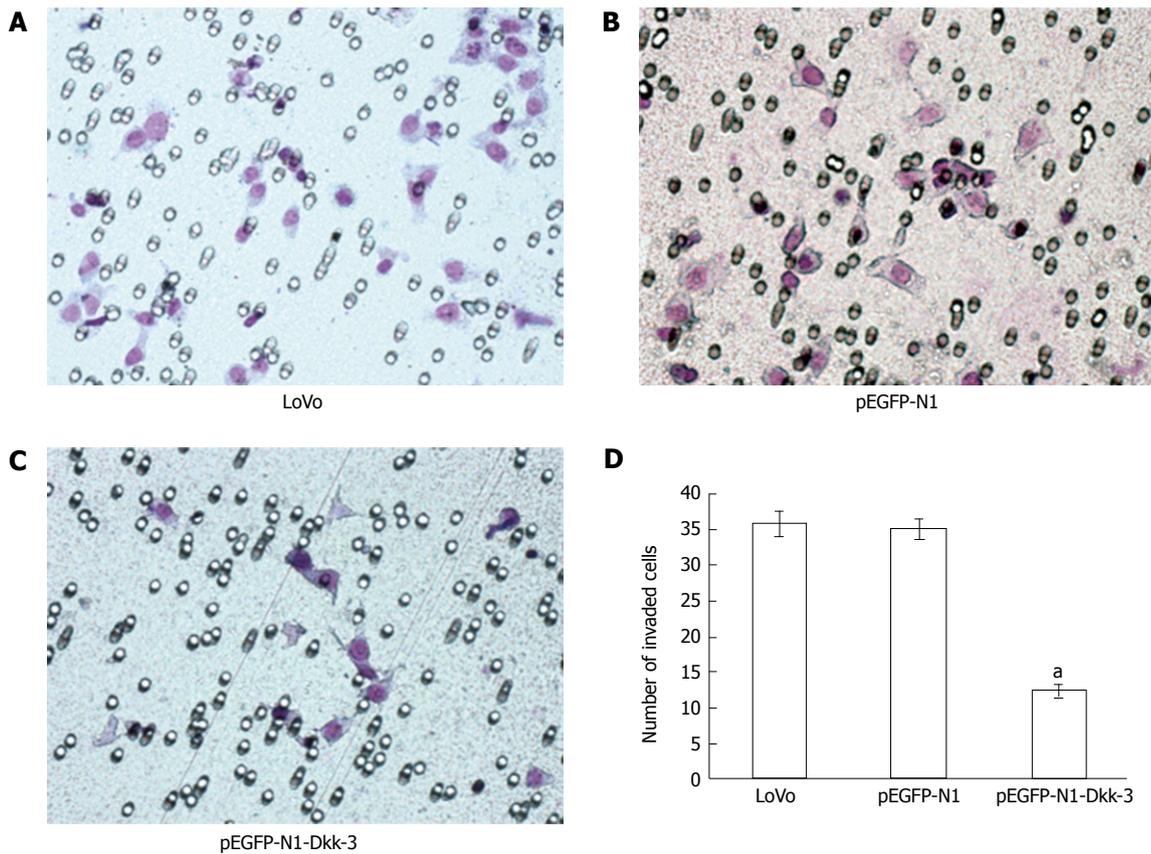
**Figure 1** Levels of Dickkopf-3 mRNA and protein expression correlate with invasive potential of human colon cancer cell lines. A: Semi-quantitative reverse transcription polymerase chain reaction of RNA extracted from colon cancer cell lines, HT-29, LoVo and SW480, respectively, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a control. <sup>a</sup> $P < 0.05$  vs HT-29 or LoVo using Student's *t* test; B: Endogenous Dickkopf-3 (Dkk-3) protein expression was examined by immunoblot analysis of total cellular protein isolated from three colon cancer cell lines: HT-29, LoVo and SW480, and actin was utilized as a loading control. <sup>c</sup> $P < 0.05$  vs HT-29 or LoVo using Student's *t* test; C: Human colon cancer cells, HT-29, LoVo and SW480 invading through the Matrigel were counted under a microscope in ten random fields at  $\times 400$  magnification. <sup>e</sup> $P < 0.05$  vs LoVo or SW480 using Student's *t* test.



**Figure 2** pEGFP-N1-Dkk-3-GFP plasmid induces overexpression of Dickkopf-3 in human colon cancer LoVo cells. A: Semi-quantitative reverse transcription polymerase chain reaction of RNA extracted from pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a control; B: Immunoblotting of total protein lysates extracted from pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells, and actin was included as a loading control; C: Green fluorescent protein (GFP) was also detected under a fluorescence microscope in pEGFP-N1-Dkk-3-GFP plasmid transfected LoVo cells (× 400). <sup>a</sup>*P* < 0.05 vs LoVo or pEGFP-N1 using Student's *t* test.

analysis. As shown in Figure 2A, analysis of the transfected cells ( $1.09 \pm 0.11$ , *P* < 0.05) for Dkk-3 expression *via* semi-quantitative RT-PCR demonstrated a specific

increase in mRNA levels for each gene relative to the pEGFP-N1 plasmid-transfected cells ( $0.14 \pm 0.02$ ) or untreated LoVo cells ( $0.10 \pm 0.02$ ). Immunoblot analysis



**Figure 3** Overexpression of Dickkopf-3 inhibits invasion in human colon cancer LoVo cells. Representative number of invading cells through the Matrigel was counted under microscope in ten random fields at × 400 magnification. Each bar represented the mean ± SD. <sup>a</sup>*P* < 0.05 vs LoVo or pEGFP-N1 using Student's *t* test. The results are representative of three separate experiments.

of cell extracts was carried out to determine whether increased mRNA expression, as observed, correlated with increased translation of the gene product. Figure 2B shows that the protein expression level of Dkk-3 was significantly increased in pEGFP-N1-Dkk-3 group ( $0.86 \pm 0.12$ , *P* < 0.05) compared with the pEGFP-N1 group ( $0.26 \pm 0.04$ ) or untreated LoVo cells ( $0.31 \pm 0.04$ ). A similar trend was observed by immunoblot analysis with the result of RT-PCR. The transient expression of GFP was observed under a fluorescence microscope after 48 h transfection (Figure 2C). Figure 2C indicates that the efficient transduction of pEGFP-N1-Dkk-3-GFP plasmid was approximately 70% after 48 h transfection.

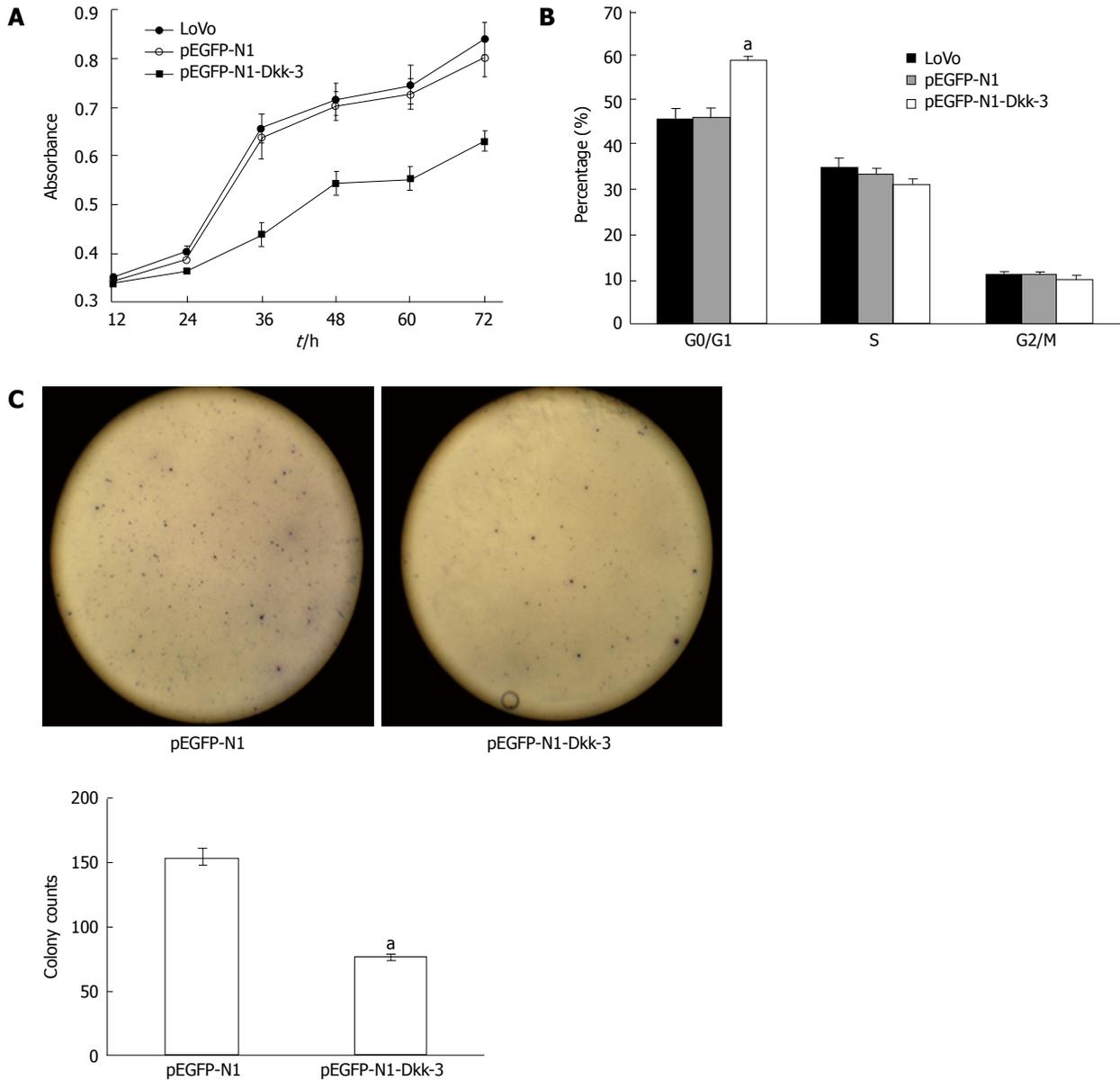
**Effect of Dickkopf-3 overexpression on invasion in human colon cancer LoVo cells**

To evaluate the impact of Dkk-3 overexpression on invasion of human colon cancer LoVo cells, a Matrigel invasion assay was performed. When compared with normal LoVo cells ( $36.00 \pm 1.85$ ) or cells transfected with pEGFP-N1 plasmid ( $36.25 \pm 1.49$ ), pEGFP-N1-Dkk-3-GFP plasmid-transfected cells ( $12.50 \pm 0.96$ , *P* < 0.05) showed a substantial reduction in invasive ability. Invasion of LoVo cells was reduced to about 70% of the controls by pEGFP-N1-Dkk-3-GFP plasmid (Figure 3). Thus, LoVo cell invasion into Matrigel was substantially regulated by

Dkk-3 function. Dkk-3 expression was required for colon cancer cell invasion leading to tumor metastasis.

**Effect of Dickkopf-3 overexpression on proliferation in human colon cancer LoVo cells**

To assess the potential effects of Dkk-3 overexpression on proliferation in human colon cancer LoVo cells, we investigated cell growth *in vitro*. Using the tetrazolium salt (WST-8) cell viability assay (see “Materials and Methods”), we generated a time-response curve by incubating cultures of transfected LoVo cells for 12, 24, 36, 48, 60 and 72 h, which showed a time-dependent inhibition of cell viability (Figure 4A). pEGFP-N1 transfection had no effect on the proliferative ability of LoVo cells, whereas pEGFP-N1-Dkk-3-GFP plasmid transfection caused a dramatic reduction in the proliferation of LoVo cells (*P* < 0.05). On the other hand, we performed colony formation assays using LoVo cells transfected with a *Dkk-3* gene construct (pEGFP-N1-Dkk-3-GFP) or with an empty vector (pEGFP-N1). The number of colonies formed was counted after 2 wk culture. When compared with the pEGFP-N1 plasmid-transfected cells ( $154.67 \pm 5.86$ ), we observed that *Dkk-3* overexpression ( $77.00 \pm 2.65$ , *P* < 0.05) decreased markedly the number of colonies (Figure 4C).



**Figure 4** Overexpression of Dickkopf-3 inhibits proliferation and induces G0/G1 arrest in human colon cancer LoVo cells. A: Dickkopf-3 (Dkk-3) inhibits proliferation in human colon cancer LoVo cells; B: Forty-eight hours after transfection, LoVo was used for cell cycle analysis using a FACSCalibur flow cytometer; C: The dickkopf homolog 3 gene Dkk-3 inhibited tumor cell colony formation. LoVo cells were transfected with pEGFP-N1-Dkk-3-GFP plasmid or with pEGFP-N1 and were maintained in the presence of G418 sulfate for 2 wk. Quantitative analysis of colony numbers are shown as the mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs pEGFP-N1 using Student's *t* test.

**Effect of Dickkopf-3 overexpression on cell cycle in human colon cancer LoVo cells**

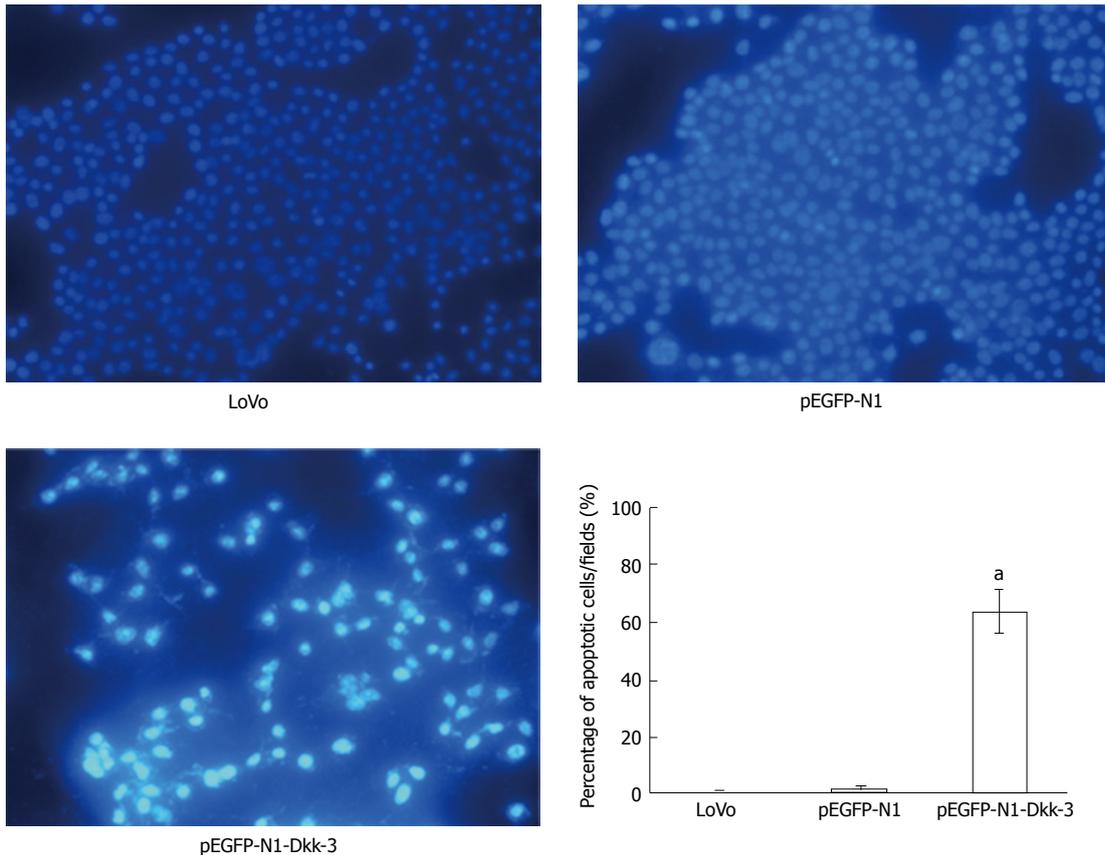
To investigate the precise mechanisms of the decreased cell viability observed in LoVo transient *Dkk-3* transfectants, we analyzed the cell cycle distribution profile by flow cytometry with propidium iodide. After 48 h transfection, the cells were fixed and stained with the DNA intercalating fluorescent dye propidium iodide. As shown in Figure 4B, untreated LoVo cells had normal cell cycle profiles with approximately 45% of cells in G<sub>0</sub>/G<sub>1</sub> phase containing 2N DNA content and 12% of cells in G<sub>2</sub>/M phase containing 4N DNA content. The percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle was significantly higher in Dkk-3 transfected LoVo cells ( $0.59 \pm 0.01$ ,  $P < 0.05$ ).

**Effect of Dickkopf-3 overexpression on apoptosis in human colon cancer LoVo cells**

The morphological changes of the apoptotic cells were detected by Hoechst 33258 staining (Figure 5). After 48 h transfection, cells were fixed and stained with Hoechst 33258 at room temperature. In the untreated LoVo cells ( $0.67 \pm 0.52$ ) and pEGFP-N1 group ( $1.33 \pm 1.21$ ), the nuclei were stained weak homogeneous blue, while in the group transfected with pEGFP-N1-Dkk-3-GFP plasmid ( $63.67 \pm 7.71$ ,  $P < 0.05$ ), bright chromatin condensation and nuclear fragmentation were found.

**Effect of Dickkopf-3 overexpression on cytoplasmic  $\beta$ -catenin accumulation in human colon cancer LoVo cells**

Dkk-3 has been reported to induce changes in  $\beta$ -catenin



**Figure 5** Detection of apoptosis by Hoechst 33285. The apoptotic feature was assessed by observing chromatin condensation and fragment staining. <sup>a</sup> $P < 0.05$  vs control cells (untreated LoVo cells or pEGFP-N1-transfected LoVo cells).

localization consistent with an increase in cell-cell adhesion<sup>[9]</sup>. To address this question in colon cancer, we examined the  $\beta$ -catenin expression level in mock and *Dkk-3* transfectants. As shown in Figure 6A, transient transfection of *Dkk-3* affected Wnt signaling by reducing the accumulation of cytosolic fraction of  $\beta$ -catenin.  $\beta$ -catenin often yields doublets on Western blot analysis, perhaps as a result of being phosphorylated (most often tyrosine or serine phosphorylation)<sup>[9]</sup>.

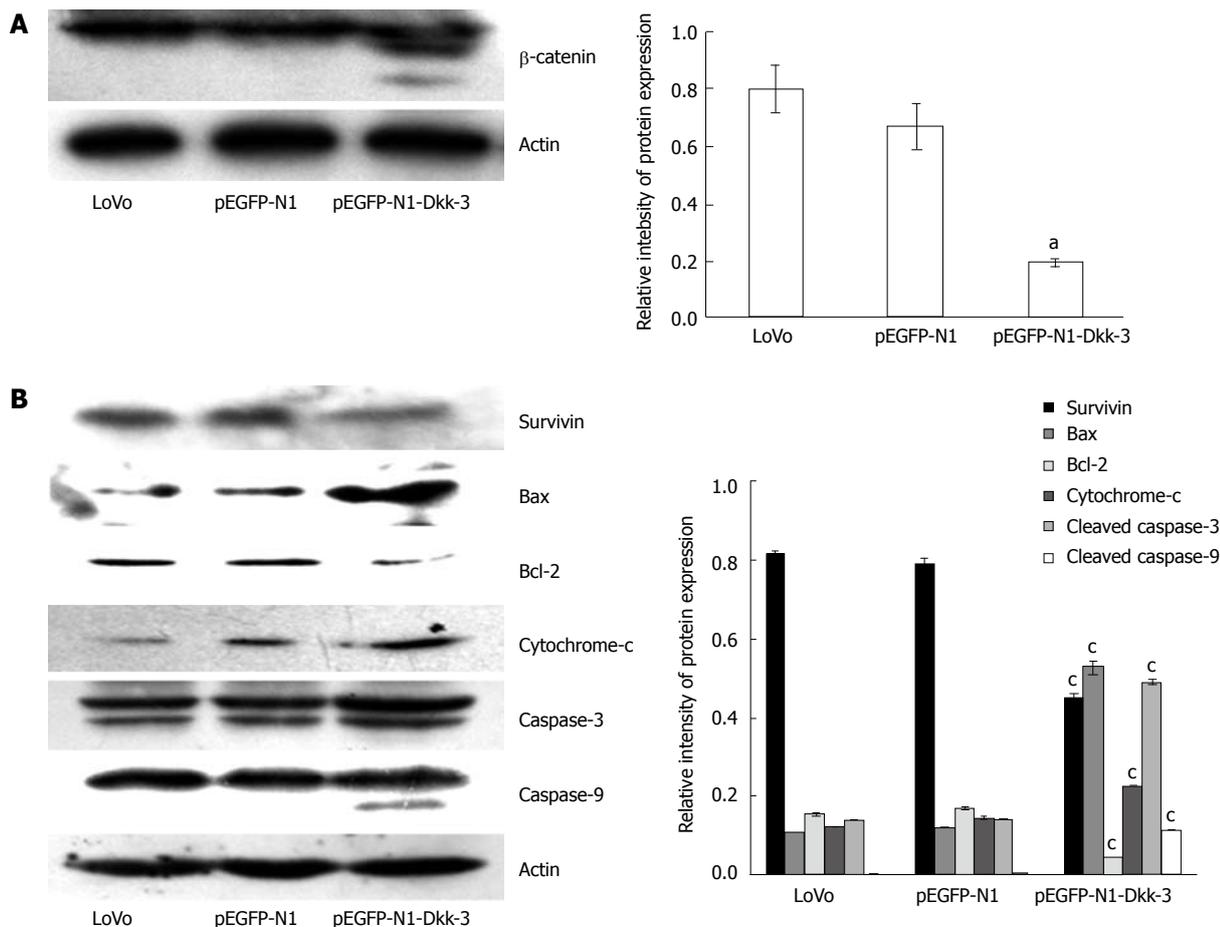
#### **Overexpression of Dickkopf-3 induces apoptosis through activation of mitochondrial pathway in LoVo cells**

To further investigate the detailed apoptotic mechanism, we examined the effect of *Dkk-3* overexpression on mitochondrial pathway. As shown in Figure 6B, *Dkk-3* overexpression caused a decline in survivin levels, a member of the inhibitors of apoptosis proteins family, which is known to block apoptosis by inhibiting caspases and mitochondria-mediated apoptosis<sup>[18]</sup>. It has been proposed that one of the main regulatory steps of programmed cell death is controlled by the ratio of anti-apoptotic and proapoptotic members of the Bcl-2 family of proteins<sup>[19,20]</sup>. The role of mitochondrial damage in apoptosis was suggested to be mediated by the release of cytochrome c<sup>[21]</sup>. Upon cleavage by upstream proteases in an intracellular cascade, the activation of caspase-3 is considered as a hallmark of the apoptotic process<sup>[22]</sup>.

*Dkk-3* overexpression also induced an increase in Bax protein levels and a decrease in Bcl-2 levels in LoVo cells, which led to a decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) ratio (Figure 6B). In addition, the expression levels of the cytosolic cytochrome c which was suggested to be involved in mitochondrial damage, the activated caspase-3 and the activated caspase-9 were significantly increased with *Dkk-3* overexpression.

## **DISCUSSION**

The Wnt signalling pathway has long been known to direct growth and patterning during embryonic development<sup>[23,24]</sup>. Recent evidence also implicates that Wnt signalling pathway is involved in the postembryonic regulation of stem-cell number in epithelia, such as those of the skin and intestine, which undergo constant renewal<sup>[24]</sup>. The pathway is composed of canonical Wnt signaling *via* Wnt/ $\beta$ -catenin and noncanonical Wnt signaling *via* the Wnt/ $\text{Ca}^{2+}$  pathway and Wnt/c-JNK (planar cell polarity)<sup>[4,5]</sup>. Wnt signaling pathway is often activated in many cancers and the expression of Wnt antagonists is often downregulated epigenetically<sup>[4,7,24-29]</sup>. The extracellular antagonists of the Wnt signalling pathway can be divided into two broad classes. Both classes of molecule prevent ligand-receptor interactions, but by different mechanisms: members of the first class, including



**Figure 6** Overexpression of Dickkopf-3 inhibits downstream signaling and induces apoptosis in LoVo cells through mitochondrial pathway. A: Dickkopf-3 (Dkk-3) reduces cytoplasmic accumulation of  $\beta$ -catenin; B: Western blotting analysis of survivin, Bcl-2, Bax, cytochrome c, caspase-3 and caspase-9 protein after 48 h transfection with pEGFP-N1-Dkk-3-GFP plasmid in LoVo cells. Actin was used as a loading control. <sup>a</sup>*P* < 0.05 vs LoVo or pEGFP-N1 using Student's *t* test.

the sFRP family, Wnt inhibitory factor-1 and Cerberus, primarily binds to Wnt proteins; the second class comprising certain members of the Dkk family, binds to one subunit of the Wnt receptor complex<sup>[6]</sup>.

Human Dkk-related genes are composed of Dkk-1, Dkk-2, Dkk-3, and Dkk-4, together with a unique Dkk-3 related protein termed Soggy (Sgy)<sup>[7]</sup>. Dkk-3, the most divergent family member, is proposed to function as a secreted tumor suppressor since it is downregulated in a number of cancer cells<sup>[28]</sup>. Dkk-3 has been reported to be silenced or down-regulated in 12 (70.6%) of 17 gastric cancer cell lines and in 3 (33.3%) of 9 colon cancer cell lines, and the loss of gene expression was associated with promoter methylation, which could be restored by demethylating agents<sup>[30]</sup>. Tissue microarrays have shown that the number of microvessels in Dkk-3-positive CRC samples was significantly higher than that in Dkk-3-negative samples (*P* = 0.001), and Dkk-3 expression was also increased with rising numbers of microvessels (*P* < 0.0001)<sup>[29]</sup>. In addition, Dkk-3 has been revealed to inhibit cancer proliferation and induce apoptosis in several cancers<sup>[25,26,31-36]</sup>. Overexpression of Dkk-3 in normal fibroblasts suppresses tumor growth *via* induction of interleukin-7<sup>[32]</sup>. In malignant glioma<sup>[37]</sup>, Dkk-3 transfection

led to apoptosis due to the activation of phosphorylated jun proto-oncogene, caspase-9, and caspase-3 and the reduction of  $\beta$ -catenin. In renal cell carcinoma<sup>[25]</sup>, prostate cancer<sup>[26,31,35]</sup>, testicular cancer<sup>[38]</sup>, bladder cancer<sup>[34]</sup> and breast cancer<sup>[33]</sup>, overexpression of Dkk-3 was found to lead to apoptotic cell death in a c-JNK phosphorylation-dependent manner and/or endoplasmic reticulum stress. In osteosarcoma<sup>[9]</sup>, transfection of Dkk-3 and dominant-negative LRP5 significantly lowers the cell invasion capacity and cell motility, and also induces changes in  $\beta$ -catenin localization consistent with an increase in cell-cell adhesion. However, Dkk-3 functional analysis and the regulation mechanism have not been reported in colorectal cancer.

In the present study, we focused on proliferation and apoptosis of colon cancer cells. We examined the anti-proliferation ability of Dkk-3 overexpression by pEGFP-N1-Dkk-3-GFP plasmid in human colon cancer LoVo cells, and measured the extent of cell proliferation by the WST-8 assay (Figure 4A). Interestingly, overexpression of Dkk-3 effectively suppressed cellular proliferation of colon cancer cells in a time-dependent fashion (Figure 4A). On the other hand, overexpression of Dkk-3 inhibited tumor cell colony formation in LoVo

cells (Figure 4C).

To investigate the precise mechanisms responsible for the Dkk-3 overexpression-mediated abortive cell divisions, we sought to examine the cell cycle distribution profile of Dkk-3 transfectants. The percentage of cells arrested in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle was also increased in Dkk-3 transfectants. It was also revealed that Dkk-3 overexpression resulted in apoptosis (Hoechst 33258) in Dkk-3 transfectants (Figure 5). The morphological features in LoVo cells of apoptotic *vs* necrotic cell death can be distinguished under microscopy<sup>[39]</sup>. Apoptotic LoVo cells were identified by observing chromatin condensation and fragment staining by Hoechst 33258. It suggests that if early apoptotic cells are not ingested by phagocytes in time, secondary necrosis would proceed<sup>[40]</sup>.

One of the main regulatory steps of programmed cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl-2 family of proteins<sup>[19,20]</sup>. Overexpression of antiapoptotic Bcl-2 family members can tip the delicate balance in favor of survival, thereby conferring drug resistance, at least in some cellular tumor model systems<sup>[41-43]</sup>. On the other hand, overexpression of proapoptotic Bax or Bak is sufficient to increase the sensitivity of malignant cancer cells to apoptosis and to overcome drug resistance<sup>[43-45]</sup>. Bcl-2 in the unphosphorylated form complexes with Bax, and thus its phosphorylation releases Bax from the Bcl-2-Bax complex<sup>[22,46-48]</sup>. Unbound Bax translocates from cytosol to the mitochondrial membrane to signal triggering of the downstream apoptotic cascade, such as release of cytochrome c and activation of executionary caspases<sup>[22,44-46]</sup>. The activation of caspase-3, upon its cleavage by upstream proteases, is considered as a hallmark of the apoptotic process<sup>[41]</sup>. In agreement with the hypothesis, activated caspase-3 and -9 were detected in Dkk-3 transfectants. Our results showed that overexpression of Dkk-3 decreased Bcl-2/Bax ratio, caused the release of cytochrome c, and the activation of caspase-3 and caspase-9 in LoVo cells (Figure 6B). Further studies are required to determine the exact mechanism whether Dkk-3 enhances apoptosis-inducing effects on human colon cancer cells, which is cross-talking activation of death receptors pathway of apoptosis.

In conclusion, Dkk-3 is anti-proliferative and proapoptotic in colon cancer LoVo cells. Overexpression of Dkk-3 caused 2N DNA accumulation in LoVo cells, suggesting that the Dkk-3 transfectants arrest in G<sub>0</sub>/G<sub>1</sub> phase preceding cell death. These abnormal cells probably trigger activation of programmed cell death that is mitochondrially-driven and executed through the activated caspase by the cleavage of downstream targets. The LoVo cell death program is also mediated through downregulation of survivin. Therefore, Dkk-3 functions as a tumor suppressor in colon cancer cells and its downregulation may be involved in colon cancer progression. Moreover, Dkk-3 may be involved in the Wnt/ $\beta$ -catenin signaling pathways in colon cancer cells.

## COMMENTS

### Background

The Wnt signal transduction pathway is activated in many cancers and the expression of Wnt antagonists are often downregulated epigenetically. Wnt antagonists can be divided into two functional classes, the secreted frizzled related proteins and the Dickkopf (Dkk). The *Dkk* gene family of secretory modulators of canonical Wnt/ $\beta$ -catenin signaling is involved in the control of proliferation, polarity and migration, cell fate specification and differentiation. Dkk-3, also known as reduced expression in immortalized cells, is the most divergent family member and proposed to function as a secreted tumor suppressor since it is downregulated in a number of cancer cells.

### Research frontiers

Recently, Wnt antagonists have received increasing and specific attention due to their potential role in carcinogenesis. Dkk-3 has been revealed to inhibit cancer proliferation and induce apoptosis in malignant glioma, breast cancer, osteosarcoma, renal cell carcinoma, prostate cancer, testicular cancer and bladder cancer.

### Innovations and breakthroughs

Few studies have described the correlation between Wnt antagonists and the development of colon cancer. The results of this study suggest that Dkk-3 may act as negative regulators of Wnts and may be involved in the Wnt/ $\beta$ -catenin signaling pathways in colon cancer cells. Dkk-3 may be a crucial Wnt signaling regulator in colon cancer and an important component of the gastrointestinal proliferative regulatory network.

### Applications

In this study, the mRNA and protein expressions of Dkk-3 were investigated in colon cancer cells and that the aberrant expression of Wnt antagonists may play an important role in carcinogenesis of colon cancer. This finding may help improve early diagnosis and new therapies by blocking this pathway in the treatment of colon cancer.

### Terminology

The Wnt signaling pathway is one of evolutionarily-conserved signal transduction pathways to direct growth and patterning during embryonic development, from Hydra to humans. Wnt signals regulate many aspects of development which include the proliferation, fate specification, polarity, and migration of cells. Moreover, overactivation of Wnt signaling by mutation is an important factor in oncogenesis in the human colon and other tissues. The pathway is composed of canonical Wnt signaling via Wnt/ $\beta$ -catenin and noncanonical Wnt signaling or pathways that are  $\beta$ -catenin independent.

### Peer review

The authors investigated the mechanisms of the biological roles of Dkk-3 in colon cancer. It revealed that Dkk-3 played an important role in mitochondria-mediated apoptosis and Dkk-3 may be involved in the Wnt/ $\beta$ -catenin signaling pathways in colon cancer cells. The article is a good attempt to work on the hypothesis and the results may represent a molecular mechanism of colon carcinogenesis.

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## Toxicarioside A inhibits SGC-7901 proliferation, migration and invasion *via* NF- $\kappa$ B/bFGF signaling

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**METHODS:** After SGC-7901 cells were treated with toxicarioside A at various concentrations (0.5, 1.5, 4.5, 9.0  $\mu$ g/mL) for 24 h or 48 h, cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay, and the motility and invasion of tumor cells were assessed by the Transwell chamber assay. Immunofluorescence staining, reverse transcription polymerase chain reaction and Western blotting were performed to detect the expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1), and nuclear factor-kappa B (NF- $\kappa$ B) activation was examined by electrophoretic mobility shift assay.

**RESULTS:** The results showed that toxicarioside A was capable of reducing cell viability, inhibiting cell growth, and suppressing cell migration and invasion activities in a time- and dose-dependent manner in SGC-7901 cells. Further analysis revealed that not only the expression of bFGF and its high-affinity receptor FGFR1 but also the NF- $\kappa$ B-DNA binding activity were effectively blocked by toxicarioside A in a dose-dependent manner compared with the control group ( $P < 0.05$  or  $P < 0.01$ ). Interestingly, application of the NF- $\kappa$ B specific inhibitor, pyrrolidinedithiocarbamate (PDTC), to SGC-7901 cells significantly potentized the toxicarioside A-induced down-regulation of bFGF compared with the control group ( $P < 0.05$ ).

**CONCLUSION:** These findings suggest that toxicarioside A has an anti-gastric cancer activity and this effect may be achieved partly through down-regulation of NF- $\kappa$ B and bFGF/FGFR1 signaling.

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**Key words:** Anti-migration; Anti-proliferation; Basic fibroblast growth factor; Gastric cancer; Nuclear factor-kappa B; Toxicarioside A

### Abstract

**AIM:** To investigate the inhibitory role of toxicarioside A on the gastric cancer cell line human gastric cancer cell line (SGC-7901) and determine the underlying molecular mechanism.

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## INTRODUCTION

*Antiaristoxicaria* (Pers.) Lesch (Moraceae) is a well known precious species widespread in the tropical rain forest of Southeast Asia. Its latex and seeds contain a complex mixture of cardenolide glycosides and is therefore toxic<sup>[1]</sup>. Representative toxicariosides A-L have recently been identified from the latex and seeds of *Antiaristoxicaria* in our laboratory and by others<sup>[2-5]</sup>. Classically, cardenolides are used to treat congestive heart failure and arrhythmia<sup>[6-8]</sup>. Additionally, certain cardenolides extracted from some plants or animals have been demonstrated to be capable of blocking tumor cell proliferation through regulation of cell signal transduction<sup>[9-15]</sup>.

Currently, gastric cancer is one of the leading malignancies in China. However, the treatment outcome is not satisfactory because early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed<sup>[16]</sup>.

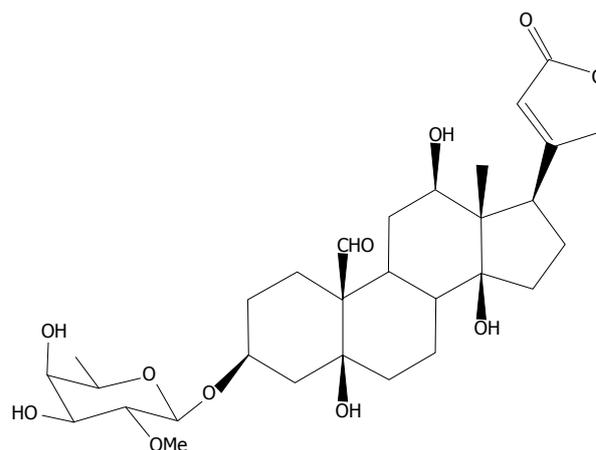
Basic fibroblast growth factor (bFGF) has been shown to be a multifunctional growth factor for tumor development<sup>[17-20]</sup>, and it exerts its biological effects mainly through interaction with its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR1)<sup>[21-24]</sup>. Compiling evidence has demonstrated that bFGF signaling is involved in the development of gastric cancer<sup>[25,26]</sup>.

Nuclear factor-kappa B (NF- $\kappa$ B) is a ubiquitous dimeric transcription factor that plays pivotal roles in regulating the expression of genes encoding cytokines and chemokines that are involved in tumor proliferation, angiogenesis, and synthesis of anti-apoptotic proteins<sup>[27,28]</sup>. It has been documented that NF- $\kappa$ B can mediate bFGF signaling<sup>[29]</sup> and some types of cardiac glycosides can block the activation of NF- $\kappa$ B<sup>[30,31]</sup>. As a result, we hypothesize that cardiac glycosides may suppress gastric tumor growth *via* a decrease in NF- $\kappa$ B activity and blocking of the bFGF signaling pathway. In the present study, we attempted to test this hypothesis in an *in vitro* cell culture model.

## MATERIALS AND METHODS

### Plant material

Latex of *Antiaristoxicaria* (Pers.) Lesch collected in Lingshui county of Hainan Province, China in November 2005 was identified with the assistance of Professor Zhunian



**Figure 1** The structure of toxicarioside A.

Wang at the Institute of Crops Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. The specimen was numbered as No. AN200511.

### Chemicals and reagents

Rabbit-anti human bFGF and FGFR1 were purchased from Santa Cruz (Santa Cruz, CA, United States). Rhodamine (TRITC)-conjugated mouse anti-rabbit immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit IgG, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue and pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma (Sigma Aldrich, St Louis, MO, United States). Fetal bovine serum (FBS), RPMI 1640 medium and trypsin were procured from Gibco (Gibco, Carlsbad, CA, United States).

### Extraction and isolation of toxicarioside A

With 95% EtOH, 4.0 L of the latex of *Antiaristoxicaria* were extracted thrice at room temperature and filtered. The combined extract was evaporated *in vacuo* to yield a syrup (263.8 g), which was fractionated sequentially with petroleum ether, EtOAc, and n-BuOH. The EtOAc fraction (8.68 g) that showed potent cytotoxic activity in the bioassay was passed through pressure-reduced column chromatography using step-wise elution with CHCl<sub>3</sub>-MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1, v/v), generating seven corresponding fractions, A1-A7. Fraction A7 (2.55 g) was further separated on silica gel column chromatography, from which compound 1 (788.1 mg) was eluted with CHCl<sub>3</sub>-MeOH (14:1, v/v). On the basis of spectral data and chemical analyses, compound 1 was defined as toxicarioside A (Figure 1).

### Cell culture

Human gastric cancer cell line (SGC-7901) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells at the logarithmic phase were

used for experiments.

### **Proliferation assay**

MTT assay and trypanblue staining were used to determine the growth and viability of SGC-7901 cells. For the MTT assay, SGC-7901 cells in logarithmic growth were trypsinized and harvested and then the cells were seeded onto a 96-well plate. After 24 h, fresh RPMI 1640 medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0  $\mu\text{g}/\text{mL}$ ) was added at 100  $\mu\text{L}$  per well, respectively, and 6 replicate wells were used for each of the concentrations. After incubation for different time intervals, 10  $\mu\text{L}$  of MTT (5  $\text{mg}/\text{mL}$ ) was added to each well and the cells were further incubated at 37  $^{\circ}\text{C}$  for 4 h. The supernatant was then removed and 100  $\mu\text{L}$  DMSO was added into each well. Absorbance (A value) at a wavelength of 490 nm was measured with a Bio-TekEXL808 microplate reader (Bio-Rad, Hercules, CA, United States). For trypanblue staining, SGC-7901 cells were trypsinized and seeded into 24-well plates at a density of  $0.5 \times 10^4/\text{mL}$ . After 4.5  $\mu\text{g}/\text{mL}$  toxicarioside A was added, the cells were collected and counted using trypan blue staining under an inverse light microscope for 3 consecutive days.

### **Invasion and migration assay**

Invasion assays were performed in a 24-well Transwell chamber (Corning, Lowell, MA, United States) as previously described<sup>[32]</sup>. Briefly, each Transwell chamber was coated with 15  $\mu\text{g}$  Matrigel,  $5 \times 10^4$  cells were seeded to pre-coated filters in 200  $\mu\text{L}$  of serum-free medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0  $\mu\text{g}/\text{mL}$ ) in triplicate. The lower parts of the chambers were filled with 500  $\mu\text{L}$  of RPMI 1640 medium containing 10% FBS. After incubation in a 5%  $\text{CO}_2$  humidified incubator at 37  $^{\circ}\text{C}$  for 24 h, the cells on the upper surface were gently removed with a cotton swab, and the filters were fixed with 95% alcohol for 15-20 min and stained with hematoxylin-eosin for 15 min. The number of cells on the lower surface of the filters was quantified under a microscope. The same procedures were followed for the migration assay except the Transwell chambers were not coated with Matrigel.

### **Immunofluorescence staining**

To detect the expression of bFGF as well as its receptor FGFR1 in SGC-7901 cells, the rabbit antibody (1:100) against bFGF and FGFR1 were used. The antigenic sites were localized by TRITC-conjugated mouse anti-rabbit IgG and FITC-conjugated mouse anti-rabbit IgG, and images of antigenic sites were captured under a laser scanning confocal microscope (FV500, Olympus, Tokyo, Japan).

### **RNA extraction and reverse transcription polymerase chain reaction**

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocols. Reverse transcription polymerase chain

reaction was carried out using pairs of primers (Invitrogen) as follows for semiquantitative assessment: bFGF (NM\_002006.4) sense, 5'-AAG AGC GAC CCT CAC ATC AA-3'; anti-sense, 5'-TCG TTT CAG TGC CAC ATC CGT CAA TA CC-3', yielding a 225 bp product; FGFR1 (M34641) sense, 5'-CTT CTGT TTC AG-3'; anti-sense, 5'-TCC ACA ATG CAG GTG TAG TT-3', yielding a 354 bp product.  $\beta$ -actin (NM\_001101) sense, 5'-GTT GCG TTA CAC CCT TTC TT-3', anti-sense, 5'-CGA AGG CTC ATC ATT CAA AA-3', yielding a 443 bp product. The products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV using the gel documentation system (Bio-Rad Gel Doc1000, Bio-Rad). The mRNA levels of bFGF, FGFR1 were calculated based on the densitometric values of the specific bFGF, FGFR1 bands after adjustment with that of the  $\beta$ -actin band.

### **Western blotting analysis**

This was performed as previously described with minor modifications<sup>[33]</sup>. Cells were homogenized and separated by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane blot (Bio-Rad). The blot was incubated with a rabbit anti-bFGF antibody (1:500) or a rabbit anti-FGFR1 antibody (1:500) at 4  $^{\circ}\text{C}$  overnight, followed by incubation with the corresponding horseradish peroxidase-conjugated anti-biotin antibody (1:2000) at room temperature for 1 h. The immunoreactive signals were visualized with enhanced chemiluminescence reagents (Pierce, Rockford, IL, United States).

### **Electrophoretic mobility shift assay**

To determine NF- $\kappa\text{B}$  activation, electrophoretic mobility shift assay (EMSA) was conducted essentially as described previously<sup>[34]</sup>. In brief, nuclear proteins (10  $\mu\text{g}$ ) were incubated with the reaction buffer for 20 min at room temperature, followed by incubation with oligonucleotide containing the consensus sequence for the NF- $\kappa\text{B}$ -DNA binding site (5'-AGAGTGGGAATT TC-CACTCA-3')<sup>[35]</sup> (synthesized by Invitrogen, Shanghai, China). The reaction mixture was separated in a non-denaturing polyacrylamide gel (6%) that was later stained by SYBR Green EMSA staining solution from Molecular Probes (Invitrogen) with continuous, gentle agitation for about 20 min, protected from light. The gel was then washed in 150 mL of  $\text{dH}_2\text{O}$  and the stained nucleic acids were visualized and the image documented under UV using the gel documentation system (Bio-Rad Gel Doc1000).

### **Statistical analysis**

All data are expressed as mean  $\pm$  SE. For a comparison between two groups, the Student's *t* test was performed. For comparisons among multiple groups, an ANOVA was carried out, followed by a Student-Newman-Keuls test. Differences were considered significant when  $P < 0.05$ .

**Table 1** The inhibition rates of human gastric cancer cell line cells treated with different concentrations of toxicarioside A for different time intervals

Groups	24 h		48 h	
	A value	Inhibitory rate (%)	A value	Inhibitory rate (%)
Control	0.879 ± 0.048	0.00 ± 0.00	0.932 ± 0.036	0.00 ± 0.00
Toxicarioside A (µg/mL)				
0.5	0.793 ± 0.062	9.79 ± 7.63	0.752 ± 0.073	19.13 ± 5.38
1.5	0.646 ± 0.041	27.83 ± 4.78 <sup>a</sup>	0.596 ± 0.113	36.19 ± 3.81 <sup>a</sup>
4.5	0.528 ± 0.078	40.18 ± 3.32 <sup>a</sup>	0.443 ± 0.056	49.32 ± 5.17 <sup>b</sup>
9.0	0.352 ± 0.092	61.84 ± 6.61 <sup>b</sup>	0.301 ± 0.049	66.94 ± 7.03 <sup>b</sup>

Data representative of six independent experiments were expressed as mean ± SE. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

**Table 2** Effect of toxicarioside A on cell migration and invasion

Groups	Dose (µg/mL)	Migration		Invasion	
		Cell number	Inhibition rate (%)	Cell number	Inhibition rate (%)
Control	0.0	69.40 ± 6.38	0.00 ± 0.00	65.60 ± 7.20	0.00 ± 0.00
Toxicarioside A	0.5	62.50 ± 8.90	10.01 ± 9.78	57.80 ± 4.32	10.89 ± 8.64
	1.5	54.80 ± 7.30	22.38 ± 10.64 <sup>a</sup>	49.70 ± 5.68	24.03 ± 9.06 <sup>a</sup>
	4.5	41.60 ± 5.88	39.58 ± 11.62 <sup>a</sup>	36.40 ± 7.94	44.68 ± 9.19 <sup>a</sup>
	9.0	35.80 ± 8.32	48.13 ± 10.12 <sup>b</sup>	30.10 ± 6.46	54.38 ± 8.17 <sup>b</sup>

Data representative of three independent experiments were expressed as mean ± SE. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

## RESULTS

### Effect of toxicarioside A on SGC-7901 cell proliferation

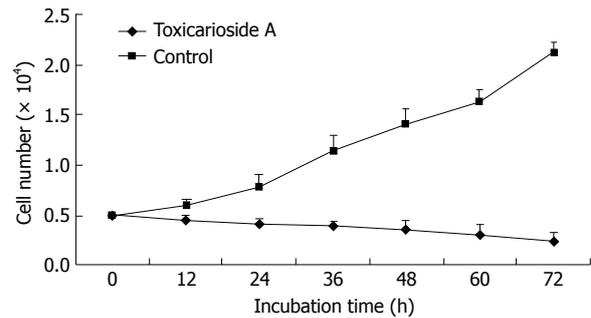
To assess the effect of toxicarioside A on the growth of gastric cancer, SGC-7901 cells were treated at various concentrations (0.5, 1.5, 4.5, 9.0 µg/mL) for 24–48 h and cell viability following these treatments was determined by MTT assays. As shown in Table 1, toxicarioside A reduced SGC-7901 cell viability in a time- and dose-dependent manner. Cell growth curves also showed that toxicarioside A significantly inhibited SGC-7901 cell growth as compared with the control (Figure 2).

### Effect of toxicarioside A on SGC-7901 cell migration and invasion

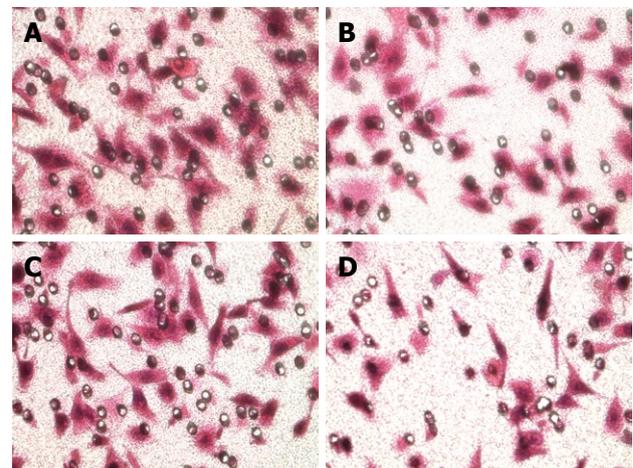
The results of Transwell cell migration and invasion are presented in Table 2 and Figure 3. Clearly, the addition of toxicarioside A to the medium in the upper chamber resulted in significant suppression of SGC-7901 migration and invasion in a dose-dependent manner at 1.5, 4.5 and 9.0 µg/mL, toxicarioside A inhibited SGC-7901 migration by 22.38% ± 10.64%, 39.58% ± 11.62% and 48.13% ± 10.12%, respectively (*P* < 0.05), and inhibited SGC 7901 invasion by 24.03% ± 9.06%, 44.68% ± 9.19% and 54.38% ± 8.17%, respectively (*P* < 0.01), as compared with the control group.

### Effect of toxicarioside A on bFGF and FGFR1 in SGC-7901 cells

At the protein level, the expression of bFGF and FGFR1 was predominantly detected in the cytoplasm of SGC-7901 cells and toxicarioside A significantly decreased this ex-



**Figure 2** Effect of toxicarioside A on the growth curve of human gastric cancer cell line cells. Cells were plated in 24-well plates at a density of  $1 \times 10^4$ /mL and treated with 4.5 µg/mL toxicarioside A for 72 h. The results shown are representative of three independent experiments.



**Figure 3** Representative figures of cell migration and invasion in non-treated and toxicarioside A-treated human gastric cancer cell line cells. A: Migration in the control group; B: Migration in the toxicarioside A-treated (4.5 µg/mL) group; C: Invasion in the control group; D: Invasion in the toxicarioside A-treated group.

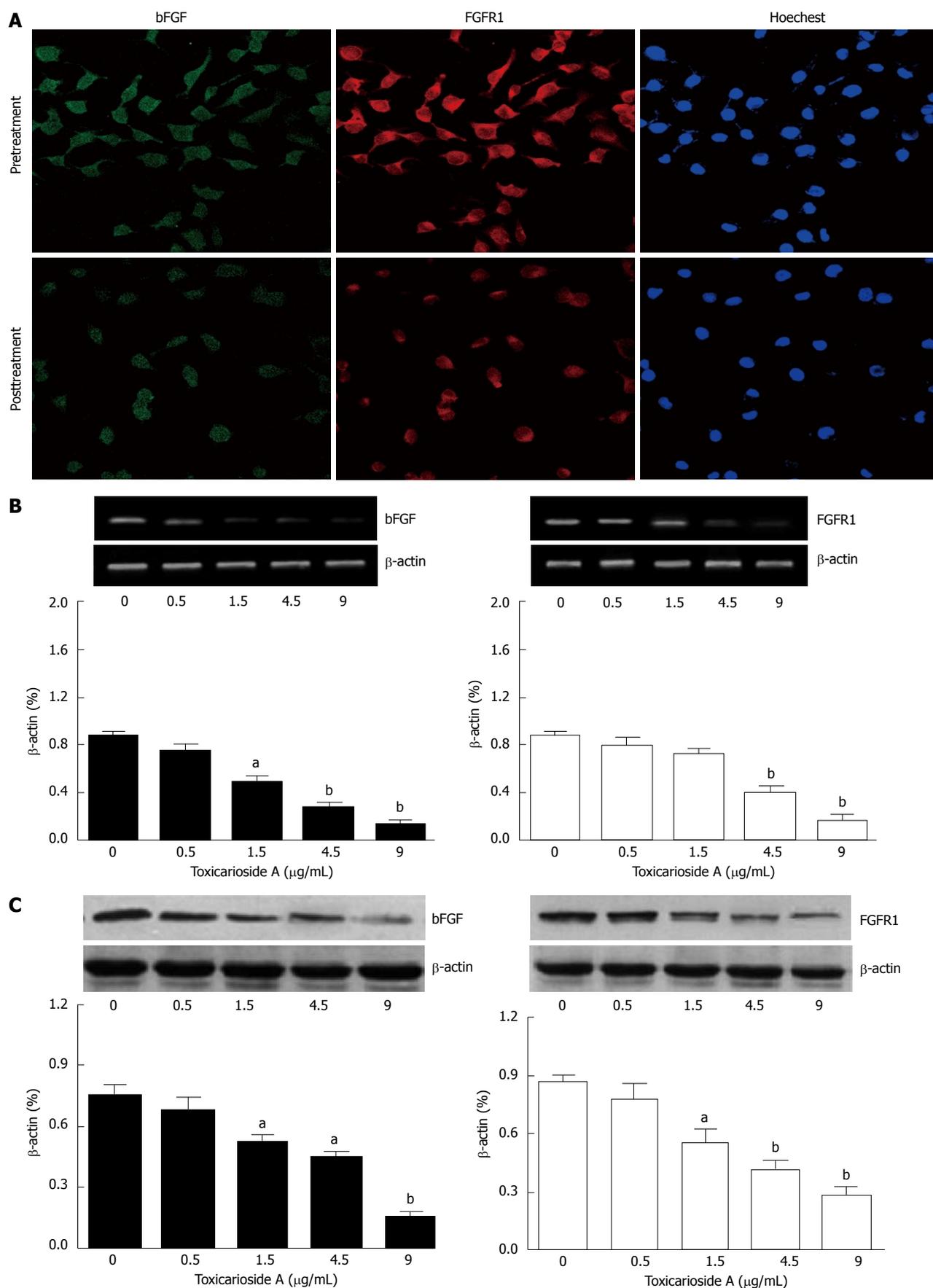
pression, as assessed by immunofluorescence staining (Figure 4A) and Western blotting analysis (Figure 4C). At the mRNA level, the expression of bFGF and FGFR1 was decreased by toxicarioside A in a dose-dependent manner in SGC-7901 cells (Figure 4B).

### Effect of toxicarioside A on NF-κB-DNA binding activity in SGC-7901 cells

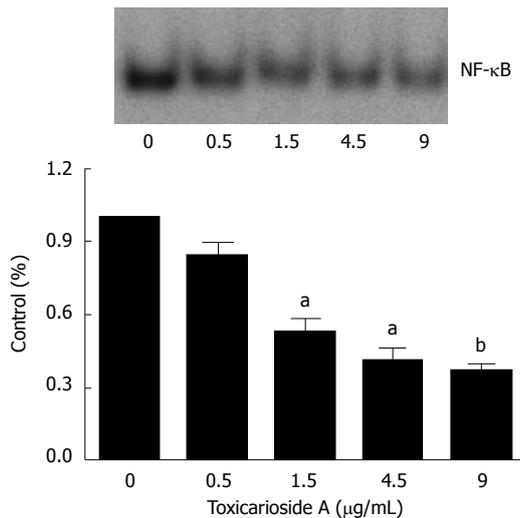
To determine the effect of toxicarioside A on NF-κB activation, the NF-κB-DNA binding activity was determined in both toxicarioside A-treated and control SGC-7901 cells by EMSA. As shown in Figure 5, after treatment with toxicarioside A at various concentrations for 48 h, the NF-κB-DNA binding activity was decreased in a dose-dependent manner as compared with the control group (*P* < 0.05 or *P* < 0.01, Figure 5).

### Effect of PDTC on toxicarioside A-induced downregulation of bFGF

To further determine whether NF-κB activation was necessary for bFGF expression, and was involved in toxicarioside A-induced downregulation of bFGF, a specific in-



**Figure 4 Basic fibroblast growth factor and fibroblast growth factor receptor-1 expression in human gastric cancer cell line cells.** A: The expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1) were detected using rhodamine and fluorescein isothiocyanate-conjugated mouse anti-rabbit immunoglobulin G in non-treated and toxicarioside A (4.5 μg/mL)-treated cells; B: bFGF and FGFR1 mRNA expression by reverse transcription polymerase chain reaction; C: bFGF and FGFR1 protein levels by Western blotting analysis. Results are depicted as mean ± SE of three independent experiments. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.



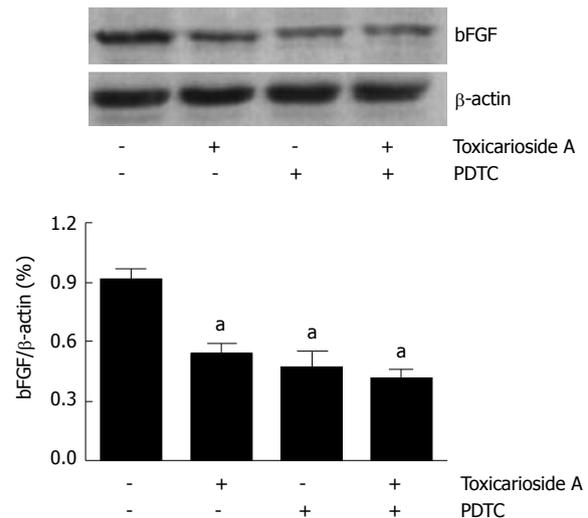
**Figure 5** Effect of toxicarioside A on nuclear factor-kappa B-DNA binding activity in human gastric cancer cell line cells. After cells were incubated with various concentrations of toxicarioside A for 48 h, nuclear proteins were isolated and electrophoretic mobility shift assay was performed to determine nuclear factor-kappa B (NF-κB)-DNA binding activity. Results are depicted as mean ± SE of three independent experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group.

hibitor of NF-κB activation, PDTC, was used. As shown in Figure 6, PDTC treatment significantly blocked bFGF expression, which was potentized when both PDTC and toxicarioside A were added to SGC-7901 cells.

## DISCUSSION

*Antiaristoxicaria* (Pers.) Lesch (Moraceae) is widespread in the tropical rain forest of southeastern Asia, and is best known for its remedial properties against injuries due to poisoned arrows, darts and blowdarts<sup>[36]</sup>. The latex-sap and seeds of *Antiaristoxicaria* consists of a complex mixture of active cardenolide glycosides, from which several cardenolides have been isolated in our laboratory and other research groups<sup>[2-5]</sup>. Besides the classical effect of the cardenolides on inhibition of the ubiquitous cell surface  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, the effect of cardiac glycosides on the growth of human malignant tumor cells has been reported in the recent past<sup>[11-15]</sup>. In the present work, we investigated the anti-cancer activity of toxicarioside A isolated from the latex of *Antiaristoxicaria*. Both the MTT assay and the growth curve analysis revealed that toxicarioside A resulted in inhibition of gastric cancer cell proliferation in a dose- and time-dependent manner. Malignant tumors are characterized by invasion and metastasis, an extremely complex process involving multi-steps. In this study, we assessed the migrating and invasive capabilities of SGC-7901 cells using the Transwell chamber assay. The results demonstrated that toxicarioside A not only suppressed cell motility, but also significantly reduced its ability to degrade the recombinant basement membrane in SGC-7901 cells.

To further investigate the molecular mechanism underlying the anti-tumor properties of cardenolides, we assessed the effect of toxicarioside A on bFGF expres-



**Figure 6** Effect of inhibitor, pyrrolidinedithiocarbamate, on basic fibroblast growth factor protein expression. Administration of pyrrolidinedithiocarbamate (PDTC) (50 μmol/L) reinforced the toxicarioside A (4.5 μg/mL)-induced downregulation of basic fibroblast growth factor (bFGF). Results are depicted as mean ± SE of three independent experiments. <sup>a</sup> $P < 0.05$  vs control group.

sion in SGC-7901 cells. It is well known that bFGF, a regulatory factor secreted from cells, is involved in a variety of biological processes including cell differentiation, cell growth, migration, angiogenesis, and tumor formation<sup>[19,20]</sup>. The biological effect of bFGF is achieved mainly through interaction with its high-affinity receptor, FGFR1<sup>[21-24]</sup>. To elucidate whether the bFGF/FGFR1 signaling pathway was a target of toxicarioside A in gastric cancer cells, we evaluated changes in the expression of bFGF and FGFR1 in SGC-7901 cells after treatment with toxicarioside A at various concentrations. The results demonstrated that toxicarioside A down-regulated the expression of bFGF and FGFR1 at both mRNA and protein levels in SGC-7901 cells in a dose-dependent manner.

Next, we sought to investigate the molecules involved in the toxicarioside A-induced down-regulation of bFGF in SGC-7901 cells. The NF-κB signaling pathway is a central common regulator for the process of inflammation, viral replication, tumorigenesis, and apoptosis<sup>[37,38]</sup>, and as a result has emerged as a potential target of numerous pharmaceutical agents<sup>[39,40]</sup>. Our results showed that toxicarioside A had an obvious suppressive effect on NF-κB-DNA binding activity in a dose-dependent manner, and treatment with an NF-κB specific inhibitor augmented the toxicarioside A-induced bFGF down-regulation in SGC-7901 cells, suggesting that the activated NF-κB may be partly necessary for bFGF expression in gastric cancer.

In summary, toxicarioside A weakened the abnormal activation of NF-κB to down-regulate the expression of bFGF, which in turn, interfered with bFGF/FGFR1 signal transduction subsequently leading to suppression of proliferation, migration and invasion in SGC-7901 cells. Future research will focus on identification of new targets to provide the theoretical basis for the potential

application of toxicarioside A in the clinical treatment of gastric cancer.

## COMMENTS

### Background

The latex and seeds of *Antiaristoxicaria* contain a complex mixture of cardenolide glycosides, and representative toxicariosides A-L have recently been identified in our laboratory and by others. Some cardenolides have been demonstrated to be capable of blocking tumor cell proliferation through regulation of cell signal transduction.

### Research frontiers

Gastric cancer is one of the leading malignancies in China. However, the treatment outcome is not satisfactory because early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed. It is important to investigate the strategies that could inhibit gastric cancer effectively.

### Innovations and breakthroughs

To date, little is known about the underlying mechanism regarding the anti-cancer effects of toxicarioside A. Therefore, this study was conducted to investigate the anti-cancer activity of toxicarioside A on gastric cancer growth and migration and the underlying molecular mechanisms *in vitro*.

### Applications

This study indicates the first evidence of the underlying molecular mechanisms of the anti-cancer activity of toxicarioside A in gastric cancer. These results provide the theoretical basis for the potential application of toxicarioside A in the clinical treatment of gastric cancer.

### Terminology

*Antiaristoxicaria* (Pers.) Lesch (Moraceae) is a well known precious species widespread in the tropical rain forest of Southeast Asia, and the latex and seeds of *Antiaristoxicaria* consist of a complex mixture of active cardenolide glycosides.

### Peer review

This manuscript showed toxicarioside A inhibits the proliferation, invasion and migration in a gastric cancer cell line, and these phenomena were correlated with down-regulation of nuclear factor- $\kappa$ B/basic fibroblast growth factor signaling. The design of study is solid and experiments were elegantly performed.

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## Medical treatment for sphincter of oddi dysfunction: Can it replace endoscopic sphincterotomy?

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### Abstract

**AIM:** To report the results of a medical management of sphincter of oddi dysfunction (SOD) after an intermediate follow-up period.

**METHODS:** A total of 59 patients with SOD (2 men and 57 women, mean age 51 years old) were included in this prospective study. After medical treatment for one year, the patients were clinically re-evaluated after an average period of 30 mo.

**RESULTS:** The distribution of the patients according to the Milwaukee's classification was the following: 11 patients were type 1, 34 were type 2 and 14 were type 3. Fourteen patients underwent an endoscopic sphincterotomy (ES) after one year of medical treatment. The median intermediate follow-up period was  $29.8 \pm 3$  mo (3-72 mo). The initial effectiveness of the medical treatment was complete, partial and poor among 50.8%, 13.5% and 35%, respectively, of the patients.

At the end of the follow-up period, 37 patients (62.7%) showed more than 50% improvement. The rate of improvement in patients who required ES was not significantly different compared with the patients treated conservatively (64.2% vs 62.2%, respectively).

**CONCLUSION:** Our study confirms that conservative medical treatment could be an alternative to endoscopic sphincterotomy because, after an intermediate follow-up period, the two treatments show the same success rates.

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**Key words:** Sphincter of oddi dysfunction; Cholecystectomy; Endoscopic sphincterotomy; Biliary scintigraphy

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### INTRODUCTION

Sphincter of oddi dysfunction (SOD) is a functional gastrointestinal abnormality characterized by pancreatobiliary pain that can be debilitating and may impair the quality of life. The cause of SOD remains speculative, but it could be due to hormonal or neurological disturbances of the sphincter of oddi (SO), leading to its intermittent obstruction despite the absence of organic

abnormalities<sup>[1-4]</sup>. The best way to establish diagnosis and manage SOD remains controversial, mainly in SOD type 2 and 3 of the Milwaukee classification<sup>[5,6]</sup>. The diagnosis of SOD is usually based on a high index of clinical suspicion. Direct endoscopic manometry of the SO is an invasive procedure that remains the gold standard for the diagnosis of SOD<sup>[7-9]</sup>. However, dynamic biliary scintigraphy has been used as a non-invasive tool for evaluation of the SO by providing indirect evidence of increased sphincter resistance. The contribution of the biliary scintigraphy appears to predict, with good effectiveness, the clinical success of biliary endoscopic sphincterotomy in SOD types 1 and 2 of the Milwaukee classification<sup>[10,11]</sup>. The management of SOD is also controversial, and it is based on the relaxation of the SO, which should improve the symptoms of SOD. The treatment can be accomplished pharmacologically either by an endoscopic procedure or surgically<sup>[12,13]</sup>. The surgical procedure is often replaced by an endoscopic procedure<sup>[14-16]</sup>. Several studies have suggested that there is a benefit from endoscopic sphincterotomy (ES) in SOD patients having high SO basal pressures at the time of manometry. However, procedural pancreatitis cannot be completely avoided, and surgical treatment will be necessary in some cases. Morbidity and mortality after ES have been reported to be as high as 9.8% and 2.3%, respectively. Moreover, long-term data regarding the rate of restenosis and complications resulting from repeated therapy are limited. If SO manometry findings are abnormal, the relief of pain after sphincterotomy occurs in 90%-95% of patients with type 1 SOD, 85% of patients with type 2 SOD, and 55%-60% of patients with type 3 SOD. However, almost uniformly and despite ES, some patients continue to have pain that is consistent with nonspecific chronic pain disorders, suggesting a multifactorial cause for SOD<sup>[17-19]</sup>. Endoscopic stenting is no longer recommended as a routine method of treatment of SOD because endoscopic stenting is associated with poor symptomatic relief and a high risk of stent-induced pancreatitis<sup>[20]</sup>. Many pharmacologic agents that are known to relax the SO have already been used in the management of SOD. However, although medical therapy may be an attractive initial approach in patients with sphincter of Oddi dysfunction, data on intermediate clinical outcomes associated with pharmacological treatment is scant<sup>[21-31]</sup>. In a recent study<sup>[32]</sup>, we showed that a one-year treatment period with trimebutine could significantly reduce pain in patients suffering from SOD, reducing the need for ES. Our aim in the current study was to determine the efficacy of medical therapy in relieving symptoms of SOD compared to ES after a prolonged follow-up period.

## MATERIALS AND METHODS

As described in our previous study<sup>[32]</sup>, fifty-nine patients complaining of SOD were included in this prospective monocentric study between 1999 and 2005. The patients

included 57 women and 2 men, with a mean age of 50.5 ± 12.3 years old (range 20-75 years old), which were followed for a mean duration of 29 mo after the end of the endoscopic or medical treatment. The main inclusion criterion was biliary or pancreatic pain after cholecystectomy after ruling out the diagnosis of residual lithiasis or the presence of a tumor. The following data were collected: age, sex, cholecystectomy, the time elapsing since the onset of the symptoms, pain and rate of SOD occurrence. All of the patients underwent biliopancreatic endoscopic ultrasonography in order to rule out the diagnosis of a residual lithiasis or the presence of a tumor (none of the patients underwent secretin-magnetic resonance cholangiopancreatography). The diameters of the main biliary pathway and the canal de Wirsung were systematically measured during these procedures. An initial biological check-up was carried out when the first painful episode occurred after the medical consultation. The biological check-up included the measurement of the following proteins: transaminases (alanine aminotransférase and aspartate aminotransférase), gamma-glutamyltransferase, alkaline phosphatases, total and conjugate bilirubin, amylase and lipase. PD-SOD was defined as the association between recurrent pancreatic pain with an increased level of serum lipase (> 3N). An initial biliary scintigraphy was carried out systematically on all of the patients. An initial hepatic and pancreatic biological assessment was done at the first event of abdominal pain. In addition, a biliary scintigraphy was performed for all patients in order to determine the isotopic hile to duodenum transit time (HDTT). According to the Milwaukee classification of SOD, the patients were subdivided into three groups. All of the patients were clearly informed about the various therapeutic modalities to manage their disease, the chances of success and the rates of complications of the medical therapy or endoscopic treatment. For each patients, we proposed an initial management of SOD by medical treatment, planned for a one-year duration; it consisted of a combined treatment, an association of trimebutine (200 mg three times per day) and nitrates taken sublingually when needed, mainly at the onset of abdominal pain. In the case of intolerance or counter-indication to the nitrates, a treatment with trimebutine alone was proposed. If previous treatment with trimebutine had been unsuccessful, only the nitrate derivatives were prescribed. If the painful attacks occurred too frequently (> 1 per week), a transdermal nitrate treatment (5 mg/d) was prescribed. The patients attended clinical follow-up consultations every four mo for a period of one year. After one year of medical therapy, an evaluation of painful symptoms was performed for each patient. The efficacy of the treatment was considered complete if there was a complete disappearance of the painful symptoms; the efficacy was considered partial if there was a greater than 50% reduction in the frequency and intensity of the pain and considered poor when the frequency and intensity of the pain decreased by less than 50%. In the cases where the medical treat-

Age, mean $\pm$ SD, yr	50.5 $\pm$ 12
Sex, M/F	2/57
Symptoms	
Biliary	47 (79.6)
Pancreatic	9 (15.2)
Biliopancreatic	3 (5)
Occurrence of painful episodes	
Once a week	9 (15.8)
Once a month	20 (35.1)
Every 3 mo	9 (15.8)
Every 6 mo	3 (5.3)
Once a year	7 (12.3)
Dilatation of biliary or pancreatic ducts (bile duct > 12 mm; pancreatic duct > 4 mm)	
Main biliary duct dilatation	29 (49.1)
Main pancreatic duct dilatation	1 (1.7)
Milwaukee classification repartition	
Type 1	11 (18.6)
Type 2	34 (57.6)
Type 3	14 (23.7)
Initial elevation of laboratory data	34 (57.6)
Lengthening of the isotopic transit time	32 (54.2)

ment was only partially successful or poor, endoscopic sphincterotomy was proposed to the patients, but not to the remaining patients. If medical treatment was successful, endoscopic treatment was not proposed. When the indications for endoscopic treatment had been chosen, the patients received clear information concerning the procedure. The endoscopic treatment was performed under general anaesthesia and consisted of an endoscopic retrograde cholangiopancreatography, with realization of a biliary sphincterotomy on patients with isolated biliary symptoms or dual sphincterotomy (biliary and pancreatic) on patients with both biliary and pancreatic symptoms. All of the patients were called by telephone to propose a follow-up examination with a hepatic biological assessment and an abdominal ultrasound in order to measure the diameter of the main biliary and pancreatic ducts; among the patients who refused this assessment, a simple evaluation of the clinical symptoms was proposed. The management was considered successful if there was an improvement greater than 50% compared to the initial symptoms; it was considered a failure if there was an absence of significant improvement by the patient. This study was approved by our local ethics committee.

### Statistical analysis

Statistical analysis was carried out on the data with the Statview software program (Abacus concept, Inc., Berkeley, CA, 1992). Quantitative data were expressed as the mean  $\pm$  SD. The significance of the differences was tested using the Student's *t* test or paired *t* tests to make comparisons between groups. Nonparametric tests (Mann Whitney tests) were performed when the Student's *t* test was not appropriate. The qualitative variables were compared using the chi-square test with Yate's correction when appropriate and with Fisher's exact test for 2  $\times$  2 contingency tables. The significance threshold was set at  $P < 0.05$ .

Global effect		
Complete or partial	38 (64.3)	$P < 0.05$
Poor	21 (35)	
Complete or partial effect according the Milwaukee group		
Type 1	5 (45)	$P = 0.31$
Type 2	23 (67)	
Type 3	10 (71)	
Complete or partial effect according to the increase of HDTT		
With prolongation of HDTT	21 (55.3)	$P = 0.77$
Without prolongation of HDTT	17 (44.7)	

HDTT: Hile to duodenum transit time.

## RESULTS

### Characteristics of the SOD and treatment

The patients developed the symptoms of SOD after a mean period of  $9.3 \pm 1.2$  years (range, 1-38 years) after the cholecystectomy. The patient's main characteristics are presented in Table 1. According to the three Milwaukee groups, there were no significant differences in age (type 1,  $56.2 \pm 2.1$  years old; type 2,  $48.1 \pm 2.1$  years old; and type 3,  $52.1 \pm 3.5$  years old,  $P = 0.22$ ) or the time elapsing from diagnosis to the cholecystectomy (type 1,  $11 \pm 3.6$  years; type 2,  $8.6 \pm 1.6$  years; and type 3,  $10 \pm 2.4$  years;  $P = 0.93$ ). Five patients were treated with nitrates only (8.4%), 12 were treated with trimebutine only (20.3%), and 42 patients had a combined treatment of nitrates and trimebutine (71.1%). The mean duration of the treatment period was  $11.5 \pm 0.8$  mo. The efficacy of the medical therapy was considered to be complete or partial in 38 patients (64.3%) and poor in 21 patients (35%). Details concerning the success of the medical treatment are presented in Table 2. Among the 21 patients with poor results from the medical management, 14 (23.7%) agreed to undergo ES, including 12 with an isolated biliary sphincterotomy and 2 with an associated pancreatic sphincterotomy (dual sphincterotomy). In patients with a dual sphincterotomy, a pancreatic duct stenting was performed. A lengthening of the isotopic time of transit was present before the ES in 11 patients of the 14 who underwent this intervention (78.5%) and in 21 patients of the 45 without ES (46.6%) ( $P = 0.02$ ). Other characteristics concerning ES are presented in Table 3. The complications of the ES were severe acute pancreatitis in two cases, which had resolved favourably with medical treatment. Among the patients requiring an ES, three patients had mixed biliary and pancreatic symptoms, and 9 patients had an isolated pancreatic pattern; among these patients, only 3 had a lengthening of the HDTT on the biliary scintigraphy corresponding to the 3 patients with isolated pancreatic symptoms as defined in the Materials and Methods section.

### Intermediate follow-up period

Five patients refused to participate in the follow-up period. The intermediate follow-up period was  $29.8 \pm 3$  mo (3-72 mo). Thirty-seven patients either did not pres-

**Table 3 Results of the endoscopic treatment (n = 14), (%)**

Time between the beginning of medical therapy and endoscopic treatment (mo)	12.2 ± 1.5
Patients concerned	
Patients with poor response to medical treatment	12 (57.1)
Patients with a partial response	2 (25)
Indication according to Milwaukee group	
Type 1	4 (36)
Type 2	9 (27)
Type 3	1 (7)
Results according to Milwaukee group	
Type 1	3/4 (75)
Type 2	6/9 (66)
Type 3	0/1 (0)

**Table 4 Results after intermediate follow-up (29.8 ± 3 mo) n (%)**

Rate of improvement		
Total rate	37 (62.7)	
Without endoscopic sphincterotomy	28 (62)	P = 0.88
With endoscopic sphincterotomy	9 (64.2)	
According to initial lengthening of HDTT		
With lengthening	19 (59.4)	
Without lengthening	18 (66.7)	P = 0.59
According to the Milwaukee group		
Type 1	6/11 (54.5)	
Type 2	23/34 (67.6)	P = 0.75
Type 3	8/14 (57.1)	

HDTT: Hile to duodenum transit time.

ent any additional painful events or improved by more than 50% (62.7%). Twenty-two patients still presented painful episodes (38.6%). The characteristics of the patients following endoscopic treatment are presented in Table 3. An abdominal ultrasound was performed in 45 patients and highlighted a dilation of the main biliary duct in 10 patients (22%). A biological laboratory assessment was also performed in these 45 patients. Among these patients, 6 still presented abnormal liver and pancreatic enzyme serum levels. At the end of the follow-up period, there was no significant difference in the size of the main biliary duct (at the beginning of the study, 49% of patients had dilation, and at the end of the study, 22% still had dilation of the main biliary duct) ( $P = 0.49$ ); these characteristics are presented in Table 4.

## DISCUSSION

The present study demonstrates that medical management with trimebutine may improve pain in patients suffering from SOD. Moreover, our study shows that after an intermediate follow-up period, the success of medical treatment (62%) does not differ from that of ES (64%). The medical treatment of SOD is usually disappointing, although nitrates or calcium-channel blockers can decrease basal pressure of the SO<sup>[32-36]</sup>. A positive effect of erythromycin on the motility of the SO has been suggested, but its clinical efficacy has not been demonstrated<sup>[29]</sup>. Somatostatin also modifies the SO activity but in the direction of an increase in the frequency of the phasic contractions of basal pressure. Therefore, somatostatin would be of little benefit for the indication of SOD and would not be suitable as a preventive measure after endoscopic retrograde cholangiopancreatography. The injection of botulinum toxin within the sphincter was tested in humans and pigs and promoted a significant reduction in the basal pressure in 50% of the cases<sup>[37-41]</sup>. On the clinical level, the injection of botulinum toxin was beneficial in 55% of the patients who suffered from biliary pain post-cholecystectomy without disturbance of the hepatic enzymes or dilation of the bile duct. However, 90% of the patients who experienced improvement presented a recurrence of symptoms after 6 mo, making the effect of botulinum toxin inconstant

and transitory<sup>[37-41]</sup>. Endoscopic management is mainly based on the ES, requiring SOD diagnosis to be formally established, i.e., based on the description of a basal high pressure of the SO. However, despite its high rate of success (86%-91%), ES is associated with high morbidity (19%-30%). Moreover, the natural course of SOD has not been well documented so far, so that the use of this endoscopic strategy remains highly controversial<sup>[42-49]</sup>.

Our research group previously described the effects of trimebutine on Oddi motility in a prospective study on patients with post-cholecystectomy pain<sup>[29]</sup>. We also reported in another study<sup>[32]</sup> that a one-year medical management could avoid the need for ES and had a success rate of 64%. Kovács *et al.*<sup>[21]</sup> previously suggested that, depending on the Milwaukee classification, medical treatment should be first attempted and its efficacy subsequently reassessed; if this treatment fails or is poorly tolerated, ES can then be proposed, especially for type 1 or 2 SOD patients, based on the presence of a lengthening of the HDTT. The lengthening of the HDTT as a factor for predicting a favourable response to ES decreases with the Milwaukee types, as mentioned by Cicala *et al.*<sup>[17]</sup>. In our study, 100% of the type 1 patients, 78% of the type 2 patients and none of the type 3 patients showed a lengthening of the HDTT and underwent ES successfully, whereas the immediate efficacy of ES was only 86%. This ES success rate is quite similar to the success rate reported in the literature, which ranged between 86% and 91%<sup>[18,50-52]</sup>. Few studies have addressed the intermediate natural history of SOD. The available data suggest that the clinical course is variable depending, in part, on the initial biliary classification. In a one-year follow-up study, seven SOD type 2 patients with abnormal SO pressure treated by a sham procedure continued to have symptoms, which ended only after subsequent ES. All patients continued to do well four years later. Five other SOD type 2 patients with abnormal SO pressure refused ES; after four years, three were unimproved while two had “fair” improvement. The clinical course was unpredictable after a sham or ES treatment in patients with SOD type 3 biliary pain. In another report, 11 such patients were followed for two years after ES. Four improved symptomatically, while

seven had no change in their symptoms. Eleven other patients had a sham procedure, five of which improved, while six had no change in their symptoms during a two-year follow-up period<sup>[53]</sup>. In our study, the monitoring of the response rate to ES after an average period of 29 mo showed a loss of efficacy of the ES in 3 patients. Therefore, the rate of patient improvement did not differ significantly in the presence or absence of a previous ES according to the performance or not of an ES.

In conclusion, our study confirms that a conservative medical treatment could be an alternative to ES because, after an intermediate follow-up period, the two treatments show the same success rates.

## COMMENTS

### Background

Sphincter of oddi dysfunction (SOD) is a symptom characterized by recurrent abdominal pain. It has been showed that endoscopic treatment called endoscopic sphincterotomy (ES) can improve symptoms but with a high level of complications. Pharmacologic agents have been tested in this pathology but are still disappointing.

### Research frontiers

Many ways of treatment (medical, endoscopic) are still studied because none of them offer a satisfying and safe rate of prolonged improvement.

### Innovations and breakthroughs

The authors here demonstrate that, after a long follow-up (29 mo), a medical treatment with trimebutine can have the same success rate than ES.

### Applications

The demonstration of a long-term efficacy of a medical conservative treatment is important because it can avoid the need for ES which is associated with morbidity and mortality after ES of 9.8% and 2.3% respectively. This study demonstrates that a medical conservative treatment with trimebutine may be an effective alternative to the endoscopic sphincterotomy since, after an intermediate follow-up, the two treatments show the same success rate.

### Terminology

The sphincter of oddi is a muscular valve that controls the flow of digestive juices (bile and pancreatic juice) through the ampulla of Vater into the second part of the duodenum. ES is an endoscopic technique developed to examine and treat abnormalities of the bile ducts, pancreas and gallbladder. The procedure was developed as an extension to the diagnostic examination, endoscopic retrograde cholangio pancreatography; with the addition of "sphincterotomy", abnormalities found during the study could be treated at the same time without the need for invasive surgery.

### Peer review

This study is about the results of a medical management of SOD after an intermediate follow-up. It is well-written.

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## Impact of comorbidities on the severity of chronic hepatitis B at presentation

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virus (HIV) (group HBV/HIV), 138 (10.2%) alcohol abuse (group HBV/alcohol); 109 (8.0%) subjects had at least two cofactors and 924 were in the cofactor-free (CF) group.

**RESULTS:** Compared with patients in group CF those in group HBV/alcohol were older and more frequently had cirrhosis ( $P < 0.001$ ), those in group HBV/HDV were younger ( $P < 0.001$ ), more frequently resided in the south of the country and had cirrhosis ( $P < 0.001$ ), those in group HBV/HCV were older ( $P < 0.001$ ) and more frequently had cirrhosis ( $P < 0.001$ ). These cofactors were all independent predictors of liver cirrhosis in HBsAg positive patients. Multivariate analysis showed that an older age [odds ratio (OR) 1.06, 95% CI: 1.05-1.08], alcohol abuse with more than 8 drinks daily (OR 2.89, 95% CI: 1.81-4.62) and anti-HDV positivity (OR 3.48, 95% CI: 2.16-5.58) are all independently associated with liver cirrhosis. This association was found also for anti-HCV positivity in univariate analysis, but it was no longer associated (OR 1.23, 95% CI: 0.84-1.80) at multivariate analysis.

**CONCLUSION:** Older age, HDV infection and alcohol abuse are the major determinants of severe liver disease in chronic HBV infection, while HCV replication plays a lesser role in the severity of hepatic damage.

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### Abstract

**AIM:** To evaluate the clinical relevance of each cofactor on clinical presentation of chronic hepatitis B.

**METHODS:** Out of 1366 hepatitis B surface antigen (HBsAg) positive subjects consecutively observed in 79 Italian hospitals, 53 (4.3%) showed as the only cofactor hepatitis D virus (HDV) infection [hepatitis B virus (HBV)/HDV group], 130 (9.5%) hepatitis C virus (HCV) (group HBV/HCV), 6 (0.4%) human immunodeficiency

**Key words:** Chronic hepatitis B; Hepatitis B virus/hepatitis D virus dual infection; Hepatitis B virus/hepatitis C virus dual infection; Alcohol abuse

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## INTRODUCTION

Seroepidemiological studies on the aetiology of chronic hepatitis in Italy performed in the 1980s, 1990s<sup>[1-3]</sup> and in 2001<sup>[4]</sup> showed a progressive reduction in the prevalence of hepatitis B surface antigen (HBsAg) positive cases from 60.7% observed in cases collected from 1976 to 1981<sup>[1]</sup> to 13% found in 2001<sup>[4]</sup>. Despite this, infection by the hepatitis B virus (HBV) is still responsible for a sizeable number of cases of chronic hepatitis and/or hepatocellular carcinoma (HCC), and the burden of HBV infection on the healthcare system in Italy is still heavy.

In a multicentre study carried out on 1829 patients with cirrhosis in 1992, the prevalence of HBV-related cases was 13.8%; in particular, HBV was the only aetiological factor in 4.2% of cases, most cases being associated with cofactors (9.6%): hepatitis D virus (HDV) in 3.4%, hepatitis C virus (HCV) in 3.2% and alcohol abuse in the remaining 3%<sup>[5]</sup>. In human immunodeficiency virus (HIV)/HBV dual infection, found in 3.7%-4.6% of HIV positive subjects in Italy but estimated around 10% in people with HIV infection worldwide<sup>[6]</sup>, the prevalence of patients with cirrhosis is reported as higher than in HIV-negative/HBV-positive cases<sup>[7]</sup>. Cofactors may therefore play a substantial role in the progression of HBsAg positive chronic hepatitis to the more severe clinical forms<sup>[8-10]</sup>.

The gradual reduction in the percentage of HBsAg positive cases was associated with a dramatic decrease in the prevalence of cases with HBV/HDV dual infection<sup>[11-13]</sup>. About a quarter of HBsAg positive patients with chronic hepatitis were anti-HDV positive in 1978-1981<sup>[14]</sup> and in 1987<sup>[15]</sup>; this prevalence was lower in 1992 (14.4%)<sup>[16]</sup>, in 1997 (8.3%)<sup>[17]</sup> and in 2001 (9.9%)<sup>[4]</sup>. No data to this effect are available on HBV/HCV dual infection and on the association of HBV infection/alcohol intake<sup>[18]</sup>.

Our survey on the largest series of patients with chronic hepatitis ever studied in Italy showed an overall low HDV prevalence in HBsAg chronic carriers (9%), a high prevalence of patients with HBV/HCV dual infection (16.9%) and alcohol abuse (10.4%)<sup>[4]</sup>. The data from this study allow us to evaluate the current main characteristics of patients with HBV/HDV or HBV/HCV chronic infection or HBV infection/alcohol abuse compared with a large control group of HBsAg chronic carriers with no evident cofactor.

## MATERIALS AND METHODS

The study design was more extensively described in a previous paper<sup>[4]</sup>. Seventy-nine centres participated in

the study, 25 in the North, 24 in the Centre and 30 in the South of Italy and the two main islands (Sicily and Sardinia).

All subjects consecutively referred from February 1 to July 31, 2001 as in-patients or out-patients to one of the 79 Italian centres were recruited; 9997 patients with chronic hepatitis were enrolled. Both tertiary and peripheral centres were randomly selected by a systematic cluster sampling procedure. For each of these three geographical areas, all of the hospitals were identified and listed numerically according to an assigned number. In each list, a single hospital was considered as a cluster. The first cluster was randomly chosen, whereas the others were selected with a probability proportional to the required number of hospitals at systematic intervals. Both prevalent and incident cases were recruited. We defined as "incident cases" all new diagnoses of chronic liver disease made during the enrolment period, and as "prevalent cases" all subjects with a previous diagnosis of chronic liver disease observed during the study period.

For each subject, the demographic, clinical and aetiological data were recorded using a pre-coded questionnaire. The amount of alcohol intake was determined using a standard questionnaire containing information on the daily intake of various alcoholic beverages and lifetime duration of alcohol consumption. An alcohol intake of more than 40 g daily for males and 30 g daily for females for at least 10 years was considered as an aetiological cofactor<sup>[19,20]</sup>.

HBV serum markers, HBsAg, anti-HBc, hepatitis B e antigen (HBeAg), anti-HBeAg and anti-HDV were determined by commercial immunoenzymatic assays. Antibodies to HCV were detected by 3rd generation commercial immunoenzymatic assays. Antibodies to HIV 1 and 2 were determined by commercial enzyme-linked immunosorbent assay (Diasorin Biomedica, Saluggia, Vercelli, Italy; Abbott Labs, North Chicago, Illinois, United States) and the positive results were confirmed by a Western Blot analysis (Genelabs Diagnostics, Science Park Drive, Singapore). Patients were enrolled in 2001 when various methods of different sensitivity were used to detect HBV viral load in different centres in Italy. For this reason, we preferred not to evaluate the predicting value of HBV viral load; data on HBV-DNA were given as positive or negative.

Data on HDV-RNA and HDV-Ag were not available. We classified patients as asymptomatic carriers when alanine aminotransferase (ALT) values were persistently normal in the absence of clinical, biochemical and ultrasound signs of chronic liver disease. Chronic hepatitis was diagnosed only on the basis of liver histology. Liver cirrhosis was diagnosed from a liver biopsy or the presence of unequivocal clinical, biochemical and ultrasound signs<sup>[20]</sup>. HCC diagnosis was based on histology, imaging techniques or biochemical parameters ( $\alpha$ -feto protein greater than 400 ng/mL)<sup>[21,22]</sup>. Patients with serum markers suggesting autoimmune liver disease and those with liver disease associated with genetic disorders were excluded from the study.

### Statistical analysis

Continuous variables were summarised as mean  $\pm$  SD or median and interquartile range, and categorical variables as absolute and relative frequencies. Differences in the means were evaluated by an unpaired Student *t* test or Kruskal-Wallis one-way analysis of variance, and the  $\chi^2$  test was applied to categorical variables. Crude odds ratios (OR) and their 95% CI for the association of liver cirrhosis with potential risk factors were calculated by univariate analysis. Adjusted OR were calculated by stepwise logistic regression analysis to identify factors independently associated with liver cirrhosis. Only factors associated with liver cirrhosis by univariate analysis were included in the logistic regression analysis. In the logistic model liver cirrhosis was the outcome variable, while age, sex, anti-HDV, anti-HCV, alcohol intake, and body mass index were the independent variables.

## RESULTS

Of the 1366 HBsAg positive patients, 924 (67.6%) lacked all the cofactors investigated (HDV, HCV, HIV and alcohol abuse); this group of patients was named the cofactor-free (CF) group. Fifty-nine (4.3%) patients showed HDV infection (anti-HDV positive) as the only cofactor (group HBV/HDV), 130 (9.5%) HCV infection (group HBV/HCV), 6 (0.4%) HIV infection (group HBV/HIV), 138 (10.2%) alcohol abuse (group HBV/alcohol) and 109 (8.0%) had more than one cofactor (group with two or more cofactors). Overall, 333 (24.4%) patients had no liver biopsy at presentation; of the remaining 1033 cases, 278 subjects (26.9%) were classified as asymptomatic carriers, 453 (43.9%) as having chronic hepatitis, 249 (24.9%) as having liver cirrhosis and 53 (5.1%) patients had a diagnosis of HCC. Interferon treatment was given in 365 (26.7%) patients and 244 (17.9%) patients received Lamivudine. Excluding cases under immunosuppressive therapy, approximately all HBeAg positive and half of HBeAg negative cases showed active HBV replication. Fifty-seven (4.2%) patients were born outside Italy, and only 4 of them were born in China.

### Characteristics of the CF group

The 924 HBsAg positive subjects with none of the cofactors investigated were more frequently males (66.5%) and observed as out-patients (81.1%) and as prevalent cases (83.2%); they aged  $48.1 \pm 14.3$  years, were infrequently HBeAg positive (12.6%) and frequently showed a mild clinical presentation: 26.9% of cases were asymptomatic carriers, 56.9% of patients showed chronic hepatitis, 12.4% liver cirrhosis and 3.8% had HCC.

### Comparison according to anti-HDV status

The 59 anti-HDV positive patients were younger than those in group CF ( $46.7 \pm 11.8$  years *vs*  $48.1 \pm 14.3$  years;  $P < 0.001$ ) and showed higher ALT levels ( $128 \pm 116$  IU/L *vs*  $98 \pm 290$  IU/L,  $P = 0.008$ ); they were more frequently observed as in-patients (25.4% *vs* 18.9%,  $P = 0.3$ ), were

more frequently born in southern Italy or on one of the two main islands (67.8% *vs* 52.9%,  $P = 0.029$ ) and more frequently had liver cirrhosis (37.3% *vs* 12.4%,  $P \leq 0.001$ ); none of the 59 anti-Delta positive patients had HCC (Table 1).

### Comparison according to anti-HCV status

The 130 subjects co-infected with HCV were older than those in group CF ( $55.2 \pm 14.7$  years *vs*  $48.1 \pm 14.3$  years,  $P < 0.0001$ ), and more frequently showed liver cirrhosis (23.1% *vs* 12.4%,  $P < 0.001$ ); this group contained the highest prevalence of patients with HCC observed in the study (6.2%) (Table 1). Active HCV replication as evaluated by positive HCV-RNA by RT-PCR was found in 115 subjects (88.5%).

### Comparison according to anti-HIV status

Only 6 patients showed HBV/HIV dual infection; all of these patients were males and younger than those in group CF. Four of them were HBeAg positive and none had liver cirrhosis (Table 1).

### Comparison according to alcohol abuse

Compared with patients in group CF, the 138 HBsAg positive patients with alcohol abuse as the only cofactor were older ( $52.6 \pm 11.8$  years *vs*  $48.1 \pm 14.3$  years,  $P < 0.0001$ ), were more frequently males (92.6% *vs* 66.5%,  $P < 0.0001$ ), had had fewer years of schooling (85.9% *vs* 67.0%,  $P < 0.001$ ) and more frequently showed liver cirrhosis (31.1% *vs* 12.4%,  $P < 0.0001$ ) and HCC (5.1%).

### Comparison according to the presence of two or more cofactors

Of the 109 patients in this group, 21 had HBV/HDV/HCV concurrent infection, 12 HBV/HDV dual infection plus alcohol abuse, 64 had HBV/HCV dual infection plus alcohol abuse, and 12 had HBV/HDV/HCV concurrent infection plus alcohol abuse. Compared with group CF, patients with two or more cofactors were more frequently males (86.8% *vs* 66.5%,  $P < 0.001$ ), in-patients (28.0% *vs* 18.9%,  $P = 0.003$ ) and had liver cirrhosis (35.8% *vs* 12.4%,  $P < 0.001$ ). They more frequently belonged to larger families (24.5% *vs* 14.6%,  $P < 0.01$ ) and had had fewer years of schooling (84.3% *vs* 67.0%,  $P < 0.001$ ). Only 4 patients showed HCC (Table 1).

### Variables associated with the presence of cirrhosis

At the univariate analysis older age, anti-HCV, HCV-RNA positivity, alcohol abuse, anti-HDV and anti-HIV positivity were all associated with liver cirrhosis. Multivariate analysis showed that age (OR 1.10, 95% CI: 1.08-1.11), alcohol abuse (between 4 and 8 drinks daily: OR 2.30, 95% CI: 1.29-4.10; more than 8 drinks daily: OR 2.42, 95% CI: 1.31-4.46), HDV positivity (OR 2.68, 95% CI: 1.56-4.61) and anti-HIV (OR 5.78, 95% CI: 1.50-22.27) were independent predictors of the development of cirrhosis, whereas anti-HCV and HCV-RNA positivity were no longer associated (Table 2).

**Table 1** Comparison of baseline features of hepatitis B surface antigen positive patients, according to different cofactors (mean  $\pm$  SD) *n* (%)

Variable	HBsAg positive ( <i>n</i> = 924)	HBsAg/anti-HDV positive ( <i>n</i> = 59)	HBsAg/anti-HCV positive ( <i>n</i> = 130)	HBsAg/anti-HIV positive ( <i>n</i> = 6)	HBsAg/alcohol abuse ( <i>n</i> = 138)	HBsAg/two or more cofactors ( <i>n</i> = 109)	<i>P</i> value
Age (yr)	48.4 $\pm$ 14.0	46.5 $\pm$ 11.7	55.2 $\pm$ 14.7	37.0 $\pm$ 3.6	52.7 $\pm$ 11.7	45.8 $\pm$ 12.1	< 0.001
Males	606 (66.5)	41 (71.9)	87 (68.0)	6 (100)	126 (92.6)	92 (86.8)	< 0.001
In-patients	171 (18.9)	15 (25.4)	29 (22.7)	0	20 (14.7)	30 (28.0)	0.060
Out-patients	732 (81.1)	44 (74.6)	99 (77.3)	6 (100)	116 (85.3)	77 (72.0)	
Prevalent cases	769 (83.2)	53 (89.8)	111 (85.4)	6 (100)	115 (83.3)	95 (87.2)	0.500
Incident cases	155 (16.8)	6 (10.2)	19 (14.6)	0	23 (16.7)	14 (12.8)	
Born in Italy							
North	192 (21.0)	11 (18.6)	36 (27.7)	3 (50.0)	32 (23.7)	35 (32.4)	0.010
Centre	195 (21.3)	5 (8.5)	16 (12.3)	0	26 (19.3)	21 (19.4)	
South/islands	484 (52.9)	40 (67.8)	76 (58.5)	2 (33.3)	73 (54.1)	49 (45.4)	
Born Abroad	44 (4.8)	3 (5.1)	2 (1.5)	1 (16.7)	4 (2.9)	3 (2.8)	
Asymptomatic carrier	233 (33.5)	3 (6.0)	17 (17.7)	1 (100)	19 (17.6)	5 (6.0)	< 0.001
Chronic hepatitis <sup>1</sup>	313 (45.0)	25 (50.0)	41 (42.7)	0	39 (36.1)	35 (42.2)	
Liver cirrhosis	115 (16.5)	22 (44.0)	30 (31.3)	0	43 (39.8)	39 (47.0)	
HCC	34 (4.9)	0	8 (8.3)	0	7 (6.5)	4 (4.8)	
ALT ( $\times$ ULN) (median, IQR)	1.0 (1.0-1.8)	2.2 (1.1-4.8)	1.4 (1.0-2.1)	1.5 (1.1-1.8)	1.1 (1.0-2.1)	1.8 (1.0-2.9)	< 0.001
BMI (kg/m <sup>2</sup> )	25.0 $\pm$ 3.4	25.5 $\pm$ 5.0	24.8 $\pm$ 3.1	22.8 $\pm$ 2.0	26.1 $\pm$ 3.5	25.6 $\pm$ 4.1	0.006
Years of schooling							< 0.001
< 6	239 (26.3)	9 (16.4)	46 (36.5)	1 (16.7)	62 (46.3)	31 (28.7)	
6-13	369 (40.7)	31 (56.4)	53 (42.1)	1 (16.7)	53 (39.5)	60 (55.6)	
> 13	299 (33.0)	15 (27.2)	27 (21.4)	4 (66.6)	19 (14.2)	17 (15.7)	
HBeAg positive	116 (12.6)	9 (15.3)	10 (7.7)	4 (66.7)	10 (7.2)	15 (13.8)	< 0.001
HBeAg negative	808 (87.4)	50 (84.7)	120 (92.3)	2 (33.3)	128 (92.8)	94 (86.2)	

<sup>1</sup>Only subjects with liver biopsy. HBsAg: Hepatitis B surface antigen; HDV: Hepatitis D virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase;  $\times$  ULN: Times the upper limit of the normal; IQR: Interquartile range; BMI: Body mass index; HBeAg: Hepatitis B e antigen.

### Comparison according to the severity of liver cirrhosis

The Child-Pugh Score was calculated for 229 (92.0%) of the 249 patients with cirrhosis: 104 cases in group CF, 43 cases in group HBV/Alcohol, 19 cases in group HBV/HDV, 30 cases in group HBV/HCV and 33 cases in the HBV/two or more cofactors group. The prevalence of patients in the Child-Pugh Classes B or C was lower in group CF (31.7%), than in group HBV/Alcohol (44.2%), in group HBV/HCV (43.3%) and in the HBV/two or more cofactors group (75.8%,  $P < 0.001$ ) (Table 3).

## DISCUSSION

This study is based on three strong points: the first, a large sample size, the second, the validity of the random selection including both tertiary and peripheral hospitals all over the country, which may have avoided the selection of “difficult-to-treat” patients, and lastly, the homogeneity of each single cofactor group and of the comparative CF group, making this study the first to investigate the clinical presentation of CF HBsAg positive chronic hepatitis and the influence of each single cofactor (HDV, HCV and alcohol intake).

Excluding the 442 patients with one or more cofactors enabled us to examine a group of almost a thousand patients with HBsAg positive chronic hepatitis and no cofactor. Most of these patients showed a mild clinical presentation and only a minority of them had liver cirrhosis (12.4%), prevalently in the Child A stage. The low

prevalence of HCC (3.9%) refers to HCC at the time of diagnosis. No information is available on the risk of occurrence of HCC, death or orthotopic liver transplantation over time, since the present study is cross-sectional and no evaluation of the clinical outcomes was made.

Despite the decreasing endemicity levels, HDV infection maintains its geographical distribution in our country, i.e., more frequent in southern Italy and on the two main islands, as previously described<sup>[15-17]</sup>. Compared with those in group CF, patients in group HBV/HDV showed a more severe clinical presentation: they were younger, more frequently hospitalised and with evidence of cirrhosis. In accordance with the low prevalence of patients with HCC in previous studies<sup>[5]</sup>, no patient in our HBV/HDV group showed HCC, most probably reflecting a more severe course of the illness with a rapid transition to death or to the need for liver transplantation before HCC becomes evident<sup>[23]</sup>. On the other hand, patients in group HBV/HCV were older than those in group CF, more frequently had liver cirrhosis and showed an almost double prevalence of HCC. These differences, although not statistically significant, may suggest that subjects with long-lasting HBV/HCV dual infection are at a higher risk of developing liver cirrhosis with or without liver cancer<sup>[24-26]</sup>.

Most patients in group HBV/Alcohol were males with a low educational level, a combination more frequently associated with a high risk of alcohol abuse. The prevalence of patients with cirrhosis was 2.5 times higher in group HBV/alcohol than in group CF, a dif-

**Table 2** Risk factors associated with cirrhosis in hepatitis B surface antigen positive patients *n* (%)

Variable	Chronic hepatitis	Cirrhosis	Crude OR (95% CI)	Adjusted OR (95% CI)	<i>P</i> value
Age (mean ± SD, yr)	44.9 ± 12.0	56.2 ± 11.6	1.06 (1.05-1.07)	1.10 (1.08-1.11)	< 0.001
Gender					
Male	371 (81.9)	192 (77.1)	1		
Female	82 (18.1)	57 (22.9)	1.34 (0.92-1.97)		
HBeAg					
Negative	387 (85.4)	225 (90.4)	1		
Positive	66 (14.6)	24 (9.6)	0.63 (0.38-1.03)		
Anti-HCV					
Negative	382 (84.3)	187 (75.1)	1	1	0.300
Positive	71 (15.7)	62 (24.9)	1.78 (1.22-2.62)	1.23 (0.84-1.80)	
HCV-RNA					
Negative	382 (86.2)	187 (77.3)	1	1	0.200
Positive	61 (13.8)	55 (22.7)	1.84 (1.23-2.76)	1.44 (0.65-1.72)	
Anti-HDV					
Negative	410 (90.5)	207 (83.1)	1	1	< 0.001
Positive	43 (9.5)	42 (16.9)	1.94 (1.23-3.06)	2.68 (1.56-4.61)	
Anti-HIV					
Negative	449 (99.1)	241 (96.8)	1	1	0.010
Positive	4 (0.9)	8 (3.2)	3.73 (1.11-12.50)	5.78 (1.50-22.27)	
Alcohol (drinks/d)					
0	247 (54.5)	117 (47.0)	1	1	
< 4	143 (31.6)	56 (22.4)	0.83 (0.57-1.21)	0.69 (0.48-1.07)	0.080
4-8	35 (7.7)	37 (14.9)	2.23 (1.34-3.72)	2.30 (1.29-4.01)	0.005
> 8	28 (6.2)	39 (15.7)	2.94 (1.73-5.01)	2.42 (1.31-4.46)	0.005
BMI (kg/m <sup>2</sup> )					
< 25	247 (54.5)	121 (48.6)	1		
25-30	173 (38.2)	107 (43.0)	1.26 (0.91-1.75)		
> 30	33 (7.3)	21 (8.4)	1.30 (0.72-2.34)		

Crude and adjusted odds ratios (OR) deriving from multiple logistic regression analysis. Patients with HCC were excluded from the analysis. HCC: Hepatocellular carcinoma; HDV: Hepatitis D virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; BMI: Body mass index.

**Table 3** Percentages of cases in different child-pugh classes in 229 of the 249 patients with liver cirrhosis, by aetiology *n* (%)

Child-pugh class	CF group ( <i>n</i> = 104)	HBV/alcohol group ( <i>n</i> = 43)	HBV/HDV group ( <i>n</i> = 19)	HBV/HCV group ( <i>n</i> = 30)	HBV/ two or more cofactors group ( <i>n</i> = 33)
A	71 (68.3)	24 (55.8)	13 (68.4)	17 (56.7)	8 (24.2)
B + C	33 (31.7) <sup>b</sup>	19 (44.2)	6 (31.6)	13 (43.3)	25 (75.8) <sup>b</sup>

<sup>b</sup>*P* < 0.001 *vs* hepatitis B virus (HBV)/two or more cofactors. Patients with hepatocellular carcinoma are not included. CF: Cofactor-free; HCV: Hepatitis C virus; HDV: Hepatitis D virus.

ference most probably due to alcohol intake rather than to the association of the two aetiological factors, since, as described in a previous paper<sup>[4]</sup>, 41.8% of 761 patients showing alcohol abuse as the only aetiological factor had liver cirrhosis. The group of patients with two or more cofactors is a miscellany of 4 subgroups that are too small to be analysed or compared with group CF or with the other cofactor groups. This miscellaneous group with more than one cofactor may be more frequently exposed to the aetiological agents of liver disease and to a higher risk of developing liver cirrhosis and/or HCC.

As observed in previous Italian studies<sup>[4,17,18]</sup>, the majority of HBsAg positive patients in this study were found to be HBeAg negative. Patients with HBV/HCV dual infection and those with HBV plus alcohol abuse showed a lower prevalence of HBeAg positive cases than those in the other aetiological groups, probably because they were

older and HBeAg loss is, at least in part, a time-dependent phenomenon.

In conclusion, HBV chronic infection was frequently associated with a mild or moderate clinical condition. Liver cirrhosis and HCC were detected in less than one sixth of cases, and viral and metabolic cofactors unfavourably influenced the clinical course in patients with chronic HBV infection since their presence was associated with an increased risk of cirrhosis, an association proven by multivariate logistic regression analysis for HDV infection and alcohol abuse.

## COMMENTS

### Background

Many cofactors play a substantial role in the progression of hepatitis B surface antigen positive chronic hepatitis to more severe clinical forms.

**Research frontiers**

The study aimed to evaluate the clinical relevance of each cofactor on the severity of the clinical presentation of chronic hepatitis B.

**Innovations and breakthroughs**

Older age, hepatitis D virus co-infection and alcohol abuse are the major determinants of severe liver disease in chronic hepatitis B virus (HBV) infection. Conversely, hepatitis C virus replication plays a lesser role in the severity of hepatic damage.

**Applications**

Removal of some risk factors may hamper the progression of chronic HBV-related liver disease in many patients.

**Peer review**

This is a large-size, multicentre study showing the importance of comorbidities in exacerbating hepatocellular necroinflammation and playing a substantial role in the progression to a more advanced stage of liver disease.

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## No evidence of circulating autoantibodies against osteoprotegerin in patients with celiac disease

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### Abstract

**AIM:** To investigate risk factors for low bone mineral density (BMD) in celiac disease (CD) patients, focusing on circulating autoantibodies against osteoprotegerin (OPG).

**METHODS:** Seventy asymptomatic CD adult patients on gluten-free diet (GFD) and harbouring persistent negative CD-related serology were recruited. Conventional risk factors for osteoporosis (e.g., age, sex, menopausal status, history of fractures, smoke, and body mass index) were checked and BMD was assessed by dual energy X ray absorptiometry. Serum calcium and parathyroid hormone (PTH) levels were evaluated. Thirty-eight patients underwent repeat duodenal biopsy. Serum samples from a selected sub-group of 30 patients, who were also typed for human leukocyte antigen (HLA) DQ2 and DQ8 haplotype, were incubated

with homodimeric recombinant human OPG and tested by western blotting with an anti-OPG antibody after immunoprecipitation.

**RESULTS:** Despite persistent negative CD-related serology and strict adherence to GFD, 49 out of the 70 (74%) patients displayed low BMD. Among these patients, 13 (24%) showed osteoporosis and 36 (76%) osteopenia. With the exception of age, conventional risk factors for osteoporosis did not differ between patients with normal and low BMD. Circulating serum calcium and PTH levels were normal in all patients. Duodenal mucosa healing was found in 31 (82%) out of 38 patients who underwent repeat duodenal biopsy with 20 (64%) still displaying low BMD. The remaining 7 patients had an incomplete normalization of duodenal mucosa with 6 (84%) showing low BMD. No evidence of circulating antibodies against OPG was found in the serum of 30 celiac patients who were tested for, independent of BMD, duodenal histology, and HLA status.

**CONCLUSION:** If any, the role of circulating autoantibodies against OPG in the pathogenesis of bone derangement in patients with CD is not a major one.

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**Key words:** Celiac disease; Osteoprotegerin; Bone mineral density; Gluten-free diet; Osteoporosis; Osteopenia

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## INTRODUCTION

Celiac disease (CD) is a permanent gluten intolerance in genetically predisposed individuals who display an inflammatory process in the small intestinal mucosa with villous atrophy, crypt hyperplasia, and increased number of lymphocytes<sup>[1]</sup>.

Evidences indicate that a low bone mineral density (BMD) is found in 20%-50% newly diagnosed patients with CD<sup>[2]</sup>. By means of dual energy X ray absorptiometry (DEXA) it can be now rapidly and easily obtained semi-quantitative values of BMD<sup>[3]</sup>.

Osteoporosis is a quantitative and qualitative alteration in the components of bone tissue, in which the process of demineralization becomes intense and prolonged and minerals are used up more quickly than they can be replaced resulting in bones fragility and increased risk of fractures<sup>[4,5]</sup>. Individual's gender, constitution and age as well as variations in endocrine systems associated with factors such as the menopause, and the presence of other pathologies, can all interact with lifestyle factors, including smoking, lack of exercise and low dietary calcium intake, to determine the onset of osteoporosis<sup>[6]</sup>.

Impaired absorption of calcium during CD is thought to result principally from loss of villous in the proximal intestine, where calcium is most actively absorbed, and also from the unabsorbed fatty acids, which bind calcium in the intestinal lumen and may reduce dietary vitamin D absorption<sup>[7]</sup>. Adherence to a strict gluten-free diet (GFD) will reverse the histological changes in the intestine and also the biochemical evidence of calcium malabsorption<sup>[8]</sup>, resulting in normal BMD in these treated patients<sup>[9]</sup>. Nevertheless, there may be long-term impairment of bone mineralization in some otherwise healthy CD patients adhering to GFD<sup>[10]</sup>. Furthermore, osteopenia has been found in treated CD patients who showed improvement or even complete healing of intestinal mucosa<sup>[11]</sup>. These findings suggest that other mechanisms of bone injury than calcium malabsorption are probably involved in patients with CD<sup>[12]</sup>.

Mediators of inflammatory immune-mediated responses (e.g., cytokines), parathyroid hormone (PTH), estrogens, androgens, corticosteroids and vitamin D are all acknowledged to affect BMD by modulating the receptor activator of nuclear factor B/receptor activator of nuclear factor B-ligand/osteoprotegerin [RANK/RANK-L/osteoprotegerin (OPG)] system<sup>[13]</sup>. Furthermore, neutralizing auto-antibodies against OPG have been recently shown in the sera of a few patients with CD, leading to the hypothesis that blocking the inhibitory effect of OPG on RANKL may have a role in the development of bone derangement in these patients<sup>[14]</sup>. Nevertheless, this finding has not yet been confirmed by others, so the role of auto-antibodies against OPG, if any, in the pathogenesis of reduced BMD in patients with CD remains to be established.

This study aimed at investigating risk factors associated with low BMD in patients with CD, focusing on circulating auto-antibodies against OPG.

## MATERIALS AND METHODS

### Patients recruitment

Seventy consecutive outpatients with CD (13M, 57F; median age 40.5 years, range 20-68 years) who reported no current symptoms, claimed to be adherent to a GFD for at least 2 years and harboured persistent (at least 18 mo) negative CD-related serology (anti-endomysium and anti-transglutaminase IgA antibodies) were recruited. Diagnosis of CD was performed on the basis of clinical presentation, positive CD-related serology and suggestive histological findings on duodenal biopsy<sup>[15]</sup>. Dietary compliance was assessed by periodic interview during follow-up visits and classified as good according to Leffler *et al.*<sup>[16]</sup>. Data on height, weight, time since diagnosis, symptoms beginning, age at menarche, cycle regularity, menopausal status, drug use, calcium intake, life style, smoking, and history of fracture were collected. Blood samples were collected in the morning after a 12 h fast in order to measure serum calcium and parathormone levels. Thirty-eight patients (8M, 30F; median age 41 years, range 20-60 years) underwent repeat duodenal biopsy after a period of at least 12 mo since GFD beginning.

A subgroup of 30 patients (8M, 22F; median age 44 years, range 21-60 years), who were typed for HLA-DQ2 and DQ8 haplotype, were selected in order to measure antibodies against OPG.

### Histology

At least four duodenal biopsies were collected during upper gastrointestinal endoscopy. Intraepithelial lymphocytes have been identified using CD3 immunostaining and a value  $\leq 25$  lymphocytes/100 epithelial cells was considered normal. Histological changes were classified according to Marsh criteria (stage 0: Normal mucosa; stage 1: Increased number of intra-epithelial lymphocytes; stage 2: Crypts proliferation; stage 3a-3b-3c: Respectively mild, moderate and severe villous atrophy)<sup>[17]</sup>.

### Measurement of BMD

All patients underwent lumbar spine and femoral neck BMD evaluation by means of DEXA. A T-score 1 to 2.5 and  $> 2.5$  distinguished osteopenia and osteoporosis, respectively.

### Measurement of antibodies against osteoprotegerin

Non-fasting serum samples were obtained from the selected subgroup of 30 CD patients. Measurement was performed according to Riches *et al.*<sup>[14]</sup>. Briefly, serum samples were incubated at a 1:100, 1:50, and 1:25 dilution with 12.5 ng of homodimeric recombinant human OPG (R and D Systems, Minneapolis, United States) and also with protein G-coated agarose beads (Calbiochem, Darmstadt, Germany) that had been pre-incubated with 5% albumin to reduce non-specific binding. After incubation for 1 h at 37 °C, the beads were washed five times with phosphate-buffered saline, suspended in 30  $\mu$ L of reducing sample buffer, and incubated at 90 °C for 5 min. After brief centrifugation,

**Table 1** Characteristics of the 70 treated celiac disease patients with negative serology according to bone mineral density as assessed by dual energy X ray absorptiometry

Variables	Overall (n = 70)	Normal BMD (n = 21)	Low BMD (n = 49)	P
Sex (M/F)	13/57	2/19	11/38	0.31
Age (yr)	40.5 ± 10.5	31.0 ± 9.7	43.0 ± 9.7	0.00
Time since diagnosis	2.8 ± 0.6	2.5 ± 0.5	2.4 ± 0.4	0.37
BMI	22.2 ± 1.4	22.5 ± 1.3	22.0 ± 1.4	0.40
Smoke	13	3	10	0.74
Fracture	0	0	0	-
Menopausal status	6	0	6	0.17

BMD: Bone mineral density; BMI: Body mass index; M: Male; F: Female.

the supernatant was loaded onto a 12% polyacrylamide gel, subjected to electrophoresis at 200 V for 60 min, transferred to membrane, and therefore probed with a mouse monoclonal antibody against human OPG (Abcam, Cambridge, United Kingdom). A peroxidase-conjugated donkey anti-mouse antibody (Jackson, Suffolk, United Kingdom) at a 1:5000 dilution was used for detection. Equal loading was assessed by probing the blot with peroxidase-conjugated goat anti-human antibody (Jackson) at a 1:5000 dilution. Immunolabeled bands were detected with the use of a chemiluminescent substrate and a chemiluminescence imager. A homodimeric recombinant human OPG (R and D Systems) was used as positive control. A 55-kDa band indicated the presence of antibodies against OPG.

The study was approved by the local research Ethical Committee, and informed consent was obtained from all participants.

### Statistical analysis

Comparison of proportions was performed using  $\chi^2$  test. A multivariate analysis was performed using Multivariate Analysis of Variance (MANOVA) to identify variables associated with low BMD. Difference was considered significant if the *P* value was < 0.05. Data were analyzed using the Statistical Package for Social Services, Version 16.0 (SPSS Inc., IL, United States).

## RESULTS

Forty-nine out of the 70 (74%) CD patients displayed low BMD, with 13 (24%) accounting for osteoporosis and 36 (76%) for osteopenia (Table 1). Multiple logistic regression analysis showed that age was the only one variable which positively correlated with low BMD (Table 1). Serum calcium and PTH levels were normal in all patients. A complete healing of duodenal mucosa was found in 31 out of 38 (82%) patients who underwent repeat intestinal biopsies. In this specific subgroup, 20 (64%) patients showed a low BMD compared to 6 out of 7 (86%) patients who were found to carry an incomplete duodenal mucosa healing (*n* = 1 Marsh 1, *n* = 2 Marsh 2, *n* = 4 Marsh 3) (Table 2, *P* = 0.4).

No evidence of the 55-kDa band was found in serum samples of the subgroup of 30 patients who were tested

**Table 2** Bone mineral density according to dual energy X ray absorptiometry in celiac disease patients with and without duodenal mucosa healing after gluten free diet

	Duodenal mucosa healing	Duodenal mucosa lesions	Total
Low BMD	20	6	26
Normal BMD	11	1	12
Total	31	7	38

BMD: Bone mineral density.

**Table 3** Characteristics of the 30 celiac disease patients who underwent measurement of serum antibodies against osteoprotegerin

Variables	Overall (n = 30)	Normal BMD (n = 6)	Low BMD (n = 24)
Sex (M/F)	8/22	1/5	7/17
Age (yr)	43.5 (21-60)	31.0 (21-54)	44.5 (32-60)
Time since diagnosis (yr)	2.8 (1.0-3.5)	2.7 (1.8-3.2)	2.9 (1.0-3.5)
BMI (kg/m <sup>2</sup> )	22.3 (19.2-25.1)	21.8 (19.2-24.7)	22.4 (20.0-25.1)
Smoke	4	0	4
Fracture	0	0	0
Menopausal status	1	0	1
Duodenal mucosa histology (n = 22)			
Healing	17	5	12
Lesions	5	1	4
HLA status			
DQ2	23	4	19
DQ8	7	2	5

BMD: Bone mineral density; BMI: Body mass index; HLA: Human leukocyte antigen.

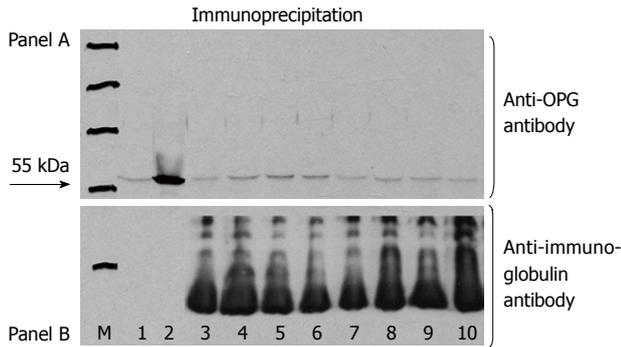
for, indicating no presence of auto-antibodies against OPG (Figure 1). The characteristics of this subgroup of CD patients, including HLA DQ2 and DQ8 status, are shown in Table 3.

## DISCUSSION

Currently, serology is employed to select individuals needing to undergo intestinal biopsy for diagnosing CD as well as to monitor adherence and response to GFD<sup>[18,19]</sup>. However, confirming a previous observation<sup>[20]</sup>, this study shows that, despite a persistent negative serology, 18% of CD patients with good adherence to GFD have an incomplete normalization of intestinal mucosa (e.g., 82% negative predictive value in detecting intestinal mucosal recovery).

A GFD normally gains mucosal damage in CD patients restoring calcium absorption, and this can support an improvement in bone mineralization in one year<sup>[21]</sup>. Nevertheless, a GFD rarely normalizes BMD in adult patients, so nutritional supplementation may be necessary<sup>[22,23]</sup>. Findings of this study show a higher prevalence (74%) of bone demineralization in adulthood diagnosed CD patients notwithstanding long-term strict adherence to GFD and persistent negative CD-related serology.

No differences in acknowledged risk factors for osteoporosis have been found between patients with low



**Figure 1** No evidence of the 55-kDa band was found in serum samples of the subgroup of 30 patients who were tested for, indicating no presence of auto-antibodies against osteoprotegerin. A: Western blotting (representative serum samples from a series of 30 celiac disease patients) showing the absence of antibodies against osteoprotegerin (OPG) after immunoprecipitation. Positive control indicates a 55 kDa band (arrow, lane 2) as the presence of antibodies against OPG. Negative control is shown in the lane 1; B: Western blotting confirms the equal loading of the gel by means of the addition of staining for immunoglobulins. Molecular-weight markers are shown in lane M.

and normal BMD except for age, suggesting that CD is a major one. Even though serum calcium levels may not adequately reflect calcium absorption, no patient showed low levels of serum calcium, and this was in accordance with the finding that calcium absorption returns to normal setting after one year GFD<sup>[24]</sup>. Furthermore, a significant proportion of patients (64%) on GFD showed low BMD, even if they displayed complete recovery of duodenal lesions as assessed by Marsh classification<sup>[25]</sup>. Nevertheless, recent observations suggested that normal Marsh grade does not exclude villous atrophy when assessed morphometrically<sup>[26]</sup>.

Despite the high frequency of low BMD, there is still not a consensus about the timing for BMD evaluation in CD patients<sup>[27]</sup>. A novel finding of this study is that DEXA performed 92% negative predictive value in detecting intestinal mucosa recovery. So, based on this finding, the use of DEXA could be proposed for its additive value in this specific issue (e.g., a non-pathological DEXA has 92% probability to predict intestinal mucosa recovery in CD patients on GFD).

Insight of the molecular mechanism regulating osteoclast formation and activation progressed a lot in the past 10 years, with the identification of the RANKL/RANK signaling system as well as the discovering of OPG, a protein that appeared to protect from excessive bone reabsorption<sup>[28,29]</sup>. Fiore *et al.*<sup>[30]</sup> demonstrated that OPG/RANKL ratio was significantly lower in CD patients with normalization of duodenal histology than in healthy controls and it positively correlated with low BMD. It has been hypothesized that in some patients OPG is bound to a plasma protein(s) and this could inactivate it<sup>[31]</sup>.

In this study, circulating antibodies against OPG were not found in 30 CD patients. This contrasts with findings of Riches *et al.*<sup>[14]</sup> who showed auto-antibodies against OPG in a man with CD on GFD presenting with severe osteoporosis and high bone turnover. As authors demonstrated, these auto-antibodies had the potential to block

the inhibitory effect of OPG on RANKL and this lead to the hypothesis that they may play a role in the development of bone derangement. In the same report, authors detected these circulating auto-antibodies in three among 15 additional patients with CD and low BMD, while there was no evidence of them in serum specimens from 10 healthy controls and 14 patients with autoimmune hypothyroidism. If these CD patients were or were not on GFD was not indicated by the authors and data on duodenal mucosa histology were not provided.

It is unlikely that discrepancy between Riches *et al.*<sup>[14]</sup> and findings of this study relies on the selection of patients neither on the used methodology. Indeed, it seems that the subgroup of 30 CD patients who were tested for antibodies against OPG is representative enough with respect to the variables that may affect the possible appearance of auto-antibodies (e.g., BMD, duodenal histology, HLA). Furthermore, Riches *et al.*<sup>[14]</sup> anti-OPG antibodies measurement methodology has been strictly followed. A positive control has been checked in order to validate the procedure and several serum sample dilutions have been tested in order to increase sensitivity. Nevertheless, that a long-term GFD, as is the case of this study, may reduce the production of circulating auto-antibodies against OPG towards undetectable levels may be a possibility. At this regard, the occurrence of a limited amount of mucosal antibodies could be taken into account. Furthermore, genetic background affecting the immune system (e.g., auto-antibody development) may be another issue. Indeed, HLA DQ2 heterodimer has been shown to be more involved than HLA DQ8 heterodimer in complicated CD<sup>[32]</sup>. While for HLA-DQ2 a single deamidation in a gluten peptide is enough to produce a CD4+ T cell response, for HLA-DQ8 it is necessary a deamidation at two positions in the gluten peptide, resulting in a more limited generation of strong antigenic gluten peptides than HLA DQ2 haplotype<sup>[33]</sup>. Furthermore, the HLA-DQ8 peptidic domain is more easily degraded limiting the availability for antigen presentation<sup>[34]</sup>. With this in mind, in this study all 30 CD patients who were screened for antibodies against OPG were also tested for HLA DQ2/DQ8 alleles. As expected<sup>[35]</sup>, a proportion of 77% and 23% for DQ2 and DQ8 haplotype, respectively, was found, indicating an unselected sample at this regard. Anyway, although the role of HLA molecules and the association to particular genotypes has been well established in CD pathogenesis, HLA is estimated to contribute only for the 35% of the genetic risk, suggesting that more genetic risk factors had to be involved in CD susceptibility<sup>[36]</sup>.

In conclusion, the negative results of this study indicate that auto-antibodies against OPG, if any, do not play a major role in the pathogenesis of bone demineralization in patients with CD, suggesting that other mechanisms should be investigated.

## ACKNOWLEDGMENTS

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## COMMENTS

**Background**

Evidences indicate that a low bone mineral density (BMD) is found in 20%-50% newly diagnosed patients with celiac disease (CD). Adherence to a strict gluten-free diet (GFD) will reverse the histological changes in the intestine and also the biochemical evidence of calcium malabsorption, resulting in normal BMD in these treated patients. Nevertheless, there may be long-term impairment of bone mineralization in some otherwise healthy CD patients adhering to GFD. Furthermore, osteopenia has been found in treated CD patients who showed improvement or even complete healing of intestinal mucosa.

**Research frontiers**

Other mechanisms of bone injury than calcium malabsorption are probably involved in patients with CD. Mediators of inflammatory immune-mediated responses (e.g., cytokines), parathyroid hormone (PTH), estrogens, androgens, corticosteroids and vitamin D are all acknowledged to affect BMD by modulating the receptor activator of nuclear factor B/receptor activator of nuclear factor B-ligand/osteoprotegerin [RANK/RANK-L/osteoprotegerin (OPG)] system. Recently, circulating neutralizing autoantibodies against OPG have been shown in CD patients with low BMD leading to the hypothesis that blocking the inhibitory effect of OPG on RANKL may have a role in the development of bone derangement in these patients.

**Innovations and breakthroughs**

This study aimed at investigating risk factors associated with low BMD in patients with CD, focusing on circulating auto-antibodies against OPG. Findings confirm a high prevalence of low BMD in CD patients despite strict adherence to GFD, persistent negative serology, and healing of duodenal lesions. Since auto-antibodies against OPG have not been found in serum samples from a subgroup of patients who were tested for, it can be argued that, if any, the role of antibodies against OPG in the pathogenesis of reduced BMD in patients with CD remains to be established.

**Applications**

The study further supports the importance of early diagnosis in order to avoid bone complications in CD patients. Furthermore, it remarks that autoantibodies against OPG do not play a major role in the pathogenesis of bone demineralization in patients with CD, suggesting that other mechanisms of bone derangement should be investigated.

**Peer review**

This is an interesting study showing antibodies to OPG are not present at least systemically to explain persistent low bone density in coeliac subjects. Authors guess there is a small possibility that mucosal antibodies could still be present which should still be alluded to.

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## Mucosa-associated bacteria in two middle-aged women diagnosed with collagenous colitis

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### Abstract

**AIM:** To characterize the colon microbiota in two women histologically diagnosed with collagenous colitis using a culture-independent method.

**METHODS:** Biopsies were taken from the ascending colon and the total DNA was extracted. Universal bacterial primers were used to amplify the bacterial 16S rRNA genes. The amplicons were then cloned into competent *Escherichia coli* cells. The clones were sequenced and identified by comparison to known sequences.

**RESULTS:** The clones could be divided into 44 different phylotypes. The microbiota was dominated by Firmicutes and Bacteroidetes. Seven phylotypes were

found in both patients and constituted 47.5% of the total number of clones. Of these, the most dominating were clones similar to *Bacteroides cellulosilyticus*, *Bacteroides caccae*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis* and *Bacteroides dorei* within Bacteroidetes. Sequences similar to *Faecalibacterium prausnitzii* and *Clostridium citroniae* were also found in both patients.

**CONCLUSION:** A predominance of potentially pathogenic *Bacteroides spp.*, and the presence of clones showing similarity to *Clostridium clostridioforme* were found but the overall colon microbiota showed similarities to a healthy one. Etiologies for collagenous colitis other than an adverse bacterial flora must also be considered.

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**Key words:** Microscopic colitis; Collagenous colitis; Lymphocytic colitis; Colonic microbiota; 16S rRNA sequencing

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### INTRODUCTION

Collagenous colitis (CC), an idiopathic inflammatory bowel disease, is a subtype of microscopic colitis (MC) together with lymphocytic colitis (LC)<sup>[1]</sup>. It is considered as a common cause of chronic diarrhea. In Sweden the incidence is approx four to five cases per 100 000<sup>[2]</sup>. The incidence for

both CC and LC in Europe and North America is almost as high as for Crohn's disease and ulcerative colitis<sup>[2]</sup>.

CC is clinically characterized by chronic non-bloody diarrhea, often combined with abdominal pain and weight loss<sup>[2]</sup>. The colonic mucosa appears macroscopically normal or near-normal and the diagnosis is made by microscopic examination of mucosal biopsies that reveals diagnostic histopathological changes. CC was first described in 1976 by Lindström<sup>[3]</sup> in a woman with chronic watery diarrhea in whom histological examination revealed a thick subepithelial collagenous deposition in the rectum. In 1989, Lazenby *et al.*<sup>[4]</sup> proposed the term lymphocytic colitis in a group of patients with chronic diarrhea and normal colonoscopy with only minor histological changes, where the microscopic evaluation of colonic biopsy specimens revealed modestly increased inflammation in the lamina propria without subepithelial collagen deposition or other mucosal changes.

The peak incidence of MC is in individuals between 55 years and 70 years of age. The female:male ratio is about 7:1 for CC. For LC the female predominance is less pronounced, with a female:male ratio of 2-3:1<sup>[5]</sup>. However, the disease can occur at all ages, and a few children with CC have been reported<sup>[6,7]</sup>. Bile acid malabsorption is found in about 27%-44% of patients with CC and 9%-60% in patients with LC<sup>[5,8-9]</sup>. Treatment with bile acid binding medications is effective in patients with bile-acid malabsorption but can also be effective in patients without bile-acid malabsorption<sup>[10]</sup>.

Both etiology and pathogenesis of MC are uncertain. The most widely held hypothesis is that a noxious agent in the lumen, probably originating from the bacterial microflora, may have a major pathogenic role in the chronic intestinal inflammation. This is supported by regression of symptoms and histopathological changes after diversion of the fecal stream, and recurrence after restoration of intestinal continuity<sup>[11,12]</sup>. Other observations supporting this hypothesis are the sudden onset of diarrhea and that treatment with antibiotics may have positive effects<sup>[2,13]</sup>. The increased infiltration of lymphocytes in the mucosa also indicates a proinflammatory component in the lumen. There are case reports of linking pathogenic bacteria such as *Clostridium difficile*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas hydrophila* to MC<sup>[2,7,14-16]</sup>.

The human microbiota in healthy persons as well as in patients with inflammatory bowel disease has been analyzed in several studies using culture-independent methods<sup>[17-19]</sup>. However, to our knowledge no such studies have been performed on patients diagnosed with CC. The aim of the present study was to characterize the mucosa-associated microbiota in the ascending colon in two women histologically diagnosed with CC, by cloning and sequencing of the bacterial 16S rRNA genes.

## MATERIALS AND METHODS

### Subjects and samples

Two female patients, 51 years and 60 years old (A and B)

with a known diagnosis of MC, took part in the study. Patient A, otherwise healthy, started to experience watery, non-bloody diarrhea after an antibiotic treatment for gastroenteritis 10 years earlier. Colonoscopy was performed and she was diagnosed with LC. She was treated with Loperamid<sup>®</sup> (Merck NM AB, Stockholm, Sweden). Two years later she had a relapse of watery, non-bloody diarrhea and a second colonoscopy was performed, still indicating LC. This time she improved spontaneously. At the time of the present study, after a period of stress and a viral gastroenteritis, she started to lose weight and had frequent, watery, non-bloody diarrhea. The present colonoscopy showed a slightly swollen mucosa and increased vascular pattern. The histological examination revealed a thickened subepithelial collagen layer as well as inflammation in the lamina propria and a damaged surface epithelial layer. Patient B had a history of chronic thyroiditis but was otherwise healthy. She was diagnosed with CC as well as with bile acid malabsorption 4 years before the study. At that time she improved spontaneously but had a recurrence after a period of major stress. Previously, she was treated with non-steroidal anti-inflammatory drugs due to muscular stiffness and actually experienced an improvement of her bowel function by this treatment. At the time of the present colonoscopy her symptoms had improved due to dietary fat reduction. Colonoscopy showed an increased vascular pattern in the right colon but was otherwise normal. Histological examination could verify a collagenous colitis.

Neither patient had any medication at the time of the colonoscopy. Celiac disease had been excluded in both women. They were both non-smokers.

The patients were asked to avoid fiber-rich foods such as fruits, vegetables, grains and seeds some days before the colonoscopy. The day before the examination they ate a plain breakfast, and no solid food was allowed after noon. Intestinal cleansing was carried out with Phosphoral<sup>®</sup> (Clean Chemical Sweden AB), a salt preparation with osmotic effects. Colonoscopy was performed and serial biopsies throughout the colon as well as two extra biopsies from the right colon were collected. The histological examination followed routine procedures. The latter were placed in tubes with TE-buffer [10 mmol Tris-HCl, 1 mmol ethylenediaminetetraacetic acid (EDTA), pH 8.0], frozen immediately in liquid nitrogen and stored at -80 °C. The study was approved by the Ethics Committee at Lund University. The women gave written, informed consent before entering the study.

### DNA extraction and amplification

Frozen tissue samples were thawed on ice and a single biopsy was transferred to a 1.5 mL tube with 190 µL Buffer G2 (DNA Tissue Kit; Qiagen, GmbH, Hilden, Germany) and 10 µL of Proteinase K (Qiagen). Eight to ten sterile glass (2 mm) beads were added and the cells were lysed at 56 °C for 3-4 h in a shaking water bath. Tubes were cooled on ice and shaken for 30 min on an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) at 4 °C

to disintegrate all bacteria. After centrifugation at  $300 \times g$  for one minute, the solution was transferred to a Qia-gen sample tube, and total DNA was extracted by using Biorobot EZ1 (Qiagen) according to the manufacturer's instructions. DNA was eluted in 200  $\mu$ L.

### Polymerase chain reaction amplification and cloning

The bacterial 16S rRNA genes were amplified by the universal primers ENV1 and ENV2 annealing to positions 8-27 and 1492-1511, respectively, according to *Escherichia coli* (*E. coli*) numbering<sup>[20]</sup>. The reaction mixture contained 5  $\mu$ L of  $10\times$  polymerase chain reaction (PCR) buffer (100 mmol Tris-HCl, 15 mmol MgCl<sub>2</sub>, 500 mmol KCl, pH 8.3), each deoxynucleotide phosphate at a concentration of 200  $\mu$ mol, 2.5 U of Tag DNA Polymerase (Roche Diagnostics, GmbH, Mannheim, Germany) and 10 pmol of each primer. To each tube, 5  $\mu$ L of extracted sample DNA was added and sterile water was added to 50  $\mu$ L. As negative controls, water was added to the reaction mixture instead of DNA. Amplification was performed on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Initially, the reaction was heated to 94 °C for 3 min, followed by 25 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 45 s and elongation at 72 °C for 2 min. Finally, the reaction was held at 72 °C for 7 min before cooling down to 4 °C. Six PCR tubes were prepared from each sample and then pooled. Forty-two  $\mu$ L of the pooled reaction mixture from one sample was separated on a 1.5% (w/v) agarose gel (Agarose Type III; Sigma Aldrich, St Louis, Mo., United States) in TBE-buffer (89 mmol Tris, 89 mmol boric acid and 2.5 mmol EDTA, pH 8.3). The agarose gel was stained with ethidium bromide (0.5 mg/L) and the band was cut out from the gel. DNA was purified by using Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, United States). For cloning Promega pGEM<sup>®</sup>-T Vector System and *E. coli* JM 109 (Promega Corp.) competent cells were used as described previously<sup>[20]</sup>. Colonies were selected randomly and recultivated on LB-agar containing ampicillin, and then harvested and stored in freezing buffer at -80 °C.

### Sequencing

Selected clones were single-strand sequenced by MWG Biotech (Ebersberg, Germany). ENV1 primer was used as sequencing primer. Sequences were edited using Bioedit Sequence Alignment editor 7.0.5.3<sup>[21]</sup>. Sequences were identified by comparing them to sequences using the option "seqmatch" available at the Ribosomal Database Project<sup>[22]</sup>. Sequences were checked for chimeric artifacts by using the Bellerophon server<sup>[23]</sup> and by creating phylogenetic trees of both 5'- and 3'- ends of the sequences. DNAdist calculations were performed using the Phylip DNAdist program using the "similarity table" option (available at: <http://mobyli.pasteur.fr/cgi-bin/portal.py?form=dnadist>)<sup>[24]</sup>. Sequences representing the different phylotypes have been submitted to Genbank

and the accession numbers are HQ992999- HQ993042.

### Diversity calculations

Shannon and Simpson's indices were used for diversity calculations. The Shannon index is based on the proportional abundance of species and accounts for both evenness and species richness. Simpson's index is the dominance measure where the abundance of commonest species is considered more than species richness<sup>[25]</sup>. The Simpson's index was expressed as 1/D.

## RESULTS

Two clone libraries were constructed, one for patient A with 87 clones and one for patient B with 90 clones. Five clones were suspected chimeras and were removed from the dataset before analysis. The lengths of the sequenced fragments were approximately 750 bp. Sequences showing > 98% similarity to each other were assigned to a single phylotype and a total of 44 phylotypes were identified (Table 1).

Sequences could be grouped into 22 phylotypes in patient A and 29 phylotypes in patient B. Shannon's and Simpson's diversity indices were calculated and both the patients showed similar values. The Shannon index was 2.61 for patient A and 2.78 for patient B, and the Simpson index was 8.13 for A and 9.29 for patient B. Firmicutes and Bacteroidetes were the dominating phyla with 50.6% and 47.2% in patient A and 57.8% and 42.2% in patient B, respectively (Figure 1).

In patient A Porphyromonadaceae constituted 1.2% of the clones and in patient B, Porphyromonadaceae and Rikenellaceae constituted 11.1% of the clones. Only two clones (2.3%) similar to Enterobacteriaceae were found in patient A.

The most common phylotypes were sequences similar to *Blautia wexlerae* (23 clones), *Faecalibacterium prausnitzii* (13 clones) and *Clostridium citroniae* (9 clones) within Firmicutes, and *Bacteroides dorei* (29 clones), *Bacteroides caccae* (16 clones) and *Bacteroides cellulosilyticus* (9 clones) within Bacteroidetes (Table 1). These phylotypes showed > 97% similarity to the closest type strain except for *C. citroniae*. Out of the 44 phylotypes identified, the two patients had 7 in common and 5 of these were assigned to Bacteroidetes and two to the Firmicutes. The phylotypes in common constituted 84 clones (47.5%) of the total number of clones. Sequences similar to *F. prausnitzii* and *C. citroniae* were found in both patients (Table 1). Within Bacteroidetes the shared phylotypes were most similar to, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *B. cellulosilyticus*, *B. caccae* and *B. dorei*.

## DISCUSSION

In the present study the microbiota of the ascending colon in the two female patients with CC showed similarities to a normal colon microbiota with Firmicutes and

Table 1 Sequences grouped into phylotypes at 98% similarity

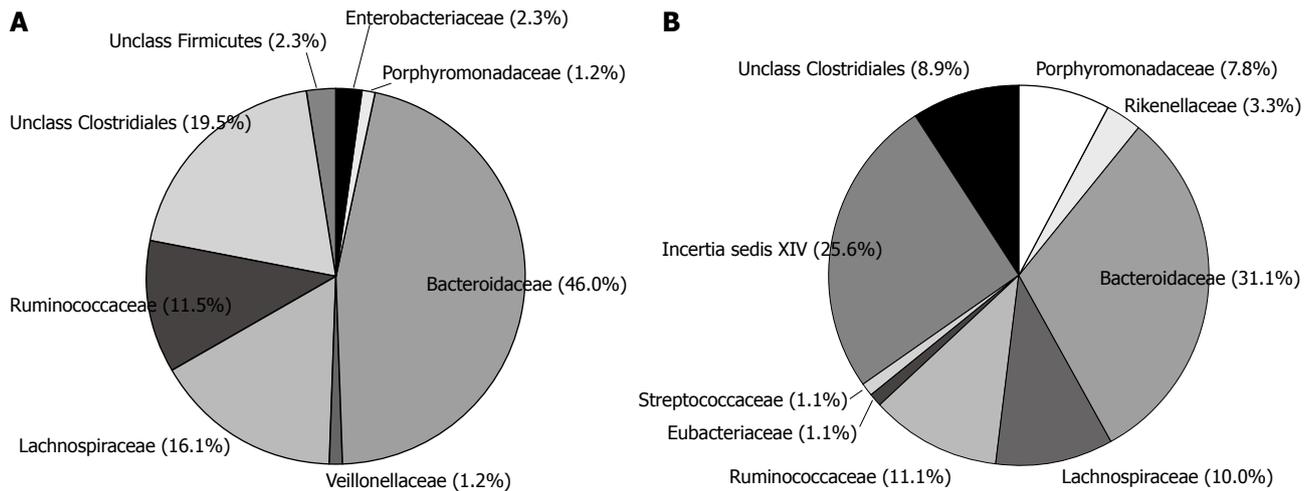
Phylotype No.	Closest type strain	Acc. No <sup>1</sup>	Similarity (%) <sup>2</sup>	No. of clones <sup>3</sup>	Distribution of clones <sup>4</sup>	Assignment of clones
1	<i>Faecalibacterium prausnitzii</i>	AJ413954	98.4-98.5	9	8 (A); 1 (B)	Ruminococcaceae
2	<i>Faecalibacterium prausnitzii</i>	AJ413954	98.4-99.1	4	4 (B)	Ruminococcaceae
3	<i>Subdoligranulum variabile</i>	AJ518869	97.0	1	1 (B)	Ruminococcaceae
4	<i>Anaerofilum agile</i>	X98011	90.6	1	1 (B)	Ruminococcaceae
5	<i>Ruminococcus lactaris</i>	L76602	94.0	1	1 (B)	Ruminococcaceae
6	<i>Oscillibacter valericigenes</i>	AB238598	92.5	1	1 (B)	Ruminococcaceae
7	<i>Ruminococcus lactaris</i>	L76602	95.9	2	2 (A)	Ruminococcaceae
8	<i>Ruminococcus lactaris</i>	L76602	95.1	2	2 (B)	Ruminococcaceae
	<i>Clostridium jejuense</i>	AY494606	94.7			Lachnospiraceae
9	<i>Marvinbryantia formatexigens</i>	AJ505973	95.7	1	1 (B)	Lachnospiraceae
10	<i>Roseburia intestinalis</i>	AJ312385	94.1	1	1 (B)	Lachnospiraceae
11	<i>Anaerostipes caccae</i>	AJ270487	99.2	2	2 (A)	Lachnospiraceae
12	<i>Anaerostipes caccae</i>	AJ270487	95.8	1	1 (B)	Lachnospiraceae
13	<i>Roseburia intestinalis</i>	AJ312385	100.0	2	2 (A)	Lachnospiraceae
14	<i>Roseburia faecis</i>	AY305310	96.9	2	2 (B)	Lachnospiraceae
	<i>Roseburia intestinalis</i>	AJ312385	97.1			Lachnospiraceae
15	<i>Pseudobutyriovibrio ruminis</i>	X95893	94.2-94.3	2	2 (A)	Lachnospiraceae
16	<i>Dorea longicatena</i>	AJ132842	94.9-95.2	3	3 (A)	Lachnospiraceae
17	<i>Dorea longicatena</i>	AJ132842	96.4-97.0	5	5 (A)	Lachnospiraceae
18	<i>Dorea longicatena</i>	AJ132842	100.0	3	3 (B)	Lachnospiraceae
19	<i>Dialister pneumosintes</i>	X82500	99.6	1	1 (A)	Veillonellaceae
20	<i>Eubacterium plautii</i>	AY724678	91.5	1	1 (B)	Eubacteriaceae
21	<i>Streptococcus thermophilus</i>	AY188354	99.9	1	1 (B)	Streptococcaceae
22	<i>Blautia wexlerae</i>	EF036467	99.1-99.9	23	23 (B)	Insertae cedis XIV
23	<i>Clostridium citroniae</i>	DQ279737	95.1-96.2	9	7 (A); 2 (B)	Unclass Clostridiales
	<i>Clostridium asparagiforme</i>	AJ582080	95.1-95.5			Unclass Clostridiales
24	<i>Clostridium clostridioforme</i>	M59089	95.0-95.2	5	5 (A)	Unclass Clostridiales
	<i>Clostridium citroniae</i>	DQ279737	95.0			Unclass Clostridiales
25	<i>Clostridium clostridioforme</i>	M59089	99.4-99.7	3	3 (A)	Unclass Clostridiales
26	<i>Clostridium aldenense</i>	DQ279736	99.1	1	1 (A)	Unclass Clostridiales
27	<i>Clostridium asparagiforme</i>	AJ582080	95.7	1	1 (A)	Unclass Clostridiales
28	<i>Clostridium asparagiforme</i>	AJ582080	96.5	1	1 (B)	Unclass Clostridiales
29	<i>Clostridium clostridioforme</i>	M59089	95.1-95.9	5	5 (B)	Unclass Clostridiales
30	<i>Clostridium ramosum</i>	X73440	100.0	2	2 (A)	Unclass firmicutes
31	<i>Escherichia fergusonii</i>	AF530475	99.7-99.9	2	2 (A)	Gammaproteobacteria
32	<i>Barnesiella intestinihominis</i>	AB267809	99.1-99.3	2	2 (B)	Porphyromonadaceae
33	<i>Barnesiella viscericola</i>	AB267809	92.1	1	1 (B)	Porphyromonadaceae
34	<i>Barnesiella viscericola</i>	AB267809	90.0	1	1 (A)	Porphyromonadaceae
35	<i>Parabacteroides distasonis</i>	AB238922	99.4-100.0	4	4 (B)	Porphyromonadaceae
36	<i>Bacteroides cellulosilyticus</i>	AJ583243	97.6-98.9	9	4 (A); 5 (B)	Bacteroidaceae
37	<i>Bacteroides caccae</i>	X83951	99.4-99.9	16	2 (A); 14 (B)	Bacteroidaceae
38	<i>Bacteroides xylanisolvens</i>	AM230650	97.7	1	1 (A)	Bacteroidaceae
39	<i>Bacteroides thetaiotaomicron</i>	AE015928	99.9	6	4 (A); 2 (B)	Bacteroidaceae
40	<i>Bacteroides thetaiotaomicron</i>	AE015930	99.3	1	1 (B)	Bacteroidaceae
41	<i>Bacteroides uniformis</i>	AB050110	99.7-100.0	6	3 (A); 3 (B)	Bacteroidaceae
42	<i>Bacteroides dorei</i>	AB242142	97.3-98.7	29	26 (A); 3 (B)	Bacteroidaceae
43	<i>Alistipes putredinis</i>	L16497	92.4-92.7	2	2 (B)	Rikenellaceae
44	<i>Alistipes onderdonkii</i>	AY974071	99.7	1	1 (B)	Rikenellaceae

The type strain showing the highest similarity to the sequence is shown. Assignment of the clones to bacterial family level was done using the "sequence match" option in the Ribosomal data base<sup>[22]</sup>. <sup>1</sup>Accession number for the type strain; <sup>2</sup>Similarity to the closest type strain; <sup>3</sup>The total number of clones assigned to the phylotype; <sup>4</sup>Number of clones found in patient A and B, respectively.

Bacteroidetes as dominating phyla, making up 97.7% and 100.0% of the clones in patient A and B, respectively. Only two clones close to Enterobacteriaceae were found in patient A. In several studies, the microbiota of healthy persons have been analyzed by sequencing of the 16S rRNA genes using either fecal samples or tissue samples from the intestinal mucosa<sup>[17,18,26-28]</sup>. All these studies showed a predominance of Firmicutes and Bacteroidetes while Verrucomicrobia, Actinobacteria and gamma proteobacteria were detected at lower frequency.

The proportion of clones belonging to *Bacteroides*

was 47.0% in patient A and 31.1% in patient B. These were higher figures than Wang *et al.*<sup>[18]</sup>, using a similar methodology, found in biopsies taken from the ascending colon from a healthy, 54-year old woman where *Bacteroides* constituted 24.4% of the clones. Hayashi *et al.*<sup>[17]</sup> analyzed fecal samples of 3 healthy men aged 27, 34 and 54 years, and the proportion of *Bacteroides* was 4.2%, 3.4% and 14.9%, respectively. In another study of fecal samples from a healthy 40-year old man, *Bacteroides* constituted 14.4% of the total number of clones<sup>[26]</sup>. Delgado *et al.*<sup>[27]</sup> analyzed clones from the descending colon from



**Figure 1** Distribution of clones at family level. Assignment of the clones were done using the Ribosomal Data Base Project Release 10 and the option "seqmatch"<sup>[22]</sup>. A: Patient A; B: Patient B.

a healthy 45-year old man and found one clone out of 20 (5%) belonging to *Bacteroides*. Of the 44 phylotypes found here, the two patients had only 7 in common. However, these shared phylotypes constituted 47.5% of the total number of clones. Within *Bacteroides* five phylotypes were common to both patients. Of these the most dominating were clones similar to *B. caccae* and *B. dorei* making up 25.4% of the total number of clones (Table 1). Both species belong to the *Bacteroides fragilis* group that are opportunistic pathogens isolated from a variety of anaerobic infections and cause about 50% of all anaerobic bacteremias<sup>[29,30]</sup>.

A subgroup of *B. fragilis*, enterotoxigenic *B. fragilis* (ETBF), that can secrete a proinflammatory enterotoxin, has been found to be implicated in traveller's diarrhea<sup>[31]</sup>. In a study by Zhang *et al.*<sup>[32]</sup>, significantly more ETBF were found in patients with watery diarrhea (26.8%) than in the control group (12.4%). ETBF was also found at a higher frequency in patients over 30 years of age compared to the control group. Additionally, it was shown that 27.0% of patients over the age of 60 carried ETBF compared to 3.7% for the control group. It has been suggested that *Bacteroides fragilis* toxin can bind to receptors on the epithelial cells, leading to a signal cascade and cleavage of cadherine promoting an increased intestinal permeability<sup>[33]</sup>. An increased intestinal permeability was shown in one patient with CC, using an Ussing chamber<sup>[12]</sup>. Permeability was measured on biopsies taken from the sigmoid colon and it was shown that the intestinal integrity was improved after a fecal diversion by an ileostomy, but after restoration of the bowel continuity the permeability increased again<sup>[12]</sup>. Some improvement has been reported when CC patients were treated with metronidazole, penicillin or erythromycin<sup>[34]</sup>. *Bacteroides* are sensitive to metronidazole and that might point to *Bacteroides* as a possible disease-provoking agent<sup>[30]</sup>. On the other hand, the positive effect shown with penicillin and erythromycin speaks against *Bacteroides*<sup>[34]</sup>.

It has been shown that *Akkermansia muciniphila* and

strains of *Clostridium*, *Prevotella* and *Bacteroides* are able to degrade mucin<sup>[35,36]</sup>. The type strain *B. thetaiotaomicron* NCTC 10582 was shown to express glycosidases and glycosulphatase and could degrade pig gastric mucin<sup>[37]</sup>. In the present study 4 clones from patient A and 3 clones from patient B showed high similarity (99.3%-99.9%) to the type strain *B. thetaiotaomicron* NCTC 10582 (Table 1). Clones belonging to *Akkermansia muciniphila* were not found. However, it has been shown that this species represents only about one percent of the microbiota in healthy children and adults<sup>[38]</sup>. One might speculate that specific components present within the microbiota of the CC patients, i.e., *Bacteroides spp.*, that has an impact both on the colonic mucin layer and the intestinal permeability, leading to an immune response.

The clones resembling *Clostridium clostridioforme*, *Clostridium citroniae*, *Clostridium aspargifforme* and *Clostridium aldenense* were distributed into 7 phylotypes showing 95%-99.7% similarity to the different type strains. Four clones from patient A showed high similarity to *C. clostridioforme* and *C. aldenense*. Also in patient B, 5 clones resembling *C. clostridioforme* were found, but they showed lower similarity to the type strain. They are all related and belong to cluster XIVa as defined by Collins *et al.*<sup>[39]</sup>, Warren *et al.*<sup>[40]</sup> and Mohan *et al.*<sup>[41]</sup>. Strains of *C. clostridioforme* and closely related species have been involved in a variety of infections<sup>[42]</sup>. In a study of autistic children, all of whom had gastrointestinal symptoms, high counts of fecal isolates showing 95% similarity to *C. clostridioforme* were found in the diseased children but not in the controls. It cannot be excluded that the presence of sequences resembling *C. clostridioforme* might play a role in the disease in the patients analyzed here.

Clones identified as *F. prausnitzii* of the Ruminococcaceae family were found in both patients and constituted about 7% of the total number of clones. These bacteria together with *Eubacterium rectale* and *Roseburia spp.* are known as butyrate producers and usually make up about 5%-10% of the human microbiota and can be re-

garded as commensals<sup>[43]</sup>. No clones resembling *Lactobacillus* nor Actinobacteria or Verrucomicrobia were found. This can probably be explained by the fact that too few clones were sequenced and that they usually constitute a minor part of the microbiota. Previously published case reports have suggested *Clostridium difficile*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas hydrophila* to CC as possible pathogens<sup>[2,7,14-16]</sup>. This could not be confirmed in the present study. As different pathogens are described, and the fact that the colonic microbiota was similar to a healthy one, the etiology to CC may not primarily depend on abnormal microbiota, and antibiotics may not be the treatment of choice in this entity, as it is sometimes considered<sup>[34]</sup>.

This study has some limitations. Only two patients were examined and the method applied here only detects the dominant bacteria. Future research needs to examine the presence of common pathogens in the bowel, but also etiologies of CC other than bacteria must be considered.

To the best of our knowledge, this is the first study of the intestinal microbiota in patients with a histologically diagnosed CC, by a culture-independent method. The overall composition of the colonic microbiota was similar to a healthy one with dominance of Firmicutes and Bacteroidetes. Due to the fact that only two patients were analyzed it is difficult to draw any conclusions, but in both patients a high proportion of potentially pathogenic species of *Bacteroides* and clones related to *C. clostridioforme* were found.

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## COMMENTS

### Background

Collagenous colitis (CC) is an idiopathic inflammatory bowel disease characterized by chronic non-bloody diarrhea. CC is regarded as a subtype of microscopic colitis. The etiology is unknown but a noxious agent, probably originating from the microbiota, in the intestinal lumen has been proposed to have a pathogenic role. However, no attempt to analyze the microbiota in diseased patients has been done.

### Research frontiers

The intestinal mucosa is colonized by a huge number of bacteria that are important for health and disease. In several studies the gut microbiota has been analyzed by culture-independent methods in patients with intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease.

### Innovations and breakthroughs

Having the opportunity to obtain histologically well-defined collagenous colitis samples, the authors have characterized the dominant microbiota in two diseased patients.

### Applications

Culture-independent methods can be used for analyzing the dominant mucosa-associated microbiota in collagenous colitis.

### Terminology

The meaning of the word microbiota here is synonymous to the bacterial flora in the intestine.

### Peer review

It is well organized. Several papers have been presented in support of micro-

biota from controls, but no speculation have been made about findings in this paper and clinical applications, limitations of the study and future of research in this field.

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## Randomized controlled trial of pancreatic stenting to prevent pancreatitis after endoscopic retrograde cholangiopancreatography

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**Author contributions:** Kawaguchi Y performed the endoscopy and led the study; Kawaguchi Y, Shimosegawa T and Mine T designed the research; Kawaguchi Y, Ogawa M and Ito H performed the research; Kawaguchi Y and Omata F analyzed the data; and Kawaguchi Y wrote the paper.

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### Abstract

**AIM:** To determine the effectiveness of pancreatic duct (PD) stent placement for the prevention of pancreatitis after endoscopic retrograde cholangiopancreatography (ERCP) in high risk patients.

**METHODS:** Authors conducted a single-blind, randomized controlled trial to evaluate the effectiveness of a pancreatic spontaneous dislodgement stent against post-ERCP pancreatitis, including rates of spontaneous dislodgement and complications. Authors defined high risk patients as having any of the following: sphincter of Oddi dysfunction, difficult cannulation, prior history of post-ERCP pancreatitis, pre-cut sphincterotomy, pancreatic ductal biopsy, pancreatic sphincterotomy, intraductal ultrasonography, or a procedure time of more than 30 min. Patients were randomized to a stent group ( $n = 60$ ) or to a non-stent group ( $n = 60$ ). An abdominal radiograph was obtained daily to assess

spontaneous stent dislodgement. Post-ERCP pancreatitis was diagnosed according to consensus criteria.

**RESULTS:** The mean age ( $\pm$  standard deviation) was  $67.4 \pm 13.8$  years and the male: female ratio was 68:52. In the stent group, the mean age was  $66 \pm 13$  years and the male: female ratio was 33:27, and in the non-stent group, the mean age was  $68 \pm 14$  years and the male: female ratio was 35:25. There were no significant differences between groups with respect to age, gender, final diagnosis, or type of endoscopic intervention. The frequency of post-ERCP pancreatitis in PD stent and non-stent groups was 1.7% (1/60) and 13.3% (8/60), respectively. The severity of pancreatitis was mild in all cases. The frequency of post-ERCP pancreatitis in the stent group was significantly lower than in the non-stent group ( $P = 0.032$ , Fisher's exact test). The rate of hyperamylasemia were 30% (18/60) and 38.3% (23 of 60) in the stent and non-stent groups, respectively ( $P = 0.05$ ,  $\chi^2$  test). The placement of a PD stent was successful in all 60 patients. The rate of spontaneous dislodgement by the third day was 96.7% (58/60), and the median (range) time to dislodgement was 2.1 (2-3) d. The rates of stent migration, hemorrhage, perforation, infection (cholangitis or cholecystitis) or other complications were 0% (0/60), 0% (0/60), 0% (0/60), 0% (0/60), respectively, in the stent group. Univariate analysis revealed no significant differences in high risk factors between the two groups. The pancreatic spontaneous dislodgement stent safely prevented post-ERCP pancreatitis in high risk patients.

**CONCLUSION:** Pancreatic stent placement is a safe and effective technique to prevent post-ERCP pancreatitis. Therefore authors recommend pancreatic stent placement after ERCP in high risk patients.

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**Key words:** Endoscopic retrograde cholangiopancreatography; Pancreatitis; Postoperative complications; Prophylaxis; Stents

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Kawaguchi Y, Ogawa M, Omata F, Ito H, Shimosegawa T, Mine T. Randomized controlled trial of pancreatic stenting to prevent pancreatitis after endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2012; 18(14): 1635-1641 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i14/1635.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i14.1635>

## INTRODUCTION

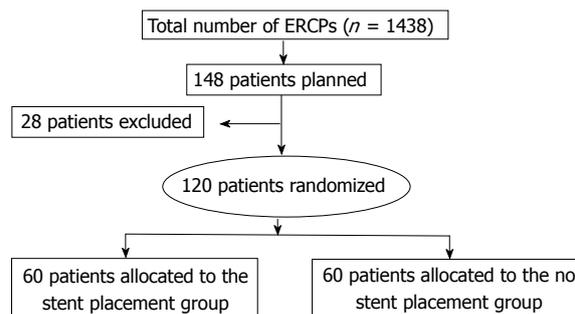
Acute pancreatitis is a serious complication of endoscopic retrograde cholangiopancreatography (ERCP). The frequency of post-ERCP pancreatitis varies between 1% and 9%<sup>[1-6]</sup> in average risk patients, and its prevention remains a critical issue. Impaired drainage of the pancreatic duct (PD), leading to acinar injury, is a commonly accepted mechanism of injury<sup>[7,8]</sup>. Possible causes of impaired drainage include mechanical, chemical and thermal injury, as well as subsequent edema and spasm of the duodenal papilla.

Several prospective studies have confirmed that PD stent placement prevents post-ERCP pancreatitis, especially in high risk patients<sup>[9-13]</sup>. However, no consensus has yet been reached on the indications for prophylactic PD stent placement or on the type of stent which should be used. Therefore, we conducted a single-blind, randomized controlled trial (RCT) to evaluate the effectiveness of spontaneous dislodgement stents in preventing post-ERCP pancreatitis in high risk patients.

## MATERIALS AND METHODS

### Study design

This RCT was conducted in Tokai University Hospital, Japan between April 2006 and June 2010. During this period, we performed 1438 ERCPs on patients with pancreatobiliary diseases. All procedures for this study were performed by one physician (Kawaguchi Y), an experienced surgeon with more than 2000 prior ERCP cases. Independent patient-related and procedure-related risk factors for post-ERCP pancreatitis have been previously published<sup>[3-5,14-16]</sup>. Patients at high risk of post-ERCP pancreatitis who met any of the following criteria were enrolled in this RCT: (1) A previous history of post-ERCP pancreatitis; (2) Sphincter of Oddi dysfunction; (3) Difficult cannulation; (4) Pre-cutting; (5) PD biopsy; (6) Intraductal PD ultrasonography; or (7) ERCP procedure time > 30 min prior to PD stent placement. Difficult cannulation was defined as > 10 min of attempted cannulation. We performed PD guidewire placement or pan-



**Figure 1** Trial profile.

creatic sphincterotomy in some cases of difficult cannulation. Exclusion criteria were as follows: (1) Inability to provide written informed consent; (2) Performance status of 4; (3) Age 19 years or younger; (4) Pregnancy or breastfeeding; (5) Inability to access duodenal papilla endoscopically; (6) Previous endoscopic sphincterotomy or endoscopic papillary balloon dilation; (7) Inability to insert a guidewire into the PD; (8) Patients requiring PD drainage; (9) Patients requiring endoscopic papillectomy; (10) Pancreatic head cancer; or (11) Pancreas divisum.

Of 148 patients screened, 120 patients who satisfied the inclusion criteria participated in this study. Patients were randomly assigned into a stent placement group (60 patients) and a non-stent placement group (60 patients) after the completion of diagnostic or therapeutic ERCP (Figure 1). Randomization was conducted by a simple randomization method without any stratification factor. After diagnostic or therapeutic ERCP was achieved and eligibility was confirmed, a research assistant assigned patients to either the stent or non-stent group, allocating patients using a uniform random number algorithm. All patients provided written informed consent before study entry. This study was approved by Institutional Review Board of Tokai University Hospital (Clinical Trials; University Hospital Medical Information Network Identification Number in Japan, 3995).

### Endoscopic procedures and patient cares

Each patient's past medical history was obtained and physical examination was performed prior to ERCP. Lidocaine gel (4%) was used for pharyngeal anesthesia. Intravenous administration of midazolam with pethidine hydrochloride was used for conscious sedation. All patients received an intravenous drip infusion of 20 mg nafamostat mesilate during the examination, starting before ERCP, with the same dose of nafamostat mesilate as well as antibiotics administered afterwards.

ERCP was performed in standard fashion with JF-240, JF-260V or TJF240 video endoscopes (Olympus, Tokyo, Japan); PR-V216Q, PR-V234Q or PR-V220Q catheters (Olympus) and Jagwire guidewires (Boston Scientific Japan, Tokyo, Japan) were used. We used 5 Fr straight polyethylene stents, 3 cm in length, unflanged on the PD side, and with two flanges on the duodenal side (GPDS-5-3; Cook Endoscopy Inc., Winston-Salem, NC,

	Non-stent	Stent	P value
No. of patients	60	60	-
Mean age (range) (yr)	68 (27-92)	66 (24-88)	0.35
Sex: female/male	25/35	27/33	0.46
Reasons of high risk			
Previous post-ERCP pancreatitis	5 (8%)	5 (8%)	0.65
Sphincter of Oddi dysfunction	0 (0%)	0 (0%)	-
A difficult cannulation	10 (17%)	10 (17%)	0.68
Pre-cut	0 (0%)	0 (0%)	-
Pancreatic sphincterotomy	5 (8%)	4 (7%)	1
Pancreatic duct biopsy	5 (8%)	6 (10%)	1
IDUS for pancreatic duct	27 (45%)	25 (42%)	0.15
Procedure time greater than 30 min	29 (48%)	33 (55%)	0.50

ERCP: Endoscopic retrograde cholangiopancreatography; IDUS: Intraductal ultrasonography.

United States). Stent dislodgment was confirmed by daily serial abdominal radiography. If the stent remained on the third day, it was removed endoscopically. In the stent group, we performed PD cannulation followed by contrast injection, guidewire insertion, and stent placement under fluoroscopy. All 120 patients were hospitalized for at least 3 d after the procedure for observation of potential pancreatitis or other complication, regardless of stent placement. On the assumption that the frequency of post-ERCP pancreatitis was 2.3% and 20% in the stent and non-stent group, respectively, 60 patients were needed in each group for 80% power and 5% type I error.

### Definitions

The definition of post-ERCP pancreatitis was based on Cotton's criteria<sup>[17]</sup>, with a modified definition of severity. Instead of the number of hospital days, we evaluated the degree of severity of pancreatitis by number of days before resuming feeding. Post-ERCP pancreatitis was defined as pancreatic pain and hyperamylasemia within 24 h post-procedure. Pancreatic pain was defined as persistent pain in the epigastric or periumbilical region. Hyperamylasemia was defined as an amylase level greater than three times the upper limit of normal in our institution.

### Outcomes

The primary outcome was frequency and severity of post-ERCP pancreatitis. As secondary outcomes, we evaluated the frequency of serum hyperamylasemia, the success rate of stent placement, time to stent dislodgement, and other complications.

### Statistical analysis

This trial was designed as a superiority study to detect differences in clinical effectiveness to prevent post-ERCP pancreatitis with the addition of PD stent placement. The effectiveness of pancreatic stenting was analyzed on an intention-to-treat basis.

The  $\chi^2$  test or Fisher's exact test were used to evaluate proportional differences. The Student *t* test was used

	Non-stent	Stent	P value
Biliary disease			
CBD stone	16 (27)	15 (25)	0.68
Cholangitis	2 (3)	2 (3)	0.49
Cholangiocarcinoma	4 (7)	3 (5)	1
Cholangiocellular carcinoma	2 (3)	1 (2)	1
Benign biliary stricture	1 (2)	2 (3)	1
Primary sclerosing cholangitis	2 (3)	1 (2)	1
GB stone	1 (2)	0 (0)	1
GB polyp	1 (2)	1 (2)	1
GB adenomyomatosis	1 (2)	1 (2)	0.46
Cholecystitis	1 (2)	1 (2)	0.46
GB carcinoma	3 (5)	3 (5)	0.47
Pancreatic disease			
IPMN	10 (17)	11 (18)	0.67
MCN	0 (0)	1 (2)	1
SCN	1 (2)	0 (0)	1
Chronic pancreatitis	3 (5)	3 (5)	0.47
Pancreatic cyst	1 (2)	2 (3)	1
Pancreatic carcinoma	11 (18)	13 (22)	0.65

CBD: Common bile duct; GB: Gallbladder; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm.

for comparing continuous variables. Univariate evaluation was made for each potential risk factor. All statistical analyses were performed with StatView Ver. 5.0 (SAS Institute, Cary, NC, United States) and StatMate 4 (ATMS, Tokyo, Japan).

## RESULTS

### Patient characteristics

Tables 1 and 2 show basic patient characteristics including risk factors for post-ERCP pancreatitis and final diagnosis in both groups. The mean age ( $\pm$  standard deviation) of all patients was  $67.4 \pm 3.8$  years and the male:female ratio was 68:52. In the stent group, the mean age was  $66 \pm 13$  years and the male:female ratio was 33:27, whereas the mean age was  $68 \pm 14$  years and the male:female ratio was 35:25 in the non-stent group. There were no significant differences between groups with respect to age, gender, final diagnosis, or type of endoscopic intervention (Table 1).

### Pancreatic stenting

The placement of the PD stent was successful in all 60 patients and no complications were observed (Table 3).

### Post-ERCP pancreatitis

The overall rate of post-ERCP pancreatitis was 7.5% (9/120). The rate of post-ERCP pancreatitis in the stent and non-stent groups was 1.7% (1/60) and 13.3% (8/60), respectively ( $P = 0.032$ , Fisher's exact test). The severity of pancreatitis was mild in all nine patients (Table 4). The rate of hyperamylasemia was 30% (18/60) and 38.3% (23/60) in the stent and non-stent groups, respectively ( $P = 0.05$ ,  $\chi^2$  test).

**Table 3 Placement of the pancreatic duct stent**

No. of patients	60
Success rate in stent placement	100%
Rate of spontaneous stent dislodgement	96.7%
Duration time to dislodgement, d, (range)	2.1 (23)
Complications	
Stent migration	0%
Post-ERCP pancreatitis	1.7%
Hyperamylasemia	30%
Hemorrhage	0%
Perforation	0%
Infection (cholangitis, cholecystitis)	0%
Others	0%
Mean serum amylase level after procedures, U/L, (range)	1246 (746-1964)

ERCP: Endoscopic retrograde cholangiopancreatography.

**Table 4 Overall post-endoscopic retrograde cholangiopancreatography pancreatitis in non-stent and stent groups**

	Non-stent	Stent	P value
No. of patients	60	60	
Hyperamylasemia	23 (38.3%)	18 (30%)	0.862
Average serum amylase level (IU/L) (range)	842.4 (381-2040)	746.2 (420-1620)	0.798
Post-ERCP pancreatitis	8 (13.3%)	1 (1.7%)	0.0322
Mild	8	1	0.0322
Moderate	0	0	-
Severe	0	0	-
Average serum amylase level in pancreatitis cases (IU/L) (range)	1720 (820-2040)	1240 (746-1964)	0.04

ERCP: Endoscopic retrograde cholangiopancreatography. The Chi-square test or Fisher's exact test was used to determine the significance of associations, and  $P < 0.05$  was regarded as significant.

### Stent dislodgement

The rate of spontaneous dislodgement by the third day was 96.7% (58/60), and the median (range) time to dislodgement was 2.1 (range, 2-3) d (Table 3).

### Other complications

The rates of stent migration, hemorrhage, perforation, infection (cholangitis or cholecystitis) or other complications were 0% (0/120) in both groups (Table 3).

### Risk factors of post-ERCP pancreatitis

Table 5 shows the final diagnoses of those patients with post-ERCP pancreatitis patients in both groups. Regarding univariate analysis of risk factors for post-ERCP pancreatitis, there were no significant differences between groups with respect to various high risk factors studied (Table 6).

## DISCUSSION

We conducted a single-blind, randomized controlled clinical study to evaluate the effectiveness of spontaneous dislodgement PD stents for preventing post-ERCP pancreatitis in high risk patients. The overall rate of post-ERCP pancreatitis was 7.5%. This was consistent

**Table 5 Final diagnoses in post-endoscopic retrograde cholangiopancreatography pancreatitis patients**

	Non-stent Pancreatitis		Stent Pancreatitis	
Biliary disease				
CBD stone	16	3 (19%)	15	0
Cholangitis	2	0	2	0
Cholangiocarcinoma	4	1 (25%)	3	0
Cholangiocellular carcinoma	2	0	1	0
Benign biliary stricture	1	0	2	0
Primary sclerosing cholangitis	2	0	1	0
GB stone	1	0	0	0
GB polyp	1	0	1	0
GB adenomyomatosis	1	0	1	0
Cholecystitis	1	0	1	0
GB carcinoma	3	1 (33%)	3	0
Pancreatic disease				
IPMN	10	1 (10%)	11	0
MCN	0	0	1	0
SCN	1	0	0	0
Chronic pancreatitis	3	0	3	0
Pancreatic cyst	1	0	2	0
Pancreatic carcinoma	11	2 (18%)	13	1 (8%)

CBD: Common bile duct; GB: Gallbladder; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm.

with reported rates of post-ERCP pancreatitis, ranging from 1% to as high as 40% in patients with risk factors<sup>[1-5,7-13,18-22]</sup>. The rate of post-ERCP pancreatitis in the stent and non-stent groups was significantly different at 1.7% and 13.3%, respectively. Thus, prophylactic PD stenting may reduce the rate of post-ERCP pancreatitis from 13.3% to 1.7% in high risk patients. Our study suggested that PD stent placement prevented post-ERCP pancreatitis in high risk patients. However the severity of pancreatitis in this study was mild in all cases and we were therefore unable to assess the efficacy of pancreatic stents for severe post-ERCP pancreatitis. Considering the sample size of this study and no cases of severe post-ERCP pancreatitis, this study had a limitation to determine any significant difference in severity between the two groups.

Several prospective studies have suggested that prophylactic PD stent placement decreases the risk of pancreatitis in high risk patients<sup>[9-14]</sup>. In contrast, Smithline *et al*<sup>[2]</sup> reported that PD stent insertion after ERCP did not have a significant beneficial effect in individuals undergoing biliary sphincterotomy for various indications. These differences may be attributed to variable levels of risk for pancreatitis in the individual study populations. However, two independent meta-analysis on the use of pancreatic stent placement for post-ERCP pancreatitis prophylaxis in patients at high risk of post-ERCP pancreatitis have demonstrated that stent placement significantly reduced the incidence of post-ERCP pancreatitis<sup>[10,23]</sup>. Based on these meta-analyses, European Society of Gastrointestinal Endoscopy guidelines recommend prophylactic pancreatic stent placement to prevent post-ERCP pancreatitis in high risk patients<sup>[24]</sup>. An updated meta-analysis of RCTs involving pancreatic stent place-

**Table 6** Analysis of risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

	Non-stent	Pancreatitis	Stent	Pancreatitis	Univariate <i>P</i> value
No. of patients	60	8	60	1	
Age (< 60 yr)	17 (28)	3 (18)	18 (30)	0 (0)	0.72
Female	25 (42)	3 (12)	27 (45)	0 (0)	0.78
Previous post-ERCP pancreatitis	5 (8)	1 (20)	5 (8)	0 (0)	0.68
Sphincter of Oddi dysfunction	0 (0)	0 (0)	0 (0)	0 (0)	-
A difficult cannulation	10 (17)	2 (20)	10 (17)	0 (0)	0.68
Pre-cut	0 (0)	0 (0)	0 (0)	0 (0)	-
Pancreatic sphincterotomy	5 (8)	1 (20)	4 (7)	0 (0)	0.68
Pancreatic duct biopsy	5 (8)	1 (20)	6 (10)	1 (17)	0.87
IDUS for pancreatic duct	27 (45)	3 (11)	25 (42)	1 (4)	0.74
Procedure time greater than 30 min	29 (48)	4 (14)	33 (55)	0 (0)	0.72

ERCP: Endoscopic retrograde cholangiopancreatography; IDUS: Intraductal ultrasonography.

ment also showed that pancreatic stent placement after ERCP reduced the risk of post-ERCP pancreatitis and was beneficial for patients at high risk compared to those who did not have stenting<sup>[25]</sup>. However, several areas requiring further clarification were noted, including the efficacy of pancreatic stents for severe post-ERCP pancreatitis, identification of risk factors and management of adverse events, optimal stent design and material, timing of both placement and removal, and comparison of stenting with wire-guided cannulation or pharmacoprophylaxis<sup>[25]</sup>. Several of these points warrant further investigation.

Regarding indications for stenting, prophylactic pancreatic stent placement has been shown to be cost-effective in patients at high risk of post-ERCP pancreatitis, but not in those at average risk<sup>[26]</sup>. Caution should be used when attempting prophylactic pancreatic stent placement due to the incidence of post-ERCP pancreatitis after failed attempts, which may be as high as 65%<sup>[27]</sup>. As such, prophylactic pancreatic stent placement in high risk patients is cost-effective only if the success rate of stent placement exceeds 75%. Careful selection of patients with risk factors for post-ERCP pancreatitis is therefore critical.

The type of stent used may also play an important role in prophylactic care. As for diameter of stents, most recent studies have used 3 Fr and 5 Fr diameter pancreatic stents. In two recent RCTs, 5 Fr stents proved equivalent to 3 Fr stents for most outcomes studied, though successful insertion of 5 Fr stents was achieved significantly more often<sup>[28,29]</sup>. Placement of 3-4 Fr stents require a small-caliber guidewire (0.018-0.025 inch), a procedure that is difficult and which requires a high level of experience<sup>[17,18,30,31]</sup>. In contrast, the 0.035-inch guidewire used for 5 Fr stents is relatively easy to use for stent placement. Regarding stent length, we used very short (3 cm) stents in this study. Long stents may be more difficult to place and may have higher rates of spontaneous dislodgement, causing damage to the intestines. We therefore recommend the use of shorter stents.

Regarding placement of PD stents, previous studies reported a 5%-10% failure rate and a low rate of complications (2%)<sup>[29,13]</sup>. It is difficult to cannulate the PD after

all the procedures, and PD cannulation itself may cause pancreatitis. Unsuccessful cases were reported to be at a higher risk of pancreatitis<sup>[7,27]</sup>. In our study, we could place PD stents successfully in all patients. PD stent-related complications, such as migration or occlusion, did not occur in any patients. Based on the previous literature and our outcomes, we recommend 5 Fr diameter and very short (3 cm) stents.

As for optimal stent design, Sofuni *et al*<sup>[12]</sup> reported that the unflanged duodenal pigtail-type stent dislodged spontaneously at a higher rate, and that handling of the short duodenal pigtail stent may be complicated, such as the sudden forward movement of the stent on release, requiring close attention and experience. In addition, an internal flange may make spontaneous PD stent dislodgement difficult. In contrast, stents without an internal flange may dislodge spontaneously into the duodenum by pancreatic juice flow or friction with passing food. Since we used 5 Fr straight type stents without an internal flange, stents dislodged spontaneously in 1 d or 2 d in most cases. In our study, the spontaneous dislodgement rate was 96% at 3 d, with a median of 2.1 d. In the absence of spontaneous migration out of the PD at 5-10 d post-ERCP, prompt endoscopic stent removal is recommended due to the increased risk of post-ERCP pancreatitis (relative risk 5.2 in patients without *vs* with spontaneous stent elimination at 2 wk) and potential for stent-induced damage to the PD<sup>[28,29]</sup>. It is our recommendation to avoid the use of stents with an internal flange and to confirm stent dislodgement, or to remove stents in a timely interval, preferably within 1-2 wk after ERCP.

Finally, prophylactic pancreatic stent placement by operator and location warrants discussion. According to survey data, only a small percentage of endoscopists utilize prophylactic pancreatic stenting. The incidence of post-ERCP pancreatitis and a high ERCP volume were independently associated with the use of prophylactic pancreatic stenting<sup>[32-35]</sup>. From these surveys, endoscopists who did not place prophylactic pancreatic stents cited lack of experience in this technique as a primary reason<sup>[32]</sup>. We anticipate that a stent insertion success rate and low rate of complications can also be achieved in other institutions by ERCP specialists. Placement of

pancreatic stents is quickly becoming standard practice. However, many endoscopists remain unfamiliar with the specific techniques required to achieve safe and effective PD guidewire access and stent placement. It is critically important that all endoscopists performing ERCP become proficient in techniques for safe and effective stent placement.

Our study has several limitations. First, this study is single-blind and observation bias is inevitable. Second, we could not evaluate the risk of post-ERCP pancreatitis in patients who failed PD stent placement. Third, we could not evaluate the efficacy of PD stenting alone because nafamostat mesilate was administered in both groups.

In conclusion, pancreatic spontaneous dislodgement stent placement decreases the risk of post-ERCP pancreatitis in patients who are likely to develop post-ERCP pancreatitis.

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## COMMENTS

### Background

Acute pancreatitis is a serious complication of endoscopic retrograde cholangiopancreatography (ERCP) and its prevention remains a critical issue. Though several studies have confirmed that pancreatic duct (PD) stent placement prevents post-ERCP pancreatitis, no consensus has yet been reached on the indications for prophylactic PD stent placement or on the type of stent.

### Research frontiers

In this study, the authors demonstrated that pancreatic stent placement was a safe and effective technique to prevent post-ERCP pancreatitis. Authors recommend pancreatic stent placement after ERCP procedures in high risk patients.

### Innovations and breakthroughs

All procedures in this study were performed by one endoscopist (Kawaguchi Y), an experienced operator with more than 2000 prior ERCP cases. Independent patient-related and procedure-related risk factors for post-ERCP pancreatitis have been previously published. This study suggests that PD stenting can significantly reduce post-ERCP pancreatitis in high risk patients.

### Applications

By the general use of the pancreatic stent placement after ERCP procedures in high risk patients, post-ERCP pancreatitis may decrease in the future.

### Terminology

The authors used 5 Fr straight polyethylene stents, 3 cm in length, unflanged on the pancreatic duct side, and with two flanges on the duodenal side. As this stent dislodges spontaneously into the duodenum as a result of pancreatic juice flow or friction with passing food, it is termed a spontaneous dislodgement stent.

### Peer review

The pancreatic spontaneous dislodgement stent placement safely prevented post-ERCP pancreatitis in high risk patients. This result is very impressive.

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## Relationship between hepatitis C virus infection and type 2 diabetes mellitus: Meta-analysis

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### Abstract

**AIM:** To investigate the association between hepatitis C infection and type 2 diabetes mellitus.

**METHODS:** Observational studies assessing the relationship between hepatitis C infection and type 2 diabetes mellitus were identified *via* electronic and hand searches. Studies published between 1988 to March 2011 were screened, according to the inclusion criteria set for the present analysis. Authors performed separate analyses for the comparisons between hepatitis C virus (HCV) infected and not infected, and HCV infected and hepatitis B virus infected. The included studies were further subgrouped according to the study design. Heterogeneity was assessed using  $I^2$  statistics. The summary odds ratios with their corresponding 95% CIs were calculated based on a random-effects model. The included studies were subgrouped according to the study design. To assess any factor that could potentially affect the outcome, results were further stratified by age group (proportion of  $\geq 40$  years), gender (proportion of male gender), body mass index (BMI) (pro-

portion of BMI  $\geq 27$ ), and family history of diabetes (i.e., self reported). For stability of results, a sensitivity analysis was conducted including only prospective studies.

**RESULTS:** Combining the electronic database and hand searches, a total of 35 observational studies (in 31 articles) were identified for the final analysis. Based on random-effects model, 17 studies ( $n = 286\ 084$ ) compared hepatitis C-infected patients with those who were uninfected [summary odds ratio (OR): 1.68, 95% CI: 1.15-2.45]. Of these 17 studies, 7 were both a cross-sectional design (41.2%) and cohort design (41.2%), while 3 were case-control studies (17.6%). Nineteen studies ( $n = 51\ 156$ ) compared hepatitis C-infected participants with hepatitis B-infected (summary OR: 1.92, 95% CI: 1.41-2.62). Of these 19 studies, 4 (21.1%), 6 (31.6%) and 9 (47.4%) were cross-sectional, cohort and case-control studies, respectively. A sensitivity analysis with 3 prospective studies indicated that hepatitis C-infected patients had a higher risk of developing type 2 diabetes compared with uninfected controls (summary odds ratio: 1.41, 95% CI: 1.17-1.7;  $I^2 = 0\%$ ). Among hepatitis C-infected patients, male patients (OR: 1.26, 95% CI: 1.03-1.54) with age over 40 years (summary OR: 7.39, 95% CI: 3.82-9.38) had an increased frequency of type 2 diabetes. Some caution must be taken in the interpretation of these results because there may be unmeasured confounding factors which may introduce bias.

**CONCLUSION:** The findings support the association between hepatitis C infection and type 2 diabetes mellitus. The direction of association remains to be determined, however. Prospective studies with adequate sample sizes are recommended.

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**Key words:** Hepatitis C; Type 2 diabetes mellitus; Observational studies; Meta-analysis

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## INTRODUCTION

Hepatitis C virus (HCV) infections has been identified as one of the leading causes of chronic liver disease with serious sequelae such as end-stage cirrhosis and liver cancer<sup>[1]</sup>. Moreover, chronic HCV infection has been associated with several extrahepatic complications<sup>[2-4]</sup>. The suggestion that HCV may be associated with type 2 diabetes mellitus (type 2 DM) was first made by Allison in 1994. Since then, scores of observational studies assessing the association between HCV and type 2 DM have been published. However, these studies have provided inconclusive results, with some studies supporting the excess type 2 DM risk with HCV infection compared to non-HCV infected controls<sup>[3,5]</sup>, and some studies showed differently<sup>[6-8]</sup>. There are narrative reviews which have assessed the association between HCV infections and type 2 DM<sup>[9-13]</sup>. In 2008, a meta-analysis of observational studies reported an excess type 2 DM risk with HCV infection<sup>[14]</sup>. After these reviews were published, new observational studies in which prevalence of type 2 DM in patients with HCV infection was assessed have been carried out in endemic countries. As the epidemiology of HCV is complex and heterogeneous, information from studies across geographic regions is important. Moreover, the current review also assesses the traditional risk factors.

The objectives were (1) to investigate the available evidence on the association between HCV infections and type 2 DM; and (2) to assess the effect of study design and traditional risk factors on the association.

## MATERIALS AND METHODS

### Data sources and search strategy

Published studies that assess the association between HCV and type 2 DM were searched in MEDLINE, EMBASE and PubMed databases covering the period from 1980 to March 2011. Literature search was carried out using the combination of terms “diabetes”, “diabetes mellitus”, “type II diabetes mellitus”, “type 2 diabetes mellitus”, “type II diabetes”, “T2D”, “T2DM”, “type 2 DM”, “non-insulin dependent diabetes”, or “NIDDM” and “hepatitis”, “hepatitis C”, “hepatitis C virus”, “HCV”, “HVC”, or “chronic hepatitis” and “risk”, “risk factor”, “case-control”, “cohort”, “clinical trial”, “cross-sectional”, “epidemiology”, “observational”, “meta-analysis”, “systematic review”, or “review”. In addition,

we searched Cochrane Database of Systematic Reviews, Cochrane Central Database of Controlled Trials, Database of Abstracts of Reviews of Effects, Google Scholar, European Association for the Study of the Liver, Eurosurveillance (<http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=695>), and GlaxoSmithKline (<http://www.gsk.com/reportsandpublications.htm>). We also searched the reference lists of the retrieved articles and reviews of this field<sup>[9,10,13,14]</sup>. Our search was limited to human studies and English publications. We also contacted the corresponding authors for any missing data or clarification.

### Study selection

Inclusion criteria for studies were: (1) An epidemiologic study design to conduct a primary or secondary data analysis; (2) At least 1 comparison group without HCV; (3) Provision of sufficient data to calculate odds ratio (OR) or relative risk (RR) comparing type 2 DM in HCV infected patients to non-HCV infected patients; (4) Controlled for at least age and gender in the study design or analysis; and (5) Conducted with not less than 20 HCV-infected patients. HCV was confirmed with the detection of anti-HCV (tested with ELIZA) or HCV RNA (detected by reverse transcriptase polymerase chain reaction). Type 2 DM was confirmed with one of the following criteria; (1) Self-reported type 2 DM (i.e., physician diagnosed); (2) Self-reported diabetes with no history of insulin medication; (3) If fasting plasma glucose exceeding 7.0 mmol/L (126 mg/dL) on two separate occasions; or (4) Impaired fasting glycaemia was between 6.1 mmol/L and 7.0 mmol/L with no insulin medication. Where available, hepatitis B virus (HBV) is confirmed with positive hepatitis B surface antigen and/or detectable serum HBV DNA. Definition of covariates such as family history of diabetes was taken directly from included studies. Studies with patients having other causes of chronic liver disease such as cirrhosis, autoimmune hepatitis, steatohepatitis, primary biliary cirrhosis, primary cholangitis, and hepatocellular carcinoma were excluded. One author (Mak JW) first screened titles and abstracts of publications using eligibility criteria.

Two authors (Naing C, Ahmed SI) independently recorded the detailed information from each primary study using piloted forms that include relevant items: author, year of publication, country, confirmation of type 2 DM, confirmation of HCV, confirmation of HBV (if presented), study design, number of controls and of cases, genotype of HCV (if provided), distribution of age and gender, family history of diabetes. Any discrepancy between these two investigators was resolved by discussion, and by consultation with another author (Maung M).

### Statistical analysis

The degree of heterogeneity between studies was assessed using chi-square and  $I^2$  test. An  $I^2$  value greater than > 50% is considered substantial heterogeneity<sup>[15]</sup>. We used the assumptions that OR from a case control

Table 1 Preferred reporting items for systematic reviews and meta-analysis reporting

Section/topic	No.	Checklist item	Reported on page
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both	Title: Meta-analysis
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known	Introduction
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes and study design	Introduction
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	Methods: Search strategy and eligibility of relevant studies
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	Methods: Search strategy and eligibility of relevant studies
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated	Search strategy
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	Methods: Eligibility of relevant studies; PRISMA flowchart provided
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	Methods: Data extraction and outcome measures
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means)	Methods: Statistical analysis
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$ ) for each meta-analysis	

PICOS: Participants, interventions, comparisons, outcomes and study design; PRISMA: Preferred reporting items for systematic reviews and meta-analysis; NA: Not available.

study approximates the RR in a cohort study. The summary OR with their corresponding 95% CI was calculated based on a random-effects model. We performed separate analysis for the comparisons between (1) HCV infected and not infected and (2) HCV infected and HBV infected. The included studies were subgrouped by the study design. In order to assess any factor that could potentially affect the outcome, results were stratified by age group (proportion of  $\geq 40$  years), gender (proportion of male gender), body mass index (BMI) (proportion of BMI  $\geq 27$ ), and family history of diabetes (i.e. self reported), where there was enough data. We also examined the funnel plots for potential publication bias among the included studies. A sensitivity analysis was conducted including only prospective studies. Data entry and analysis was performed using RevMan 5.1<sup>[16]</sup>. The methods and findings of the present review have been reported based on the preferred reporting items for systematic reviews and meta-analysis checklist (PRISMA) (Table 1)<sup>[17]</sup>.

## RESULTS

### Study selection

Figure 1 provides a flowchart of the present review. We retrieved 57 full articles. Of these, 26 publications were excluded because: (1) They were reviews<sup>[10-12]</sup>; (2) They did not adequately distinguish type 2 DM from diabetes<sup>[18,19]</sup>; (3) They were conducted in a special population such as transplant patients<sup>[20,21]</sup>, patients with Human immunodeficiency virus<sup>[22]</sup>, with cryoglobulinemia<sup>[23]</sup>, or with thalassemia<sup>[24]</sup>; (4) They did not include or provide data on patients without HCV infection<sup>[25-31]</sup>; (5) They were conducted in patients with known chronic liver disease<sup>[32-35]</sup>; (6) It had less than 20 HCV infected patients<sup>[36-38]</sup>; and (7) It had duplicate data<sup>[39]</sup>. The remaining 31 publications of 35 independent studies<sup>[5,7,8,40-67]</sup> were eligible for inclusion in the present meta-analysis. Four publications<sup>[8,55,56,66]</sup> assessed both HCV positive *vs* negative and HCV positive *vs* HBV positive.

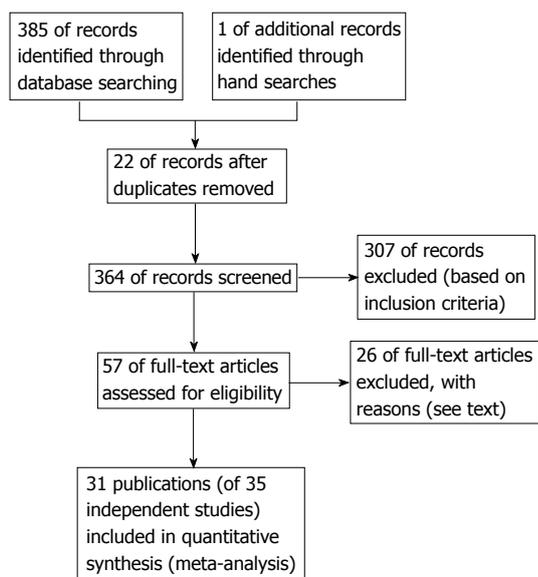
### Study characteristics

A summary of study characteristics in the present analy-

**Table 2** Characteristics of the included studies

Study	Country	Type of study	Age, yr (mean ± SD)	Confirmation of HCV	Confirmation of T2D
Akbar <i>et al</i> <sup>[40]</sup>	Saudi Arabia	CC	94% had > 40 <sup>2</sup>	Anti-HCV	FBS
Antonelli <i>et al</i> <sup>[41]</sup>	Italy	CC	65 ± 10	Anti-HCV, HCV RNA	FPG
Arao <i>et al</i> <sup>[42]</sup>	Japan	CC		Anti-HCV, HCV RNA	Random, FBG
Boschi-Pinto <i>et al</i> <sup>[43]</sup>	Japan	Cohort	65% had > 54 <sup>2</sup>	Anti-HCV	Nil
Butt <i>et al</i> <sup>[44]</sup>	United States	Cohort	50.8 <sup>2</sup>		ICD-9
Caronia <i>et al</i> <sup>[45]</sup>	Italy	CC	57.5 ± 8	Anti-HCV	FPG
Chehadeh <i>et al</i> <sup>[8]</sup>	Kuwait	Cohort	51 (23-73) <sup>3</sup>	HCV RNA	FPG
Chen <i>et al</i> <sup>[46]</sup>	Taiwan	CS		Anti-HCV	
Gulcan <i>et al</i> <sup>[47]</sup>	Turkey	CC	56.89 ± 11.9	Anti-HCV, HCV RNA	Guideline <sup>5</sup>
Howard <i>et al</i> <sup>[48]</sup>	United States	CS	51 (37-75) <sup>3</sup>	Anti-HCV, HCV RNA	Patient reported, FPG
Huang <i>et al</i> <sup>[49]</sup>	Taiwan	CC	52.7 ± 0.73	Anti-HCV, HCV RNA	FPG
Imazeki <i>et al</i> <sup>[50]</sup>	Japan	CC	45 ± 16.5	Anti-HCV, HCV RNA	FBS
Jadoon <i>et al</i> <sup>[51]</sup>	Nigeria	Cohort	48.19 ± 10.32	Anti-HCV	Clinic diagnosed
Kaabia <i>et al</i> <sup>[52]</sup>	Tunisia	CS	55.6 <sup>2</sup>	Anti-HCV, HCV RNA	Patient reported
Knobler <i>et al</i> <sup>[53]</sup>	Israel	CC	54 ± 14	HCV RNA	FPG
Lecube <i>et al</i> <sup>[54]</sup>	Spain	Cohort	52.9 ± 14.1	Anti-HCV, HCV RNA	FPG
Li-Ng <i>et al</i> <sup>[55]</sup>	United States	Cohort	30-79 <sup>4</sup>	HbsAg <sup>1</sup>	ICD-9
Mason <i>et al</i> <sup>[5]</sup>	United States	CS	72% had > 37	Anti-HCV	FPG, Random
Marzouk <i>et al</i> <sup>[56]</sup>	Egypt	Cohort	> 25 <sup>2</sup>	Anti-HCV, HCV RNA	FBS
Mehta <i>et al</i> <sup>[57]</sup>	United States	CS	> 20 <sup>2</sup>	Anti-HCV	FPG
Nwokediuko <i>et al</i> <sup>[58]</sup>	Nigeria	CS	55.8 ± 11.84	Anti-HCV	FPG
Okan <i>et al</i> <sup>[59]</sup>	Turkey	CS	51.9 <sup>2</sup>	Anti-HCV, HCV RNA	Nil
Olokoba <i>et al</i> <sup>[60]</sup>	Nigeria	CS	51.5 ± 12	Anti-HCV	FBS
Papathodoridis <i>et al</i> <sup>[7]</sup>	Greece	Cohort	48.1 ± 15.3	Anti-HCV, HCV RNA	FPG
Qureshi <i>et al</i> <sup>[61]</sup>	Pakistan	CS	42 ± 13	Anti-HCV	Random
Rouabhia <i>et al</i> <sup>[62]</sup>	Pakistan	CS	55 ± 9	Anti-HCV, HCV RNA	FPG
Ryu <i>et al</i> <sup>[63]</sup>	Korea	Cohort	44 ± 14	Anti-HCV	FPG
Sangiorgio <i>et al</i> <sup>[64]</sup>	Italy	CS		Anti-HCV	
Simó <i>et al</i> <sup>[65]</sup>	Spain	CC	46.4 ± 21.2	Anti-HCV	WHO
Wang <i>et al</i> <sup>[66]</sup>	Taiwan	Cohort	50.9 ± 14.2	Anti-HCV	FPG
Wang <i>et al</i> <sup>[67]</sup>	China	CC	50.9 ± 14.2	HCV RNA	FBS

<sup>1</sup>For HBV infection; <sup>2</sup>Mean; <sup>3</sup>Mean and range; <sup>4</sup>Range only; <sup>5</sup>American Diabetes Association Guideline. CC: Case-control study; CS: Cross-sectional study; FPG: Fasting plasma glucose; FBS: Fasting blood sugar; IFG: Impaired fasting glycaemic; HCV: Hepatitis C virus; T2D: Type 2 diabetes mellitus; HbsAg: Hepatitis B surface antigen; ICD-9: International Classification of Diseases, Ninth Revision; WHO: World Health Organization.



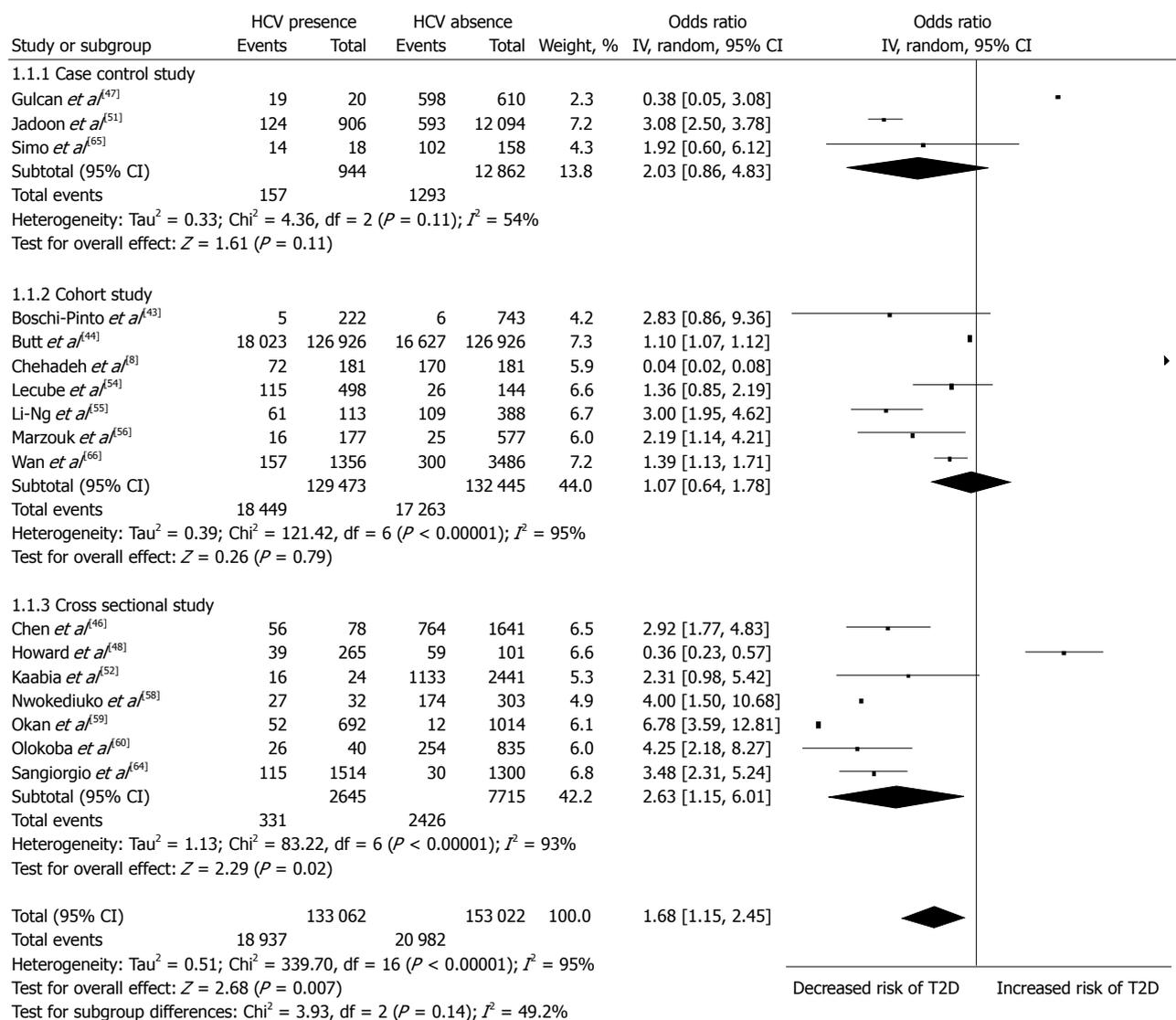
**Figure 1** Flowchart of studies identified for the present review.

sis is presented in Table 2. Five studies were carried out in United States<sup>[5,44,48,55,57]</sup>, and three each in Italy<sup>[41,45,64]</sup>, Japan<sup>[42,43,50]</sup> and Taiwan<sup>[46,49,66]</sup>, among others. Notably,

4 studies<sup>[51,60,62,67]</sup> identified for the present analysis were published between 2010 and March 2011. Of the included studies, 17 studies ( $n = 286\ 084$ ) compared HCV-infected participants with those uninfected; 7 were both a cross-sectional design (41.2%) and cohort design (41.2%), while 3 (17.6%) were case-control studies (Figure 2). Nineteen studies ( $n = 51\ 156$ ) compared HCV-infected participants with HBV-infected; 4 (21.1%), 6 (31.58%) and 9 (47.4%) were cross-sectional, cohort and case-control, respectively (Figure 3). The sample size of the included studies widely varied from 135<sup>[52]</sup> to 126 926 participants<sup>[43]</sup>.

**Main results**

Of the included studies, 17 studies ( $n = 286\ 084$ ) compared HCV-infected participants with those uninfected and the pooled OR was 1.68 (95% CI: 1.15-2.45). There was, however, substantial heterogeneity among studies ( $I^2 = 95\%$ , heterogeneity  $P < 0.001$ ). Nineteen studies ( $n = 51\ 156$ ) compared HCV- infected participants with HBV-infected and the pooled OR was 1.92 (95% CI: 1.41-2.62). There was evidence of considerable heterogeneity among studies ( $I^2 = 91\%$ , heterogeneity  $P < 0.001$ ). Among HCV-infected patients, based on available data, male patients



**Figure 2 Forest plot of comparison: Hepatitis C virus-infected patients vs hepatitis C virus-noninfected patients, outcome is type 2 diabetes mellitus.** HCV: Hepatitis C virus; IV: Inverse variance; T2D: Type 2 diabetes mellitus.

**Table 3 Stratified analysis of type 2 diabetes mellitus in hepatitis C virus-infected participants**

Description	Cases	OR	95% CI
Age (k = 3; n = 599)	455 vs 144	7.39	5.82-9.38
≥ 40 yr			
< 40 yr			
BMI (k = 3; n = 190)	65 vs 190	0.87	0.08-9.19
≥ 27			
< 27			
Gender (k = 8; n = 757)	401 vs 356	1.26	1.03-1.54
Male			
Female			
Family history of diabetes (k = 3; n = 580)	420 vs 164	4.64	0.57-38.04
Yes			
No			

OR: Odds ratio; BMI: Body mass index; k: Number of primary studies; n: Number of participants.

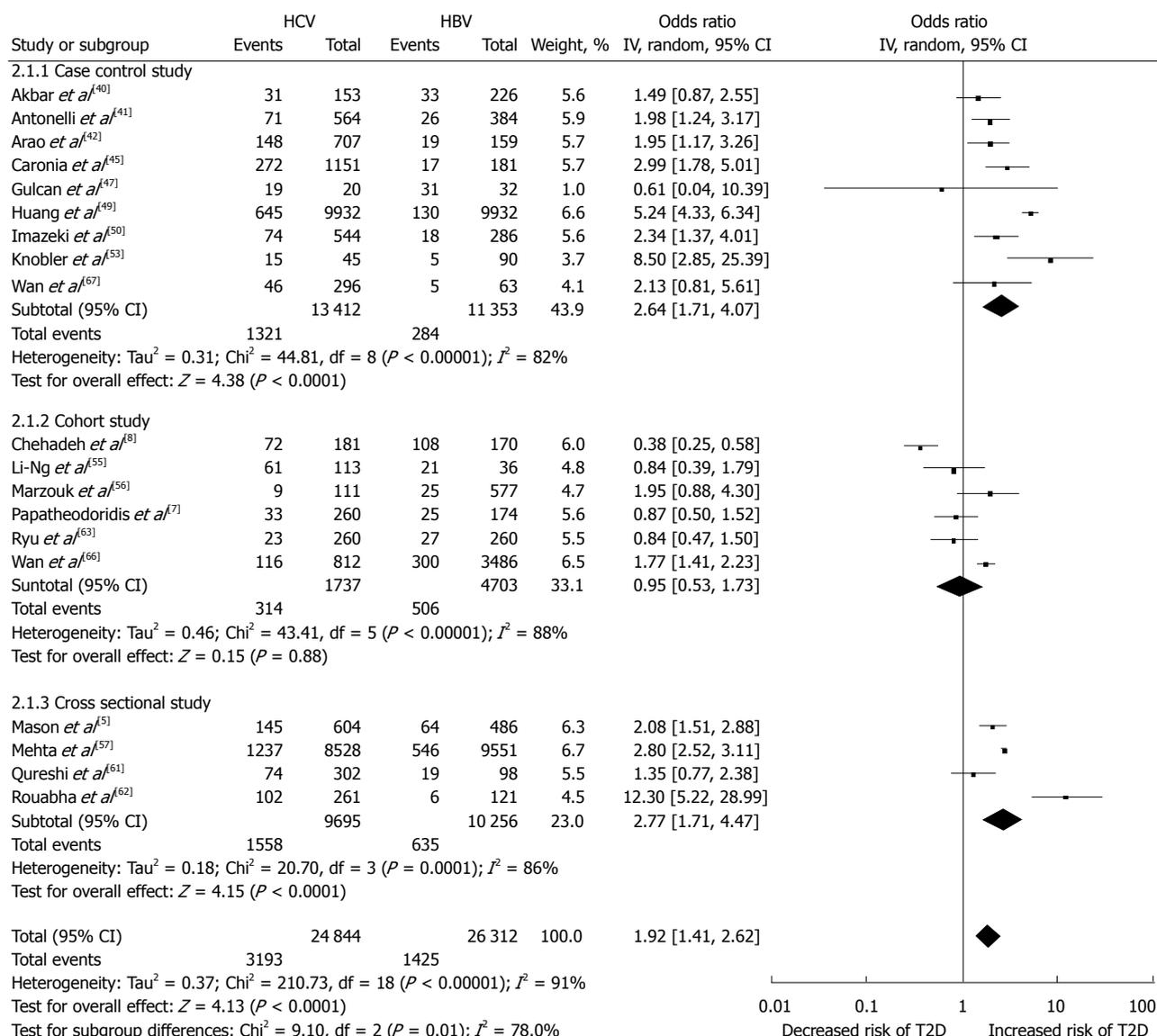
(summary OR: 1.26, 95% CI: 1.03-1.54) with age over 40

years (summary OR: 7.39, 95% CI: 5.82-9.38) had significantly increased type 2 DM prevalence (Table 3). Funnel plots of the associations between HCV and type 2 DM were investigated, providing little evidence of publication bias (Figure not shown).

For better stability of the results, sensitivity analysis with three prospective studies (n = 6449)<sup>[43,54,66]</sup> provided the pooled OR: 1.41 (95% CI: 1.17-1.7, I<sup>2</sup> = 0%), supporting the increased frequency of type 2 DM in HCV (Figure 4).

## DISCUSSION

This review indicates that patients with HCV infections were at higher risk of developing type 2 DM compared with patients with HBV infection. Findings of this review are comparable with a previous review<sup>[14]</sup>, and a large sample-individual study<sup>[56]</sup>. In the 2008 review<sup>[14]</sup> an excess risk of type 2 DM in HCV-infected cases was observed in



**Figure 3 Forest plot of comparison: Hepatitis C virus-infected patients vs hepatitis B virus-infected patients, outcome is type 2 diabetes mellitus.** HCV: Hepatitis C virus; HBV: Hepatitis B virus; IV: Inverse variance; T2D: Type 2 diabetes mellitus.

comparison to HBV-infected controls (summary OR: 1.63, 95% CI: 1.11-2.39). In the present analysis, this is also encountered (summary OR: 1.92, 95% CI: 1.41-2.62) and the association becomes stronger over time.

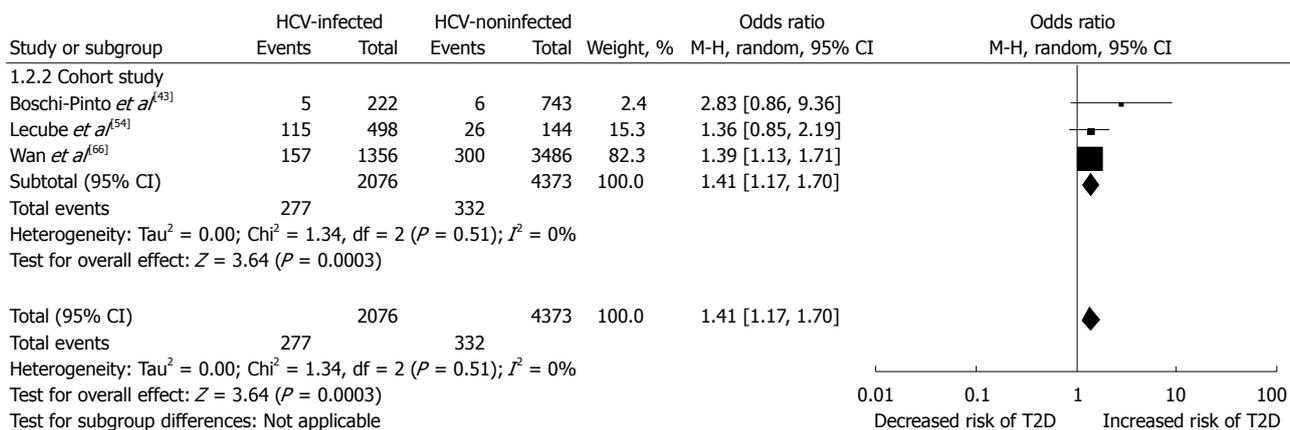
As both these viruses can replicate in extra-hepatic sites they can produce  $\beta$ -cell damage resulting in diabetes<sup>[10,61]</sup>. The lower risk in HBV infection could be explained by two factors: (1) Hepatitis B has been controlled in most developed countries, with active HBV vaccination programme; the occurrence of chronic HBV and its complications in these countries is very low; and (2) The disease progression is rather fast in HBV infection and therefore very few patients reach the level of cirrhosis and thus diabetes frequency is lower in this population<sup>[61]</sup>.

An excess risk of type 2 DM in HCV infected cases was also observed in comparison to non-HCV infected controls in the present analysis (summary OR: 1.63, 95%

CI: 1.11-2.39). The evidence of heterogeneity in these studies could be explained by variation in definition of case and control subjects and in the sample size of the primary studies.

Available studies had suggested that an expression of the HCV core protein induces hepatic insulin resistance through alterations in signaling in the insulin receptor substrate-1 pathway. This, along with other factors such as diet and obesity, can result in expression of the diabetic phenotype<sup>[38,61,68-70]</sup>. When insulin resistance reaches extents no longer compensated by the  $\beta$ -cell, insulin secretion declines and hyperglycemia emerges<sup>[10,68,70]</sup>. The complex interaction of chronic HCV infection with the host hepatic glucose and lipid metabolism has not been fully understood<sup>[10,65,67,70]</sup> and it remains to be determined.

In the studies identified, anti-HCV antibody was assessed only at the entry point of the study. Anti-HCV is considered as time-varying<sup>[43]</sup>. As such, there may be



**Figure 4 Sensitivity analysis with prospective studies: Hepatitis C virus-infected patients vs hepatitis C virus-non-infected patients, outcome is type 2 diabetes mellitus.** HCV: Hepatitis C virus; M-H: Mantel-Haenszel; T2D: Type 2 diabetes mellitus.

a likelihood of changes in serological status of anti-HCV over the study period. Liver disease and endocrine disorders, both common in the general population, have a bidirectional and complex relationship<sup>[69]</sup>. In addition, it is conceivable that patients with an earlier stage of chronic HCV infection have  $\beta$ -cell dysfunction but that diabetes does not become established until cirrhosis has supervened. Thus, a combination of  $\beta$ -cell dysfunction and insulin resistance is required for overt expression of diabetes mellitus<sup>[2,10,45]</sup>. Patients in some of the primary studies were not confirmed for the absence of cirrhosis by liver biopsy which is the best predictor of disease progression<sup>[2]</sup>. As such, we were unable to rigorously exclude cirrhosis individuals from the present analysis, and including these patients in the analysis may have exaggerated the association estimated. Of interest, it has been postulated that HCV has a permissive rather than a direct effect on the development of diabetes and acts in concert with other determinants to lead to diabetes<sup>[70]</sup>. Recent animal model evidence suggests a more direct effect of HCV infection on insulin resistance in the liver<sup>[38]</sup> indicating the role of hepatic tumor necrosis factor- $\alpha$  in affecting insulin signaling in this animal model of HCV infection<sup>[71]</sup>. In the present review, as cross-sectional and longitudinal prospective studies both show the same evidence, an excess type 2 DM risk in HCV-infected persons suggests a direct role of HCV in inducing derangement of glucose metabolism<sup>[9,10,45]</sup>. Further, there may be other factors influencing the development of type 2 DM in HCV infected patients which is not possible to address in the present analysis.

There are limitations to the present study. Most, if not all, observational studies have the potential for ascertainment bias<sup>[10,70]</sup> particularly for the studies in which diabetes status was defined by self report. Thus, there may be biased estimates of association. Moreover, recall bias is a factor in case-control studies. Although confounding factors were addressed in many of these observational studies, it is likely that there may be unmeasured confounding factors which may introduce bias into our findings. Further, as patient level data were not available

for each study, we could not make further adjustments for important factors such as genotype that were not included in most of the primary studies.

**Biological plausibility**

Findings of those prospective studies<sup>[42,53,66]</sup> which have measured HCV prior to diagnosis of type 2 DM support evidence for a temporal relationship between exposure and outcome. In a study<sup>[43]</sup>, a significant link between viral load and diabetes was found and it supported the diabetogenic role of HCV infection. The influence of viral load on the progression rate of type 2 DM was not examined in most of the studies. More research is needed to assess a dose-response association. It is also recommended that surveillance of HCV could indicate whether trends in its incidence continue to reflect changes in the prevalence of type 2 DM in the defined group.

**Public health implications**

If the associations do support temporality, the early detection and provision of aggressive antiviral treatment for HCV could prevent the development of type 2 DM, particularly in patients at high risk of HCV.

Nevertheless, the findings of the current analysis, to a certain extent, represent the HCV endemic countries. The present study has significant strengths in two ways: (1) It is comprehensive, including most recent studies; and (2) It addresses traditional risk factors (age, gender, BMI, family history of diabetes) which could potentially affect the outcome. As the prevalence of obese patients obtained in the group of HCV-positive patients with type 2 DM was significantly lower than that in diabetic HCV-negative patients found in an independent study<sup>[8]</sup> and also in the present meta-analysis, it is suggesting the pathogenesis of diabetes in HCV infection could be different from that in the general population.

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## COMMENTS

### Background

Several observational studies assessing the association between hepatitis C virus (HCV) infection and type 2 diabetes mellitus (type 2 DM) have been published. However, these studies have provided inconclusive results, with some studies supporting the excess type 2 DM with HCV infection compared to non-infected controls, and some studies showing differently. The authors, thus, performed a meta-analysis to synthesise the available evidence on the association between HCV infections and type 2 DM.

### Research frontiers

Based on the available evidence, the present study aimed to investigate the association between HCV infection and type 2 DM, and also the effect of study design and traditional risk factors on the association

### Innovations and breakthroughs

Combining the electronic database and hand searches, a total of 35 observational studies (in 31 articles) were identified for the final analysis, based on the inclusion criteria set for the present analysis. The findings support the association between HCV infection and type 2 DM. However, the direction of association remained to be determined.

### Applications

The results support an association between HCV infection and type 2 DM. Findings of this review are comparable with previous reviews, and a large sample-individual study. An early detection and provision of aggressive antiviral treatment for HCV could prevent the development of type 2 DM.

### Peer review

The authors show the review of the association between HCV infection and diabetes by meta-analysis. This paper is an interesting and instructive manuscript.

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## A population-based cohort study of symptomatic gallstone disease in diabetic patients

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ease (GSD) and to evaluate the risk of symptomatic GSD among diabetic patients.

**METHODS:** The study was conducted by analyzing the National Health Research Institutes (NHRI) dataset of ambulatory care patients, inpatient claims, and the updated registry of beneficiaries from 2000 to 2008. A total of 615 532 diabetic patients without a prior history of hospital treatment or ambulatory care visits for symptomatic GSD were identified in the year 2000. Age- and gender-matched control individuals free from both GSD and diabetes from 1997 to 1999 were randomly selected from the NHIR database ( $n = 614\ 871$ ). The incidence densities of symptomatic GSD were estimated according to the subjects' diabetic status. The distributions of age, gender, occupation, income, and residential area urbanization were compared between diabetic patients and control subjects using Cox proportion hazards models. Differences between the rates of selected comorbidities were also assessed in the two groups.

**RESULTS:** Overall, 60 734 diabetic patients and 48 116 control patients developed symptomatic GSD and underwent operations, resulting in cumulative operation rates of 9.87% and 7.83%, respectively. The age and gender distributions of both groups were similar, with a mean age of 60 years and a predominance of females. The diabetic group had a significantly higher prevalence of all comorbidities of interest. A higher incidence of symptomatic GSD was observed in females than in males in both groups. In the control group, females under the age of 64 had a significantly higher incidence of GSD than the corresponding males, but this difference was reduced with increasing age. The cumulative incidences of operations for symptomatic GSD in the diabetic and control groups were 13.06 and 9.52 cases per 1000 person-years, respectively. Diabetic men exhibited a higher incidence of operations for symptomatic GSD than did their counterparts in the control group (12.35 vs 8.75 cases per 1000 person-years).

### Abstract

**AIM:** To investigate the prevalence of gallstone dis-

**CONCLUSION:** The association of diabetes with increased symptomatic GSD may provide insight to the treatment or management of diabetes in clinical settings.

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**Key words:** Gallstone disease; Diabetes; Symptomatic; Incidence density; Hazard ratio

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## INTRODUCTION

Gallstone disease (GSD) is one of the most common and costly digestive diseases worldwide, and it is more prevalent in Europe and America than in Asia and Africa. Symptomatic GSD and its related complications inflict heavy economic costs and social burdens<sup>[1-3]</sup> because surgical gallbladder removal, usually by laparoscopic cholecystectomy, is often required. GSD affects 10%-15% of the United States population (over 25 million people). Approximately 25% of the patients require treatment, at a cost of several billion dollars annually<sup>[4]</sup>. The prevalence of GSD in Taiwan is 4.3%-10.7% and increases significantly with age, which is consistent with reports from other countries<sup>[5]</sup>. In addition, an increasing trend in the incidence of severe GSD among patients aged 20-39 years has been reported in Taiwan<sup>[6]</sup>. Published epidemiological studies of GSD have revealed a steady upward trend in the admission rates for treatment of gallstones since the 1990s<sup>[7]</sup>. However, the reported prevalence of GSD varies considerably depending on study design, patient ethnicity, and geographic region<sup>[8-11]</sup>. A number of factors, including old age, female gender, genetics, diet, obesity, diabetes, and the use of oral contraceptives or hormone therapy, have been associated with increased risk of GSD<sup>[12,13]</sup>.

Diabetic patients appear to have an increased risk of developing gallstones<sup>[14,15]</sup>. Although previous studies reported mixed results regarding the temporal relationship between GSD and diabetes, the reciprocal relationship between GSD and diabetes suggests a common etiological or biological mechanism<sup>[16]</sup> that may be reflected in gallstone composition. Gallstones are generally classified

as either cholesterol stones or pigment stones according to their morphology and composition. The composition of cholesterol stones varies widely across different populations. For example, gallstones consist of more than 50% cholesterol in Western patients and an overwhelming 95% in Germany<sup>[17-20]</sup>. A mechanistic link between diabetes and GSD was recently defined using an animal model with increased cholesterol secretion and insulin resistance<sup>[21]</sup>.

The inconsistent reports of the prevalence of GSD in diabetic patients have been attributed primarily to variations in study design<sup>[22-26]</sup>. One case-control study reported an estimated GSD rate of 32.7% in patients with diabetes and 20.8% in corresponding non-diabetic controls<sup>[14]</sup>. In contrast, Persson *et al*<sup>[22]</sup> reported no differences in the prevalence of GSD between diabetic patients and controls. Another case-control study reported that diabetes increased the prevalence of gallstones in females but not in males (47% *vs* 26%)<sup>[23]</sup>. To our knowledge, few studies have investigated the incidence of GSD in diabetic and non-diabetic patients using a cohort design. Furthermore, the majority of studies have been hospital-based or community-based, which might compromise the representativeness of the study sample, thus reducing the statistical power for comparisons of GSD risk in patients with and without diabetes. A population-based follow-up study conducted in Kinmen, Taiwan, reported that the incidence of GSD was 3.56% per year among type 2 diabetics<sup>[24-27]</sup>. However, it seems to be inappropriate to generalize the results from this small sample ( $n = 281$ ) to the entire Taiwanese population.

Examining the co-occurrence of medical conditions related to diabetes and GSD may shed light on a common etiology and enable the identification of common biological mechanisms or pathways, which may greatly contribute to clinical interventions for GSD. Furthermore, diabetic patients with a number of complications must be aware of the symptoms and treatment for all of these diseases<sup>[28]</sup>. Given that most patients with GSD are asymptomatic and are not aware that they have gallstones, although the assessment of GSD risk among diabetic patients may add disease burden to policy makers responsible for planning health care resources, this may draw attention to the importance of managing diabetes *per se* and its related complications in clinical settings<sup>[1,29,30]</sup>.

This study aimed to examine the risks of developing GSD among diabetic patients. The presence of comorbidities associated with diabetes was also evaluated in the representative diabetic cohorts retrieved from the Taiwanese National Health Research Insurance (NHRI) database.

## MATERIALS AND METHODS

### Data source

The Department of Health in Taiwan created the universal National Health Insurance (NHI) system in 1995, and approximately 96% of the Taiwanese population

had been covered in the NHI program by the end of 1996<sup>[28]</sup>. The Bureau of NHI (BNHI) has contracts with 97% of hospitals and 90% of clinics across the island<sup>[31,32]</sup>. To ensure the accuracy of the claim data, the BNHI conducts expert reviews of a random sample of 50-100 ambulatory and inpatient claims from each hospital and clinic quarterly. The computerized administrative claims and datasets compiled by the NHRI are made available to investigators for research purposes after the individual health information is encrypted to ensure privacy<sup>[33]</sup>. This study was conducted using the NHRI dataset of ambulatory care claims, inpatient claims, and the updated registry of beneficiaries from 2000 to 2008.

### Study cohorts and comorbidities

Diabetic ambulatory care claims record the patients with diabetes-related diagnoses (ICD-9: 250 or A-code: A181). An individual was classified as a diabetic patient if she or he had an initial diabetes-related diagnosis at any time in 2000 and then experienced one or more additional diagnoses within the subsequent 12 mo. The first and last outpatient visits within a given year must be at least 30 d to avoid the accidental inclusion of miscoded patients<sup>[34]</sup>. To detect newly diagnosed gallstone cases, we excluded patients who sought hospital or ambulatory care treatment for gallstones (ICD-9: 574) from 1997 to 1999. A total of 615 532 diabetic patients were identified in the year 2000.

Subjects in the control group were selected from all beneficiaries insured in 2000 who were free from both diabetes and GSD from 1997 to 2000. A total of 614 871 control individuals were randomly selected to generate an age- and gender-matched control population for the diabetic group.

Once the study subjects were identified, we examined the ambulatory care visits and hospitalization claims for selected comorbidities including hypertension (ICD-9: 401, 405), gout (ICD-9:274), hyperlipidemia (ICD-9: 272.0-272.9, A182), cystic fibrosis (ICD-9: 277.0), sickle cell anemia (ICD-9: 282.6), cirrhosis (ICD-9: 571.2, 571.5, 571.6), cholangitis (ICD-9: 576.1), Caroli's disease (ICD-9: 576.2), Crohn's disease (ICD-9: 555.9), and hemolytic anemia (ICD-9: 282-283). The comorbidities mentioned above were counted only when the initial diagnosis had been made during the study period (2000-2008).

### Study endpoints

The study subjects from the diabetic and control groups were linked to ambulatory care visits and hospitalization claims from 2000 to 2008 for possible gallstone episodes (ICD-9-CM 574). Person-years (PYs) of follow-up were calculated for each diabetic patient from the time of his/her first diagnosis of diabetes in 2000 to the date of the first ambulatory care visit or hospitalization due to gallstones prior to the end of 2008. The PYs for control subjects were defined as the period between the first day of insurance coverage by NHI in 2000 and the date that the first gallstone symptoms developed and

were diagnosed.

### Statistical analysis

The age, sex, occupation, income, and residential urbanization level were compared between diabetic patients and control subjects. The differences in the rates of selected comorbidities were assessed in the two groups. We also estimated the incidence densities of symptomatic GSD according to the subjects' diabetic status.

Cox proportion hazards models were generated to assess the gender- and age-specific effects of diabetes on the risk of developing gallstones. Hazard ratios (HR) and 95% CI were calculated to estimate the relative risk of developing symptomatic GSD. All analyses were performed using SAS statistical software (version 9.1 for Windows; SAS Institute, Inc., Cary, NC), and the results were considered to be statistically significant when two-tailed *P* values were less than 0.05.

## RESULTS

A total of 615 532 diabetic patients and 614 817 control participants who were initially free of symptomatic GSD were included in this study (Table 1). The two groups had similar baseline age and gender distributions, with a mean age of 60 years and a greater proportion of females. Although the average insurance premium was lower for patients in the diabetic group, the Charlson score was extraordinarily higher. The geographical distributions and urbanization scores of diabetic patients were also similar to those of the control group.

The diabetic group exhibited significantly higher baseline rates for all comorbidities of interest (Table 2). The largest discrepancy in prevalence was noted for hyperlipidemia (73.4% *vs* 37.8%), followed by hypertension (86.7% *vs* 61.8%) and gout (32.4% *vs* 23.3%).

Over the 8-year follow-up period, 60 734 diabetic patients and 48 116 controls developed symptomatic GSD and underwent operations. The cumulative operation rates for the diabetic and control groups were 9.87% and 7.83%, respectively (Table 3). A higher incidence of symptomatic GSD was also found in females than in males in both groups. Particularly, females under the age of 64 in the control group had a significantly higher incidence of GSD (20% and 22% more) than the corresponding males, but this difference decreased with increasing age. A similar but less significant pattern was also observed in the diabetic group (Table 3). Figure 1 shows that the cumulative incidence rates of operations for symptomatic GSD in the diabetic and control groups were 13.06 and 9.52 cases per 1000 PYs between 2000 and 2008. Diabetic men had a higher incidence of operations for symptomatic GSD than did their control counterparts (12.35 *vs* 8.75 cases per 1000 PYs), representing a significantly increased adjusted hazard ratio of 1.12 (95% CI: 1.07-1.16) for diabetic men. Furthermore, diabetic women also had a modest but significant additional risk of developing GSD during the 8-year follow-up period (HR = 1.05; 95% CI: 1.01-1.08).

Table 1 Characteristics of diabetic and control groups in this study, 2000-2008, Taiwan, China

Variables <sup>1</sup>	Control group		Diabetic group	
	<i>n</i>	%	<i>n</i>	%
Age, yr				
< 45	69 617	11.3	69 825	11.3
45-64	296 810	48.3	297 142	48.3
> 64	248 444	40.4	248 562	40.4
Mean age ( $\pm$ SD)	60.0 $\pm$ 12.8		60.1 $\pm$ 12.7	
Sex				
Female	319 308	51.9	319 310	51.9
Male	295 563	48.1	295 566	48.1
Insurance premium (NTD) <sup>2</sup>				
Dependent	156 296	25.4	169 761	27.6
< Median (19 200)	135 948	22.1	137 408	22.3
$\geq$ Median	322 627	52.5	308 363	50.1
Mean premium ( $\pm$ SD) <sup>3</sup>	20 142.6 $\pm$ 15 269.4		19 307.7 $\pm$ 14 454.7	
Charlson score				
0	551 094	89.6	0	0.0
1	49 777	8.1	444 658	72.2
$\geq$ 2	14 000	2.3	170 874	27.8
Mean score ( $\pm$ SD)	0.1 $\pm$ 0.4		1.4 $\pm$ 0.8	
Geographic area				
Northern	269 239	44.2	269 920	44.4
Central	151 693	25.0	141 321	23.2
Southern	168 995	27.8	178 627	29.4
Eastern	17 938	3.0	17 944	3.0
Urbanization status				
Metropolis	243 808	39.8	255 467	42.0
Satellite city/town	163 515	26.8	159 687	26.2
Rural area	202 343	33.2	193 949	31.8
Total	614 871	100.0	615 532	100.0

<sup>1</sup>The inconsistencies between the total population and the sums of the populations for individual variables are due to missing information; <sup>2</sup>NTD: New Taiwan Dollars; <sup>3</sup>Dependent insurers were not included.

## DISCUSSION

This study aimed to explore the risk of developing symptomatic GSD in diabetic patients compared with the general population and to examine the risk of comorbidities related to diabetes. The study revealed a higher incidence of symptomatic GSD in patients with diabetes in all age groups. Furthermore, the cumulative incidence trends were more marked in women than in men. The prevalence of selected comorbidities was higher in the diabetic group with symptomatic gallstones than in those without gallstones.

A previous study in an Italian population reported that the cumulative incidence of GSD was 0.67% per year, and GSD was more common in females than in males<sup>[13]</sup>. Another study reported that the 5-year incidence of gallstone disease was approximately 2%-3% among Danish individuals over the age of 40<sup>[12]</sup>. Our analysis of the Taiwanese NHRI datasets revealed that the incidence and the incidence density of symptomatic GSD in non-diabetic patients were approximately 7% and 9% per year, respectively. These incidence estimates are slightly higher than those measured in Western countries. Previous evidence has shown that both incidence and prevalence increase with age<sup>[1,9-12]</sup>. Therefore, one

Table 2 Prevalence of selected comorbidities at baseline in diabetic and control groups, 2000-2008, Taiwan, China

Variables <sup>1</sup>	Control group		Diabetic group		<i>P</i> value <sup>2</sup>
	<i>n</i>	%	<i>n</i>	%	
Hypertension					< 0.001
No	235 138	38.2	81 913	13.3	
Yes	379 733	61.8	533 619	86.7	
Gout					< 0.001
No	471 346	76.7	416 400	67.6	
Yes	143 525	23.3	199 132	32.4	
Hyperlipidemia					< 0.001
No	382 560	62.2	163 598	26.6	
Yes	232 311	37.8	451 934	73.4	
Cystic fibrosis					0.006
No	614 836	99.9	615 470	99.9	
Yes	35	0.1	62	0.1	
Cirrhosis					< 0.001
No	589 838	95.9	569 024	92.4	
Yes	25 033	4.1	46 508	7.6	
Cholangitis					< 0.001
No	605 197	98.4	602 604	97.9	
Yes	9674	1.6	12 928	2.1	
Caroli's disease					< 0.001
No	612 979	99.7	613 326	99.6	
Yes	1892	0.3	2206	0.4	
Crohn's disease					< 0.001
No	590 912	96.1	590 404	95.9	
Yes	23 959	3.9	25 128	4.1	
Hemolytic anemia					< 0.001
No	612 100	99.6	612 111	99.4	
Yes	2771	0.4	3421	0.6	
Total	614 871	100.0	615 532	100.0	

<sup>1</sup>Hypertension (ICD-9: 401-405, A260, A269), gout (ICD-9: 274), hyperlipidemia (ICD-9: 272.0-272.4, A182), cystic fibrosis (ICD-9: 277.0), cirrhosis (ICD-9: 571.2, 571.5, 571.6), cholangitis (ICD-9: 575.8, 576.1), Caroli's disease (ICD-9: 576.2), Crohn's disease (ICD-9: 555.0, 555.1, 555.9), hemolytic anemia (ICD-9: 282-283); <sup>2</sup>Based on  $\chi^2$  test.

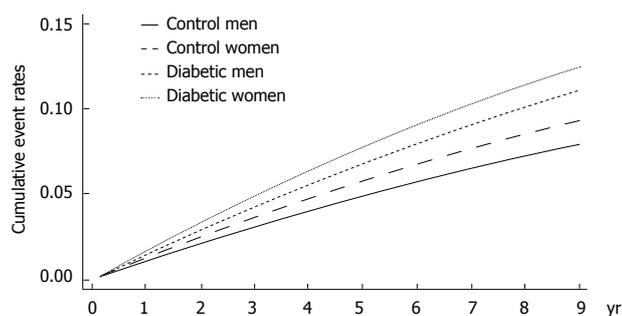
potential explanation for this discrepancy might be that the control group in this study, which was age- and gender-matched to the diabetic group, was older than the study cohorts in previous reports.

Despite the controversies about the prevalence of GSD in diabetic *vs* non-diabetic groups, GSD is not uncommon in patients with diabetes. Previous studies have reported that roughly 14%-30% of diabetic patients develop GSD<sup>[14,22-25]</sup>. One population-based follow-up study indicated that 3.56% of type 2 diabetic patients developed GSD per year. However, no conclusions about relative risk can be made based on these estimated incidence rates because no control groups were included in these studies<sup>[27]</sup>. Our study, based on a population-based dataset in Taiwan, illustrated that approximately 9.52% of the Taiwanese population developed symptomatic GSD annually, and the incidence density was higher in diabetic group than in the general population (13.06% *vs* 9.52%). In addition, females and older individuals were more likely to develop symptomatic GSD, regardless of their diabetes status. The results were consistent with the previously described epidemiology of GSD. It is noteworthy that gender differences in symptomatic GSD in-

**Table 3** Overall age- and sex-specific incidence densities and relative hazards of gallstone disease (ICD-9: 574) in diabetic and control groups, 2000-2008, Taiwan, China

Variables <sup>1</sup>	Control group			Diabetic group			aHR <sup>2</sup> (95% CI) <sup>2,4</sup> in association with diabetic group		
	No. of patients	No. of events	ID <sup>2</sup> (per 1000 patient-years) (95% CI) <sup>2,3</sup>	No. of patients	No. of events	ID <sup>2</sup> (per 1000 patient-years) (95% CI) <sup>2,3</sup>			
<b>Men</b>									
< 45	40 537	1467	4.25 (4.24-4.25)	40 537	2360	7.06 (7.06-7.07)	0.80 (0.66-0.96) <sup>4</sup>		
45-64	141 899	9184	7.59 (7.58-7.59)	141 899	12 561	11.23 (11.22-11.23)	1.03 (0.96-1.09) <sup>4</sup>		
> 64	113 127	10 241	12.31 (12.30-12.31)	113 129	12 224	16.40 (16.39-16.40)	1.20 (1.14-1.27) <sup>4</sup>		
Total	295 563	20 892	8.75 (8.75-8.76)	295 566	27 145	12.35 (12.34-12.35)	1.12 (1.07-1.16) <sup>5</sup>		
<b>Women</b>									
			Increased % vs males				Increased % vs males		
< 45	29 080	1292	5.11 (5.11-5.12)	+ 20.8%	29 079	1898	7.69 (7.69-7.70)	+ 8.9%	0.75 (0.60-0.94) <sup>4</sup>
45-64	154 911	12 596	9.30 (9.29-9.30)	+ 22.5%	154 911	15 989	12.59 (12.58-12.59)	+ 12.1%	0.96 (0.90-1.02) <sup>4</sup>
> 64	135 317	13 336	12.61 (12.60-12.61)	+ 20.8%	135 318	15 685	16.81 (16.81-16.82)	+ 2.5%	1.13 (1.07-1.18) <sup>4</sup>
Total	319 308	27 224	10.21 (10.21-10.22)	+ 2.4%	319 310	33 572	13.70 (13.70-13.71)	+ 10.9%	1.05 (1.01-1.08) <sup>5</sup>
Overall	614 871	48 116	9.52 (9.52-9.53)		615 532	60 734	13.06 (13.06-13.07)		1.08 (1.05-1.10) <sup>6</sup>

<sup>1</sup>Inconsistencies between the total population and the sums of populations for individual variables are due to missing information; <sup>2</sup>ID: Incidence density; aHR: Adjusted hazard ratio; <sup>3</sup>Based on poisson assumption; <sup>4</sup>Based on Cox proportional hazard regression adjusted for all variables, except for age and sex; <sup>5</sup>Based on Cox proportional hazard regression adjusted for all variables, except for sex; <sup>6</sup>Based on Cox proportional hazards regression adjusted for age, sex, insurance premium, Charlson score, geographic area, urbanization status, and status of diabetes, hypertension, gout, hyperlipidemia, cystic fibrosis, cirrhosis, cholangitis, Caroli's disease, Crohn's disease and hemolytic anemia.

**Figure 1** Cumulative incidence of gallstone disease in patients with or without diabetes over the study period.

incidence were not significant in patients over 64 years of age in either the diabetic or the control group, suggesting that age is a more important variable than gender.

Our findings indicate that subjects in the diabetic group suffer from more comorbidities than those in the control group, which was not surprising, but the significantly higher incidence rates of hypertension and hyperlipidemia were particularly noteworthy. Gallbladder function and bile acid metabolism are the two major factors associated with gallstone formation<sup>[35]</sup>. Diabetes or insulin resistance may affect gallbladder motility or contractility, further promoting the formation of gallstones<sup>[36-40]</sup>. This may be explained by the fact that diabetes tends to lower the levels of high-density-lipoprotein cholesterol and raise the triglyceride and low-density-lipoprotein levels, that may subsequently affect gallbladder dysmotility<sup>[35,37,39]</sup>. Previous evidence has shown that hypersecretion of hepatic cholesterol and altered lipid profiles derived from diabetic dyslipidemia may also be linked to the super-saturation of bile with cholesterol, thereby altering bile acid metabolism and cholesterol crystallization<sup>[40,41]</sup>. The higher incidence of symptomatic

GSD in diabetes may be attributable to the higher prevalence of hypertension and hyperlipidemia among diabetic patients.

Considerable clinical evidence has indicated that an array of abdominal manifestations is likely to be associated with GSD and diabetes, including cystic fibrosis, cirrhosis, cholangitis, Caroli's disease, and Crohn's disease<sup>[5,11,42-48]</sup>. Calcium salts of unconjugated bilirubin in the enterohepatic circulation have been suggested to underlie these co-occurring manifestations. For instance, bilirubin excretion may be related to an increased risk of calcium bilirubinate precipitation, especially in chronic hemolytic disorders. Chronic bacterial infections of the bile ducts may also contribute to gallstone formation by increasing the combination of unconjugated bilirubin with calcium<sup>[41,46]</sup>. Based on the results of this study (Table 2), we strongly suggest that gallstones developed in diabetic patients were primarily cholesterol stones; this hypothesis will be examined directly in future studies.

Gallstone formation may be caused by many etiological factors, each of which may produce different clinical consequences. Most patients remain asymptomatic for a long period, frequently for life. Gallstones may traverse the cystic duct with or without symptoms of obstruction. Transient cystic duct obstruction causes periodic painful episodes, whereas persistent obstruction usually produces inflammation and acute cholecystitis, leading to the onset of symptomatic GSD. However, there is little information regarding the direct mechanisms that underlie the increased onset of GSD in diabetes. Because elderly patients are more likely to develop symptomatic GSD, it is important to diagnose GSD early in patients with diabetes. Patients may benefit from the early detection of GSD and the underlying comorbidities that may promote both GSD and diabetes, which could subsequently enhance the effectiveness of diabetes management.

Urban-rural differences were also detected in this study. Diabetic patients in metropolitan areas had a higher incidence of symptomatic GSD than patients from rural areas. The observed urbanization-level differences likely reflect the differences in the distribution of medical resources and/or treatment-seeking behaviors<sup>[49]</sup>. Future research is needed to explain the urban-rural variations and enable policy-makers to promote policies that will reduce or eliminate these differences.

There were several limitations that should be noted in this study. First, potential misclassification might arise due to our exclusive reliance on claims datasets. A previous report showed that the accuracy of diabetes diagnoses in the NHI claims data was only 74.6%<sup>[50]</sup>. In order to avoid this bias, we included and analyzed only the patients that had been diagnosed with diabetes at least twice, with the first and the last outpatient visits at least 30 d but less than one year apart. It is also possible that newly diagnosed or undiagnosed diabetic cases without records of ambulatory care visits were included in the control group. Therefore, the overall difference in incidence of GSD could have been underestimated<sup>[51]</sup>. Second, type 1 and type 2 diabetes were not differentiated in this dataset, which limited our interpretation of the study findings. However, other studies have shown that type 2 diabetes accounted for the majority of diabetic patients in Taiwan; as a result, the interpretations of our results are likely to be most relevant for type 2 diabetes<sup>[52,53]</sup>. Third, other factors that might confound our results were not available, such as body mass index, socioeconomic status, duration and treatment of diabetes, smoking, alcohol use, family history of GSD, *etc.* Fourth, the incidence of symptomatic GSD among diabetic patients with/without comorbidities was not reported; therefore, these differences in prevalence could not be analyzed.

Despite the above-mentioned limitations, this is one of very few studies to examine the risk of symptomatic GSD in patients with diabetes using a Taiwanese population-based cohort study design. Given the high coverage rate of National Health Insurance in Taiwan, the likelihood of non-response and loss to follow-up was relatively limited, ensuring the representativeness of the sample. In addition, we took advantage of the longitudinal nature of the NHI dataset to follow up the incidence rate of symptomatic GSD and related comorbidities in diabetic and control groups.

In conclusion, an increased risk of symptomatic GSD in diabetic patients over an 8-year study period was observed in this study. Diabetes and GSD may share a number of common risk factors or etiologies. A crucial link between insulin resistance and increased cholesterol predisposed the diabetic patients to gallstone formation<sup>[21]</sup>. These results may provide insight into the treatment or management of diabetes in clinical settings. Future research is needed to facilitate public health prevention or intervention programs to reduce the incidence of symptomatic GSD<sup>[54]</sup>.

## COMMENTS

### Background

Gallstone disease (GSD) is one of the most common of all digestive diseases worldwide. Symptomatic GSD and related complications necessitate surgical removal of gallbladder, inflicting a heavy economic costs and social burdens. Most patients with GSD are asymptomatic and unaware of having gallstones. Diabetic patients with complications particularly require an adequate awareness for care management of GSD. The apparent incongruity for GSD prevalence in diabetic patients should be attributable to varied study designs.

### Research frontiers

This study aimed to investigate the incidence of GSD and examined the risks of developing symptomatic GSD among the diabetes using a cohort design and retrieving data from the National Health Insurance Research database of Taiwan.

### Innovations and breakthroughs

The results showed that the cumulative operation rates for diabetes and controls were 9.87% and 7.83% among 615 532 diabetic patients and 614 817 control participants over the 8-year follow-up period. Diabetic patients also tended to have significantly higher prevalence in developing comorbidities, most notably hyperlipidemia and hypertension. Higher incidence of symptomatic GSD was found in females than in males in both groups. Females aged < 64 years in control group had a significantly higher incidence than corresponding males, but the difference reduced with increasing age. A similar but less significant pattern was also observed in diabetic group. Both diabetic men and women were characterized with higher incidence of symptomatic GSD operation than their corresponding counterparts after 8-year follow-up. The authors concluded that higher incidence of symptomatic GSD was found in patients with diabetes in all age groups and the trends of cumulative incidence were more marked for women than men. In addition, the prevalence of selected comorbidities in diabetic group with gallstone was also higher than those without symptomatic GSD.

### Terminology

GSD is caused by gallstones that block the normal flow of bile if they lodge in any of the ducts that carry bile from the liver to the small intestine. Severe damage or infections affecting the gallbladder, liver, or pancreas can occur if any of these ducts remain blocked for a significant period of time, which necessitate surgical removal of gallbladder, usually by laparoscopic cholecystectomy.

### Peer review

This is a good study in which authors analyzed incidence of symptomatic GSD in patients with diabetes by using a representative database over a long period of time. The results are interesting and suggest that diabetes is associated with increased risk of developing symptomatic GSD. The investigators also drew a conclusion that patients with diabetes developed more symptomatic GSD in all age groups and were more notable for women than men. This study was based on a nationwide population-based datasets, which illustrated that about 9.52% population in Taiwan developed symptomatic GSD per year and the incidence density was higher in diabetic patients (13.06%). Diabetes associated with increased risk of developing symptomatic GSD was observed over an 8-year study period. Being female and with older ages were associated with increased incidence of symptomatic GSD, however, the potential importance of gender was overridden by aging.

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## Swab culture monitoring of automated endoscope reprocessors after high-level disinfection

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### Abstract

**AIM:** To conduct a bacterial culture study for monitoring decontamination of automated endoscope reprocessors (AERs) after high-level disinfection (HLD).

**METHODS:** From February 2006 to January 2011, authors conducted randomized consecutive sampling each month for 7 AERs. Authors collected a total of 420 swab cultures, including 300 cultures from 5 gastro-scope AERs, and 120 cultures from 2 colonoscope AERs. Swab cultures were obtained from the residual water from the AERs after a full reprocessing cycle. Samples were cultured to test for aerobic bacteria, anaerobic bacteria, and mycobacterium tuberculosis.

**RESULTS:** The positive culture rate of the AERs was 2.0% (6/300) for gastro-scope AERs and 0.8% (1/120) for colonoscope AERs. All the positive cultures, including 6 from gastro-scope and 1 from colonoscope AERs, showed monofloral colonization. Of the gastro-scope

AER samples, 50% (3/6) were colonized by aerobic bacterial and 50% (3/6) by fungal contaminations.

**CONCLUSION:** A full reprocessing cycle of an AER with HLD is adequate for disinfection of the machine. Swab culture is a useful method for monitoring AER decontamination after each reprocessing cycle. Fungal contamination of AERs after reprocessing should also be kept in mind.

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**Key words:** Automated endoscope reprocessor; Gastrointestinal scope; High-level disinfection; Swab culture; Monitoring; Decontamination

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### INTRODUCTION

Gastrointestinal (GI) scopes are complex reusable instruments that require unique consideration with respect to decontamination. Most of the guidelines with updated guidance emphasize decontamination of these scopes<sup>[1-3]</sup>. While decontamination has been reviewed by several working groups in Britain, problems related to preventing contamination of rinse water, and procedures to monitor contamination have not been addressed thus far. In a recent study, we reported that GI scope contamination might be the result of a contaminated automated

endoscope reprocessor (AER)<sup>[4]</sup>. There is currently no literature on the quality of disinfection of AERs after reprocessing with high-level disinfection (HLD). Therefore, we conducted this bacterial culture study on AERs after HLD in order to monitor the quality of disinfection.

## MATERIALS AND METHODS

From February 2006 to January 2011, a 5-year prospective bacterial study was conducted with randomized consecutive sampling every month in GI scope unit, Chang Gung Memorial Hospital, Kaohsiung Medical Center. We took a total of 420 swab cultures, including 300 cultures from gastroscopy AERs and 120 cultures from a colonoscopy AER. The swab cultures were obtained from the dependent part of the inner surface of the AER after a full reprocessing cycle. Collected samples were cultured to test for aerobic and anaerobic bacteria and mycobacterium tuberculosis. The samples were incubated at 37 °C and examined for bacterial growth at 24 h and 48 h and for mycobacterium growth at 6 wk, and then the results were analyzed.

Culture results were reported as positive or negative. If a culture was positive, the specific AER was reprocessed and could only be used again for clinical use after repeated cultures were found negative according to our previous method<sup>[2]</sup>. GI scope decontamination was performed in accordance with the guidelines of the European Society of GI Endoscopy (ESGE)<sup>[3]</sup>. Manual cleaning was performed by trained GI nurses, with tap water, enzymatic soap, brushing, and irrigation, followed by AER, performed by a trained health technician. The liquid disinfectant used was 2.4% alkaline glutaraldehyde, and disinfectant-soaking duration was 20 min. If the cultures were positive, the soaking duration was prolonged to 25 min. The disinfectant was forced into the working channels and the GI scope was completely submerged. Then, the GI scopes were flushed with sterile filtered water prior to forced air-drying. The disinfectant solution, 2.4% alkaline glutaraldehyde, was stored at a temperature of 15 °C-30 °C and changed every 2 wk despite overstorage<sup>[4]</sup>.

### Reprocessing cycle of AER

After each scope procedure, thorough manual cleaning with Endozime Premium (Ruthof Corporation, NY, United States), including brushing and flushing of all accessible endoscope channels, was performed before automatic endoscope disinfection. We used the EW-30 AER (Aizu Olympus Co., Ltd, Tokyo, Japan) for reprocessing. Manual cleaning and reprocessing was performed by a fully trained scope nurse using accredited standards of practice as defined by the Digestive Endoscopy Society of Taiwan. HLD involved total immersion of the scope in 2.4% alkaline glutaraldehyde solution (Cidex 14, Ethicon, Inc., NJ, United States) for 20 min at a preset temperature of 25 °C and an additional washing cycle of 30 min

**Table 1** Rate of positive swab culture from the automated endoscope reprocessor after gastroscopy and colonoscopy reprocessing *n* (%)

Category	AER	P value
Gastroscopy ( <i>n</i> = 300)	6 (2.0)	NS
Colonoscopy ( <i>n</i> = 120)	1 (0.8)	NS
Total ( <i>n</i> = 420)	7 (1.7)	NS

AER: Automated endoscope reprocessor; NS: Not significant.

**Table 2** Organisms from swab culture of automated endoscope reprocessor after a full cycle of reprocessing with high-level disinfection

Category	Gastroscopy	Colonoscopy	Total
GNGN Bacteria <sup>1</sup>	2	1	3
<i>Moraxella osloensis</i>	1	-	1
Yeast-like organisms	2	-	2
<i>Candida glabrata</i>	1	-	1
Total positive culture	6	1	7

<sup>1</sup>All of the positive cultures had aerobic bacteria and mono-floral colonization. GNGN: Glucose-nonfermenting gram-negative bacteria.

in each reprocessing. The disinfectant was forced into the suction channels and the scope was completely submerged. The normal relief valve pressure of the AER was  $1.85 \pm 0.05$  kgf/cm<sup>2</sup>, and the water supply requirements were 17 L/min. Subsequent flushing with 200 cc of 90% alcohol for 10 min, rinsing, and drying were essential steps to remove the chemical solution and prevent bacterial colonization during storage. The rinse cycle used reverse osmosis-treated water for decontamination.

### Statistical analysis

The  $\chi^2$  test was used to analyze independent and paired samples. Statistical analyses were performed using the SPSS statistical software for Windows, version 19.0 (Chicago, IL, United States). *P* values less than 0.05 were considered statistically significant.

## RESULTS

The overall positive culture rate was 1.7% (7/420) in swab cultures from AERs after a full reprocessing cycle with HLD. For gastroscopy and colonoscopy AERs, the positive swab culture rates were 2.0% (6/300) and 0.8% (1/120) respectively, without a statistically significant difference in the culture rate between the upper and lower GI scope AERs (Table 1). All 7 positive swab cultures, including 6 gastroscopy reprocessing culture and 1 colonoscopy reprocessing culture, showed monofloral colonization. None of the cultures was positive for mycobacterium tuberculosis, and no anaerobic bacteria were found in any swab cultures. Among the cultures from gastroscopy reprocessing, 50% (3/6) were positive for aerobic bacteria, while the remaining 50% (3/6) showed fungal contamination (Table 2).

## DISCUSSION

The British Society of Gastroenterology Endoscopy Committee first published recommendations on endoscope decontamination practices in 1988, and recommendations from the fourth working group were published in the journal *Gut* in 1998<sup>[1]</sup>. Some of these decontamination recommendations are based on microbiological studies<sup>[5-8]</sup>. Most of the decontaminating guidelines are directed towards GI scopes and associated devices, but no literature is available on AER decontamination. According to our previous report, leakage of the inflow water valve of an AER could be one of the reasons for failure of decontamination of GI scopes and associated devices, even after subjecting them to a full reprocessing cycle<sup>[4]</sup>. Therefore, in this study, we aimed to monitor proper disinfection of AERs after HLD; to the best of our knowledge, this is the first study to do so. The overall positive culture rate of swab cultures from AERs after a full reprocessing cycle with a HLD process was 1.7% (7/420). Surprisingly, the rate was lower than the previously reported 18.4%-24% contamination rate for GI scope culture<sup>[5-8]</sup>. This suggests the contamination of GI scopes is not fully caused by AER contamination. We would like to clarify that since drying has been shown to be an important component of GI scope decontamination, the same is true of AERs as well?

The importance of drying in decontamination to make this point clearer is performing in our ongoing study. On the other hand, controlled trials in the field of GI scope decontamination are lacking because of a reluctance to expose “placebo control” patients to the risk of an infection. A controlled study to clarify the relationship between AER and GI scope contamination is necessary and is ongoing in our lab. An AER should be used for all GI scope decontamination following manual cleaning. Effective disinfection is difficult to achieve due to the complex nature of the internal structures of these long and narrow diagnostic instruments<sup>[4,9,10]</sup>. Manual disinfection is unacceptable. Inflow water used in an AER should be free of particulate contamination and microorganisms. This can be achieved either by using bacteria-retaining filters or by reverse osmosis. In our GI scope units, we used water treated by reverse osmosis in AER reprocessing<sup>[10]</sup>. The final rinse water should be sampled from the AER and regularly tested for microbiological quality in accordance with the current Health Technical Memorandum (HTM)<sup>[11]</sup>. A glutaraldehyde-based disinfectant (Cidex<sup>®</sup>) that was widely used in the past has been withdrawn from the United Kingdom market by its manufacturer. This is not only because there have been advances in the development of disinfectants with superior bactericidal activity but also because glutaraldehyde is chemically related to formaldehyde and has similar toxic effects on the skin and mucous membranes as formaldehyde does. The resulting adverse effects include severe dermatitis, conjunctivitis, sinusitis, asthma, and even chemical colitis. A further problem with glutaraldehyde-based disinfectants is their potential to cross-link residual protein material. The resulting amalgam is very difficult to remove from the working channels

of endoscopes that have been repeatedly flushed with aldehydes<sup>[3]</sup>. This again underscores the importance of manual pre-cleaning and brushing of all accessible internal channels and valve chambers before disinfection. Glutaraldehyde and its derivatives kill most bacteria and viruses (including human immunodeficiency virus and hepatitis B) in less than 5 min. Mycobacteria are more resistant to 2% glutaraldehyde, and earlier guidelines recommended that endoscopes be immersed in 2% glutaraldehyde for 20 min at room temperature<sup>[1]</sup>. Although we did not detect mycobacterial contamination in our study, we found that of the 1.7% positive cultures from AERs, 50% (3/6) were positive for fungal contamination. The high rate of fungal contamination is most likely due to failure to properly dry the AER after completion of reprocessing. Other than manual pre-cleaning and reprocessing disinfection, the last of the major processes of decontamination of a scope is drying before storage<sup>[3]</sup>. This step can prevent contamination by fungus or bacterial colonization on the surface of the GI scope after disinfection. It has been recommended that, before the start of each list, each scope to be used should undergo a full reprocessing cycle unless last used and decontaminated within the preceding 3 h. Many GI units are now using drying and storage chambers built purposefully for these scopes, some of which have been shown to prevent colonization of endoscope channels for up to 72 h. Therefore, all AERs should be validated and tested in accordance with guidance provided in the DoH Estates and Facilities HTM publications and relevant standards<sup>[12]</sup>. AERs should also include flow monitoring for each individual channel to detect blockages.

Furthermore, variant Creutzfeldt-Jacob disease (vCJD) is a rare and fatal condition caused by the consumption of beef contaminated by the bovine spongiform encephalopathy agent<sup>[13]</sup>. In contrast to the traditional forms of CJD, vCJD contaminated in GI tract, conventional HLD with AER was reported hard to full decontamination. ESGE guideline suggested that endoscope study is not recommended in possible patients<sup>[14]</sup>. Fortunately, there is no patient with suspicion of vCJD infection before endoscope examination, and there was no positive culture for vCJD in our series. The further study is necessary.

In conclusion, a full reprocessing cycle of an AER with HLD is adequate for disinfection of the machine. Swab culture is a useful method for monitoring AER decontamination after each reprocessing cycle. Fungal contamination of AER after reprocessing should be considered.

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## COMMENTS

### Background

Swab culture is a simple method for the detection of bacterial contamination. In the real world, it is always used to monitor the clearing effectiveness such

as the button of the elevator. But up to now, there are still now ideal methods to monitor the decontaminated effect of automated endoscopy reprocessor itself in clinical practices.

### Research frontiers

Automated endoscopy reprocessor is a very important washing machine for the endoscopy decontamination in daily clinical practice. Authors apply the swab culture method to monitor the examined endoscopy, which is the source of the reprocessor contamination.

### Innovations and breakthroughs

In fact, decontamination of the automated endoscopy reprocessor is limited description before. Swab culture is a common method for the identification of the pathological organisms from the wound infection. For the quality of the infection control and the hospital identification with high standard monitoring, the results of the swab culture from the automated endoscopy reprocessor should be a standard score of a hospital identification and guideline in the clinical practice in the future.

### Applications

The study results suggest that the method of swab culture from the inner surface of automated endoscopy reprocessor is a useful method that could be used in monitoring decontamination after a complete endoscopy reprocessing cycle.

### Terminology

Automated endoscopy reprocessor: Automated endoscopy reprocessor is a automatic washing machine for the decontamination of the practically used endoscopy. Accompanist with high-level disinfection, it is effective prevention the hospital acquired microbiological infection.

### Peer review

This is an interesting prospective study. Which has dealt with an important topic not properly covered before. The authors analyze the monitoring effect of swab culture from the inner surface of automated endoscopy reprocessor after the end of daily decontamination. The results are interesting and suggest that swab culture is a potential monitoring method that could be used in preventing not complete disinfection or contamination induced by hospital acquired infectious outbreak.

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## Prognostic significance of PTEN, Ki-67 and CD44s expression patterns in gastrointestinal stromal tumors

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### Abstract

**AIM:** To develop a prognostic approach for gastrointestinal stromal tumors (GISTs) using a cluster of indicators and follow-up information.

**METHODS:** One hundred and four GISTs that had not been subjected to targeted therapies were collected and classified by NIH risk assessment and anatomic location. By immunohistochemistry, the expressions of PTEN, Ki-67, CD44s matrix metalloproteinase (MMP)-9 and TIMP-1 were detected on tissue microarray. Univariate and multimarker survival analyses were performed and then a COX hazard proportion model was constructed to evaluate a cluster of predictors of GIST.

**RESULTS:** Our data showed small intestinal GIST are more aggressive than gastric GIST. The NIH risk assessment correlated with disease-free survival for

either gastric GIST or small intestinal GIST. Immunohistochemical analysis revealed that Ki-67 labeling indexes (LIs) < 5% predicted higher disease-specific survival (DSS) in gastric and small intestinal GIST. CD44s positivity and PTEN LIs  $\geq$  50% correlated with higher DSS in gastric GIST. MMP-9 and TIMP-1 had no correlation with survival. Multimarker analysis revealed that the expression pattern of PTEN LIs  $\geq$  50% combined with Ki-67 LIs < 5% and CD44s positivity reliably predicted favorable outcomes for gastric GIST ( $P = 0.009$ ), as did the combination of PTEN LIs  $\geq$  50% and Ki-67 LIs < 5% for small intestinal GIST ( $P = 0.011$ ). Authors also found that high NIH risk grade was correlated with DSS in patients with gastric GIST and disease-free survival in patients with small intestinal GIST.

**CONCLUSION:** PTEN LIs  $\geq$  50%, Ki-67 LIs < 5% and CD44s positivity provides an accurate, favorable prognosis for gastric GIST. PTEN LIs  $\geq$  50% and Ki-67 LIs < 5% does the same for small intestinal GIST. Ki-67 LIs enhances the NIH assessment.

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**Key words:** Gastrointestinal stromal tumor; Prognosis; PTEN; Ki-67; CD44s

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### INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most com-

mon mesenchymal tumor in the alimentary tract. The principle pathogenesis of GIST has been identified as the continuous activation of KIT, caused by gain-of-function mutation in c-kit<sup>[1]</sup> or platelet derived growth factor  $\alpha$  (PDGFRA)<sup>[2]</sup>. Based on this finding, the molecular targeted therapy of imatinib<sup>[3]</sup> or sunitinib<sup>[4,5]</sup> has achieved great success.

Clinicians are trying to improve the survival rate of GIST patients by prophylactic interference of imatinib or sunitinib, based on the prognosis. Because of the expense of the targeted therapy, an accurate prognosis is very important, but prognoses of GIST are highly variable. Although the NIH risk assessment is currently used as a standard guideline for localized tumors<sup>[6]</sup>, many researchers consider that evaluation of large cohorts is necessary for reliable data. Some modifications have been made to the NIH assessment, to enhance prediction accuracy<sup>[7,8]</sup>. Current researchers have also found some molecular indicators, such as p16<sup>INK4a</sup><sup>[9]</sup>, PTEN<sup>[10]</sup>, p53<sup>[11,12]</sup>, p27<sup>[13,14]</sup>, CD44s<sup>[15,16]</sup> and some other cell-cycle regulators<sup>[17]</sup>, but their utility is debated. Despite the mixed opinion, there are limitations in using one marker for a prognosis. A tumor is resulted from the accumulation of numerous molecular incidents, and one prognostic marker may only be applicable to a minority of patients. Our objective was to find a multi-marker indicator to improve prognoses for all patients. PI3K/Akt has been found to be a major signal transduction pathway in GIST<sup>[18]</sup>. Accordingly, we selected PTEN, the inhibitor of PI3K/Akt, for analysis. The markers related to adhesion and metastasis, such as CD44s, matrix metalloproteinase (MMP)-9 and TIMP-1, were also selected. Ki-67 was chosen to analyze the prognostic value of cell proliferation. All five markers were detected on tissue microarray of 104 GISTs. Follow-up survival data was collected. The prognostic values of these five markers were analyzed and compared to the NIH risk assessment.

## MATERIALS AND METHODS

### Case collecting

A total of 155 gastric and small intestinal GISTs were collected from the archive of the Pathology Department of Chinese PLA General Hospital. All the tumors were reassessed by the immunohistochemical panel of CD117, CD34, SMA and S-100 protein. The cases with CD117 positivity were diagnosed as GIST. One hundred and four cases were completely followed-up. One hundred and twenty one samples were obtained from these 104 cases. These tumor samples included primary, recurrent and metastatic GIST. All the tumor paraffin blocks were used to construct the tissue microarray.

### Risk assessment

Formalin fixed, paraffin embedded, HE stained slides were reviewed by two experienced pathologists. The mitotic index was determined by counting 50 adjacent high-power fields in the most active areas. Using the NIH

risk assessment<sup>[6]</sup>, all the tumors were classified into four grades of risk: very low, low, intermediate and high.

### Tissue microarray and immunohistochemistry

Three tissue cores, 1 mm in diameter, were sampled from each tumor specimen to construct the tissue microarray (TMA). The TMA blocks were sectioned at 4  $\mu$ m and stained with hematoxylin-eosin sequentially. Antigen retrieval was carried out with EDTA (pH 8.0; Santa Cruz Biochemistry, Calif) for 15 min by microwave. The primary antibody was incubated for 1 h at room temperature and subsequently detected using a Two-step PicTure™ kit (PV6000, Invitrogen Co. Carlsbad California United States). The primary antibodies, sources, and dilutions were as follows: CD117, A4502, polyclonal, Dako A/S Co. Ltd., Denmark, 1:200; SMA, clone 1A4, Invitrogen Co. Carlsbad California, United States, 1:100; S-100, clone 4C4.9, Invitrogen Co. Carlsbad California, United States, 1:50; Ki-67, clone K-2, Invitrogen Co. Carlsbad California, United States, 1:50; PTEN, clone 28H6, Invitrogen Co. Carlsbad California, United States 1:100; CD44s, clone 156-3c11, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:50; MMP-9, clone 15W2, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:25; TIMP-1, clone 6F6a, Novocastra Laboratories Ltd. Newcastle, United Kingdom, 1:50.

The labeling indexes (LIs, %) of PTEN and Ki-67 were determined by counting 1000 cells in the most active area. For CD117, SMA, S-100, CD44s, MMP-9 and TIMP-1, staining of more than 10% of the tumor cells was considered positive.

### Statistical analysis

All the patients were followed up after the resection of the tumor. The death of patients with GIST, untreated with imatinib, was selected as the end point. We analyzed follow-up data using Stata Statistical Software (Intercooled Stata 7.0, Stata Co., College Station, TX). The  $\chi^2$  test was used to analyze the expression status of the selected markers. Survival analysis was performed using Kaplan-Meier plots and the log-rank test to reveal the prognostic usefulness of the selected markers. Univariate and multivariate COX proportional hazard models with both backward and forward elimination of variables were also constructed to find the most significant factor for prognosis. Statistical significance was set at  $P < 0.05$ .

## RESULTS

### Clinicopathologic features and the follow-up data

In a total of 155 patients, having a male-to-female ratio of 2.5:1 (111 *vs* 44), there were 83 gastric and 72 small intestinal GISTs. A total of 104 cases had complete follow-up data. The median follow-up time was 33 mo, within the range of 3 to 230 mo. Fifty one cases without follow-up data were excluded from the subsequent survival analysis.

In 83 gastric patients, the male to female ratio was 2.1:1 (55 *vs* 26). The age ranged from 13 to 82 years (mean: 55.4

years; median: 57 years). The 62 followed-up cases included 42 males and 20 females. Thirteen patients died of GIST, four were alive with GIST and 45 were disease-free. The survival time of the 13 died patients ranged from 6 to 132 mo. The 3-year disease-specific survival rate (DSS) was 80.77%  $\pm$  11.5% and the 5-year DSS was 66.51%  $\pm$  17.06%.

In 72 small intestinal patients, the male to female ratio was 3.3:1 (55 *vs* 17). The age ranged from 20 to 77 years (mean: 50.6 years; median: 51.5 years). Forty-two followed-up cases included 31 males and 11 females. Fifteen patients died of GIST, four were alive with GIST and 23 were event-free. The survival time of the died patients ranged from 3 to 230 mo. The 3-year DSS was 73.65%  $\pm$  14.24% and the 5-year DSS was 61.76%  $\pm$  18.30%. There was no significant difference between the DSS of patients with gastric and small intestinal GIST ( $P = 0.274$ ).

There were 23 patients that suffered recurrence, nine with gastric GIST and 14 with small intestinal GIST. Most of them were noted to have intra-abdominal spreading. A total of 16 patients developed metastasis, eight with gastric GIST and eight with small intestinal GIST. Small intestinal GIST presented a higher liability to recurrence and metastasis than gastric GIST (22/42 *vs* 17/60,  $P = 0.013$ ). GIST metastasized to the liver in 15 cases, indicating that the liver was another common metastatic site. One of these 15 patients suffered multi-organic metastases to the bone, brain and lung at the same time. Beside liver metastasis and abdominal spread, one patient also suffered metastasis to subcutaneous tissue. Detailed information on the 155 GISTs is provided in Table 1.

Five patients suffered a secondary malignant tumor. Two patients with gastric GIST had early and advanced gastric adenocarcinoma, respectively. Three small intestinal GIST patients were found to have early gastric carcinoma, sigmoid colonic adenocarcinoma and bile duct adenocarcinoma, respectively.

### Comparison of selected immunohistochemical markers in gastric and small intestinal GISTs

Sixty-one gastric and 42 small intestinal GIST patients were included in the analysis of survival data based on the expressions of selected immunohistochemical markers. These results are summarized in Table 2 and displayed in Figure 1. All 103 GISTs were CD117 positive. Among these 103 cases, 83 were positive for CD34, 32 for SMA and four for S-100. The expressions of CD44s and MMP-9 were statistically predominant in small intestinal GISTs. TIMP-1 and Ki-67 had no significant difference between the gastric and the small intestinal GIST. The expression of Ki-67 was negatively correlated to PTEN ( $P = 0.027$ ) and CD44s ( $P = 0.02$ ). In patients that died of gastric GIST, the expressions of MMP-9 and Ki-67 were statistically higher than in those who survived. In contrast, CD44s and PTEN were significantly lower. The difference in TIMP-1 was not significant.

In patients that died of small intestinal GIST, Ki-67 LIs were much higher than in those who survived, while PTEN was significantly lower. The other markers, includ-

**Table 1** Clinical and pathological parameters in 155 cases of gastrointestinal stromal tumor

	In general	Gastric GIST	Small intestinal GIST
Total case	153	81	72
Followed-up cases	104	62	42
Age (yr)			
< 50	38	14	24
$\geq 50$	64	46	18
Gender			
Male	72	41	31
Female	30	19	11
Tumor size (cm)			
< 2	1	1	0
2.1-5	31	20	11
5.1-10	46	29	17
> 10	24	10	14
Mitotic index			
$\leq 5/50$ HPF	54	31	23
5-10/50HPF	15	9	6
> 10/50HPF	33	20	13
Risk grade			
Very low risk	2	2	0
Low risk	23	14	9
Intermediate Risk	28	17	11
High risk	49	27	22
Recurrence	22	8	14
Metastasis	15	7	8
Survival rate (%)			
3-yr disease specific	76.64 $\pm$ 9.38	80.77 $\pm$ 11.50	73.65 $\pm$ 14.24
5-yr disease specific	69.05 $\pm$ 11.18	66.51 $\pm$ 17.06	61.76 $\pm$ 18.30

GIST: Gastrointestinal stromal tumor.

ing CD44s, MMP-9, and TIMP-1, showed no significant difference.

### Significance of PTEN, Ki-67 and CD44s on GISTs prognosis

For gastric GISTs, PTEN LIs  $< 50\%$  was significantly correlated with lower specific survival rate ( $P = 0.006$ , Figure 2B), as was Ki-67 LIs  $\geq 5\%$  ( $P = 0.004$ , Figure 2C) and CD44s negativity ( $P = 0.006$ , Figure 2D). In a total of 13 patients who died of GIST, there were five with PTEN LIs  $< 50\%$ , six with Ki-67 LIs  $\geq 5\%$  and eight patients who lost the expression of CD44s. For small intestinal GISTs, Ki-67 LIs  $\geq 5\%$  significantly correlated with worse outcomes. Multivariate analysis did not reveal an independent factor for gastric or small intestinal GISTs. In a total of 15 patients who died of GIST, there were 10 with Ki-67 LIs  $\geq 5\%$ .

No statistically significant difference was observed in disease specific survival for the patients grouped by the expressions of MMP-9 or TIMP-1. The balance between MMP-9 and TIMP-1 also did not correlate with disease specific survival. We failed to find an independent prognosis indicator after constructing the univariate and multivariate COX hazard proportional model (Table 3).

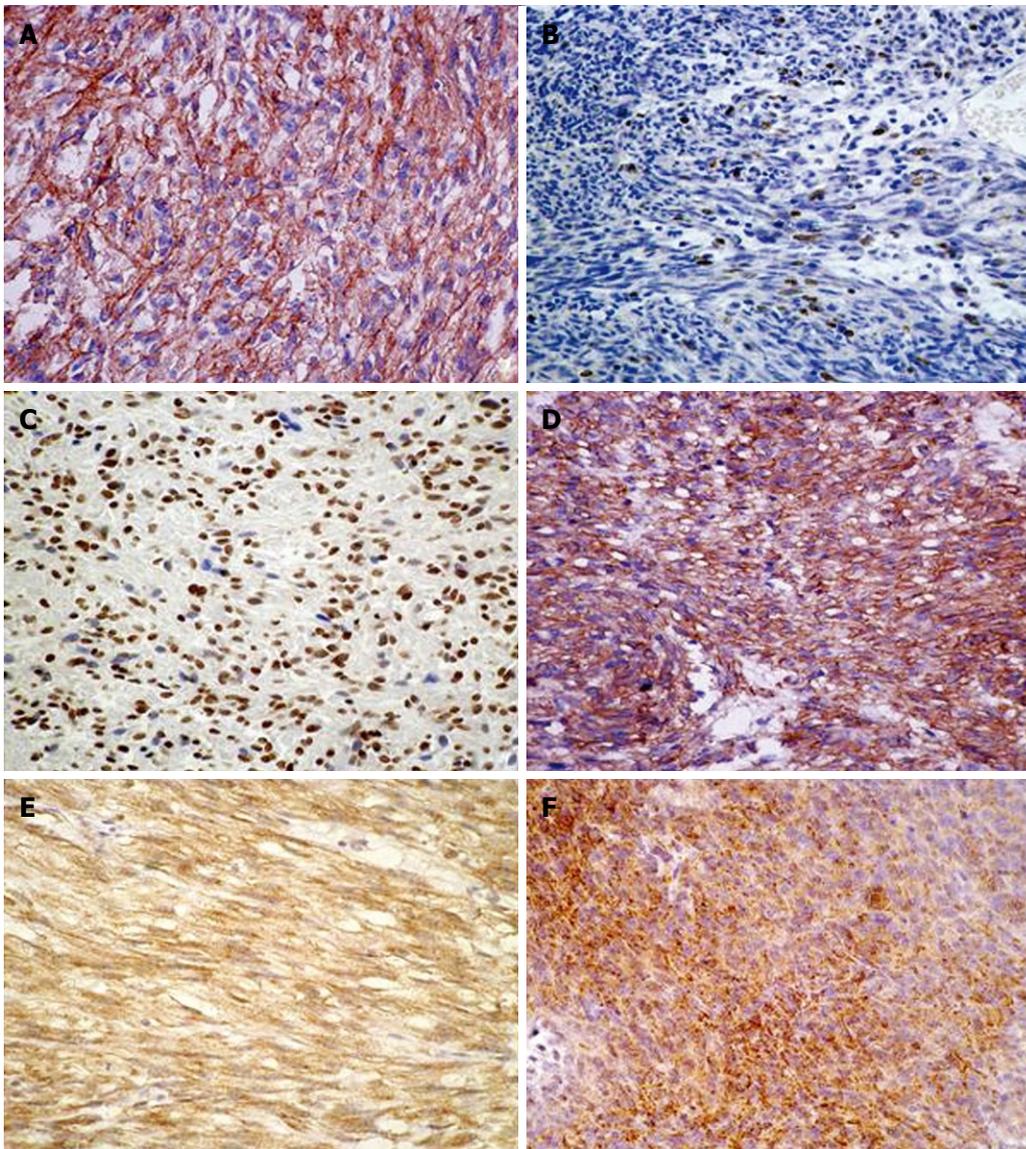
### Combined analysis of PTEN, Ki-67 and CD44s

Combined analysis revealed that for gastric GISTs, the

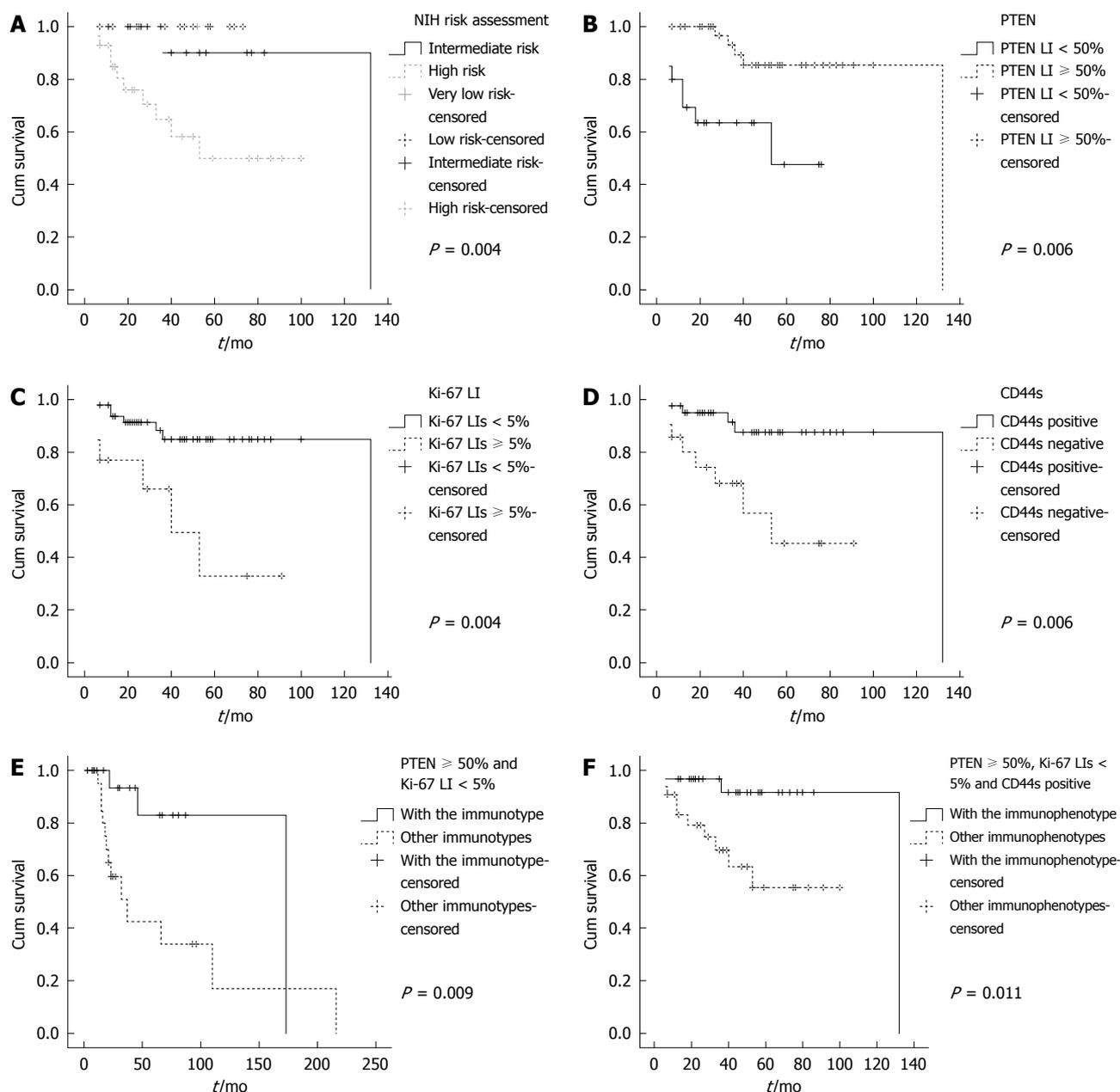
Table 2 Expressions of 5 molecular markers in gastric and small intestinal gastrointestinal stromal tumor

Protein	Gastric GISTs		P value	Small intestinal GISTs		P value
	Positive (%)			Positive (%)		
	DOD (n = 13)	Alive (n = 48)		DOD (n = 15)	Alive (n = 27)	
CD44s	5 (38.4)	35 (72.9)	0.020	15 (100)	23 (85.2)	0.397
MMP-9	11 (84.6)	23 (47.9)	0.018	11 (73.3)	22 (81.5)	0.339
TIMP-1	7 (53.8)	22 (45.8)	0.608	3 (20.0)	14 (51.9)	0.085
PTEN						
< 50%	5 (38.5)	8 (8.3)	0.014	11 (73.3)	6 (22.2)	0.003
≥ 50%	8 (61.5)	44 (91.7)		4 (26.7)	21 (77.8)	
Ki-67						
< 5%	7 (53.8)	41 (85.4)	0.022	5 (33.3)	24 (88.9)	0.0001
≥ 5%	6 (46.2)	7 (14.6)		10 (66.7)	3 (11.1)	

GIST: Gastrointestinal stromal tumor; MMP: Matrix metalloproteinase.



**Figure 1** Examples of the selected markers expressed in gastrointestinal stromal tumor. A: The tumor cells were strongly positive for CD117 with diffuse membrane staining; B: Nuclear positivity of Ki-67 in the tumor cells; C: Nuclear positivity of PTEN in gastrointestinal stromal tumor; D: CD44s was diffusely positive in tumor cell membrane; E: Matrix metalloproteinase 9 was diffusely positive in cytoplasm; F: TIMP-1 was diffusely positive in cytoplasm. Immunostains counterstained with hematoxylin-eosin, original magnifications  $\times 200$ .



**Figure 2** Kaplan-Meier cumulative survival plots of patients with gastric gastrointestinal stromal tumor. The end point was death due to gastrointestinal stromal tumor (GIST). A: High NIH risk assessment correlated significantly with worse outcomes in patients with gastric GIST ( $P = 0.004$ ); B: PTEN labeling indexes (LIs)  $\geq 50\%$  correlated significantly with favorable outcomes in patients with gastric GIST ( $P = 0.006$ ); C: In patients with gastric GIST, those with Ki-67 LIs  $< 5\%$  had significantly more favorable outcomes than those with Ki-67 LIs  $\geq 5\%$  ( $P = 0.004$ ); D: CD44s positivity was a significant, favorable indicator for patients with gastric GIST ( $P = 0.006$ ); E: For patients with small intestinal GIST, the immunophenotype of PTEN LIs  $\geq 50\%$  and Ki-67 LIs  $< 5\%$  was a favorable indicator ( $P = 0.009$ ); F: Gastric GIST patients with immunophenotype of PTEN LIs  $\geq 50\%$ , Ki-67 LIs  $< 5\%$  and CD44s positivity had significantly more favorable outcomes than those with other immunophenotypes ( $P = 0.011$ ).

survival rate of the patients with the expression pattern of PTEN LIs  $\geq 50\%$ , Ki-67 LIs  $< 5\%$  and CD44s positivity was significantly higher than those with other immunophenotypes ( $P = 0.009$ , Figure 2E). In the 13 patients who died of gastric GIST, 11 did not show the above immunophenotype. Of the two patients with the phenotype, one survived over 10 years after the resection of GIST and the other survived 53 mo.

For patients with small intestinal GIST, the survival rate was significantly higher in those with the combined

expressions of PTEN LIs  $\geq 50\%$  and Ki-67 LIs  $< 5\%$  relative to those with other immunophenotypes ( $P = 0.011$ , Figure 2F). In the 15 patients who died of small intestinal GIST, only two showed this favorable immunophenotype.

### Prognostic impact of NIH risk assessment

The NIH risk assessment showed excellent correlation with disease-specific survival. The patients were classified as higher risk with worse prognosis ( $P = 0.001$ ).

**Table 3** Univariate and multivariate COX regression analyses of molecular markers in gastric gastrointestinal and small intestinal stromal tumor

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Gastric GIST				
PTEN	0.23 (0.07-0.74)	0.013	0.35 (0.10-1.20)	0.100
Ki-67	4.54 (1.45-14.16)	0.009	2.69 (0.73-9.87)	0.130
CD44s	0.21 (0.07-0.73)	0.013	0.43 (0.83-9.98)	0.320
PTEN, Ki-67, CD44s	5.92 (1.29-27.08)	0.022	1.35 (0.15-11.80)	0.780
MMP-9	4.22 (1.92-19.31)	0.042		
TIMP-1	0.90 (0.29-2.82)	0.862		
MMP-9/TIMP-1	0.72 (0.22-2.38)	0.586		
Small intestinal GIST				
Ki-67	3.29 (1.14-9.46)	0.019		
PTEN, Ki-67	4.02 (1.25-16.09)	0.021		

MMP: Matrix metalloproteinase; GIST: Gastrointestinal stromal tumor; OR: Odds ratio.

GIST arising from gastric and small intestine showed different recurrent potential; we subsequently analyzed the prognostic value of risk assessment in these two anatomic sites. For gastric GIST, high risk stratification was correlated with significantly worse prognoses ( $P = 0.004$ , Figure 2A). But for small intestinal GIST, the risk stratification had no significant correlation with DSS ( $P = 0.205$ ). Considering the disease-free survival rate, the risk stratification was significantly correlated with survival not only in small intestinal GISTs ( $P = 0.011$ ), but also in gastric GISTs ( $P = 0.044$ ).

### Ki-67 and risk assessment

In our series, 29 gastric patients who were graded as high risk had shorter survival time than the other three grades ( $61.31 \pm 17.28$  mo *vs*  $121.64 \pm 17.06$  mo). Fourteen gastric patients with Ki-67 LIs  $\geq 5\%$  had shorter survival time than those with Ki-67 LIs  $< 5\%$  ( $49.75 \pm 10.84$  mo *vs*  $111.46 \pm 15.26$  mo). Ten of these 14 cases were classified as high risk grade, four were not in this group but suffered relapse or metastasis.

For small intestinal GISTs, two cases with Ki-67 LIs  $\geq 5\%$  suffered relapse and one died of the GIST in 32 mo. Neither of these two cases were classified as high risk grade by NIH risk assessment.

## DISCUSSION

In this study based on follow-up data, we developed a cluster of immunohistochemical markers that can facilitate the assessment of GIST prognosis in Chinese patients. The expression patterns of PTEN, Ki-67 and CD44s can help clinicians evaluate the clinical outcome of the patients with gastric GIST, as can the combination of PTEN and Ki-67 for those with small intestinal GIST.

Although PTEN, Ki-67 and CD44s can individually assist the prognosis, there are still apparent limitations because each marker just focuses on some of the patients. When tested by follow-up data, although eight patients expressed PTEN LIs  $\geq 50\%$ , seven of them died of GIST within 5 years after the surgery resection. Such a

conflict phenomenon is also very common in patients with gastric GIST when the prediction is based only on Ki-67 or CD44s. This also occurs for Ki-67 or PTEN in patients with small intestinal GIST. Combining the expressions of PTEN, Ki-67 and CD44s can improve the specificity and accuracy of the prognosis.

Our results showed that Ki-67 LIs is negatively correlated with PTEN and CD44s. PTEN and Ki-67 are both involved in cell proliferation. PTEN can upregulate p27<sup>kip1</sup> resulting in apoptosis and cell cycle arrest, suppression of cell proliferation by a mechanism independent of Akt activity in nuclei<sup>[19]</sup>. Also, PTEN upregulates p53 activity by maintaining the high acetylation of p53<sup>[20]</sup>. In our study, PTEN was exclusively located in nuclei, and PTEN LIs  $< 50\%$  was statistically correlated with a worse outcome of gastric GIST. A similar result was also reported by Ricci, regarding the correlation of decreased PTEN expression with worse outcomes<sup>[10]</sup>.

Ki-67 is widely used to predict the proliferation potential of malignant tumors. Many reports have confirmed the prognostic value of Ki-67 in GIST<sup>[21-23]</sup>. The differences in these reports are the cut-off value of the Ki-67 index, which varied from 4.5% to 10%. Whether Ki-67 is one of best predictors is debatable. Nakamura proposed that Ki-67 LIs and risk assessment were useful for predicting GIST outcome<sup>[17]</sup>, while Wong considered that mitotic count<sup>[24]</sup>, not Ki-67 LIs, remained the best predictor of gastric GIST. Our results revealed that higher Ki-67 LIs were associated with worse outcomes. Interestingly, there were four cases of low risk gastric GISTs with high Ki-67 LIs that suffered worse outcomes. Another two cases of low risk intestinal GISTs that had high Ki-67 LIs also resulted in worse outcomes. This indicates that Ki-67 may enhance the NIH consensus criteria, especially when applied to patients of low risk. Though the absolute cut-off of Ki-67 LIs is difficult to define, Ki-67 may become one of the most robust indicators of GIST.

CD44 is now widely accepted as a stem cell marker in many kinds of carcinomas, including gastric cancer, colorectal cancer<sup>[25]</sup>, and breast cancer. While only a minority of the cancer cells have the capability of car-

cinogenesis, this portion of the cell population is stem cells; in Du's series, no more than 5% of the cells were positive for CD44. In GISTs, once CD44 is positive, it is diffusely expressed by nearly all the cells. Based on this finding, CD44 may not be a suitable stem cell marker for GIST. CD117 and CD34 are stem cell markers for the haematopoietic stem cells. In GIST, CD117 is the crucial diagnostic marker; CD34 is one of the most important differential diagnostic markers. The expression of these stem cell markers in GIST may support the hypothesis that GIST originates from mesenchymal stem cells. The suitable robust stem cell markers for GIST needs further investigation. CD44 and its associated partner proteins monitor changes in the extra-cellular matrix that influences cell growth, survival and differentiation. It can be a molecular switch between growth and arrest depending on the extra-cellular conditions. As reported for CD44-deficient fibroblasts, the recruited CD44s suppress metastasis and proliferation<sup>[26]</sup>. Furthermore, CD44 can promote tumor invasion by recruiting MMP-9<sup>[27]</sup>. In small intestinal GIST, the expressions of CD44s and MMP-9 are significantly higher than in gastric GISTs. The co-expression of CD44s and MMP-9 may be responsible for the high metastatic liability of small intestinal GIST.

The anatomic site of GIST is now believed to be a prognostic factor. Some experts believe that when GIST is considered as a location-specific entity, multiple clinicopathologic parameters can be used together to define the biological behavior. In the revised version of NIH risk stratification, anatomic location was considered to be an important factor in the assessment of moderate and high risk<sup>[6]</sup>. In our series, the small intestinal GIST was more aggressive than gastric GIST. Of the patients with small intestinal GIST, 52.38% suffered recurrence or metastasis, while only 25% of gastric GIST patients did. The molecular markers expressed in gastric and small intestinal GIST were also different, as were the prognostic indicators. CD44 had no impact on the prognoses for patients with small intestinal GIST, and a similar result has been reported for rectal GIST<sup>[28]</sup>. The dissimilar anatomic locations may be responsible for these difference. Based on these facts, we believe that GIST should be sub-grouped by anatomic location first and then assessed by other factors.

In conclusion, the expression patterns of PTEN, Ki-67 and CD44s are useful for the prognosis of patients with gastric GIST, and PTEN and Ki-67 are valuable outcome indicators for patients with small intestinal GISTs, Ki-67 LIs can enhance the NIH consensus criteria.

## COMMENTS

### Background

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract. Because the targeted therapy of imatinib and sunitinib has achieved great success, accurate prediction of GIST outcomes is becoming more and more important. The biological behavior of GIST is highly variable. NIH risk assessment is now widely used as a prognostic indicator, but further investigation of the prognostic factors is still needed.

### Research frontiers

NIH risk assessment is widely used for GIST risk stratification; the revised version is based on tumor location, diameter and mitotic index. Many investigators have analyzed the prognostic value of other factors including stage, grade, KIT mutation and immunohistochemical markers.

### Innovations and breakthroughs

Based on follow-up information on patients without targeted therapy, we comprehensively analyzed anatomic location, NIH risk assessment and a number of immunohistochemical markers including PTEN, Ki-67, CD44s, matrix metalloproteinase 9 and TIMP-1.

### Applications

This study provides a new cluster of prognostic markers for GIST. PTEN LIs  $\geq 50\%$ , Ki-67 LIs  $< 5\%$  and CD44s positivity correlates with favorable outcomes for gastric GISTs, as does PTEN LIs  $\geq 50\%$  and Ki-67 LIs  $< 5\%$  for small intestinal GISTs. Anatomic location is a prognostic indicator of GISTs; depending on location, GISTs may have different biological features. The intrinsic nature needs further investigation.

### Terminology

GIST is the most common mesenchymal tumor of digestive tract. Gain-of-function mutation of in *c-kit* or platelet derived growth factor  $\alpha$  are hypothesized as the principle molecular pathogenesis. Based on this point, the targeted therapy of imatinib or sunitinib has achieved great success. But the biological behavior of GIST is highly variable; many experts are investigating improvements in prognostic markers.

### Peer review

This is an interesting, well done and well written paper adding useful insights in the field.

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## Vitamin D receptor gene polymorphisms and colorectal cancer risk: A systematic meta-analysis

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### Abstract

**AIM:** To investigate the relationship between polymorphisms present in the vitamin D receptor (*VDR*) gene and colorectal cancer risk, a systematic meta-analysis of population-based studies was performed.

**METHODS:** A total of 38 relevant reports published between January 1990 and August 2010 were identified, of which only 23 qualified for this meta-analysis based on our selection criteria. Five polymorphic variants of the *VDR* gene, including *Cdx-2* (intron 1e) and *FokI* (exon 2) present in the 5' region of the gene, and *BsmI* (intron 8), *ApaI* (intron 8), and *TaqI* (exon 9) sites present in the 3' untranslated region (UTR), were evaluated for possible associations with colorectal

cancer risk. Review manager 4.2 was used to perform statistical analyses.

**RESULTS:** In the meta-analysis performed, only the *BsmI* polymorphism was found to be associated with colorectal cancer risk. In particular, the *BsmI* B genotype was found to be related to an overall decrease in the risk for colorectal cancer [*BB vs bb*: odds ratio (OR) = 0.87, 95% CI: 0.80-0.94,  $P = 3 \times 10^{-4}$ ; *BB vs Bb + bb*: OR = 0.90, 95% CI: 0.84-0.97,  $P = 5 \times 10^{-4}$ ]. Moreover, in subgroup analyses, the *BsmI* B genotype was significantly associated with colon cancer, and not rectal cancer. An absence of between-study heterogeneity was also observed.

**CONCLUSION:** A meta-analysis of 23 published studies identified the *BsmI* polymorphism of the *VDR* gene to be associated with an increased risk of colon cancer.

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**Key words:** Vitamin D receptor; Polymorphism; Meta-analysis; Colorectal cancer

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### INTRODUCTION

Colorectal cancer represents the third most common cancer worldwide, second only to lung cancer and gastric

cancer<sup>[1]</sup>. Furthermore, it is estimated that there are more than 370 000 cases of colon and rectal cancer diagnosed in Europe every year, with 200 000 cases resulting in death<sup>[2]</sup>. However, the underlying etiology of colorectal cancer, including cancerous growths of the colon, rectum, and appendix, remains poorly understood. It has been proposed that some categories of external agents, including physical, chemical, and biological carcinogens, may contribute to the development of this disease, and the role of these factors in carcinogenesis would depend largely on genetic factors. Correspondingly, a recent study showed that insufficient levels of vitamin D may result in colorectal cancer<sup>[3]</sup>. Furthermore, genetic variations in genes controlling vitamin D activity would be hypothesized to play an important role in determining susceptibility to colorectal cancer.

*In vivo*, vitamin D helps bones and muscles grow, and may also help prevent many diseases, such as prostate cancer and breast cancer. The biological activity of vitamin D is mediated by the vitamin D receptor (VDR)<sup>[4]</sup>, which interacts with other cell signaling pathways to influence cell behavior. Expression of VDR has been detected in various organs and tissues of the human body, including the kidney and bone cells. VDR is also expressed in normal colon mucosa<sup>[5]</sup>. In the intestine, VDR plays an important role in regulating cell proliferation, differentiation, and the induction of apoptosis<sup>[6]</sup>. Furthermore, VDR may be associated with the effects of calcium on colorectal epithelial proliferation<sup>[7]</sup>.

Molecular epidemiological studies have shown that polymorphisms in the VDR gene may be linked to biological functions of vitamin D. At the 5' end of the VDR gene, a *FokI* polymorphism (rs2228570/rs10735810, exon 2) has been associated with a frameshift in the VDR protein<sup>[8]</sup>. Moreover, polymorphisms in the 3' untranslated region (UTR), including *BsmI* (rs1544410, intron 8), *ApaI* (rs7975232, intron 9), and *TaqI* (rs731236, exon 9) sites, have been shown to influence gene transcription and mRNA stability<sup>[9]</sup>. Additionally, these polymorphisms have exhibited the potential for strong linkage disequilibrium (LD)<sup>[10,11]</sup>, and functional differences have been associated with the associated haplotypes<sup>[11,12]</sup>. Given that polymorphisms in the VDR gene could potentially influence the binding of 1, 25(OH)<sub>2</sub>D<sub>3</sub> and the anti-proliferative effects of vitamin D, VDR polymorphisms have been hypothesized to be associated with colorectal cancer risk.

In 2001, the first report of an association between colorectal cancer and the VDR gene was published by Kim and colleagues<sup>[13]</sup>. They identified a random subset of 393 cases of colorectal adenomas and 406 colonoscopy-negative controls from a clinic-based, case-control study conducted in the United States between 1991 and 1994. Based on their analysis, the *BsmI* BB genotype was found to be associated with a reduced risk of colorectal adenoma when intake of calcium and vitamin D was reduced. In addition to the *BsmI* site<sup>[12,14-23]</sup>. Other polymorphic sites present in the VDR gene, including *Cdx-2*<sup>[12,19,21,24,25]</sup>, *FokI*<sup>[12,15,16,18,19,21-24,26-33]</sup>, *ApaI*<sup>[12,16,19,21,34]</sup>, and

*TaqI*<sup>[12,16,19,23,24,29,31,33-35]</sup>, have been evaluated in genetic association studies. However, the results are inconsistent. Since it can be difficult for individual studies to achieve sufficient statistical power to detect associations between VDR polymorphisms and colorectal cancer risk, a meta-analysis that combines data from all published studies may detect genetic associations more accurately. In addition, a reduced probability of false-negatives might also be achieved<sup>[36]</sup>. Therefore, a systematic meta-analysis of population-based studies was performed to investigate the association between VDR polymorphisms and the risk of colorectal cancer. Based on the search strategy and criteria used, 23 studies were analyzed which identified several important polymorphic variants.

## MATERIALS AND METHODS

### Search strategy and data extraction

To examine the association between VDR polymorphisms and colorectal cancer risk, a search of the MEDLINE database (from January 1990 to August 2010) and the US National Library of Medicine's PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>) was performed. In addition, various scientific research tools available on the web were used to search relevant references such as Google (<http://scholar.google.com/>) and Scirus (<http://www.scirus.com/>). In particular, data relevant to five well-characterized polymorphic variants was identified, including: *Cdx-2*, *FokI*, *BsmI*, *ApaI*, and *TaqI* sites within VDR. Keywords used in searches included "vitamin D receptor" in combination with "polymorphism", "vitamin D", "genotype", "allele", "colorectal cancer", or "risk".

Papers selected for this meta-analysis included a case-control study and complete data, including the authors' names; the subjects' region/country; year of publication; numbers of cases and controls; mean age (or range) of the case/control group; diagnostic criteria used; and number of subjects with the VDR genotype in both case and control groups. All relevant references that met these inclusive criteria and that were published as articles or abstract containing original data, were included in this study. In contrast, case-only studies, studies with incomplete data, or studies with inadequate control groups were excluded. In addition, the data extracted needed to conform to the guidelines of MOOSE, a proposal for reporting meta-analyses of observational studies<sup>[37]</sup>. If the same or overlapping data were reported in multiple publications, the most recent publication was selected<sup>[38]</sup>.

### Statistical analysis

For each data set included in this study, the odds ratios (ORs) and corresponding 95% CI for the incidence of cancer in subjects with or without particular restriction sites (lowercase *vs* uppercase lettering), was compared. Furthermore, deviations from the Hardy-Weinberg equilibrium for each control group were assessed using the goodness-of-fit test. To estimate associations with colorectal cancer risk, various genotypic models were

Table 1 Characteristics of case-control studies included in the meta-analysis

First author	Year	Country	Racial descent	Mean age in cases	Mean age in controls	Cases/controls	Genotyping method	Quality control	Adjusted	Studied polymorphisms
Ingles <i>et al</i> <sup>[11]</sup>	1998	United States	American	62.3	62.2	373/394	PCR-RFLP	Yes	Yes	<i>FokI</i>
Kim <i>et al</i> <sup>[13]</sup>	2001	United States	American	58.0 ± 9.7	53.0 ± 11.0	393/406	TaqMan	Yes	Yes	<i>BsmI</i>
Peters <i>et al</i> <sup>[27]</sup>	2001	United States	American	18-74	18-74	208/184	PCR-RFLP	Yes	NR	<i>FokI</i>
Slatter <i>et al</i> <sup>[23]</sup>	2001	United States	American	NR	NR	424/366	PCR-RFLP	NR	Yes	<i>FokI, BsmI, TaqI</i>
Speer <i>et al</i> <sup>[14]</sup>	2001	Hungary	European	64	63	56/112	PCR-RFLP	NR	NR	<i>BsmI</i>
Grau <i>et al</i> <sup>[29]</sup>	2003	United States	American	60.8 ± 9.0	60.9 ± 9.0	372/379	PCR-RFLP	Yes	Yes	<i>FokI, TaqI</i>
Wong <i>et al</i> <sup>[28]</sup>	2003	Singapore	Asian	66	56.5	217/890	PCR-RFLP	Yes	Yes	<i>FokI</i>
Peters <i>et al</i> <sup>[35]</sup>	2004	United States	American	62.9	62.3	763/774	PCR-RFLP	Yes	NR	<i>TaqI</i>
Slattery <i>et al</i> <sup>[15]</sup>	2004	United States	American	30-79	30-79	1936/2130	PCR-RFLP	NR	Yes	<i>FokI, BsmI</i>
Murtaugh <i>et al</i> <sup>[30]</sup>	2006	United States	American	30-79	30-79	2450/2821	PCR-RFLP	NR	Yes	<i>FokI</i>
Park <i>et al</i> <sup>[16]</sup>	2006	South Korea	Asian	55	55	190/354	PCR-RFLP	NR	Yes	<i>FokI, BsmI, ApaI, TaqI</i>
Flügge <i>et al</i> <sup>[12]</sup>	2007	Germany	European	61.9 ± 10.0	62.2 ± 11.2	256/256	PCR-RFLP	Yes	Yes	<i>Cdx-2, FokI, BsmI, ApaI, TaqI</i>
Kadiyska <i>et al</i> <sup>[19]</sup>	2007	Bulgaria	European	59	59 ± 5	133/94	PCR-RFLP	NR	Yes	<i>BsmI</i>
Slattery <i>et al</i> <sup>[18]</sup>	2007	United States	American	30-79	30-79	2380/2990	TaqMan	Yes	Yes	<i>FokI, BsmI</i>
Yaylim-Eraltan <i>et al</i> <sup>[31]</sup>	2007	Turkey	European	59.1 ± 4.0	52.0 ± 0.8	26/52	PCR-RFLP	NR	Yes	<i>FokI, TaqI</i>
Grünhage <i>et al</i> <sup>[32]</sup>	2008	Germany	European	65 ± 9	63 ± 8	192/220	PCR-RFLP	NR	Yes	<i>FokI</i>
Hubner <i>et al</i> <sup>[17]</sup>	2008	United Kingdom	European	NR	NR	137/409	TaqMan	Yes	Yes	<i>Cdx-2, FokI, BsmI, ApaI, TaqI</i>
Ochs-Balcom <i>et al</i> <sup>[24]</sup>	2008	United States	American	62.8 ± 10.2	58.5 ± 12.1	250/246	TaqMan	Yes	Yes	<i>Cdx-2, FokI, TaqI</i>
Parisi <i>et al</i> <sup>[20]</sup>	2008	Spain	European	NR	NR	170/120	PCR-RFLP	NR	Yes	<i>BsmI</i>
Theodoratou <i>et al</i> <sup>[21]</sup>	2008	United Kingdom	European	62.0 ± 10.8	62.4 ± 10.5	3005/3072	Microarray	Yes	Yes	<i>Cdx-2, FokI, BsmI, ApaI</i>
Wang <i>et al</i> <sup>[33]</sup>	2008	China	Asian	38-78	19.6 ± 1.3	69/218	PCR-RFLP	NR	Yes	<i>FokI</i>
Jenab <i>et al</i> <sup>[22]</sup>	2009	Europe	European	NR	NR	1248/1248	TaqMan	Yes	Yes	<i>FokI, BsmI</i>
Mahmoudi <i>et al</i> <sup>[34]</sup>	2010	Iran	Asian	52.7 ± 14.0	44.4 ± 17.7	160/180	PCR-RFLP	Yes	Yes	<i>ApaI, TaqI</i>

NR: Not reported.

selected, including codominant, additive, recessive, and dominant. Both the Peto Mantel-Haenszel fixed-effects model and the DerSimonian Laird random-effects model (with weights based on the inverse variance) were used to calculate summary ORs, and both within- and between-study variations were considered<sup>[39]</sup>. A *P*-value less than 0.10 was considered statistically significant when comparing trials showing heterogeneity, and random-effects analysis was selected. In contrast, fixed-effects analysis was used for comparing trials exhibiting homogeneity. Inverted funnel plots were also used to examine asymmetry, in which the ORs were plotted on a logarithmic scale against the inverse of their corresponding standard errors<sup>[40]</sup>. In the presence of publication bias, the funnel plot was asymmetric and the data showed remarkable skewness. There may be many reasons for this, most notably that some studies with negative findings are not published. In contrast, the plots were symmetric when bias was absent.

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 13.0) and Review Manager (version 4.2, The Cochrane Collaboration), and all *P*-values were two-sided.

## RESULTS

### Characteristics of case-control studies included in the meta-analysis

According to the criteria defined above, 38 published studies relevant to the *VDR* gene and colorectal cancer

risk were reviewed. Fifteen of these papers were excluded due to insufficient clarity in data presentation, repeated literature, or significant differences were present in their study design compared with the other papers identified<sup>[41]</sup>. The remaining 23 eligible case-control studies are listed in Table 1, and were included in a meta-analysis to investigate possible associations between *Cdx-2*, *FokI*, *BsmI*, *ApaI*, and *TaqI* polymorphisms present in the *VDR* gene and the risk of colorectal cancer.

In 21/23 studies, data regarding the 5' end of the *VDR* gene were provided. In four of these studies, 2639 cases and 2948 controls were analyzed for the *Cdx-2* polymorphism, while 17 studies included 13 301 cases and 15 942 controls analyzed for the *FokI* polymorphism. In addition, the 3' UTR region of the *VDR* gene has been analyzed. For example, 12 studies containing 10 083 cases and 11 242 controls analyzed the *BsmI* polymorphism, 5 studies including 2739 cases and 3200 controls analyzed the *ApaI* polymorphism, and 9 studies including 2580 cases and 3016 controls analyzed the *TaqI* polymorphism.

Among controls, the frequency of the *c* allele at the *Cdx-2* site ranged from 65.6% in Berlin-Bush populations of Germany, to 80.0% in a United Kingdom population<sup>[12,19,21,24,25]</sup>. In contrast, the frequency of the *f* allele of *FokI* among controls ranged from 31.7% in Turkey, to 47.2% in a Singapore population<sup>[12,15,16,18,19,21-24,26-33]</sup>. The frequency of the *b* allele at *BsmI* among controls ranged from 56.1% in a Bulgarian population, to 94.7% in a Korean population<sup>[12,14-23]</sup>, while the frequency of the *a* allele at *ApaI*

**Table 2 Summary odds ratios and 95% CI in the vitamin D receptor gene**

SNP	Model	Total No. cases	Total No. controls	OR (95% CI) <sup>1</sup>	P value <sup>2</sup>	P value <sup>3</sup>
<i>Cdx-2</i>	Codominant (CC vs cc)	152/1561	146/1820	1.25 (0.98-1.59)	0.07	0.26
	Codominant (Cc vs cc)	926/2487	982/2802	1.09 (0.97-1.22)	0.15	0.64
	Codominant (C vs C)	1230/4048	1279/4622	1.10 (1.01-1.21)	0.03	0.47
	Dominant (CC + Cc vs cc)	1078/1561	1208/1820	0.98 (0.88-1.09)	0.72	< 0.001
	Recessive (CC vs Cc + cc)	152/2487	146/2802	1.22 (0.96-1.54)	0.10	0.23
<i>FokI</i>	Codominant (ff vs FF)	1844/5068	2377/5982	0.94 (0.87-1.01)	0.09	0.001
	Codominant (Ff vs FF)	6189/11 257	7583/13 565	0.98 (0.93-1.03)	0.34	0.001
	Codominant (f vs F)	9867/16 320	12 190/19 329	0.97 (0.94-1.00)	0.07	< 0.001
	Dominant (ff + Ff vs FF)	8033/5068	9960/5982	0.96 (0.92-1.01)	0.15	< 0.001
	Recessive (ff vs FF + Ff)	1844/11 257	2377/13 565	0.95 (0.89-1.02)	0.13	0.01
<i>BsmI</i>	Codominant (BB vs bb)	1512/3838	1817/4122	0.87 (0.80-0.94)	< 0.001	0.65
	Codominant (Bb vs bb)	4733/8571	5303/9425	0.94 (0.88-0.99)	0.03	0.64
	Codominant (B vs b)	7757/12 409	8937/13 577	0.93 (0.90-0.97)	< 0.001	0.85
	Dominant (BB + Bb vs bb)	6245/3838	7120/4122	0.92 (0.87-0.97)	0.003	0.84
	Recessive (BB vs Bb + bb)	1512/8571	1817/9425	0.90 (0.84-0.97)	0.006	0.28
<i>ApaI</i>	Codominant (AA vs aa)	748/578	1004/603	0.85 (0.73-0.99)	0.03	0.06
	Codominant (Aa vs aa)	1378/1956	1593/2196	0.91 (0.79-1.04)	0.18	0.39
	Codominant (A vs a)	2944/2534	3601/2799	0.92 (0.85-0.99)	0.02	0.04
	Dominant (AA + Aa vs Aa)	2161/578	2597/603	0.89 (0.78-1.01)	0.07	0.12
	Recessive (AA vs Aa + aa)	783/1956	1004/2196	0.89 (0.80-1.00)	0.05	0.21
<i>TaqI</i>	Codominant (tt vs TT)	382/1112	398/1320	1.05 (0.89-1.24)	0.58	0.07
	Codominant (Tt vs TT)	1086/2198	1298/2618	0.93 (0.83-1.05)	0.23	0.30
	Codominant (t vs T)	1850/3310	2098/3938	0.99 (0.92-1.08)	0.86	0.10
	Dominant (tt + Tt vs TT)	1468/1112	1696/1320	0.95 (0.85-1.07)	0.42	0.37
	Recessive (tt vs Tt + TT)	382/2198	398/2618	1.07 (0.92-1.25)	0.38	0.01

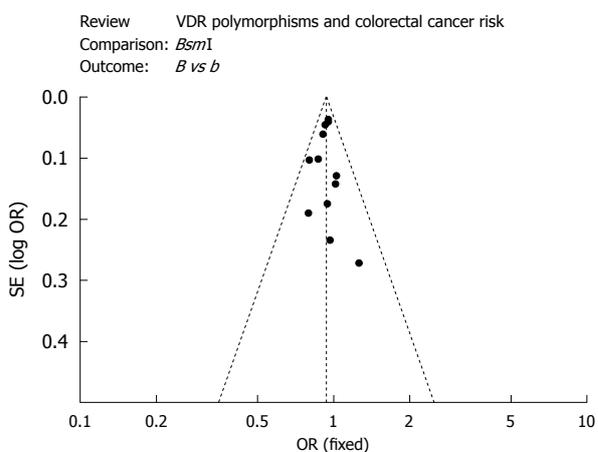
<sup>1</sup>Based on fixed effects model; <sup>2</sup>Test for overall effect; <sup>3</sup>Test for heterogeneity. OR: Odds ratios.

among controls ranged from 23.0% in a Korean population to 49.3% in a population of the United Kingdom<sup>[12,16,19,21,34]</sup>. Lastly, the frequency of the *t* allele at *TaqI* among controls ranged from 8.8% in a Korean population to 43.6% in a population of the United States<sup>[12,16,19,23,24,29,31,33-35]</sup>.

**Qualitative assessment of included studies**

Genotyping of the *Cdx-2*, *FokI*, *BsmI*, *ApaI* and *TaqI* polymorphisms was performed using the polymerase chain reaction-restriction fragment length polymorphism technique in 75% of the studies included in this meta-analysis. Due to the low sensitivity of this classic technology, quality control of this genotyping was required, and included blindness to the case-control status, random repeats of samples, or validation using a different genotyping method. However, only 38.9% (7/18) of the eligible studies provided sufficient quality control. Regarding sample size, only 5/24 (20.8%) studies employed more than 1000 cases or controls. Moreover, most of these studies were associated with poor statistical power due to sample sizes that were less than 500 and in some cases contained less than 100 cases or controls.

Assessment of Hardy-Weinberg proportion is regarded as an important criterion for evaluating genetic association studies<sup>[38]</sup>. Most of the studies included in this meta-analysis reported genotype frequencies in their control groups that were consistent with Hardy-Weinberg proportions ( $P > 0.05$ ). For example, deviations from Hardy-Weinberg proportions in controls were observed in three studies for *FokI*<sup>[29,31,32]</sup>, two studies of *BsmI*<sup>[19,21]</sup>, one study of *ApaI*<sup>[19]</sup>, and two studies of *TaqI*<sup>[23,24]</sup>.



**Figure 1 A funnel plot was used to estimate the publication bias of the studies included in the meta-analysis performed.**

Funnel plotting was performed to evaluate whether publication bias was present in the meta-analysis performed. As shown in Figure 1, the shapes of the funnel plots obtained appear to be symmetrical in codominant, dominant, and recessive models, suggesting that publication bias is absent in the meta-analysis performed.

***Cdx-2*, *FokI*, *BsmI*, *ApaI*, *TaqI* polymorphisms and colorectal cancer risk**

A heterogeneity test of potential associations between the *Cdx-2*, *FokI*, *BsmI*, *ApaI*, and *TaqI* polymorphisms and risk of colorectal cancer are presented in Tables 2 and 3.

Table 3 The *BsmI* effect odds ratios stratified by anatomical site

Model	Colon cancer			Rectal cancer		
	Total No. cases/controls	OR (95% CI)	<i>P</i> value <sup>1</sup>	Total No. cases/controls	OR (95% CI)	<i>P</i> value <sup>1</sup>
Codominant ( <i>BB vs bb</i> )	1365/1581	0.80 (0.68-0.93)	0.004/0.21	659/790	0.92 (0.73-1.15)	0.46/0.90
Codominant ( <i>Bb vs bb</i> )	2257/2438	0.99 (0.88-1.12)	0.92/0.95	1029/1240	0.94 (0.80-1.11)	0.48/0.21
Codominant ( <i>B vs b</i> )	5328/5972	0.91 (0.84-0.98)	0.01/0.25	2440/2966	0.95 (0.85-1.06)	0.38/0.99
Dominant ( <i>BB + Bb vs bb</i> )	2664/2986	0.94 (0.84-1.05)	0.25/0.65	1220/1483	0.94 (0.77-1.16)	0.59/0.40
Recessive ( <i>BB vs Bb + bb</i> )	2664/2986	0.80 (0.69-0.92)	0.002/0.19	1220/1483	0.93 (0.80-1.09)	0.40/0.51

<sup>1</sup>Test for overall effect and heterogeneity, respectively. OR: Odds ratios.

***Cdx-2* polymorphism:** Currently, only four studies have investigated the relationship between the *VDR Cdx-2* polymorphism and colorectal cancer risk, and all of these studies were in Hardy-Weinberg equilibrium<sup>[12,19,21,24,25]</sup>. Furthermore, in the overall and subgroup analyses performed, the *Cdx-2* polymorphism did not appear to be linked to colorectal cancer risk.

***FokI* polymorphism:** Seventeen studies included in the meta-analysis performed found the *FokI* polymorphism to be statistically heterogeneous in all genetic models ( $P \leq 0.01$ )<sup>[12,15,16,18,19,21-24,26-33]</sup>. Moreover, no significant association was found between *FokI* and colorectal cancer risk in overall and subgroup analyses.

***BsmI* polymorphism:** A total of 12 studies examined the association between colorectal cancer and the *BsmI* polymorphism, and there was little statistical evidence of heterogeneity among the studies ( $P \geq 0.28$ )<sup>[12,14-23]</sup>. Individuals with the *BB* genotype (OR = 0.87; 95% CI = 0.80-0.94,  $P = 3 \times 10^{-4}$ ;  $P = 0.65$  for heterogeneity), or the *Bb* genotype (OR = 0.94; 95% CI: 0.88-0.99,  $P = 0.03$ ;  $P = 0.48$  for heterogeneity), were associated with a significant decrease in colorectal cancer risk compared with patients carrying the *bb* genotype. The Dominant model (*BB + Bb vs bb*) and the recessive model (*BB vs Bb + bb*) also showed a significant association with colorectal cancer risk, with the associated ORs being 0.92 (95% CI: 0.87-0.97,  $P = 0.003$ ;  $P = 0.84$  for heterogeneity) and 0.90 (95% CI: 0.84-0.97,  $P = 0.006$ ;  $P = 0.28$  for heterogeneity), respectively (Figure 2). Although two of these studies were not consistent with Hardy-Weinberg proportions<sup>[17,21]</sup>, the effect was negligible. In addition, the *BB* genotype showed a decreased risk for colon cancer compared with the *bb* (OR = 0.80, 95% CI: 0.68-0.93,  $P = 0.004$ ;  $P = 0.21$  for heterogeneity), or *Bb + bb* genotypes (OR = 0.80, 95% CI: 0.69-0.92,  $P = 0.002$ ;  $P = 0.19$  for heterogeneity). However, no significant differences were observed between these polymorphisms and rectal cancer risk (Table 3).

***ApaI* polymorphism:** The association between the *ApaI* polymorphism and colorectal cancer was investigated in five studies, of which only one study was not consistent with Hardy-Weinberg proportions<sup>[12,16,19,21,34]</sup>. Moreover, although the codominant model (*AA vs aa*, OR = 0.83; 95% CI: 0.71-0.97,  $P = 0.02$ ) showed a

significant association with colorectal cancer risk, the *P* value of 0.05 suggested that this genetic model was statistically heterogeneous.

***TaqI* polymorphism.** Except for the recessive model (*tt vs Tt + TT*,  $P = 0.06$ ), there was little evidence of statistical heterogeneity among the nine studies that investigated an association between the *TaqI* polymorphism and colorectal cancer risk ( $P \geq 0.35$ )<sup>[12,16,19,23,24,29,31,33-35]</sup>. When the two studies in which controls were not in Hardy-Weinberg equilibrium were excluded, the pooled ORs for all genetic models for *TaqI* were shifted, yet the results remained null<sup>[23,24]</sup>.

## DISCUSSION

This study was undertaken to assess whether *VDR* polymorphisms in both the 5' (*Cdx-2* and *FokI*) and 3' (*BsmI*, *ApaI* and *TaqI*) regions of the *VDR* gene are associated with colorectal cancer risk. A total of 38 reports had previously evaluated a possible genetic association, and only 23 of these were eligible for this study based on the selection criteria employed. The pooled ORs (95% CI) for these studies were identical according to both fixed- and random-effects models. Moreover, only the polymorphic variant, *BsmI*, was found to be associated with increased risk for colorectal cancer. The meta-analysis performed also showed that the *BsmI B* genotype was related to a significant decrease in overall risk for colorectal cancer, with the co-dominant *BB* and the *BB + Bb vs bb* dominant model exhibiting 0.87- and 0.92-fold increases in the risk for disease, respectively. The *BB vs Bb + bb* recessive model also had a 90% decreased risk for colorectal cancer.

In addition, subgroup analyses by anatomical site identified the *BsmI* polymorphism to be significantly associated with colon cancer, and not rectal cancer. Furthermore, compared with the *bb* or *Bb + bb* genotypes, the *BB* genotype was associated with a 90% decrease in the risk for colon cancer. However, it remains unclear why the *BsmI* site is related to the risk of colon cancer, and not rectal cancer. It is possible that the difference in epithelial cells between the two cancers plays a role, with ciliated columnar epithelial cells being present in the lining of the colon, while squamous epithelial cells are present in the rectum. The role of the micro-environment may also be a contributing factor, since physical damage as a result of oxygen or poisonous food residues is more likely to influence the rectum than the colon. In addition,

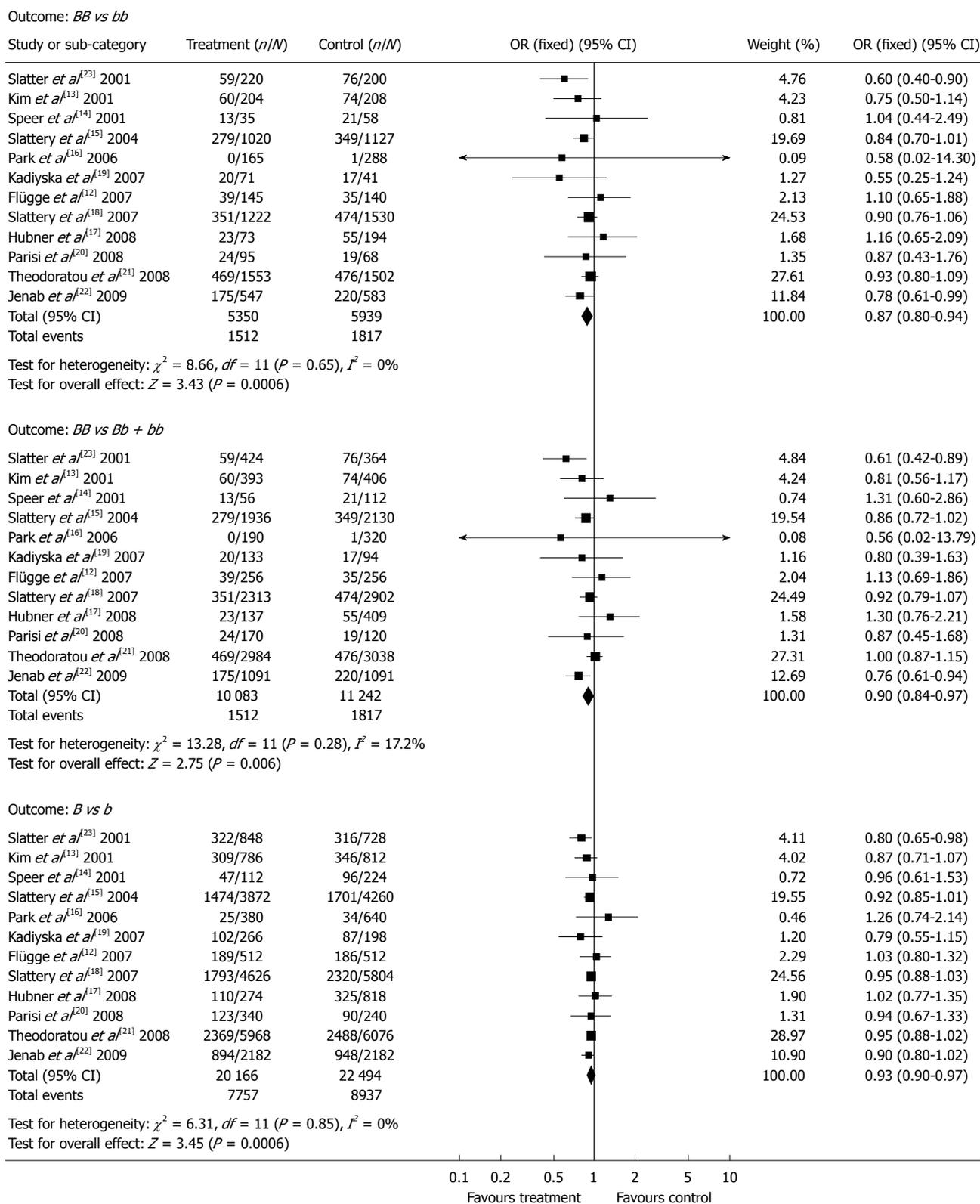


Figure 2 Forest plot of the meta-analysis performed to investigate the association between the *BsmI* polymorphism of the vitamin D receptor gene and colorectal cancer risk (fixed-effects model).

genetic factors may have a more important role in colon cancer than rectal cancer.

Based on the distribution differences observed between cases of colorectal cancer and controls, we hypothesized that the *BsmI* B allele might have a protective

effect against tumorigenesis. Correspondingly, of the 12 relevant reports reviewed, 9 studies supported this hypothesis. In these studies, the populations analyzed were from Asia ( $n = 1$ ), Europe ( $n = 4$ ), and the United States ( $n = 4$ ). In addition, Jenab *et al*<sup>[22]</sup> evaluated different Cau-

casian populations from 23 centers in Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. In this study, the *BB* genotype, rather than the wild-type *bb* genotype, was associated with a reduced risk of colorectal cancer. The *BsmI BB* genotype was also found to be associated with a reduced risk of colorectal cancer among non-aspirin/NSAID users<sup>[42]</sup>. Thus, multiple lines of evidence support the hypothesis that the *BsmI B* allele mediates a protective effect against the development of cancers in the digestive tract, especially colon cancer.

The *BsmI* polymorphism is located in the 3' UTR of the *VDR* gene, and does not alter the amino acid sequence of the *VDR* protein. Thus, for a single *BsmI* polymorphism, there is a low probability that it directly influences *VDR* function<sup>[9]</sup>. The *BsmI* polymorphism also does not affect mRNA or protein levels of *VDR*<sup>[20]</sup>, or levels of 25(OH)<sub>2</sub>D<sub>3</sub> or 125(OH)<sub>2</sub>D<sub>3</sub><sup>[43]</sup>. However, the *BsmI* site does exhibit strong LD with other *VDR* polymorphisms, including *eTru9I*, *ApaI*, *TaqI*, and *Poly(A)* microsatellites. Based on these results, it appears that the *BsmI* polymorphism affects some type of biological function, and these could potentially include regulation of *VDR* transcription, translation, or RNA processing<sup>[9]</sup>. Other unidentified SNPs in the *VDR* gene, as well as SNPs in other genes such as *CYP3A5*<sup>[44]</sup>, may also affect the function of the *BsmI*. Furthermore, patients carrying the *BsmI* allele have also been shown to have significantly higher levels of *erbB-2* expression, suggesting other tumor-related molecules may also be involved in the function of the *BsmI* polymorphism<sup>[14]</sup>.

Although the *BsmI B* allele has been associated with a protective effect, the frequency of this effect has been found to be lower in Asian populations than in Caucasian populations. However, this is inconsistent with the incidence of colorectal cancer identified in recent epidemiological data<sup>[2]</sup>. Moreover, in the meta-analysis performed in the present study, no significant association was found between *VDR* genotypes and the risk of colorectal cancer in group analyses (not shown). In combination, these results suggest that other factors may be involved. For example, environment, food, and lifestyle may play a more significant role, in combination with genetic factors, in the occurrence and development of colorectal cancer than previously thought, which would potentially account for the inconsistent results obtained from previous studies.

## COMMENTS

### Background

Colorectal cancer is one of the most common cancers worldwide, and its incidence is increasing with each year. However, the underlying etiology of colorectal cancer remains unclear. Several epidemiologic studies have reported that 1, 25(OH)<sub>2</sub>D<sub>3</sub> can reduce the risk of colorectal cancer, and thus, vitamin D receptor (*VDR*), a crucial mediator of the cellular effects of 1, 25(OH)<sub>2</sub>D<sub>3</sub>, may play an important role in the occurrence and development of colorectal cancer.

### Research frontiers

Recently, several polymorphic variants of the *VDR* gene have been reported to be associated with the risk of colorectal cancer. However, the published findings remain inconsistent. In this study, the authors conducted a systematic meta-analysis to evaluate the evidence regarding this association.

## Innovations and breakthroughs

In the present study, all relevant reports published between January 1990 and August 2010 were reviewed, with a focus on five well-characterized polymorphic variants of *VDR*: *Cdx-2*, *FokI*, *BsmI*, *ApaI*, and *TaqI*. In the meta-analysis performed, *BsmI* was found to be associated with colorectal cancer, while the *Cdx-2*, *FokI*, *ApaI*, and *TaqI* sites did not exhibit any significant association. Moreover, the *BsmI* 'B' genotype was associated with a significant decrease in the risk of colorectal cancer, especially colon cancer. Based on these results, it is hypothesized that *BsmI* may mediate a protective effect on tumorigenesis.

## Applications

The results of this meta-analysis have the potential to partly explain the genetics that influence the pathogenesis of colorectal cancer. This study also helps provide a basis for clinical diagnosis and methods for early intervention.

## Peer review

The authors performed a systematic meta-analysis of population-based studies to investigate the association between *VDR* polymorphisms and colorectal cancer risk. The authors found a *BsmI* site in the 3' UTR of the *VDR* gene to be associated with colon cancer risk, and then analyzed the underlying mechanism. The results are interesting and may help explain the genetic mechanism of colorectal carcinogenesis.

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## Heme oxygenase-1 prevents liver fibrosis in rats by regulating the expression of PPAR $\gamma$ and NF- $\kappa$ B

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### Abstract

**AIM:** To investigate the effects of heme oxygenase (HO)-1 on liver fibrosis and the expression of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and nuclear factor-kappa B (NF- $\kappa$ B) in rats.

**METHODS:** Sixty Wistar rats were used to construct liver fibrosis models and were randomly divided into 5 groups: group A (normal, untreated), group B (model for 4 wk, untreated), group C (model for 6 wk, untreated), group D [model for 6 wk, treated with zinc protoporphyrin IX (ZnPP-IX) from week 4 to week 6], group E (model for 6 wk, treated with hemin from week 4 to week 6). Next, liver injury was assessed by measuring serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin levels. The degree of hepatic fibrosis was evaluated by measuring serum hyaluronate acid (HA), type IV collagen (IV-C) and by histological examination. Hydroxyproline (Hyp) content in the liver homogenate was determined. The expres-

sion levels of alpha-smooth muscle actin ( $\alpha$ -SMA) in liver tissue were measured by real-time quantitative polymerase chain reaction (RT-PCR). The expression levels of PPAR $\gamma$  and NF- $\kappa$ B were determined by RT-PCR and Western blotting.

**RESULTS:** The expression of HO-1 increased with the development of fibrosis. Induction of HO-1 by hemin significantly attenuated the severity of liver injury and the levels of liver fibrosis as compared with inhibition of HO-1 by ZnPP-IX. The concentrations of serum ALT, AST, HA and IV-C in group E decreased compared with group C and group D ( $P < 0.01$ ). Amount of Hyp and  $\alpha$ -SMA in the liver tissues in group E decreased compared with group C ( $0.62 \pm 0.14$  vs  $0.84 \pm 0.07$ ,  $1.42 \pm 0.17$  vs  $1.84 \pm 0.17$ , respectively,  $P < 0.01$ ) and group D ( $0.62 \pm 0.14$  vs  $1.11 \pm 0.16$ ,  $1.42 \pm 0.17$  vs  $2.56 \pm 0.37$ , respectively,  $P < 0.01$ ). The expression of PPAR $\gamma$  at levels of transcription and translation decreased with the development of fibrosis especially in group D; and it increased in group E compared with groups C and D ( $0.88 \pm 0.15$  vs  $0.56 \pm 0.19$ ,  $0.88 \pm 0.15$  vs  $0.41 \pm 0.11$ , respectively,  $P < 0.01$ ). The expression of NF- $\kappa$ B increased with the development of fibrosis especially in group D; and it decreased in group E compared with groups C and D ( $1.43 \pm 0.31$  vs  $1.89 \pm 0.29$ ,  $1.43 \pm 0.31$  vs  $2.53 \pm 0.54$ , respectively,  $P < 0.01$ ).

**CONCLUSION:** Our data demonstrate a potential mechanism that HO-1 can prevent liver fibrosis by enhancing the expression of PPAR $\gamma$  and decreasing the expression of NF- $\kappa$ B in liver tissues.

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**Key words:** Heme oxygenase-1; Peroxisome proliferator-activated receptor gamma; Nuclear factor-kappa B; Liver fibrosis; Hemin

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Yang H, Zhao LF, Zhao ZF, Wang Y, Zhao JJ, Zhang L. Heme oxygenase-1 prevents liver fibrosis in rats by regulating the expression of PPAR $\gamma$  and NF- $\kappa$ B. *World J Gastroenterol* 2012; 18(14): 1680-1688 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i14/1680.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i14.1680>

## INTRODUCTION

Liver fibrosis is the mechanism of compensation and reparation after chronic hepatic injury, which is a necessary pathological stage for the development of chronic hepatitis. Heme oxygenase-1 (HO-1), also known as heat shock protein 32, is a microsomal enzyme and rate-limiting enzyme that catalyzes the degradation of heme into biliverdin, iron atoms and carbon monoxide (CO)<sup>[1]</sup>. HO-1 and its breakdown products play vital physiological roles in anti-inflammation, anti-oxidation and regulation of apoptosis according to reports<sup>[2,3]</sup>. Many researchers have recently confirmed that HO-1 has protective effects on liver cells under such conditions as acute liver injury, alcoholic liver disease, liver transplantation and ischemia/reperfusion injury<sup>[4-8]</sup>. In chronic liver disease, induction of HO-1 is important to prevent the development of liver fibrosis<sup>[9]</sup>. However, the underlying molecular mechanisms are still unknown.

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor which is widely distributed in tissues<sup>[10]</sup>. Three PPAR subtypes have been identified, namely  $\alpha$ ,  $\beta$  and  $\gamma$ . PPAR $\gamma$  is mainly expressed in hepatic stellate cells (HSC). Studies have shown that the expression of PPAR $\gamma$  benefits the maintenance of HSC static phenotype<sup>[11]</sup>. Up-regulation of PPAR $\gamma$  resulted in a significant reduction of HSC activation, and reversed the development of liver fibrosis.

Nuclear factor-kappa B (NF- $\kappa$ B) is an important nuclear transcription factor, which plays an important role in the regulation of gene transcription such as cytokines, chemokines, adhesion molecules and other inflammatory mediators<sup>[12]</sup>. Up-regulating the activation of NF- $\kappa$ B promotes HSC proliferation and decreases HSC apoptosis. Therefore, inhibiting the activation of NF- $\kappa$ B resulted in reduction of HSC activation, promoting HSC apoptosis and decreasing extracellular matrix production<sup>[13,14]</sup>.

In the present study, we have evaluated the role of HO-1 in liver fibrosis caused by carbon tetrachloride (CCl<sub>4</sub>) in rat models, then observed the expression of PPAR $\gamma$  and NF- $\kappa$ B in liver after up-regulation of HO-1 by ferriprotoporphyrin IX chloride (hemin) or inhibition of HO-1 by zinc protoporphyrin IX (ZnPP-IX) pretreatment, and we finally hypothesize about a potential mechanism for the cytoprotection by HO-1.

## MATERIALS AND METHODS

### Reagents

ZnPP-IX is a selective HO inhibitor which can suppress the activity of HO-1 by blocking CO production and

restricting the transformation of heme to biliverdin<sup>[15]</sup>. Hemin is a well-known physiological substrate and potent inducer of HO activity<sup>[16]</sup>. Both ZnPP-IX and hemin were purchased from Sigma Chemical Co. (St. Louis, MO, United States). Polyclonal antibodies HO-1 and PPAR $\gamma$  were purchased from Cell Signaling (Danver, MA, United States). Anti-phospho-NF- $\kappa$ B p65 monoclonal antibody was purchased from Santa Cruz (Santa Cruz, CA, United States). Anti-beta actin antibody was purchased from Biogenesis (Bournemouth, United Kingdom). All other chemicals were of analytical grade and commercially available.

### Animals and experimental design

Male Wistar rats (Medical University Laboratory Animal Center, Shanxi, China) weighing 220 g-250 g were used to establish fibrogenesis models<sup>[17-19]</sup>. All procedures used in this study were approved by the Ethics Committee for the use of experimental animals at Shanxi Medical University. All rats were kept at 21 °C-25 °C under a 12 h dark/light cycle, drank normal water and were fed with 79.5% corn meal, 20% lard and 0.5% cholesterol for the first two weeks, then 99.5% corn meal and 0.5% cholesterol thereafter. Sixty rats were randomly divided into five groups (twelve rats/group); groups B, C, D and E received subcutaneous injections of 40% CCl<sub>4</sub> (a mixture of pure CCl<sub>4</sub> and olive oil, 0.3 mL/100 g) every four days for six weeks. The rats in group A were fed with normal diet and received a 0.9% NaCl subcutaneous injection. In the fourth week, Group B and some of group A were killed, while group D and group E began to be peritoneally injected with ZnPP-IX (20  $\mu$ mol/kg) or hemin (30  $\mu$ mol/kg) every other day until the sixth week when they were killed with group C and the remnants of group A. The dose and preparation of ZnPP and hemin solution were based on our preliminary studies and references<sup>[20-22]</sup>. The numbers of rats were reduced to 11, 9, 9 and 10 in groups B, C, D, and E, respectively, due to deaths during the process. A small portion of the liver was removed for histological analysis by fixation with 10% formalin and subsequent embedding in paraffin. The remaining liver was cut into pieces and frozen in liquid nitrogen and kept at -80 °C until it was used for extraction of total RNA and proteins.

### Serum biochemical and liver fibrosis indicator measurements

Markers of hepatic damage such as serum alanine aminotransferase (ALT), aspartate transaminase (AST) and albumin (ALB) levels were measured by using an automated biochemistry clinical analyzer (Hitachi, Japan) according to an automated procedure. The levels of serum hyaluronic acid (HA) and type IV collagen (IV-C) were determined using Chemiluminescence Quantitative Immunoassay Kit (Beijing Yuande Bio-Medical Engineering Co., Ltd.).

### Quantification of hydroxyproline assay

Hydroxyproline (Hyp) content in the liver specimens represented the total amount of collagen in livers, which

was quantified to evaluate the degree of liver fibrosis by using a colorimetric method<sup>[23]</sup>. Kits for the measurement were purchased from Nanjing Jiancheng Bioengineering. In brief, 100 mg of freeze-dried liver specimens were weighed and tested according to the manufacturer's directions. At the end of the experiment, absorbance of each sample was read at 550 nm using a spectrophotometer. The content was obtained according to a formula and expressed as micrograms Hyp/milligram liver. Each sample was analyzed in triplicate.

### Histologic evaluation

Liver tissues were fixed in 10% neutral formalin solution overnight, embedded in paraffin blocks, and then were sectioned at 4  $\mu$ m thickness for staining with hematoxylin and eosin (HE) or Masson by using standard techniques. The results were analyzed by light microscopy. Representative views of liver sections are shown.

### RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from liver tissue using the RNA Trizol isolation reagent kit (Invitrogen, United States). cDNA was obtained by reverse transcription of RNA by using random primer and Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Merelbeke, Belgium). The conditions were 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min, finally cooling at 5 °C for use. Amplification reactions were performed with a SYBR green polymerase chain reaction (PCR) master mix (Applied Biosystems). Three microliters of diluted cDNA samples were used for quantitative analysis. The cycle conditions were 95Mul for 10 min for denaturation, followed by 50 cycles of 15 s at 95Mul, 30 s at 55Mul and 45 s at 72Mul. Each sample was analyzed in triplicate. The primers were as follows: HO-1, (forward) 5'-CAC GCA TAT ACC CGC TAC CT-3' and (reverse) 5'-AAG GCG GTC TTA GCC TCT TC-3'; PPAR $\gamma$ , (forward) 5'-CCC TGG CAA AGC ATT TGT AT-3' and (reverse) 5'-ACT GGC ACC CTT GAA AAA TG-3'; NF- $\kappa$ B, (forward) 5'-AAC ACT GCC GAG CTC AAG AT-3' and (reverse) 5'-CAT CGG CTT GAG AAA AGG AG-3'; alpha-smooth muscle actin ( $\alpha$ -SMA), (forward) 5'-TGT GCT GGA CTC TGG AGA TG-3' and (reverse) 5'-GAA GGA ATA GCC ACG CTC AG-3'; Beta-actin, (forward) 5'-GTC AGG TCA TCA CTA TCG GCA AT-3' and (reverse) 5'-AGA GGT CTT TAC GGA TGT CAA CGT-3'. Beta-actin was used as an internal control.

### Western blotting analysis

Proteins were extracted from frozen liver samples with radioimmuno-precipitation buffer containing 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1% Nonidet P-40, 2  $\mu$ g/mL aprotinin, 1 mmol/L phenyl-methylsulphonyl fluoride, 1% sodium dodecyl sulfate (SDS) and 0.5% deoxycholate, then centrifuged at 10 000 *g* for 10 min at 4 °C. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes. The membranes were blocked

overnight in 5% bovine serum albumin in tris buffer saline Tween20 buffer at 4 °C, and then incubated with a 1:1000 dilution of anti-HO-1 polyclonal antibody, a 1:1000 dilution of anti-PPAR $\gamma$  polyclonal antibody, a 1:1000 dilution of anti-NF- $\kappa$ B monoclonal antibody or anti-beta-actin monoclonal antibody overnight at 4 °C. Then the membranes were treated with horseradish peroxidase-conjugated secondary antibody. Blots were visualized using the Super ECL detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Relative densities of the bands were analyzed using the Kodak Digital Science Imaging System.

### Statistical analysis

Data were expressed as mean  $\pm$  SE and were analyzed with SPSS Version 13.0. Differences between experimental groups were analyzed using one-way analysis of variance or Student's *t* test. All differences were considered statistically significant when *P* < 0.05.

## RESULTS

### Expression of HO-1 in the liver of rats in different groups

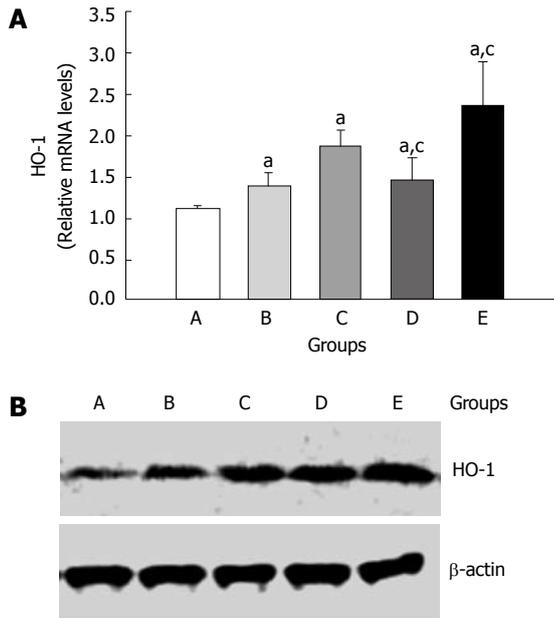
Long-term application of CCl<sub>4</sub> can induce hepatic fibrogenesis not only in humans but also in rats. We established CCl<sub>4</sub> rat models to evaluate the effect of HO-1 expression on liver fibrogenesis (Figure 1). The process lasted for 6 wk. In this study, we used two opposite reagents, i.e., hemin (induction of HO-1) and ZnPP-IX (inhibition of HO-1) from week 4 to week 6 to observe the regulation and mechanism of HO-1 in rat liver fibrosis. The mRNA and protein expressions of HO-1 in groups B and C were significantly higher than group A and increased with the severity of fibrosis, but all values were lower than in group E (*P* < 0.01); while those in group D were lower than in group C (*P* < 0.01), but still higher than in group A (*P* < 0.01).

### Effects of HO-1 expression on rat model of hepatic fibrogenesis

The rat model for group B exhibited inflammatory infiltration, hepatic steatosis and slight fibrosis (Figure 2B and G), while group C showed obvious fibrosis (Figure 2C and H). Treatment with hemin from week 4 to week 6 markedly reduced the severity of hepatic inflammatory infiltration and fibrosis (Figure 2E and J), whereas hepatic steatosis, inflammatory infiltration, especially fibrosis in hepatic portal areas, varying degrees of fibrosis around the central vein and extension to the hepatic lobule were aggravated in groups treated with ZnPP-IX (Figure 2D and I). These results indicate that HO-1 induction could protect rats from CCl<sub>4</sub>-induced liver injury and fibrosis.

### Effects of HO-1 on the levels of serum ALT, AST and ALB

The levels of serum ALT and AST increased with the development of fibrosis, and were higher in group C than in group B (Table 1). The increase in serum ALT and AST was markedly augmented by ZnPP-IX in group D (*P* < 0.01), but attenuated by hemin in group E com-



**Figure 1** Effects of hemin and zinc protoporphyrin on the expression of heme oxygenase-1 in the liver of rats with fibrosis caused by CCl<sub>4</sub>. Group A (normal, untreated); group B (model for 4 wk, untreated); group C (model for 6 wk, untreated); group D (model for 6 wk, treated with zinc protoporphyrin IX from week 4 to week 6); group E (model for 6 wk, treated with hemin from week 4 to week 6). A: Heme oxygenase-1 (HO-1) mRNA levels, determined by real-time quantitative polymerase chain reaction; B: HO-1 protein levels, detected by Western blotting. Data are expressed as the mean ± SE (*n* = 4 per group). <sup>a</sup>*P* < 0.01 vs the levels in group A; <sup>c</sup>*P* < 0.01 vs the levels in group C.

pared to group C (*P* < 0.01). The levels of ALB in group E increased significantly compared to group C (*P* < 0.01). Meanwhile, ALB levels decreased in group D but did not differ significantly from group C.

**Effects of HO-1 on the expression of fibrosis-related indicators**

To evaluate the effect of HO-1 induction on fibrosis, we assessed the expression levels of hepatic fibrosis-related indicators, i.e., HA, IV-C, Hyp and α-SMA. Rats in group D injected with ZnPP-IX showed enhanced expression of hepatic α-SMA and Hyp, which correlated with the levels of serum HA and IV-C. Meanwhile mice in group E injected with hemin exhibited depressed expression of these compared with group C (*P* < 0.01), which still did not recover to the levels of group B (*P* > 0.05) (Figure 3).

**Role of HO-1 in the expression of PPARγ and NF-κB at mRNA and protein levels**

Studies have shown that up-regulating the activation of PPARγ resulted in a significant reduction of type I collagen and α-SMA expression, inhibited HSC proliferation and even reversed the development of liver fibrosis<sup>[2-4]</sup>. Studies also have found that up-regulating the activation of NF-κB inhibited HSC apoptosis and promoted the release of inflammatory response factors<sup>[24]</sup>. To evaluate the mechanism of the effect of HO-1 on fibrosis, we explored the expression of PPARγ and NF-κB at levels of transcription and translation. Unlike the trends of HO-1,

**Table 1** Quantitation of alanine aminotransferase, aspartate aminotransferase and albumin in the serum from different groups of rats (mean ± SE)

Groups	<i>n</i>	ALT (U/L)	AST (U/L)	ALB (g/L)
A	12	20.80 ± 5.49	29.40 ± 4.45	40.2 ± 1.789
B	11	102.00 ± 24.54 <sup>b</sup>	122.00 ± 31.43 <sup>b</sup>	37.33 ± 2.42
C	9	166.50 ± 19.47 <sup>b</sup>	211.83 ± 29.16 <sup>b</sup>	28.50 ± 2.59 <sup>b</sup>
D	9	265.67 ± 43.61 <sup>b,d</sup>	323.83 ± 40.22 <sup>b,d</sup>	26.17 ± 2.93 <sup>b</sup>
E	10	114.40 ± 22.99 <sup>b,d</sup>	152.20 ± 25.65 <sup>b,d</sup>	32.80 ± 2.68 <sup>b,d</sup>

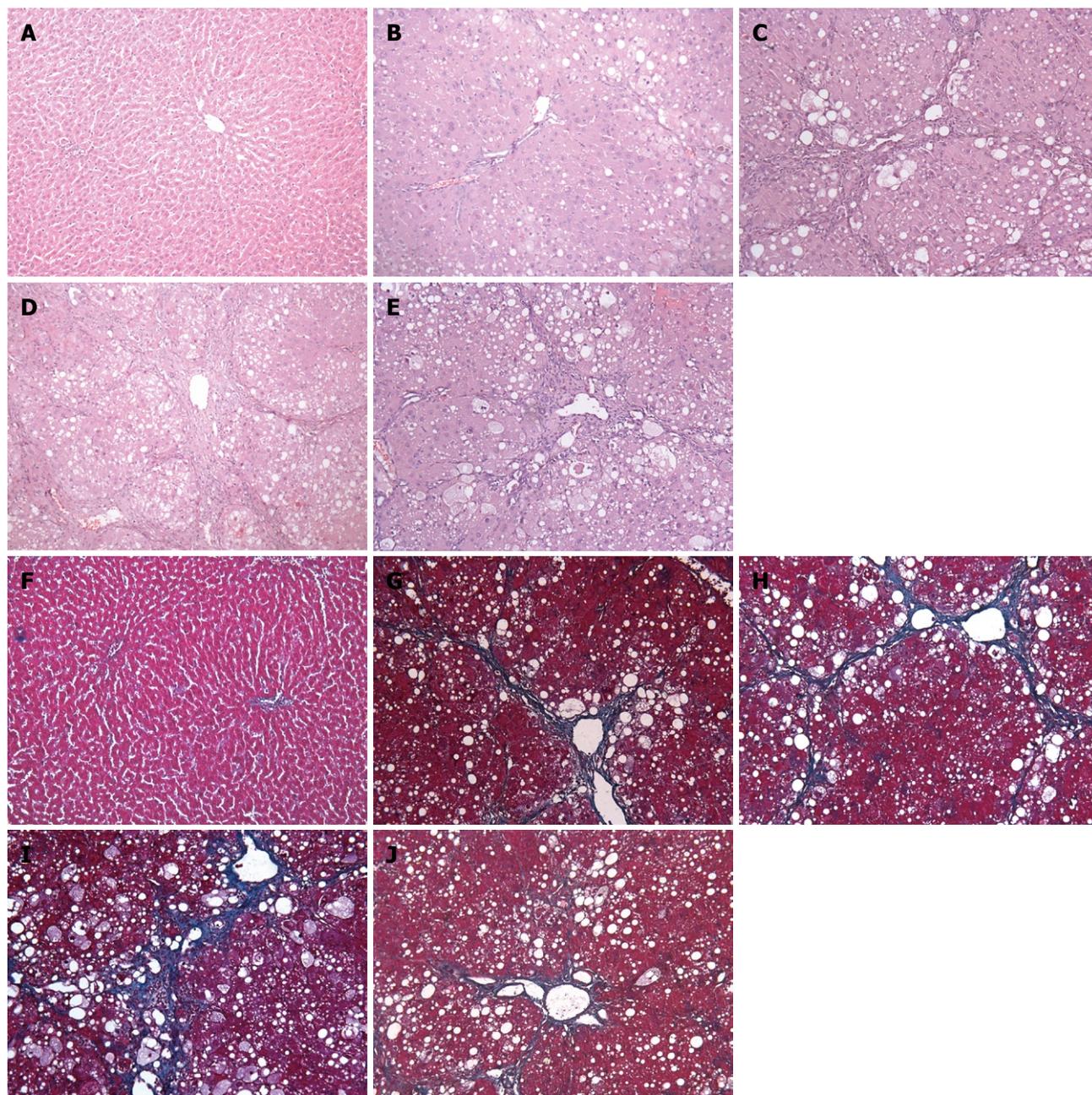
<sup>b</sup>*P* < 0.01 vs the levels in group A; <sup>d</sup>*P* < 0.01 vs the levels in group C. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin.

real-time PCR and Western blotting showed that the expression of PPARγ decreased with the development of liver fibrosis (Figure 4A). The expression of PPARγ decreased more obviously after application of HO-1 inhibitor in group D as compared with group C (*P* < 0.05). On the contrary, PPARγ increased significantly after pretreatment with hemin in group E, and was higher than group C (*P* < 0.01). However, the expression of NF-κB gradually increased with the development of liver fibrosis, which was consistent with the change of HO-1 (Figure 4B). After using the inhibitor of HO-1 in group D, the expression of NF-κB increased as compared with group C, whereas expression decreased more significantly than group D and even group C when HO-1 was induced in group E (*P* < 0.01).

**DISCUSSION**

Liver fibrosis is the mechanism of compensation and reparation after chronic hepatic injury, which is a necessary pathologic stage from chronic hepatitis to cirrhosis. Previous studies have found that 25%-40% of liver fibrosis will eventually develop to cirrhosis and even liver cancer<sup>[25]</sup>. Therefore, it is essential to further clarify the mechanism of liver fibrosis in order to block and reverse the process of liver disease. We constructed liver fibrosis models in rats by using composite factors which had been confirmed successfully in the Department of Pathophysiology, Shanxi Medical University<sup>[18,19]</sup>. At the fourth week we observed inflammatory infiltration, hepatic steatosis and slight fibrosis in livers, and obvious liver cirrhosis could be seen at the sixth week.

HO-1 is the rate-limiting enzyme for heme degradation in a wide range of human and mammalian tissues. Prior clinical and animal research has confirmed that an external irritant could up-regulate the expression of HO-1 with increasing levels of oxygen-derived free radicals in cells<sup>[26]</sup>. It has previously been reported that induction of HO-1 is an important defense mechanism against many kinds of liver injuries. In chronic liver disease, especially liver fibrosis, induction of HO-1 can reduce the secretion of type I collagen, thus effectively preventing the development of liver fibrosis<sup>[27-30]</sup>. In this study, we also examined the effects of HO-1 inhibitor or inducer, and found that with the development of liver fibrosis, the expression of HO-1 was significantly

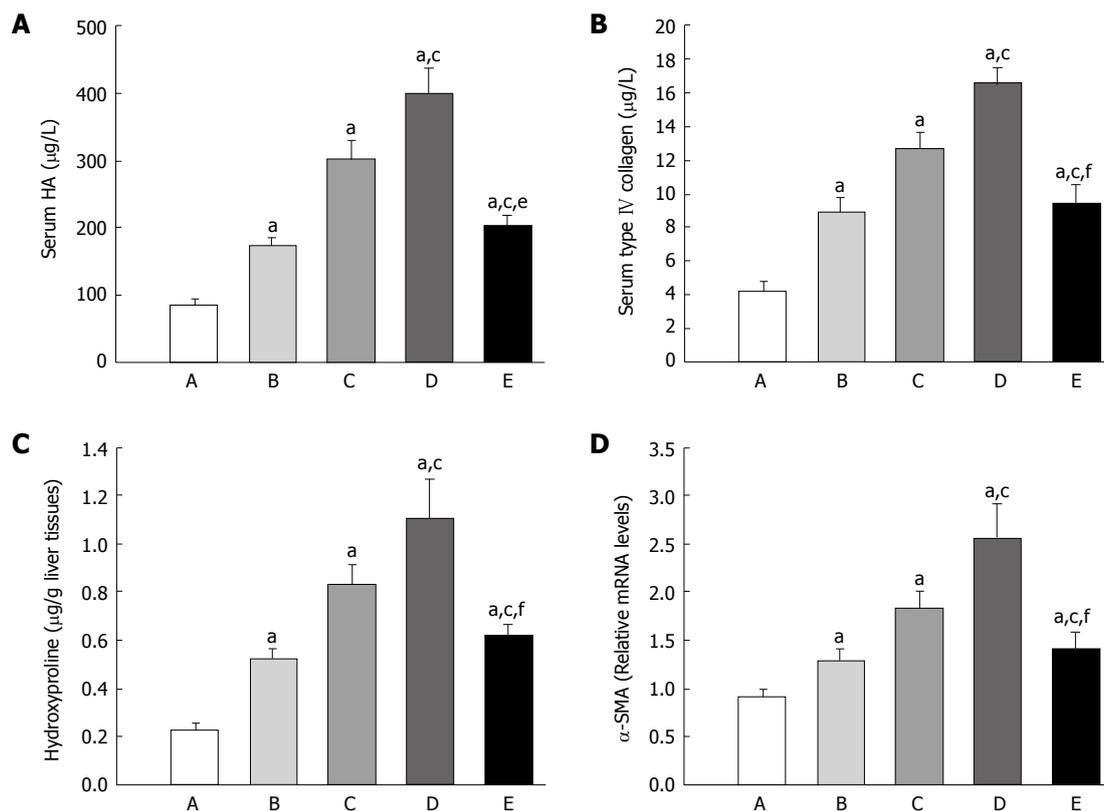


**Figure 2** Effects of heme oxygenase-1 on the histopathology of rat liver fibrosis induced by CCl<sub>4</sub>. A, F: Group A (normal, untreated); B, G: Group B (model for 4 wk, untreated); C, H: Group C (model for 6 wk, untreated); D, I: Group D (model for 6 wk, treated with zinc protoporphyrin IX from week 4 to week 6); E, J: Group E (model for 6 wk, treated with hemin from week 4 to week 6). A-E: Hematoxylin and eosin staining (magnification × 200); F-J: Masson staining of collagens (magnification × 200).

enhanced in liver of rats, whereas hemin pretreatment made this induction more prominent. We analyzed the biochemical parameters reflecting liver damage related to function and structure, such as serum ALT and AST levels, which indicated a remarkable decrease after HO-1 induction. Liver histopathology also clearly showed that HO-1 induction markedly reduced the severity of hepatic inflammatory infiltration and fibrosis. To further validate the protection by HO-1, we pretreated rats with concomitant ZnPP-IX (a competitive HO-1 inhibitor) and observed that the liver damage was more serious than in the control group and hemin-treated group. Then we assessed the levels of HA and IV-C in serum

and detected the content of Hyp and  $\alpha$ -SMA mRNA in rat liver tissues of the different groups, in order to determine the proliferation levels of fibrosis. Results showed that the induction of HO-1 could reduce all the biochemical indicators of fibrosis and attenuate the degree of fibrosis detected pathologically, while the inhibitor of HO-1 caused an opposite result. Taken together, we concluded that induction of HO-1 in hepatic tissues could produce anti-inflammatory effects and slow the process of liver fibrosis effectively; however the inhibition of HO-1 could enhance the liver fibrosis.

PPAR, including  $\alpha$ ,  $\beta$  and  $\gamma$  subtypes, are new steroid hormone receptors and ligand-activated transcription



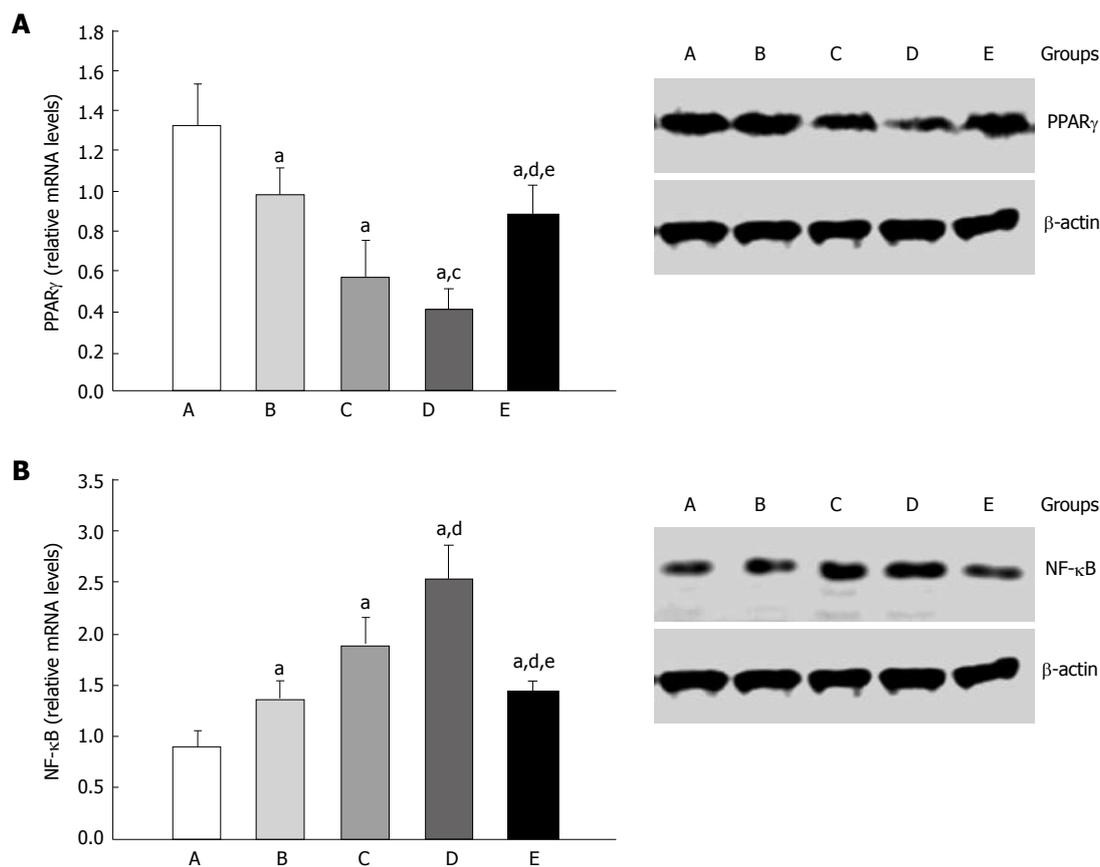
**Figure 3** Effects of heme oxygenase-1 on hyaluronate acid, type IV collagen, hydroxyproline and  $\alpha$ -smooth muscle actin expression. A: Levels of serum hyaluronate acid (HA); B: Levels of serum type IV collagen (IV-C); C: Quantity of liver hydroxyproline; D: Levels of liver alpha-smooth muscle actin ( $\alpha$ -SMA) mRNA. Values are expressed as mean  $\pm$  SE. <sup>a</sup> $P < 0.01$  vs the levels in group A; <sup>c</sup> $P < 0.01$  vs the levels in group C; <sup>b</sup> $P < 0.05$  vs the levels in group B; <sup>f</sup> $P > 0.05$  vs the levels in group B.

factors. PPAR $\gamma$  plays an important role in many biological processes, including adipogenesis, inflammatory reaction, cell growth regulation and cell differentiation<sup>[31]</sup>. The expression of PPAR $\gamma$  is beneficial in maintaining the quiescent phenotype of HSC; however the inhibition of PPAR $\gamma$  may be an early event in HSC transformation from quiescent to activated state<sup>[11]</sup>. Studies found that the PPAR $\gamma$  agonist rosiglitazone could be used to increase the expression of PPAR $\gamma$  in activated HSC, which could reduce oxidative stress, decrease the expression of  $\alpha$ -SMA and the synthesis of type I collagen, inhibit cell proliferation and promote cell apoptosis<sup>[32]</sup>. Recent studies also found that PPAR $\gamma$  activation reduced TGF- $\beta$ 1-induced CTGF expression at both transcriptional and posttranscriptional levels in HSC<sup>[33]</sup>. Enhancement of PPAR $\gamma$  activity might interrupt the signaling pathways for platelet-derived growth factor and epidermal growth factor, and then suppress hepatic fibrogenesis<sup>[34]</sup>.

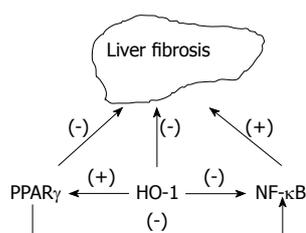
NF- $\kappa$ B is a nuclear transcriptional activator that plays a central role in stress response and inflammation<sup>[35]</sup>. Activation of NF- $\kappa$ B can promote HSC proliferation, reduce HSC apoptosis and increase the production of collagen and inflammatory chemokines in the process of liver fibrosis. But inhibiting the activation of NF- $\kappa$ B can induce apoptosis of HSC. Studies have found that a decrease of PPAR $\gamma$  was accompanied with an increase of NF- $\kappa$ B in lung tissues, which played an important role in the development of lung fibrosis<sup>[36]</sup>. PPAR $\gamma$  can inhibit the transcription and DNA synthesis of NF- $\kappa$ B by binding

p50/p65 subunits to form transcriptional repressor complexes directly or by binding p300 and CBP co-activating factors to inhibit the transcription and expression of NF- $\kappa$ B competitively<sup>[37]</sup>. Other research showed that a specific inducer of PPAR $\gamma$  such as troglitazone could interfere with NF- $\kappa$ B signaling pathway by activating PPAR $\gamma$ <sup>[38]</sup>.

Studies in other areas have shown that co-regulation exists between HO-1 and PPAR $\gamma$ . Research regarding the interaction of HO-1 and PPAR $\gamma$  in human vascular endothelial cells demonstrated that HO-1 enzymatic activity mediated antiinflammatory and antiproliferative effects exerted by PPAR ligands, and that a clinically relevant (GT)n dinucleotide length polymorphism within the human HO-1 promoter significantly influenced the transcriptional regulation of HO-1 by both PPAR $\alpha$  and PPAR $\gamma$ <sup>[39]</sup>. Li *et al.*<sup>[40]</sup> reported that induction of HO-1 could mediate the effect of PPAR $\gamma$  in suppressing the proliferation of rat pulmonary artery smooth muscle cells, but that this effect was blocked by knockdown of HO-1 through siRNA transfection. Recent studies demonstrated that HO-1, as an identifier of novel trophoblast invasion-related genes, controlled motility *via* up-regulation of PPAR $\gamma$ ; researchers found that up-regulation of PPAR $\gamma$  protein and activity by HO-1 was required to down-regulate cell motility, but blocking of PPAR $\gamma$  largely abolished the effect of HO-1<sup>[41]</sup>. Studies also found that there was an NF- $\kappa$ B binding site in the HO-1 promoter region. The activity of HO-1 was directly related to NF- $\kappa$ B<sup>[42]</sup>. HO-1 played an important



**Figure 4** Levels of peroxisome proliferator-activated receptor gamma mRNA and nuclear factor-kappa B mRNA in rat liver analyzed by real-time polymerase chain reaction assay and associated protein assessed by Western blotting. A: Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) mRNA and protein. The molecular weight of PPAR $\gamma$  is 55 kD; B: Nuclear factor-kappa B (NF- $\kappa$ B) mRNA and protein. The molecular weight of NF- $\kappa$ B is 50 kD.  $\beta$ -Actin was used as an invariant control. Values are expressed as mean  $\pm$  SE ( $n = 4$  per group). <sup>a</sup> $P < 0.01$  vs the levels in group A; <sup>c</sup> $P < 0.05$  vs the levels in group C; <sup>d</sup> $P < 0.01$  vs the levels in group D; <sup>e</sup> $P < 0.01$  vs the levels in group E. Control for equal loading.



**Figure 5** The regulatory pathway between heme oxygenase-1 and peroxisome proliferator-activated receptor gamma or nuclear factor-kappa B in liver fibrosis of rats. (+) indicate promoting; (-) indicate inhibiting. PPAR $\gamma$ : Peroxisome proliferator-activated receptor gamma; HO-1: Heme oxygenase-1; NF- $\kappa$ B: Nuclear factor-kappa B.

role in the down-regulation of NF- $\kappa$ B activation. Yeh *et al*<sup>[43]</sup> showed that HO-1 activation could attenuate the surge of inflammation-related cytokines and decrease the occurrence of cardiomyocytic apoptosis *via* inhibition of NF- $\kappa$ B and AP-1 translocation. Liu *et al*<sup>[44]</sup> indicated that up-regulation of HO-1 could alleviate severe acute pancreatitis-associated lung injury in rats by decreasing NF- $\kappa$ B activity drastically and inhibiting the serum levels of tumour necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 significantly. Overexpression of HO-1 could protect against TNF- $\alpha$ -mediated airway inflammation by dimin-

ishing NF- $\kappa$ B activation in both cultured human tracheal smooth muscle cells and the airways of mice<sup>[45]</sup>.

In this study, we found that the expression of PPAR $\gamma$  was decreased in group D, which was treated with ZnPP-IX to inhibit the expression of HO-1, whereas the expression of NF- $\kappa$ B was increased. But PPAR $\gamma$  was overexpressed in group E by treating with hemin to induce the expression of HO-1, whereas the expression of NF- $\kappa$ B was reduced. By examining the HE stained liver histology, we found that the degree of liver fibrosis was significantly higher in group D than in groups C and E. Masson staining for collagen showed the same results: that the collagen content in group D was significantly increased compared to group C, but was markedly reduced in group E compared with groups D and C. Thus we hypothesize that induction of HO-1 could alleviate the liver injury and reverse the process of liver fibrosis by up-regulating the expression of PPAR $\gamma$  and down-regulating the expression of NF- $\kappa$ B, and then affect the releasing of inflammatory cytokines such as TNF- $\alpha$  in NF- $\kappa$ B-related signal pathways or induce HSC apoptosis. Among these, the inhibition of NF- $\kappa$ B may be regulated directly by HO-1 on the one hand, or on the other hand be regulated by the expression levels of PPAR $\gamma$  which are associated with the expression of HO-1 (Figure 5). In conclusion, our data

demonstrate that the ability of HO-1 to alleviate liver fibrosis is correlated with the regulation of PPAR $\gamma$  and NF- $\kappa$ B, which construct a complex network system. Further study with regard to this mechanism will help us to form new strategies for the effective treatment of liver fibrosis.

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## COMMENTS

### Background

Heme oxygenase-1 (HO-1) is a microsomal enzyme and rate-limiting enzyme. HO-1 and degradation products are important defense mechanisms against many kinds of liver injuries. In chronic liver disease, induction of HO-1 is important to prevent the development of liver fibrosis effectively. However, the underlying molecular mechanisms are still unknown.

### Research frontiers

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is a ligand-activated transcription factor which benefits the maintenance of the hepatic stellate cell (HSC) static phenotype. Nuclear factor-kappa B (NF- $\kappa$ B) plays an important role in the regulation of gene transcription which can promote HSC proliferation and decrease HSC apoptosis, then aggravate liver fibrosis. Studies have shown that co-regulation exists between HO-1 and PPAR $\gamma$ . HO-1 can mediate the effect of PPAR $\gamma$ . Studies have also found that there is an NF- $\kappa$ B binding site in the HO-1 promoter region. HO-1 plays an important role in the down-regulation of NF- $\kappa$ B activation.

### Innovations and breakthroughs

In this study, by establishing a model of liver fibrosis in rats, the authors attempted to investigate the effects of HO-1 on liver fibrosis, and to evaluate whether the role of HO-1 in liver protection was achieved by regulating the expression of PPAR $\gamma$  and NF- $\kappa$ B, which are both important in the process of liver fibrosis.

### Applications

This study further clarified one of the mechanisms of HO-1 in liver protection, which could help provide a new strategy for treating liver fibrosis.

### Terminology

HO-1, also known as heat shock protein 32, is a microsomal enzyme and rate-limiting enzyme that catalyzes heme degradation into biliverdin, iron atoms and carbon monoxide. HO-1 and its breakdown products play vital physiological roles in anti-inflammation, anti-oxidation and regulation of apoptosis according to reports. PPAR is a ligand-activated transcription factor which is widely distributed in the tissues. Three PPAR subtypes have been identified, namely  $\alpha$ ,  $\beta$  and  $\gamma$ . NF- $\kappa$ B is an important nuclear transcription factor, which plays an important role in the regulation of gene transcription such as cytokines, chemokines, adhesion molecules and other inflammatory mediators. Zinc protoporphyrin-IX is a selective HO inhibitor which can suppress the activity of HO-1 by blocking carbon monoxide production and restricting the transformation of heme to biliverdin. Hemin is a well-known physiological substrate and potent inducer of HO activity.

### Peer review

The authors explored the protective effect of HO-1 in the CCl $_4$  rat model of liver fibrosis. In order to determine the mechanism of such protection, the authors evaluated the expression of two important transcription factors, PPAR $\gamma$  and NF- $\kappa$ B. These transcription factors are involved in regulation of hepatic stellate cell activation, the primary cell responsible for liver fibrosis. This study proposes a pathway for the protective action of HO-1 in liver fibrosis. If reproduced by other investigators, this pathway could provide information that helps in designing new therapies for prevention and treatment of liver fibrosis.

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## Treatment of cholecystitis with Chinese herbal medicines: A systematic review of the literature

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### Abstract

**AIM:** To analyze the literature on the use of Chinese herbal medicines for the treatment of cholecystitis.

**METHODS:** The literature on treatment of cholecystitis with traditional Chinese medicines (TCM) was analyzed based on the principles and methods described by evidence-based medicine (EBM). Eight databases including MEDLINE, EMBASE, Cochrane Central (CCTR), four Chinese databases (China Biological Medicine

Database, Chinese National Knowledge Infrastructure Database, Database of Chinese Science and Technology Periodicals, Database of Chinese Ministry of Science and Technology) and Chinese Clinical Registry Center, were searched. Full text articles or abstracts concerning TCM treatment of cholecystitis were selected, categorized according to study design, the strength of evidence, the first author's hospital type, and analyzed statistically.

**RESULTS:** A search of the literature published from 1977 through 2009 yielded 1468 articles in Chinese and 9 in other languages; and 93.92% of the articles focused on clinical studies. No article was of level I evidence, and 9.26% were of level II evidence. The literature cited by Science Citation Index (SCI), MEDLINE and core Chinese medical journals accounted for 0.41%, 0.68% and 7.29%, respectively. Typically, the articles featured in case reports of illness, examined from the perspective of EBM, were weak in both quality and evidence level, which inconsistently conflicted with the fact that most of the papers were by authors from Level-3 hospitals, the highest possible level evaluated based on their comprehensive quality and academic authenticity in China.

**CONCLUSION:** The published literature on TCM treatment of cholecystitis is of low quality and based on low evidence, and cognitive medicine may function as a useful supplementary framework for the evaluation.

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**Key words:** Cholecystitis; Traditional Chinese medicine; Literature analysis; Randomized controlled trials; Cognition-based medicine

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Dong ZY, Wang GL, Liu X, Liu J, Zhu DZ, Ling CQ. Treatment of cholecystitis with Chinese herbal medicines: A systematic review of the literature. *World J Gastroenterol* 2012; 18(14): 1689-1694 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i14/1689.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i14.1689>

## INTRODUCTION

Cholecystitis, defined as a type of acute or chronic inflammation occurring in the gallbladder caused by infection, bile stimulus, reflux of pancreatic juice to the biliary passage, as well as bilirubin and lipoid metabolic disorders *etc.* Cholecystitis is often secondary to previously asymptomatic gallstone disease<sup>[1]</sup>. Around 90%-95% of cholecystitis cases are claimed to be caused by gallstone disease, the incidence of which is 8%-10% in America and 3%-11% in China<sup>[2,3]</sup>. Recent epidemiological studies have shown that the incidence of cholelithiasis has been continuously rising, and the rate is doubling every 10 years<sup>[4]</sup>. The incidence rate of cholelithiasis grows steadily with age, varies by race, and occurs more frequently in female patients than in male patients<sup>[2]</sup>.

For symptomatic cholecystitis, antibiotics and antispasmodic treatment are conventional therapy while cholecystectomy or laparoscopic cholecystectomy are also appropriate modalities of treatment<sup>[5]</sup>. However gallstone disease of this type may recur within several months. Gallstones may also recur in the biliary tract after cholecystectomy<sup>[2]</sup>. Therefore, it is important to identify effective treatment options and adjuvant therapeutic methods for cholecystitis. Traditional Chinese medicines (TCM) has a long history of use for treating cholecystitis and has developed an integrate system of medical examination and treatment. Classic TCM works such as Huang Di Nei Jing and Shang Han Za Bing Lun have both expounded on this disease in depth. In TCM, cholecystitis is categorized as a type of illness with symptoms such as aching over the lateral torso, jaundice, hepatic distention, gallbladder distention and abdominal pain, *etc.*<sup>[6,7]</sup>. Cholecystitis is considered by TCM to be caused mainly by unrestrained food and drink, exogenous heat and moisture, chronic illness and/or injury<sup>[8]</sup>.

The large quantity of research literature on the TCM treatment of cholecystitis in China stimulates the development of innovative and improved therapeutic methods for the treatment of the disease. However, even basic information about the literature such as the level of evidence, quantity, trends in publication, and existence of research institutes remains unclear since they have not been sufficiently studied or evaluated outside of China due to barriers by language and access. Thus, a comprehensive analysis of this large quantity of literature is urgently required.

Based on the principles and methods described by evidence-based medicine (EBM), this study conducted an examination and statistical analysis of current literature on the treatment of cholecystitis with TCM, aiming to

discuss the necessity for a systematic review as well as producing a reference to enable better research of TCM.

## MATERIALS AND METHODS

### Literature search

Electronic literature searches were conducted on the following databases: China Biological Medicine Database (CBM), Chinese National Knowledge Infrastructure Database (CNKI), Database of Chinese Science and Technology Periodicals (VIP), Database of Chinese Ministry of Science and Technology (Wanfang), The Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE (*via* PubMed) and EMBASE. The databases were searched from the earliest possible date until June 1, 2009. The search terms included (“Cholecystitis” or “Acalculous Cholecystitis” or “Emphysematous Cholecystitis” or “Cholecystitis, Acute” or “Cholecystitis, Chronic”) and (“Chinese herbs” or “TCM” or “Chinese medicine” or “Integrated TCM WM” or “herb” or “herbs” or “traditional Chinese medicine” or “Drugs, Chinese Herbal”). The search terms were adjusted depending on the database being searched. Titles and abstracts of all citations were screened independently by two reviewers (Dong ZY and Wang GL).

### Literature selection criteria

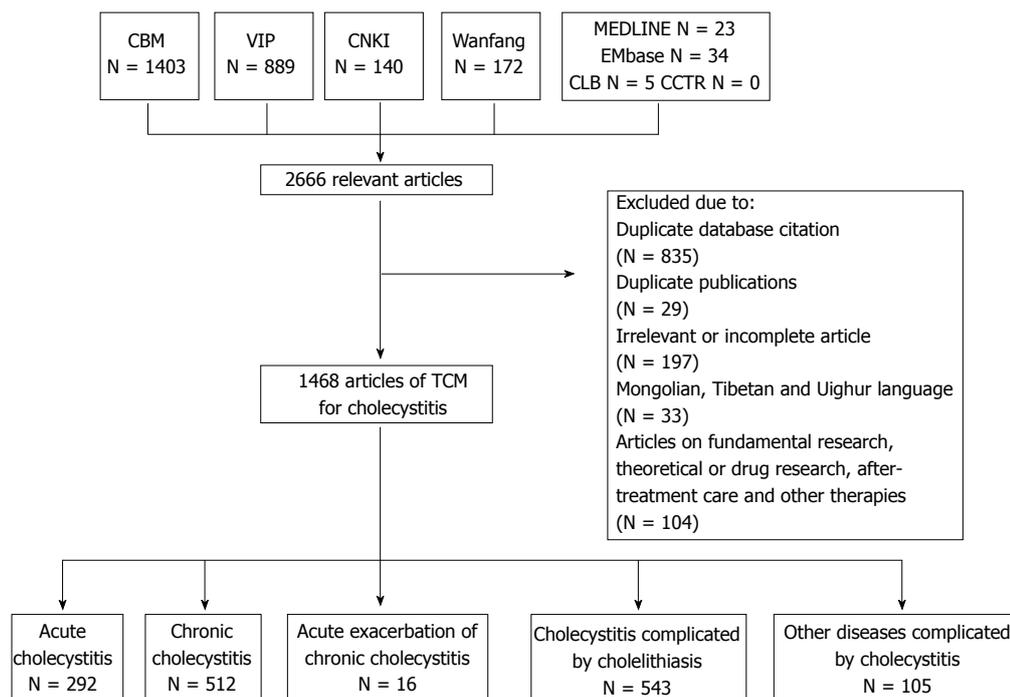
Articles on TCM treatment of cholecystitis were included; and articles on cholecystitis treated by integrated traditional Chinese and modern medicine were included.

### Data acquisition and quality assessment

The full-text of articles that met all the selection criteria was retrieved. The data were screened independently by two reviewers (Dong ZY and Wang GL) using a self-made data extraction form which collected the following information: year of publication, first author, the organization of the first author, the hospital level of the first author, titles of authors, study design, type of article, journal name, and indexed/citation situation by medical indexing databases. The first author of each article was contacted if there were any missing data. Articles that did not meet the inclusion criteria were excluded by reading the titles and summaries. Disagreements whether a paper was to be included were resolved by discussion.

### Methodology of data classification

Methodology of data classification were listed below. (1) Classified by types of study<sup>[9]</sup>; (2) Classified according to indexed/citation situation (evaluated according to 2008 edition of “Guide to the Core Journals”, and the list of MEDLINE contains Chinese journals 2008)<sup>[10,11]</sup>; (3) Classified according to the first authors’ hospital-level<sup>[12]</sup>: Level-3 hospital - The national, provincial, municipal large hospitals and affiliated hospitals of medical colleges; Level-2 hospital - General hospitals of cities, counties, districts, hospitals affiliated to factories, mining enterprises and institutions; Level-1 hospital - Country-



**Figure 1 Prism flow diagram.** CBM: China Biological Medicine Database; VIP: Database of Chinese Science and Technology Periodicals; CNKI: Chinese National Knowledge Infrastructure Database; CLB: The Cochrane Library; CCTR: Cochrane Central; TCM: Traditional Chinese medicines; N: Number.

side primary hospitals, townships or neighborhood community and private clinics and hospitals; and (4) Classified according to the strength of evidence (Grading quality of evidence and strength of recommendations, strength grading standards by Cochrane Collaboration)<sup>[13]</sup>.

### Statistical analysis

Statistical data were collected and recorded in Excel and SPSS 17.0. Percentages, percentage bar charts and trends lines were produced to analyze the situation and trends, while the constituent ratios were expressed in terms of percentages in order to perform a descriptive analysis.

## RESULTS

### Results of literature screening

Altogether 2666 potentially relevant articles were retrieved from eight databases, among which 1822 were written in Chinese and 835 were duplicate citations between databases. Twenty-nine duplicate articles were excluded; there were 197 irrelevant or incomplete articles; 33 articles were written in Mongolian, Tibetan and Uighur; and 104 articles were on fundamental research, theoretical or drug research, after-treatment care and other therapies. Thus, there were 1468 articles on the TCM treatment of cholecystitis in total. Among these articles, there were 292 concerning cholecystitis, 512 regarding chronic cholecystitis, 16 regarding acute exacerbation of chronic cholecystitis, 543 regarding cholecystitis complicated by cholelithiasis, and 105 on other diseases complicated by cholecystitis. Among the retrieved articles, 9 were foreign articles, including 2 in Bulgarian, 1 in Russian and 6 in English (Figure 1).

We also searched Evidence Based Complementary and Alternative Medicine (eCAM), The American Journal of Chinese Medicine (AJCM), Journal of Chinese Integrative Medicine (J Chin Integr Med), Chinese Journal of Integrated Traditional and Western Medicine and Alternative Medicine Review (Altern Med Rev). No relevant articles were found in any of these sources.

### Literature type

In total, 1468 articles on TCM treatment of cholecystitis were retrieved: 15 articles on treatment and care, 24 on animal and fundamental experimental research, 25 on theoretical research, 9 on relevant drug research and 22 on other therapies including massage, ear points, diet, infrared ray, acupuncture and ultrasonic therapy, *etc.* The data revealed that researches on clinical treatment covered the majority of the relevant literature, with the percentage as high as 93.92%, and that TCM was applied to treat almost all types of cholecystitis (Table 1).

### Evidence level of the literature

The 1468 articles were categorized according to the Cochrane collaboration criteria as shown in Table 2. No article was included into the category of the highest evidence strength, namely level I, while 136 (9.26%) articles were categorized into level II, 101 articles (6.88%) into level III, 961 articles (65.46%) into level IV; and 270 articles (18.39%) into level V. This revealed that, with randomized controlled trial (RCT) forming a low percentage, the evidence level of the research literature on TCM treatment of cholecystitis appears to be relatively low, requiring a further systematic evaluation of RCT in

**Table 1 Literature type**

	Acute cholecystitis	Chronic cholecystitis	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis	Other diseases complicated by cholecystitis	Total
Clinical trial study	292	512	16	543	105	1468
Treatment and care	3	0	0	12	0	15
Animal and fundamental experimental research	3	3	0	17	1	24
Theoretical research	3	10	0	10	2	25
Other therapies	3	12	0	7	0	22
Relevant drug research	2	4	1	2	0	9
Total	306	541	17	591	108	1563

**Table 2 Evidence strength analysis**

	Acute cholecystitis (H/M)	Chronic cholecystitis (H/M)	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis (H/M)	Other diseases complicated by cholecystitis (H/M)	Total	Evidence level
Systemic review	0	0	0	0	0	0	I
RCT	35 (2/2)	62 (6/0)	6 (1/0)	28 (1/1)	5 (1/0)	136	II
Case-control	19 (1/0)	40 (2/1)	1	37 (4/1)	4 (1/0)	101	III
Case report	203 (9/3)	336 (26/0)	7 (1/0)	344 (27/1)	71 (7/0)	961	IV
Experience reports/ Masters experience	31 (2/0)	54 (4/0)	2	109 (7/2)	20 (1/0)	216	V
Review	5 (1/0)	19	0	25 (2/0)	5 (1/0)	54	V
Total	293	511	16	543	105	1468	

H: Core Chinese Journals; M: MEDLINE; Foreign literatures number = 9: 2 pieces of Literatures in Bulgarian, 1 in Russian, 6 in English (SCI), 1 RCT Chronic Cholecystitis, 2 case reports, 1 case comparison, 1 review (cholecystitis), 1 case report (other complicated cholecystitis). RCT: Randomized controlled trial.

**Table 3 Distribution of the first authors' hospital level**

	Acute cholecystitis	Chronic cholecystitis	Acute exacerbation of chronic cholecystitis	Cholecystitis complicated by cholelithiasis	Other diseases complicated by cholecystitis	Total
Level-3 hospitals	107	149	10	163	35	464
Level-2 hospitals	106	197	5	193	37	538
Level-1 hospitals and others	76	132	1	163	28	400
Medical schools and universities	4	26	0	16	5	51
Research institutes	0	7	0	6	2	15

order to determine the efficacy and safety of the TCM treatment of cholecystitis.

**General status of the literature included by databases**

Among the 1477 articles on TCM treatment of cholecystitis both in Chinese and English, 107 (7.24%) were included by core Chinese journals. The 10 articles included in MEDLINE and the 6 in SCI represented 0.68% and 0.41% of the articles, respectively. This reflects a seemingly inadequate writing quality, low research level of the literature, and a generally low international recognition.

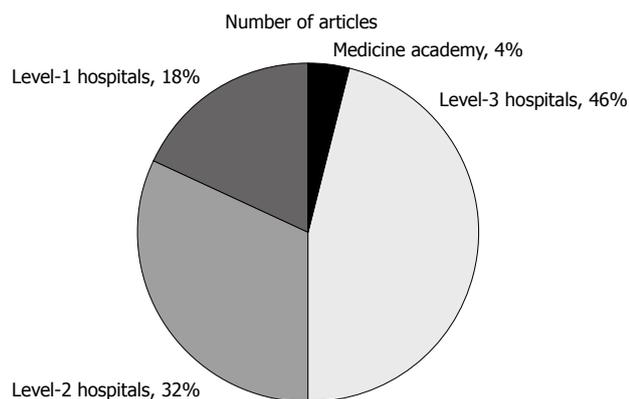
**Distribution of the first authors' hospitals**

The 1468 Chinese articles on TCM treatment of cholecystitis were categorized according to the first authors' hospital levels as shown in Table 3. Among the literature, authors from level-3 hospitals contributed 464 articles (31.61%); level-2 hospitals 538 (36.65%); medical schools

and universities 51 (3.47%); research institutes 15 (1.02%); and level-1 hospitals, township hospitals and private clinic/hospitals contributed 400 (27.25%). This shows that the authors of the research literature in this study mainly came from level-3 and level-2 hospitals, which accounted for 68.26% of the total. The RCT distributed as such: level-3 hospitals contributed 62 articles (45.59%); level-2 hospitals 43 (31.62%), level-1 hospitals and others 25 (18.38%); medical schools and universities 6 (4.41%) (Figure 2). Literature with a higher evidence level was also mainly contributed by authors from level-3 hospitals and medical schools/universities.

**DISCUSSION**

From the above analysis, two major weaknesses were revealed by the selected literature on TCM treatment of cholecystitis. The first one is that only few papers were



**Figure 2** Distribution of the hospitals where the first authors of the randomized controlled trial research on traditional Chinese medicines treatment of cholecystitis come from.

included by SCI and MEDLINE. Although it is not appropriate to evaluate the quality of papers merely according to whether they are included by SCI, MEDLINE and other core medical databases, it is an objective index to certain degrees, based on the strict selection rules and expert evaluation system of SCI and MEDLINE<sup>[14]</sup>.

The second weakness of the literature in general lies in its low evidence level. The concept of evidence levels originated from the emergence and development of EBM, and was first proposed by the clinical epidemiologist Dave Sackett from McMaster University of Canada in 1990<sup>[15-17]</sup>. High-quality papers and RCTs require an adequate high-quality research platform. Is the large quantity of papers with low evidence level given rise to by an inadequate platform for the research of TCM? To answer this question, after analyzing the distribution of the hospitals, institutions or universities of the first authors, we discovered that 35.08% of the papers were contributed by authors from level-3 hospitals and universities. This accounts for all the articles included by SCI or published in core journals not included in SCI, while 64.92% of the articles were from level-1 and level-2 hospitals with a relatively low research capacity, which indicates a close correlation between the quality of published papers and the research platform. We also found from the data that RCTs hold only less than 10% of all TCM clinical studies, which is an outstanding difference compared to the 70% share of RCTs in the clinical study of modern medicine. Why such a low percentage TCM clinical study is from RCTs is a significant question that deserves further consideration<sup>[18]</sup>.

EBM is a young discipline; the first International EBM Research Center was established in the United Kingdom in 1993, and the first EBM Research Center in China was set up three years later and quickly motivated the spread of EBM theory within China. Although the preference of Chinese TCM researchers for clinical study models somewhat limits the strict RCT research to a certain degree, TCM has its own unique treatment models and standards which deserves proper recognition. For example, it advocates individualized treatment and medication. Namely,

different treatment methods and medication might be applied to the same disease and even the same syndrome according to different physical conditions of the individual patient or even according to the different time and place that the illness occurs. This makes it hard for TCM to conduct a high-quality RCT which might be the major cause resulting in the low evidence level of these Chinese papers.

By examining the literature on the treatment of cholecystitis with TCM according to the principles of EBM, we hope to expose the problems and weakness in current TCM clinical studies so as to raise the quality of TCM research.

Here arises the question, how to evaluate the therapeutic effectiveness of TCM more scientifically? This is the right and urgent question that not only TCM but also the entire alternative and complementary medicine should address. EBM experts are trying to further perfect the research standard of RCTs and drafting research guidelines that can better meet the characteristics of TCM. Besides, more scholars are trying to improve the present frame of EBM. Professor Keine<sup>[18]</sup> from the Institute for Applied Epistemology and Medical Methodology in Germany has conducted a study of cognition-based medicine. Cognition-based medicine is a newly-developed methodological system of scientific medicine. Its primary element is the criteria-based assessment of therapeutic causality at the level of the individual patient<sup>[19]</sup>. Principles and criteria of single-case causality assessment have been analyzed and explained. Cognition-based medicine enables a methodological professionalization of clinical judgment as well as the explication of physician experience and expertise. Cognition-based medicine study design expands the current range of clinical research, extending from criteria-based causality assessment in single cases to new forms of cohort evaluations. Though cognition-based medicine studies only started recently, this trend is inspiring and promising. It will not only facilitate the evaluation of TCM, which greatly emphasize individualized medical treatment solution, but also accord with the trend of medical development which stresses the significance of individualized treatment, and cognition-based medicine, a beneficial complement to EBM, may play a significant role in clinical research<sup>[20,21]</sup>.

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## COMMENTS

### Background

Epidemiological studies have shown that the incidence of cholelithiasis in recent years has been continuously rising and doubling every 10 years. For symptomatic cholecystitis, antibiotics and antispasmodic treatment are adopted clinically as conventional therapy while cholecystectomy or laparoscopic cholecystectomy are also considered as surgical modalities. However, gallstone disease of this type may recur within several months. Gallstones may also recur in the biliary tract after cholecystectomy. Therefore, it is important to identify effective treatment options and adjuvant therapeutic methods for cholecystitis.

### Research frontiers

By analyzing, from the perspective of evidence-based medicine, the substantial amount of Chinese literature over the past 10 years concerning the use of traditional Chinese medicine in the treatment of cholecystitis, the authors discovered the problems existing in these relevant studies, and proposed that cognitive medicine could provide supplementary methodology for future research.

### Innovations and breakthroughs

When reviewing the large amount of relevant Chinese literature according to the evaluation standards provided by evidence-based medicine, most of the articles appear to be of poor design, quality and evidence level. However, many of these studies showed that traditional Chinese medicine functions effectively in treating cholecystitis. Cognition-based medicine, a beneficial complement to evidence-based medicine, may play a significant role in clinical research.

### Applications

More appropriate randomized controlled trials with large samples should be designed and conducted in order to reasonably evaluate the efficacy of traditional Chinese medicine in the treatment of cholecystitis, and cognitive medicine also functions as a useful supplementary framework for the evaluation.

### Terminology

Evidence-based Medicine (EBM) aims to apply the best available evidence gained from the scientific method to clinical decision making. It seeks to assess the strength of evidence of the risks and benefits of treatments and diagnostic tests. Cognition-based medicine is a newly-developed methodological system of scientific medicine. Its primary element is the criteria-based assessment of therapeutic causality at the level of the individual patient. Principles and criteria of single-case causality assessment have been analyzed and explicated. Cognition-based medicine enables a methodological professionalization of clinical judgment as well as the explication of physician experience and expertise. Cognition-based medicine study designs expand the current range of clinical research, extending from criteria-based causality assessment in single cases to new forms of cohort evaluations. Though cognition-based medicine study only started in recent years, this trend is inspiring and promising.

### Peer review

The main stay of cholecystitis treatment is either laparoscopic cholecystectomy while it is a "hot" or conservative management with antibiotics and analgesia followed by laparoscopic cholecystectomy approximately 8 wk later. However, alternative medical therapies are used when the patient is unfit for surgical intervention. The article is suitable for publication in WJG.

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## High resolution impedance manometric findings in dysphagia of Huntington's disease

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### Abstract

Conventional manometry presents significant challenges, especially in assessment of pharyngeal swallowing, because of the asymmetry and deglutitive movements of oropharyngeal structures. It only provides information about intraluminal pressure and thus it is difficult to study functional details of esophageal motility disorders. New technology of solid high resolution impedance manometry (HRIM), with 32 pressure sensors and 6 impedance sensors, is likely to provide better assessment of pharyngeal swallowing as well as more information about esophageal motility disorders. However, the clinical usefulness of application of HRIM in patients with oropharyngeal dysphagia or esophageal dysphagia is not known. We experienced a case of Huntington's disease presenting with both oropharyngeal and esophageal dysphagia, in which HRIM revealed the mechanism of oropharyngeal dysphagia and provided comprehensive information about esophageal dysphagia.

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**Key words:** Dysphagia; Esophagus; High resolution impedance manometry; Huntington's disease; Oropharynx

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Lee TH, Lee JS, Kim WJ. High resolution impedance manometric findings in dysphagia of Huntington's disease. *World J Gastroenterol* 2012; 18(14): 1695-1699 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i14/1695.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i14.1695>

### INTRODUCTION

Conventional manometry presents considerable challenges in evaluation of oropharyngeal dysphagia because of the asymmetry and deglutitive movements of oropharyngeal structures<sup>[1]</sup>. Introduction of high resolution manometry (HRM), which employs pressure sensors at 1 cm intervals across the entire anatomic region from the oropharynx to the stomach represented a significant improvement in data recording and diagnostic yield, especially in cases of functional dysphagia over conventional manometry<sup>[1,2]</sup>. However identifying a manometric abnormality does not equate to identifying a disease and thus achalasia and (perhaps) diffuse esophageal spasm are relevant to manometric findings, which have a functional correlate and can cause dysphagia<sup>[3]</sup>. Most recently, high resolution impedance manometry (HRIM) has been introduced to combine the benefits of HRM and impedance-based bolus transit assessment. Actually patients with normal manometry can have abnormal bolus transit, and patients with abnormal manometry can have normal bolus transit<sup>[4]</sup>. Koya *et al*<sup>[5]</sup> reported that abnormal impedance even in patients with normal manometry may be a sensitive indicator of esophageal functional abnormality as represented by the symptom of dysphagia in these patients.

To our knowledge, little is known with regard to in-

investigation of dysphagia using the HRIM technique. We experienced a case of Huntington's disease presenting with both oropharyngeal and esophageal dysphagia, in which HRIM revealed the mechanism of oropharyngeal dysphagia, and provided comprehensive information about esophageal dysphagia.

## CASE REPORT

### Present illness

A 65-year-old male was admitted to the hospital because of a 5-year history of progressive dysphagia. Five years before admission, he began to have difficulty in swallowing liquids such as tea and thin soup. Three years later he complained of difficulty in eating liquids as well as solid foods, pain extended up to the manubrium, and he had lost 12 kg in weight. Ten years before admission, the patient became aware of involuntary movements of all his limbs and face. In his family history, his mother and younger brother complained of the same symptoms.

### Physical study in general and neurological study

The patient had a history of pulmonary tuberculosis, which had been cured 30 years previously. He had smoked cigarettes for 40 years. He had drunk moderate amounts of alcohol for 40 years, but he had stopped drinking 3 years before admission. His body temperature was 36.5 °C, pulse was 85 bpm, and respirations were 18 breaths/min. Blood pressure was 125/85 mmHg. Physical examination revealed mild dysarthria, chorea, and limitation of down gaze in eye movement. No muscle rigidity, muscle atrophy or pathologic reflexes were noted. Brain magnetic resonance imaging exhibited ventriculomegaly and mild atrophy in all regions of his brain. The patient was finally diagnosed with Huntington's disease by the result that the CAG repeat numbers in the Huntington gene were 44 in comparison with the normal numbers of 10-35.

### Endoscopic study

The whole mucosa of the hypopharynx and esophagus was normal except for a mucosal break less than 5 mm from the esophagogastric junction. Endoscopy revealed relatively normal opening of the upper esophageal sphincter (UES) and low esophageal sphincter, and no presence of residual food in the esophagus. However, spastic contraction of the mid esophagus was noticed.

### Esophagogram study

Esophagography demonstrated barium retention with significant delay of contrast passage into the stomach. There were frequent irregular contractions between the mid and distal esophagus. There were no typical signs of primary esophageal motility disorder such as bird-beak appearance or corkscrew appearance.

### Video fluoroscopic swallowing study

On swallowing of a spoonful of pudding mixed with

barium powder, the patient had a tendency to eat rapidly. Labial closure was normal but disorganized tongue movement, as well as postural instability induced by chorea resulted in residual bolus in the vallecula and pyriform sinuses.

### HRIM study

A solid-state HRIM manometry assembly (Sandhill Scientific Instruments Inc. United States) was used to evaluate dysphagia in the patient. A HRIM study was also performed in 26 healthy persons to compare the characteristics of pharyngeal motility. The HRIM catheter was 4.0 mm diameter with 32 solid pressure sensors and 6 impedance sensors. The 4 active impedance channels were located in the traditional locations for analysis, i.e., 5, 10, 15 and 20 cm above the high pressure zone of the lower esophageal sphincter (LES). There were 32 pressure sensors which spanned the esophagus from UES to the LES to allow for evaluation of swallows from initiation of the swallow to closure of the LES. The zero mark on the probe was located at the channel used for LES analysis. The study was performed in a sitting position after at least 6-h fasting. The HRIM assembly was passed transnasally and positioned to record from the hypopharynx to the stomach with about 3-5 intragastric sensors. The catheter was fixed in place by taping it to the nose. The manometric protocol included a 5-min period to assess basal sphincter pressure, 10 5-mL saline swallows and 10 5-mL viscous swallows (so called standard method). Manometric data were acquired and stored using software (Sandhill Scientific Instruments Inc. United States). The HRIM catheter was pulled back by 10 cm and the same sessions were repeated because of inability to assess all the pharyngeal manometric information and bolus transit of pharyngoesophageal segment (so called modified method). Takasaki *et al*<sup>[6]</sup> reported that vocalizing "kakakaka" in investigation of pharyngeal swallow using high resolution manometry easily identified the locations of the velopharyngeal swallowing pressure. Therefore vocalizing "kakakaka" was added to the HRIM study using modified method.

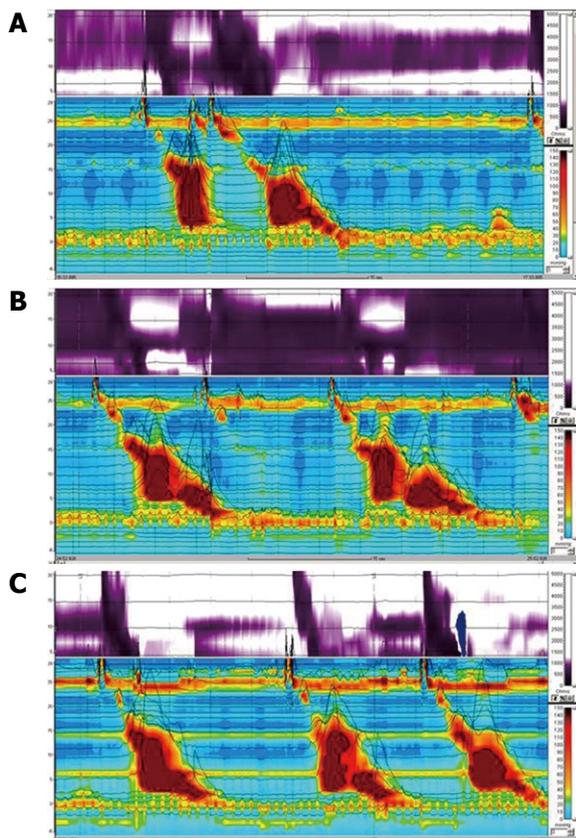
### Initial HRIM findings by the standard method

The results of HRIM by the standard method are summarized in Table 1. Impedance results demonstrated 100% swallowing with incomplete bolus transit of both the liquid and the viscous solution. Manometric results revealed high LES pressure with incomplete relaxation (Figure 1A). For liquid bolus, distal esophageal high pressure simultaneous repetitive contraction was observed in 70% of swallows on the manometric topographic view and abnormal liquid transit was noted on the impedance contour view. For viscous bolus, contractile pressures were higher, and simultaneous repetitive contractions were noted on the manometric topographic view, which was associated with the sensation of retrosternal bolus hold-up, chest pain, and abnormal viscous transit (Figure 1B).

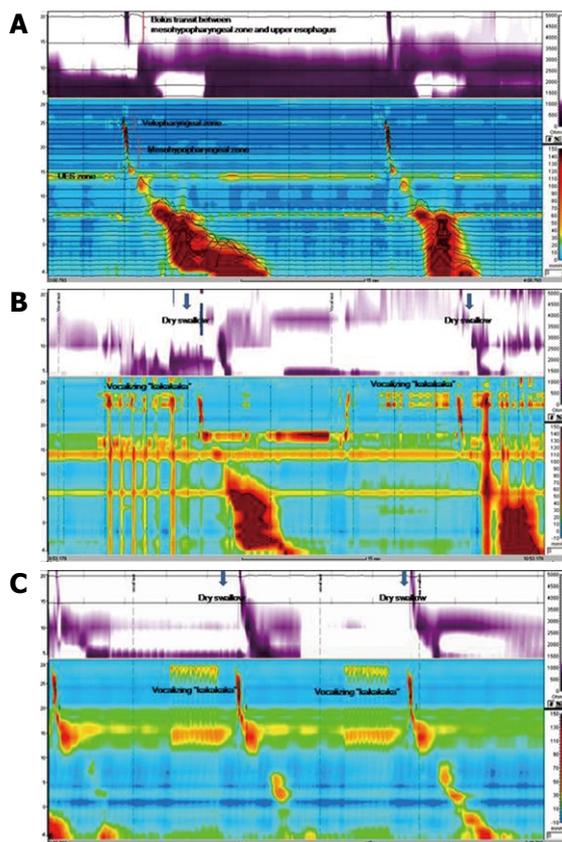
**Table 1 High resolution impedance manometric findings by the standard method**

	Initial finding	After botulinum toxin injection
<b>Manometry</b>		
LES pressure (mmHg)	44.2	37.2
<b>Relaxation of LES</b>		
Residual pressure (mmHg)	12.1	10.8
Duration (s)	10.1	9.3
Relaxation percent (%)	74	71
<b>Body peristalsis</b>		
Simultaneous contraction (%)	70	60
Peristaltic contraction (%)	30	40
Aperistalsis (%)	0	0
Amplitude of lower esophagus (mean, mmHg)	441	382
<b>Impedance</b>		
<b>Liquid swallow</b>		
Incomplete bolus transit (%)	100	70
Complete bolus transit (%)	0	30
<b>Viscous swallow</b>		
Incomplete bolus transit (%)	100	70
Complete bolus transit (%)	0	30

LES: Lower esophageal sphincter.



**Figure 1 High resolution impedance manometry findings using the standard protocol.** A: Liquid swallowing at admission reveals high amplitude, simultaneous contractions of esophageal body with incomplete lower esophageal sphincter relaxation, and incomplete bolus transit; B: Viscous swallowing at admission demonstrates higher amplitude, repetitive contractions and incomplete bolus transit; C: After injection of botulinum toxin, the isocontour of impedance shows considerably improved bolus transit compared with those at admission during saline swallows (Figure 1A), but spasms are still seen on the isocontour of manometry.



**Figure 2 High resolution impedance manometry findings using modified protocol.** A: Patient; unremarkable peristaltic contraction and bolus transit between the hypopharynx and upper esophagus are seen after pull back of the catheter; B: Patient; irregular contractions are seen at the velopharyngeal zone, and simultaneous contraction between velopharyngeal and mesohypopharyngeal zone accompanying impaired bolus transit of pharyngo-upper esophageal segment after vocalizing “kakakaka”; C: Healthy subject without dysphagia; regular contractions are noted at the velopharyngeal zone, and peristaltic contractions of pharyngo-upper esophageal segment are normal as well as bolus transit after vocalizing “kakakaka”.

**HRIM findings by the standard method after post-botulinum toxin injection**

Given the esophageal dysmotility with a spastic component, subsequent trials of a proton pump inhibitor, a nitrate, a calcium channel blocker, and a phosphodiesterase inhibitor were made for 1 mo. There was no response to these drugs in the patient. Treatment with 100 U botulinum toxin injected around the lower esophagus showed considerable improvement in the impedance results and in symptoms such as retrosternal bolus hold-up and chest pain despite no change in the manometry results (Figure 1C).

**Initial HRIM findings by the modified method**

HRIM using the standard method demonstrated normal UES relaxation and peristaltic pharyngeal pressure (UES pressure 41.1 mmHg, residual pressure -1.1 mmHg, duration 0.6 s, 100% relaxation). After withdrawal of the catheter by 10 cm, HRIM revealed unremarkable bolus transit and peristalsis between the meso hypopharynx and upper esophagus (Figure 2A). However, HRIM using the modified method and vocalizing “kakakaka”

revealed irregular contractions of the velopharyngeal zone, simultaneous contraction between the velopharyngeal and mesohypopharyngeal zone, and impaired bolus transit of the pharyngo-upper esophageal segment (Figure 2B). In contrast to the HRIM findings of our case, HRIM findings using the modified method from all healthy persons showed regular contractions of the velopharyngeal zone and normal bolus transit between mesohypopharyngeal zone and the upper esophagus (Figure 2C).

## DISCUSSION

To our knowledge, this report is the first study of Huntington's disease using HRIM, which indicated the combined oropharyngeal and esophageal dysphagia. The patient had difficulty in initiating swallowing and had retrosternal bolus hold-up. Insidious onset of dysphagia associated with some neurologic symptoms such as chorea and dysarthria suggested oropharyngeal dysphagia from a neurologic basis. A video fluoroscopic swallowing study provided the diagnosis of oropharyngeal dysphagia which resulted from a lack of coordination between the oral and pharyngeal stage. Unexpected involuntary movement in the oral cavity induced oropharyngeal incoordination. Oropharyngeal dysphagia in Huntington's disease results from tachyphagia, or rapid uncontrolled swallowing, secondary to impaired sensory and cognitive function<sup>[7]</sup>. Furthermore, it is caused by buccolingual chorea resulting in food being transferred impulsively<sup>[7]</sup>. Respiratory chorea, marked by involuntary respiratory movements, occurs in approximately 40% of patients, and interrupts the normal respiratory-deglutition cycle<sup>[8]</sup>. Given the anatomical structures involved in vocalization, the HRIM findings using the modified method may reflect the mechanism of oropharyngeal dysphagia in the Huntington's disease patient. In other words, the modified test may disclose oropharyngeal incoordination related to buccolingual chorea, which cannot be detected even with HRM or HRIM using the usual protocol such as the liquid and viscous swallow test. Further investigation should be carried out to confirm the role of the vocal test in either HRM or HRIM studies for assessing oropharyngeal dysphagia related to chorea.

HRIM results concerning esophageal dysphagia appeared to indicate diffuse esophageal spasm (DES) such as simultaneous contraction associated with > 10% of wet swallows, mean simultaneous contraction amplitude > 30 mmHg, and repetitive contractions. Esophageal dysmotility of the patient, however, can be better classified as an atypical disorder of LES relaxation because there was incomplete relaxation of the LES<sup>[9]</sup>. The esophageal dysmotility in this case may be an intermediate form between DES and achalasia because a few case reports have suggested a transition from DES to achalasia in some patients<sup>[10-12]</sup>.

Kagel *et al.*<sup>[13]</sup> reported esophageal dysphagia was relatively uncommon in Huntington's disease. It was most

likely secondary to the disruptive effects of chorea in the aerodigestive tract. To our knowledge there has been no report regarding spastic esophageal dysmotility in Huntington's disease. The cause of the spastic esophageal motility disorder in the patient was unknown. There are a few studies documenting acid reflux-induced esophageal spasm<sup>[12,14,15]</sup>. Simultaneous contractions from gastroesophageal reflux should be treated first by a proton pump inhibitor<sup>[16]</sup>. Reflux esophagitis in this case may be considered the cause of the esophageal dysmotility with a spastic component. However, there was no improvement under therapy with a proton pump inhibitor.

Considering dysphagia associated with chest pain attributable to esophageal spasm, subsequent trials of a nitrate, a calcium channel blocker, and a phosphodiesterase inhibitor were performed. There was no response to these drugs in the patient. Actually current treatments for esophageal spasm, including calcium channel blockers and nitrate donors, are limited by poor efficacy and side effects<sup>[17]</sup>. Recently botulinum toxin injections in the lower esophagus and at the level of the gastroesophageal junction have been reported to have a beneficial effect in patients suffering from DES<sup>[18-20]</sup>. These beneficial effects of botulinum toxin are perhaps related to the improved manometric results. Interestingly, injection of botulinum toxin in this case showed improvement in impedance results and symptoms despite no improved manometry results. It suggests that the esophageal symptoms are more closely related to disturbed bolus transport and impaired clearance than esophageal dysmotility *per se*<sup>[4]</sup>. It may also suggest that esophageal dysmotility in terms of abnormal contractility and impaired bolus transit is limited in explaining the dysphagia. Dysphagia symptoms are related to factors other than esophageal dysmotility, e.g., esophageal sensitivity disorder.

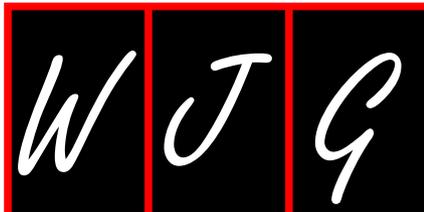
In conclusion, HRIM enabled us to obtain more detailed and important information in the Huntington's disease patient with both oropharyngeal dysphasia and esophageal spastic dysmotility. Further investigation of HRIM will be needed to assess its role in either oropharyngeal or esophageal dysphagia.

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## Rifaximin for the prevention of spontaneous bacterial peritonitis

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### Abstract

According to a review article by Biecker *et al* published in a previous issue of *World Journal of Gastroenterology* in March 2011, intestinal decontamination with norfloxacin remains the mainstay of primary prophylaxis of spontaneous bacterial peritonitis (SBP) at the expense of development of quinolone-resistant bacteria after long-term use. In our research, the administration of a 4-wk regimen with rifaximin 1200 mg/d reduced significantly the ascitic neutrophil count in cirrhotic patients with sterile ascites in line with a significant decrease in plasma endotoxin levels. Our observations concur with recent findings, showing a significantly reduced 5-year probability of SBP in cirrhotic patients taking rifaximin.

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**Key words:** Rifaximin; Cirrhosis; Ascites; Spontaneous bacterial peritonitis

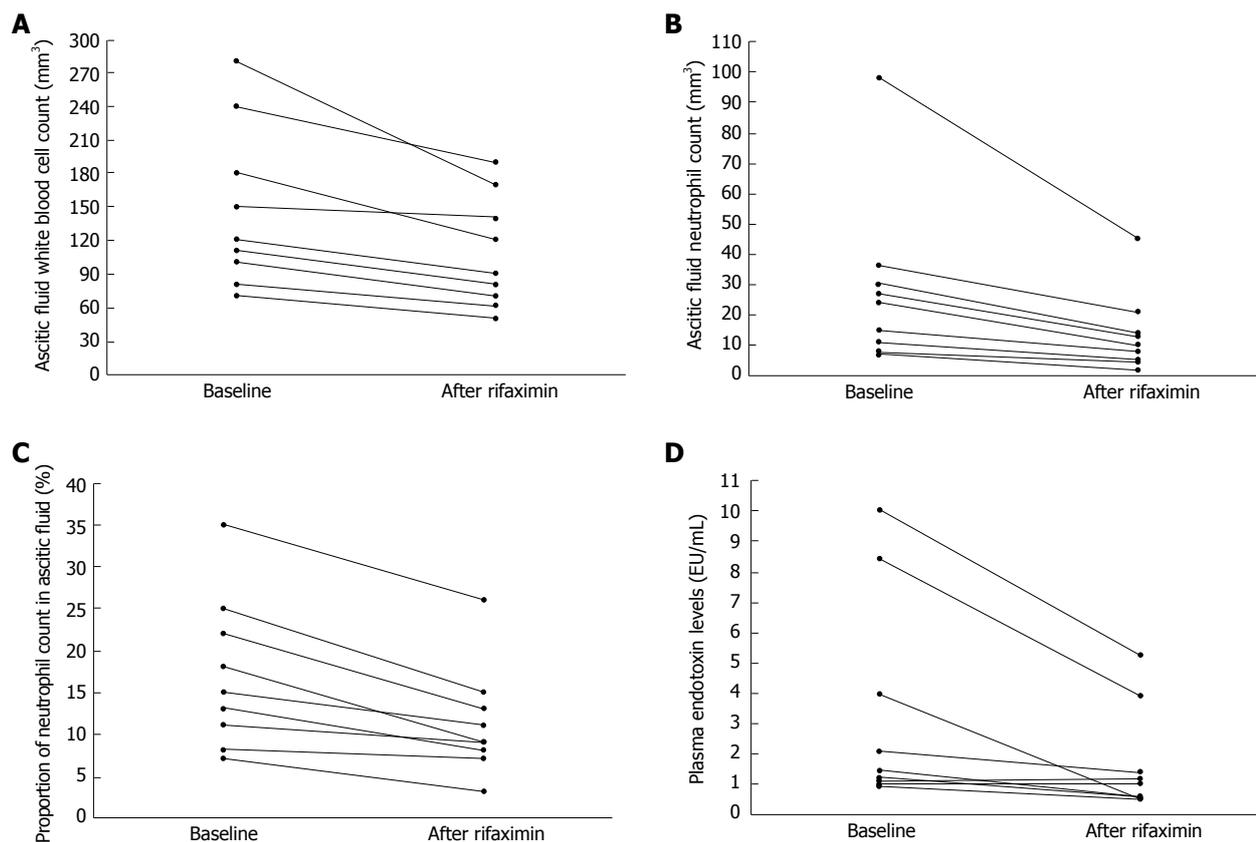
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### TO THE EDITOR

We read with great interest the article by Biecker *et al*<sup>[1]</sup> regarding management of ascites published on *World J Gastroenterol* 2011; 17: 1237-1248. Development of spontaneous bacterial peritonitis (SBP) is a major complication of ascites and possibly the final step in a series of events, including intestinal bacterial overgrowth (IBO), bacterial translocation (BT) resulting in bacteremia, endotoxemia, and colonization of mesenteric lymph nodes, and finally seeding of bacteria into the ascitic fluid (AF). Indeed, SBP in non-hospitalized patients is mostly caused by gram-negative bacteria of intestinal origin. It has been hypothesized that patients at risk of SBP have probably sustained multiple episodes of colonization and resolution before they present with the first clinically apparent infection. In this respect, a previous study showed that higher neutrophil counts in sterile AF are associated with higher risk of subsequent development of SBP<sup>[2]</sup>. The high mortality of SBP warrants its prevention with administration of antibiotics aimed at decreasing the burden of gut bacteria, thus interrupting the sequence of events leading to AF infection. Norfloxacin is widely used for primary prophylaxis of SBP; however its extensive long-term use has increased the incidence of quinolone-resistant and gram-positive SBP<sup>[1]</sup>.

Rifaximin is an antibiotic with a broad-spectrum activity against gram-positive and gram-negative microorganisms within the gastrointestinal tract. The main



**Figure 1** Individual changes in ascitic fluid white blood cell (A), neutrophil count (B) and proportion of neutrophils (C), and plasma endotoxin levels (D) after administration of rifaximin for 4 wk.

advantage of rifaximin is that it is virtually unabsorbable, which minimizes the antimicrobial resistance and adverse events and renders the drug safe in all patient populations. In addition, rifaximin has a better activity against gram-positive organisms than norfloxacin<sup>[3]</sup>.

We investigated whether rifaximin can reduce the burden of gut flora and BT, which are the requisite effects of a drug used for SBP prophylaxis, by studying its effects on circulating endotoxin levels and AF neutrophil counts in cirrhotic patients with sterile ascites.

Sixteen cirrhotic patients with ascites with no history of previous SBP episodes who required regularly a large-volume paracentesis were included in our study. Cirrhosis was established by non-invasive and/or histological criteria; all patients were Child Pugh class C. The patients were studied at baseline and after a 4-wk regimen with rifaximin 1200 mg/d (Group 1, *n* = 9; alcohol/viral etiology: 7/2) or an observational period (Group 2, *n* = 7; alcohol/viral etiology: 5/2). Exploratory paracentesis was performed in association with each therapeutic paracentesis to exclude ascitic fluid infection. All patients were included after written informed consent was obtained from them and the local scientific-ethical committee approved the study. Criteria for inclusion were: (1) Abstinence from alcohol for at least 6 mo before inclusion; (2) Absence of clinical and laboratory signs of bacterial infections; (3) No history of variceal bleeding within the 2 wk preceding the study; and (4) No

treatment with antibiotics during the last 8 wk before inclusion. For ethical reasons, only patients with AF total protein concentration >1 g/dL were studied. AF white blood cell (WBC) and neutrophil count, the proportion of neutrophils in AF (AF% neutrophils), and plasma endotoxin levels were measured at baseline and at the end of observational or treatment period. For the detection of plasma endotoxin levels, the Limulus amoebocyte lysate chromogenic endpoint assay (Hycult biotech, Uden, The Netherlands) was used as instructed by the manufacturer. The Wilcoxon matched pairs test was used for comparing variations within the same group. Results were expressed as mean ± SE. Statistical significance was designated as *P* < 0.05.

Rifaximin caused significant reductions in AF WBC, neutrophil count, AF% neutrophil count, and plasma endotoxin levels (Table 1); the values of the abovementioned parameters decreased uniformly in all patients (Figure 1). No significant changes in the AF cytological characteristics or plasma endotoxin levels were noted in Group 2. No patient developed AF infection during the study period and no side-effects were noted by the use of rifaximin.

Our findings strongly suggest that rifaximin suppresses IBO, which in turn reduces BT and the subclinical activation of AF defence mechanisms from prior silent colonisations with bacteria in cirrhotic patients with sterile ascites. The reduction of endotoxemia by ri-

Table 1 Ascites cytological characteristics and plasma endotoxin levels in patients after rifaximin treatment or in observational period

	Group 1 (n = 9)			Group 2 (n = 7)		
	Baseline	4 wk	P value	Baseline	4 wk	P value
WBC count (per mm <sup>3</sup> )	147.7 ± 24.1	107.7 ± 16.6	0.004	164.5 ± 30.2	175 ± 20.8	NS
Neutrophil count (per mm <sup>3</sup> )	28.4 ± 9.3	13.5 ± 4.3	0.01	34.6 ± 6.4	37.9 ± 7.2	NS
AF% neutrophils	17.1 ± 3	11.2 ± 2.1	0.0008	21.6 ± 3.5	22.1 ± 2.5	NS
Plasma endotoxin (EU/mL)	3.3 ± 1.1	1.6 ± 0.5	0.03	2.9 ± 0.9	3 ± 0.8	NS

Data are expressed as mean ± SE. WBC: White blood cell; NS: Not significant; AF: Ascitic fluid. Group 1: 9 cirrhotic patients with refractory ascites at baseline and after 4-wk rifaximin treatment; Group 2: 7 patients in the observational period.

faximin may further reduce BT by causing a fall in portal pressures<sup>[4]</sup> considering that portal hypertension induces structural abnormalities in intestinal mucosa leading to an enhanced permeability<sup>[1]</sup>. Overall, the effects of rifaximin on IBO and BT in our study are consistent with recent findings, showing a significantly reduced 5-year probability of SBP in cirrhotic patients taking rifaximin<sup>[5]</sup>. In conclusion, the role of rifaximin as an alternative mean of preventing SBP deserves further attention in prospective studies.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States

**GENERAL INFORMATION**

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*World Journal of Gastroenterology*

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Redit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only

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### Acknowledgments

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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. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar 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]

*No volume or issue*

- 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

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- 15 Morse SS. Factors in the emergence of infectious dis-

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## Role of ileostomy in restorative proctocolectomy

Gianluca Pellino, Guido Sciaudone, Silvestro Canonico, Francesco Selvaggi

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### Abstract

Restorative proctocolectomy (RP) is the treatment of choice in patients affected with refractory ulcerative colitis or familial adenomatous polyposis. Surgery in elective settings is often performed in 2 stages, fashioning an ileostomy which is closed 2-3-mo later. It is still debated whether omitting ileostomy could offer advantages in the management of patients undergoing RP.

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**Key words:** Restorative proctocolectomy; Ileostomy; Ulcerative colitis; Familial adenomatous polyposis; Ulcerative colitis; Familial adenomatous polyposis; Ileopouch-anal anastomosis

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### INTRODUCTION

Restorative proctocolectomy (RP) with ileopouch-anal anastomosis (IPAA) is considered the treatment of choice for patients affected by ulcerative colitis (UC) and familial adenomatous polyposis (FAP) who require surgery. RP removes the disease, reduces the long-term risk of carcinogenesis and preserves transanal defecation<sup>[1]</sup>. Short-term results demonstrate excellent functional outcomes with good quality of life, while some deterioration of function is reported in the long term. Pelvic sepsis is the most serious complication of RP, leading to pouch failure or malfunction<sup>[2,3]</sup>. Since the first description of IPAA<sup>[4]</sup>, many techniques have been introduced to prevent or limit the consequences of an IPAA leak; the necessity of fashioning a covering ileostomy at the time of RP remains controversial. The aim of this review is to establish the role of a covering ileostomy in patients undergoing RP for UC or FAP.

### ILEOSTOMY AND PELVIC SEPSIS/POUCH FAILURE

RP is generally performed in two stages in elective settings, but it can be performed as a single-stage procedure in order to avoid ileostomy and its complications<sup>[5,6]</sup>. Sugerman *et al*<sup>[5]</sup> reported a high rate of pelvic sepsis (12%) in 192 patients undergoing IPAA without covering ileostomy, with excellent function retained in 19 out of the 23 patients who developed pelvic sepsis. Teixeira *et al*<sup>[7]</sup> suggested that ileostomy does not eliminate the risk of pelvic sepsis; they found that an IPAA stricture was more frequent in patients undergoing IPAA without a covering ileostomy (31.3% *vs* 4.7%). Furthermore, Ikeuchi *et al*<sup>[8]</sup> found that the incidence of post-operative complications after the first intervention did not differ significantly between patients with or without an ileostomy: pouch-related complications affected 12 patients (8%) without ileostomy, with surgery being required in 5 cases (3.3%), and 4 patients (4.3%) with ileostomy, all of who were managed conservatively. Despite this, the authors reported that the

overall incidence of post-operative complications was significantly higher in the group with ileostomy than in the non-ileostomy group: 55.4% *vs* 32%.

It needs to be remembered that omitting ileostomy appears to expose the patient to a high risk of pouch failure due to early anastomotic leakage. In St. Mark's Hospital the rate of pouch failure was higher in patients undergoing IPAA without a covering ileostomy: 15% *vs* 8%<sup>[9]</sup>. Data from the Cleveland Clinic of 1965 patients undergoing RP revealed that anastomotic leak occurred in 5.3% of the patients with ileostomy, but in > 14% when ileostomy was omitted<sup>[10]</sup>. The global rate of complications after 1504 ileostomy closures was as high as 11.4%<sup>[11,12]</sup>.

## COMPLICATIONS/CONSEQUENCES OF ILEOSTOMY

Fashioning a covering ileostomy can be burdened by complications due to diversion, such as dehydration from excessive stoma output, the need for a second intervention and hospitalization to undergo ileostomy closure, the risk of anastomotic leak and a presumed increased risk of subsequent small-bowel occlusion, which some authors attribute to either internal hernias around the stoma or to adhesions in the proximity of the stoma site<sup>[5]</sup>. Complications such as irritation of the peristomal skin or stoma prolapse are well described in the literature<sup>[13]</sup>. Ileostomy closure is associated with longer hospitalization, and some authors report that this can increase the cost of the procedure<sup>[14]</sup>. Moreover, the preternatural anus, even if temporary, can have psychological effects and affect the patient's perception of their body<sup>[15,16]</sup>.

## ILEOSTOMY AND PRE-TAKEDOWN ASSESSMENT (SECOND-STAGE SURGERY)

A covering ileostomy after RP should be taken down after a minimum of 8 wk to allow IPAA healing. Takedown is usually performed after a clinical examination (often including pouchoscopy) and a pouch enema (pouchogram/pouchography)<sup>[17]</sup>; the latter can reportedly enable the detection of pouch or IPAA leaks and IPAA strictures<sup>[18,19]</sup>. Few studies have investigated the role of pouchography in the assessment of patients with IPAA scheduled for ileostomy closure. It has been hypothesized that a discontinuous ileoanal anastomotic ring at pouchography is sensitive (88%) but not specific (57%) in predicting a subsequent pelvic sepsis, whereas a leak is quite specific (81%) but not sensitive (56%) in predicting pelvic collections<sup>[20]</sup>. These data suggest that tiny tracts often heal spontaneously, and justify delaying ileostomy closure if a leak is observed. Pouch sinuses are tiny blind tracts originating from IPAA, and are seen at pouchography in < 8% of patients undergoing RP<sup>[21]</sup>. A leak leading to pelvic sepsis can reportedly occur even after radiological healing of a sinus<sup>[21]</sup>. A study from the Mayo Clinic suggests that pouch function is not affected by occult sinuses, ques-

tioning the utility of detecting asymptomatic defects<sup>[13]</sup>. Moreover, managing these tiny tracts aggressively could result in overtreatment. Previous studies have not clearly compared clinical data - obtained through anamnesis plus clinical examination - and pouchography. In our experience, a preoperative pouchogram in patients at risk of developing complications adds valuable information<sup>[20]</sup>, but it should not be intended as a routine examination in symptomatic UC patients with negative results of a clinical/endoscopic examination.

Comparison of data from two groups of clinically negative UC pouch patients who did ( $n = 37$ ) or did not ( $n = 33$ ) undergo pouchography before ileostomy takedown in our centre revealed that 10.8% of patients in the former group exhibited an asymptomatic radiological abnormality that was otherwise undetected (four pouch sinuses). However, the failure rate was similar in the two groups (3% *vs* 2.7%,  $P = \text{NS}$ ), as was the overall rate of complications. In addition, patients in the pouchography group who experienced failure had a normal pouchogram at the time of ileostomy closure<sup>[22]</sup>. We follow the following algorithm in our practice: a clinical/endoscopic assessment is always applied to patients scheduled to undergo ileostomy takedown, and a pouchography study is carried out if symptoms or the clinical examination make us suspect that a complication has occurred. The ileostomy of asymptomatic, clinically negative patients is closed without radiological evaluation of the pouch.

## ILEOSTOMY AND BASELINE DISEASE

Intuitively, patients undergoing a pelvic pouch procedure for non-inflammatory disease are at lower risk of developing septic complications, because they do not need to be treated with steroid medications. The findings of several studies suggest that a loop ileostomy can be safely omitted, even in patients undergoing IPAA for UC<sup>[8,23-30]</sup>. However, a recent retrospective multivariate analysis of patients undergoing RP at the Cleveland Clinic and at St. Mark's Hospital revealed that FAP is associated with a higher probability of a safe one-stage RP (odds ratio, 2.6)<sup>[31]</sup>.

## ILEOSTOMY AND STEROID DRUGS

Therapy for UC includes the use of steroid drugs, sometimes administered at a high dosage, and these patients are at risk of becoming steroid-dependent or steroid-refractory. Matikainen *et al*<sup>[23]</sup> stated that steroid therapy at the time of RP does not contraindicate one-stage surgery. However, most experienced centres consider a daily prednisone dose of  $\geq 20$  mg an exclusion criterion for a one-stage procedure<sup>[11,32]</sup>. Although not agreeing with this cut-off value, Sugerma *et al*<sup>[5]</sup> suggested that treatment with steroids is relevant to evaluating patients scheduled for RP. Ziv *et al*<sup>[33]</sup> considered that omission of ileostomy is an unacceptable procedure in patients treated with steroids at the time of RP. Ikeuchi *et al*<sup>[8]</sup> showed that patients taking steroids at the time of IPAA are more likely to develop

complications. Although they did not establish a cut-off value for steroid therapy, those authors suggest that steroid treatment is a relevant factor in selecting patients who are suitable for a one-stage procedure.

## ILEOSTOMY AND TYPE OF ILEOPOUCH-ANAL ANASTOMOSIS

During RP, the IPAA can be either hand-sewn (with or without mucosectomy) or stapled using a circular stapler introduced transanally. Some authors have reported that the type of IPAA can affect the outcome of patients undergoing RP with or without ileostomy. A hand-sewn IPAA is technically more difficult to perform, which has resulted in there being few reports of patients undergoing RP with a hand-sewn IPAA in the literature. Ikeuchi *et al*<sup>[8]</sup> reported one of the largest series, since they routinely use a hand-sewn technique. They compared 150 patients undergoing hand-sewn IPAA without ileostomy with 92 hand-sewn IPAA patients operated on with a two-stage procedure. While the rate of pouch-related complications was high, it did not differ significantly between the two groups. Some authors have reported that fashioning a hand-sewn IPAA leads to an unacceptably high risk of complications if a covering ileostomy is omitted, especially if steroid medications are taken at the time of RP. However, there is no definitive evidence regarding the impact of this factor on patient selection<sup>[11,33,34]</sup>.

With respect to the pouch shape, patients with a W-pouch are at higher risk of complications when ileostomy is omitted than those with a stapled J-pouch<sup>[31]</sup>.

## ILEOSTOMY AND TECHNOLOGICAL ADVANCES IN SURGERY

Surgical technology has made huge advances in recent years. Laparoscopic RP is widely used nowadays. Pouch surgery can be performed as a totally laparoscopic three-/four-trocar access procedure, as a laparoscopic-assisted procedure (with the pouch being constructed outside the body through a Pfannenstiel incision<sup>[35]</sup>), as a hand-assisted laparoscopic proctocolectomy (HALP), by means of a special port designed to allow the surgeon to introduce his or her hand inside the body while preserving the pneumoperitoneum<sup>[36]</sup>, and as single-access laparoscopic surgery (SILS)<sup>[37]</sup>. A recently introduced technique uses a laparoscopy-assisted robotic surgery approach to RP<sup>[38]</sup>. In the context of a minimally invasiveness, the omission of a protective ileostomy has definite advantages regarding the overall cosmetic result. Kienle *et al*<sup>[35]</sup> performed 59 laparoscopic RP procedures with extracorporeal pouch construction, omitting a primary ileostomy in 16 patients: 3 FAP (18.75%) and 6 UC (37.5%) patients developed complications (7 pelvic sepsis) requiring reoperation and secondary ileostomy. Agha *et al*<sup>[36]</sup> reported that HALP is a safe procedure with a rapid learning curve; in their experience of 19 patients undergoing RP, a protective ileostomy

was always fashioned. SILS RP is a viable procedure that produces good results if the surgeon is highly skilled in laparoscopic surgery. Gash *et al*<sup>[37]</sup> found that fashioning a protective ileostomy at the site of SILS trocar introduction seemed to be a prudent choice in their series of ten patients.

The minimally invasive approach *per se* probably does not reduce the need for a protective ileostomy. The selection criteria for the addition or omission of a protective ileostomy in minimally invasive RP remain to be clearly defined. In our opinion further studies are needed before definitive conclusions can be drawn about the advisability of avoiding an ileostomy using the most recent techniques<sup>[36-38]</sup>, with this also being dependent on the acquisition of better technical skills by surgeons and the use of longer follow-ups.

## ILEOSTOMY AND SAMPLE SIZE/STATISTICAL POWER

It should be noted that studies promoting a one-stage procedure generally lack significant statistical power, which thus renders evaluation of the pelvic sepsis rate unreliable. Heuschen *et al*<sup>[24]</sup> compared the results of 57 one-stage procedures with 114 controls and found that the rate of IPAA stricture was higher in patients with a covering ileostomy, but this finding could have been due to the selection of case patients.

Failure was more common at St. Mark's Hospital<sup>[9]</sup> when higher ileostomy was omitted (15% *vs* 8%). Conversely, in concurrent experience MacRae *et al*<sup>[39]</sup> found that the pouch failure rate was < 1% in patients undergoing one-stage RP. Recent multicentre studies<sup>[30,31]</sup> have involved wider series but only retrospective analyses. The lack of randomization, which is often incompatible with acceptable ethical conduct in such patients, represents a limitation that has yet to be overcome.

## ILEOSTOMY AND EVIDENCE BASED MEDICINE

Several factors make it difficult to perform randomized controlled trials around this topic: (1) the difficulty in enrolling sufficient patients for revealing statistically relevant differences; (2) **omitting a stoma is not indicated** in some patients because of surgical safety and (3) the unethical problems of randomizing patients at risk. With regard to UC, the most recent Consensus Paper of the European Crohn's and Colitis Organization stated that a diverting ileostomy is generally recommended, but that it can be avoided in selected patients (Evidence Level 3b, Recommendation Grade C)<sup>[40]</sup>. Currently the best way to select patients remains unclear.

## ILEOSTOMY AND PATIENTS

The responsibility regarding surgical outcomes has gained

such importance that it is now deemed necessary to involve the patient in surgical decision-making. Obese patients (body mass index  $\geq 30 \text{ kg/m}^2$ ) could benefit from stoma omission, since fashioning an ileostomy in these patients could be technically more difficult<sup>[5]</sup>. Most importantly, the patient's will to avoid ileostomy should be considered in light of the psychological implications, such that if complications do arise following one-stage surgery, the eventual subsequent need for an ileostomy will certainly affect a patient who has also undergone reintervention in an emergency setting<sup>[16]</sup>.

## PERSONAL EXPERIENCE

Between 1987 and 2011, we performed 241 RPs for UC, FAP or cancer (1 S-pouch, 121 W-pouches, and 119 J-pouches); 23 (9.5%) of these patients underwent one-stage RP. The global rates of pelvic sepsis were 7.7% and 13% among patients with or without ileostomy, respectively, while failure occurred in 5% and 4.3% of case. However, there were no statistically significant differences. Since our series covered a very long period of time (24 years), and considering the recent technical advancements, we stratified our experience into two periods, focusing on stoma omission. Between 1987 and 2000, we performed nine one-stage RPs, with a pelvic sepsis rate of 22.2% and a failure rate of 11.1%; these figures are higher than those for staged procedures (9.2% and 7%, respectively), although the difference did not reach statistical significance. Between 2001 and 2011, we performed 14 straight RPs: 7.1% of patients developed pelvic sepsis, while none of them experienced failure. Again, these data did not differ significantly from those of patients undergoing two-/three-stage procedures (pelvic sepsis in 6% and failure in 4%).

In addition to the increased level of expertise among surgeons, these improved findings are due to increasingly careful patient selection among those who could benefit from stoma omission. Most of the selected patients were female, overweight, young, affected with FAP and undergoing stapled J-pouch with stapled IPAA. Patients taking steroids were not considered suitable for a one-stage procedure. The type of surgery (laparoscopic, open or HALP) was not *per se* a selection criterion, since the choice was patient-tailored. Any bias could be attributable to us omitting ileostomy more frequently as our experience with the pelvic pouch procedure increased, especially when the surgeon had reached the plateau of the learning curve for the RP surgical technique. This is relevant, since we believe - in agreement with the great majority of the reported studies - that it is as now impossible to predict preoperatively whether a patient would be suitable for one-stage surgery. A straightforward surgery (e.g., contained blood loss, short operative time, lack of tension and good vascularization of the IPAA, no contamination, intraoperative testing of the anastomosis) is a desirable condition when choosing to omit ileostomy. These factors are definitively surgeon-related.

## CONCLUSION

At the present time, given the information in the literature and wider described series under the critical light of the EBM, the decision to omit ileostomy substantially represents an exercise in risk management. Most surgeons opt for a protective ileostomy because this is reported to diminish the risk of detrimental pelvic contamination, even if it does not abolish the risk of pelvic sepsis. Centres specializing in pelvic pouch procedures have reported that the rate of patients undergoing one-stage RP ranges between 15% and 25%<sup>[29,30]</sup>. By adopting stringent selective criteria, the rate of patients undergoing RP without an ileostomy did not reach 10% in our series. It is our opinion that omitting ileostomy is a viable alternative in a very limited number of patients who fit specific selection criteria; they must be highly motivated to avoid a stoma and well informed regarding the eventual subsequent risks. This option could be proposed to them, but it is only at the end of each pelvic pouch procedure that the surgeon will be able to take the final decision.

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## Etiology of inflammatory bowel disease: A unified hypothesis

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### Abstract

Inflammatory bowel disease (IBD), including both ulcerative colitis (UC) and Crohn's disease (CD), emerged and dramatically increased for about a century. Despite extensive research, its cause remains regarded as unknown. About a decade ago, a series of findings made me suspect that saccharin may be a key causative factor for IBD, through its inhibition on gut bacteria and the resultant impaired inactivation of digestive proteases and over digestion of the mucus layer and gut barrier (the Bacteria-Protease-Mucus-Barrier hypothesis). It explained many puzzles in IBD such as its emergence and temporal changes in last century. Recently I further found evidence suggesting sucralose may be also linked to IBD through a similar mechanism as saccharin and have contributed to the recent worldwide increase of IBD. This new hypothesis suggests that UC and CD are just two symptoms of the same morbidity, rather than two different diseases. They are both caused by a weakening in gut barrier and only differ in that UC is mainly due to increased infiltration of gut bacteria and the resultant recruitment of neutrophils and formation of crypt abscess, while CD is mainly due to increased infiltration of antigens and particles from gut lumen and the resultant recruitment of macrophages and formation of granulomas. It explained the delayed appearance but accelerated increase of CD over UC and many other phenomena. This paper aims to provide a detailed description of a unified hypothesis regarding

the etiology of IBD, including the cause and mechanism of IBD, as well as the relationship between UC and CD.

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**Key words:** Etiology; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Dietary chemicals; Saccharin; Sucralose

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### INTRODUCTION

As we know, inflammatory bowel disease (IBD) refers to ulcerative colitis (UC) and Crohn's disease (CD), two highly related debilitating diseases of the digestive tract with similar clinical, pathological, and epidemiological features<sup>[1,2]</sup>. Although some descriptions in ancient books had been suspected as symptoms of IBD, clustered cases only started to emerge around the end of the 19th century<sup>[1]</sup>. Right now, IBD has become one of the most common chronic inflammatory conditions only after rheumatoid arthritis, with millions of patients all over the world<sup>[3]</sup>. It is most prevalent in young adults and remains regarded as incurable, with the patients usually requiring lifelong heavy medication and multiple devastating surgeries like bowel resection, proctocolectomy, ileostomy, and ileal pouch-anal anastomosis, *etc.*<sup>[2,4]</sup>. As stated by Dr. Kirsner, "ulcerative colitis and Crohn's disease today represent two of the more challenging diseases in all of medicine"<sup>[1]</sup>.

Since its appearance, people had been puzzled by the constant changes of manifestations of IBD in age, gender, ethnic, temporal and geographical distributions<sup>[3,5,6]</sup>. Great efforts have been taken to find out its cause. Many factors had been suspected, including bacteria such as

*Bacillus coli*, *Bacillus proteus*, *Bacillus pyocyaneus*, *Bacillus lactis aerogenes*, diplostreptococci, dysentery bacillus, *Spherophorus necrophorus*, *Bacillus morgagni*, *Escherichia coli*, spirochetes, Mycobacteria (*Mycobacteria tuberculosis*, *Mycobacteria paratuberculosis* and *Mycobacteria kansasii*), *Pseudomonas maltophilia*, *Bacillus vulgatus*, *Aerobacter aerogenes*, *Aerobacter coprococcus*, *Aerobacter bifidobacteria*, *Campylobacter fetus ssp. jejuni*, *Yersinia enterocolitica*, and *Chlamydia trachomatis*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Edwardsiella tarda*, *Blas-tocystis hominis*, *Bacteroides necrophorum*, *Bacteroides fragilis*, *Pseudomonas maltophilia*, *Helicobacter hepaticus* or pylori species<sup>[1,7,8]</sup>; fungi like *Histoplasma* and *Monilia*<sup>[1]</sup>; virus such as lymphopathia venereum, Behcet's virus, cytomegalovirus, Echo A, B adenovirus, Epstein-Barr, rotavirus, Norwalk virus, influenza, mumps, measles, herpes, Coxsackie A and B, Reovirus, Polio virus, Paramyxovirus<sup>[1,7,8]</sup>; protozoa and parasites like *Escherichia histolytica*<sup>[1]</sup>; vaccines such as the trivalent measles, mumps and rubella and **Bacillus Calmette-Guérin**<sup>[9,10]</sup>; microparticles of aluminum, titanium, silicon oxides, calcium phosphate from the diet, tooth paste, dust or soil<sup>[8,10-12]</sup>; drugs like oral contraceptives and non-steroid anti-inflammatory drugs (NSAIDs)<sup>[10,13]</sup>; dietary components like protein, fat, sugar, fruits and vegetables, margarine, dairy products, coffee, coca cola, fast food<sup>[10]</sup>, or glycoalkaloids in potato<sup>[14]</sup>, and carrageenan in seaweeds<sup>[15]</sup>; smoking<sup>[1]</sup>; and other factors like refrigeration (cold chain)<sup>[8,10,16]</sup>. Despite that, the cause of IBD remains virtually unknown, as none of them can well explain the dynamically changed profiles of IBD. For instance, smoking is currently regarded as the most determined environmental factor for IBD: it reduces the risk of UC, while exacerbates CD<sup>[2]</sup>. Despite that, the low prevalence of CD in heavily smoking countries like China and high prevalence of CD in the low smoking countries like Canada suggest the contribution of smoking in the general population being negligible and other factors in the environment would have played the predominant role<sup>[2]</sup>. Another example would be the Mycobacterium avium subspecies paratuberculosis (MAP), a bacteria that causes Johne's disease in cattle, that had been suspected the causative factor for CD as early as 1913<sup>[17]</sup>. Despite a century long research and debate, a causal relationship between MAP and CD still cannot be established<sup>[18,19]</sup>. MAP hypothesis also failed to explain the cause of UC, which has been the main form of IBD in most circumstances.

About a decade ago, I found that digestive proteases like trypsin and chymotrypsin can be inactivated by free (unconjugated or deconjugated) bilirubin but not conjugated bilirubin or biliverdin. Further pursuit in the literature led me to suspect that impairment in this process due to inhibition of gut bacteria (thus the major source of  $\beta$ -glucuronidase that is needed for deconjugation of the mostly conjugated biliary bilirubin) by dietary chemicals like saccharin may have played an important causative role in IBD, as the result of damage of the protective mucus layer and the underlying gut tissue by the poorly-inactivated digestive proteases<sup>[20]</sup>. Recently, I further found that sucralose, the new generation of arti-

ficial sweetener, may exert an even potent impact on gut bacteria than saccharin and have probably contributed to the record high incidence of IBD seen recently in many countries<sup>[21]</sup>. Based on the evidences gathered and thoughts evolved and developed in the last decade, this paper aims to provide a detailed description of a unified hypothesis regarding the etiology of IBD, including the cause and mechanism of IBD, as well as the relationship between UC and CD.

## LARGE COMMERCIAL MARKETING OF SACCHARIN IN 1887 AND THE EMERGE OF CLUSTERED CASES OF ULCERATIVE COLITIS SINCE 1888, STARTED FROM THE UNITED KINGDOM

### ***The discovery of saccharin in 1878 from coal tar and its large-scale production and marketing since 1887***

Saccharin was discovered in 1878 by Constantin Fahlberg, a young chemist from Germany who engaged in research in Professor Ira Remsen's laboratory at Johns Hopkins University in Baltimore, Maryland, the United States<sup>[22-24]</sup>. One evening, Fahlberg found extra sweetness of bread and his hand during dinner, and tracked to its source to a coal tar product in the lab. Later, it was revealed that this chemical was 300 to 500 times as sweet as sugar and had little toxicity<sup>[22-25]</sup>. In 1882, Constantin Fahlberg himself consumed 10 g of the chemical and experienced no adverse reactions<sup>[26]</sup>. Most of it passed the body unchanged, through urine or feces<sup>[22,25]</sup>. In 1884, associated with his uncle, Adolph List, of Leipzig, Germany, Fahlberg tried pilot experimental production of this chemical in New York and named it as saccharin (also called saccharine at some occasions)<sup>[23]</sup>. Due to the high expenses of labor and materials in New York, Fahlberg, together with his cousin, established the firm Fahlberg, List and Co. and built a factory in Salbke, Germany in 1886 for the commercial production of saccharin<sup>[22-24,27]</sup>. Large quantities of saccharin were produced and reached the market in 1887<sup>[28]</sup>. After that, more factories were established in Germany<sup>[22]</sup>. The production of saccharin in Germany before its ban by the end of 1902 is showed in Table 1<sup>[29-33]</sup>. Since later 1890s, factories were also built in other countries like France<sup>[34]</sup> and the Switzerland<sup>[31,35]</sup>. In 1901, John Francis Queeny established Monsanto in St. Louis, Missouri, the United States for the sole purpose of saccharin production<sup>[27,36]</sup>. For the first several years, all the saccharin it produced was sold to Coco Cola, then a small company in Atlanta<sup>[27,36]</sup>.

### ***The favorite use of saccharin in United Kingdom since 1887 but dislike or ban of saccharin in Germany and most of the other western countries in the early years***

Although saccharin was only produced in Germany in the early years after its marketing in 1887, only about 3% of saccharin was actually consumed in Germany<sup>[37]</sup>,

**Table 1 Saccharin production in Germany before its ban by the end of 1902**

Year	Number of factories	Production (tons)
1888	1	5.2 <sup>[29]</sup>
1889	1	14.6 <sup>[30]</sup>
1896	3	33.5 <sup>[31]</sup>
1897	4	34.7 <sup>[31]</sup>
1898	5	78.4 <sup>[31]</sup>
1899	6	130.3 <sup>[31]</sup>
1900	6	159.4 <sup>[32]</sup>
1901	6	189.7 <sup>[31]</sup>
1902	6	174.8 <sup>[31]</sup>
1903	1	40 <sup>[33]</sup>

mainly due to the bad image of this coal tar product in that country. Saccharin was regarded as being inferior and only consumed by people who could not afford the luxury of sugar<sup>[38]</sup>. A domestic servants' club even advocated a commitment not to working for people who sweetened their coffee with saccharin instead of sugar<sup>[38]</sup>. In 1902, saccharin production was eventually brought under strict control in Germany<sup>[24]</sup>. Only one firm, Fahlberg, List and Co, among the six factories was allowed to continue producing saccharin<sup>[24]</sup>, and it was regulated that saccharin can only be used for medicinal purpose and available through pharmacies<sup>[24]</sup>. The production of saccharin in Germany reduced from nearly 190 tons in 1901 to about 40 tons in 1903<sup>[33]</sup>, among which only 3 tons were consumed within Germany, with the remaining being exported to other countries<sup>[33]</sup>. Similar as Germany, many other countries like France, Italy, Spain, Belgium, Holland, Portugal, Russia, Austro-Hungary also put strict regulations on the importation, production, and use of saccharin during these early years<sup>[39,40]</sup>. These restrictions greatly stimulated the smuggling of saccharin in Europe, with saccharin being hidden in chocolate or match boxes, oil or milk cans, artificial stones or candles, coats, vests, suits with secret pockets, feed bags for horses, carousel, or even coffin<sup>[41]</sup>.

In contrast to the countries above, saccharin was greatly appreciated in the United Kingdom. Even before its appearance on the market, saccharin had been highly praised by some eminent authorities like Sir Henry E. Roscoe, who had been a member of the parliament, a fellow of royal society, and presidents of Chemical Society, the Society of Chemical Industry, and the British Association<sup>[42-44]</sup>. In the presidential inaugural address on August 27, 1886, he stated that: "the most remarkable instance is the production of an artificial sweetening agent, termed saccharine, prepared by a complicated series of reactions from coal tar"<sup>[42,43]</sup>. People were assured by official analysts and doctors that saccharin was harmless and enjoyable<sup>[45,46]</sup>. Pamphlets with detailed descriptions of many formulae and uses of saccharin were written by professor and editor and distributed to households<sup>[46,47]</sup>. As stated in a publication in early twenty century: "Here we now have a coal oil product deliberately recommended in England as a valuable and suitable agent for sweetening mineral

waters and, presumably, for use wherever it could take the place of genuine sugar. It is altogether different in France, where it has been entirely contraband and even in Germany, where it is manufactured to so considerable an extent, efforts are made to hold it under control"<sup>[48]</sup>. Therefore, it would be not surprising that, shortly after its marketing, saccharin soon appeared in almost any family grocer, instead of chemists' shop in United Kingdom<sup>[49]</sup>. As the result, United Kingdom was the biggest buyer of saccharin at that time. As stated in the publication: "the American trade (of saccharin) has been inconsequential. In 1891 the export to New York was only about eight hundred kilograms (1800 pounds approximately), while during the same period 7200 kg were shipped to England"<sup>[50]</sup>.

### **Emergence of clustered cases of ulcerative colitis since 1888, started from United Kingdom**

Although it was suspected that some forms of diarrhea described in ancient books could be sporadic case of UC<sup>[1]</sup>, it appeared that clustered cases of UC only started to emerge after 1888. As stated by Dr. Sidney Philips in his discussion during the first symposium on IBD in the world in 1909 that presented a collection of more than three hundreds of UC patients from 9 hospitals in London: "Ulcerative colitis appeared to be much more common now in this country than formerly. There was no mention of it in any of the published reports of any of the London hospitals before 1888, when Dr. Hale White published cases in Guy's Hospital Reports. It was not mentioned in St. Bartholomew's or Westminster Hospital Reports before 1893, nor in the London Hospital Reports till 1897. And the textbooks used twenty or thirty years ago, such as Bristowe's and Hilton Fagge's, made no allusion to it. The speaker himself had seen many cases at St. Mary's Hospital and elsewhere since 1888, but not before then"<sup>[51]</sup>. In addition, Dr. Philips even suspected that the increase in UC might be caused by some food additives. He stated: "Possibly the cause of acute ulcerative colitis was connected with our food supply; tinned or preserved foods might have something to do with it"<sup>[51]</sup>. Interestingly, saccharin was largely used in early years in canned foods to preserve vegetables, fruits and meats, taking the advantage of both its sweet and antiseptic properties<sup>[25,52]</sup>.

### **THE WIDESPREAD USE OF SACCHARIN DURING THE TWO WORLD WARS AND THE SPREAD OF ULCERATIVE COLITIS IN WESTERN COUNTRIES**

During World War I, the shortage of sugar caused great demand for saccharin<sup>[53,54]</sup>. Figure 1 demonstrated this dramatic increase of saccharin consumption in Germany<sup>[38,54]</sup>. The ban on saccharin was lifted in Germany and other countries, accompanied by a striking increase in saccharin production<sup>[53,54]</sup>. In 1916, saccharin production

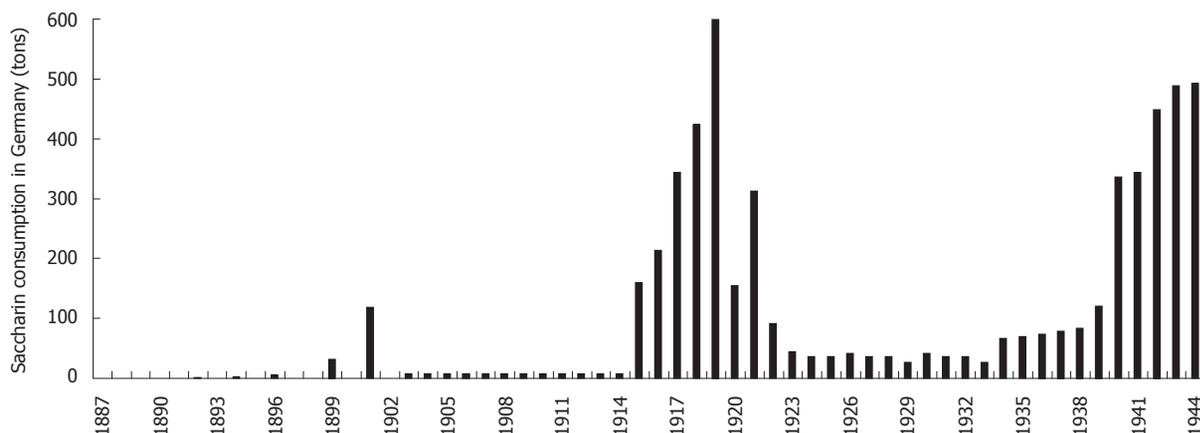


Figure 1 Saccharin consumption in Germany over time (1888-1944).

in Germany resumed to 1 ton per day<sup>[53]</sup>. In France, four more factories were equipped to produce saccharin<sup>[55]</sup>. In the United States, saccharin became allowed using in soft drinks and foods<sup>[56]</sup>. In addition to increased production, the importation of saccharin in the United States increased from 8 pounds in 1914 to 5617 pounds in 1915 and 12954 pounds in 1916<sup>[57]</sup>. Despite the increased production and importation, the price of saccharin in New York market increased from \$1.15-1.25 per pound in 1914 to \$2.85-11.50 in 1915, \$11.50-21.50 in 1916, and \$20.50-46.00 in 1917<sup>[53]</sup>, reflected the great increases in the demand and consumption of saccharin during this period.

In accordance with the spread use of saccharin since World War I, UC cases were also more frequently seen in countries other than United Kingdom. As stated by Dr. Evans: "During the recent war (1914-1918), while acting as surgeon to an improvised hospital for Turkish prisoners in Mesopotamia, and later as civil surgeon of Baghdad, an opportunity arose of observing a large number of cases of colitis; the majority were chronic and were complicated by scurvy. The combination of these two diseases made the colitis extremely intractable, and in consequence large numbers died. While in Mesopotamia I performed appendicostomy for intractable ulcerative colitis in ten patients-Turks, Arabs, and Indians"<sup>[58]</sup>. This helped the recognition of UC as an independent entity other than, for instance, dysentery. As stated by Lups S: "In the latter part of the nineteenth century most clinicians considered this affection which later was called 'ulcerative colitis' belonging to the dysentery group, even after 1903, when Boas expressed the view that ulcerative colitis was an independent disease. About 1914, however, there was a marked change in the viewpoints of many observers on this question although many still felt that it was of dysenteric origin. They knew full well that only in a few cases true dysenteric organisms were found"<sup>[59]</sup>. In another paper published in 1928, Dr. Thorlakson stated that: "the subject of ulcerative colitis has received a great deal of attention in the medical literature of all countries during the past decade. In reviewing the English, German, French and American

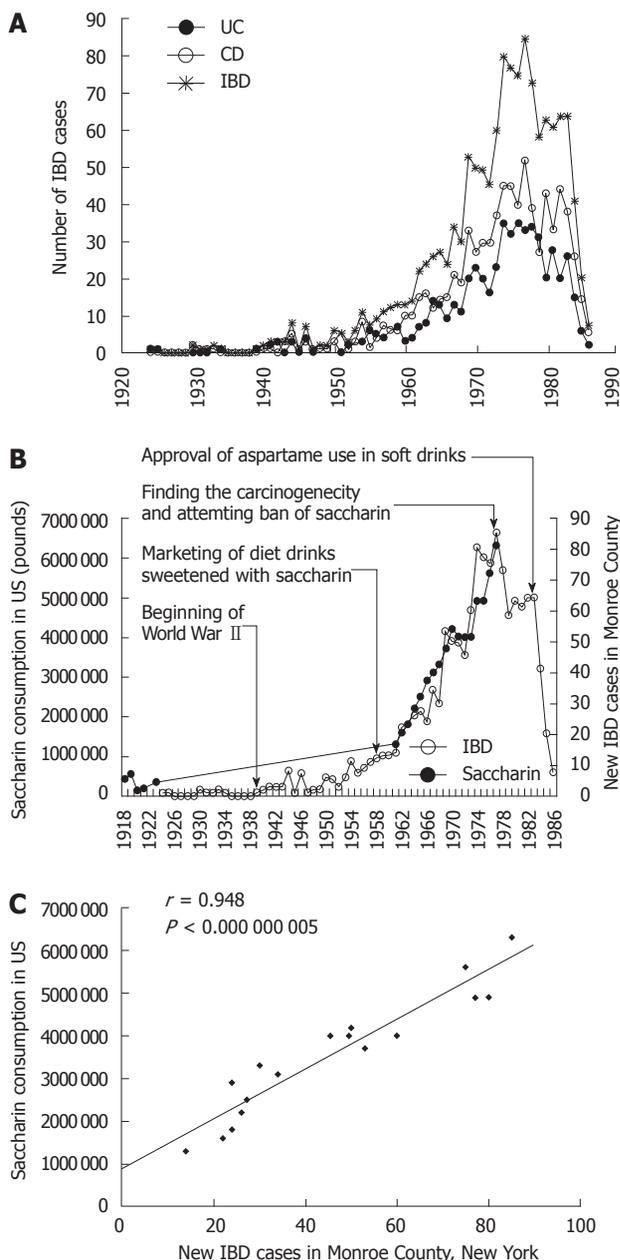
literature on this disease, one is struck by the similarity of the articles. It seems obvious that these writers from various countries are all dealing with the same condition, and not, as has been suggested, with different diseases brought under the same name-ulcerative colitis"<sup>[60]</sup>.

In the United States, more cases of UC were seen since World War I. For instance, Logan reported 117 cases of UC treated in Mayo Clinic up to 1918, with 19 cases being before 1915, 18 cases during 1915, 23 cases during 1916, 57 cases during 1917 and to April 1, 1918<sup>[61]</sup>, while there were 693 cases between 1923-1928<sup>[62,63]</sup>. In 1922, Dr. Yeomans in the Department of Surgery, Columbia University College of Physicians and Surgeons reported 65 cases of UC mostly observed during 1916 and 1921 with only 6 cases before that<sup>[64]</sup>. During this period, many UC patients were also reported in California<sup>[65]</sup>, Massachusetts<sup>[66]</sup>, and other places<sup>[67]</sup>.

World War II resulted in another jump in saccharin consumption (Figure 1). In the United States, Sigma Chemical Co. was formed to manufacture saccharin and Monsanto resumed large-scale production to meet the high demand<sup>[68]</sup>. Interestingly, record high incidence of IBD was also seen during this period. For instance, 525 cases of UC were diagnosed in male United States Army in 1944, with a rate as high as 12 per 100 000 in 40-45 age group<sup>[69]</sup>. This may relate to the preferred use of saccharin in the United States army. As early as in 1896, United States army had chosen saccharin rather sugar as the emergence ration for sweetening coffee or tea, taking the advantage of its immaterial weight as well as the anti-septic property to reduce the prevalence of diarrhea<sup>[70]</sup>.

## THE DRAMATIC INCREASE IN SACCHARIN CONSUMPTION SINCE 1950S AND THE REMARKABLE INCREASE IN INFLAMMATORY BOWEL DISEASE DURING 1950S AND 1970S IN COUNTRIES LIKE THE UNITED STATES

Although the shortage of sugar in World War I and II



**Figure 2** The dramatic increase in saccharin consumption since 1950s and the remarkable increase in inflammatory bowel disease during 1950s and 1970s in countries like the United States. A: Occurrence of ulcerative colitis (UC), Crohn's disease (CD), and inflammatory bowel disease [inflammatory bowel disease (IBD) = UC + CD] in Monroe County, New York during 1920s to 1980s; B: A comparison of the temporal change of IBD in Monroe County, New York and the saccharin consumption in the United States; C: Correlation between IBD in Monroe County, New York and the saccharin consumption in the United States.

led to increased consumption of saccharin as a sugar substitute, a huge increase only occurred since late 1950s, when low calorie high intensity sweetener began to be used in foods and drinks designated for special diets<sup>[56,71]</sup>. In 1976, approximately 7 million pounds of saccharin were consumed in the United States, with soft drinks accounted for 74 percent of those used in foods and beverages<sup>[72]</sup>. Saccharin had been used in juices and drinks, sauces and dressings, canned fruits, dessert toppings, coo-

kies, cereals, gums, jams, candles, ice cream and puddings, in addition to as a non-nutritive tabletop sweetener<sup>[72]</sup>. It was also used in drugs, toothpaste, mouthwashes, and cosmetics<sup>[72]</sup>.

In accordance with this dramatic increase in saccharin consumption, the incidence of IBD also showed a striking increase during this period. This was clearly demonstrated in the study by Stowe *et al*<sup>[73]</sup>, which showed the annual incidence of both UC and CD in Monroe County, New York between 1920s and 1986 (Figure 2A). From Figure 2B we can see the increase in IBD for up to later 1970s paralleled neatly with the increased consumption of saccharin during the same period<sup>[74]</sup>, with a very significant correlation (Figure 2C). The correlation coefficient between saccharin consumption in the United States and the new cases of UC, CD and IBD (UC + CD) in Monroe County were 0.930, 0.935 and 0.948, and the *P* value being  $3.43 \times 10^{-8}$ ,  $1.90 \times 10^{-8}$ , and  $3.85 \times 10^{-9}$ , respectively.

### DISCOVERY OF CARCINOGENICITY OF SACCHARIN IN LABORATORY ANIMALS IN 1970S AND THE LEVELING OFF OR DECREASE OF INFLAMMATORY BOWEL DISEASE OBSERVED IN MANY COUNTRIES SINCE THE SAME PERIOD

From Figure 2A, we can see the incidence of both UC and CD in Monroe County reached a peak in 1978, followed by a mysterious rapid decrease after that. Again, this change was in accordance with the finding of the carcinogenicity of saccharin in animals and the attempted ban for its use in the United States in 1977<sup>[26]</sup>. Due to the protest from the public the congress imposed a two year moratorium instead of a complete ban on saccharin, but passed the Saccharin Study and Labeling Act that required further studies on saccharin and putting a warning label on products containing saccharin<sup>[26]</sup>. Study showed that these events indeed affected saccharin consumption, especially for those with high education and families with children<sup>[75]</sup>. Not only in Monroe County in New York, the leveling off or decrease in IBD in later 1970s and 1980s was also seen in other cities of the United States such as Olmsted County, Minnesota<sup>[76]</sup>, as well as in many other countries such as Canada<sup>[77]</sup>, Denmark<sup>[78,79]</sup>, Germany<sup>[80]</sup>, Japan<sup>[81]</sup>, Israel<sup>[82]</sup>, Sweden<sup>[83-86]</sup> and United Kingdom<sup>[87-90]</sup> (Figure 3). In 1981 aspartame was approved by Food and Drug Administration (FDA) for use in dry food products<sup>[91]</sup>. On July 8, 1983 FDA further approved the use of aspartame in carbonated beverages and syrups<sup>[91]</sup>. Aspartame soon became the main high intensity sweetener in the market, with a sharp decline in saccharin use and consumption<sup>[91]</sup>, which was in accordance with the remarkable decrease in the incidence of UC and CD observed in Monroe County at this period (Figure 2A).

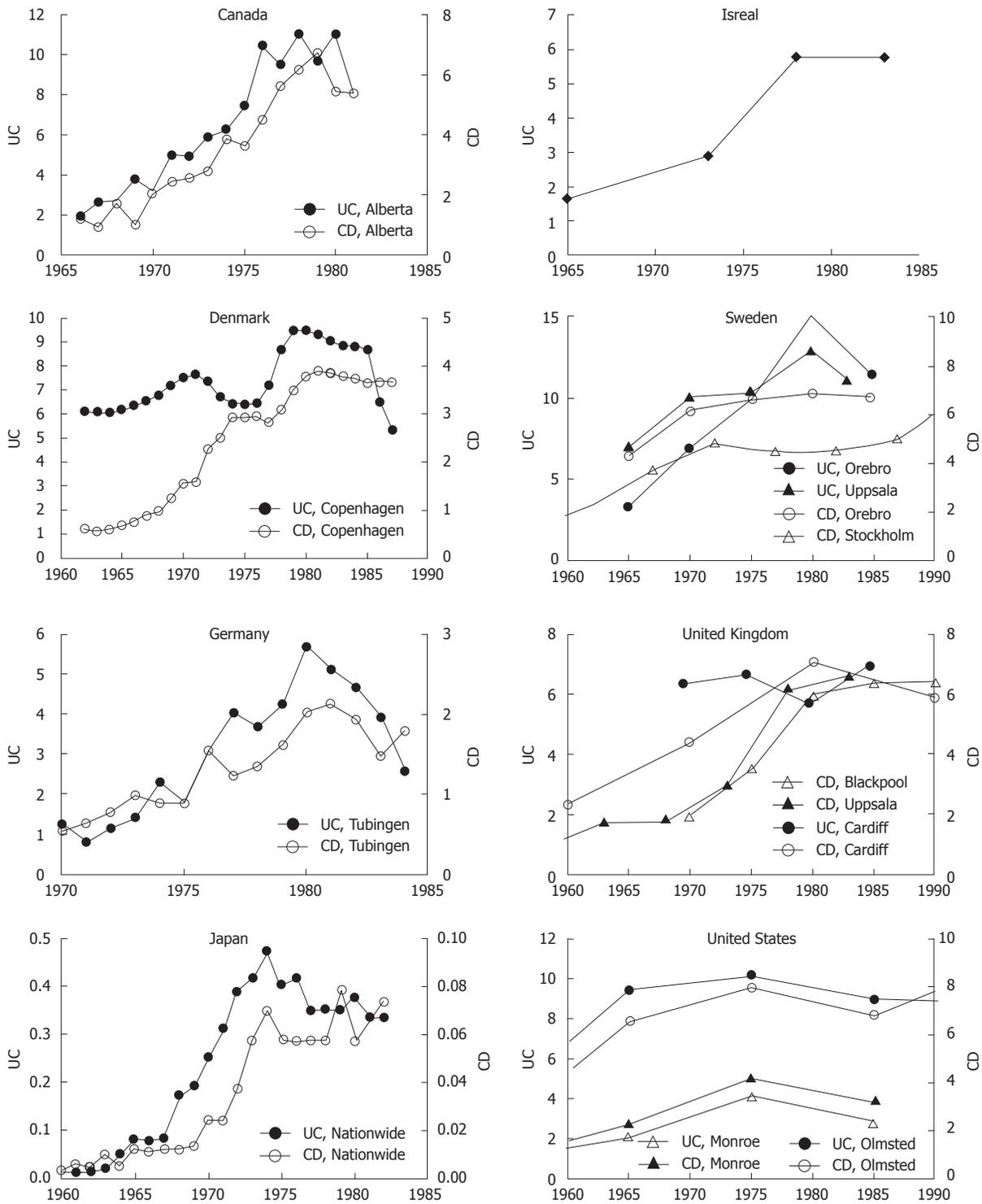


Figure 3 A leveling off or decrease of ulcerative colitis or Crohn's disease during 1970s and 1980s in the different countries such as Canada, Denmark, Germany, Japan, Israel, Sweden, United Kingdom, and United States. UC: Ulcerative colitis; CD: Crohn's disease.

### THE REBOUNDED USE OF SACCHARIN AND THE INCREASE AGAIN OF INFLAMMATORY BOWEL DISEASE SINCE 1990S

Although a leveling off or decrease in IBD was observed in many places (Figure 3) during the later 1970s and early

1980s, the increase again of IBD since 1990s was observed in many of these places such as Denmark<sup>[92]</sup>, Sweden<sup>[86,93,94]</sup>, United Kingdom<sup>[89]</sup>, and the United States<sup>[76]</sup>. This was again in accordance with the rebounded use of saccharin. After finding the carcinogenicity of saccharin in animals in 1970s, many studies were carried out. As the result, most studies failed to show a link between saccharin consumption and bladder cancer in humans<sup>[95,96]</sup>.

People gradually regained confidence in saccharin consumption. Saccharin production boomed again, largely because saccharin is very cheap and also very stable thus can be used in a wide range of drink and food products and put on the shelf for a long time<sup>[97,98]</sup>. In China, saccharin production increased from about 8000 tons in middle 1980s<sup>[99]</sup>, to 13 126 tons in 1991 and 29 175 tons in 1998, accompanied by dramatic increases in both domestic use and exportation<sup>[100]</sup>. The great adverse impact on sugar industry led the Chinese government adopting strict measures to limit the production and domestic use of saccharin. In the United States, after multiple times of renewal of the two year moratorium, the National Institute of Environmental Health Sciences finally delisted saccharin from carcinogen list in 2000 and the “Sweetest Act” of the Congress eliminated the requirement for putting the warning label on saccharin products<sup>[26]</sup>. This is accompanied by a dramatic increase in saccharin importation from 2 772 000 pounds in 2000 to 8 346 000 pounds in 2008<sup>[101]</sup>. In 2007, 52 212 000 pounds of saccharin were exported world wide, with millions of pounds of saccharin being imported in countries like Germany, Spain, United Kingdom, South Korea, Japan, India and Brazil<sup>[101]</sup>. These massive use of saccharin may have contributed to the worldwide increase of IBD in recent years<sup>[102]</sup>.

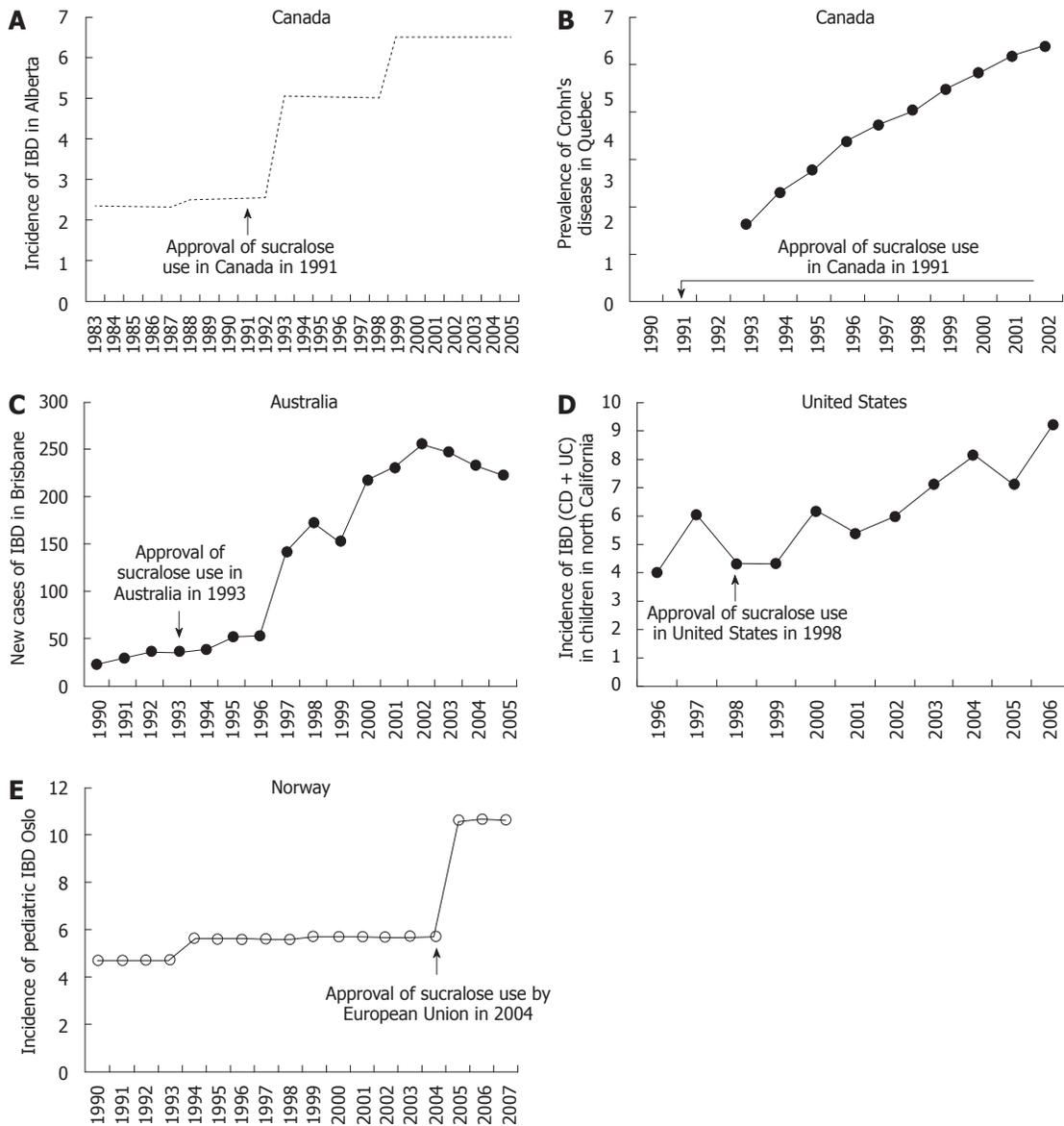
## **SUCRALOSE, A NEW GENERATION OF ARTIFICIAL SWEETENER, MIGHT BE ANOTHER IMPORTANT RISK FACTOR THAT CONTRIBUTED TO THE RECORD HIGH INCIDENCE OF INFLAMMATORY BOWEL DISEASE SEEN RECENTLY IN MULTIPLE COUNTRIES**

The evidences demonstrated above provided a simple explanation for many puzzles of IBD such as the emergence and temporal changes of IBD in last century. It suggests saccharin might be the key causative factor for IBD, by primarily its inhibition on gut bacteria<sup>[20]</sup>. This notion is supported by the recent large-scale studies showing antibiotics greatly increased the risk of IBD<sup>[103,104]</sup>. Although both saccharin<sup>[105-107]</sup> and antibiotics can inhibit bacteria, saccharin would have a much more great impact on the general population due to the wide extensive use<sup>[108]</sup>. When United States FDA attempted a ban on saccharin in 1977, saccharin was used as the only non-nutritive sweetener by up to 70 million Americans<sup>[74]</sup>. Saccharin was the first and oldest artificial sweetener. From its marketing in 1887 until a temporary decline in the market due to the heavy use of aspartame since 1980s saccharin had been the only or the predominant artificial sweetener, which made it a key dietary chemical in last century. However, more and more chemicals were introduced and became heavily used as food additives in modern society. It would be no surprising that some of them may also have a significant impact on gut bacteria,

thus IBD. Recently, I found evidences suggesting sucralose, the new generation of artificial sweetener, might be just an example. Sucralose is synthesized by replacing three hydroxyl groups of sucrose with three chlorides, which makes it 600 times as sweet as sucrose<sup>[109]</sup>. Like saccharin, sucralose is stable under heat and over a broad range of pH, and most of it passes through the body without further metabolism<sup>[97,109]</sup>. However, sucralose has a much lower absorption rate but a much high acceptable daily intake and thus a more potent impact on gut bacteria<sup>[97,110]</sup>. Sucralose was first approved for use in drinks and foods in Canada in 1991, which was in accordance with the dramatic increase of IBD seen in Alberta<sup>[111]</sup> and CD in Montreal<sup>[112]</sup> since early 1990s, as well as the finding in recent studies that Canada suddenly became a country with the highest incidence of IBD<sup>[113,114]</sup>. After Canada, sucralose was approved in Australia in 1993, in New Zealand in 1996, in the United States in 1998, and in the European Union in 2004, which was again in accordance with the dramatic increase or the record high incidence of IBD in Australia<sup>[115]</sup>, New Zealand<sup>[116]</sup>, the United States<sup>[117]</sup>, and Norway<sup>[118]</sup> (Figure 4)<sup>[21]</sup>. Currently, sucralose has been approved for use in more than 80 countries<sup>[119]</sup>, and started to appear in rivers and other surface waters of many countries<sup>[120]</sup>. In countries like the United States artificial sweeteners are now used by more than half of the population, with sucralose by far the number one in the market and being used in thousands of food and drink products<sup>[121]</sup>. This unrestricted use of sucralose may be also the explanation for the recent remarkable increase of IBD in children as observed in many studies<sup>[122]</sup>. We may expect to see more reports on record-breaking incidence of IBD, both in adults and children.

## **THE POSSIBLE MECHANISM OF INFLAMMATORY BOWEL DISEASE: THE BACTERIA-PROTEASE-MUCUS-BARRIER HYPOTHESIS**

After the emergence of IBD about a century ago, numerous hypotheses had been suggested as the possible mechanism, which included infection, toxicants, psychogenic disturbances, nutritional deficiencies, allergy to pollens or foods, abdominal trauma, impaired vascular or lymphatic circulation, lysozymes and other enzymes<sup>[1,123,124]</sup>, or the excessive or deficient immune response due to reduced exposure to bacteria or helminthes<sup>[125,126]</sup>. Most of them were invalidated and forgotten. Up to date, a full coherent mechanistic explanation for IBD is still lacking, but people start to realize that the pathogenesis of IBD involves four fundamental components: the environment, gut microbiota, the immune system and the gene<sup>[127-129]</sup>. Currently, the dominant theory regarding the increase of IBD (as well as other autoimmune and allergic diseases) in modern society is the “hygiene hypothesis”, which proposes that these diseases are caused by an aberrant



**Figure 4** Relationship between the increase of inflammatory bowel disease and approval of sucralose in countries like Canada, Australia, the United States and Norway. UC: Ulcerative colitis; CD: Crohn's disease.

development and response of the immune system due to the reduced exposure to microorganisms such as the microbes in the gut<sup>[127,128]</sup>. However, this theory neglected another fact: the increased intestinal permeability, which has been observed not only in these patients and their healthy relatives<sup>[130,131]</sup>, but also in their spouse<sup>[130,132]</sup>, suggesting likely a prerequisite condition for these diseases. As the gut contains such a large amounts of bacteria that are ten times of the number of cells of our body and can kill the host thousands of times over, I believe it would be the permeability of the gut rather than the absolute number of the bacteria in gut lumen that determined the level of exposure<sup>[133]</sup>. Therefore, the enhanced immune activities seen in these patients may just be a normal response to the increased infiltration of bacterial and dietary components from the gut lumen<sup>[133]</sup>. Then the key to the mystery would be to know what caused the increased intestinal permeability in modern society. Here I propose a mecha-

nism for the increased intestinal permeability as well as IBD, featured by the Bacteria-Proteases-Mucus-Barrier hypothesis.

As shown in Figure 5, this hypothesis proposes that the increased intake of dietary chemicals like saccharin and sucralose caused a significant reduction in gut bacteria, along with a failure for prompt replenishment due to the improved hygiene in modern society. This led to a remarkable decrease in  $\beta$ -glucuronidase in gut lumen, resulting in impaired deconjugation of the biliary bilirubin and the subsequent inactivation of digestive proteases. Then these poorly inactivated proteases work synergistically with the glycosides from the remaining bacteria to cause an accelerated degradation of the mucus layer, resulting in damage of the gut barrier (the Bacteria-Protease-Mucus-Barrier hypothesis). This will further result in infiltration of bacteria and their components (mainly in the large intestine) and recruitment of neutrophils lead-

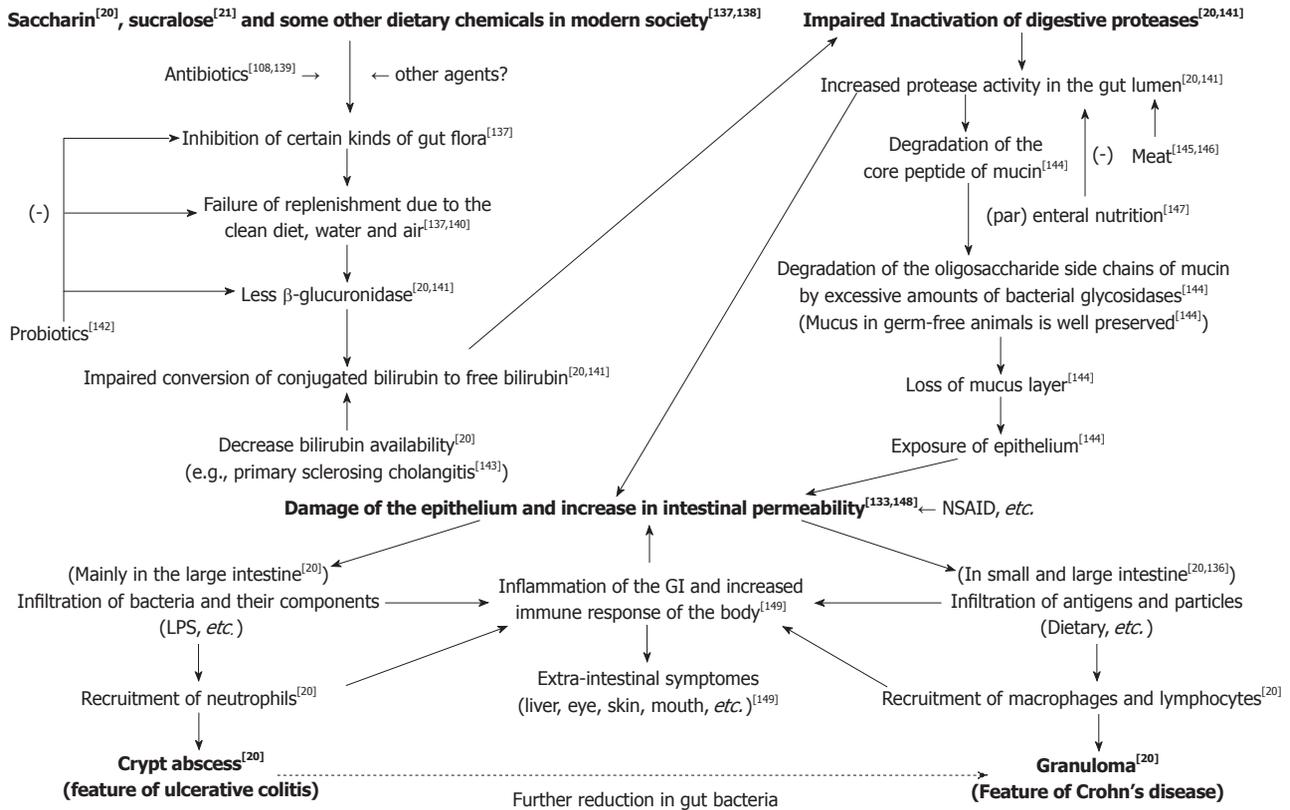


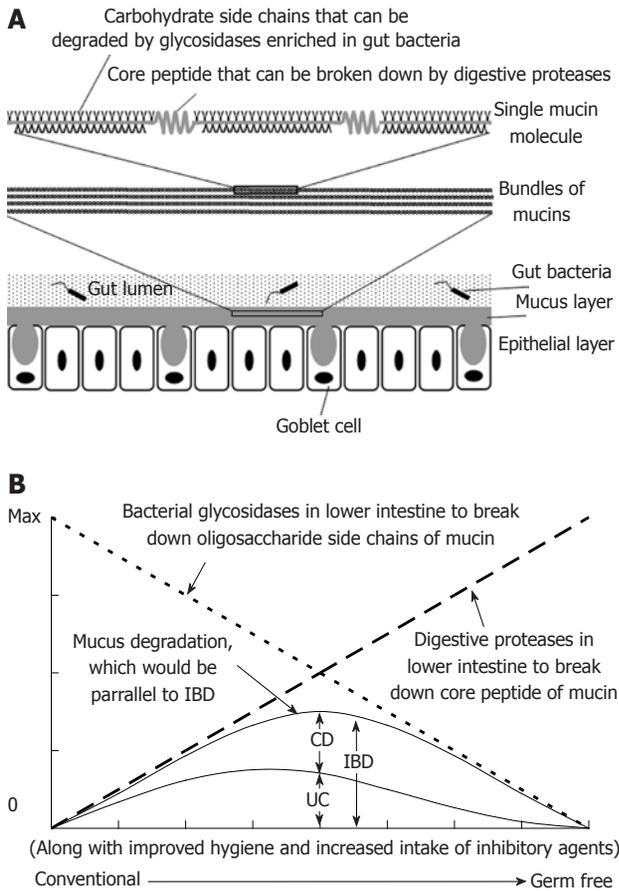
Figure 5 An overall hypotheses for the cause and mechanism of inflammatory bowel disease (both Crohn's disease and ulcerative colitis).

ing to the formation of crypt abscess<sup>[20]</sup>, the characteristic change of UC<sup>[134]</sup>; while at places or situations being relative sterile, the increased infiltration of antigens and particles from gut lumen would result in accumulation of macrophages and formation of inflammatory granulomas<sup>[20]</sup>, the hallmark of CD<sup>[134]</sup> (Figure 5). This would further induce enhanced immune response of the body, leading to further damage of the gut as well as extra-intestinal manifestations in the joints, skin, eyes, and mouth, *etc.*<sup>[135,136]</sup>. More detailed descriptions regarding the proposed mechanism can be found in the corresponding references<sup>[20,21,108,133,136-151]</sup>.

## ULCERATIVE COLITIS AND CROHN'S DISEASE ARE LIKELY JUST TWO SYMPTOMS OF THE SAME MORBIDITY RATHER THAN TWO DIFFERENT DISEASES

The Bacteria-Protease-Mucus-Barrier hypothesis and mechanism of IBD as described above and illustrated in Figure 5 suggest that UC and CD share virtually the same cause, thus UC and CD would be just two symptoms of the same morbidity rather than two different diseases. This notion contradicts the current main stream of thoughts that are trying to dissect UC and CD into multiple subtypes with different causes and diverse mechanisms<sup>[152]</sup>. Nevertheless, this new perception is in accor-

dance with many facts. Figure 6 further illustrated the mechanism of IBD as well as the relationship between UC and CD. It explained why gut damage and IBD started to appear and became more prevalent along with the improved sanitary condition but failed to develop under both conventional and germ-free condition<sup>[144]</sup>, and predicted a shift of predominance from the bacteria-mediated UC to antigen/particle-mediated CD along with the decrease in gut bacteria in modern society or other circumstances (Figure 6B). It provided a simple explanation for many big puzzles in IBD such as: (1) the discrepancy between the temporal changes of UC *vs* CD: Many studies had revealed a regular pattern that UC emerged first, then reached a plateau or even started to decrease, while CD showed a delayed appearance but accelerated increase with an eventual tendency to pass UC<sup>[2]</sup>. This would be just the pattern demonstrated in Figure 6B; (2) The increase in colonic CD over time: Studies in Stockholm County, Sweden found that colonic CD increased from 15% during 1959-1964 to 32% during 1980-1989, and further to 52% during 1990-2001, while the ileocaecal CD decreased from 58% during 1959-1964 to 41% during 1980-1989, and to 28% during 1990-2001<sup>[86,93]</sup>. Similar changes were also observed in other long-term studies such as the one conducted in Cardiff, United Kingdom<sup>[89]</sup>. Again, Figure 6B illustrated the anticipated shift of UC to colonic CD over time; (3) the inverse relationship between age and colonic CD in children: It is found that the younger the children, the more likely they had colonic CD<sup>[153]</sup>. This may be just due to the younger the children,



**Figure 6** Ulcerative colitis and Crohn's disease are likely just two symptoms of the same morbidity rather than two different diseases. A: The structure of mucin; B: Mechanistic sketch of the temporal changes of ulcerative colitis (UC) and Crohn's disease (CD) and their relationship. A reduction in gut bacteria along with the improved hygiene and increased intake of dietary chemicals like saccharin and sucralose will result in impairment in digestive proteases inactivation. The poorly inactivated proteases will work together with glycosidases from the gut bacteria to cause accelerated degradation of the mucus layer that is proposed here paralleling the risk of developing inflammatory bowel disease (IBD = UC + CD). UC and CD differ in that UC is caused by the increased infiltration of bacteria and the resultant recruitment of neutrophils and formation of crypt abscess, while CD is caused by increased infiltration of luminal antigens and particles and the resultant recruitment of macrophages and formation of granulomas. Thus the reduction in gut bacteria along with the modernization or other factors will result in a shift of predominance from the bacteria-mediated UC to antigen/particle-mediated CD. IBD: Inflammatory bowel disease.

the less population of bacteria in their gut; (4) the more pronounced increase in the risk of CD than UC along with the use of antibiotics<sup>[103]</sup>: again, this would be just the result of shift from UC to colonic CD along with the dramatic decrease in gut bacteria by the antibiotics; and (5) the proposed mechanisms as demonstrated in Figure 5 and Figure 6 may even provide some explanation for the similarities and discrepancies in the susceptible genes between UC and CD: UC and CD as well as other diseases like psoriasis and ankylosing spondylitis shared some common genes related to chronic inflammation such as interleukin-23 pathway, while CD being more associated with genes related to the function of macrophages such as NOD2 and autophagy, but UC being more associated

with genes related to neutrophil extravasation and epithelial defense such as LSP1<sup>[154]</sup>.

## CONCLUSION

Currently, tremendous efforts have been taking for research on the genes, the microbiota and the immune system: genome-wide association studies to find out the susceptible loci among the tens of thousands of genes in our body; metagenome, metaproteome, and metabolome analyses of gut microbiota that contains more than 100 times of genes than our own genome<sup>[155]</sup> to find out the bacteria responsible; and extensive studies on the many cells, signal pathways, mediators and cytokines of the immune system to find out aberrant immune response<sup>[127,128,156-158]</sup>. IBD emerged and became epidemic for about a century, suggesting the factors in environment rather than the gene or other factors within the body would be the primary cause. Increased risk of IBD in twins, families, or the same ethnic groups may attribute to not only the gene, but also environmental factors they shared such as the type of diet<sup>[159,160]</sup>. This article proposes saccharin being the key causative factor that contributed greatly to the emergence and epidemic of IBD in last century. As described above, there were big variations in saccharin regulation and consumption among the different countries and also in the different periods or even different parts (such as the different states in the United States<sup>[161]</sup>) within a country. This may explain the constant temporal changes and big geographical variations in IBD, and why similar changes were more likely seen within the border of a country rather than the closeness between cities<sup>[3,5]</sup>. In the developed countries, saccharin may be more frequently used by white collars working in the office, while saccharin may be more likely consumed by people in lower class in the developing countries due to its cheapness. This may contributed to the high incidence of IBD in high social, economical, educational status in the developed countries<sup>[162]</sup>, while some early study in India found almost all the patients belonged to the middle and poorer classes under poor hygiene condition<sup>[163]</sup>, in accordance with a heavy saccharin consumption in that country<sup>[164]</sup>. This would suggest improved hygiene itself might facilitate but still not be sufficient, while some dietary chemicals might be enough to cause significant impact on gut bacteria and IBD. This notion is also in accordance with the close relationship of IBD with westernization rather industrialization, as demonstrated by the much low incidence of IBD in the developed countries like Japan, Singapore and Hong Kong<sup>[138,156]</sup>. The recent increase again of IBD in the developed countries is also unlikely attribute to a further improvement in hygiene condition. At early times, saccharin was mainly used as a tabletop sweetener in the restaurants, tea houses or coffee shops, thus men may have more chance to consume than housewives. However, in modern society, dietary drinks or foods may be more consumed by young ladies for concerns on their body weight. This may account for the

changes and variations in gender and age of IBD over time<sup>[3,5,6]</sup>. The recent finding of the even stronger impact of sucralose on gut bacteria further provided a possible explanation for the record high IBD observed recently in multiple countries<sup>[21,114]</sup>, as well as the remarkable increase of IBD in children<sup>[122]</sup>. Epidemiological study revealed spotted areas with high IBD<sup>[165]</sup>. Probably we should add another thing to the checklist: to see if there is any famous bakery, restaurant, or bar where foods or drinks are sweetened heavily by these artificial sweeteners.

Although it is proposed here that saccharin and sucralose might be the key causative factors for IBD, it does not mean to rule out that some other genetic or environmental factors may also be capable of affecting or contributing to IBD one way or the other.

However, the peculiar changes of IBD such as the recent worldwide increase of IBD, especially in the developed countries in children, seems unlikely to be explained by any of the currently suspected factors like the genes, smoking, NSAIDs, conceptive, appendectomy, sunshine and vitamin D, refrigeration, reduced exposure to bacteria, virus or worms, *etc.* Therefore, a fundamental breaking through in IBD would largely depend on finding out the key causative factors in the environment and thus the root mechanism of IBD. This paper proposed that impaired inactivation of digestive proteases due to the inhibition of gut bacteria by dietary chemicals like saccharin and sucralose being the primary mechanism. This would suggest it is not any pathogen but the digestive proteases produced by our body to digest the food for survival being the principal culprit for IBD. Under conventional conditions, the commensal bacteria (the microbiota) would be a valuable partner of our body by helping promptly inactivating these destructive proteases, probably by simply providing their enriched  $\beta$ -glucuronidase needed for deconjugation of biliary bilirubin. Thus the gut microbiota should be treated as an “microbial organ” of the body and taken into consideration when assessing the toxicity of chemicals or the adverse effects and efficacy of drugs<sup>[150]</sup>. However, the microbiota could be beneficial and also could be detrimental. Once there were not enough gut bacteria to help maintaining the function of gut barrier, they would start to leak into the body and become the driving force for the chronic inflammation seen in IBD. Once getting into the body, none of the commensal bacteria will remain our friend and our body will fight desperately against it. It seems unlikely and might also be unnecessary to pin down to one or a couples strains of bacteria exclusively responsible for IBD by screening the tens of thousands of species of bacteria in gut microbiota<sup>[149]</sup>. The enhanced immune activity in IBD would be just the normal reaction to the infiltrated bacterial and dietary components from gut lumen rather than an unbalanced or aberrant immune response, which may explain the remarkable increase of both the Th1-mediated CD and Th2-mediated UC in modern society<sup>[133]</sup>. It is proposed here that UC and CD are just two symptoms of the same morbidity rather than two different diseases. Some recent studies revealed that colonic CD had be-

come the main form of CD<sup>[166-168]</sup>. Apparently, the CD we are talking about today is no more the “regional ileitis” when this disease was defined by Crohn *et al*<sup>[169]</sup> in 1932. These CD cases are also no more the resemblance of Johne’s disease in cattle, but rather IBD frequently seen in dogs and cats<sup>[146]</sup>. Many of the colonic CD diagnosed today would be just UC cases early. On the other hand, the advance in endoscopies and other technologies led to reveal that a substantial portion of UC patients have ileitis (backwash ileitis) and the prevalence of inflammation seen in the esophagus, stomach, and duodenum is comparable among CD and UC<sup>[170]</sup>, further suggesting the intimate similarities between UC and CD. As stated above, the discrepancy between the temporal changes of UC *vs* CD would actually reflect their intimate connection. Elucidating the true relationship between UC and CD would be the crucial step for a full understanding of IBD.

This paper proposed a unified hypothesis regarding the etiology for IBD, including the cause and mechanism of IBD as well as the relationship between UC and CD. It provides a simple explanation for many puzzles of IBD. However, just like all other hypothesis and even existing theories well written in textbooks, it must be tested against facts. In the last decade, I have contacted multiple national and international organizations and IBD professionals suggesting checking out the possible link between saccharin and IBD, but failed to raise any action. I have also tried multiple times to apply grants from different agents, but remain unsuccessful. Hope this article may draw more attention and efforts. IBD just emerged and became epidemic for about a century and would be preventable and also likely curable, but first we may need to find out the key causative factors and thus the primary and fundamental mechanism<sup>[171]</sup>.

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## Epidemiology and clinical course of Crohn's disease: Results from observational studies

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### Abstract

The authors review the clinical outcome in patients with Crohn's disease (CD) based on studies describing the natural course of the disease. Population-based studies have demonstrated that the incidence rates and prevalence rates for CD have increased since the mid 1970s. The authors search for English language articles from 1980 until 2011. Geographical variations, incidence, prevalence, smoking habits, sex, mortality and medications are investigated. An increasing incidence and prevalence of CD have been found over the last three decades. The disease seems to be most common in northern Europe and North America, but is probably increasing also in Asia and Africa. Smoking is associated with an increased risk of developing CD. Age < 40 at diagnosis, penetrating/stricturing complications, need for systemic steroids, and disease location in terminal ileum are factors associated with higher relapse rates. A slight predominance of women diagnosed with CD has been found. Ileocecal resection is the most commonly performed surgical procedure, and within the first five years after the diagnosis about one third of the patients have had intestinal surgery. Smoking is associated with a worse clinical course and with increased

risk of flare-ups. In most studies the overall mortality is comparable to the background population. To date, the most effective treatment options in acute flares are glucocorticosteroids and Tumor Necrosis Factor (TNF)- $\alpha$ -blockers. Azathioprine/methotrexate and TNF- $\alpha$ -blockers are effective in maintaining remission.

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**Key words:** Crohn's disease; Epidemiology; Diagnosis; Smoking; Extra-intestinal manifestations; Therapy

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### INTRODUCTION

The incidence rates for Crohn's disease (CD) and ulcerative colitis (UC) in Western countries have increased since the mid-1970s. The same trend, although less pronounced, is also seen in the developing world<sup>[1-3]</sup>. The reported geographical variations in incidence may in part be due to differences in diagnostic tools and study design, and many of the epidemiological studies have been retrospective and hospital-based<sup>[4]</sup>. CD is a disease with a broad spectrum of clinical manifestations, and the initial presentation is seldom a good predictor of the clinical course<sup>[5-7]</sup>. Patients with newly diagnosed CD often ask about expectations related to the course of the disease. To answer this question, information on the natural course of CD based on observational, population-based

cohort studies is crucial. The “natural course” in CD might be different in 2011 compared to the situation for instance in the 1980s, and there are at least two reasons for this: we now have better tools to diagnose the condition in an earlier phase, and we have new therapeutic agents that hopefully will alter the course of the disease.

The primary aim of this article is to review the clinical outcome in patients with CD based on population-based studies conducted over the last thirty years, focusing predominantly on studies describing the natural clinical course in representative cohorts of patients with CD.

The factors investigated include incidence, prevalence, age, sex, smoking, geographical differences, surgical aspects, extra-intestinal manifestations, mortality, medication, clinical course with focus on relapse and surgery, the location and behavior of the disease, and finally, the need for sick leave/unemployment. A Medline search (1980 to 2011) for English language articles was conducted.

The MESH term “Crohn disease” was combined with free text search for “population based” and “clinical”. This search yielded sixty-seven articles. All potential relevant articles were evaluated.

## EPIDEMIOLOGICAL ASPECTS OF CROHN'S DISEASE

### *Incidence, prevalence and time-trends*

The incidence of CD differs depending on the region studied. The United Kingdom, North America and the northern part of Europe are the areas with the highest incidence<sup>[8-10]</sup>. A Danish study from 1997 found that the mean incidence rate for men per 100 000 person years increased from 3.3 in 1981-1984 to 4.1 in 1989-1992<sup>[11]</sup>. For women in the same area and in the same time intervals, the incidence rate increased from 4.6 to 6.2. A peak incidence rate was found among 15-29-year-olds, with an incidence rate among men and women of 5.3 and 9.1, respectively. A recent study in which all new CD cases in Finland between 2000 and 2007 were included revealed an overall incidence rate of 9.2 per 100 000 inhabitants<sup>[12]</sup>. The incidence rate in Olmsted County, Minnesota, United States was 5.7 cases per 100 000 person years between 1940 and 1993<sup>[10]</sup>. During the study period, a marked increase in incidence was found: in 1940-1943 the incidence rate was 1.0, while the rate in 1984-1993 was 6.9. Traditionally, the incidence has been low in Asia and Africa. Studies from these areas suggest that the incidence of CD is increasing<sup>[2,13]</sup>.

The prevalence of CD in Europe varies from less than 10 to about 150 per 100 000 inhabitants<sup>[9,14]</sup>. An adjusted prevalence of 133 per 100 000 was found in Minnesota, United States in 1991<sup>[10]</sup>. One study from South Korea indicated prevalence of 11.2<sup>[15]</sup>. From the existing data, one can conclude that the incidence and prevalence rates of CD have increased over last decades.

### *Sex and age at diagnosis and smoking*

In a retrospective study from the United Kingdom on patients diagnosed with CD<sup>[16]</sup>, the patient population was

divided in three groups by year of diagnosis: 1986-1991, 1992-1997 and 1998-2003. Of a total of 341 subjects diagnosed with CD, 62% were females. In all time intervals, the median age at diagnosis was 30 years. In a prospective study from Denmark, all patients in Copenhagen County diagnosed with UC and CD from 2003 to 2005 were followed for 11.3 mo (median); 54% of the CD patients were females, and median age at diagnosis was 31 years<sup>[8]</sup>. A retrospective study from Olmsted County, Minnesota, United States showed that 54% of the patients were females, and the median age at diagnosis was 29.5 years<sup>[10]</sup>.

In Norway, the Inflammatory Bowel South-Eastern Norway (IBSEN) group, in a prospective study, has followed patients with inflammatory bowel disease (IBD) since the beginning of the 1990s<sup>[17]</sup>. This study reported a slight predominance of women diagnosed with CD, with a male/female-ratio of 0.95. The median age at diagnosis was 30 years. In Finland, no significant difference between the genders was found<sup>[12]</sup>. A population-based Canadian study<sup>[18]</sup> found a female predominance: 58% of the patients were women. Cigarette smoking is associated with increased risk of developing CD<sup>[19]</sup>. Smoking negatively influences the clinical course of CD and is also associated with the clinical recurrence of CD after surgical resection in CD patients<sup>[20]</sup>. Cosnes *et al*<sup>[21]</sup> found that smoking, particularly heavy smoking, markedly increased the risk of flare-ups of the disease.

Based on these studies, the conclusion is that there is a slight predominance of women diagnosed with CD, that the age at diagnosis is approximately 30, and that cigarette smoking is harmful in patients with CD.

### *Appendectomy*

The relationship between appendectomy and the risk of developing CD has been debated, and in 2009, a systematic review of the literature was performed<sup>[22]</sup>. The authors found that the relative risk (RR) for having CD diagnosed following an appendectomy was significantly elevated. Within the first year after the surgery, the RR was 6.69 (95% CI 5.42-8.25). An increase in the risk of developing CD also was found 1-4 years after the appendectomy, but thereafter the risk was not increased. Another study confirmed these results, but no increased risk of developing CD was found in patients who underwent appendectomy before the age of ten<sup>[23]</sup>. It seems that there was an inverse relationship between appendectomy and the development of UC, at least in patients who underwent appendectomy before the age of 20<sup>[24]</sup>.

### *Geographical differences*

The occurrence of CD seems to vary according to geographical location. A north-south axis has been found in both Europe and in the United States, with higher incidence and prevalence in the northern regions. In a study from 1996<sup>[25]</sup> on the incidence of IBD across Europe, incidence rates were found to be 80% higher in northern centers than in southern. A French study from 2006<sup>[26]</sup> also demonstrated a north-south gradient within France. Data from Columbia support the clinical experience that

CD is rare in South America<sup>[27]</sup>. Based on a prospective European population-based inception cohort of 380 CD patients, a difference in management was observed between northern and southern centers, indicating that CD patients in the north had a more severe disease course than did those in the south<sup>[28]</sup>. One problem is that there are still huge differences in diagnostic facilities. South Asians who live in Europe are more likely to develop IBD than South Asians who do not. In some regions of the world, there are diagnostic challenges due to overlap with intestinal tuberculosis<sup>[11]</sup>. In Brazil, Argentina, Puerto Rico and Panama, the prevalence of CD and UC together is between 20-100/100 000 inhabitants, but very few reports exist, and the ratio between CD and UC is uncertain. This is in marked contrast to the numbers from the United States and Canada, where the prevalence numbers vary between 320/100 000 and 511/100 000 inhabitants<sup>[27]</sup>. The reasons for these differences are not fully elucidated.

Hispanics in the United States are less prone to develop IBD than the non-Hispanic population. It is known that the *NOD2* gene on chromosome 16 is a marker for the susceptibility to CD. A recent study showed that 4.4% of Hispanics and 9.1% of the white population have *NOD2*<sup>[29]</sup>. This indicates that there are real differences in incidence/prevalence between North and South America.

### Clinical important risk factors

In many studies, risk factors predicting a disabling course of CD are described. The IBSEN group<sup>[30]</sup> has described the relapse rates and need for surgery one, five, and ten years after the diagnosis. Age < 40 at diagnosis and the need for systemic steroids to treat the first flare-up were factors associated with higher relapse rates. Age < 40 at diagnosis, disease location in terminal ileum and penetrating/stricturing complications were associated with higher risk for surgery (Tables 1 and 2). Beaugerie *et al*<sup>[31]</sup> also found that age < 40 at diagnosis of CD, presence of perianal disease, and initial requirement for steroids were independent factors predicting disabling disease during the first five years after the diagnosis. Henriksen *et al*<sup>[32]</sup> did not find any association between CRP levels and a risk of surgery for the CD group as a whole, but a significant linear association between CRP levels and a risk of surgery was found with L1 localization (disease localization in terminal ileum).

## SURGERY

### Bowel resection

Stenoses, fistulas and abscesses are the main reasons for bowel resection in patients with CD.

In a Danish study from 2003-2005, 12% of CD patients underwent bowel resection performed within one year after the diagnosis, and the median time from diagnosis to resection was one month (range 0-8 mo)<sup>[8]</sup>. The authors compared the results to what was seen in patients diagnosed with CD in the same area between 1962 and

1987. In the earlier period, as many as 35% of the CD patients underwent bowel resection performed within the first year of diagnosis. A shorter delay from the onset of symptoms to diagnosis and the introduction of more intensive immunosuppressive therapy might be among the explanations for this decreased risk of surgical resection.

The Montreal classification of CD includes the location (L) of the disease, where L1 is location in the terminal ileum, L2 is in the colon, L3 is an ileocolonic location, and L4 is an upper GI location<sup>[33]</sup>. In one study, patients with L1 location at diagnosis had increased likelihood of intestinal surgery<sup>[5]</sup>. Oral corticosteroid use within three months of diagnosis, stricturing disease and low age at diagnosis were also associated with increased likelihood of resection. In the IBSEN study, the cumulative probability of surgery was 13.6%, 27.0% and 37.9% at one, five, and ten years after diagnosis<sup>[30]</sup>, respectively. In this cohort, L1 location was strongly associated with surgery compared to L2 and L3 locations (Table 1).

A United Kingdom study of changes in medical treatment and surgical resection rates from 1986 to 2003 found a marked reduction in the proportion of patients needing intestinal surgery<sup>[16]</sup>. Within five years of diagnosis of CD, 59% of the patients diagnosed from 1986-1991 had intestinal surgery. In patients diagnosed between 1992-1997 and 1998-2003, 37% and 35%, respectively, had intestinal surgery within five years of diagnosis. In addition, there was a significant reduction in patients undergoing any surgical procedure (surgery for perianal disease or intestinal surgery) during an advanced stage of disease. Ileocecal resection was the most commonly performed procedure. The most striking reductions were seen in the numbers of ileocecal resections and in the numbers of panproctocolectomies.

A French retrospective study with 2573 CD patients<sup>[34]</sup> divided the cohort in five groups according to the year of diagnosis (1978-1982, 1983-1987, 1988-1992, 1993-1997 and 1998-2002). The main outcome criterion was the time to first intestinal resection. The cumulative probability to receive immunosuppressants [azathioprine (AZA), methotrexate (MTX)] increased from 0 in the 1978-1982 cohort to 0.56 in the 1998-2002 cohort. Interestingly, in contrast to the study from the United Kingdom<sup>[16]</sup>, one found that the year of diagnosis did not have any significant effect upon the need for surgery.

### Extra-intestinal manifestations

Extra-intestinal manifestations of CD include musculoskeletal, dermatologic, ocular, hepatobiliary, vascular and renal complications<sup>[35]</sup>. About 25%-46% of the patients with CD will experience extra-intestinal manifestations<sup>[36,37]</sup>. Primary sclerosing cholangitis (PSC) is in many ways the most serious, and the most serious complication from this condition is cholangiocellular carcinoma (CCC)<sup>[37]</sup>; 7% to 15% of the patients with PSC eventually develop CCC<sup>[38,39]</sup>, 60%-70% of the PSC patients are male, and the age at diagnosis is about 40 years. There is a close relationship between UC and PSC. The relationship

**Table 1** Solberg *et al*<sup>[30]</sup>, with permission

Variables at diagnosis	Total in each subgroup	Relapse during the 1st year		Relapse between 1-5 yr		Relapse between 5-10 yr	
		cum% (CI)	P value	cum% (CI)	P value	cum% (CI)	P value
Age groups							
A1 < 40 yr	148	54 (50-58)	0.7	80 (77-83)	0.6	61 (57-65)	0.03
A2 ≥ 40 yr	49	51 (44-58)		76 (70-82)		43 (36-50)	
Gender							
Female	95	54 (49-59)	0.9	76 (72-80)	0.4	56 (51-61)	0.9
Male	102	53 (48-58)		81 (77-85)		57 (52-62)	
Location							
L1: Terminal ileum	51	54 (47-61)	0.4	78 (72-84)	0.8	57 (50-64)	0.1
L2: Colon	94	57 (52-64)		76 (72-80)		49 (44-54)	
L3: Ileocolon	48	44 (37-51)		81 (75-87)		69 (62-76)	
L4: Upper GI	4	75 (53-97)		100 (-)		75 (53-97)	
Behavior							
B1: Inflammatory	127	56 (52-60)	0.7	79 (75-83)	0.6	54 (50-58)	0.4
B2: Stricturing	50	49 (42-56)		74 (68-78)		64 (57-71)	
B3: Penetrating	20	50 (39-61)		85 (77-93)		55 (44-66)	
Systemic steroids							
No	86	47 (42-52)	0.08	69 (64-74)	0.008	47 (42-52)	0.02
Yes	109	59 (54-64)		85 (82-88)		63 (58-68)	
Missing	2	-		-		-	
Smoking status							
Never	82	52 (47-57)	0.5	79 (75-84)	0.9	59 (54-64)	0.09
Current smoker	82	57 (52-62)		79 (75-84)		61 (56-66)	
Ex-smoker	29	45 (36-54)		76 (68-84)		38 (29-47)	
Missing	4	-		-		-	
Total	197	54 (50-57)		79 (76-82)		56 (53-60)	

Cumulative rate (cum%) of Crohn's disease patients with relapsing disease during the first year and in the periods 1-5 years and 5-10 years after diagnosis.  $\chi^2$  comparisons within each subgroup; CI: Confidence interval; GI: Gastrointestinal.

**Table 2** Solberg *et al*<sup>[30]</sup>, with permission

Variables at diagnosis	Number in analysis	Number with surgery (%)	Unadjusted			Adjusted		
			HR	95% CI	P value	HR	95% CI	P value
Age								
A1: < 40 yr	165	69 (42)	1	[Ref]	0.03	1	[Ref]	0.03
A2: ≥ 40 yr	72	16 (22)	0.5	0.3-0.9		0.5	0.3-0.9	
Gender								
Female	118	40 (34)	1	[Ref]	0.9	Not included		
Male	119	45 (38)	1	0.7-1.6				
Location								
L1: Terminal ileum	64	38 (59)	1	[Ref]	< 0.001	1	[Ref]	
L2: Isolated colonic	115	26 (23)	0.2	0.1-0.4		0.3	0.2-0.6	0.001
L3: Ileocolon	54	17 (32)	0.3	0.2-0.6		0.3	0.2-0.5	< 0.001
L4: Upper GI <sup>1</sup>	4	4 (100)	1.4	0.5-3.8		1.6	0.5-4.4	0.4
Behavior								
B1: Inflammatory	147	32 (22)	1	[Ref]	< 0.001	1	[Ref]	
B2: Stricturing	64	36 (56)	3.5	2.1-5.6		2.3	1.3-4.1	0.004
B3: Penetrating	26	17 (65)	4.9	2.7-8.8		5.4	3.0-9.9	< 0.001
Smoking status <sup>2</sup>								
Never	103	38 (37)	1	[Ref]	0.2	Not included		
Current < 10 cigarettes/d	57	18 (32)	0.8	0.4-1.4				
Current > 10 cigarettes/d	36	18 (50)	1.9	0.8-2.6				
Ex-smoker	35	11 (31)	0.8	0.4-1.6				
Systemic steroids <sup>3</sup>								
No	106	36 (34)	1	[Ref]	0.8	Not included		
Yes	129	48 (37)	1.1	0.7-1.6				

<sup>1</sup>Difficult to conclude because of an insufficient number of patients; <sup>2</sup>data unknown in 6 cases. There were none in the operated group; <sup>3</sup>data unknown in 2 cases; There was one in the operated group. Risk factors at diagnosis associated with surgery during follow-up analyzed by Cox regression. Ref: Reference variable; HR: Hazard ratio; CI: Confidence interval;

between CD and PSC is less pronounced but marked. Studies from Sweden and Holland showed that 72% and 73%, respectively, of the PSC patients with IBD had UC, and 7% and 25%, respectively, of the PSC patients with IBD had CD<sup>[40,41]</sup>.

In both studies, a certain proportion of patients with PSC did not have a diagnosis of IBD. To date, no medical treatment has been established to be effective. A hydrophilic dihydroxy bile acid, ursodeoxycholic acid, is used in the treatment of PSC, but the efficacy of the treatment is not well established. A recent study even concluded that serious adverse events were more common in the drug-treated group than in the placebo group<sup>[42]</sup>.

Five-year follow up data from the IBSEN study showed that the cumulative occurrence of peripheral arthritis related to CD is 14%; 6% had ankylosing spondylitis; 1% had psoriatic arthritis; and 19% had undifferentiated spondyloarthropathy<sup>[43-45]</sup>. In Canada, one study following the patients from 1984 to 1997 showed that 6027 patients suffering from IBD had a 40% increased risk of fractures compared to the control group<sup>[46]</sup>. The incidence rate ratio (IRR) for fracture at the hip was 1.59 (95% CI: 1.27-2.00,  $P < 0.001$ ); the IRR for fracture of the spine was 1.74 (95% CI: 1.34 -2.24,  $P < 0.001$ ); while the IRR for rib fracture was 1.25 (95% CI: 1.02-1.52,  $P = 0.03$ ). No differences were found in the IRR between CD and UC patients. A study from Olmsted County, United States, showed that the relative risk for osteoporotic fractures in CD patients was 1.4 (95% CI: 0.7-2.7), and the risk ratio for thoracolumbar vertebral fracture was 2.2 (95% CI: 0.9-5.5)<sup>[47]</sup>.

### Mortality

During the decades from 1970 to 1990, population-based studies have shown a slightly decreased life expectancy in CD patients<sup>[48-50]</sup>. Because these studies are from the era before the introduction of the immunomodulating agents, the applicability might be of limited value.

A meta-analysis from 2007<sup>[51]</sup> identified 13 papers that reported standardized mortality ratios (SMR). Most of these papers included patients diagnosed in the 1950s to the 1970s, although four of them included patients diagnosed from 1980 to 1985. In this study, an age-adjusted mortality risk in CD patients was more than 50% greater than in the general population, but three of the studies actually reported an SMR below 1.0. A meta-analysis from 2010<sup>[52]</sup> also confirmed slightly increased mortality in CD patients (SMR 1.39, 95% CI: 1.30-1.50). In a recent report, a complete 10-year follow-up was achieved in 197 of 237 patients<sup>[30]</sup>. Two deaths during follow-up were probably CD-related. Another study did not show any decrease in survival curves for the total group of 373 CD patients followed for five years compared to the background population, although a small subgroup of patients diagnosed at the age of 20-29 and a subgroup with extensive small bowel disease displayed slightly increased mortality<sup>[53]</sup>. In the Netherlands, 1187 patients diagnosed with IBD during a 12-year period from 1991 were included<sup>[54]</sup>. The mortality in CD, UC, and indeterminate colitis

was comparable to the background population, but the disease-specific mortality risk was significantly increased for gastrointestinal causes in both CD and UC patients. Overall, a slight increase in mortality was found in CD patients. This is mainly caused by malignant diseases in the gastrointestinal tract and in the lungs<sup>[52]</sup>.

### Medication

CD is a chronic disorder that, at least so far, is not curable. The induction and maintenance of symptom improvement and, at best, the induction and maintenance of mucosal healing are the goals of treatment<sup>[55]</sup>. Disease location, disease severity and complications should be taken into consideration when therapy is to be decided.

Even at high doses and prolonged administration, glucocorticosteroids (GCSs) induce endoscopic remission in less than one third of the patients with colonic CD<sup>[56]</sup>. Use of GCSs has a favorable effect on the symptoms of Crohn's disease of the small intestine but will not achieve a significant reduction in endoscopically observed inflammation<sup>[57]</sup>.

A few decades ago, a large clinical trial<sup>[58]</sup> showed that CD patients with colonic involvement were especially responsive to sulfasalazine, but a European multicenter double-blind study from the 1980s did not show any beneficial effects from sulfasalazine as compared to 6-methylprednisolone<sup>[59]</sup>. Oral mesalamine has been, and still is, widely used in the treatment of CD. A meta-analysis of three large, double-blind, randomized studies in the treatment of active CD showed that mesalamine 4 g/d was better than placebo in reducing the Crohn's Disease Activity Index, but the clinical significance was unclear<sup>[60]</sup>. The recently published European evidence-based consensus on the diagnosis and management of CD<sup>[61]</sup> concluded that active colonic CD may be treated with sulfasalazine if only mildly active, and that mesalamine should be considered no more effective than placebo in the treatment for active ileal or colonic CD.

The rationale for the use of antibiotics in the treatment of mild to moderate CD is the hypothesis that bacteria may cause or exacerbate CD. Metronidazole, 10 or 20 mg/kg per day, compared to placebo, did not show any difference in the ability to induce remission in patients with mild/moderate disease<sup>[62]</sup>. Comparison of ciprofloxacin and mesalamine did not reveal any consistent pattern<sup>[63]</sup>. About 50% in each group achieved clinical remission. A recent meta-analysis<sup>[64]</sup> concludes that long-term treatment with nitroimidazoles or clofazimine appeared to be effective in CD patients.

A recent review on the effect of AZA or 6-mercaptopurine for the maintenance of remission in CD<sup>[65]</sup> concluded that both AZA and 6-mercaptopurine had a positive effect on maintaining remission. The study reported weak evidence for a steroid-sparing effect of AZA. In a recent single-center study<sup>[66]</sup>, the authors found that MTX is efficient as a second-line immunomodulator in chronic active CD. In steroid-dependent CD patients, complete remission and steroid withdrawal were seen in 77% of the cases after 22.9 mo of treatment. After six months,

one, two, and three years on MTX, 95.3%, 89.5%, 70.6% and 62.8%, respectively, were in remission<sup>[67]</sup>. However, a high proportion of the patients developed side-effects (79% and 39%, respectively), including hepatotoxicity and hair loss<sup>[66,67]</sup>. Side effects associated with the use of AZA and 6-mercaptopurine include leucopenia, thrombocytopenia, pancreatitis and an increased risk of developing lymphoma<sup>[65]</sup>. The introduction of anti-tumor necrosis factor (TNF) agents has changed the treatment of refractory CD. Although the causes of CD are not known, many of the molecules involved in the disease process have been identified and can act as targets of biological treatment. TNF is a cytokine that promotes inflammatory responses in many diseases, including CD<sup>[68]</sup>. Infliximab is effective in both luminal and fistulizing CD<sup>[69,70]</sup> and is highly effective and safe in children<sup>[71]</sup>. The combination of infliximab and AZA is more effective in moderate-to-severe CD than infliximab alone<sup>[72]</sup>. One problem is that, each year, about 10% of patients, for different reasons, drop out of treatment<sup>[73]</sup>. Adalimumab is a human monoclonal antibody against TNF. It is administered subcutaneously and has proven effective in the treatment of luminal CD<sup>[74]</sup>. Certolizumab pegol is approved in the United States for the treatment of CD<sup>[68]</sup>. No trials exist that compare the three different anti-TNF agents, but it seems that infliximab, adalimumab and certolizumab pegol are comparable in efficacy. Anti-TNF agents definitely deserve to be considered as a treatment option for patients with CD; it is therefore widely discussed when patients should be introduced to these agents, and further studies are needed to establish this aspect of the approach to treatment.

In a review article, Vermeire *et al.*<sup>[75]</sup> summarized the therapies that have been shown to alter the natural history of CD. Mucosal healing, the need for hospitalizations/surgery together with decreased recurrence after surgery, are surrogate markers of changes in the natural course of the disease. Anti-TNF agents have shown the ability to induce mucosal healing and to reduce the need for surgery in randomized, placebo-controlled studies. It is not known, however, if they can reduce the risk of recurrence after surgery.

## CLINICAL COURSE

Markov models will show the probability of changes in state from one time-point to another. This model was used in a Danish study from 1995<sup>[6]</sup>. One found that the disease activity course in CD is not dependent on age, sex, or localization of the disease. The United Kingdom study and the French study<sup>[16,31]</sup> showed conflicting results, at least in the proportion of patients requiring surgery. The IBSEN study showed that ten years after the diagnosis was made, the course was generally better than in earlier reports<sup>[30]</sup>. The need for immunosuppressives and GCSs declined from the first five-year period to the second five-year period. The probability of surgery was 37.9%, and fewer patients than expected developed complicated disease behavior; however, the cumulative relapse

rate was as high as 90% (Table 2). Because we have had the opportunity for biological treatment for some years now, we are looking forward to evaluating new epidemiological studies on the clinical course of the disease.

## Sick leave/unemployment

There are just a few reports on sick leave/unemployment in patients with CD. In 2006, Bernklev *et al.*<sup>[76]</sup> found that 24.6% of women with CD were on a disability pension (DP) five years after the diagnosis was established, which was a three-fold increase compared to the background population (8.8%). Five years after the diagnosis, 53% of the CD patients reported taking sick leave during the prior six months; 23% of the sick leaves were CD-related. A Dutch study from 2002 also concluded that CD patients had a significantly higher frequency of sick leave than the controls (odds ratio 1.7, 95% CI: 1.2-2.6)<sup>[77]</sup>. Both studies concluded that having CD is correlated to an increased unemployment rate. An earlier Danish study did not find any differences in the state of employment between CD patients nine years after the diagnosis compared to a control group, and as few as 3% were on DP<sup>[78]</sup>.

## CONCLUSION

Population-based studies have demonstrated that the incidence and prevalence of CD have increased over the last three decades. CD is most common in northern Europe and North America, and there is a slight predominance of women diagnosed with the disease. The majority of patients experience progression from inflammatory disease to the development of strictures and fistulas. Within the first five years of the disease, at least one third of the patients have had intestinal surgery, where ileocecal resection is the most commonly performed procedure. Appendectomy is associated with an increased risk of having CD diagnosed within the first four years after the surgery. A rising incidence and a slight female predominance is found in CD. The diagnosis is made when the patients are approximately thirty years old. Smoking is associated with an increased risk of developing CD, with a negative clinical course in patients with CD, and with increased risk of flare-ups of the disease in both operated and non-operated patients.

The overall mortality in most studies is comparable to the background population, although subgroups of CD patients seem to have slightly increased mortality. New therapeutic approaches are promising. To date, the most effective treatment options in acute disease are GCSs and TNF- $\alpha$ -blockers. TNF- $\alpha$ -blockers and AZA/MTX are effective in maintaining remission.

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## Macrophage secretory products induce an inflammatory phenotype in hepatocytes

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### Abstract

**AIM:** To investigate the influence of macrophages on hepatocyte phenotype and function.

**METHODS:** Macrophages were differentiated from THP-1 monocytes *via* phorbol myristate acetate stimulation and the effects of monocyte or macrophage-conditioned medium on HepG2 mRNA and protein expression determined. The *in vivo* relevance of these findings was confirmed using liver biopsies from 147 patients with hepatitis C virus (HCV) infection.

**RESULTS:** Conditioned media from macrophages, but not monocytes, induced a transient morphological change in hepatocytes associated with upregulation of vimentin ( $7.8 \pm 2.5$ -fold,  $P = 0.045$ ) and transforming growth factor (TGF)- $\beta$ 1 ( $2.6 \pm 0.2$ -fold,  $P < 0.001$ ) and downregulation of epithelial cadherin ( $1.7 \pm 0.02$ -fold,  $P = 0.017$ ) mRNA expression. Microarray analysis revealed significant upregulation of lipocalin-2 (17-fold,  $P < 0.001$ ) and pathways associated with inflammation, and substantial downregulation of pathways related to hepatocyte function. In patients with chronic HCV, real-time polymerase chain reaction and immunohistochemistry confirmed an increase in lipocalin-2 mRNA (F0  $1.0 \pm 0.3$ , F1  $2.2 \pm 0.2$ , F2  $3.0 \pm 9.3$ , F3/4  $4.0 \pm 0.8$ ,  $P = 0.003$ ) and protein expression (F1  $1.0 \pm 0.5$ , F2  $1.3 \pm 0.4$ , F3/4  $3.6 \pm 0.4$ ,  $P = 0.014$ ) with increasing liver injury. High performance liquid chromatography-tandem mass spectrometry analysis identified elevated levels of matrix metalloproteinase (MMP)-9 in macrophage-conditioned medium, and a chemical inhibitor of MMP-9 attenuated the change in morphology and mRNA expression of TGF- $\beta$ 1 ( $2.9 \pm 0.2$  vs  $1.04 \pm 0.1$ ,  $P < 0.001$ ) in macrophage-conditioned media treated HepG2 cells. In patients with chronic HCV infection, hepatic mRNA expression of CD163 (F0  $1.0 \pm 0.2$ , F1/2  $2.8 \pm 0.3$ , F3/4  $5.3 \pm 1.0$ ,  $P = 0.001$ ) and MMP-9 (F0  $1.0 \pm 0.4$ , F1/2  $2.8 \pm 0.3$ , F3/4  $4.1 \pm 0.8$ ,  $P = 0.011$ ) was significantly associated with increasing stage of fibrosis.

**CONCLUSION:** Secreted macrophage products alter the phenotype and function of hepatocytes, with increased expression of inflammatory mediators, suggesting that hepatocytes actively participate in liver injury.

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**Key words:** Macrophages; Hepatic fibrosis; Lipocalin-2; Transforming growth factor- $\beta$ 1; Matrix metalloproteinase-9

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Melino M, Gadd VL, Walker GV, Skoien R, Barrie HD, Jothmani D, Horsfall L, Jones A, Sweet MJ, Thomas GP, Clouston AD, Jonsson JR, Powell EE. Macrophage secretory products induce an inflammatory phenotype in hepatocytes *World J Gastroenterol* 2012; 18(15): 1732-1744 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i15/1732.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i15.1732>

## INTRODUCTION

Most of the morbidity/mortality from chronic liver diseases occurs in subjects with advanced fibrosis or cirrhosis, who are at risk of developing complications of end-stage liver disease including hepatocellular cancer. Activated liver macrophages have a key role in the progression of liver injury and repair, and knowledge about their interaction with hepatocytes and other cells in the liver microenvironment may provide new targets for antifibrotic therapy. In experimental models of liver disease, an increase in the number of macrophages correlates with the extent of injury, and damage can be attenuated by depletion of these cells<sup>[1-6]</sup>. Similarly, in diseased human liver, particularly chronic viral hepatitis, there is an increase in the density and size of macrophages<sup>[7,8]</sup>. This is seen largely at inflammatory sites with prominent mononuclear cell infiltration or within inflamed portal tracts. These tissue-resident macrophages appear to be derived from circulating monocytes even in steady state conditions, although there is a marked increase in recruitment of these cells to the liver following inflammatory insults<sup>[9]</sup>. A recent study has demonstrated that monocytes increase in the circulation as well as in the liver of patients during progression of chronic liver disease, and that they are activated and release high amounts of proinflammatory cytokines and reactive oxygen species<sup>[10]</sup>.

Secreted products from activated macrophages contribute to stellate cell activation and fibrosis. However, relatively little is known about the influence of macrophages on hepatic epithelial cell phenotype and function. Early studies have shown that secretions from activated macrophages influence hepatocyte DNA synthesis and cytochrome P-450 metabolism<sup>[11-13]</sup>. In a more recent study

using a human cell co-culture model, macrophages triggered secretion of proinflammatory cytokines from bile duct epithelial cells, as well as apoptosis<sup>[14]</sup>. The authors speculated that this cellular interaction provided a mechanism to amplify chronic inflammation and bile duct destruction in vanishing bile duct syndromes<sup>[14]</sup>. The role of activated macrophages in modulating the hepatocyte inflammatory response to injury has not been determined. Accumulating data also suggest that during inflammatory liver injury, some hepatocytes and cholangiocytes may lose epithelial markers and acquire partial mesenchymal characteristics, although the direct role of macrophages and the contribution of this process to fibrogenesis have not been determined<sup>[15,16]</sup>.

The aim of this study was to investigate the influence of macrophages on hepatocyte phenotype and function, and in particular, to determine whether macrophage-secreted products induce a proinflammatory response in hepatocytes. In order to address this, the effect of monocyte and macrophage-conditioned medium on cell morphology and gene expression was examined in two hepatocyte cell lines, along with the macrophage-secreted products that modulated the hepatocyte phenotype. We also evaluated human liver samples from patients with chronic hepatitis C virus (HCV) infection. Our data indicate that secreted products from activated macrophages induce an inflammatory phenotype in hepatocytes, which may have implications for persistent inflammation and fibrogenesis.

## MATERIALS AND METHODS

### Cell culture

The human hepatoma-derived cell lines, HepG2 and Huh7, and the human acute monocytic leukemia cell line, THP-1, were purchased from American Type Culture Collection (ATCC) (Manassas, VA, United States). Cells were maintained at 37 °C and 5% CO<sub>2</sub>. Unless otherwise indicated, cells were cultured in “complete medium” comprising Dulbecco’s Modified Eagle’s Medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), 2 mmol GlutaMAX™ (Invitrogen) and 20 µmol MEM non-essential amino acids (Invitrogen).

Macrophages were generated as described previously<sup>[17,18]</sup>. Briefly, THP-1 monocytes were seeded in six-well plates (Nunc, Roskilde, Denmark) at a density of  $4 \times 10^6$  cells per well in 3 mL of complete medium and incubated for 2 h. Cells were treated with phorbol myristate acetate (PMA) (200 nmol; Sigma-Aldrich, St. Louis, MO, United States) for 24 h, washed three times with  $1 \times$  phosphate buffered saline (PBS) and cultured for 42 h in 2 mL of fresh complete medium. The resulting macrophage-conditioned media (M $\phi$ CM) was collected, clarified by centrifugation at  $400 \times g$  and stored at -20 °C until use. Conditioned media from THP-1 monocytes (MonoCM) was harvested in a similar fashion. For some experiments, M $\phi$ CM was generated in the presence of matrix metalloproteinase (MMP)-9 Inhibitor I, (100 µmol, Cat# 444278, Calbiochem, Merck Pty Ltd, Kilsyth, Victoria, Australia).

**Table 1** Demographic and clinical features of patients with chronic hepatitis C virus infection

No. of patients	<i>n</i>	147
Age (yr)	mean ± SEM	42.3 ± 0.9
Sex	M/F	98/49
Viral genotype	1/ 2/ 3	80/6/61
Stage of fibrosis	0/1/2/3-4	13/65/44/25
Grade of steatosis	0/1/2-3	73/44/29
Necroinflammatory score	1-3/4-5/6-8	56/61/30
BMI (kg/m <sup>2</sup> )	mean ± SEM	26.2 ± 0.4
BMI	Lean, overweight, obese <sup>1</sup>	66/51/24
Alcohol - current (g/d)	Median (range)	1 (0-120)
Alcohol - past (g/d)	Median (range)	30 (0-500)
Creatinine (μmol/L)	mean ± SEM	77.8 ± 1.5
Platelets (× 10 <sup>9</sup> /L)	mean ± SEM	213 ± 6
Red blood cells (× 10 <sup>12</sup> /L)	mean ± SEM	4.8 ± 0.3
Total WBC (× 10 <sup>9</sup> /L)	mean ± SEM	7.2 ± 0.2
Neutrophils (× 10 <sup>9</sup> /L)	mean ± SEM	4.0 ± 0.1
Lymphocytes (× 10 <sup>9</sup> /L)	mean ± SEM	2.3 ± 0.06
Monocytes (× 10 <sup>9</sup> /L)	mean ± SEM	0.6 ± 0.02

<sup>1</sup>Lean: ≤ 25 kg/m<sup>2</sup>; overweight: > 25 and < 30 kg/m<sup>2</sup>; obese: ≥ 30 kg/m<sup>2</sup>. BMI: Body mass index; WBC: White blood cell.

HepG2 and Huh7 cells were seeded in six-well plates at a density of  $2 \times 10^5$  cells per well in 3 mL of complete medium. After 24 h, cells were washed and cultured with 50% MφCM or 50% MonoCM in complete media for 24 h unless indicated otherwise. Cell morphology was observed by phase contrast microscopy using the Nikon Eclipse TS100. Culture in CM did not influence cell viability as determined by trypan blue exclusion.

#### Microarray analysis of HepG2 cells treated with macrophage-conditioned media or MonoCM

Total RNA was extracted from untreated, monocyte CM and MφCM-treated HepG2 cells using TRI Reagent® (Sigma-Aldrich). RNA quality was assessed with an Agilent 2100 BioAnalyser (Agilent Technologies, Santa Clara, CA, United States) and only samples with a RNA integrity number above 8.0 were included. cRNA was generated from 500 ng total RNA using the Illumina TotalPrep cRNA Amplification Kit (Applied Biosciences, Carlsbad, CA, United States) and hybridised to Human HT-12 V3 Expression BeadChips (Illumina, San Diego, CA, United States). Array data were processed using the Illumina GenomeStudio software, transformed by variance stabilization transformation<sup>[19]</sup> and normalized by robust spline normalization<sup>[20]</sup> with Lumi<sup>[21]</sup>. Differential gene expression patterns between MφCM-treated and untreated HepG2 cells were identified using class comparison corrected for multiple testing with BRB ArrayTools (National Cancer Institute, Bethesda, MD, United States)<sup>[22]</sup>. Gene Ontology (GO), KEGG and BioCarta analyses were applied to the differentially expressed gene sets to identify altered pathways and cell functions. Least-squares (LS)/Kolmogorov-Smirnov (KS) permutation tests, Efron-Tibshirani's gene set analysis maxmean test and Goeman's global test were used to identify relevant pathways and a significance threshold of 0.005 was applied.

#### Patients and clinical data

The study involved 147 consecutive patients with chronic hepatitis C, who had undergone a liver biopsy at the Princess Alexandra Hospital, Brisbane, Australia. Informed consent was obtained from each patient and the protocol was approved by the University of Queensland and Princess Alexandra Hospital Research Ethics Committees. Diagnosis of chronic HCV infection was based on standard serological assays and abnormal serum aminotransferase levels for at least 6 mo. All patients were positive for HCV antibody by the third-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL, United States), with infection confirmed by detection of circulating HCV RNA by polymerase chain reaction (PCR) using the Amplicor HCV assay (Roche, New Jersey, United States). Viral genotyping was performed using the Inno-Lipa HCV II assay (Innogenetics, Zwijnaarde, Belgium). Patients with other forms of chronic liver disease or antibodies to human immunodeficiency virus were not considered for the analysis. Patients with comorbidity such as acute coronary artery disease, unstable angina, congestive heart failure, significant renal impairment, acute and chronic infectious diseases, autoimmune and rheumatic diseases, cancer, endocrine diseases or inflammatory bowel diseases were not included in the study. Demographic and clinical details for the patients are presented in Table 1.

A fragment of liver tissue (2-3 mm) was immediately frozen in liquid nitrogen at the time of biopsy and stored at -80 °C until extraction of RNA was performed. The remaining core was fixed in buffered formalin and embedded in paraffin. The degree of inflammation was graded according to the method of Ishak<sup>[23]</sup>, and fibrosis was staged (0-4) according to the method of Scheuer<sup>[24]</sup>. Steatosis was graded as follows: 0 (< 5% hepatocytes affected); 1 (5%-33% of hepatocytes affected); 2 (34%-66% of hepatocytes affected); or 3 (> 66% of hepatocytes affected).

#### Real-time polymerase chain reaction

Total RNA was extracted from liver biopsy tissue, Huh7, HepG2, Mφ and THP-1 cells using TRI Reagent® (Sigma-Aldrich) according to the manufacturer's instructions and reverse-transcribed to cDNA by SuperScript® III Reverse Transcriptase (Invitrogen). Liver tissue RNA quality was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies) and the RNA 6000 Nano LabChip according to the manufacturer's instructions. The median RNA Integrity Number (RIN) of RNA extracted from the liver biopsy samples was 8.0 (range: 6.0-9.2). Semi-quantitative real-time PCR (qPCR) for genes of interest was performed using Platinum® SYBR® Green qPCR SuperMix (Invitrogen) and analysed with MxPro QPCR software for MxPro 3000P QPCR systems (Stratagene, La Jolla, CA, United States) as previously described<sup>[25]</sup>. The expression of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase, human acidic ribosomal protein and 18S ribosomal RNA was determined using a multiplex real-time PCR protocol as previously described<sup>[25]</sup>. The relative mRNA expression of genes of interest was normalized to

**Table 2** Primer and probe sequences used in real-time polymerase chain reaction assays

E-cadherin	for	5'-ATTGCAAATTCCTGCCATTC-3'
	rev	5'-GCTGGCTCAAGTCAAAGTCC-3'
Vimentin	for	5'-GTTTCCAAGCCTGACCTCAC-3'
	rev	5'-GCTTCAACGGCAAAGTTCTC-3'
TGF- $\beta$ 1	for	5'-AAGTGGACATCAACCGGTTTC-3'
	rev	5'-TGCGGAAGTCAATGTACAGC-3'
MMP-9	for	5'-TTCGACGTGAAGGCGCAGATGG-3'
	rev	5'-AACTCACGCGCCAGTAGAAGCG-3'
CD163	for	5'-CCAACAAGATGCTGGAGTGAC-3'
	rev	5'-TGACAGCACTTCCACATTCAAG-3'
Lipocalin-2	for	5'-TCACCCTCTACGGGAGAACCAAGG-3'
	rev	5'-TGTGCACTACGCCGTGATACAC-3'
GAPDH	for	5'-TGCACCACTCAACTGCTTACG-3'
	rev	5'-GGCATGGACTGTGGTCATGAG-3'
HuPO	probe	5'-CCTGGCCAAGGTCATCCATGACAACCTT-3'
	for	5'-GCTTCTGGAGGGTGTCC-3'
	rev	5'-GGACTCGTTTGTACCCGTTG-3'
18s	probe	5'-TGCCAGTGTCTGCTGAGATTGG-3'
	for	5'-GCCCCAAGCGTTTACTTTGA-3'
	rev	5'-TCCATTATTCCTAGCTGCGGTATC-3'
	probe	5'-AAAGCAGGCCCGAGCCGCC-3'

E-cadherin: Epithelial cadherin; TGF- $\beta$ 1: Transforming growth factor- $\beta$ 1; MMP-9: Matrix metalloproteinase-9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; 18s: 18S ribosomal RNA; HuPO: Human acidic ribosomal protein; for: Forward; rev: Reverse.

the geometric mean of the expression of the three house-keeping genes. Primers for SYBR<sup>®</sup> Green assays were custom made by Geneworks (Thebarton, SA, Australia). Custom made primer-probe sets were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Specific primer and probe sequences are shown in Table 2.

### Immunofluorescence and immunohistochemistry

To visualise epithelial cadherin (E-cadherin) and vimentin protein expression, HepG2 and Huh7 cells were cultured, as described above, on sterile glass cover slips (Deckglaser, Freiburg, Germany). After treatment, cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, United States), washed with 1% glycine in 1  $\times$  PBS and permeabilized for 5 min with 0.2% Triton X-100 (Sigma-Aldrich). Non-specific binding was blocked with 10% heat-inactivated goat serum (Sigma-Aldrich) for 30 min. Cells were incubated with primary antibodies (1/50 dilution in 1% heat-inactivated goat serum) against E-cadherin (mouse monoclonal, ab1416; Abcam, Cambridge, MA, United States) or vimentin (mouse monoclonal, ab8069; Abcam) for 1 h at room temperature. After washing, goat anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen; 1/200 dilution) was added for 1 h at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen) and observed using the Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, North Ryde, NSW, Australia).

Immunofluorescence was performed on sections of formalin-fixed, paraffin-embedded liver from patients with chronic HCV using monoclonal antibodies to MMP-9 (diluted 1/400, ab76003; Abcam) and CD163 (diluted 1/300, NB110-59935; Novus Biologicals, Littleton, CO, United

States). Antigen retrieval was performed with 10 mmol Tris/1 mmol EDTA, pH 9.0 solution and sections were blocked with 20% heat-inactivated goat serum (Sigma-Aldrich) to prevent non-specific binding. Positively stained macrophages were observed using the Zeiss LSM 510 Meta confocal microscope.

Immunohistochemistry was also performed on nine biopsy specimens using an antibody directed against lipocalin-2 (LCN2) (diluted 1/35, ab23477; Abcam). Immunoreactivity was revealed using the Novolink Polymer detection system (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia) according to the manufacturer's instructions, and sections were photographed using the Nano-Zoomer (Olympus, Centre Valley, PA, United States). The sections were assessed for intensity of staining (weak, 1; moderate, 2; heavy, 3) and multiplied by the proportion of cells stained (0%-24%, 1; 25%-49%, 2; 50%-74%, 3; 75%-100%, 4) to give a value between 1 and 12.

### Identification of macrophage-secreted products

CM from M $\phi$  and THP-1 cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4%-20% polyacrylamide gels (Invitrogen) and stained with SimplyBlue<sup>™</sup> Safestain (Invitrogen). Bands differentially displayed in M $\phi$ CM ( $n = 10$ ) were precisely excised, de-stained, in-gel-trypsin-digested and analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) and database searching<sup>[26,27]</sup>. One hundred and thirty-six proteins were identified. Following pruning to eliminate proteins of incorrect molecular weight or proteins not known to be secreted, database and literature searching identified probable candidates, of which MMP-9 was further investigated.

### Western blotting analysis

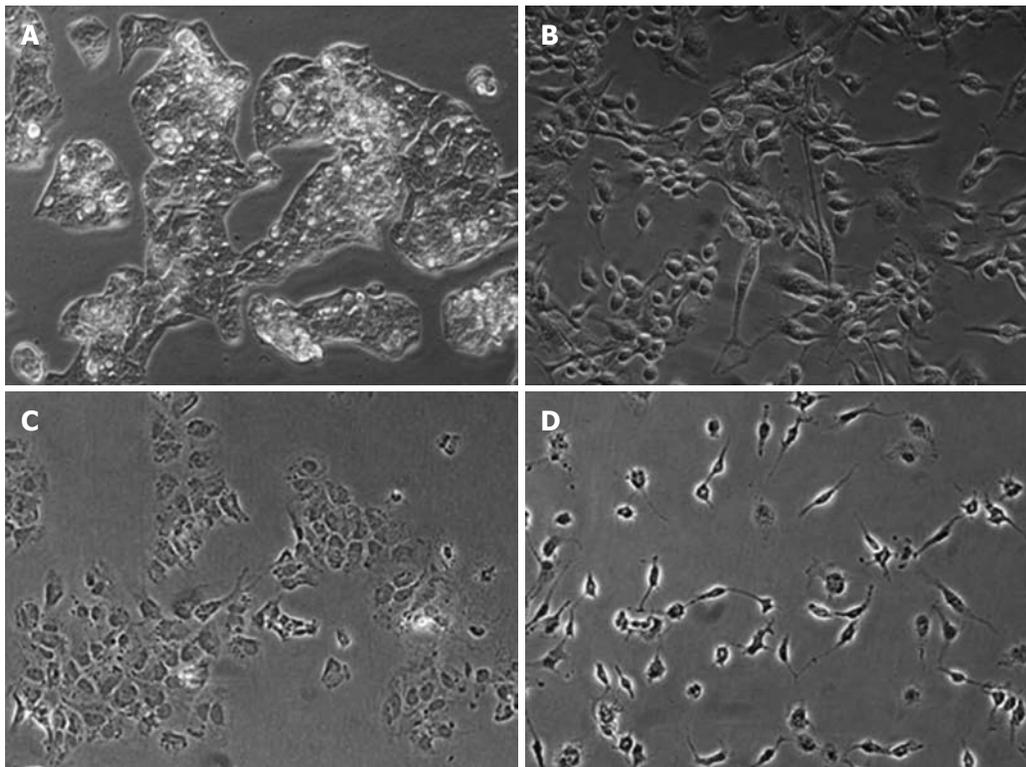
M $\phi$ CM (PMA-differentiated THP-1 cells) and MonoCM (THP-1 cells) was subjected to SDS-PAGE on 4%-20% polyacrylamide gels (Invitrogen) and transferred to Immobilon P membrane (Millipore) using standard methods. Blots were incubated overnight at 4  $^{\circ}$ C with monoclonal anti-MMP-9 (diluted 1/5000, ab76003; Abcam) followed by Envision horseradish-peroxidase-linked anti-mouse polymer (Dako Australia, Botany, NSW, Australia; dilution 1/30). Blots were washed as previously described<sup>[28]</sup>. Proteins were detected with the enhanced chemiluminescence (ECL+) system (GE Healthcare Bio-Sciences, Rydalmere, Sydney, NSW, Australia).

### Zymography

Zymography was performed on CM from M $\phi$ s (PMA-differentiated THP-1 cells) and monocytes (THP-1 cells) using Novex<sup>®</sup> 10% Zymogram (Gelatin) Gel (Invitrogen) according to the manufacturer's instructions. Gels were stained with SimplyBlue<sup>™</sup> Safestain (Invitrogen) to visualize bands of protease activity.

### Statistical analysis

Continuous normally distributed variables were represented graphically as mean  $\pm$  SEM. Grade of steatosis,



**Figure 1** Macrophage-conditioned media induces a fibroblast-like morphological change in hepatocytes. Phase contrast microscopy of HepG2 (A and B) and Huh7 cells (C and D) grown in complete media (A and C) or 50% macrophage-conditioned media in complete medium (B and D). The images shown are representative of six experiments of cells after 24 h of culture in the indicated medium.

grade of hepatic inflammation, stage of fibrosis, alcohol consumption and RIN were summarized as median and range. For normally distributed variables, analysis of variance (ANOVA) or Student's *t* test was performed to compare the means between groups. To determine differences between groups not normally distributed, medians were compared using the Kruskal-Wallis or Mann-Whitney *U* test.

Multivariate analysis was performed, including terms for age at biopsy, sex, viral genotype, stage of fibrosis, body mass index (BMI), alcohol consumption, total inflammatory score and grade of steatosis. Independent effects of normally distributed variables were assessed by ANOVA using general linear models. A backward elimination approach was used to remove non-significant variables and determine the most parsimonious model. All analyses were performed using SPSS version 17.0 (SPSS Inc, Chicago, IL, United States) and  $P < 0.05$  was considered significant.

## RESULTS

### **Macrophage-conditioned media induces transient morphological change in hepatocytes**

We began by assessing the effects of macrophage culture supernatants on hepatocyte morphology and gene expression. HepG2 and Huh7 cells typically form epithelial clusters with well-developed cell junctions (Figure 1A-C). Upon treatment with M $\phi$ CM, these hepatocytes displayed an observable change (Figure 1B-D), acquiring an elongated

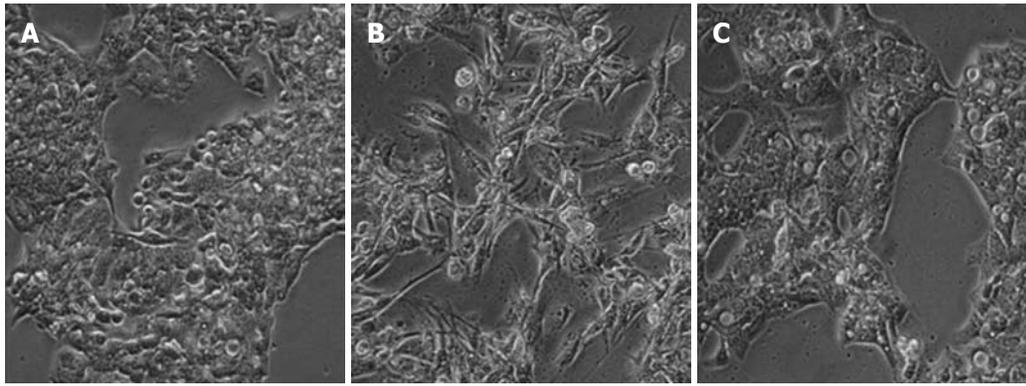
spindle shape with loss of cell-cell contact. To determine if the morphological change in hepatocytes was permanent, after the initial M $\phi$ CM treatment, HepG2 and Huh7 cells were washed and cultured in fresh growth medium for a further 2 d. Following release from treatment with M $\phi$ CM, cells that were initially elongated and spindle-shaped after 24 h of treatment, formed epithelial clusters similar to untreated hepatocytes (Figure 2).

In association with the observable morphological change, HepG2 and Huh7 cells treated with M $\phi$ CM demonstrated a substantial reduction in mRNA and protein expression of the epithelial marker E-cadherin (Figure 3A-C). mRNA and protein levels of vimentin, an intermediate filament and a marker of mesenchymal cells, were substantially enhanced in M $\phi$ CM-treated hepatocytes (Figure 3D-F). mRNA expression of the pro-fibrogenic cytokine transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) was also increased (HepG2, 2.9-fold induction,  $P < 0.001$ ; Huh7, 3.2-fold induction,  $P = 0.002$ ), whereas collagen and  $\alpha$ -smooth muscle actin (SMA) levels were not significantly altered (not shown).

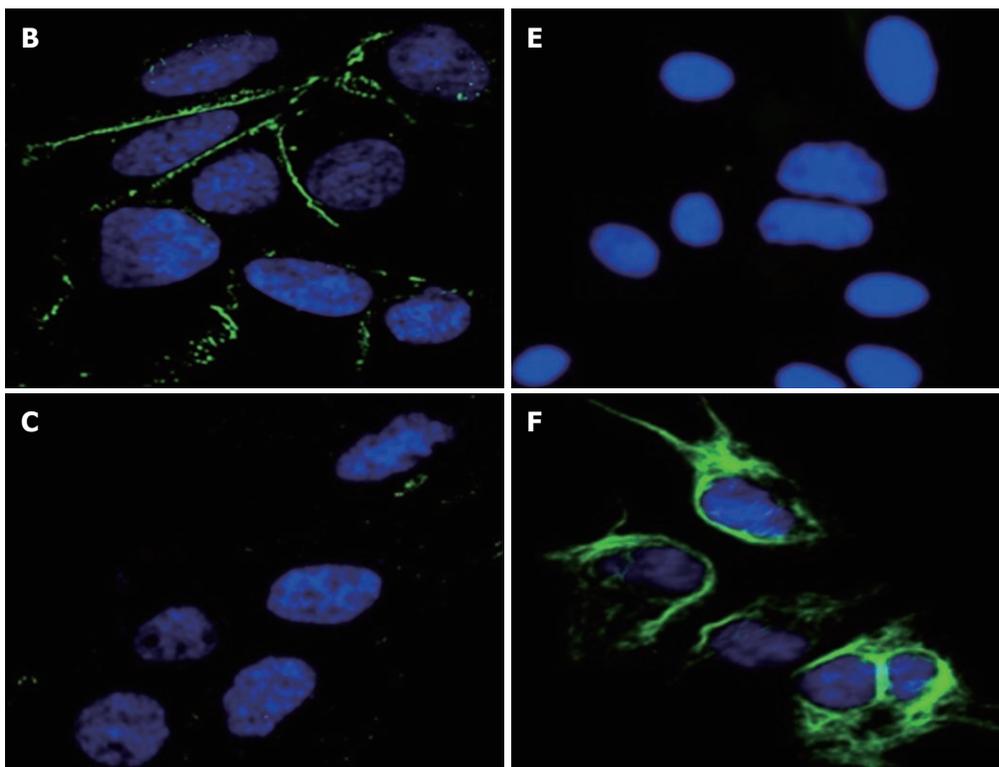
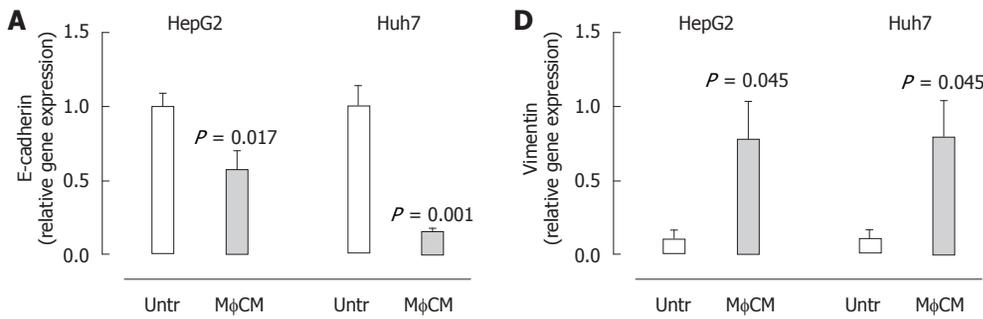
In contrast to M $\phi$ CM, no morphological changes or alteration in the levels of epithelial and mesenchymal markers were observed in hepatocytes treated with THP-1 monocyte CM (data not shown).

### **Differential gene expression in macrophage-conditioned media-treated HepG2 cells**

Our initial analysis of expression of epithelial and mesenchymal marker genes was next extended more globally



**Figure 2** Macrophage-conditioned media induces a transient change in hepatocytes. Phase contrast microscopy of HepG2 cells grown in complete media (A) for 72 h, macrophage-conditioned media (M $\phi$ CM) for 72 h (B) or M $\phi$ CM for 24 h followed by washing and culture in complete medium for a further 48 h (C). The images shown are representative of cells after 72 h of culture in the indicated medium.



**Figure 3** Expression of E-cadherin is reduced and vimentin is increased in hepatocytes treated with macrophage-conditioned media. A, D: Following culture in complete media or 50% macrophage-conditioned media (M $\phi$ CM) for 24 h, E-cadherin (A) and vimentin (D) mRNA levels in HepG2 and Huh7 cells were measured by real-time polymerase chain reaction. Results are expressed as fold of untreated cells (mean  $\pm$  SEM,  $n = 8$ ) ( $P < 0.05$  vs untreated); B, C: Immunofluorescence staining for E-cadherin (green) in untreated (B) and M $\phi$ CM-treated (C) Huh7 cells; E, F: Immunofluorescence staining for vimentin (green) in untreated (E) and M $\phi$ CM-treated (F) Huh7 cells. 4',6-diamidino-2-phenylindole stained nuclei blue (63  $\times$  magnification). Untr: Untreated.

**Table 3** Selected altered gene ontology, KEGG and BioCarta pathways

	ID	Biological process (no. genes)	Differential expression	P value <sup>a</sup>
Gene ontology	0034097	Response to cytokine stimuli (80)	↑	0.00125
	0034341	Response to interferon- $\gamma$ (11)	↑	0.00165
	0000302	Response to reactive oxygen species (73)	↑	0.00123
	0031663	LPS-mediated signalling pathway (12)	↑	0.00144
	0019882	Antigen processing and presentation (58)	↑	0.00172
	0032393	MHC class I receptor activity (15)	↑	0.00160
	0019217	Regulation of fatty acid metabolic process (48)	↓	0.00127
KEGG	hsa00071	Fatty acid metabolism (51)	↓	0.00001
	hsa00120	Bile acid biosynthesis (41)	↓	0.00001
BioCarta	h_tnfr2Pathway	TNFR2 signaling pathway (17)	↑	0.00130
	h_ctlPathway	CTL mediated immune response against target cells (15)	↑	0.00157
	h_nkcellsPathway	Ras-independent pathway in NK-cell-mediated cytotoxicity (22)	↑	0.00179
	h_cd40Pathway	CD40L signaling pathway (15)	↑	0.00221
	h_fxrPathway	FXR and LXR regulation of cholesterol metabolism (7)	↓	0.00135

<sup>a</sup>Average *P*-value < 0.005 as measured by least-squares permutation, Kolmogorov-Smirnov permutation, Efron-Tibshirani's gene set analysis test and Goe-man's global test. LPS: Lipopolysaccharide; TNFR2: Tumour necrosis factor receptor 2; NK: Natural killer; CTL: Cytotoxic lymphocyte; FXR: Farnesoid X receptor; LXR: liver X receptor.

**Table 4** The 21 most differentially expressed genes between control and macrophage-conditioned media-treated HepG2 cells as determined by microarray

Gene		Fold change	Differential expression	P valueE
LCN2	Lipocalin-2	17.8	↑	< 1E-07
TIMP1	Metallopeptidase inhibitor 1	11.8	↑	< 1E-07
UBD	Ubiquitin D	10.9	↑	< 1E-07
SERPINA3	Serpin peptidase inhibitor clade A member 3	7.5	↑	< 1E-07
IGFBP1	Insulin-like growth factor binding protein 1	7.5	↑	< 1E-07
S100A3	S100 calcium binding protein A3	6.6	↑	< 1E-07
RASD1	RAS dexamethasone-induced 1	6.4	↑	< 1E-07
CEBPD	CCAAT/enhancer binding protein delta	6.2	↑	< 1E-07
SERPINE1	Serpin peptidase inhibitor clade E member 1	5.9	↑	9.00E-07
NDRG1	N-myc downstream regulated gene 1	4.8	↑	< 1E-07
EMP3	Epithelial membrane protein 3	4.6	↑	< 1E-07
DUSP5	Dual specificity phosphatase 5	4.6	↑	< 1E-07
SOD2	Superoxide dismutase 2, mitochondrial	4.4	↑	< 1E-07
F2RL1	Coagulation factor II receptor-like 1	4.4	↑	< 1E-07
CCL20	Chemokine ligand 20	4.2	↑	< 1E-07
SDC4	Syndecan 4	4.1	↑	6.00E-07
TGM2	Transglutaminase 2	4.1	↑	< 1E-07
NR1H4	Nuclear receptor subfamily 1, group H, member 4	4.2	↓	< 1E-07
LIME1	Lck interacting transmembrane adaptor 1	4.2	↓	1.00E-07
DDC	Dopa decarboxylase	4.3	↓	< 1E-07
ANKRD38	Ankyrin repeat domain 38	5.1	↓	< 1E-07

through expression profiling. The microarray contained 48 000 probes; 34 693 of which were found to be expressed in at least one sample. Using this probe set, unsupervised hierarchical clustering showed good delineation between untreated and M $\phi$ CM-treated HepG2 cells. MonoCM-treated HepG2 cells did not cluster differently from untreated cells and were thus not included for further analysis.

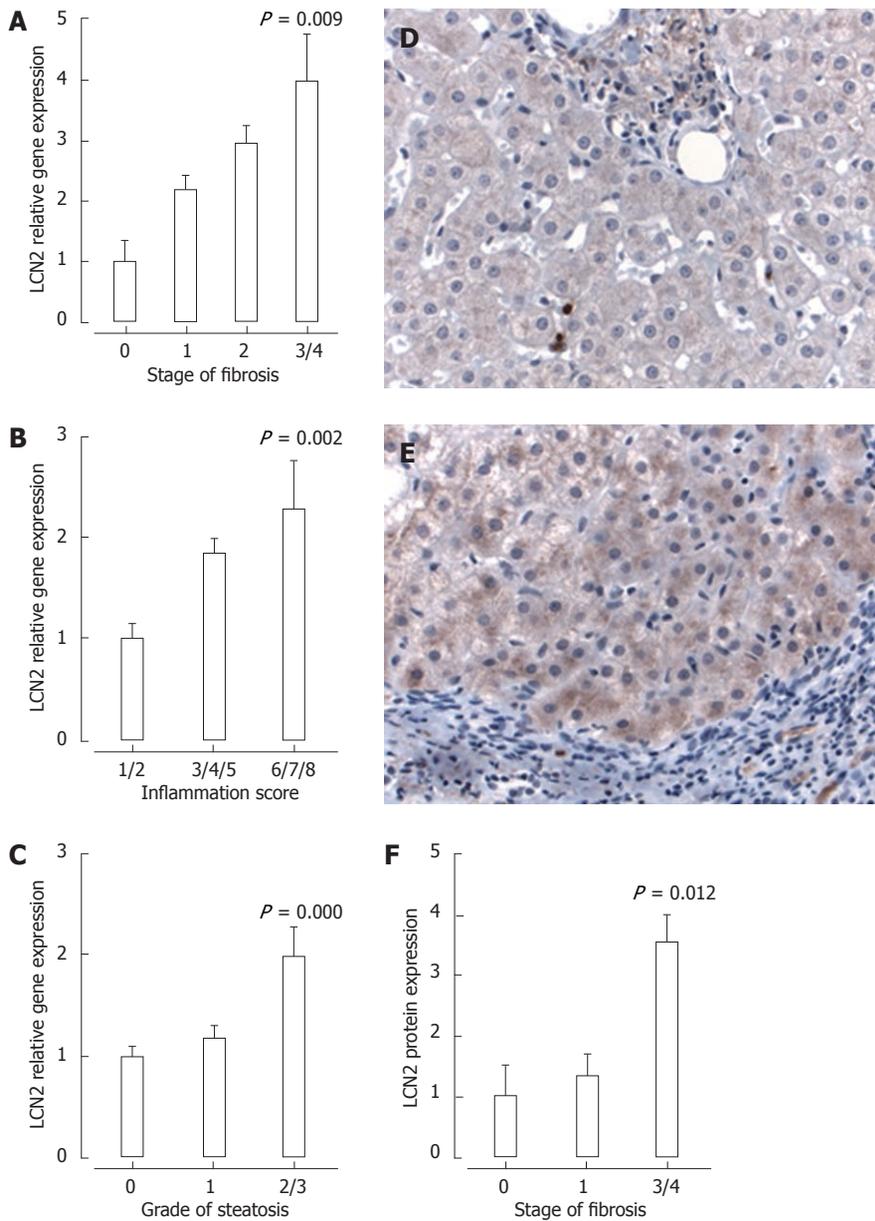
GO, KEGG and BioCarta analyses revealed significant upregulation of genes associated with inflammatory pathways; the majority of which included responses to cytokines and reactive oxygen species in addition to antigen processing and presentation (Table 3). Pathway analysis also identified a number of significantly downregulated hepatocyte functional processes including fatty acid and

cholesterol metabolism and bile acid biosynthesis (Table 3).

To identify genes regulated by M $\phi$ CM in HepG2 cells, we carried out a two-sample *t* test (with random variance model) corrected for multiple comparisons. 2665 genes were identified as significantly differentially expressed between untreated and M $\phi$ CM-treated HepG2 cells with a *P* value  $\leq$  0.005 (corrected for false discovery rate). A significant > 4-fold change in expression was seen for 21 genes (17 upregulated and four downregulated) (Table 4). The most differentially expressed gene was LCN2 with an ~18-fold change in expression.

#### **Fibrosis, inflammation and steatosis are associated with increased expression of lipocalin-2**

To validate observations from the *in vitro* studies, we ex-



**Figure 4** Lipocalin-2 mRNA expression was increased in hepatitis C virus-infected patients with increasing stage of fibrosis (A), grade of inflammation (B) or grade of steatosis (C). D and E: Lipocalin-2 (LCN2) staining by immunohistochemistry was minimal in hepatitis C virus (HCV)-infected patients with stage 1 fibrosis (D; 400× magnification) compared with stage 4 fibrosis (E; 400 × magnification); F: Increased LCN2 protein expression in HCV-infected patients with stage 3/4 fibrosis.  $P < 0.05$  vs fibrosis stage 0 (A), inflammation grade 1/2 (B) steatosis grade 0 (C) or fibrosis stage 1 (D).

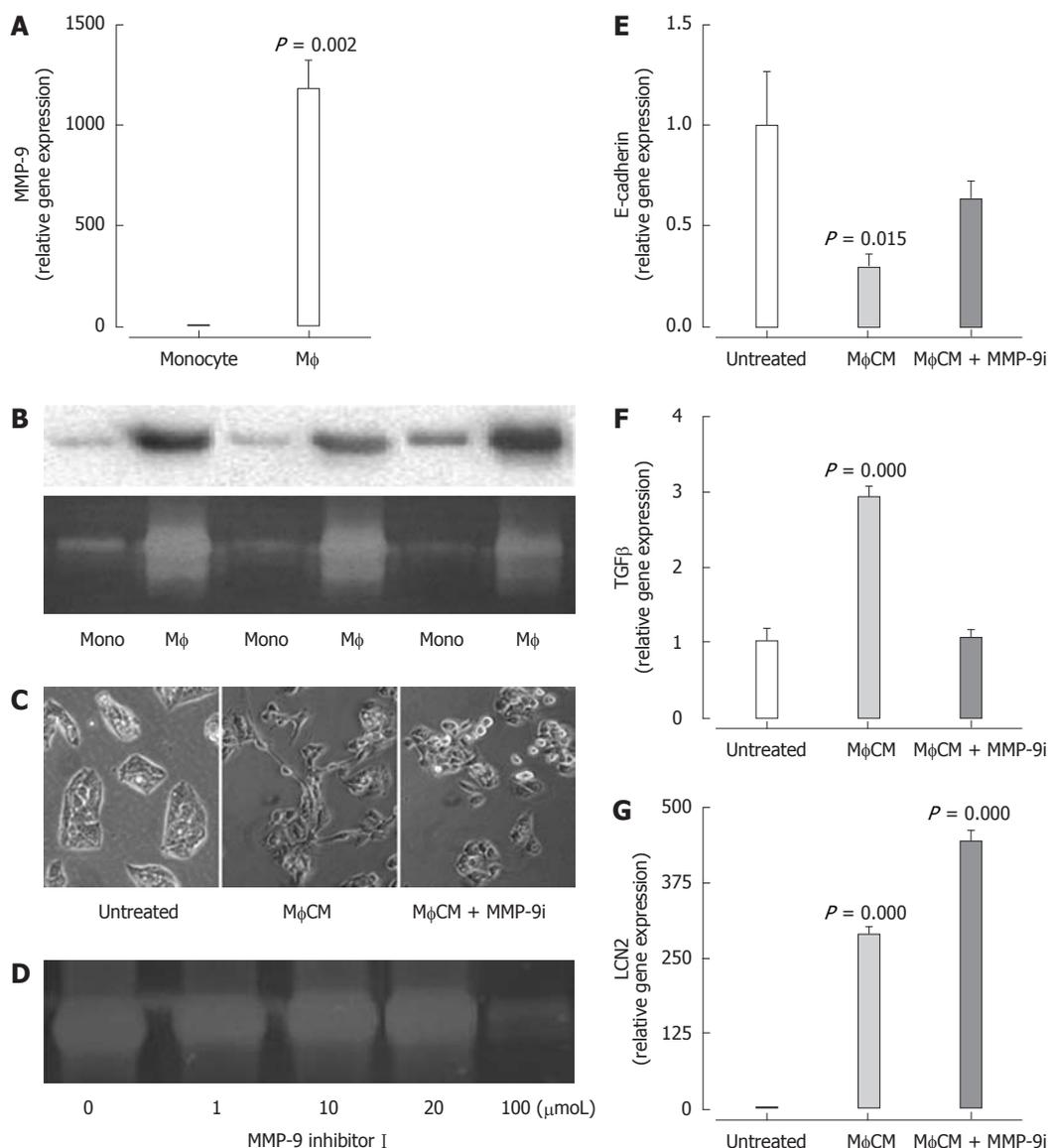
amined the expression of LCN2 in liver biopsies from patients with chronic HCV. A significant increase in hepatic mRNA levels of LCN2 was seen with increasing stage of fibrosis (Figure 4A), grade of inflammation (Figure 4B) and grade of steatosis (Figure 4C). Following multivariate analysis (correcting for BMI, age, sex, alcohol consumption, viral genotype, grade of inflammation and presence of stainable iron), stage of fibrosis (OR: 1.6, 95% CI: 1.1-2.2) and grade of steatosis (OR: 1.7, 95% CI: 1.1-2.5) remained independently associated with hepatic mRNA expression of LCN2.

Immunohistochemistry was performed on a subset of liver biopsies selected due to their varying levels of LCN2 mRNA expression. Although negligible LCN2 staining was observed in patients with minimal fibrosis,

enhanced protein expression was evident with increasing stage of fibrosis, predominantly within the hepatocyte cytoplasm, sparse sinusoidal neutrophils and some portal mononuclear cells (Figure 4D and E). To quantify LCN2 expression, tissue sections were scored for intensity of staining (1, weak; 2, moderate; 3, heavy) and multiplied by the proportion of cells stained (1, 1%-24%; 2, 25%-49%; 3, 50%-74%; 4, 75%-100%). In support of the mRNA data, enhanced LCN2 protein expression was observed in patients with stage 3-4 fibrosis (Figure 4F).

**Activated Mφs secrete matrix metalloproteinase-9 that contributes to changes in hepatocyte phenotype and function**

To address mechanisms by which macrophages may regulate hepatocyte function, we surveyed proteins present in

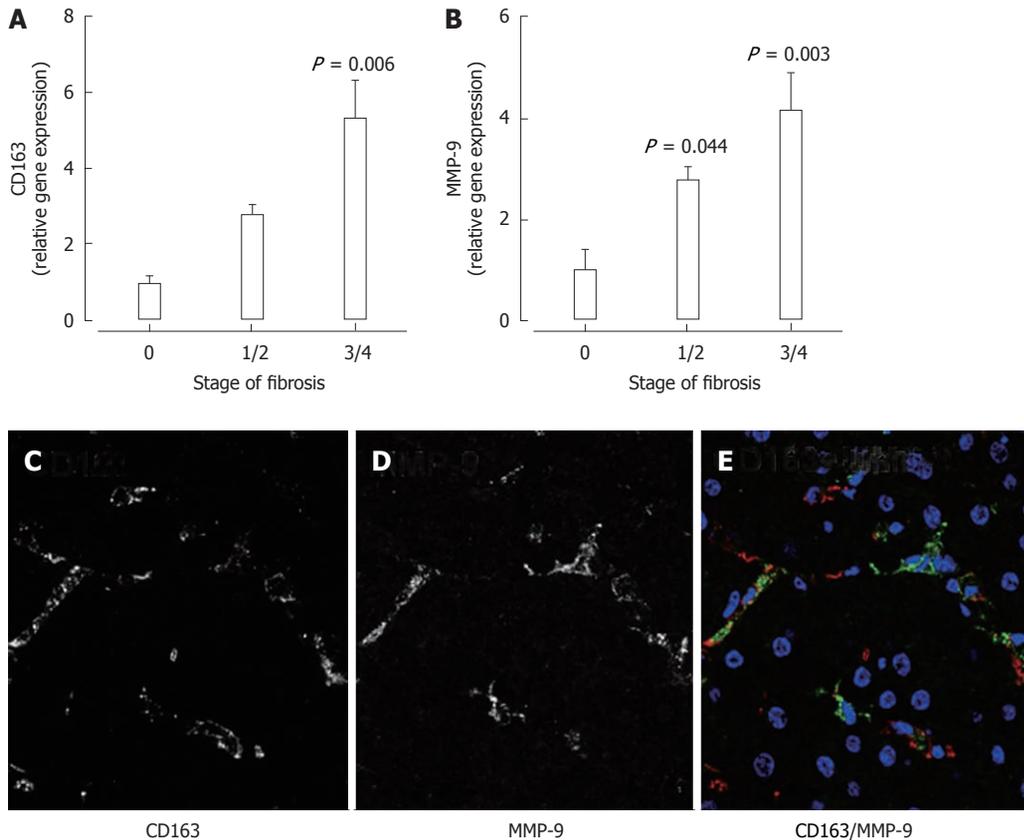


**Figure 5 Matrix metalloproteinase-9 mRNA expression is significantly increased in macrophages compared with THP-1 monocytes.** A: Results are expressed as fold of monocytes (mean  $\pm$  SEM,  $n = 6$ ) ( $P < 0.05$  vs monocytes); B: Western blotting analysis (top) and zymography (bottom) of matrix metalloproteinase (MMP)-9 in MonoCM and macrophage-conditioned media (M $\phi$ CM) from three independent experiments; C: Generation of M $\phi$ CM in the presence of MMP-9 inhibitor I (100  $\mu$ mol) prevented the M $\phi$ CM-induced morphological change in HepG2 cells; D: Zymography gel confirming MMP-9 inhibitor I reduces MMP-9 activity in M $\phi$ CM at 100  $\mu$ mol; E-G: MMP-9 Inhibitor I significantly attenuated downregulation of E-cadherin (E) and upregulation of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (F) but not lipocalin-2 (LCN2) (G) mRNA expression in response to M $\phi$ CM. Results are expressed as fold of untreated cells (mean  $\pm$  SEM,  $n = 5$ ),  $P < 0.05$  vs untreated.

M $\phi$ CM (generated in serum-free medium). HPLC/MS/MS analysis identified the presence of MMP-9 in M $\phi$ CM but not MonoCM. qPCR (Figure 5A), Western blotting and zymography (Figure 5B) confirmed the significantly enhanced levels of MMP-9 expression in M $\phi$ CM. Generation of M $\phi$ CM in the presence of MMP-9 Inhibitor I (100  $\mu$ mol) prevented the M $\phi$ CM-induced morphological change in HepG2 cells (Figure 5C). The efficacy of MMP-9 inhibitor I (100  $\mu$ mol) was confirmed by zymography (Figure 5D). This reduction in MMP-9 activity significantly attenuated the downregulation of E-cadherin (Figure 5E) and the upregulation of TGF- $\beta$ 1 (Figure 5F). In contrast, inducible LCN2 expression was not attenuated by the MMP-9 inhibitor (Figure 5G).

**Fibrosis is associated with increased expression of CD163 and matrix metalloproteinase-9**

To validate observations from the *in vitro* studies, we examined expression of MMP-9 and the macrophage marker CD163 by qPCR and immunofluorescence in liver biopsies from patients with chronic HCV. Hepatic mRNA expression of the macrophage marker CD163 (Figure 6A) as well as MMP-9 (Figure 6B) was significantly associated with increasing stage of fibrosis. Immunofluorescence in liver biopsies from patients with chronic HCV demonstrated MMP-9 expression in CD163<sup>+</sup> macrophages (Figure 6C-E). Thus, macrophage-expressed MMP-9 may contribute to hepatocyte dysfunction during chronic liver disease, but macrophages are also likely to regulate hepatocyte



**Figure 6** In patients with chronic hepatitis C, increased hepatic mRNA expression of the macrophage marker CD163 (A) as well as matrix metalloproteinase-9 (B) was significantly associated with increasing stage of fibrosis ( $P < 0.05$  vs F0). C-E: Immunofluorescence in liver biopsies from patients with chronic hepatitis C demonstrated matrix metalloproteinase (MMP)-9 expression in CD163<sup>+</sup> macrophages.

function independently of this pathway, as demonstrated by the failure of the MMP-9 inhibitor to antagonize inducible LCN2 expression.

## DISCUSSION

Macrophages are a prominent feature of chronic inflammatory liver diseases and have a pivotal role in hepatic stellate cell activation and fibrogenesis<sup>[29]</sup>. This study was undertaken to determine whether macrophages also have a proinflammatory or profibrogenic effect on other cell populations within the hepatic microenvironment, specifically hepatocytes. Indeed, macrophage-secreted products induced a morphological change in hepatocytes accompanied by an altered gene expression program associated with the production of inflammatory mediators and fibrogenic agonists. This hepatocyte phenotypic change was transient, at least *in vitro*, with prompt reversal following removal of M $\phi$ CM.

Relatively little is known about the contribution of liver epithelial cells to the local inflammatory response to injury. Studies performed more than a decade ago showed that human hepatocytes secrete a narrow repertoire of inflammatory cytokines and chemokines in response to stimulation with interleukin (IL)-1 $\beta$  or tumor necrosis factor (TNF)- $\alpha$ <sup>[30]</sup>, and primary hepatocytes isolated from rat liver produce increased IL-8 in the presence of

conditioned medium from lipopolysaccharide-stimulated Kupffer cells<sup>[31]</sup>. In a more recent study, exposure of hepatocytes to bile acids led to increased production of inflammatory mediators, including cytokines, chemokines, adhesion molecules and other proteins that may modulate immune cell accumulation and function<sup>[32]</sup>. Analogous to hepatocytes, cholangiocytes alter their phenotype in response to co-culture with macrophages, with increased secretion of cytokines involved in inflammation as well as apoptosis<sup>[14]</sup>. These studies suggest that liver epithelial cells are not simply targets of injury, but actively participate in propagating liver injury by amplifying the inflammatory response.

In the current study, hepatocytes treated with M $\phi$ CM showed downregulation of genes associated with hepatic metabolism and biosynthetic functions, such as bile acid biosynthesis, fatty acid and cholesterol metabolism. In contrast, genes associated with a number of inflammatory pathways, including the CD40L, interferon- $\gamma$  and TNF receptor 2 signaling pathways were significantly upregulated. Our data therefore suggest that macrophages both perturb normal homeostatic hepatocyte functions and promote a proinflammatory phenotype within these cells.

M $\phi$ CM was a potent inducer of LCN2. LCN2 is a small glycoprotein that is secreted by macrophages and antagonizes the actions of bacterial siderophores. It therefore plays a crucial role in innate immunity by limiting

iron availability to bacterial pathogens<sup>[33]</sup>. It also has direct effects on inflammatory cells<sup>[34]</sup>, and can facilitate mucosal regeneration<sup>[35]</sup>. Some studies have also linked LCN2 expression to chronic disease. During chronic kidney disease progression, epidermal growth factor (EGF) receptor activation leads to LCN2 expression, which subsequently mediates the mitogenic effect of EGF<sup>[36]</sup>. Circulating LCN2 levels are elevated in obese subjects<sup>[37]</sup> and patients with non-alcoholic fatty liver disease<sup>[38]</sup>, and mRNA levels of the gene are markedly upregulated in the liver of db/db obese/diabetic mice compared with their lean littermates<sup>[37]</sup>. A recent study using chronic liver injury models in the rat showed increased expression of LCN2, *via* an IL-1 $\beta$ -nuclear factor- $\kappa$ B-dependent pathway, in hepatocytes and proliferative bile duct epithelia<sup>[39]</sup>. The authors suggested that measurement of this protein may have diagnostic value as a biomarker of inflammatory liver damage. In keeping with this view, we demonstrated for the first time that LCN2 was expressed in liver biopsy samples from subjects with chronic HCV infection, and that the level of expression was significantly associated with the extent of liver injury. Furthermore, immunohistochemistry demonstrated LCN2 protein expression in hepatocytes, neutrophils and other inflammatory cells, and immunoreactivity in hepatocytes was particularly prominent in subjects with severe fibrosis. The pathophysiological function of LCN2 in liver disease remains unclear. It is not known at present whether LCN2 has an anti-inflammatory, protective role or whether it contributes to injury. Nevertheless, it appears to be a very good surrogate marker of a proinflammatory state.

Together with the induction of a proinflammatory profile, hepatocytes exposed *in vitro* to M $\phi$ CM underwent a reversible change in cell shape. This was accompanied by a decrease in the epithelial marker E-cadherin, and increases in the expression of the mesenchymal marker vimentin, and the profibrogenic cytokine TGF- $\beta$ 1. These epithelial cells did not acquire  $\alpha$ -SMA or type 1 collagen expression however, consistent with the concept that the observed morphological change is a response to injury, rather than a permanent transition into a mesenchymal cell<sup>[40]</sup>. Recent studies in human chronic liver disease have shown dual expression of epithelial and mesenchymal markers in bile duct cells and in some hepatocytes adjacent to portal tracts<sup>[16]</sup>. In addition, strong expression of TGF- $\beta$ 1 mRNA was seen in epithelial cells comprising the ductular reaction at the interface with parenchyma. In a carbon-tetrachloride-induced liver fibrosis model, abundant TGF- $\beta$ 1 protein expression was seen, not only in inflammatory cells and myofibroblasts, but also in cells with the morphology of hepatocytes immediately adjacent to the scars<sup>[41]</sup>. Similarly, in liver biopsies from patients with chronic hepatitis, TGF- $\beta$ 1 was detected in the cytoplasm of hepatocytes at the portal tract interface and in close proximity to areas of fibrosis<sup>[42]</sup>. Our study confirms expression of another secreted protein, LCN2, in hepatocytes adjacent to inflammatory/fibrotic areas in patients with chronic liver disease. Hence, accumulating data suggest that, in response to injury or inflammatory stimuli,

hepatocytes are not simply bystanders but may directly contribute to the inflammatory/fibrogenic milieu. Importantly, our *in vitro* results provide support for the plasticity of this process, with reversal of the phenotypic change following withdrawal of the inflammatory stimulus. This is an important finding from a therapeutic perspective because it implies that, in a chronic setting, it may be possible to reverse the propagation of inflammation.

The inflammatory cell or signal driving the hepatocyte inflammatory response *in vivo* remains unclear. In the current study, MMP-9 was differentially expressed in M $\phi$ CM *vs* MonoCM, and its inhibition prevented the morphological change in hepatocytes and the increase in TGF- $\beta$ 1 production (although chemical inhibitors of MMPs may also inhibit other unknown proteins and so may not be as specific as claimed). In contrast, MMP-9 inhibition led to a paradoxical increase in hepatocyte LCN2 mRNA levels. LCN2 has been shown to co-localize with MMP-9 in chronic vascular disease<sup>[43]</sup> and in the urine of patients with cirrhosis<sup>[44]</sup>, where it may modulate proteolytic activity by binding to and preventing the degradation of MMP-9<sup>[45]</sup>. Further studies are required to determine the additional factors responsible for inducing the phenotypic change in hepatocytes.

In conclusion, this study provides evidence that macrophage-secreted products can induce transient phenotypic changes in hepatocytes that may contribute to chronic inflammation and fibrogenesis. Importantly, the data suggest that hepatocytes contribute to LCN2 production during inflammatory liver injury with recruitment and activation of liver macrophage populations. In the future, strategies aimed at blocking macrophage-mediated hepatocyte phenotypic changes could be considered as potential therapeutic approaches for diseases in which liver inflammation contributes to pathology.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Activated liver macrophages have a key role in the progression of chronic liver injury and repair, and knowledge about their interaction with hepatocytes and other cells in the liver microenvironment may provide new targets for antifibrotic therapy.

### Research frontiers

Macrophages are a prominent feature of chronic inflammatory liver diseases. Secreted products from activated macrophages contribute to stellate cell activation and fibrosis. Macrophages also contribute to secretion of proinflammatory cytokines from bile duct epithelial cells, as well as apoptosis. Currently, there is limited knowledge about the effects of macrophage secreted products on hepatic epithelial cell function.

### Innovations and breakthroughs

The current study found that macrophage-secreted products induced an altered gene expression program in hepatocytes associated with the production of inflammatory mediators and fibrogenic agonists. Macrophage-conditioned medium is a potent inducer of hepatocyte lipocalin-2 (LCN2) expression. In patients with chronic hepatitis C, fibrosis, inflammation and steatosis are associated with increased expression of LCN2.

### Applications

Strategies aimed at blocking macrophage-mediated hepatocyte phenotypic changes could be considered as potential therapeutic approaches for diseases in which liver inflammation contributes to pathology.

### Terminology

LCN2 is a small glycoprotein that has been implicated in the innate immune response to bacterial infection, obesity and in the regulation of chronic inflammatory diseases. However, its pathophysiological functions in liver disease remain unclear.

### Peer review

This is a well written and implemented research paper that explores the relationship between Kupffer cells and hepatocytes in human liver disease, using a model of cell lines (the effects of THP-1 conditioned medium, with or without differentiation to macrophages, on HepG2 or Huh7 cells). The importance of the findings with regard to LCN2 was confirmed using samples from human tissues. This is an emerging area of research that has not been studied in great detail. The study is well implemented and the findings are refreshingly not over-interpreted. The only change we would suggest is a comment or two on the fact that chemical inhibitors of matrix metalloproteinases may also inhibit other unknown proteins (so called specific inhibitors tend to not always be as specific as claimed), so the findings with regard to the inhibitors need to be interpreted with caution.

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## Wnt5a participates in hepatic stellate cell activation observed by gene expression profile and functional assays

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### Abstract

**AIM:** To identify differentially expressed genes in quiescent and activated hepatic stellate cells (HSCs) and explore their functions.

**METHODS:** HSCs were isolated from the normal Sprague Dawley rats by *in situ* perfusion of collagenase and pronase and density Nycodenz gradient centrifugation. Total RNA and mRNA of quiescent HSCs, and culture-activated HSCs were extracted, quantified and reversely transcribed into cDNA. The global gene expression profile was analyzed by microarray with Affymetrix rat genechip. Differentially expressed genes were annotated with Gene Ontology (GO) and analyzed with Kyoto encyclopedia of genes and genomes (KEGG) pathway using the Database for Annotation, Visualization and Integrated Discovery. Microarray data were validated by quantitative real-time polymerase chain reaction (qRT-PCR). The function of Wnt5a on human HSCs line LX-2 was assessed with lentivirus-mediated Wnt5a RNAi. The expression of Wnt5a in fibrotic liver of a carbon tetrachloride (CCl<sub>4</sub>)-induced fibrosis rat model was also analyzed with Western blotting.

**RESULTS:** Of the 28 700 genes represented on this chip, 2566 genes displayed at least a 2-fold increase or decrease in expression at a  $P < 0.01$  level with a false discovery rate. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated HSCs. These differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing, regulation of cell growth, vasculature development and actin cytoskeleton organization. KEGG pathway analysis revealed that Wnt5a signaling pathway participated in the activation of HSCs. Wnt5a was significantly increased in culture-activated HSCs as compared with quiescent HSCs. qRT-PCR validated the microarray data. Lentivirus-mediated suppression of Wnt5a expression in activated LX-2 resulted in significantly impaired proliferation, downregulated expressions of type I collagen and transforming growth factor- $\beta$ 1. Wnt5a was upregulated in the fibrotic liver of a CCl<sub>4</sub>-induced fibrosis rat model.

**CONCLUSION:** Wnt5a is involved in the activation of HSCs, and it may serve as a novel therapeutic target in the treatment of liver fibrosis.

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**Key words:** Hepatic stellate cells; Wnt5a; Microarray; Bioinformatics analyses; Liver fibrosis

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Xiong WJ, Hu LJ, Jian YC, Wang LJ, Jiang M, Li W, He Y. Wnt5a participates in hepatic stellate cells activation observed by gene expression profile and functional assays. *World J Gastroenterol* 2012; 18(15): 1745-1752 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i15/1745.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i15.1745>

## INTRODUCTION

Liver fibrosis is a common pathological change characterized by excessive deposition of extracellular matrix (ECM) that occurs in most types of chronic liver diseases. Hepatic stellate cells (HSCs) are a major source of ECM and activation of HSCs is one of the key steps in the development of liver fibrosis<sup>[1,2]</sup>. However, the molecular mechanisms underlying the activation of HSCs are not fully understood.

Previous studies have reported the differentially expressed gene profiles in quiescent and activated HSCs with gene chip. De Minicis *et al.*<sup>[3]</sup> determined gene expression changes in three different models of HSC activation: culture-activated HSCs, HSCs isolated from CCl<sub>4</sub>-treated mice and mice underwent bile duct ligation (BDL). They found that the gene expression pattern of culture-activated HSCs was different from that of BDL- and CCl<sub>4</sub>-activated HSCs. Woo *et al.*<sup>[4]</sup> performed a time-based study to investigate the differences in gene expression patterns during the activation of HSCs with microarrays and revealed a number of newly discovered genes involved in fibrogenesis during the activation of HSCs. Sancho-Bru *et al.*<sup>[5]</sup> investigated the differences in phenotypic, genomic and functional characteristics between HSCs from human cirrhotic livers and HSCs from normal livers after prolonged culture. However, these three studies are lack of intense bioinformatics analyses.

In this study, we performed gene expression profile analyses using oligonucleotide microarrays to identify the genes involved in HSCs activation. These analyses revealed the altered expression of 2566 genes. On this basis, bioinformatics analyses were done to classify the function and explore the involved signaling pathway of these differentially expressed genes. Bioinformatics analyses revealed that these differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing, regulation of cell growth, vasculature development, and actin cytoskeleton organization. It is reported that Wnt5a signaling takes part in wound healing, regulation of cell proliferation, vasculature development and the formation of cell polarity<sup>[6-9]</sup>. After intensive analyses of Kyoto encyclopedia of genes and genomes (KEGG) pathways, we found that Wnt5a signaling pathway participated in the HSCs activation, an observation verified by quantitative real-time polymerase chain reaction (qRT-PCR).

Because the role of Wnt5a in HSCs activation is unknown and the direct evidence has not been reported, we treated human HSCs line LX-2 with lentivirus-mediated Wnt5a ShRNA and found that knockdown of Wnt5a gene expression in LX-2 led to significantly impaired proliferation, downregulated expressions of type I collagen and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). Wnt5a was upregulated in fibrotic liver of a carbon tetrachloride (CCl<sub>4</sub>)-induced fibrosis rat model. Collectively, our data suggest that Wnt5a may play a role in HSCs activation and liver fibrogenesis. We expect that our results will

help illustrate the molecular mechanisms involved in liver fibrogenesis and provide preliminary experimental evidence for the feasibility of targeting Wnt5a in gene therapy of liver fibrogenesis.

## MATERIALS AND METHODS

### Cell isolation, identification and culture

HSCs were isolated from normal Sprague Dawley (SD) rats by collagenase-pronase perfusion and subsequent density centrifugation on Nycodenz gradients as described previously<sup>[10]</sup>. Purity was tested by retinoid autofluorescence and exceeded 95% in all isolations. HSCs were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and antibiotics. After 1 (quiescent HSCs) and 7 (activated HSCs) d of culture, total RNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA, United States). Human HSCs line LX-2 was cultured in DMEM (Invitrogen) supplemented with 10% FBS.

### Microarray experiments and bioinformatics analysis

We used rat genome 430 2.0 array gene chips (Affymetrix, Santa Clara, CA). The arrays were hybridized, washed and scanned according to the standard Affymetrix protocol. The gene chip tests were performed by professional staffs of Shanghai Biochip Company. Chips were scanned with a Genechip Scanner 3000 7G (Affymetrix). Data analysis was performed using GCOS1.2 software. After background correction, we performed normalization for each array and gene. Data were filtered based on both signal intensity and detection call. Gene activity was considered to differ between quiescent HSCs and culture-activated HSCs ( $P < 0.01$ ) when compared by the unpaired Student's *t* test using multiple testing correction (Benjamini and Hochberg False Discovery Rate). Molecular function and classification of differentially expressed genes were analyzed with GO and KEGG pathway using the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7<sup>[11]</sup> (<http://david.abcc.ncifcrf.gov>).

### Quantitative real-time polymerase chain reaction

Five differentially expressed genes were selected for qRT-PCR analysis, with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a control, to verify the reliability of the array data. The primer sequences of analyzed genes are shown in Table 1. Super Script III reverse transcriptase (Invitrogen) was used for reverse transcription reactions. PCR amplification was performed using TaqMan universal Master Mix (Applied Biosystems, Foster City, CA, United States). Amplified reactions were quantified using an ABI PRISM 7900 Sequence detection system (Applied Biosystems, Foster City, CA, United States). Experiments were performed in triplicate. Relative gene quantities were obtained using the comparative Ct method after normalization to GAPDH control genes.

Table 1 Primer sequences of genes validating the microarray analysis by quantitative real-time polymerase chain reaction

Gene name	Sense primer	Antisense primer
Wnt5a	AGGGCTCCTACGAGAGTGCT	GACACCCCATGGCACTTG
Frizzled2	TCTCTGAGGACGGTTATCGCA	CAGAATCACCCACCAGATGGA
Calcium calmodulin mediated kinase II delta	TCGGCTCACACAGTACATGGA	CCCCGAACGATGAAAGTGAA
$\alpha$ -1 type I collagen	ACAGACTGGCAACCTCAAGAAG	AAGCGTGCTGTAGGTGAATCG
Connective tissue growth factor	GTTGGCGAACAAATGGCCTT	TGCCTCCA AACCAGTCATAG
Glyceraldehydes-3-phosphate dehydrogenase	TCTGCACCACCAACTGCTTAG	AGTGGCAGTGATGCCATGGACT

### Construction of lentivirus-mediated Wnt5a RNAi

Lentiviral vector pII3.7 expressing human Wnt5a ShRNA targeting 5'-GGACUUUCUCAAGGACAGA-3' (538-556) was constructed by Shanghai Biyang Biotechnology. Plasmids from correct clones were amplified by transforming into DH5 $\alpha$  *Escherichia coli* cells. We used tronolab lentiviral vector system (pRsv-REV, pMDlg-pRRE and pMD2G) to perform lentivirus-mediated Wnt5a RNAi. An additional scrambled sequence was produced as a negative siRNA control. The scrambled sequence was not homologous with any known gene in mammalian cells as determined by a Basic Local Alignment Search Tool. 293T cells were transfected with polybrene. The crude virus lysates were amplified in 293T cells to generate higher titre viral stocks. Virus particles were purified and concentrated. The lentivirus titer was determined by a limited dilution plaque assay. LX-2 transfected with lentivirus-mediated pII3.7 Wnt5a ShRNA at concentrations of MOI 10 and MOI 20 was used as study cells; cells transfected with negative siRNA were negative controls, and cells without transfection were blank controls. The effect of Wnt5a knockdown was validated with Western blotting. Blots were developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology Inc., CA, United States), exposed on Kodak Xmat blue XB-1 film and quantified by BandsScan 5.0 software using  $\beta$ -actin as internal control.

### Effect of Wnt5a RNAi on cell proliferation

Cells were spread onto 96-well flat-bottom plates at a density of  $5 \times 10^4$ /mL, 100  $\mu$ L per well, and cultured in DMEM including 5% fetal calf serum. Subgroups included: control group, negative siRNA control group and Wnt5a ShRNA group. Each group was repeated for 6 wells. The plates were incubated in a 5% CO<sub>2</sub> humidified incubator at 37 °C for 24 h and 200  $\mu$ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) was added into each well. The plates were then incubated for 4 h and the supernatants were removed. After 150  $\mu$ L DMSO was added into each well, the plates were agitated for 20 min to dissolve the crystals. Finally, the A values were measured using the enzyme-linked immunoassay at a wave-length of 450 nm.

### Effect of Wnt5a RNAi on the expression of collagen I and transforming growth factor- $\beta$ 1

Effects of Wnt5a RNAi on the expression of collagen I and TGF- $\beta$ 1 in LX-2 were analyzed with RT-PCR

*in vitro*. Experiments were performed in triplicate. The primer of  $\alpha$ -1 type I collagen (COL1A1) : sense primer: 5'-TGCCAAGGGAGATGCTGGTC-3', antisense primer: 5'-TTCACCCTTAGCACCAACAGCA-3', product size 238 bp; TGF $\beta$ 1: sense primer: 5'-TGCAAGAC-TATCGACATGGAG-3', antisense primer: 5'-ACTT-GAGCCTCAGCAGACGCA-3', product size 389 bp. The amplified products were applied to electrophoresis on 1% agarose gel analysis. The relative expression levels of COL1A1 and TGF $\beta$ 1 were assessed based on the  $\beta$ -actin control by calculating the average ratios of light density using symmetry computerized gel imaging system.

### Experimental fibrotic model

Liver fibrosis was induced by subcutaneous injection at hind leg of 40% CCl<sub>4</sub>-tea oil, 0.5 mL/100 g body weight for the first time and then 0.3 mL/100 g body weight twice a week for 8 wk. Control rats received only tea oil. The amount of CCl<sub>4</sub> administered was adjusted for body weight each week. Liver tissue samples obtained from CCl<sub>4</sub>-treated and non-treated control rats were dehydrated, embedded in paraffin, sectioned and stained with HE and Masson staining. The expression of Wnt5a was analyzed with Western blot with antibodies to Wnt5a (sc-30224, Santa Cruz, United States). The bands were semi-quantitatively evaluated by densitometric analysis. Protein expression levels of Wnt5a were thereby normalized to those of the housekeeping gene  $\beta$ -actin.

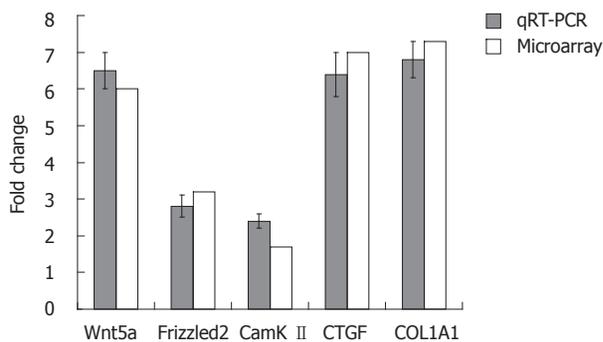
### Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Two-way analysis of variance was performed to detect the effects of Wnt5a knockdown on cell proliferation and Student *t* test was used to detect the difference between any two groups. The results were considered statistically significant at  $P < 0.05$ .

## RESULTS

### Microarray analysis and validation of microarray data

Culture-activated HSCs exhibited myofibroblast-like appearance. cRNAs, prepared from quiescent HSCs and culture-activated HSCs, were hybridized to a rat genome 430 2.0 array gene chips. Of the 28 700 genes represented therein, 2566 genes displayed at least a 2-fold increase or decrease in expression at the  $P < 0.01$  level with a false discovery rate. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated



	Wnt5a	Frizzled2	CamK II	CTGF	COL1A1
qRT-PCR	6.5	2.8	2.4	6.4	6.8
Microarray	6	3.2	1.7	7	7.3
SD	0.5	0.3	0.2	0.6	0.5

**Figure 1 Validation of microarray data by quantitative real-time polymerase chain reaction.** Comparison between expression values of five selected differently expressed genes estimated by quantitative real-time polymerase chain reaction (qRT-PCR) and gene expression data. The detected changes measured by qRT-PCR reflected different changes in gene expression between quiescent and activated hepatic stellate cells obtained by microarray analysis. SD: Sprague Dawley; CTGF: Connective tissue growth factor; CamK II: Calcium calmodulin mediated kinase II.

HSCs. As expected, genes such as COL1A1 and connective tissue growth factor (CTGF) were significantly upregulated. We were particularly interested in Wnt proteins in view of their potential role in HSCs activation. Wnt5a was upregulated by approximately 6-fold in activated HSCs in comparison with quiescent HSCs, its downstream factors Frizzled2 was upregulated by 3.2-fold and calcium calmodulin mediated kinase II (CamK II) was upregulated by 1.7-fold. Changes in gene expression profiles observed in the microarrays were validated by qRT-PCR analysis using the same total RNAs analyzed in the microarrays. We verified the upregulation of Wnt5a, Frizzled2 and CamK II as well as COL1A1, CTGF (Figure 1). This verification confirmed that all the qRT-PCR results were in agreement with the microarray data.

### Gene ontology analysis

To elucidate the relationship between gene differential expression patterns of quiescent HSCs and culture-activated HSCs, we examined the functional bias of 2566 differentially expressed transcripts according to Gene Ontology (GO) classifications. These differentially expressed transcripts were grouped into 545 GO based on biological process GO terms. The most enriched GO terms included response to wounding, wound healing and regulation of cell growth (Table 2). Analyses of GO also indicated that there were 102 GO terms identified by cellular component classification, and 90 GO terms identified by molecular function classification.

### Kyoto encyclopedia of genes and genomes pathway analysis

Analysis of KEGG pathways revealed many enrichment-related pathways including focal adhesion, ECM-receptor

interaction, regulation of actin cytoskeleton, pathways in cancer, dilated cardiomyopathy, hypertrophic cardiomyopathy, TGF- $\beta$  signaling pathway, chemokine signaling pathway, p53 signaling pathway, renal cell carcinoma, Wnt signaling pathway, leukocyte transendothelial migration, MAPK signaling pathway in HSCs activation. The top 20 significantly perturbed pathways are listed in Table 3. Of these, Wnt signaling pathway is shown in Figure 2. It revealed that Wnt5a/Frizzled2/PLC/CamK II pathway participated in the activation of HSCs.

### Effect of Wnt5a knockdown on LX-2 cells

LX-2 cells were infected with lentiviral vector encoding GFP at a multiplicity of infection (MOI) of 10, resulting in GFP expression in the majority of cultured cells. Western blot analysis showed that the protein expression levels of Wnt5a were lower in the Wnt5a ShRNA group than in the control and negative siRNA group ( $P < 0.01$ ) (Figure 3). MTT assay indicated that the proliferation of LX-2 cells was inhibited significantly after transfection with Wnt5a ShRNA as compared with the control and negative siRNA group ( $P < 0.05$ ) (Figure 4). RT-PCR showed that Wnt5a gene silencing remarkably decreased the levels of COL1A1 and TGF- $\beta$ 1 mRNA ( $P < 0.05$ , respectively) as shown in Figure 5.

### Upregulation of Wnt5a in a CCl<sub>4</sub>-induced fibrosis model

Next, we investigated whether Wnt5a was induced during liver fibrosis *in vivo*. Compared with the normal group, 8 wk after CCl<sub>4</sub> treatment, collagen deposition was increased, pseudolobules formed, and numerous inflammatory cells infiltrated the portal area. Western blotting revealed that Wnt5a was upregulated as significantly as  $\alpha$ SMA after 8 wk of CCl<sub>4</sub> treatment ( $P < 0.01$ ) (Figure 6).

## DISCUSSION

To discover novel genes involved in activation of HSCs, we compared gene expression profile between quiescent and culture activated HSCs. We showed that 2566 genes displayed at least a 2-fold increase or decrease in expression. Of these, 1396 genes were upregulated, while 1170 genes were downregulated in culture-activated HSCs. GO classification showed the most enriched GO terms included response to wounding, wound healing and regulation of cell growth. Analysis of KEGG pathways revealed that Wnt signaling pathway participates in HSCs activation. We were particularly interested in Wnt proteins in view of their potential role in HSCs activation.

The Wnt signaling pathway is a network of proteins best known for their roles in embryogenesis and cancer, but also involved in normal physiological processes in adult animals<sup>[12]</sup>. There are at least three different Wnt pathways: the canonical pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca<sup>2+</sup> pathway. In the canonical Wnt pathway, Wnt ligands bind to the receptor of frizzled family, which transduce signal to  $\beta$ -catenin causing  $\beta$ -catenin to enter the nucleus and activate the target

Table 2 Top 20 enriched Gene Ontology terms based on Gene Ontology classifications

Biological process	Count	Percent	Molecular function	Count	Percent	Cellular component	Count	Percent
Response to wounding	78	7.24	Cytoskeletal protein binding	73	6.78	Extracellular region part	125	11.61
Wound healing	46	4.27	Actin binding	52	4.83	Extracellular region	180	16.71
Regulation of cell growth	43	3.99	Growth factor binding	24	2.23	Extracellular matrix	69	6.41
Vasculature development	50	4.64	Glycosaminoglycan binding	24	2.23	Proteinaceous extracellular matrix	61	5.66
Actin cytoskeleton organization	42	3.90	Calcium ion binding	76	7.06	Extracellular matrix part	34	3.16
Actin filament-based process	43	3.99	Extracellular matrix structural constituent	14	1.30	Cytoskeleton	125	11.61
Blood vessel development	48	4.46	Heparin binding	19	1.76	Basement membrane	25	2.32
Cell migration	51	4.73	Polysaccharide binding	24	2.23	Extracellular space	71	6.59
Cell adhesion	72	6.69	Pattern binding	24	2.23	Cell projection	92	8.54
Biological adhesion	72	6.69	Insulin-like growth factor binding	11	1.02	Cytoskeletal part	88	8.17
Regulation of growth	55	5.11	Integrin binding	12	1.11	Cell surface	53	4.92
Regulation of cellular component size	46	4.27	Carbohydrate binding	39	3.62	Actin cytoskeleton	39	3.62
Extracellular matrix organization	27	2.51	Chemokine activity	10	0.93	Actin filament bundle	14	1.30
Regulation of cell proliferation	87	8.08	Chemokine receptor binding	10	0.93	Plasma membrane	216	20.06
Cytoskeleton organization	54	5.01	Protein complex binding	29	2.69	Collagen	12	1.11
Response to organic substance	109	10.1	Identical protein binding	57	5.29	Actomyosin	14	1.30
Response to hormone stimulus	72	6.69	Collagen binding	8	0.74	Stress fiber	13	1.21
Response to endogenous stimulus	77	7.15	Growth factor activity	20	1.86	Microtubule cytoskeleton	53	4.92
Cell motion	62	5.76	Platelet-derived growth factor binding	5	0.46	Cell leading edge	26	2.41
Blood vessel morphogenesis	38	3.52	Phospholipid binding	20	1.86	Vesicle lumen	15	1.39

Table 3 Signaling pathway related to activation of hepatic stellate cells

Terms	Count	Percent	P value	Fold enrichment	Benjamini
rno04510: Focal adhesion	51	4.73	6.34E-19	4.16	9.90E-17
rno04512: ECM-receptor interaction	27	2.50	9.93E-13	5.30	7.75E-11
rno04810: Regulation of actin cytoskeleton	36	3.34	5.18E-08	2.74	2.69E-06
rno05200: Pathways in cancer	40	3.71	2.74E-05	2.00	0.001066
rno05414: Dilated cardiomyopathy	18	1.67	3.27E-05	3.18	0.00102
rno05410: Hypertrophic cardiomyopathy	17	1.57	5.01E-05	3.22	0.001302
rno04350: TGF-beta signaling pathway	17	1.57	6.76E-05	3.148	0.001505
rno04540: Gap junction	15	1.39	4.27E-04	2.94	0.008302
rno04110: Cell cycle	19	1.76	7.69E-04	2.40	0.013254
rno04666: Fc gamma R-mediated phagocytosis	15	1.39	0.001011	2.71	0.015653
rno05222: Small cell lung cancer	14	1.29	0.001758	2.68	0.024645
rno04062: Chemokine signaling pathway	22	2.04	0.002121	2.04	0.027229
rno04115: p53 signaling pathway	12	1.11	0.002359	2.89	0.027945
rno05211: Renal cell carcinoma	12	1.11	0.003385	2.76	0.03708
rno04310: Wnt signaling pathway	19	1.76	0.003846	2.08	0.039281
rno04670: Leukocyte transendothelial migration	16	1.48	0.005063	2.21	0.048281
rno04010: MAPK signaling pathway	28	2.59	0.008006	1.67	0.071107
rno05219: Bladder cancer	7	0.64	0.022754	3.09	0.172201
rno04270: Vascular smooth muscle contraction	14	1.29	0.0235	1.97	0.169299
rno05212: Pancreatic cancer	10	0.92	0.027028	2.30	0.184168

genes. Wnt-Ca<sup>2+</sup> signaling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the kinase protein kinase C and CamK II and the phosphatase calcineurin<sup>[13-15]</sup>.

With KEGG pathway analyses, we found that the canonical pathway, the PCP pathway and the Wnt/Ca<sup>2+</sup> pathway participated in the activation of HSCs. In recent years, researches have shown that activation of Wnt signaling pathway was closely related with the development and progress of fibroproliferative diseases, such as pul-

monary fibrosis<sup>[16-19]</sup>, renal fibrosis<sup>[20-22]</sup>, post-infarct cardiac remodeling<sup>[23]</sup> and hypertrophic scars<sup>[24,25]</sup>, which makes it easy to think that Wnt pathway may have a definite effect on liver fibrosis. It is reported that Wnt/ $\beta$ -catenin signaling contributes to “antiadipogenic” activation of HSCs and may be used as a new target for liver fibrogenesis<sup>[26]</sup>.

The study about the role of Wnt5a in HSCs activation is still in its initial stage. Jiang<sup>[27]</sup> reported that expressions of Wnt5a and its receptor Frizzled 2 were highly upregulated in culture-activated HSCs *in vitro*. In addition,

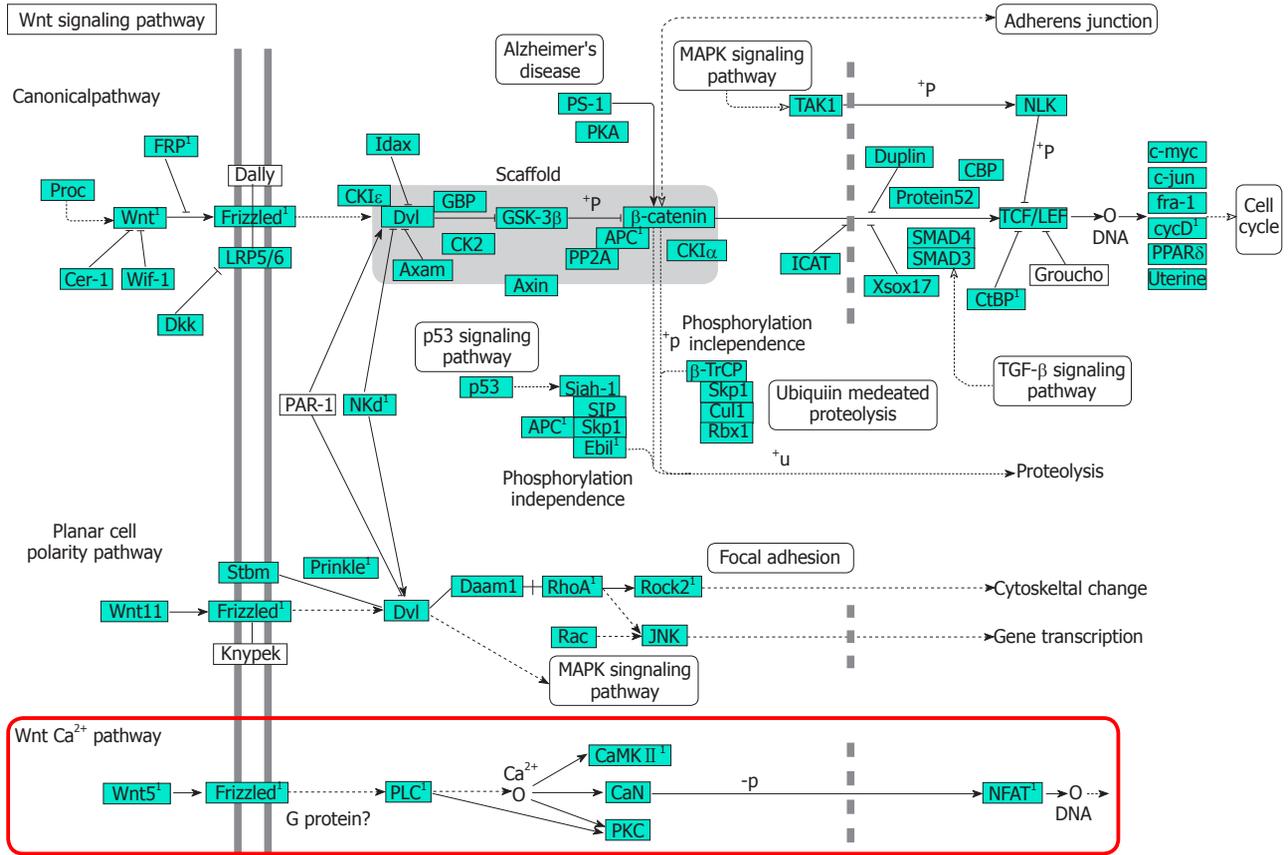


Figure 2 Wnt signaling pathway participated in the activation of hepatic stellate cells. <sup>1</sup>?? TGF-β1: Transforming growth factor-β1; LRP: GBP: APC: CBP: PKC: NFAT: PLC:

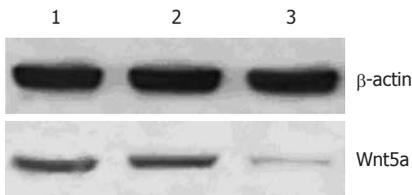


Figure 3 Expression levels of Wnt5a proteins in three groups detected by Western blotting. 1: Control; 2: Negative siRNA; 3: Wnt5a ShRNA.

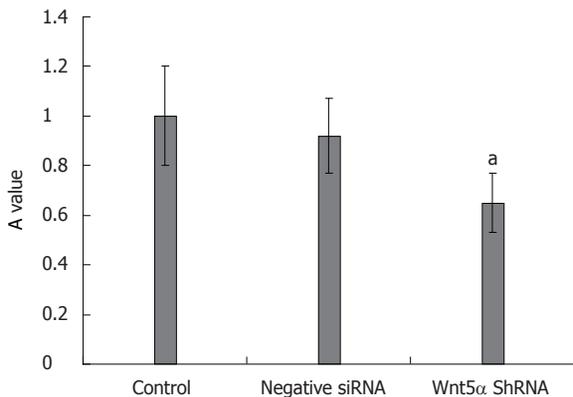


Figure 4 Knock down of Wnt5a decreases the cell proliferation. The role of Wnt5a in regulating LX-2 proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Compared with the negative siRNA and control group, Wnt5a ShRNA could inhibit the proliferation of LX-2, <sup>a</sup>P < 0.05 vs control group.

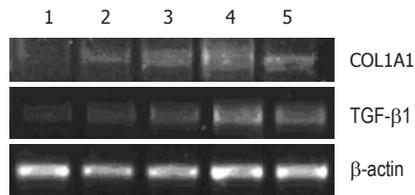


Figure 5 Knockdown of Wnt5a decreases expressions of α-1 type I collagen and transforming growth factor-β1. Experiments were repeated three times and representative results are shown. Compared with the negative siRNA, vector and control groups, the expressions of α-1 type I collagen (COL1A1) and transforming growth factor-β1 (TGF-β1) mRNA were downregulated remarkably in the Wnt5a shRNA group (P < 0.05). 1: Wnt5a ShRNA multiplicity of infection (MOI) 20; 2: Wnt5a ShRNA MOI 10; 3: Negative siRNA; 4: Vector; 5: Control.

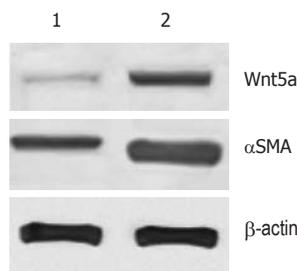


Figure 6 Expression of Wnt5a in fibrotic livers determined with Western blotting. The bands were semi-quantitatively evaluated by densitometric analysis. Wnt5a protein was upregulated as significantly as αSMA in the CCl4-induced liver fibrosis model when compared with the normal rats (P < 0.01). 1: Normal group; 2: Model group.

the expressions of Wnt5a and Frizzled2 in HSCs isolated from the mouse liver fibrosis model induced by bile duct ligation were higher as compared with HSCs isolated from normal mice. It suggests that Wnt5a signaling pathway may be involved in differentiation of quiescent HSCs into myofibroblasts. Shackel<sup>[28]</sup> reported the highly expression of Wnt5a in primary biliary cirrhosis. We found that Wnt5a was upregulated in activated HSCs in comparison with quiescent HSCs as well as its downstream factors such as Frizzled2 and CamK II. Changes in gene expression profiles observed in the microarrays were validated by qRT-PCR analysis. The direct evidence of Wnt5a on the activation of HSCs has not been reported. Because of this, we treated human HSCs line LX-2 with Wnt5a ShRNA, and found that knockdown of *Wnt5a* gene expression in LX-2 led to significantly impaired proliferation, downregulated expressions of type I collagen and TGF- $\beta$ 1. Wnt5a was upregulated in fibrotic liver of a CCl<sub>4</sub>-induced fibrosis rat model.

In summary, our data suggest that Wnt5a may play a role in HSCs activation and liver fibrogenesis, thus revealing the mechanism of HSCs activation from a new perspective, which may provide a novel means and therapeutic target for the prevention and treatment of liver fibrosis.

## COMMENTS

### Background

Hepatic stellate cells (HSCs) activation is one of the key steps in the development of liver fibrosis. However, the molecular mechanisms underlying the activation of HSCs are not fully understood. It is reported that the expression levels of Wnt5a and its downstream signaling molecules were significantly increased in activated HSCs and experimental liver fibrosis model, indicating that Wnt5a signaling pathways may be involved in liver fibrosis. But the direct evidence of Wnt5a on the activation of HSCs has not been reported. In this study, the authors aimed to identify differentially expressed genes in quiescent and activated HSCs and explore the role of Wnt5a in the activation of HSCs.

### Research frontiers

Chip technology is rapid and concise in detecting mRNA expression. Previous studies have reported the differentially expressed gene profiles between quiescent and activated HSCs with gene chip. But, intense bioinformatics analyses and functional assays are deficient. No study has provided the direct evidence of Wnt5a in the mechanism of HSCs activation.

### Innovations and breakthroughs

Bioinformatic analysis of the differentially expressed gene profiles between quiescent and activated HSCs reveals the role of Wnt5a signaling pathway in HSCs activation. This is the first study addressing the role of Wnt5a in the mechanism of HSCs activation.

### Applications

This study suggests that Wnt5a participates in the HSCs activation and liver fibrogenesis, which may provide a novel means and therapeutic target for the prevention and treatment of liver fibrosis.

### Terminology

Wnt signaling molecules are involved in diverse developmental and pathological processes. Based on the different intracellular events activated after Wnt-Frizzled binding, Wnt signaling is divided into two subclasses: the canonical pathway and the non-canonical pathway. Wnt5a is a non-canonical member of the Wnt family. In the Wnt5a signaling pathway, Wnt5a and Frizzled binding triggers the intra-cytoplasmic release of calcium. Increased intracellular calcium concentration activates calcineurin and calcium calmodulin mediated kinase II. Wnt5a signaling takes part in wound healing, regulation of cell proliferation, vasculature development and the formation of cell polarity.

### Peer review

The research is the in vitro experiments showing that Wnt5a ShRNA decreases proliferation, downregulates expression of collagen type I and transforming growth factor beta, that probes into a direct Wnt5a participation in the mechanism of HSC activation.

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## Pro-apoptotic effects of tectorigenin on human hepatocellular carcinoma HepG2 cells

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### Abstract

**AIM:** To investigate the effects of tectorigenin on human hepatocellular carcinoma (HCC) HepG2 cells.

**METHODS:** Tectorigenin, one of the main components of rhizome of *Iris tectorum*, was prepared by simple methods, such as extraction, filtration, concentration, precipitation and recrystallization. HepG2 cells were incubated with tectorigenin at different concentrations, and their viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Apoptosis was detected by morphological observation of nuclear change, agarose gel electrophoresis of DNA ladder, and flow cytometry with Hoechst 33342, Annexin V-EGFP and propidium iodide staining. Generation of reactive oxygen species was quantified using DCFH-DA. Intracellular  $Ca^{2+}$  was monitored by Fura 2-AM. Mitochondrial membrane potential was monitored using Rhodamine 123. Release of cytochrome c from mitochondria to cytosol was detected by Western blotting. Activities of caspase-3, -8 and -9 were investigated by Caspase Activity Assay Kit.

**RESULTS:** The viability of HepG2 cells treated by tectorigenin decreased in a concentration- and time-dependent manner. The concentration that reduced the number of viable HepG2 cells by 50% ( $IC_{50}$ ) after 12, 24 and 48 h of incubation was 35.72 mg/L, 21.19 mg/L and 11.06 mg/L, respectively. However, treatment with tectorigenin at 20 mg/L resulted in a very slight cytotoxicity to L02 cells after incubation for 12, 24 or 48 h. Tectorigenin at a concentration of 20 mg/L greatly inhibited the viability of HepG2 cells and induced the condensation of chromatin and fragmentation of nuclei. Tectorigenin induced apoptosis of HepG2 cells in a time- and dose-dependent manner. Compared with the viability rate, induction of apoptosis was the main mechanism of the anti-proliferation effect of tectorigenin in HepG2 cells. Furthermore, tectorigenin-induced apoptosis of HepG2 cells was associated with the generation of reactive oxygen species, increased intracellular  $[Ca^{2+}]_i$ , loss of mitochondrial membrane potential, translocation of cytochrome c, and activation of caspase-9 and -3.

**CONCLUSION:** Tectorigenin induces apoptosis of HepG2 cells mainly *via* mitochondrial-mediated pathway, and produces a slight cytotoxicity to L02 cells.

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**Key words:** Tectorigenin; *Iris tectorum* maxim; Apop-

tosis; Hepatocellular carcinoma; HepG2; Mitochondria; Liver cancer

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Jiang CP, Ding H, Shi DH, Wang YR, Li EG, Wu JH. Pro-apoptotic effects of tectorigenin on human hepatocellular carcinoma HepG2 cells. *World J Gastroenterol* 2012; 18(15): 1753-1764 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i15/1753.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i15.1753>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide, with 600 000 deaths per year<sup>[1]</sup>. It often develops in patients with chronic liver diseases associated with hepatitis B virus or hepatitis C virus infections<sup>[1]</sup>. Although surgical techniques have been improved and several non-surgical treatment modalities have been developed, there is still no effective therapy for significant improvement of extremely poor prognosis of HCC patients<sup>[2]</sup>. Therefore, efforts have been made to search for mechanism-based agents, such as sorafenib<sup>[3]</sup>, chelidonine<sup>[4]</sup>, 5-allyl-7-gendifluoromethylenechrysin<sup>[5]</sup>, troglitazone<sup>[6]</sup>, chaga mushroom extract<sup>[7]</sup> and curcumin<sup>[8]</sup>, for treatment of HCC. Sorafenib is the first substance that proved to significantly prolong the survival of HCC patients. And multikinase inhibitor has shown to have anti-proliferative and anti-angiogenic properties<sup>[3]</sup>.

Apoptosis is a physiological process leading to cell deletion and regulates the balance between cell proliferation and death. The hallmark of cancer cells is the dysregulation of cell proliferation and apoptosis. The tumor growth depends on the cell proliferation rate and apoptosis. Therefore, induction of apoptosis of tumor cells has become a strategy in cancer treatment<sup>[5-7]</sup>. The integration of multiple survival and death signals determines whether a cell survives or undergoes apoptosis. In recent years, mitochondrial pathways and death receptor pathways have been identified as the two major mechanisms for induction of apoptosis<sup>[9,10]</sup>.

*Iris tectorum* (*I. tectorum*), a traditional Chinese medicine, has been widely used for treating liver-related diseases, including hepatitis, liver fibrosis, liver cirrhosis and liver cancer. As one of the main ingredients of *I. tectorum* rhizome<sup>[11]</sup>, tectorigenin has been reported to have *in vivo* and *in vitro* anti-angiogenic activities<sup>[12]</sup>. A previous study also showed that tectorigenin possessed anti-tumor activities in mice implanted with murine Lewis

lung carcinoma or bearing sarcoma 180<sup>[12]</sup>. Our previous studies revealed that tectorigenin possessed anti-proliferative and pro-apoptotic effects on hepatic stellate cells (HSCs)<sup>[13]</sup>. However, until now there has been no report about the anti-tumor effect of tectorigenin on human HCC HepG2 cells and the associated mechanisms. Considering that tectorigenin is one of the main components in rhizome of *I. tectorum* which had been used for the treatment of liver cancer for centuries, we hypothesized that tectorigenin may have anti-proliferation and pro-apoptosis effects on human HCC HepG2 cells. The aim of the present study was to examine whether tectorigenin could suppress the proliferation of HepG2 cells and induce apoptosis of HepG2 cells.

## MATERIALS AND METHODS

### Reagents

All reagents and solvents were purchased from commercial suppliers and were used without further purification. Roswell Park Memorial Institute (RPMI)-1640 medium was purchased from HyClone (Hyclone, UT, United States). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Annexin V-EGFP was purchased from PharMingen (San Diego, CA, United States). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst H33258, DCFH-DA, ethidium bromide (EB), Proteinase K, RNase A and Rhodamine 123 were purchased from Sigma Aldrich Co. (St. Louis, MO, United States). Fura 2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). The kits used for caspase activity assays were obtained from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). All other chemicals were of analytic grade.

### Plant materials

The rhizomes of *I. tectorum* were collected at Dafeng, Jiangsu Province, China. The voucher specimen (the registration number NJU-603) was identified by Prof. Gong ZN, Nanjing Normal University and deposited at the herbarium of Nanjing University, Nanjing, Jiangsu Province, China.

### Preparation of tectorigenin

The rhizomes (400 g) of *I. tectorum*, after ground on an electrical grinder, were extracted twice with 2000 mL of 80% ethanol under reflux for 3 h. The filtrate of the obtained 80% ethanol extract was condensed in vacuum to afford a dark-brown residue called Crude-Extract (73.1 g). The Crude-Extract was dissolved in 4000 mL 3% Na<sub>2</sub>CO<sub>3</sub> solution at 85 °C, and then the solution was filtrated. The pH of the filtrate was regulated to 3-4 by adding 18% HCl, and then the crude isoflavone part was precipitated from the acid liquor. The formed crude isoflavone (23.2 g) was collected by filtration and dissolved in 2000 mL 5% NaHCO<sub>3</sub> solution at 85 °C. The 5%

NaHCO<sub>3</sub> solution was extracted with 2000 mL EtOAc twice. The EtOAc extract was condensed in vacuum to afford a light-brown residue (11.5 g). The residue was washed by 2000 mL hot distilled water and 2000 mL ligarine successively, and sequentially dried at 50 °C. The dried residue (7.3 g) was recrystallized in ethanol twice to afford pure compound (4.45 g).

### Cell culture and treatment

Human liver carcinoma HepG2 cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). These cells were grown in RPMI 1640 medium with 10% FBS in the presence of 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>. L02 cells (a human hepatocyte cell line), purchased from Xiangya Central Experiment Laboratory, Central South University, China, were cultured in Dulbecco's Modified Eagle Medium (Gibco, NY, United States) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS, in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub> at 37 °C. Tectorigenin was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL as a stock solution and diluted to the required concentration with fresh medium immediately before use. The final DMSO concentration in cultures was < 0.1% (v/v), which did not influence cell growth when compared with the vehicle-free controls. Cells grown in the media containing an equivalent amount of DMSO without tectorigenin served as control.

### Cell viability assay

Cell viability was assessed by MTT method. Briefly, cells were seeded in 96-well plate at a density of  $1 \times 10^4$  cells/well. After 24 h incubation, tectorigenin at different concentrations was added to the cells while only DMSO (solvent) was added as a negative control. After growing for 12, 24 and 48 h, cells were incubated with MTT (0.5 mg/mL) for 4 h at 37 °C. During this incubation period, water-insoluble formazan crystals were formed, which were dissolved by the addition of 100 µL/well DMSO. The optical densities (*A*) at 570 nm were measured using an enzyme-linked immunosorbent assay plate reader. Wells containing culture medium and MTT but no cells acted as blanks. The percentage of cell viability was calculated as follows:  $A_{\text{drug-blank}}/A_{\text{control-blank}} \times 100\%$ .

### Morphological observation of nuclear change

Cell morphological assay was carried out according to Wu *et al.*<sup>[13]</sup> with minor modification. In this assay, cells were seeded in 24-well plates and treated with tectorigenin or vehicle (control). After 48 h incubation, cells were washed carefully with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min at room temperature, then washed with pre-chilled PBS three times and exposed to 5 µg/mL Hoechst 33258 at room temperature in the dark for 20 min. Samples were observed under a fluorescent microscope (Nikon UFX-II, Japan). Cells showing cytoplasmic and nuclear shrink-

age, chromatin condensation or fragmentation, were defined as apoptotic cells.

### Determination of DNA fragmentation

The integrity of DNA was assessed by agarose gel electrophoresis. Cells were harvested after 48 h incubation with 20 µg/mL tectorigenin by centrifugation (300 × *g*, 5 min) and lysed in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.1% of Triton X-100. Then cells were incubated with RNase A at 37 °C for 60 min and proteinase K at 50 °C for 120 min, respectively. After centrifugation (2000 × *g*, 10 min), supernatants were transferred to new tubes and precipitated by the addition of 0.5 volume of 7.5 mol/L ammonium acetate and 2.5 volumes of ethanol overnight. After centrifugation at 600 × *g* for 5 min, the pellets were dissolved in Tris-HCl EDTA buffer (TE buffer) (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) and loaded on 1.5% agarose gel for electrophoresis. The gel was stained with EB and photographed with ultraviolet illumination.

### Assessment of apoptosis

Apoptosis could be determined by staining cells with Annexin V-EGFP and propidium iodide labeling. In this study, apoptosis was assessed according to Hai *et al.*<sup>[14]</sup>. Briefly, cells were harvested after having exposed to the indicated concentrations of tectorigenin for 24 or 48 h, washed twice with cold PBS and then resuspended in 1 mL binding buffer (10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub>) at a concentration of  $1 \times 10^6$  cells/mL. The cells were incubated with 5 µL Annexin V-EGFP (300 mg/L) for 10 min, and then 10 µL of 20 mg/L PI for 30 min in the dark. Cell fluorescence was measured on FACScan flow cytometer (Becton Dickinson) using an argon ion laser (488 nm).

### Measurement of reactive oxygen species generation

The cellular reactive oxygen species (ROS) was quantified using DCFH-DA according to Shi *et al.*<sup>[15]</sup>. Cells were seeded in black 96-well plates and incubated with tectorigenin at indicated concentrations for 1, 3, 6 or 24 h. Then cells were washed with PBS twice and incubated with 100 µmol/L DCFH-DA in the loading medium in 5% CO<sub>2</sub>/95% air at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed with PBS and DCFH-DA-loaded cells were read in a Safire (Tecan, Crailsheim, Germany) fluorescence plate reader (excitation, 485 ± 12 nm; emission, 530 ± 12 nm). The fold increase in fluorescence per well was calculated by the formula  $[F_t/F_0]$ , where *F*<sub>0</sub> is the fluorescence without tectorigenin treatment and *F*<sub>t</sub> is the fluorescence with the tectorigenin treatment at the indicated concentration.

### Measurement of intracellular Ca<sup>2+</sup>

Intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) was monitored using the fluorescent Ca<sup>2+</sup>-sensitive dye, Fura 2-acetoxymethyl ester (Fura 2-AM)<sup>[15]</sup>. Cells were seeded in black 96-well plates

and incubated with tectorigenin at the indicated concentration for 3, 6 or 24 h. The cells were washed with PBS twice and incubated with 1  $\mu\text{mol/L}$  Fura 2-AM which was dissolved in HEPES buffer saline (20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L  $\text{CaCl}_2$ , 0.8 mmol/L  $\text{MgCl}_2$ , 13.8 mmol/L glucose, pH 7.4) for 30 min in the dark at 37 °C in a humidified incubator. They were then gently rinsed with HEPES buffer saline twice and incubated with HEPES buffer saline for 60 min at 37 °C in a humidified incubator. The fluorescence was measured at an emission wavelength of 510 nm and an excitation wavelength of 340 and 380 nm on a Safire (Tecan, Crailsheim, Germany) fluorescence plate reader. The ratio of fluorescence intensity of 340-380 nm (F340/F380) was used to estimate intracellular free calcium.

#### Measurement of mitochondrial membrane potential

As an index to determine mitochondrial dysfunction, mitochondrial membrane potential (MMP) was monitored using Rhodamine 123<sup>[16]</sup>. Cells were treated with tectorigenin at indicated concentration for 3, 6 or 24 h in a 6-well culture plate at  $1 \times 10^5$  cells/mL. The medium was then removed and washed three times with serum-free RPMI 1640 medium followed by incubation in fresh serum-free medium containing 3 mg/L Rhodamine 123 at 37 °C in dark for 30 min. Finally, the cells were collected and washed twice with PBS and then analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States).

#### Western blotting

HepG2 cells were seeded into 60-mm dishes ( $1 \times 10^6$  cells/dish). On the next day, after treated for 48 h with tectorigenin at 0, 5, 10 and 20 mg/L, respectively, HepG2 cells were harvested, resuspended in an ice-cold lysis buffer containing 50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 5 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.5% Triton X-100, and protease inhibitor cocktail tablets (Roche, IN), incubated for 10 min on ice, disrupted in a micro-ultrasonic cell disrupter for 10 s and centrifuged at  $750 \times g$  for 15 min at 4 °C. The supernatant (cytosolic fraction) was removed and maintained at -80 °C. The pellet containing mitochondria was resolved in a lysis buffer. Protein level was measured using a standard colorimetric assay kit (BCA kit). Proteins were separated by polyacrylamide/SDS gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Roche, IN). The membranes were probed with antibody (cytochrome c diluted at 1:1000, Cell Signaling Technology, MA, United States) overnight at 4 °C, and incubated with a Horseradish peroxidase (HRP)-coupled secondary antibody (HRP; 1:5000, Cell Signaling Technology, MA, United States). Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, United Kingdom).  $\beta$ -actin (1:200, Boster, Wuhan, China) as a loading control. Results were quantified with a scan-

ning densitometer (Bio-Rad, United States).

#### Caspase-3, -8 and -9 activity assay

Caspases activities were measured using Caspase Activity Assay Kit (Beyotime, C1115, C1151 and C1157) according to the manufacturer's instructions. Briefly, cultured HepG2 cells ( $5 \times 10^6$ ) were washed with cold PBS twice, resuspended in lysis buffer and left on ice for 20 min. The lysate was centrifuged at  $16\,000 \times g$  at 4 °C for 3 min. Supernatants were collected and protein concentrations were measured with a BCA kit. Caspase-3, -8 and -9 activities were measured by reaction buffer (containing DTT) and caspase substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively. The release of p-nitroanilide (pNA) was qualified by determining the absorbance with Tecan SUNRISE at 405 nm. The fold increase in absorbance was calculated by the formula  $[\text{OD}_i/\text{OD}_0]$ , where  $\text{OD}_0$  is the absorbance without tectorigenin treatment and  $\text{OD}_i$  is the absorbance with tectorigenin treatment at the indicated concentration.

#### Statistical analysis

All data were expressed as mean  $\pm$  SD. Origin Pro 7.0 statistical package was used to determine statistical significance. Difference between two groups was analyzed by two-tailed Student's *t* test, and difference among three or more groups was analyzed by one-way analysis of variance multiple comparisons.  $P < 0.05$  was considered statistically significant.

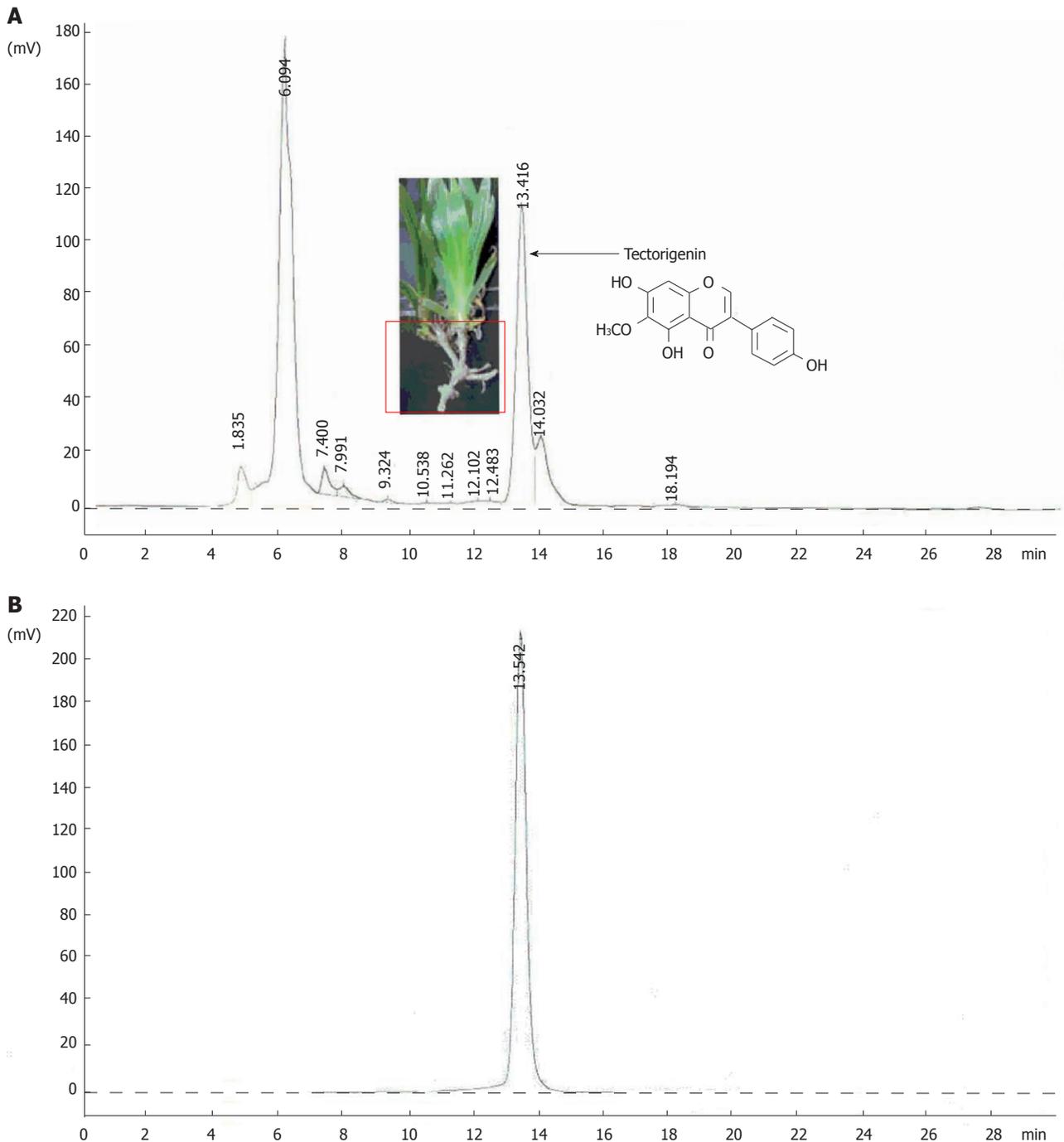
## RESULTS

#### Preparation of tectorigenin

As illustrated in Figure 1A, the high performance liquid chromatography (HPLC) profile for Crude-Extract revealed that tectorigenin is one of the main components of the rhizomes of *I. tectorum*. The compound was identified as tectorigenin (CAS registry number: 548-77-6) by chemical and spectral approaches. The structure is shown in Figure 1A. The purity of tectorigenin was above 98% (HPLC analysis, Figure 1B).

#### Effect of tectorigenin on viability of HepG2 and L02 cells

The effect of different concentrations of tectorigenin on the viability of HepG2 cells for 12, 24 and 48 h was assessed by the MTT assay. The number of viable HepG2 cells treated by tectorigenin decreased in a concentration- and time-dependent manner (Figure 2A). When HepG2 cells were treated with tectorigenin at 5, 10 and 20 mg/L for 24 h, the viability rate was 91%, 79% and 62%, respectively (Figure 2A). Whereas when HepG2 cells were treated with tectorigenin at 5, 10 and 20 mg/L for 48 h, the viability rate was reduced to 83%, 57% and 33%, respectively (Figure 2A). The concentration that reduced the number of viable HepG2 cells by 50% ( $\text{IC}_{50}$ ) after 12h, 24 h and 48h of incubation was 35.72, 21.19 and 11.06 mg/L, respectively. However, treatment with tectorigenin at 20 mg/L resulted in a very slight cytotoxicity to



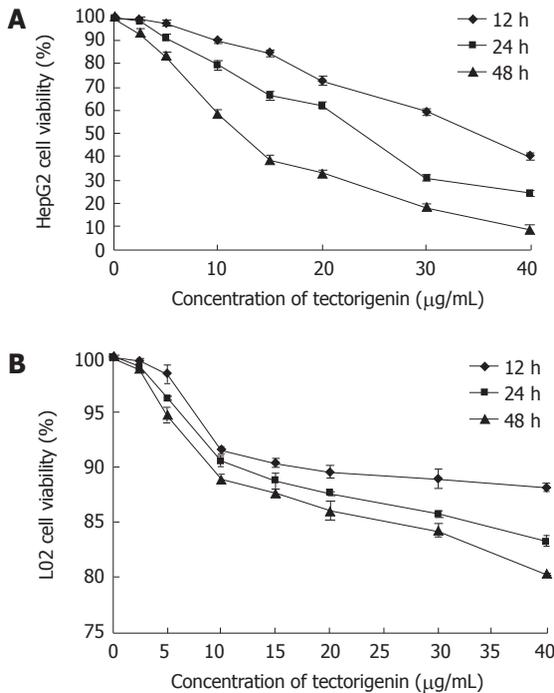
**Figure 1** The high-performance liquid chromatography profiles of the aqueous ethanol (80%) extract of *Iris tectorum* rhizomes (A) and tectorigenin (B). High-performance liquid chromatography analysis was accomplished at 25 °C on an instrument consisting of L-7110 (Hitachi) pump, L-7420 (Hitachi) ultraviolet detector (set at 254 nm) and Alltech Apollo C18 (4.6 mm × 250 mm) column using MeOH:EtOAc:H<sub>2</sub>O (60:1:40) mixture as a mobile phase at a flow rate of 1.0 mL/min. Sample injection: 10 μL of extract (2.5 g/L) or tectorigenin (100 mg/L) in MeOH.

L02 cells after incubation for 12, 24 or 48 h (Figure 2B).

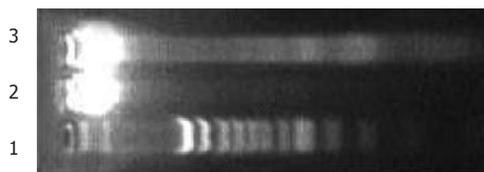
**Morphological changes of HepG2 cells after exposure to tectorigenin**

To verify tectorigenin-induced apoptosis of HepG2 cells, we observed the changes in cell morphology after tectorigenin exposure by Hoechst 33258 staining. As shown in Figure 3, regular and round-shaped nuclei were observed in the control HSC-T6 cells (Figure 3A). After

treated with 5, 10 and 20 mg/L (Figure 3B-D) tectorigenin for 48 h, the blue emission light became much brighter in apoptotic cells than in control cells. Condensed chromatin could also be found in many tectorigenin-treated cells and the structure of apoptotic bodies was formed in some cells, which is one of the classic characteristics of apoptotic cells. Moreover, after treated with 20 mg/L tectorigenin, the nuclei of HSC-T6 cells were further condensed with the number of apoptotic



**Figure 2** Effects of tectorigenin on the viability of HepG2 (A) and L02 cells (B). Cells were treated for 12, 24 and 48 h with tectorigenin at 2.5, 5, 10, 15, 20, 30 and 40 mg/L, respectively, followed by assessing the cell viability relative to that of untreated cells (control). Bars represent mean  $\pm$  SD.



**Figure 4** DNA fragmentation of HepG2 cells induced by tectorigenin. Cells were treated with tectorigenin at 20 mg/L for 48 h, then total DNA was isolated as described in Methods and analyzed by electrophoresis for detection of DNA fragmentation. 1: Marker; 2: Control; 3: Tectorigenin.

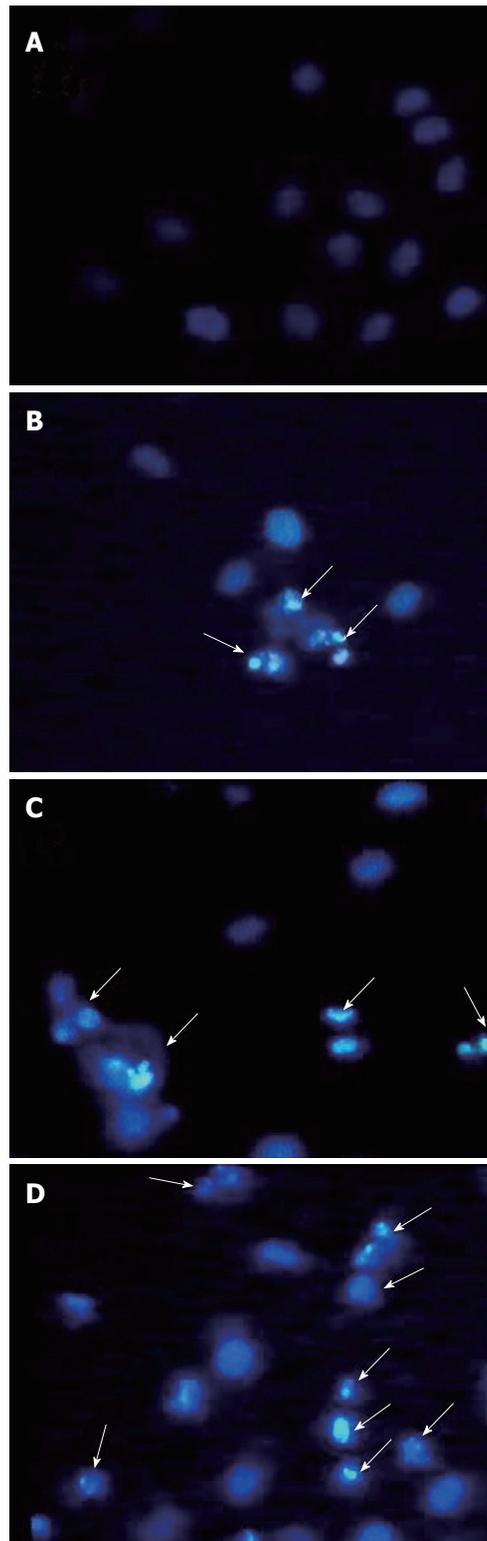
bodies sharply increased.

**DNA ladder of HepG2 cells treated with tectorigenin**

Genomic DNA was prepared from HepG2 cells that had been incubated in the presence or absence of 20 mg/L tectorigenin for 48 h. The integrity of the DNA was assessed by agarose gel electrophoresis. DNA isolated from HepG2 cells cultured with 20 mg/L tectorigenin for 48 h showed a “ladder” pattern of apoptosis (Figure 4). A comparison with molecular weight markers indicated that the fragments were multiples of approximately 180-200 base pairs.

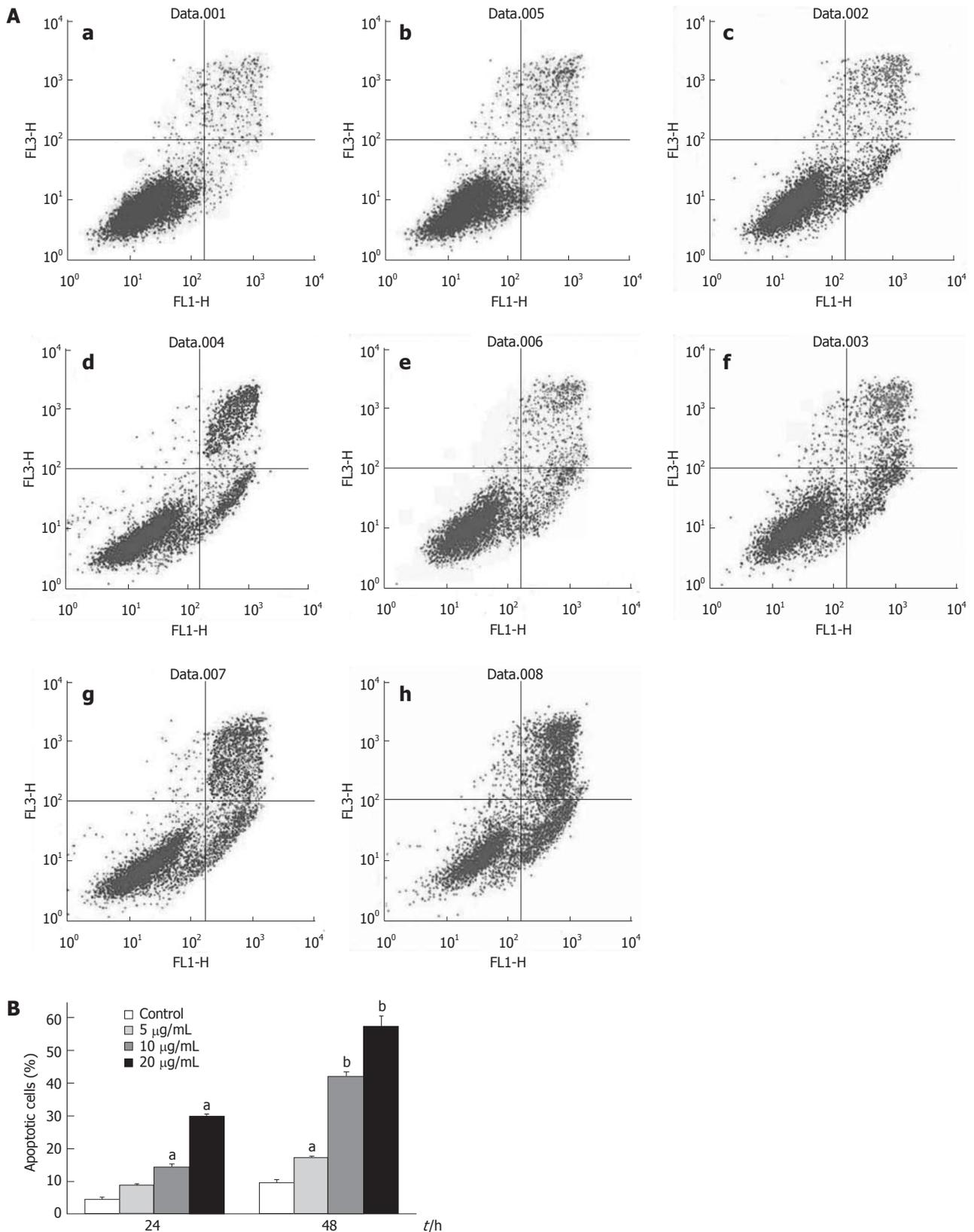
**Apoptotic rate of HepG2 cells treated with tectorigenin**

Treatment of HepG2 cells with tectorigenin resulted in significant increase of apoptotic cells in a time- and dose-dependent manner compared with that of non-treated cells quantified by Annexin V analysis (Figure 5B), as shown in Figure 5A. Apoptotic rate of HepG2 cells treated with 5, 10 and 20 mg/L tectorigenin for 24

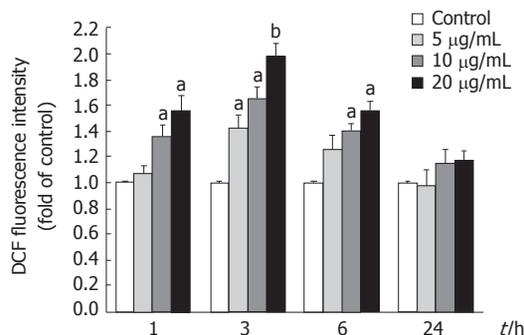


**Figure 3** Morphological changes of HepG2 cells after exposure to tectorigenin. HepG2 cells were treated with vehicle (A) and tectorigenin at 5 (B), 10 (C), 20 (D) mg/L for 48 h, the cells were then fixed and stained with Hoechst 33258 and observed under a fluorescent microscope (Leica DM IRB) (Magnification  $\times$  400). Tectorigenin significantly induced condensed chromatin and fragmented nuclei. The arrows in B-D indicate cells undergoing apoptosis.

h was 8%, 14%, and 30%, respectively. However, when HepG2 cells were treated with 5, 10 and 20 mg/L tectorigenin for 48 h, its apoptotic rate was 16%, 42% and



**Figure 5** Flow cytometric analysis of apoptosis in HepG2 cells treated with tectorigenin. HepG2 cells were incubated for 24 and 48 h with tectorigenin at 0, 5, 10, 20 mg/L, respectively. And then the cells were stained with EGFP-conjugated Annexin V and propidium iodide (PI). The EGFP and PI fluorescence was measured using flow cytometer with FL1 and FL3 filters, respectively. A: Representative dot plots of Annexin V/PI staining. a: Control, 24 h; b: 5 mg/L, 24 h; c: 10 mg/L, 24 h; d: 20 mg/L, 24 h; e: Control, 48h; f: 5 mg/L, 48 h; g: 10 mg/L, 48 h; h: 20 mg/L, 48 h. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the early apoptotic (Annexin V+/PI-) population and upper right quadrant contains the late apoptotic/necrotic (Annexin V+/PI+) population; B: Data pooled from three independent experiments show the percentage of apoptotic cells. Difference was considered statistically significant when <sup>a</sup>P < 0.05 and <sup>b</sup>P < 0.01 vs control.



**Figure 6 Stimulation by tectorigenin of reactive oxygen species generation in HepG2 cells.** Reactive oxygen species was measured using an oxidation-sensitive fluorescent probe, DCFH-DA. Cells were treated with tectorigenin for 1, 3, 6 and 24 h, and then incubated for 30 min with DCFH-DA at 37 °C. After washing with phosphate buffered saline, the fluorescence intensity of the cells was analyzed with a fluorescence plate reader. Data are expressed as the mean of three individual experiments  $\pm$  SD. Difference was considered statistically significant when  $^aP < 0.05$  and  $^bP < 0.01$  vs control. DCF: Dichlorodihydrofluorescein.

58%, respectively. Compared with the viability rate in Figure 2, these results suggested that induction of apoptosis was the main mechanism of the anti-proliferative effect of tectorigenin in HepG2 cells.

#### Intracellular accumulation of ROS induced by tectorigenin

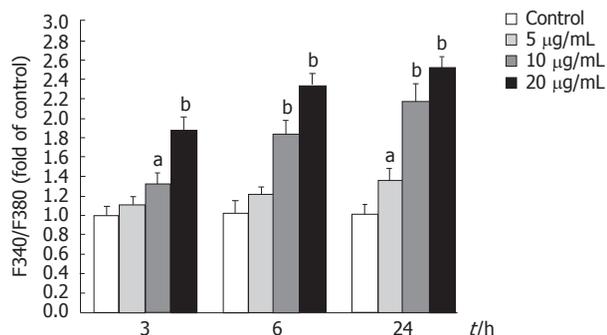
To determine whether tectorigenin could induce intracellular ROS generation, levels of ROS production in HepG2 cells were determined using the fluorescence probe DCFH-DA. As shown in Figure 6, HepG2 cells exposed to tectorigenin at 10 and 20 mg/L for 1 or 6 h displayed a significant increase in the intracellular level of ROS as compared with that in the control cells. When HepG2 cells were treated with tectorigenin at 5, 10 and 20 mg/L for 3 h, they displayed a maximal increase in the intracellular level of ROS. However, HepG2 cells incubated with tectorigenin at 5, 10 and 20 mg/L for 24 h, showed a mild but not significant increase in the intracellular level of ROS compared with that in the control cells.

#### Tectorigenin-induced increase of $[Ca^{2+}]_i$ in HepG2 cells

To determine whether tectorigenin influences the level of intracellular  $Ca^{2+}$ , the level of intracellular  $Ca^{2+}$  was measured with Fura 2-AM staining. As shown in Figure 7, treatment of HepG2 cells with tectorigenin at the concentrations of 5, 10 and 20 mg/L for 3 h increased the fluorescence ratio (F340/F380) by  $1.11 \pm 0.09$ ,  $1.32 \pm 0.11$  and  $1.86 \pm 0.13$  fold as compared with the control. When HepG2 cells were treated with tectorigenin for 6 and 24 h, they displayed a higher increase of the fluorescence ratio (F340/F380) in a dose- and time-dependent manner (Figure 7). Moreover, when the incubation time was prolonged from 3 h to 24 h with the incubation concentration of 20 mg/L, the F340/F380 value increased from  $1.86 \pm 0.13$  to  $2.52 \pm 0.17$ .

#### Tectorigenin-induced loss of MMP in HepG2 cells

To assess the effect of tectorigenin on the changes of



**Figure 7 Effects of tectorigenin on the increase of intracellular  $Ca^{2+}$  in HepG2 cells.** Cells were treated with tectorigenin at 5, 10 or 20 mg/L for different time points, and then loaded with Fura 2-acetoxymethyl ester. Intracellular  $Ca^{2+}$  was measured by fluorescence analysis. The data were presented as mean  $\pm$  SD ( $n = 6$ ). Difference was considered statistically significant when  $^aP < 0.05$  and  $^bP < 0.01$  vs control.

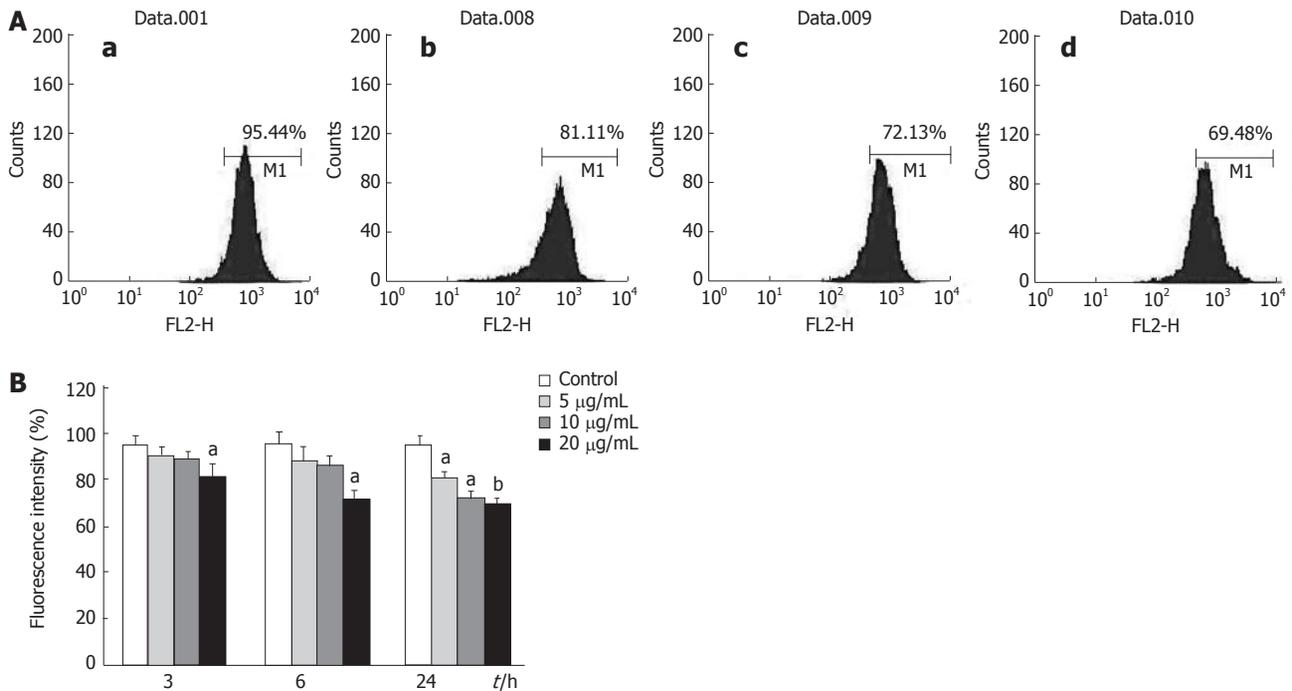
MMP in HepG2 cells, flow cytometric analysis was carried out to detect the fluorescence intensity of Rhodamine 123. As shown in Figure 8B, treatment of HepG2 cells with tectorigenin at the concentrations of 5 and 10 mg/L for 3 h and 6 h caused a moderate depolarization of MMP. However, HepG2 cells treated with tectorigenin at the concentrations of 5, 10 and 20 mg/L for 24 h (Figure 8A and B), as well as for 3 and 6h, displayed a remarkable depolarization of MMP corresponding to a much lower fluorescence intensity compared with the control, suggesting the collapse of the inner mitochondrial membrane and mitochondrial dysfunction. Compared with the control cells (without treatment of tectorigenin), HepG2 cells treated with 5, 10 and 20 mg/L tectorigenin for 24 h decreased the MMP from  $95.39\% \pm 5.47\%$  to  $81.39\% \pm 4.28\%$ ,  $72.55\% \pm 3.97\%$  and  $69.45\% \pm 3.28\%$ , respectively.

#### Effect of tectorigenin on cytochrome c release in HepG2 cells

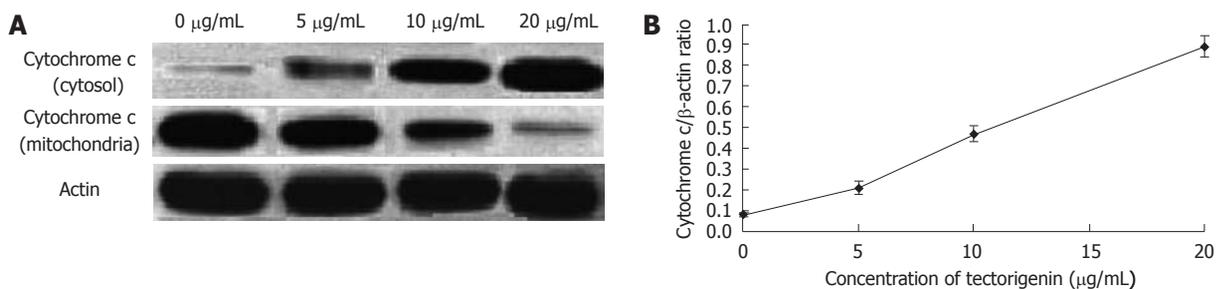
The cytosolic and mitochondrial levels of cytochrome c were measured to confirm apoptosis *via* the mitochondrial pathway in tectorigenin-treated HepG2 cells. The results indicated that mitochondrial cytochrome c was released into the cytosol in a dose-dependent manner (Figure 9A). Compared with control cells (without treatment of tectorigenin), HepG2 cells treated with tectorigenin at 5, 10 and 20 mg/L for 48 h showed an increase in levels (cytochrome c/ $\beta$ -actin ratio) of cytosolic cytochrome c from  $0.08 \pm 0.007$  to  $0.21 \pm 0.03$ ,  $0.47 \pm 0.04$  and  $0.89 \pm 0.05$ , respectively (Figure 9B).

#### Effect of tectorigenin on caspase-3, -8 and -9 activities in HepG2 cells

The apoptotic process included the activation of cysteine proteases, which represent both initiators and executors of cell death. Tectorigenin treatment caused a significant time- and dose-dependent increase in caspase-3 and -8 proteolytic activity in HepG2 cells (Figure 10A and C). However, HepG2 cells treated with tectorigenin displayed



**Figure 8** Effects of tectorigenin on integrity of mitochondrial membrane in HepG2 cells. Cells were treated with tectorigenin for different time points, then incubated with Rhodamine 123 and analyzed by flow cytometry. A: HepG2 cells were treated with 0 (a), 5 (b), 10 (c) and 20 (d) mg/L tectorigenin for 24 h. Mitochondrial membrane potential was measured by Rh123, a fluorescent probe, on FACSCalibur at FL2 channel with an excitation wavelength of 488 nm. Each test was performed 3 times and images presented were typical of 3 independent experiments; B: The data were presented as mean ± SD (n = 3). Difference was considered statistically significant when <sup>a</sup>P < 0.05 and <sup>b</sup>P < 0.01 vs control.



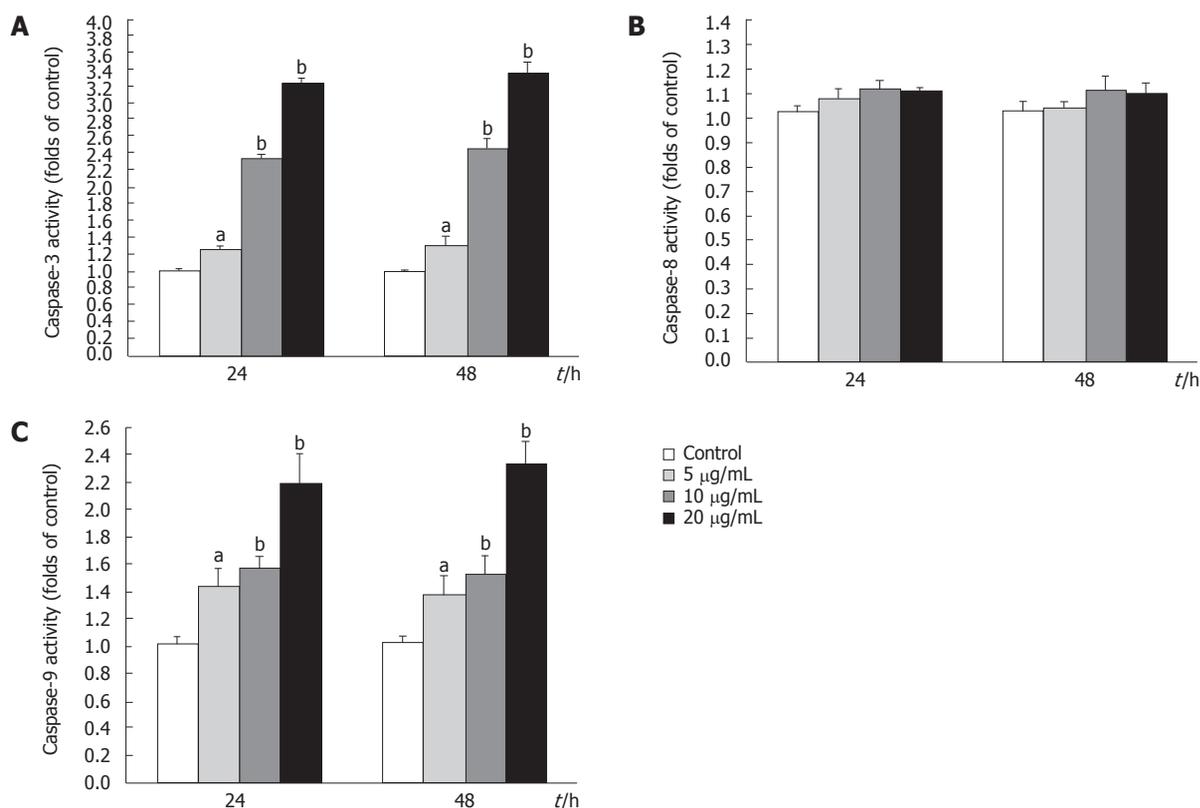
**Figure 9** Western blotting analysis. After 48-h exposure to tectorigenin at 0, 5, 10 and 20 mg/L, respectively, levels of cytochrome c in HepG2 cells (A), along with those of cytochrome c (B) in cytosol, were evaluated. Protein (50 μg) from each sample was resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and β-actin was used as a loading control.

a very slight increase in caspase-8 activity (Figure 10B).

## DISCUSSION

*I. tectorum* is a kind of widely planted flower, which has been used in traditional Chinese medicine as a folk proven prescription for curing liver-related illnesses. By chemical analysis, we found that tectorigenin is one of the main components in the rhizome of *I. tectorum* (Figure 1) and another main component was tectoridin<sup>[11,17]</sup>. However, it has been reported that tectorigenin could be transformed from tectoridin by intestinal microflora<sup>[17]</sup>. Therefore, we hypothesized that the main active ingredient in the rhizome of *I. tectorum* might be tectorigenin. In fact, tectorigenin has been reported to have *in vivo* hepatoprotective<sup>[18]</sup>, estrogenic<sup>[19]</sup> and anti-inflammation

activities<sup>[20]</sup>, and pro-apoptotic effect on hepatic stellate cells<sup>[13]</sup>. Previous studies also have demonstrated a role for tectorigenin in regulating prostate cancer cell number by inhibiting proliferation through cell cycle regulation<sup>[21]</sup>. Tectorigenin also has pro-apoptotic effects and decreases tissue invasion by up-regulation of tissue inhibitor of metalloproteinase-3 in prostate cancer<sup>[22]</sup>. Therefore, its hepatoprotective activity and pro-apoptotic effect on hepatic stellate cells made us believe that tectorigenin is the substance base of the utilization of *I. tectorum* rhizome in the treatment of liver-related diseases. Considering that tectorigenin is one of the main components in the rhizome of *I. tectorum* (Figure 1) and *I. tectorum* has been used as a folk proven prescription for the treatment of liver cancer, we supposed that tectorigenin could be used for the treatment of HCC. The present study revealed



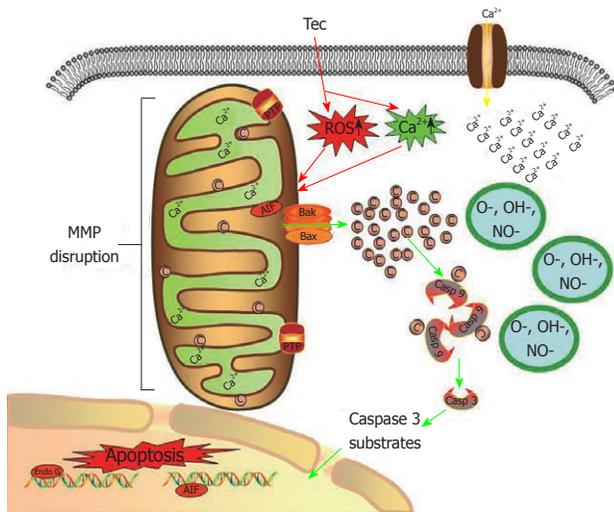
**Figure 10** Caspase activities in tectorigenin-treated HepG2 cells. Cells were treated with tectorigenin at the indicated concentrations for 24 and 48 h. Cytosolic extracts were prepared and assayed for caspase activities as described in "Materials and Methods". A: Alteration of caspase-3 activity in tectorigenin-induced HepG2 cells for indicated time; B: Effect of tectorigenin on caspase-8 activity in HepG2 cells; C: Changes of caspase-9 activity in tectorigenin-treated HepG2 cells at 5, 10 and 20 mg/L for 24 and 48 h. Data are expressed as the mean of three individual experiments  $\pm$  SD. Difference was considered statistically significant when  $^aP < 0.05$  and  $^bP < 0.01$  vs control.

that tectorigenin possessed considerable pro-apoptotic effects on human HCC HepG2 cells and reinforced our hypothesis that tectorigenin is the substance base of the utilization of *I. tectorum* rhizome in the treatment of liver cancer. Base on the results in the present study, we will further investigate the *in vivo* anticancer effects of tectorigenin. After 48 h of incubation,  $IC_{50}$  of tectorigenin against HepG2 cells was 11.06 mg/L and treatment with tectorigenin at 10 mg/L resulted in about 10% cell death of the normal L02 cells (Figure 2). The dosage of tectorigenin 10-15 mg/kg body weight is large enough to induce *in vivo* apoptosis of cancer cells.

In this study, compared with the viability rate (Figure 2), the high apoptotic cell percentage (Figure 5) suggested that induction of apoptosis was the main mechanism of the anti-proliferative effect of tectorigenin in HepG2 cells. The upstream apoptotic mechanism of tectorigenin in HepG2 cells was investigated based on the intracellular accumulation of ROS. A previous study has found that mitochondria are the major organelle where ROS was generated and excessive ROS could lead to lipid peroxidation, oxidation of proteins and DNA damage<sup>[23]</sup>. Theoretically, as a consequence of excessive ROS generation in cells, mitochondrial dysfunction should occur<sup>[24]</sup>. The intracellular ROS peaked when HepG2 cells were treated with different concentrations of tectorigenin for 3 h, then declined and when treated for 24 h it

declined to the approximately normal level (Figure 6). As expected, significant dissipation of MMP (Figure 8) was observed in the current study, indicative of mitochondrial dysfunction. Excessive ROS could raise the  $Ca^{2+}$  concentration in the cytoplasm<sup>[25,26]</sup>. Marked elevation in  $Ca^{2+}$  then causes the new ROS formation<sup>[27-29]</sup>. On the other hand, the increased  $Ca^{2+}$  may also impair mitochondrial function<sup>[29-31]</sup>, leading to a significant decrease in MMP. In this study, tectorigenin could significantly increase the  $[Ca^{2+}]_i$  in HepG2 cells.

In normal situation, cytochrome *c* resides in the mitochondrial intermembrane and serves as a transducer of electrons in the respiratory chain. However, the increment of ROS and  $[Ca^{2+}]_i$ , and subsequent mitochondrial dysfunction result in cytochrome *c* release<sup>[32]</sup>. In the present study, release of cytochrome *c* from mitochondria to cytosol was detected (Figure 9). After release from mitochondria, cytochrome *c* could bind with Apaf-1 and participate in the activation of caspase-9 (the initiator). The activated caspase-9 then activates caspase-3 (the effector). Initiator caspases and effector caspases act together to augment the death signal and finally lead to apoptosis<sup>[31-36]</sup>. In this study, we detected enhanced caspase-3 and -9 activities in tectorigenin-treated HepG2 cells (Figure 10A and C), which validated mitochondria-mediated apoptosis pathway. Similarly, in the study of Zhou *et al*<sup>[6]</sup>, troglitazone inhibited growth and induced



**Figure 11** Possible mechanisms by which tectorigenin induces apoptosis in human hepatocellular carcinoma HepG2 cells. Tectorigenin produces reactive oxygen species and increases  $Ca^{2+}$  concentration in cytoplasm of HepG2 cells, leading to the depletion of mitochondrial membrane potential and release of cytochrome c from mitochondria. Cytochrome c facilitates apoptosis of HepG2 cells by activating caspase cascade. Tec: Tectorigenin; ROS: Reactive oxygen species; C: Cytochrome c; Casp: Caspase; AIF: Apoptosis inducing factor; MMP: Mitochondrial membrane potential.

apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Caspase-8 is an initiating caspase, which modulates the death receptor-dependent pathway. We found very mild increase in caspase-8 activity in tectorigenin-treated HepG2 cells (Figure 10B), which suggested that a death receptor-mediated pathway might be excluded in the apoptosis of HepG2 cells induced by tectorigenin. In the study of Tsagarakis *et al.*<sup>[37]</sup>,  $10^{-8}$  mol/L octreotide could significantly inhibit the proliferation of HepG2 cells and significantly increase caspase-3, caspase-8 and caspase-2 activities. In this study, tectorigenin increased the ROS production and  $Ca^{2+}$  concentration in cytoplasm of HepG2 cells, the intracellular accumulation of ROS and  $Ca^{2+}$  further induced the loss of MMP. The disruption of MMP caused release of cytochrome c from mitochondria to cytosol. Cytosolic cytochrome c activated the pro-caspase-9 and subsequently, caspase-9 activated the downstream effector caspases-3, eventually triggered apoptosis of HepG2 cells (Figure 11). In the study of Tan *et al.*<sup>[5]</sup>, 5-allyl-7-gen-difluoromethylenechrysin was found to exert its apoptotic effect by activation of Peroxisome proliferator-activated receptor gamma, down-regulation of nuclear factor kappa B and Bcl-2 protein expression, up-regulation of Bax protein expression, and reduction of the ratio of Bcl-2 to Bax.

Our previous research showed that tectorigenin suppressed the proliferation of HSC-T6 cells and induced apoptosis of HSC-T6 cells in a time- and dose-dependent manner<sup>[13]</sup>. Tectorigenin at a concentration of 100 mg/L greatly inhibited the viability of HSC-T6 cells and induced the condensation of chromatin and fragmentation of nuclei<sup>[13]</sup>. In this study, tectorigenin could inhibit the viability of HepG2 cells and induce apoptosis at a

lower concentration of 20 mg/L. Furthermore, tectorigenin-induced apoptosis of HSC-T6 cells was associated with the generation of ROS, increased intracellular  $[Ca^{2+}]$ , loss of mitochondrial membrane potential, translocation of cytochrome c, and activation of caspase-9 and -3<sup>[13]</sup>. In this study, tectorigenin could induce apoptosis of HepG2 cells by similar mechanisms. In contrast, in the study of Zhou *et al.*<sup>[6]</sup>, troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin, while it did not affect expression of apoptosis-promoting factor Bax.

In conclusion, tectorigenin significantly inhibits the proliferation and induces apoptosis in human HCC HepG2 cells mainly *via* mitochondrial-mediated pathway. These observations indicated that tectorigenin is a promising chemotherapeutic and chemopreventive agent for the treatment of HCC.

## COMMENTS

### Background

Human hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. Unfortunately, the disease is often diagnosed at a late stage. The hallmark of cancer cells is the dysregulation of cell proliferation and apoptosis. The growing of a tumor is determined by the rate of cell proliferation and the apoptosis. Therefore, the induction of apoptosis in tumor cells has become a strategy in cancer treatment and the mitochondrial-mediated pathways have been identified as one of the major mechanisms for induction of apoptosis. *Iris tectorum* (*I. tectorum*), a traditional Chinese medicine, has been widely used for treating liver-related diseases, including hepatitis, liver fibrosis, liver cirrhosis and liver cancer.

### Research frontiers

Recent studies have revealed that tectorigenin possessed anti-proliferative and pro-apoptotic effects on hepatic stellate cells (HSCs) and have potent *in vivo* anti-liver fibrosis activity. However, until now there is no report about the anti-tumor effect of tectorigenin on human HCC HepG2 cells and the associated mechanisms. In this study, tectorigenin was found to suppress the proliferation of HepG2 cells and induce apoptosis of HepG2 cells.

### Innovations and breakthroughs

This study revealed that tectorigenin possessed considerable pro-apoptotic effects on human HCC HepG2 cells, which confirmed that tectorigenin is the substance base of the utilization of *I. tectorum* rhizome in the treatment of liver cancer. To be known, this is the first study to report the apoptosis induction effects of tectorigenin on human HCC HepG2 cells.

### Applications

Tectorigenin significantly inhibits the proliferation and induces apoptosis in human HCC HepG2 cells mainly *via* mitochondrial-mediated pathway, and produces a slight cytotoxicity to L02 cells. These observations indicated that tectorigenin is a promising chemotherapeutic and chemopreventive agent for the treatment of HCC.

### Peer review

The paper investigates the effect of tectorigenin on proliferation and apoptosis in human hepatocellular carcinoma HepG2 cells. The study is of interest.

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## Diazoxide attenuates ischemia/reperfusion injury *via* upregulation of heme oxygenase-1 after liver transplantation in rats

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### Abstract

**AIM:** To evaluate the effects of diazoxide on ischemia/reperfusion (I/R)-injured hepatocytes and further elucidated its underlying mechanisms.

**METHODS:** Male Sprague-Dawley rats were randomized (8 for donor and recipient per group) into five groups: I/R group (4 h of liver cold ischemia followed by 6 h of reperfusion), Heme oxygenase-1 (HO-1) small interfering RNA (siRNA) group (injection of siRNA via donor portal vein 48 h prior to harvest), diazoxide (DZ) group (injection of DZ *via* donor portal vein 10 min prior to harvest), HO-1 siRNA + DZ group, and siRNA control group. Blood and liver samples were collected at 6 h after reperfusion. The mRNA expressions and protein levels of HO-1 were determined by reverse transcription polymerase chain reaction and Western blotting, and tissue morphology was examined by light and transmission electron microscopy. Serum transaminases level and cytokines concentration were also measured.

**RESULTS:** We observed that a significant reduction of HO-1 mRNA and protein levels in HO-1 siRNA and HO-1 siRNA + DZ group when compared with I/R group, while the increases were prominent in DZ group. Light and transmission electron microscopy indicated severe disruption of tissue with lobular distortion and mitochondrial cristae damage in HO-1 siRNA and HO-1 siRNA + DZ group as compared with DZ group. Serum alanine aminotransferase, aspartate transaminase, tumor necrosis factor- $\alpha$  and interleukin-6 levels increased in HO-1 siRNA and HO-1 siRNA + DZ group, and decreased in DZ group.

**CONCLUSION:** The protective effect of DZ may be induced by upregulation of HO-1. Inhibiting the expression of HO-1, this protection pretreated with DZ was abolished.

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**Key words:** Ischemia/reperfusion injury; Diazoxide; Heme oxygenase-1; Liver transplantation; Rat

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### INTRODUCTION

The ATP-sensitive potassium (KATP) channel was identified in cardiac muscle<sup>[1]</sup>. Later, a similar channel was described in liver<sup>[2]</sup>, brain<sup>[3,4]</sup> and skeletal muscle mitochondria<sup>[5]</sup>. The primary function of this channel is to allow K<sup>+</sup> transport into the mitochondrial matrix and this phe-

nomenon could be involved in maintaining mitochondrial volume homeostasis<sup>[6]</sup>. KATP channels that exist in the inner membrane of mitochondria<sup>[2]</sup> has been implicated to mediate ischemic preconditioning's protective effects in ischemic heart<sup>[7,8]</sup>. Diazoxide (DZ) is a selective mitochondria ATP-sensitive potassium (mitoKATP) channel opener, which has been reported to have protective effect on the heart<sup>[9,10]</sup>, brain<sup>[11,12]</sup> and spinal cord<sup>[13]</sup> following ischemia/reperfusion (I/R) injury. Several recent studies have suggested that mitoKATP channels may be only a trigger, but not a mediator of protection<sup>[14]</sup>. The exact mechanisms have not been fully clarified.

Ischemic preconditioning (IPC) is a well-known phenomenon in which brief episodes of ischemia and reperfusion confers a state of protection against subsequent sustained long-term I/R injury<sup>[15,16]</sup>. Although the exact underlying mechanisms of IPC are unknown, activation of mitoKATP channels has been proposed to play a pivotal role in preconditioning<sup>[17]</sup>. More studies have indicated that pretreatment with mitoKATP channel openers induces IPC-like protective effects<sup>[18]</sup>, and that IPC-induced protection is antagonized by mitoKATP channel inhibitors<sup>[19,20]</sup>. These findings support the hypothesis that IPC is mediated through the opening of mitoKATP channels.

Heme oxygenase-1 (HO-1) is the rate-limiting step in the oxidative degradation of heme. Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplant model<sup>[21-23]</sup>. Recent studies suggested that IPC may protect against systemic inflammatory response *via* enhanced HO-1 overexpression<sup>[24-27]</sup>. The purpose of our study was to investigate that the protective action of DZ on I/R injury and expression of HO-1 after liver transplantation in rats. We explored the potential molecular mechanisms for DZ to reduce I/R injury. Using HO-1 small interfering RNA (HO-1 siRNA), we further observed the roles of HO-1 in DZ-induced action.

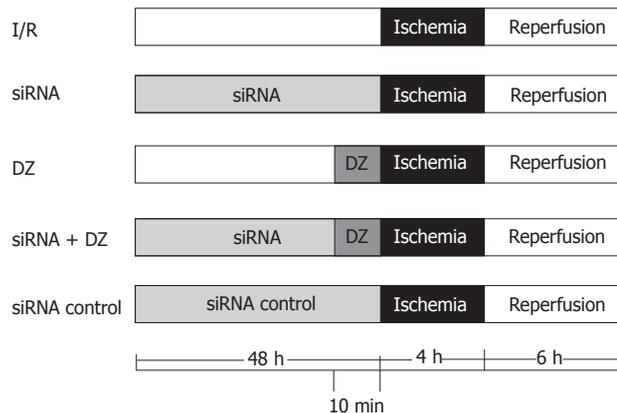
## MATERIALS AND METHODS

### Animals

Adult male Sprague-Dawley (S-D) rats (200-250 g) (Kunming Medical College Laboratory Animal Center, China) were used. Rats were maintained on a standard rodent chow and water *ad libitum* in a room according to the local animal welfare guidelines. All experiments were approved by the ethics committee for the use of experimental animals at Kunming Medical College.

### Heme oxygenase-1 siRNA design

The design of HO-1 siRNA was based on the characterization by Zhang *et al.*<sup>[28]</sup>. Additionally, a scrambled sequence was designed as a Negative Control siRNA. The targeted sequence of rat HO-1 siRNA was 5'-AAGC-CACACAGCACUAUGUdTdT-3' (sense) and 5'-ACAU-AGUGCUGUGGCCUdTdT-3' (antisense). siRNA duplexes were chemically synthesized by Guangzhou Rui Bo Biological Technology Co (Guangzhou, China).



**Figure 1** Experimental protocol. DZ: Diazoxide; siRNA: Small interfering RNA.

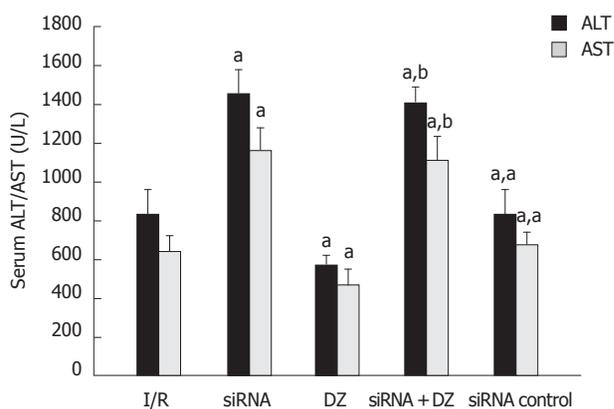
### Experimental protocol

S-D rats underwent ether anesthesia. The basic techniques of liver harvesting and orthotopic transplantation without hepatic arterial reconstruction were according to the method described previously by Kamada *et al.*<sup>[29]</sup>. After organ harvest, liver grafts were stored for 4 h in cold University of Wisconsin solution at 4 °C. Subsequently, syngeneic orthotopic liver transplantation (OLT) was performed and the anhepatic phase was estimated to be 11-13 min for all recipients. All transplant experiments in this study were performed by a single person. Separate groups of rats were killed at 6 h after their vessels were unclamped, then samples of blood and liver tissue were taken for further analysis.

Eighty rats (16 for every group) were divided randomly into five groups (Figure 1): (1) I/R group: OLT was performed according to method described previously; (2) siRNA group: Same treatment as I/R group, but donors were injected with HO-1 siRNA (200 nmol/kg of body weight) *via* portal vein 48 h before liver harvest; (3) DZ group: Same as I/R group, but donors were injected with DZ (5 mg/kg of body weight), a mitoKATP channel opener, *via* portal vein 10 min before liver harvest; (4) siRNA + DZ group: Same treatment as I/R group, donors were treated with HO-1 siRNA and DZ; and (5) siRNA control group: Same as I/R group, donors were injected with Negative Control siRNA (200 nmol/kg of body weight). DZ was purchased from Sigma Chemical Co. (St Louis, MO, United States) and dissolved in dimethyl sulfoxide (DMSO) (sigma, United States) before addition to experimental solutions. The final concentration of DMSO in the solution was less than 0.05%.

### Serum enzymes

At 6h following by liver reperfusion, blood was collected *via* abdominal aorta. After centrifugation of whole blood (3000 rpm, 15 min), serum was extracted and stored at -70 °C until analysis. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using a clinical chemistry system (7060 automatic analyzer, Hitachi, Japan).



**Figure 2** Serum alanine aminotransferase and aspartate transaminase levels after reperfusion. <sup>a</sup> $P < 0.05$  vs ischemia/reperfusion (I/R) group, <sup>b</sup> $P < 0.01$  vs diazoxide (DZ) group. ALT: Alanine aminotransferase; AST: Aspartate transaminase.

### Reverse-transcriptase polymerase chain reaction

Animals were sacrificed 6 h after transplantation. Liver samples were obtained, shock-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further RNA isolation. Total RNA was extracted from 100 mg liver tissues with Trizol reagents (Invitrogen, United States) according to the manufacturer's guidelines and quantified by UV absorption. Following DNase I (Invitrogen, United States) digestion, 1  $\mu\text{g}$  of total RNA was subjected to reverse transcription polymerase chain reaction (RT-PCR) with M-MLV reverse transcriptase (Promega, United States), using oligo-dT as primers. The mixture was inactivated at  $42^{\circ}\text{C}$  for 60 min, and then the reverse transcriptase was inactivated by heating at  $70^{\circ}\text{C}$  for 10 min. Specific primers for rat HO-1 and  $\beta$ -actin (an internal standard) were designed by Primer Premier 5.0. For HO-1, the primers were 5'-TGGAAGAGGAGATAGAGCGA-3' (sense) and 5'-TGTTGAGCAGGAAGGCCGGTC-3' (antisense), generating a 451-base pair fragment. For  $\beta$ -actin, the primers used were 5'-CACGATGGAGGGGCCGGAC TCATC3' (sense) and 5'-TAAAGACCTCTATGC-CAACACAGT-3' (antisense), generating a 240-base pair fragment. PCR reaction constituents were as follows: 2  $\mu\text{L}$  of cDNA mixture was subjected to amplification in 20  $\mu\text{L}$  of final volume.  $94^{\circ}\text{C}$ , 2 min; then  $94^{\circ}\text{C}$ , 1 min;  $60^{\circ}\text{C}$ , 1 min;  $72^{\circ}\text{C}$ , 2 min, for 40 cycles; and  $72^{\circ}\text{C}$  5 min to end the reaction. The PCR products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide and visualized by UV illumination. This semi-quantitative measure for HO-1 was expressed as ratios to  $\beta$ -actin.

### Western blotting analysis

Proteins extracts were isolated from liver tissue with radioimmunoprecipitation containing phenylmethyl sulfonylfluoride. Protein concentration was determined by a bicinchoninic acid protein assay reagent (Pierce Rockford, IL). Proteins (20  $\mu\text{g}$ /sample) in SDS-loading buffer were heated to  $100^{\circ}\text{C}$  for 5 min, and separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using an electroblotting ap-

paratus. The membrane was blocked overnight at  $4^{\circ}\text{C}$  in 5% nonfat dry milk and TBST buffer to block non-specific binding sites. Western blots were probed with primary antibodies (anti-HO-1, 1:200 dilution, Millipore, United States) at room temperature (RT) for 2 h. After being washed, blots were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibodies (dilution, 1:50 000, Pierce Biotechnology) for 2 h at RT. Thereafter the proteins were visualized by ECL detection system (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States) and analyzed by the Quantity One Analysis Software (Bio-Rad, United States).  $\beta$ -actin was used as protein loading control.

### Histology and electron microscopic examination

Livers were excised rapidly and fixed in conventional fixing solutions (10% neutral-buffered formalin) after 6 h reperfusion. Livers were paraffin embedded and sectioned into 3  $\mu\text{m}$ -thick slices with a tissue chopper. The sections were examined by hematoxylin and eosin staining. The histological severity of I/R injury was graded according to Suzuki's classification<sup>[30]</sup>. All slides were judged by the same investigator who had been blinded to the corresponding study group. The excised liver samples were cut into small pieces, and immersed in excessive volumes of 2.5% glutaraldehyde with 0.1 mol phosphate buffer (pH 7.2). Following fixation for 24 h, samples were further immersed in 2% osmium tetroxide ( $\text{OsO}_4$ ) in 0.1 mol cacodylate buffer (pH 7.2) for 2 h at  $4^{\circ}\text{C}$ , dehydrated, and embedded in epoxide resin (EPON812). Ultrathin sections were stained with lead citrate and uranylacetate, and examined using a JEM-1200EX transmission electron microscope.

### Enzyme-linked immunosorbent assay

Serum interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels of different groups were measured using commercially available ELISA kits (R and D System, United States) according to the test protocols. All samples, including standard and control solution, were assayed in duplicate. Values were expressed as pg/mL.

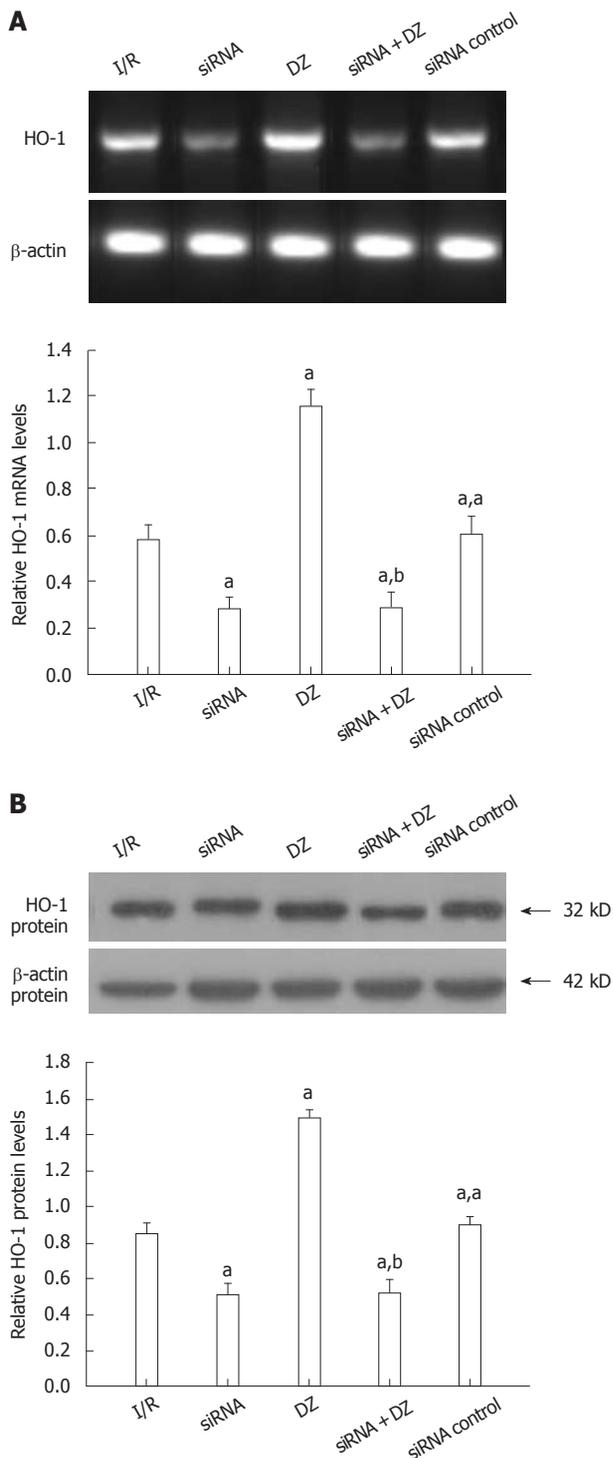
### Statistical analysis

Statistical analysis was performed using SPSS Version 13.0 for Windows (SPSS, Inc, Chicago, IL). Values are expressed as means  $\pm$  SD. Difference between experimental groups were analyzed by one-way analysis of variance.  $P$  values  $< 0.05$  were considered statistically significant.

## RESULTS

### Hepatic transaminases

The hepatocellular damage was measured with the determination of ALT and AST levels. Rats treatment with siRNA showed a significant increase in ALT and AST compared with I/R rats. Liver function measurement in the DZ group was significantly lower than those in the I/R group. There was no statistical difference between siRNA and siRNA + DZ group ( $P > 0.05$ ) (Figure 2).



**Figure 3** Liver heme oxygenase-1 mRNA and protein levels at 6 h after transplantation. <sup>a</sup>*P* < 0.05 vs ischemia/reperfusion (I/R) group, <sup>b</sup>*P* < 0.01 vs diazoxide (DZ) group.

### Heme oxygenase-1 mRNA and protein expression levels

The PCR products were separated on agarose gel and the relative expression of HO-1 were shown. As shown in Figure 3A, HO-1 mRNA expression was decreased in both siRNA and siRNA + DZ groups compared with the I/R group at 6 h after I/R injury. In contrast, DZ treatment significantly enhanced the hepatic HO-1 mRNA level. No statistical difference was observed between si-

RNA and siRNA + DZ group. HO-1 protein level was determined by western blotting. The results were almost the same as those obtained in the RT-PCR analyses. Compared with the I/R group, HO-1 protein expression was markedly downregulated in both siRNA and siRNA + DZ groups. The level of HO-1 was significantly higher in the DZ group than all other groups. Moreover, the difference between siRNA and siRNA + DZ group did not attain statistical significance (Figure 3B).

### Histology

Necrosis and liver damage were assessed with H and E-stained liver tissue 6 h after reperfusion. Liver specimens from rats subjected to I/R showed macrovesicular fatty changes, nuclear fragmentation, and sinusoidal congestion and congested with many red blood cells. In siRNA and siRNA + DZ groups, histological tissue damage was significantly aggravated. Moreover, multiple and extensive ballooning/hepatocellular necrosis and massive infiltration of neutrophils were observed. However, these pathological changes were significantly decreased in the DZ group (data not shown). There was no significant difference between I/R and siRNA control group (Figure 4A).

### Transmission electron microscopy

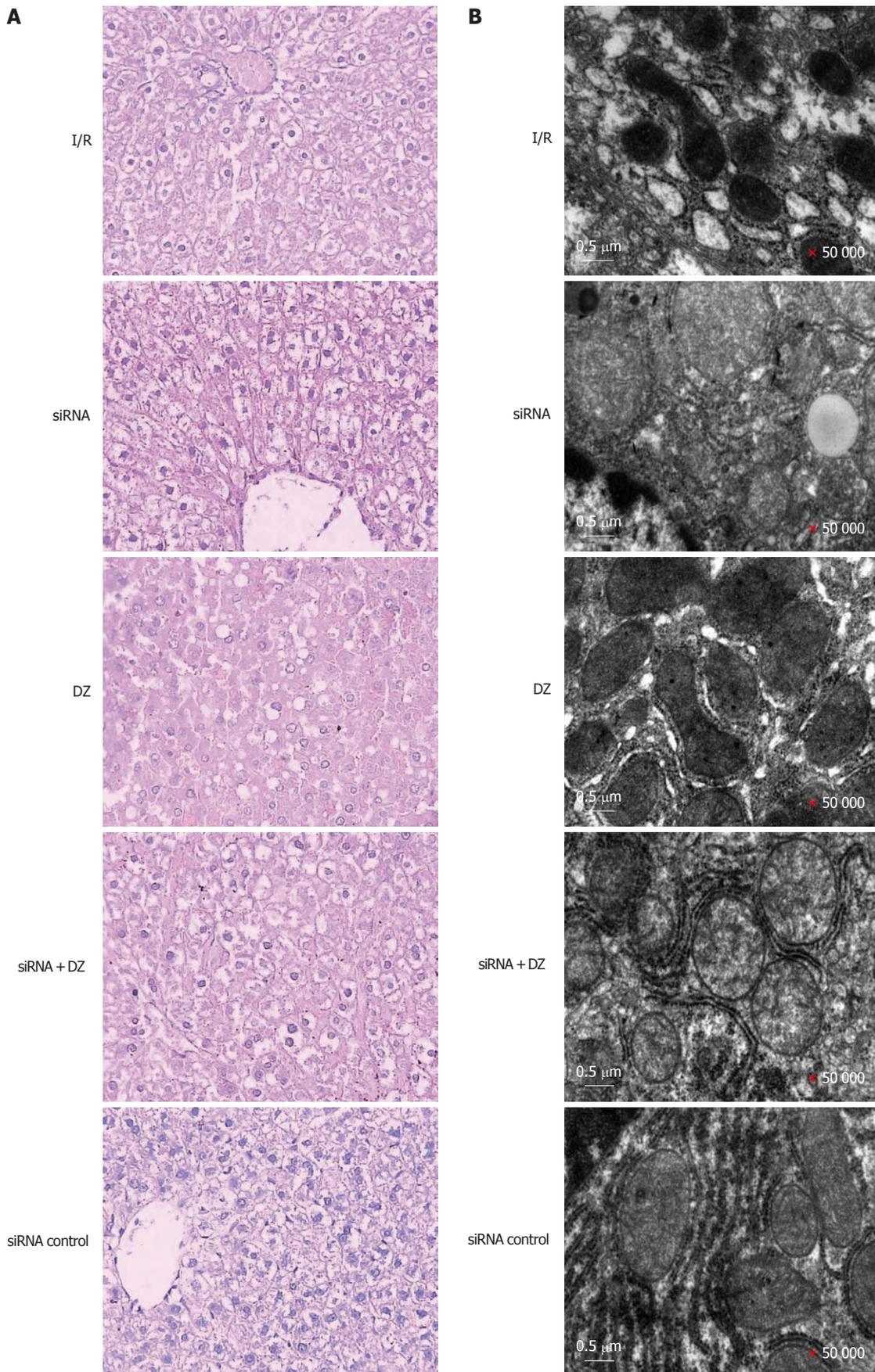
To further confirm hepatocytes ultrastructure alterations, we examined the liver grafts during cold ischemia and reperfusion by electron microscopy. In siRNA group, the nuclei of the hepatocytes were severe irregular in shape and contained damaged nuclear chromatin in the nucleoplasm. The hepatocytes appeared largely intact vacuolar structures with lipid droplets were present in the cytoplasm of the cells. The mitochondria and the smooth endoplasmic reticulum were dilated and even destroyed, and the mitochondria cristae were disorganized. By contrast, mitochondria from rats treated with DZ appeared markedly better with less mitochondrial membrane damage and swelling in the rat hepatocytes after ischemia and reperfusion. However, siRNA + DZ group treatment had markedly aggravated these damages as compared with DZ group. The I/R group and siRNA control group revealed lower hepatocytes damages than the siRNA group (Figure 4B).

### Cytokines assay

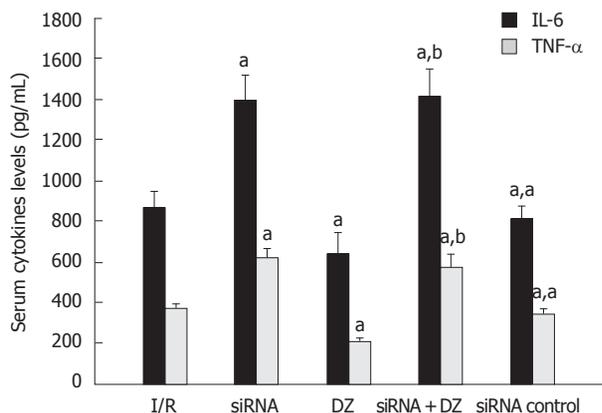
Serum cytokine levels (IL-6/TNF- $\alpha$ ) were determined by ELSA. The IL-6/TNF- $\alpha$  levels revealed a substantial increase in the siRNA group as compared with the I/R group. But groups pretreated with DZ had markedly decreased these products. Moreover, the cytokine levels (IL-6/TNF- $\alpha$ ) in the siRNA + DZ group were significantly higher, when compared to the DZ group. There was no statistical difference between I/R group and siRNA control group (*P* > 0.05) (Figure 5).

## DISCUSSION

HO-1 is up-regulated in response to oxidative stress and



**Figure 4** Liver histology and ultrastructural changes of hepatocytes. A: Ischemia/reperfusion (I/R) treated livers showed vacuolization, nuclear fragmentation, sinusoidal congestion, and hepatocyte necrosis. However, histological tissue damage was aggravated in the small interfering RNA (siRNA) and siRNA + diazoxide (DZ) group. In contrast, DZ treatment relieved liver damages (hematoxylin and eosin, × 400); B: The mitochondria and the smooth endoplasmic reticulum were dilated and even destroyed, and the mitochondria cristae were disorganized in I/R treated hepatocytes.



**Figure 5** Serum cytokine levels (interleukin-6/ tumor necrosis factor- $\alpha$ ) were measured. <sup>a</sup> $P < 0.05$  vs ischemia/reperfusion (I/R) group, <sup>b</sup> $P < 0.01$  vs diazoxide (DZ) group. IL-6: Interleukin-6; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

catalyzes the degradation of pro-oxidant heme to carbon monoxide (CO), iron and bilirubin<sup>[31]</sup>. Three HO isoforms have been identified to date: HO-1, HO-2 and HO-3. HO-1 is inducible and distributed ubiquitously in mammalian tissue<sup>[32]</sup>, while HO-2 is expressed constitutively and found mainly in the central nervous system<sup>[33]</sup>. HO-3 is only described in the rat brain and has no activity. HO-1 expression is an adaptive and protective response to oxidative stress in a wide variety of cells<sup>[34,35]</sup>. HO-1 is induced by a variety of stimuli, including non-heme inducers and various agents that cause oxidative stress, and prevents the oxidative DNA damage caused by heat shock and reactive oxygen species (ROS).

Mitochondrial ATP-sensitive potassium channel opener is responsible for more effective oxidative phosphorylation<sup>[36]</sup> and regulation of ROS generation<sup>[37,38]</sup> in ischemic preconditioning. Prevention of mitochondrial failure improves cellular survival after an ischemic event. Pharmacological agents that maintain mitoKATP channels can prevent mitochondrial failure in a manner similar to that induced by ischemic preconditioning<sup>[13,39]</sup>. MitoKATP is shown to have protective effect on the heart, kidney and brain following I/R injury<sup>[40,41]</sup>. However, the detailed effect of mitoKATP in liver I/R injury has not fully studied. Diazoxide is mainly used as a vasodilator in the treatment of clinical hypertension. But in our study, we used DZ, the putative mitoKATP channel opener to evaluate the effects of mitoKATP channels in liver I/R injury in OLT.

In the present investigation, rats pretreated with DZ results in enhanced resistance to liver cold I/R injury and increased HO-1 expression levels. To examine whether HO-1 was responsible for the protective effect of DZ in liver I/R injury, we used HO-1 siRNA, which interferes with the enzymatic expression of HO-1 to examine its possible effect. Inhibition of the expression of HO-1 almost completely blocked the protection afforded by DZ. These results strongly indicated that the protection of DZ was mediated through upregulation of HO-1. We speculate that DZ induces an increase in HO-1 levels that protect liver I/R injury. The K<sup>+</sup> channel-independent ef-

fects of diazoxide to inhibit succinate oxidation<sup>[42]</sup> and succinate dehydrogenase<sup>[43]</sup> or inhibit opening of the mitochondrial membrane pore<sup>[44]</sup> may be responsible for its protective effects. When we inhibited the expression of HO-1 and the above effects of DZ remains, but the protection of DZ to hepatic I/R injury in rats almost disappeared. It is possible that DZ, through opening KATP channels, activated an unknown pathway, which directly or indirectly upregulated the expression of HO-1. Since HO-1 plays a critical role in protect liver I/R injury in rats OLT, inhibition of HO-1 activity may exaggerate I/R injury.

We measured serum cytokine levels (IL-6/TNF- $\alpha$ ) by ELISA to determine the effect on anti-inflammatory. Rats pretreated with DZ significantly decreased serum IL-6/TNF- $\alpha$  levels. However, they were markedly elevated in siRNA + DZ group than those in DZ group. Xu B *et al*<sup>[45]</sup> showed that DZ inhibit the production of the proinflammatory cytokines TNF- $\alpha$  and IL-6 by peripheral blood mononuclear cells (PBMCs) in normal pregnancy. But our study shows that DZ was able to reduce the release of cytokines. But when the expression of HO-1 was inhibited, the release of cytokines could not reduce. HO-1 has been shown to be expressed principally in Kupffer cells<sup>[21,46,47]</sup>. Our study suggests that the potential utility of HO-1 overexpression in preventing I/R injury results from inhibition of Kupffer cells activation<sup>[48]</sup>. Activated Kupffer cells release a large amount of proinflammatory cytokines<sup>[49-51]</sup> which lead to aggravation of I/R injury. Devey *et al*<sup>[52,53]</sup> reported that depletion of Kupffer cells using liposomal clodronate led to loss of hepatic HO-1 expression and much more severe injury. It has been demonstrated that overexpression of HO-1 played an important role in a number of I/R injury and liver transplant models. Therefore, we could think that the protective effect of DZ was by increasing HO-1 expression in our study.

In summary, DZ can attenuate hepatic I/R injury in rat liver transplantation and induce hepatic HO-1 expression. In addition, inhibiting HO-1 expression with a specific siRNA, this protection pretreated with DZ was abolished. Thus, the mechanism of the protective effects of DZ is dependent on HO-1. The upregulation of HO-1 results in an alleviation of hepatic I/R injury, as well as a decrease of the production of inflammatory cytokine such as IL-6 and TNF- $\alpha$ . Our results provide an insight into induction of HO-1, and suggest that DZ may have the potential to be developed as a hepatic HO-1 inducer for therapeutic purposes. Our work also raises an interest that HO-1 can be induced by DZ to stimulate protective process attenuating I/R injury.

## COMMENTS

### Background

Diazoxide (DZ) is a selective mitochondria ATP-sensitive potassium channel opener, which has been reported to have protective effect on the heart, brain and spinal cord following ischemia/reperfusion (I/R) injury. But the protective action of DZ on liver I/R injury are unknown and the exact underlying mechanisms are poorly understood.

### Research frontiers

Heme oxygenase-1 (HO-1) is the rate-limiting step in the oxidative degradation of heme. Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplant model. HO-1 expression is an adaptive and protective response to oxidative stress in a wide variety of cells.

### Innovations and breakthroughs

The data represent a major advance in our understanding of ATP-sensitive potassium channel. DZ, can attenuate hepatic I/R injury in rat liver transplantation and induce hepatic HO-1 overexpression. In addition, the authors clearly demonstrated a direct correlation between expression of HO-1 and DZ. Thus, DZ may have the potential to be developed as a hepatic HO-1 inducer for therapeutic purposes.

### Applications

The study results suggest that the mechanism of the protective effects of DZ is dependent on HO-1. Further studies are needed to evaluate the safety and efficacy of DZ used in clinical.

### Peer review

This was a well designed study with excellent results. It was well written. It seemed almost too perfect. However, as long as they have a history of academic integrity.

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## Safety and efficacy of Profermin<sup>®</sup> to induce remission in ulcerative colitis

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### Abstract

**AIM:** To test the efficacy and safety of Profermin<sup>®</sup> in inducing remission in patients with active ulcerative colitis (UC).

**METHODS:** The study included 39 patients with mild to moderate UC defined as a Simple Clinical Colitis Activity Index (SCCAI) > 4 and < 12 (median: 7.5), who were treated open-label with Profermin<sup>®</sup> twice daily for 24 wk. Daily SCCAI was reported observer blinded *via* the Internet.

**RESULTS:** In an intention to treat (ITT) analysis, the mean reduction in SCCAI score was 56.5%. Of the 39 patients, 24 (62%) reached the primary endpoint, which was proportion of patients with  $\geq 50\%$  reduction in SCCAI. Our secondary endpoint, the proportion of patients in remission defined as SCCAI  $\leq 2.5$ , was in ITT analysis reached in 18 of the 39 patients (46%). In a repeated-measure regression analysis, the estimated mean reduction in score was 5.0 points (95% CI: 4.1-5.9,  $P < 0.001$ ) and the estimated mean time taken to obtain half the reduction in score was 28 d (95% CI: 26-30). There were no serious adverse events (AEs) or withdrawals due to AEs. Profermin<sup>®</sup> was generally well tolerated.

**CONCLUSION:** Profermin<sup>®</sup> is safe and may be effective in inducing remission of active UC.

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**Key words:** Ulcerative colitis; Diet; Probiotic; Profermin<sup>®</sup>; Inflammatory bowel disease; Dietary management; Medical foods

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### INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing bowel dis-

ease characterized by colonic mucosal inflammation. The goal of treatment in UC is to induce and maintain remission of the disease. Failure to induce remission occurs in 20%-30% of patients on current treatments, leaving colectomy as the only alternative in a proportion of patients<sup>[1,2]</sup>. Furthermore, many patients find the side effects of treatment with corticosteroids and other drug therapies unacceptable. Accordingly, new treatment alternatives are being sought. The pathogenesis of UC has still not been determined in detail; however, a major hypothesis is suggested to be an aggressive immune response to the intestinal content including, a subset of nonpathogenic enteric bacteria in genetically predisposed individuals. Clinical and experimental studies point towards alterations in the relative balance of aggressive and protective bacterial species in these disorders<sup>[3,4]</sup>. Interventions to alter the intestinal microflora in order to decrease proinflammatory stimuli and increase anti-inflammatory signaling are under investigation<sup>[5,6]</sup>. Some studies have suggested an effect of probiotics in the treatment of UC<sup>[5,7-9]</sup>. The effects of probiotics on the microflora may only be limited and transient because colonization and survival of the probiotics are difficult to achieve. However, remission from inflammatory bowel diseases may be induced by food for special medical purposes (FSMPs), e.g., elemental diets. In this study we investigated a new dietary product Profermin®, which is intended to be registered as a FSMP for the dietetic management of UC. It consists of fermented oats, *Lactobacillus plantarum* (*L. plantarum*) 299v, barley malt, lecithin and water. Our aim was to investigate the safety and possible efficacy of Profermin® in patients with mild to moderate UC. We also assessed the usefulness of a new online daily symptom registration system.

## MATERIALS AND METHODS

### Patients

The study was conducted between 2008 and 2009. Patients were eligible if they were between 18 and 50 years of age and had an established diagnosis of UC based on clinical, endoscopic and histological features. Active disease was assessed by Simple Clinical Colitis Activity Index (SCCAI) (Table 1) score  $> 4$  and  $< 12$ <sup>[10]</sup>. Patients who initiated treatment with azathioprine, 6-mercaptopurine, cyclosporin or methotrexate within 8 wk prior to inclusion or tumor necrosis factor- $\alpha$  inhibitors within 12 wk before inclusion or had changes in UC treatment within 2 wk before inclusion were ineligible for the study. Concomitant celiac disease, lactose intolerance and irritable bowel syndrome were also exclusion criteria. In addition any malignant or premalignant condition or recent gastroenteritis and irritable bowel syndrome rendered patients ineligible. Patients were recruited through advertisement on the website of the local patients' association, in local newspapers and through Google ads. The advertisement showed a link to a website that briefly described the trial and encouraged patients who were

Table 1 Simple Clinical Colitis Activity Index<sup>[10]</sup>

Symptom	Score
Bowel frequency (day)	
1-3	0
4-6	1
7-9	2
> 9	3
Bowel frequency (night)	
1-3	1
4-6	2
Urgency of defecation	
Hurry	1
Immediately	2
Incontinence	3
Blood in stool	
Trace	1
Occasionally frank	2
Usually frank	3
General wellbeing	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extracolonic features	1 per manifestation

interested and had the relevant disease characteristics to contact the trial nurse. The patients had to sign a declaration stating when and where they had been diagnosed with UC and describing the course of their disease including the fulfillment of the inclusion criteria. The data were confirmed by cross checking the patients' medical records. A specialist had diagnosed all patients.

Patients were excluded if UC medication was modified during the study period, however, a dose reduction of ongoing drugs was accepted. The patients were also excluded if new medication that may affect UC symptoms was prescribed for other conditions.

### Outcome measures

Our primary endpoint was to estimate the proportion of patients with a  $\geq 50\%$  reduction in SCCAI. Our secondary endpoint was to estimate the proportion of patients in remission defined as  $SCCAI \leq 2.5$ <sup>[11]</sup>.

### Study design and assessment of outcome measures

A prospective open-label study design was used to gain experience with time to response and remission and compliance for the later design of a comprehensive controlled study. After a run-in period of 6-14 d, the patients were followed for 24 wk with daily SCCAI score assessment. The SCCAI was chosen for the following reasons. The SCCAI score has been shown to correlate well with the more complex and invasive scoring systems and it facilitates daily and observer blinded symptoms registration<sup>[10,11]</sup>. Furthermore, colonoscopy may be experienced as painful and therefore compromise the patients' willingness to participate in a clinical trial. Internet software was developed by a company specialized in software development for interactive Internet solutions

(Franklyweb, Copenhagen, Denmark). The Internet platform was created according to protocol instructions and used for individual patient registration of SCCAI parameters. Regular access to a computer with Internet access was a criterion for inclusion. Each patient received a username and a password and was instructed to register the SCCAI parameters daily on the trial website. Each SCCAI parameter was formulated as a question e.g. "How many defecations have you experienced during daytime today? Click on the appropriate answer "1-3", "4-6", "7-9" or "> 9". Each SCCAI question needed to be answered before the patient could continue to the next question. After registration of the last SCCAI question, the patient was shown an overview of the answers to every SCCAI question and was asked to confirm or amend the information. After confirmation, the patient was shown a graph with the daily SCCAI scores from the first day of the run-in period up to the present date. The patients could communicate on the trial website with the nurse who checked the status of each patient at least twice a week and could send reminders if patients failed to register the daily symptoms. The symptoms were registered on a daily basis. When a patient failed to register a day's symptoms, the patient was reminded by the nurse about the lacking registration. The registration rate (registered days out of total number of days) was > 95%. The data were transferred from the patients *via* the Internet in encrypted form (Secure Sockets Layer) and stored on a secure server. The data were instantly copied - in raw and unprocessed form - to a similar server at the Technical University of Denmark, Department of Informatics and Mathematical Modelling, in order to secure the authenticity of the data and to analyze the data statistically.

Occasionally some patients did not have access to a computer with an Internet connection. In such cases, these patients received paper SCCAI questionnaires to be completed for each day of the relevant period. When access to the Internet was again established, the patients transferred the SCCAI parameters noted on the questionnaires to the website. During the screening process, 30 of the included patients (77%) had a face-to-face meeting with the trial nurse. There were no other face-to-face contacts with the patients. All other communication was electronic (phone or e-mail). The patients were instructed to report adverse events (AEs) *via* the trial website. Safety of Profermin® was assessed by analyzing the AE reports.

### Description of the intervention

Profermin® is manufactured as follows. Oat gruel is produced by mixing oats, water and a small amount of barley malt. The mixing process lasts for 1 h at 88 °C. The gruel is then cooled to 38 °C, and a *L. plantarum* 299v starter culture is added. The mixture is kept at 38 °C for 15 h with constant gentle stirring. The resulting oat-fermented gruel is cooled to about 8 °C. Lecithin is then added while the mixture is gently stirred and the resulting Pro-

fermin® is packed in 250-mL cartons under sterile conditions. The product is tested for pH and colony forming units (CFU) of Enterobacteriaceae, yeasts/moulds and *L. plantarum* 299v. The pH must be between 3.6 and 4.2 and the CFU of Enterobacteriaceae, yeasts/moulds must each be < 100/mL. The CFU of *L. plantarum* must be > 10<sup>8</sup>/mL.

After 6-14 d of run in, the Profermin® intervention was initiated by scaling the patient into a daily oral intake of Profermin®. The initial daily Profermin® dose was 125 mL as the first meal and 125 mL as the last meal of the day. After 2 d, the Profermin® dose was increased to 250 mL as the first meal and 250 mL as the last meal of the day. However, the protocol was open for periodical changes of the total dose of Profermin® in the interval of 25 mL to 500 mL taken once or twice daily, for example, if a patient experienced AEs during the introduction, the low Profermin® dose was prolonged for up to 2 wk. The median dose was 445 mL/d with an interquartile range of 408-500 mL/d. The patients reported their intake of Profermin® on a daily basis through the trial website and the mean self-reported adherence therapy was > 95%.

Patients were recommended to be cautious with consumption of dairy products and concentrated sugar products in accordance with routine dietetic recommendations widely used in Danish IBD clinics<sup>[6]</sup>. Compliance with this recommendation was not monitored. Patients continued their usual UC medication and clinical follow-up with gastroenterologists.

### Ethics, approvals and patient consent

The trial was approved by the Danish Data Protection Agency (2008-41-2961) and has been cleared with The Ethical Committees of the Copenhagen Region and registered (H-B-2008-FSP-20). As Profermin® is an FSMP and not a medicinal product, no authorization by the Danish Medicines Agency was required. All patients gave written informed consent according to the Helsinki declaration. The study was registered on www.clinicaltrials.gov (NCT01245465).

### Statistical analysis

In all analyses, we applied the principle of intention-to-treat (ITT). Data for patients who dropped out or who were excluded during the study period were included in the analysis by using the principle of last value carried forward. Unadjusted estimates for the primary and secondary endpoints were presented as total numbers and percentages. To gain further insight into the longitudinal effect of Profermin® on the possible decline in SCCAI over time adjusted for the starting value, we applied the following non-linear regression model:

$$Score_{ij} = \beta_0 + \beta_1 \times 2^{-Day_{ij}/\theta}$$

Where the dependent variable  $Score_{ij}$  was the score for person  $j$  on Day  $i$ . Time was denoted by the independent variable  $Day_{ij}$ . In the model, the parameters to be estimated had the following interpretation:  $\beta_0$  was the ultimate

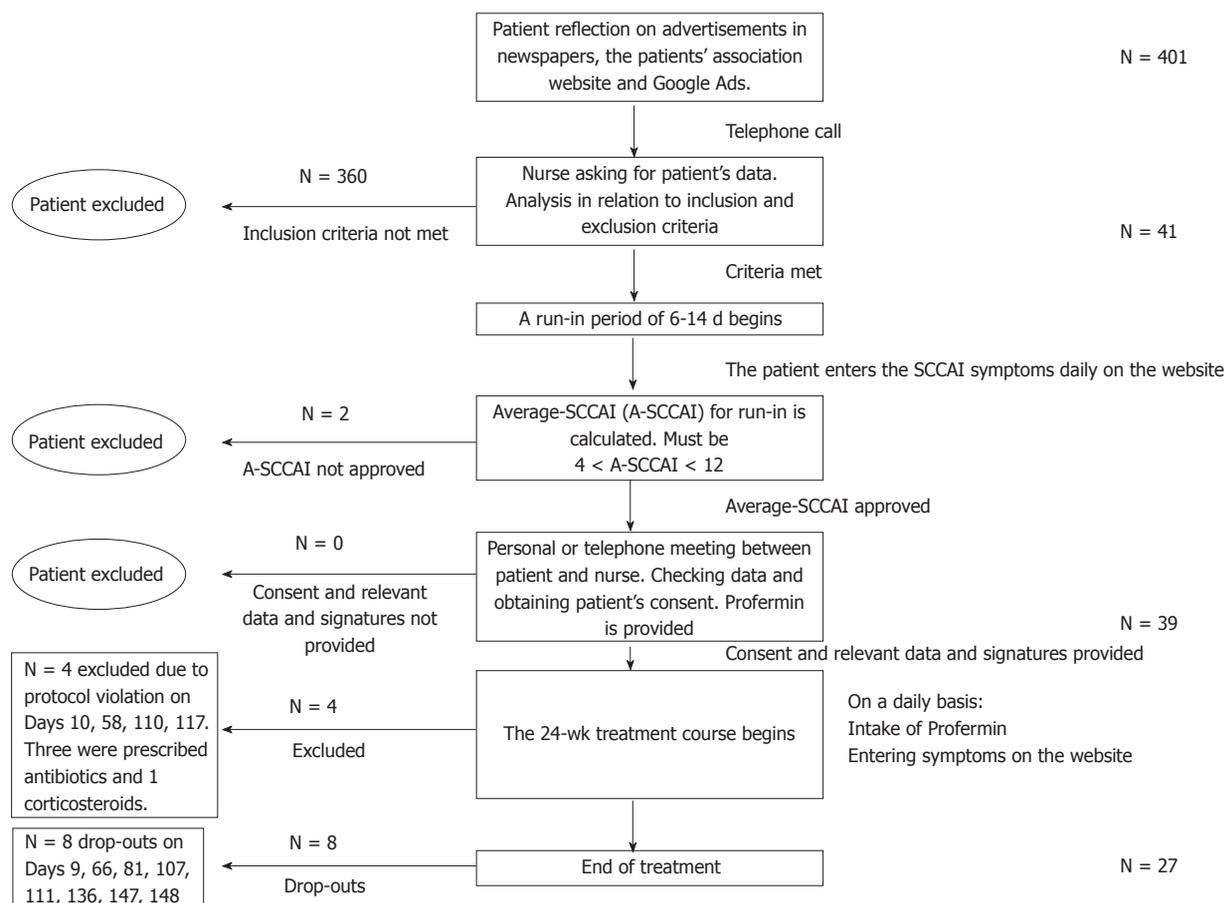


Figure 1 Patient flow diagram. SCCAI: Simple Clinical Colitis Activity Index.

score (or asymptote),  $\beta_1$  was the total reduction in score and  $\theta$  was the time taken to obtain half the reduction in score. Besides giving the parameters a marginal interpretation, it was of interest to describe the between-patient variation. Thus, we applied a nonlinear mixed-effects model, assuming that all three parameters in question followed a Gaussian distribution, i.e., each parameter was person-specific:

$$Score_{ij} = \beta_{0j} + \beta_{1j} \times 2^{-Day_i/\theta_j}$$

Here, we assumed that  $\beta_{0j} = N(\beta_0, \sigma_{\beta_0})$ ,  $\beta_{1j} = N(\beta_1, \sigma_{\beta_1})$  and  $\theta_j = N(\theta, \sigma_\theta)$ . The mixed-effect model led to the following interpretation: the estimated parameter  $\sigma_{\beta_0}$  was the between-person SD regarding the asymptote. Similarly,  $\sigma_{\beta_1}$  was the between-person SD related to the total reduction in score, and  $\sigma_\theta$  was the between-person SD related to the time taken to obtain half the reduction in score.

Before making inference, we examined both standardized residuals and estimated random effects for marginal normality by applying normal probability plots. In all statistical analysis, the software R and the package nlme were used. All tests were done as likelihood ratio tests with a significance level of 5%.

## RESULTS

The advertisements attracted 401 respondents, of whom 360 were excluded before the run-in period because the

inclusion criteria were not met (Figure 1). Reasons for exclusions were primarily SCCAI < 5 or recent change in oral corticosteroid treatment. During the run-in period, two additional patients were excluded because the SCCAI criteria were not met. The study comprised 39 patients who were available for ITT analysis: four were excluded and eight dropped out during the 24-wk treatment period (Figure 2 and Table 2).

Baseline characteristics and use of concomitant medications are shown in Tables 3 and 4.

### Safety and tolerability

No major AEs were reported and there were no drop-outs due to AEs. An increased number of bowel movements were reported by 11 patients (28%), bloating by four (10%) and an increased number of bowel movements and bloating by three (8%). All AEs were self-limiting or managed by dose adjustments. For example, if a patient experienced a presumable AE during the introduction of Profermin®, the period with the low Profermin® dose was prolonged for up to 2 wk. None of the eight dropout or four excluded patients left the trial due to deterioration in UC symptoms.

### Clinical response and remission

Of the 39 patients, 27 completed the entire study. For those completing the study, the mean follow-up was

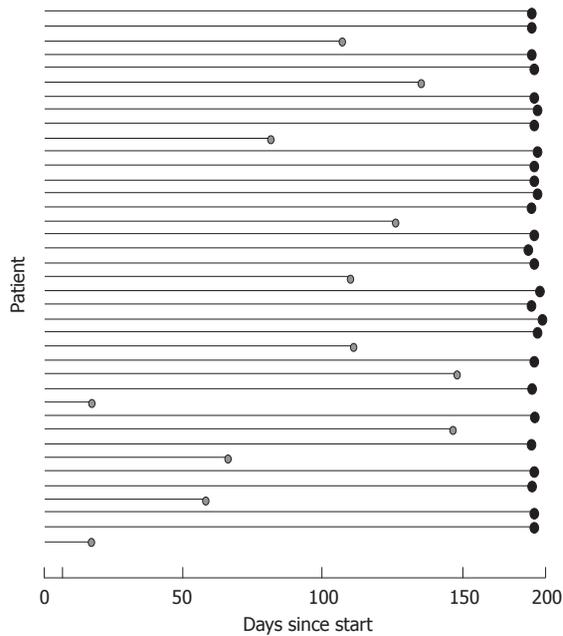


Figure 2 Point indicates that patient has dropped out.

176 d (range: 174-179 d). For those not completing the study, the mean follow-up was 94 d (range: 9-141 d). An ITT analysis showed that the mean reduction in SCCAI score was 56.5%. For the primary endpoint, ITT analysis showed that 24 of the 39 patients (62%) achieved a  $\geq 50\%$  reduction in SCCAI score (Figure 3A). In per protocol (PP) analysis, 85% reached the primary endpoint. In ITT analysis, four patients (10%) experienced deterioration in SCCAI but 13 (33%) experienced  $> 75\%$  improvement in SCCAI during the study (Figure 3B). Of the 39 patients, 18 reached the secondary endpoint and obtained remission defined as SCCAI score  $\leq 2.5$ , with an ITT success rate of 46% (Figure 3C). In PP analysis, 67% reached the secondary endpoint remission. Applying only the four defecation scores in the SCCAI (Table 1), four patients (10%) had deterioration and 17 (44%) had  $> 75\%$  improvement in defecations scores (Figure 3D).

When applying the nonlinear model for the ITT decline in SCCAI over time, the estimated ultimate score (or asymptote) was 2.8 points (95% CI: 92.0-3.6), the estimated mean reduction in score was 5.0 points (95% CI: 4.1-5.9,  $P < 0.0001$ ) and the estimated mean time taken to obtain half of the reduction in score was 28 d (95% CI: 26-30) (Figure 4).

**Descriptions of exclusions and dropouts**

Protocol violations accounted for the exclusion of four patients (10%) - days 10, 45, 100 and 116 (Table 4); three were prescribed antibiotics for pneumonia, salmonella infection and gastroenteritis, respectively, and one was prescribed corticosteroids for UC. Among those excluded, the mean SCCAI on inclusion was 9.0 (range: 8.0-11) and at exclusion 4.6 (range: 3.6-6.6). There were eight dropouts (20%) (Table 2). Among these, the mean SCCAI at inclusion was 7.0 (range: 4.6-9.0) and at drop-

Table 2 Description of four excluded patients and eight dropouts

Day	SCCAI run in	Reason for discontinuation	SCCAI dropout	Reduction	Increase
Excluded patients					
10	8.9	1	6.6	2.3	
45	8.0	1	4.1	3.9	
100	8.2	1	4.1	4.1	
116	11	2	3.6	7.4	
Drop-outs					
9	8.9	3	6.7	2.2	
54	4.6	3	5.1		0.5
73	5.9	3	7.3		1.4
100	5.6	3	8.4		2.8
102	9.0	3	6.7	2.3	
127	6.6	3	5.6	1.0	
140	6.7	3	5.3	1.4	
141	8.3	3	6.3	2.0	

Reasons for discontinuation in the study. 1: Excluded because of new medication prescribed for unrelated diseases; 2: Excluded because of new medication prescribed for colitis; 3: Dropouts main reason: Not satisfied with effect of treatment. SCCAI: Simple Clinical Colitis Activity Index.

Table 3 Demographic and baseline clinical characteristics of patients

Characteristic	Profermin, n = 39
Sex, male:female	15/24
Age, yr, median and range	35 (19-50)
Mean duration of disease, yr, median and range	7 (1-21)
Disease location, n (%)	
Proctitis	17 (44)
Left-sided colitis/procto-sigmoiditis	11 (28)
Pancolitis	11 (28)
Extraintestinal manifestations, n (%)	
Initial CRP, mg/L, median and range	15 (< 1-156)
Initial albumin, g/L, median and range	41 (23-47)

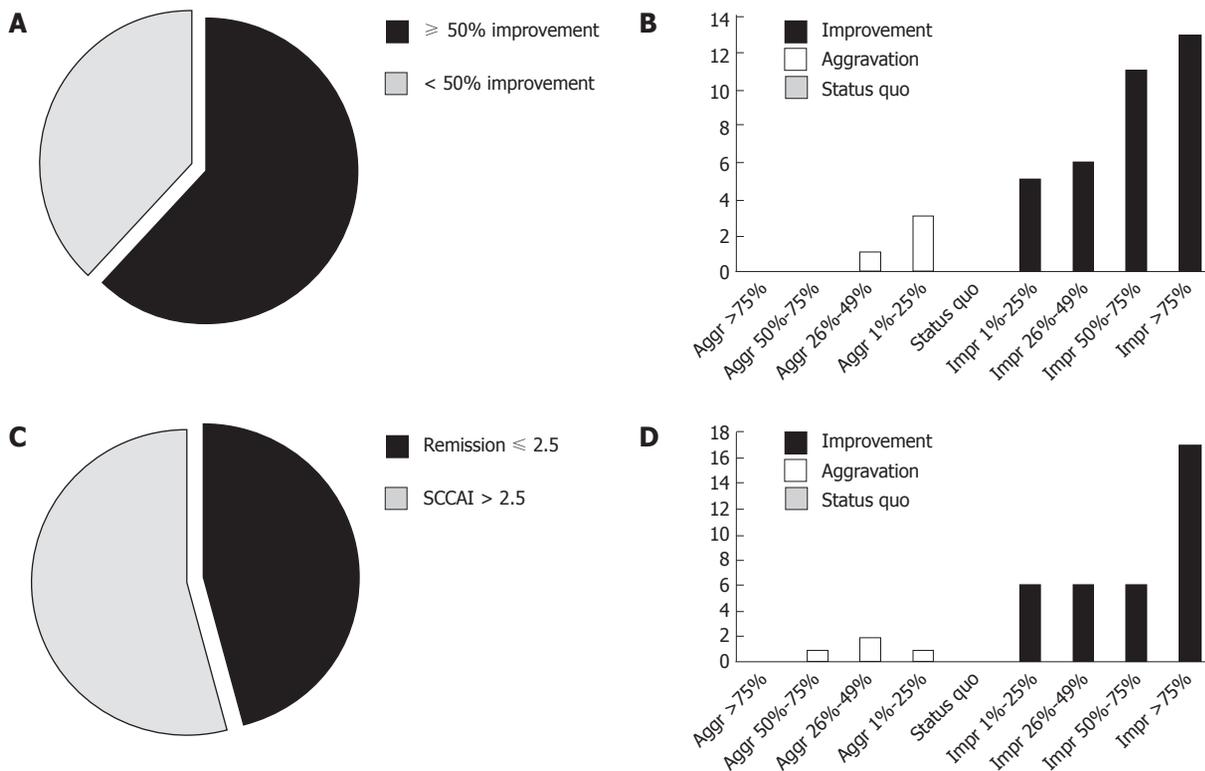
CRP: C-reactive protein.

Table 4 Concomitant medications

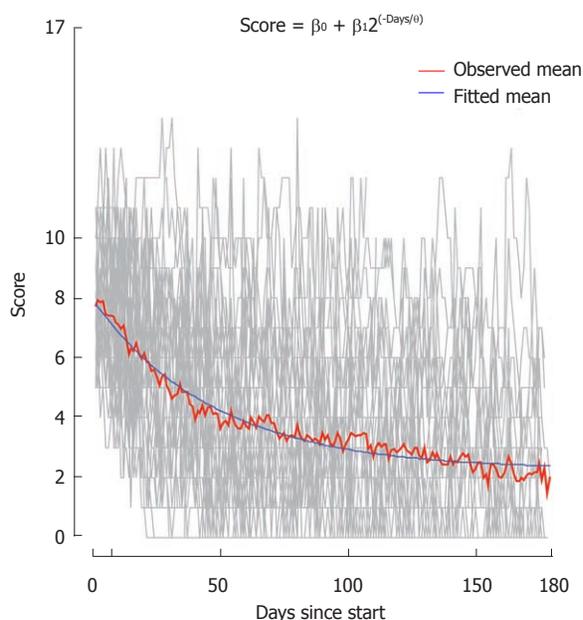
	n (%)
Mesalamine oral	
Alone	21 (54)
Combined with immunosuppressants	8 (21)
Mesalamine enema or suppository	6 (15)
Azathioprine or 6-mercaptopurine	3 (8)
Corticosteroids oral	0 (0)
Corticosteroids enema	1 (3)
Antibiotics	0 (0)
TNF- $\alpha$ inhibitors	3 (8)
None	3 (8)

TNF- $\alpha$ : Tumor necrosis factor alpha.

out 6.4 (range: 5.1-8.4). Among the dropouts, three experienced an increase in SCCAI (mean: 1.6) while in the study and five experienced a decrease (mean: 1.8). The three patients with an increase in their SCCAI score represented treatment failures.



**Figure 3** Intention to treat analysis of the primary (A, B) and secondary (C, D) endpoint. A, B: Proportion of patients with  $\geq 50\%$  reduction in Simple Clinical Colitis Activity Index (SCCAI) at week 24 and the relative development in SCCAI score; A: The primary endpoint was in intention to treat (ITT) analysis reached by 24 in 39 patients (62%); B: Illustrates the relative development in SCCAI in the last week of observation compared to the run-in week; C, D: Remission at week 24 and the relative development in the 4 defecation scores; C: The secondary endpoint was in ITT analysis reached by 18 in 39 patients (46%); D: Illustrates the relative development in the 4 defecation scores in the SCCAI (Table 1) in the last week of observation compared to the run-in week.



**Figure 4** Development in Simple Clinical Colitis Activity Index score.

## DISCUSSION

In ITT analysis, 62% of the patients reached the primary endpoint, defined as  $\geq 50\%$  reduction in SCCAI, and 46% reached the secondary endpoint of remission, defined as an SCCAI score  $\leq 2.5$ . A study on endpoints

for clinical improvement and remission in UC found a relevant clinical improvement to be a decrease of 1.5 SCCAI points and the best cut-off to establish remission as an SCCAI of 2.5<sup>[11]</sup>. In ITT analysis, the average reduction was  $> 1.5$  SCCAI points 12 d after the intervention was initiated. After 32 d, the average reduction increased to  $> 3$  SCCAI points and after 84 d to  $> 4.5$  (Figure 4). In a repeated measure regression analysis, the mean reduction in SCCAI after 24 wk of Profermin® treatment, adjusted for baseline value, was 5.0 and the estimated ultimate score was calculated to be 2.8 (Figure 4), suggesting a clinically relevant and significant effect. A meta-analysis of response rates in the placebo arms of UC trials estimated the placebo rates of remission and response to be 13% (95% CI: 9-18) and 28% (95% CI: 23-33), respectively<sup>[12]</sup>. The ITT remission and response rates in our study were 33 and 34 percentage points above these standard placebo rates, supporting a possible clinical effect of Profermin® when compared with standard placebo rates of response and remission. The safety profile of Profermin® appears favorable with no major AEs and no withdrawals due to AEs. Mild AEs were observed mainly in the initial days of treatment and ceased within a few days or after dose adjustments. Safety and tolerability of Profermin® are comparable to those of probiotics<sup>[7-9]</sup>.

Profermin® is a complex product and the mode of action is probably complex as well. However, dietary management with Profermin® uses a novel approach and

cannot be categorized as a probiotic, prebiotic or symbiotic product<sup>[13]</sup>. The dietary effect may be related to the fermented oats components such as the relatively large amounts of secondary metabolites from the fermentation process and the composition of oats *per se*. The short chain fatty acids of secondary metabolites have been shown to serve as a major source of energy for colonocytes, and  $\beta$ -glucans have been described as biological response modifiers<sup>[14,15]</sup>. *L. plantarum* is a common species in the human gastrointestinal microbiota<sup>[16-18]</sup>. *L. plantarum* 299v survives passage through the gastrointestinal tract and has been isolated from feces and rectal and jejunal biopsies 11 d after 10 d administration to healthy volunteers, indicating at least transient colonization of the gut mucosa<sup>[19-21]</sup>. It has been shown that oat gruel fermented with *L. plantarum* 299v increases iron absorption by 50%, suggesting an important dietary effect for UC<sup>[22]</sup>. In addition, the phosphatidylcholine (PC) in lecithin may serve as an important food component because the content of PC in the colonic mucus of patients with UC is significantly lower compared with the content in healthy controls<sup>[23]</sup>. Lastly, the daily intake of relatively large quantities of fermented oats may change the intestinal contents and environment and thereby establish an altered platform for microbial activity.

In this study, we introduced a new online self-reporting Internet-based system for assessing daily activity in UC and for monitoring response to treatment. The system allows electronic monitoring of data and enables daily reporting with minimal interruption to the patient's daily life. The risk of patients being influenced in their self-assessment by health personnel is limited. Independent monitoring and statistical analyses can be achieved easily. In Denmark, where most people have Internet access, this system is a very useful research tool in a population of UC patients.

Our study had some limitations: (1) it was an uncontrolled study and the results need to be confirmed in a randomized trial; and (2) patients were informed to consider their consumption of dairy products and concentrated sugar products, in particular fresh milk and confectionary. This may have had an effect on UC or may have reinforced the effect of Profermin®.

The present study demonstrates that Profermin® is safe and may be effective in inducing remission of active UC. Randomized controlled studies are planned to explore further the clinical efficacy of Profermin®.

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## COMMENTS

### Background

Ulcerative colitis (UC) is a chronic relapsing bowel disease. The goal of treat-

ment in UC is to induce and maintain remission of the disease, however, failure to induce remission occurs in 20%-30% of patients on current treatments. Accordingly, new treatment alternatives and additives are being sought. Dietetic interventions may improve symptoms in UC.

### Research frontiers

Clinical and experimental studies point towards alterations in the relative balance of aggressive and protective bacterial species in UC. Interventions to alter the intestinal microflora in order to decrease disease activity are under investigation and prebiotics, probiotics and symbiotics have been investigated in UC but the reported efficacies have varied.

### Innovations and breakthroughs

This is believed to be the first study to assess Profermin® in UC. The study was an open label study that investigated safety and efficacy of Profermin® in patients with moderate active UC. Profermin® is a new developed product for the dietary management of UC. It is a fermented oat gruel with *Lactobacillus plantarum* 299v, barley malt, lecithin and water.

### Applications

Profermin® is safe and well tolerated and may be effective in reducing symptoms and inducing remission in UC. Larger randomized trials should be performed to investigate further the efficacy of Profermin® in UC.

### Terminology

Simple Clinical Colitis Activity Index (SCCAI) assessed active disease. SCCAI is a clinical scoring system for UC, based on bowel frequency day and night, urgency, fecal blood, general well being and extracolonic manifestations.

### Peer review

The authors have reported a study on the safety of Profermin®, a combination of fermented oats, probiotics and lecithin, in patients with mild to moderate UC. This is primarily an open-label safety study in patients. The agent was well-tolerated and may be efficacious.

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## Glycer-AGEs-RAGE signaling enhances the angiogenic potential of hepatocellular carcinoma by upregulating VEGF expression

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### Abstract

**AIM:** To investigate the effect of glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) on hepatocellular carcinoma (HCC) cells.

**METHODS:** Two HCC cell lines (Hep3B and HepG2 cells) and human umbilical vein endothelial cells (HUVEC) were used. Cell viability was determined using the WST-8 assay. Western blotting, enzyme linked immunosorbent assay, and real-time reverse transcription-polymerase chain reactions were used to detect protein and mRNA. Angiogenesis was evaluated by assessing the proliferation, migration, and tube formation of HUVEC.

**RESULTS:** The receptor for AGEs (RAGE) protein was detected in Hep3B and HepG2 cells. HepG2 cells were

not affected by the addition of Glycer-AGEs. Glycer-AGEs markedly increased vascular endothelial growth factor (VEGF) mRNA and protein expression, which is one of the most potent angiogenic factors. Compared with the control unglycated bovine serum albumin (BSA) treatment, VEGF mRNA expression levels induced by the Glycer-AGEs treatment were  $1.00 \pm 0.10$  vs  $1.92 \pm 0.09$  ( $P < 0.01$ ). Similarly, protein expression levels induced by the Glycer-AGEs treatment were  $1.63 \pm 0.04$  ng/mL vs  $2.28 \pm 0.17$  ng/mL for the 24 h treatment and  $3.36 \pm 0.10$  ng/mL vs  $4.79 \pm 0.31$  ng/mL for the 48 h treatment, respectively ( $P < 0.01$ ). Furthermore, compared with the effect of the control unglycated BSA-treated conditioned medium, the Glycer-AGEs-treated conditioned medium significantly increased the proliferation, migration, and tube formation of HUVEC, with values of  $122.4\% \pm 9.0\%$  vs  $144.5\% \pm 11.3\%$  for cell viability,  $4.29 \pm 1.53$  vs  $6.78 \pm 1.84$  for migration indices, and  $71.0 \pm 7.5$  vs  $112.4 \pm 8.0$  for the number of branching points, respectively ( $P < 0.01$ ).

**CONCLUSION:** These results suggest that Glycer-AGEs-RAGE signaling enhances the angiogenic potential of HCC cells by upregulating VEGF expression.

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**Key words:** Advanced glycation end-products; Angiogenesis; Glyceraldehyde; Hepatocellular carcinoma; Nonalcoholic steatohepatitis

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## INTRODUCTION

Advanced glycation end-products (AGEs) are formed by the Maillard reaction, a non-enzymatic reaction between the ketones or aldehydes of sugars and the amino groups of proteins, which contributes to aging and the pathological complications of diabetes<sup>[1,2]</sup>. Recent studies have suggested that AGEs can be formed not only from sugars, but also from carbonyl compounds derived from the autoxidation of sugars and other metabolic pathways<sup>[3,4]</sup>. Among the different AGEs, there is evidence that glyceraldehyde-derived AGEs (Glycer-AGEs) are associated with the complications of diabetes, as well as Alzheimer's disease, nonalcoholic steatohepatitis (NASH), and cancer<sup>[5-8]</sup>.

NASH is recognized as a component of metabolic syndrome and is associated with insulin resistance and abnormalities in glucose and lipid metabolism<sup>[9-11]</sup>. NASH is one of a group of nonalcoholic fatty liver diseases (NAFLD) that range from simple steatosis to steatohepatitis<sup>[12]</sup>. However, although simple steatosis appears to be a benign and non-progressive condition, NASH is a potentially progressive disease that can lead to cirrhosis, liver failure, and hepatocellular carcinoma (HCC)<sup>[13,14]</sup>. In fact, several case series of NASH-associated HCC have been reported<sup>[15,16]</sup>. HCC, which accounts for more than 90% of all primary liver cancers, is one of the most common malignancies worldwide<sup>[17]</sup>. Its incidence is particularly high in the Asian population<sup>[18]</sup>.

A recent study suggested that expression of the receptor for AGEs (RAGE) mRNA was lower in normal liver cells than in those of hepatitis and HCC patients<sup>[19]</sup>. Furthermore, we have demonstrated that Glycer-AGEs are present in significantly high concentrations in the sera of patients with NASH<sup>[8]</sup>. In addition, the interaction of Glycer-AGEs with the RAGE was found to increase C-reactive protein expression in Hep3B cells<sup>[20]</sup>. However, the effects of Glycer-AGEs on HCC cells remain poorly understood.

In the present study, we examined the effects of Glycer-AGEs on HCC cells and showed that Glycer-AGEs-RAGE signaling enhances the angiogenic potential of HCC cells by upregulating vascular endothelial growth factor (VEGF) expression.

## MATERIALS AND METHODS

### Preparation of glyceraldehyde-derived advanced glycation end-products

All chemicals were commercial samples of high purity and were used as supplied. Glycer-AGEs were prepared as described previously<sup>[21]</sup>. Briefly, 25 mg/mL of bovine serum albumin (BSA; A0281, Sigma-Aldrich) was incubated at 37 °C for 7 d under sterile conditions with 0.1 mol/L glyceraldehyde and 5 mmol/L diethylenetriaminepentaacetic acid (Dojindo Laboratories, Kumamoto, Japan) in 0.2 mol/L phosphate buffer (pH 7.4). As a control, unglycated BSA was incubated under the same con-

ditions, but without glyceraldehyde. The unglycated and glycated albumin were purified using a PD-10 column (GE Healthcare UK Ltd., Buckinghamshire, England) and dialysis against PBS. All preparations were tested for endotoxin using the Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan). Protein concentrations were determined using the Dc protein assay reagent (Bio-Rad Laboratories, Richmond, CA, United States), using BSA as a standard. In all experiments, control unglycated BSA and Glycer-AGEs were used at culture medium concentrations of 100 µg/mL.

### Cell cultures

Hep3B and HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, United States) under standard cell culture conditions (humidified atmosphere, 5% CO<sub>2</sub>, 37 °C). Cells ( $1.5 \times 10^4$  cells/cm<sup>2</sup>) were then seeded in various plates or culture dishes (BD Biosciences, Franklin Lakes, NJ, United States) and incubated for 48 h before the start of all experiments, except the migration assay. The control unglycated BSA and Glycer-AGEs (100 µg/mL) treatments were carried out in serum free DMEM.

Human umbilical vein endothelial cells (HUVEC) were grown in endothelial cell growth medium (GM; Cell Applications, San Diego, CA, United States) under standard cell culture conditions.

### Preparation of cell lysate

Cells were washed with ice-cold Ca<sup>2+</sup> and Mg<sup>2+</sup> free PBS [PBS (-)] and subjected to lysis buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L sodium chloride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 × protease inhibitor cocktail (complete, Mini; Roche)]. Subsequently, cell lysates were passed through a syringe several times for further homogenization and centrifuged at 10 000 × *g* for 10 min at 4 °C. Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories).

### Western blotting

Cell lysates (30 µg of proteins/lane) were dissolved in SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.01% bromophenol blue] containing 5% 2-mercaptoethanol, boiled for 3 min at 95 °C, separated by SDS-polyacrylamide gel electrophoresis, and then electro-transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States). Biotinylated markers (Cell Signaling, Beverly, MA, United States) were used as molecular weight markers. Membranes were blocked for 1 h using 5% skimmed milk in phosphate buffered saline (PBS) containing 0.05% polyoxyethylene sorbitan monolaurate (PBS-T). After being washed twice with PBS-T, membranes were incubated overnight with goat anti-RAGE antibody (N-16), mouse anti-β-actin antibody (Santa Cruz, Santa Cruz, CA, United States), or rabbit anti-cyclooxygenase-2 (anti-COX-2)

antibody (Cayman Chemical, Ann Arbor, MI, United States). Subsequently, membranes were washed twice with PBS-T and incubated with anti-goat IgG antibody (Santa Cruz), anti-mouse Ig's antibody (Biosource, Camarillo, CA, United States), or anti-rabbit IgG and anti-biotin antibodies (Cell Signaling) for 1 h. After being washed a further three times with PBS-T, immunoreactive proteins were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare) using a luminescent image analyzer (LAS-1000UVmini; Fujifilm, Tokyo, Japan). The density of the bands was analyzed using a Multi Gauge version 3.0 (Fujifilm).

### Cell viability

Cell viability was determined using the WST-8 assay, which measures metabolic activity. After removing the medium from a 96-well microplate that had been used to culture cells as above, 100  $\mu$ L/well of 10% FBS/DMEM and 10  $\mu$ L/well of WST-8 solution (Dojindo Laboratories) were added, and cells were incubated for 2 h. Absorbance was then measured at 450 nm and 650 nm using a microplate reader (Labsystems Multiskan Ascent, Model No. 354; Thermo Fisher Scientific, Kanagawa, Japan). The net difference ( $A_{450}-A_{650}$ ) was used as a measure of cell viability.

### Real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from cells with ISOGEN (Nippon Gene, Tokyo, Japan), and 50 ng of RNA were reverse transcribed into cDNA with the PrimeScript™ reverse transcription (RT) reagent kit (Takara, Shiga, Japan) using a GeneAmp® 9700 polymerase chain reaction (PCR) System (Perkin-Elmer Applied Biosystems, Foster City, CA, United States). Real-time polymerase chain reaction was performed with SYBR Premix Ex Taq™ (Takara) using a Smart Cycler® II System (Takara). The reaction mixture (25  $\mu$ L) contained 1  $\times$  SYBR Premix Ex Taq™, 0.2  $\mu$ mol/L PCR forward primers, 0.2  $\mu$ mol/L PCR reverse primers, and 10 ng of cDNA as a template. The primers used were as follows: COX-2: 5'-GAGTACCGCAAACGCTTTATGC-3' and 5'-GCCGAGGCTTTTC-TACCAGAA-3', VEGF: 5'-TGCAGATTATGCGGATCAAACC-3' and 5'-TGCAITTCACATTTGTTGTGCTGTAG-3', and  $\beta$ -actin: 5'-TCCACCTCCAGCAGATGTGG-3' and 5'-GCATTTGCGGTGGACGAT-3'. All processes were performed according to the manufacturer's instructions. Expression levels of the target genes were calculated using a relative quantification method.  $\beta$ -Actin was used as an endogenous control gene to normalize target gene expression values. Product specificity was determined by a melting curve analysis.

### Enzyme linked immunosorbent assay

Cells were incubated with control unglycated BSA or Glycer-AGEs for 24 h or 48 h. The culture medium was collected and centrifuged at 200  $\times g$  for 10 min to remove any particles, and the resultant supernatant was analyzed

using the VEGF enzyme-linked immunosorbent assay kit (Ray Biotech, Norcross, GA, United States). All processes were performed according to the manufacturer's instructions.

### Migration assay

The migratory capacity of Hep3B cells was evaluated using the Oris™ Cell Migration Assay (Platypus Technologies, Madison, WI, United States). Cells ( $1.5 \times 10^5$  cells/mL) were incubated with 10% FBS/DMEM for 24 h. After removing the stopper covering the center of the well, fluorescently-labeled cells were incubated with control unglycated BSA or Glycer-AGEs for 24 h. The number of cells that had migrated to the center was assessed at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using a fluorescence microplate reader (Labsystems Fluoroskan Ascent CF, Type 374; Thermo Fisher Scientific).

### Preparation of conditioned medium

Hep3B cells were incubated with control unglycated BSA or Glycer-AGEs for 48 h. The culture medium was collected and filtered to remove any particles. The CM was then frozen at -80 °C until it was used in the experiments.

### Human umbilical vein endothelial cells proliferation assay

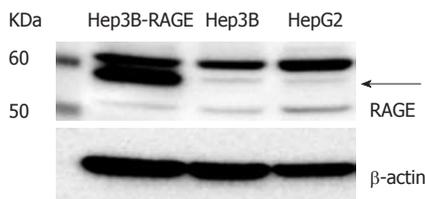
HUVEC ( $0.75 \times 10^4$  cells/cm<sup>2</sup>) were incubated with GM for 24 h, before being cultured in DMEM or CM in 10% FBS for 72 h. HUVEC proliferation was determined using the WST-8 assay.

### Human umbilical vein endothelial cells migration assay

The migratory capacity of HUVEC was evaluated using the BD BioCoat™ Angiogenesis System-Endothelial Cell Migration assay (BD Biosciences). In this assay, the upper and lower culture compartments were separated by fluorescence blocking polyethylene terephthalate filters (3  $\mu$ m pore size) coated with human fibronectin. Cells ( $3.3 \times 10^5$  cells/mL) in serum-free DMEM were added to each of the upper chambers for 20 h. Then, 0.5% FBS/CM was added to the lower chamber and used as a chemoattractant. The number of fluorescently-labeled cells that had migrated to the opposite side of the chamber was then assessed at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using a fluorescence microplate reader (Thermo Fisher Scientific).

### Tube formation assay

The tube formation assay was performed with the BD BioCoat™ Angiogenesis System-Endothelial Cell Tube Formation assay (BD Biosciences). Before the start of the assay, the Matrigel matrix was polymerized for 30 min at 37 °C under a 5% CO<sub>2</sub> environment. Cells ( $4 \times 10^5$  cells/mL) were incubated with 0.5% FBS/CM for 12 h and then photographed under a microscope, and the number of branch points was counted in five randomly chosen fields.



**Figure 1** The receptor for advanced glycation end-products expression in hepatocellular carcinoma cells. The receptor for advanced glycation end-products (RAGE) expression as measured by Western blotting. Cell lysates (30  $\mu$ g of proteins/lane) were loaded onto a 10% polyacrylamide gel. Size markers (kDa) are shown on the left. Equal protein loading was verified using an anti- $\beta$ -actin antibody. The arrow indicates full-length RAGE.

### Statistical analysis

All experiments were performed in duplicate and repeated at least two or three times, with each experiment yielding essentially identical results. Data are expressed as the mean  $\pm$  SD. The significance of the differences between group means was determined by a one-way analysis of variance. *P* values of less than 0.05 were considered significant.

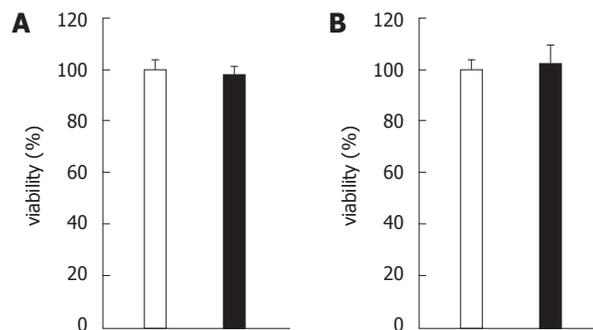
## RESULTS

To investigate whether RAGE proteins were present in Hep3B and HepG2 cells, we carried out Western blotting using an anti-RAGE antibody. RAGE proteins of different molecular weights were detected in Hep3B and HepG2 cells (Figure 1). In full-length RAGE cDNA-transfected Hep3B cells, the major band (57 kDa) (indicated by an arrow in Figure 1) represented the full-length RAGE protein. Likewise, the full-length RAGE protein was also detected in Hep3B and HepG2 cells, and there was no difference in its expression level between the two cell types.

We examined the effect of Glycer-AGEs on the viability of Hep3B and HepG2 cells. Cell viabilities resulting from treatment with the control unglycated BSA or the Glycer-AGEs for 24 h were  $100\% \pm 3.5\%$  *vs*  $97.8\% \pm 3.3\%$  in Hep3B cells (Figure 2A), and  $100\% \pm 4.3\%$  *vs*  $102.3\% \pm 6.7\%$  in HepG2 cells (Figure 2B). Thus, Glycer-AGEs did not have any effect on the viability of Hep3B and HepG2 cells.

To investigate whether Glycer-AGEs affected the malignancy of HCC cells, we examined COX-2 mRNA and protein expression. COX-2 mRNA expression levels induced by the control unglycated BSA or the Glycer-AGEs treatment were  $1.00 \pm 0.27$  *vs*  $2.16 \pm 0.34$  ( $P < 0.01$ ) (Figure 3A), and COX-2 protein expression levels were increased by Glycer-AGEs at 24 h in Hep3B cells (Figure 3B), whereas no such change was detected in HepG2 cells (Figure 3B).

We also evaluated the influence of Glycer-AGEs on cell migration, which is an index of malignancy in Hep3B cells. However, migration indices for the control unglycated BSA or the Glycer-AGEs treatment were  $18.2 \pm 0.6$  *vs*  $18.7 \pm 0.5$  (Figure 3C), and Glycer-AGEs did not



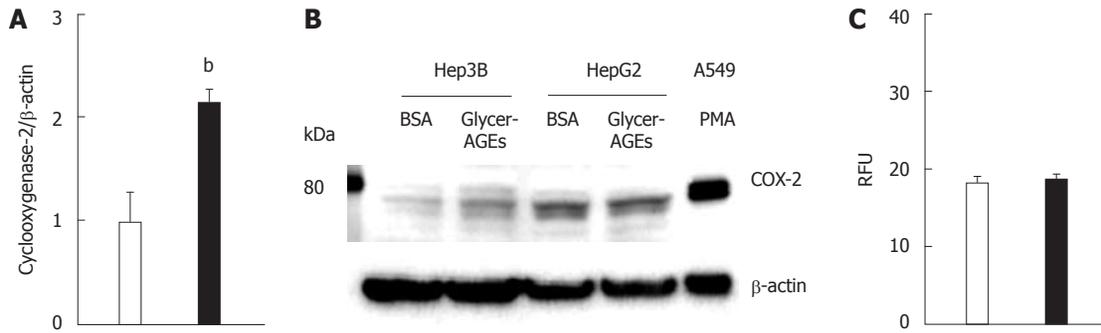
**Figure 2** Effect of glyceraldehyde-derived advanced glycation end-products on the viability of hepatocellular carcinoma cells. Cell viability was determined using the WST-8 assay. Hep3B (A) and HepG2 (B) cells were incubated with control unglycated bovine serum albumin (BSA) or glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) (100  $\mu$ g/mL) for 24 h. The open and filled bars represent results for cells treated with control unglycated BSA and Glycer-AGEs, respectively. Data are shown as the mean  $\pm$  SD ( $n = 6$ ).

affect the migratory capacity of Hep3B cells; i.e., Glycer-AGEs did not increase the malignancy of Hep3B cells.

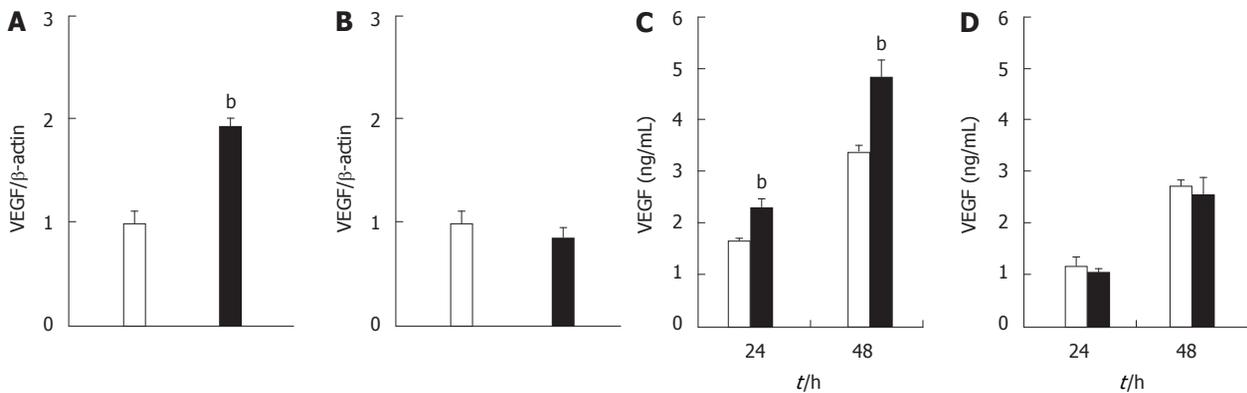
To investigate whether Glycer-AGEs affected the angiogenesis of HCC cells, we examined the expression levels of VEGF mRNA and protein. VEGF mRNA expression levels induced by the control unglycated BSA or the Glycer-AGEs treatment were  $1.00 \pm 0.10$  *vs*  $1.92 \pm 0.09$  in Hep3B cells ( $P < 0.01$ ) (Figure 4A), and  $1.00 \pm 0.11$  *vs*  $0.87 \pm 0.10$  in HepG2 cells (Figure 4B). The expression levels of the VEGF protein induced by the control unglycated BSA or the Glycer-AGEs treatment for 24 and 48 h were  $1.63 \pm 0.04$  ng/mL *vs*  $2.28 \pm 0.17$  ng/mL (24 h,  $P < 0.01$ ), and  $3.36 \pm 0.10$  ng/mL *vs*  $4.79 \pm 0.31$  ng/mL (48 h,  $P < 0.01$ ), respectively, in Hep3B cells (Figure 4C). In HepG2 cells, the results were  $1.15 \pm 0.19$  ng/mL *vs*  $1.04 \pm 0.03$  ng/mL (24 h), and  $2.70 \pm 0.10$  ng/mL *vs*  $2.53 \pm 0.32$  ng/mL (48 h), respectively, (Figure 4D). Thus, the VEGF mRNA expression of Hep3B cells was increased by Glycer-AGEs at 24 h, and VEGF protein expression levels in these cells were also increased by Glycer-AGEs at 24 and 48 h. However, no such changes were observed in HepG2 cells.

We then examined the effect of CM-Glycer-AGEs on the viability of HUVEC. Cell viabilities resulting from treatment with the control unglycated BSA or the Glycer-AGEs for 72 h were  $100\% \pm 6.4\%$  *vs*  $96.2\% \pm 5.4\%$ , and the control unglycated BSA-treated CM (CM-BSA) or the CM-Glycer-AGEs for 72 h were  $122.4\% \pm 9.0\%$  *vs*  $144.5\% \pm 11.3\%$  ( $P < 0.01$ ) (Figure 5). There was no difference in viability between the Glycer-AGEs-treated and control unglycated BSA-treated cells, whereas cell viability in cells treated with CM-Glycer-AGEs was significantly higher than that of those treated with CM-BSA.

Finally, we examined the effect of CM-Glycer-AGEs on the migration and tube formation of HUVEC. Migration and tube formation of endothelial cells play key roles in tumor angiogenesis. Migration indices for the CM-BSA or the CM-Glycer-AGEs treatment were  $4.29 \pm 1.53$  *vs*  $6.78 \pm 1.84$  ( $P < 0.05$ ) (Figure 6A), and cell migration



**Figure 3** Effect of glycer-AGEs on the malignancy of hepatocellular carcinoma cells. Hep3B and HepG2 cells were incubated with control unglycated bovine serum albumin (BSA) or glycer-AGEs for 24 h. A: In Hep3B cells, COX-2 mRNA expression levels were analyzed using real-time reverse transcription-polymerase chain reactions, and results were normalized to the  $\beta$ -actin mRNA level ( $n = 3$ );  $^bP < 0.01$  vs control unglycated BSA. B: COX-2 expression as measured by Western blotting. Cell lysates (30  $\mu$ g of proteins/lane) were loaded onto a 10% polyacrylamide gel. Size markers (kDa) are shown on the left. Equal protein loading was verified using an anti- $\beta$ -actin antibody. As a positive control, A549 cells were incubated with phorbol 12-myristate 13-acetate (PMA: 100 nmol) for 6 h; C: The migratory capacity of Hep3B cells was evaluated using the Oris cell migration assay ( $n = 8$ ). Cells were incubated with control unglycated BSA or Glycer-AGEs for 24 h, and the number of migrating cells was then assessed using a fluorescence microplate reader. RFU: Relative fluorescence units. The open and filled bars represent results for cells treated with control unglycated BSA and Glycer-AGEs, respectively. Data are shown as the mean  $\pm$  SD.



**Figure 4** Effect of glycer-AGEs on the angiogenesis of hepatocellular carcinoma cells. A and B: Hep3B and HepG2 cells were incubated with control unglycated bovine serum albumin (BSA) or glycer-AGEs for 24 h. Vascular endothelial growth factor (VEGF) mRNA expression was analyzed using real-time reverse transcription-polymerase chain reactions, and results were normalized to the  $\beta$ -actin mRNA level; C and D: Hep3B and HepG2 cells were incubated with control unglycated BSA or Glycer-AGEs for 24 or 48 h. The conditioned medium was collected, and VEGF expression levels of the cells were determined by enzyme-linked immunosorbent assay. The open and filled bars represent results for cells treated with control unglycated BSA and Glycer-AGEs, respectively. Data are shown as the mean  $\pm$  SD ( $n = 3$ );  $^bP < 0.01$  vs control unglycated BSA.

with the CM-Glycer-AGEs treatment was significantly higher than that of the CM-BSA treatment. In tube formation, the CM-Glycer-AGEs-treated HUVEC showed an increased number of tube-like structures and larger tube networks (Figure 6B). Furthermore, the number of branching points for the CM-BSA or the CM-Glycer-AGEs treatment were  $71.0\% \pm 7.5\%$  vs  $112.4\% \pm 8.0\%$  ( $P < 0.01$ ) (Figure 6C), and the number of branching points with the CM-Glycer-AGEs treatment was significantly higher than that of the CM-BSA treatment (Figure 6C).

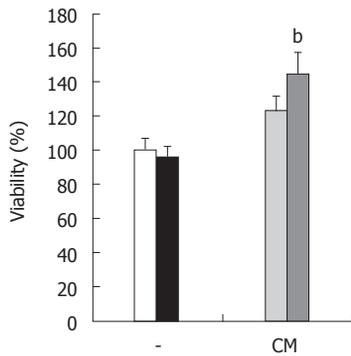
These results showed that Glycer-AGEs enhance the angiogenic potential of HCC cells by upregulating VEGF expression.

## DISCUSSION

The incidence of HCC in developed countries has been increasing over the last 20 years<sup>[22]</sup>. Although hepatitis C

virus is responsible for half of the recent increase in the prevalence of HCC, the etiologies of 15%-50% of new HCC cases remain unclear<sup>[23]</sup>. NASH, a component of metabolic syndrome, is thought to be responsible for some of these cases<sup>[15,16]</sup>. AGEs are one possible mechanistic link between metabolic syndrome and NASH, and Glycer-AGEs have been reported to be involved in NASH<sup>[8,24]</sup>; however, their role in HCC has barely been investigated.

A recent study suggested that the expression of RAGE mRNA was lower in normal liver tissue than in the liver cells of hepatitis and HCC patients. In addition, in HCC, RAGE mRNA expression was high in well and moderately differentiated tumors, but declined as the tumors dedifferentiated to poorly differentiated HCC<sup>[19]</sup>. The Hep3B and HepG2 cells used in this experiment were well-differentiated HCC cell lines. RAGE protein was detected in both Hep3B and HepG2 cells and there was no difference in its expression between the two cell lines.

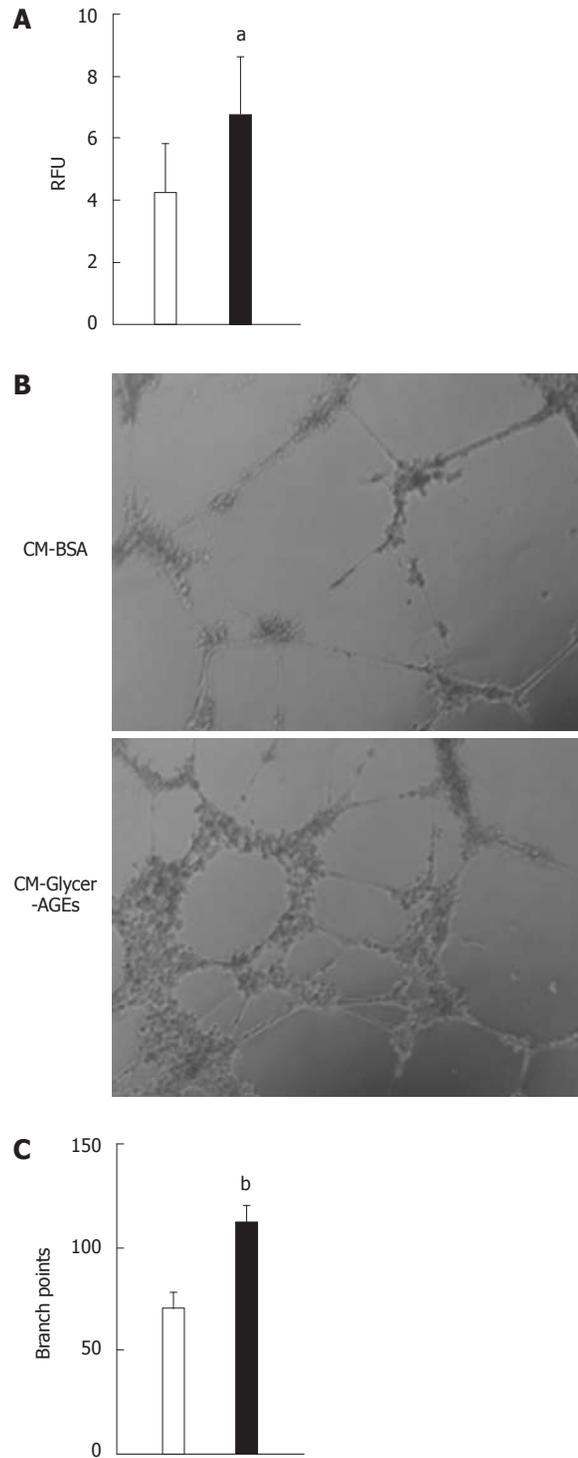


**Figure 5** Effect of glycer-aldehyde-derived advanced glycation end-products-treated CM on human umbilical vein endothelial cells proliferation. Cell viability was determined with the WST-8 assay. Human umbilical vein endothelial cells were incubated with control unglycated bovine serum albumin (BSA), glycer-aldehyde-derived advanced glycation end-products (Glycer-AGEs) (100  $\mu$ g/mL), CM-BSA, or CM-Glycer-AGEs for 72 h. The open and filled bars represent results for cells treated with control unglycated BSA and Glycer-AGEs, respectively, and the light grey and the black grey bars represent results for cells treated with CM-BSA and CM-Glycer-AGEs, respectively. Data are shown as the mean  $\pm$  SD ( $n = 6$ ); <sup>b</sup> $P < 0.01$  vs CM-BSA.

However, the effects of Glycer-AGEs differ between Hep3B and HepG2 cells. A previous report found that Hep3B cells activate AGEs signaling through RAGE<sup>[20]</sup>. On the other hand, it was reported that HepG2 cells did not express the RAGE protein on their cell surfaces and were not affected by Glycer-AGEs<sup>[25]</sup>. HepG2 cells were also not affected by Glycer-AGEs in our study.

In this study, Glycer-AGEs did not increase cell growth or migration of Hep3B cells. However, Glycer-AGEs slightly increased COX-2 protein expression levels in these cells. This protein plays important roles in HCC malignancy by producing prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), including cell growth, migration, and invasion<sup>[26,27]</sup>, and PGE<sub>2</sub> promotes the migration of HCC cell lines in  $> 1.5 \mu$ g/mL<sup>[28-30]</sup>. Indeed, PGE<sub>2</sub> protein expression levels induced by COX-2 increased by Glycer-AGEs, but the quantity was  $< 6$  pg/mL (data not shown). The results imply that although Glycer-AGEs induced increases in the expression levels of COX-2 and PGE<sub>2</sub> protein, they were not sufficient to increase cell growth or migration.

On the other hand, Glycer-AGEs markedly increased the VEGF protein expression levels of Hep3B cells. VEGF is one of the most potent angiogenic factors<sup>[31]</sup>, and angiogenesis plays a significant role in HCC progression<sup>[32-34]</sup>. Proliferation, migration, and tube formation of endothelial cells are important events in angiogenesis<sup>[35]</sup>. In addition, CM-Glycer-AGEs significantly increased the proliferation, migration, and tube formation of HUVEC. The results suggested that Glycer-AGEs indirectly increased angiogenesis in HCC. The formation of new blood vessels is initiated by hypoxic or ischemic conditions<sup>[36]</sup>. Interestingly, it was reported that HCC cell lines that are resistant to hypoxia displayed higher levels of RAGE expression, and RAGE expression in these cell lines increased under hypoxic conditions<sup>[19]</sup>. These results suggest that Glycer-AGEs-RAGE signaling is increased



**Figure 6** Effect of glycer-aldehyde-derived advanced glycation end-products-treated CM on human umbilical vein endothelial cells angiogenesis. A: The migratory capacity of human umbilical vein endothelial cells (HUVEC) was evaluated using the endothelial cell migration assay. Cells were incubated with CM-bovine serum albumin (BSA) or glycer-aldehyde-derived advanced glycation end-products-treated CM (CM-Glycer-AGEs) for 22 h, and the number of migrating cells was assessed using a fluorescence microplate reader. RFU: relative fluorescence units ( $n = 8$ ); B and C: The tube formation of HUVEC was evaluated using the endothelial cell tube formation assay ( $n = 5$ ). Cells were incubated with CM-BSA or CM-Glycer-AGEs for 12 h and then photographed under a microscope (B); the number of branch points was counted (C). Magnification = 100  $\times$ . The open and filled bars represent results for cells treated with CM-BSA and CM-Glycer-AGEs, respectively. Data are shown as the mean  $\pm$  SD; <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs CM-BSA.

during the early stages of tumorigenesis, which occurs in hypoxic conditions.

In summary, we have demonstrated that Glycer-AGEs-RAGE signaling enhances the angiogenic potential of HCC cells by upregulating VEGF expression. These results suggest that Glycer-AGEs-RAGE signaling plays a critical role in the progression of HCC, and hence, is a potential target for therapeutic intervention.

## COMMENTS

### Background

Advanced glycation end-products (AGEs) are formed by the Maillard reaction, a non-enzymatic reaction between the ketones or aldehydes of sugars and the amino groups of proteins that contributes to aging and the pathological complications of diabetes. Among the different AGEs, there is evidence that Glycer-AGEs are associated with the complications of diabetes, as well as nonalcoholic steatohepatitis (NASH) and cancer. NASH is a potentially progressive disease that can lead to cirrhosis, liver failure and hepatocellular carcinoma (HCC). In fact, several case series of NASH-associated HCC have been reported. However, the effects of Glycer-AGEs on HCC cells remain poorly understood.

### Research frontiers

Angiogenesis plays a significant role in HCC progression, and vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors. In this study, the authors examined the effects of Glycer-AGEs on the angiogenesis of HCC cells.

### Innovations and breakthroughs

This study reported the Glycer-AGEs enhanced the angiogenic potential of HCC cells by upregulating VEGF expression.

### Applications

The experimental data can be used in further studies as a potential target for therapeutic intervention.

### Terminology

AGEs: AGEs are formed by the Maillard reaction, a non-enzymatic reaction between the ketones or aldehydes of sugars and the amino groups of proteins that contributes to aging and the pathological complications of diabetes; RAGE: RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules, and interacts with distinct molecules implicated in homeostasis, development, and inflammation properties; NASH: NASH is a disease having the histopathological findings typical of alcoholic liver disease in patients without a history of significant alcohol abuse; VEGF: VEGF, which is also known as vascular permeability factor, is a specific mitogen to endothelial cells.

### Peer review

The work presented is very interesting, and I am sure further work in the future will extend to clinical studies as a potential target for therapeutic intervention.

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## Prospective controlled study on the effects of polyethylene glycol in capsule endoscopy

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### Abstract

**AIM:** To prospectively confirm whether a small amount of polyethylene glycol (PEG) ingested after swallowing endoscopy capsule improves image quality and completion rate.

**METHODS:** Forty-four consecutive patients referred to us for capsule endoscopy (CE) were randomized to two groups. All patients were restricted to clear fluids for 12 h before the examination. Patients in group A (22 cases) received no additional preparation, while those in group B (20 cases) ingested 500 mL of PEG within a 2 h period starting 30 min after swallowing the capsule. Clear fluids and meals were allowed 2 h and 4 h after capsule ingestion, respectively. Image quality was assessed as the percentage of visualized bowel surface area as follows: 1: < 25%; 2: 25%-49%; 3: 50%-74%; 4: 75%-89%; 5: > 90%. The small bowel record was divided into five segments by time, and the score for each segment was evaluated. All CE examinations were performed with the Pillcam SB capsule endoscopy sys-

tem (Given Imaging Co. Ltd., Yoqnem).

**RESULTS:** This study ended in December 2009, because sample size was considered large enough. A total of 44 patients were enrolled. Two patients in group B were excluded from the analysis because small bowel images could not be obtained from these patients; one had a full stomach, while the other presented with a massive gastric bleed. Thus, 22 patients from group A and 20 patients from group B completed the study. There was no significant difference in age ( $P = 0.22$ ), sex ( $P = 0.31$ ), and indication for CE. No significant adverse events occurred in any of the study patients. In group A, image quality deteriorated as the capsule progressed distally. However, in group B, image quality was maintained to the distal small bowel. In each of the five segments, the visibility score was significantly higher in group B than in group A (segment 1:  $4.3 \pm 0.7$  vs  $4.7 \pm 0.5$ ;  $P = 0.03$ , segment 2:  $4.2 \pm 0.9$  vs  $4.8 \pm 0.4$ ;  $P = 0.01$ , segment 3:  $4.0 \pm 1.0$  vs  $4.6 \pm 0.7$ ;  $P = 0.04$ , segment 4:  $3.6 \pm 1.1$  vs  $4.5 \pm 0.6$ ;  $P = 0.003$ , segment 5:  $2.7 \pm 1.0$  vs  $4.4 \pm 0.8$ ;  $P = 0.00004$ ). Thus, the use of PEG during CE examination significantly improved image quality in all time segments, and this effect was more pronounced in the distal ileum. The completion rate to the cecum was not significantly different between groups A and B (81.8% vs 85.0%;  $P = 0.89$ ). There was no difference in the gastric transit time between groups ( $36.2 \pm 35.0$  min vs  $54.0 \pm 56.6$  min;  $P = 0.23$ ), but the small bowel transit time was significantly longer in group A than in group B ( $246.0 \pm 107.0$  min vs  $171.0 \pm 104.0$  min;  $P = 0.04$ ).

**CONCLUSION:** The ingestion of a small amount of PEG after the swallowing of an endoscopy capsule significantly improved CE image quality, but did not enhance the completion rate to the cecum.

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**Key words:** Capsule endoscopy; Completion rate; Image quality; Polyethylene glycol; Preparation

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## INTRODUCTION

Capsule endoscopy (CE) is the first-line procedure to explore small bowel pathology<sup>[1-4]</sup>. The preparation for CE suggested by the manufacturers of CE systems consists only of a clear liquid diet and an 8 h fast. Preparing the bowel with simethicone has been reported to bring about significantly better visibility by reducing intraluminal bubbles<sup>[5,6]</sup>. The diagnostic yield of CE, however, can be limited due to reduced visibility of the mucosal surface caused by residual material or dark colored bile, especially in the distal small bowel. In addition, CE sometimes fails to reach the cecum within the battery life of the capsule, resulting in a failure to visualize the distal small intestine<sup>[7]</sup>. It has been suggested that ingesting a small amount of polyethylene glycol (PEG) after the ingestion of the capsule may improve both the quality of the CE images and the likelihood that the cecum will be visualized before the capsule's battery life ends (completion rate to the cecum)<sup>[8,9]</sup>. This study aimed to prospectively confirm whether a small amount of PEG solution ingested after the swallowing of the endoscopy capsule improves CE image quality and the completion rate to the cecum.

## MATERIALS AND METHODS

This study was a single-center, prospective, single-blind, parallel, randomized controlled trial that was approved by the ethical committee at Kanto Medical Center NTT EC. This study was registered on February 9, 2009 in the UMIN-Clinical Trials Registry with a test ID of UMIN 000001696 (<https://upload.umin.ac.jp/cgi-bin/ctr/ctr.cgi?function=brows&action=brows&type=detail&receptno=R000002039&admin=0&language=J>) and was carried out in our hospital during a period from March 2009 to December 2009. Written informed consent was obtained from all patients, and the study was carried out in accordance with the Declaration of Helsinki (1989). There was no funding or other support for this study.

Patients referred to our institution for CE during the aforementioned period were prospectively randomized by the envelope method to group A or B. Randomization was done by means of sealed opaque envelopes.

Envelopes were provided by the study coordinator at our institute. Gastroenterologists enrolled the participants, and an assistant at the Endoscopy Center of our institute assigned patients to group A or B by the envelope method. All patients were restricted to clear fluids for 12 h before the examination, ingested 80 mg of simethicone at the time of capsule ingestion, and were then placed in the right lateral position for an hour. Patients in group A received no further preparation, but 30 min after swallowing the endoscopy capsule, the patients in group B ingested 500 mL of a PEG solution within a 2 h period. Clear fluids and meals were allowed 2 h and 4 h after capsule ingestion, respectively. After that, patients were free to leave the hospital, with instructions to return at the end of the 8 h study period to have the data recorder removed. All CE examinations were performed with the Pillcam SB capsule endoscopy system (Given Imaging Co. Ltd., Yoqnem). A single experienced reviewer blinded to the preparation analyzed each capsule study using Rapid Reader 5 software (Given Imaging Co. Ltd.). The primary outcome measures of the study were CE image quality and completion rate to the cecum, while the secondary outcome measures were gastric transit time (GTT) and small bowel transit time (SBTT). The image quality was evaluated only in cases in which the capsule reached the cecum within the examination period. The visibility of the mucosal surface was assessed as the percentage of visualized bowel surface area as follows: 1: < 25%; 2: 25%-49%; 3: 50%-74%; 4: 75%-89%; 5: > 90% (8). The small bowel record was divided into five segments by time, and the score for each segment was evaluated. The completion rate to the cecum was assessed based on the CE images.

Based on the preliminary examination, the sample size was determined based on the number of subjects needed to detect a 15% change in the mean visualization score and a 30% change in the mean transit time between the two groups at  $P$  (alpha) = 0.05 and power = 0.70. Interpretation of significance was adjusted for multiple comparisons by the Bonferroni's method. Parametric data were expressed as mean  $\pm$  SD. The Students' two-sided  $t$ -test was used to compare parametric data between the groups. Nonparametric data were expressed as frequencies and were compared between the groups with the  $\chi^2$  test. A  $P$  value of less than 0.05 was regarded as statistically significant in all tests. Patients in group B were excluded from the analysis because small bowel images could not be obtained from these patients; one had a full stomach, while the other presented with a massive gastric bleed. Thus, 22 patients from Group A and 20 patients from group B completed the study. The demographic data of the 2 groups, as well as the indication for each CE, are shown in Table 1. There was no significant difference in age ( $P = 0.22$ ), sex ( $P = 0.31$ ), and indication for CE. No significant adverse events occurred in any of the study patients. In group A, the image quality deteriorated as the capsule progressed distally. However, in group B, image quality was maintained to the distal small bowel (Figure 1). In each of the 5 segments, the visibility

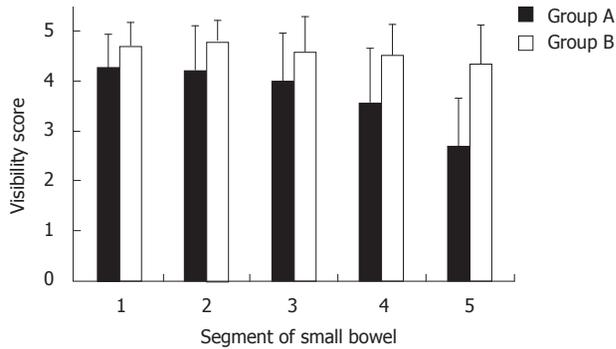


Figure 1 Visualization scores of groups A and B.

score was significantly higher in group B than in group A (segment 1:  $4.3 \pm 0.7$  vs  $4.7 \pm 0.5$ ;  $P = 0.03$ , segment 2:  $4.2 \pm 0.9$  vs  $4.8 \pm 0.4$ ;  $P = 0.01$ , segment 3:  $4.0 \pm 1.0$  vs  $4.6 \pm 0.7$ ;  $P = 0.04$ , segment 4:  $3.6 \pm 1.1$  vs  $4.5 \pm 0.6$ ;  $P = 0.003$ , segment 5:  $2.7 \pm 1.0$  vs  $4.4 \pm 0.8$ ;  $P = 0.00004$ ). The completion rate to the cecum was not significantly different between groups A and B (81.8% vs 85.0%;  $P = 0.89$ ) (Table 2). There was no difference in the GTT between groups ( $36.2 \pm 35.0$  min vs  $54.0 \pm 56.6$  min;  $P = 0.23$ ), but the SBTT was significantly longer in group A than in group B ( $246 \pm 107$  min vs  $171 \pm 104$  min;  $P = 0.04$ ).

## DISCUSSION

Some studies have suggested that bowel preparation that utilizes large amounts of purgatives can lead to higher quality CE images and/or accelerate the gastrointestinal transit of the CE<sup>[10-15]</sup>. On the other hand, others have failed to demonstrate image quality improvement in studies utilizing a large amount of purgatives in the bowel preparation<sup>[16-20]</sup>. All of these studies examined the effects of bowel preparation given prior to CE. In the present study, we examined the effects of PEG on CE image quality and completion rate to the cecum when PEG is given during, not before, the CE procedure. One great advantage of CE is its extreme non-invasiveness. However, it is a considerable burden for patients to have to take the large amount of purgatives needed as preparation for CE. Thus, we previously tried to see whether a small amount of PEG given during the CE procedure could improve the CE image quality and completion rate. In that retrospective study, we suggested that such an enhancement may improve both CE image quality and cecal completion rate<sup>[8]</sup>. The present study was undertaken to confirm those effects in a prospective randomized controlled study. PEG passes smoothly through the intestine. As such, it is assumed to move much faster through the intestine than an endoscopy capsule. Therefore, if PEG is given before capsule ingestion, it may well be completely cleared from an area of the intestine by the time the endoscopy capsule reaches that area. Thus, we administered the solution after capsule ingestion. In addition, PEG is completely transparent. As such, the clarity of a view through PEG was thought to be superior to

that of a view through natural intestinal fluid. The PEG solution was given starting 30 min after the swallowing of the capsule because the median pylorus passing time of an endoscopic capsule is approximately 20 min in our department (data not shown).

The present study confirmed that a small amount of PEG given after endoscopy capsule ingestion significantly improves image quality, especially in the lower segments of the small intestine. As CE imaging is disturbed by residual material or dark colored bile, especially in the distal small bowel, we feel that the image enhancing effect of this intervention is of significant worth. Furthermore, this procedure causes almost no discomfort to patients.

In regards to the completion rate to the cecum, conflicting results have been reported. Some have suggested that purgatives have completion enhancing effects<sup>[10,11]</sup> while others have failed to show such an effect<sup>[12,16,18,19]</sup>. Our previous retrospective study described above suggested that a small amount of PEG given after capsule ingestion may improve the cecal completion rate of CE<sup>[8]</sup>, but the present prospective single blinded randomized study failed to confirm that effect. However, the present study did show that the SBTT is shortened by this enhancement. Therefore, the administration of an additional intervention that can shorten the GTT in conjunction with a small amount of PEG given after capsule ingestion may be useful in improving completion rate. One intriguing possibility for such an intervention would be prokinetics, as they have been suggested to shorten the GTT and improve completion rate to the cecum<sup>[21-25]</sup>.

In conclusion, we show that as little as 500 mL of a PEG solution given after the swallowing of an endoscopy capsule can significantly improve the quality of the CE images in a prospective randomized study. Therefore, we recommend the ingestion of a small amount of PEG after the swallowing of the capsule as a standard enhancement of CE.

## COMMENTS

### Background

Capsule endoscopy (CE) is the first-line procedure to explore small bowel pathology. The preparation for CE suggested by the manufacturers of CE systems consists only of a clear liquid diet and an 8 h fast. Preparing the bowel with simethicone has been reported to bring about significantly better visibility by reducing intraluminal bubbles. The diagnostic yield of CE, however, can be limited due to reduced visibility of the mucosal surface caused by residual material or dark colored bile, especially in the distal small bowel. In addition, CE sometimes fails to reach the cecum within the battery life of the capsule, resulting in a failure to visualize the distal small intestine.

### Research frontiers

It has been suggested that ingesting a small amount of polyethylene glycol (PEG) after the ingestion of the capsule may improve both the quality of the CE images and the likelihood that the cecum will be visualized before the capsule's battery life ends (completion rate to the cecum). This study aimed to prospectively confirm whether a small amount of PEG solution ingested after the swallowing of the endoscopy capsule improves CE image quality and the completion rate to the cecum.

### Innovations and breakthroughs

In the present study, the beneficial effects of a small amount of PEG solution on CE image quality were demonstrated in a prospective single-blinded random-

ized controlled study. In the study group without preparation, the image quality deteriorated as the capsule progressed distally. However, in the group that took 500 mL of PEG solution after capsule ingestion, image quality was maintained to the distal small bowel.

### Applications

Ingestion of as little as 500 mL of PEG solution brings about an excellent clarity in CE images without any discomfort. This study prospectively confirmed the suggested beneficial effect of PEG solution on CE images, and can provide evidence for an effective, safe, cost-effective and minimally invasive preparation of capsule endoscopy in clinical practice.

### Peer review

Here is an interesting single-blinded prospective randomized controlled study regarding the effects of PEG on capsule endoscopy image quality and completion rate to the cecum when PEG is given during, and not before, the capsule endoscopy procedure. Data on this topic is limited and therefore the study is welcome. What is more, the study has an excellent methodology and the results are really interesting.

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## Cyclooxygenase-2 expression as a predictor of outcome in colorectal carcinoma

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### Abstract

**AIM:** To correlate cyclo-oxygenase-2 (COX-2) expression profile with clinical and pathological variables to assess their prognostic/predictive value in colorectal carcinoma (CRC).

**METHODS:** Archival tumor samples were analyzed using immunohistochemistry for COX-2 expression in 94 patients with CRC. Patients were diagnosed and treated

at the Departments of Surgery and Oncology, King Abdulaziz University Hospital, Saudi Arabia.

**RESULTS:** Fifty-six percent of the tumors showed positive cytoplasmic COX-2 expression, whereas 44% of cases were completely COX-2-negative. There were no significant correlations between COX-2 expression and sex, age, grade or tumor location. However, COX-2 expression revealed a significant correlation with tumor stage ( $P = 0.01$ ) and distant metastasis ( $P = 0.02$ ), and a borderline association with lymph node involvement ( $P = 0.07$ ). Tumors with high COX-2 expression showed a higher recurrence rate than tumors with no expression ( $P < 0.009$ ). In univariate Kaplan-Meier survival analysis, there was a significant ( $P = 0.026$ ) difference in disease-free survival between COX-2-positive and negative tumors in favor of the latter. COX-2 expression did not significantly predict disease-specific survival, which was much shorter for COX-2-positive tumors. In multivariate (COX) models, COX-2 did not appear among the independent predictors of disease-free survival or disease-specific survival.

**CONCLUSION:** COX-2 expression seems to provide useful prognostic information in CRC, while predicting the patients at high risk for recurrent disease.

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**Key words:** Colorectal carcinoma; Cyclo-oxygenase-2; Immunohistochemistry; Disease outcome; Adjuvant therapy

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## INTRODUCTION

Numerous epidemiological and clinical studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the number and size of adenomas in patients with familial adenomatous polyposis and decrease the incidence of colorectal cancer (CRC)<sup>[1,2]</sup>. Recent studies have shown that regular use of aspirin after the diagnosis of CRC is associated with a lower risk of mortality, particularly when the tumors overexpress cyclooxygenase-2 (COX-2)<sup>[3,4]</sup>. It is suggested that this effect is mediated through inhibition of COX-2, an enzyme involved in prostaglandin metabolism, inflammation and carcinogenesis<sup>[4,5]</sup>. Elevated levels of COX-2 have been detected in 85%-95% of CRC samples, and in 40%-50% of adenomas, and elevated mRNA levels of COX-2 in 86% of CRC and 43% of adenomas, in contrast to no or weak expression of COX-2 protein or mRNA in normal colorectal mucosa<sup>[6,7]</sup>. There is some recent evidence that COX-2 is related to apoptosis inhibition, induction of Bcl-2 expression, as well as stimulation of angiogenesis<sup>[8-10]</sup>. However, the role of COX-2 in colorectal carcinogenesis remains unclear.

Increased COX-2 expression has been reported in several human malignancies including uterine cervix<sup>[11]</sup>, non-small cell lung carcinoma<sup>[12]</sup>, prostate<sup>[13]</sup>, stomach<sup>[9]</sup> and breast cancer<sup>[14]</sup>, as well as in nonepithelial tumors such as gastrointestinal stromal tumors<sup>[15]</sup> and Hodgkin's lymphoma<sup>[16]</sup>. The aim of the present study was to explore the relationship between COX-2 expression profile and clinicopathological features of CRC as well as its value as a predictor of disease outcome.

## MATERIALS AND METHODS

The material of the present study consists of a series of 94 CRC specimens, retrospectively collected from the archives of Anatomical Pathology Laboratory in King Abdulaziz University, Jeddah, Saudi Arabia, covering the period from January 2005 to December 2009. Serial sections were cut from paraffin blocks, stained with hematoxylin and eosin for routine histological examination, classification, grading and staging following the American Joint Committee on Cancer staging system<sup>[17]</sup>. The pertinent clinicopathological data (sex, age, stage, grade and lymph node status), and follow-up results were retrieved from the patients' records after obtaining the relevant ethical approval (Table 1). The mean age of the patients was 58 years (median: 59 years, range: 24-90 years).

### Immunohistochemical procedures

Four-micrometer-thick tissue sections were cut from the paraffin blocks (containing both tumor and benign tissues), mounted on charged poly-L-lysine-coated slides,

**Table 1** Correlation between cyclooxygenase-2 expression and clinicopathological features of colorectal carcinoma

Features	n (%)	COX-2 expression		P value
		Negative	Positive	
Sex				0.97
Male	48 (51)	21	27	
Female	46 (49)	20	26	
Age group (yr)				0.59
< 60	47 (50)	22	25	
> 60	47(50)	19	28	
LN involvement				0.07
Yes	60 (64)	22	38	
No	34 (36)	19	15	
Distant metastasis				0.02
Yes	33 (35)	11	22	
No	61 (65)	30	31	
Tumor stage				0.01
I / II	32 (34)	20	12	
III / IV	62 (66)	21	41	
Tumor grade				0.50
Well	25 (27)	11	14	
Moderate	56 (60)	26	30	
Poor	11 (13)	3	8	
Tumor location				0.80
Right colon	38 (40)	16	22	
Left colon	56 (60)	25	31	
Recurrence				0.22
Yes	31 (33)	11	20	
No	38 (40)	19	19	
Unknown	25 (27)			

LN: Lymph node; COX-2: Cyclooxygenase-2.

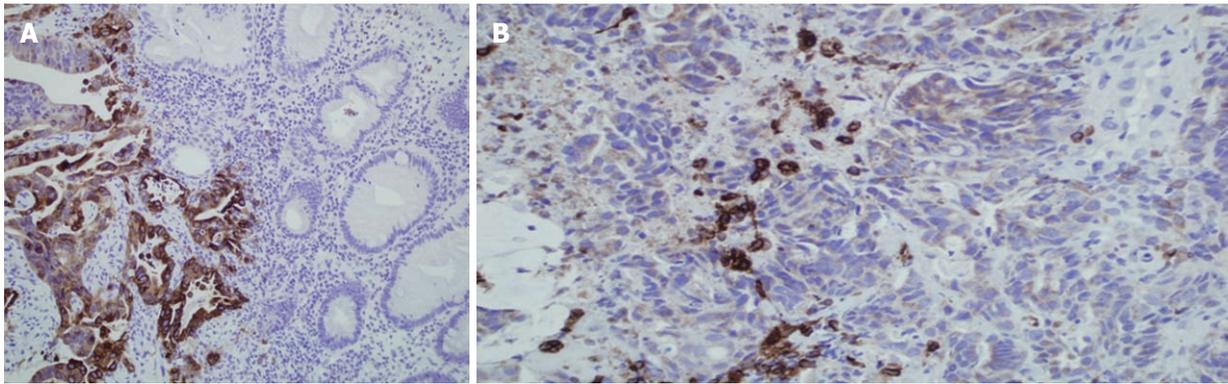
and subjected to immunohistochemistry using the Avidin Biotin detection system, following the manufacturer's instructions. The antibody used was a mouse anti-human COX-2 monoclonal antibody (Dako Cytomation Norden A/S, Glostrup, Denmark; dilution 1:50). Immunohistochemistry was carried out by an automatic immunostainer (Ventana Bench Mark XT; Ventana Inc., Tucson, AZ, United States). In each analysis, positive controls were used consisting of CRC samples previously shown to stain with this antibody. Tris-buffered saline in place of the primary antibody was used as a negative control.

### Interpretation of immunohistochemical staining

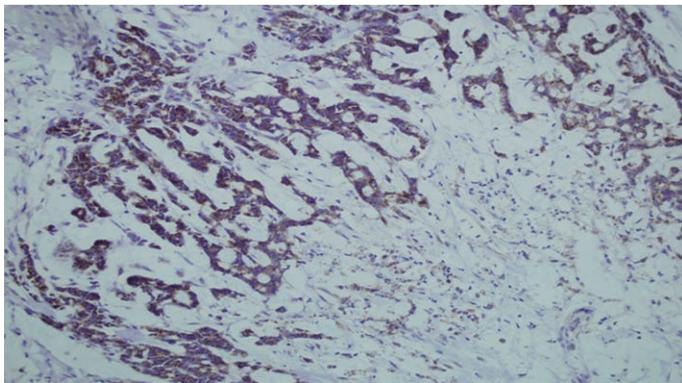
Cells were considered positive for COX-2 when distinct cytoplasmic yellow to brown staining was identified. The extent and intensity of the staining were recorded on a scale from 0 to +++; +++ implied strong staining that was maximally intense throughout the specimen, and 0 implied negative staining. When dichotomized for statistical risk assessment [odds ratio (OR) calculation], negative (-) and weak (+) staining were defined as low expression, whereas moderate (++) and intense (+++) staining were included in the high expression category.

### Statistical analysis

Statistical analyses were performed using SPSS (IBM, New York, NY, United States) and Stata (Stata Corp., College Station, TX, United States) software (PASW Sta-



**Figure 1 Cyclooxygenase-2 expression.** A: Strong diffuse brown cytoplasmic cyclooxygenase-2 (COX-2) expression in colorectal adenocarcinoma cells, in contrast to negative expression in normal colonic cells; B: Negative COX-2 expression in poorly differentiated colorectal adenocarcinoma. Note the positive expression of stromal inflammatory cells.



**Figure 2 Diffuse positive cytoplasmic cyclooxygenase-2 expression of mucoid carcinoma.**

istics for Windows, version 18.0.2 and STATA/SE version 11.1). Frequency tables were analyzed using the  $\chi^2$  test, with likelihood ratio or Fischer's exact test to assess the significance of the correlation between the categorical variables. OR and 95% confidence interval (CI) were calculated when appropriate, using the exact method. Differences in the means of continuous variables were analyzed using nonparametric tests (Mann-Whitney or Kruskal-Wallis) for two and multiple independent samples, respectively. Analysis of variance was only used to derive the mean values (and 95% CI) of each individual stratum. Univariate survival analysis for the outcome measure [disease-specific survival (DSS) and disease-free survival (DFS)] was based on the Kaplan-Meier method, with log-rank (Mantel-Cox) comparison test. To assess the value of COX-2 as an independent predictor, multivariate survival analysis was performed, using the Cox proportional hazards regression model, controlling for the confounding by the following variables: age, sex, tumor localization, tumor stage, grade, (for DFS), and recurrence as additional variable for DSS. In all tests,  $P < 0.05$  was regarded as statistically significant.

## RESULTS

### **Cyclo-oxygenase-2 expression profiles**

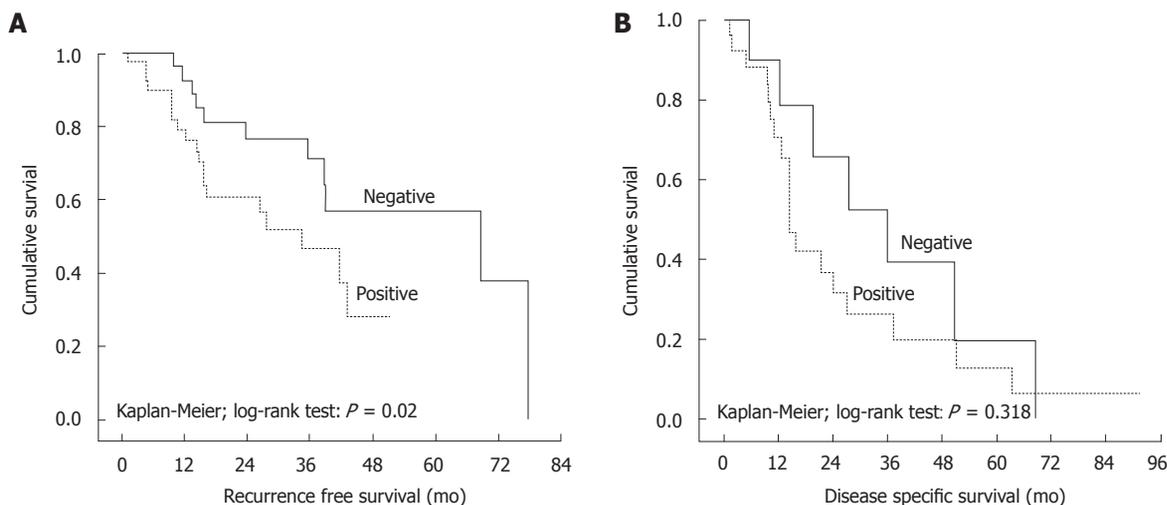
Normal colorectal mucosa showed no or weak COX-2

expression, but weak cytoplasmic expression was detected in a few inflammatory mononuclear cells (Figure 1). In cancer cells, COX-2 expression appeared as yellow-brown staining and was observed mainly in the cytoplasm and occasionally in the nuclear envelope (Figures 1 and 2). Expression was high in 53 (56%) cases, and low in 41 cases (44%).

### **Correlation of cyclo-oxygenase-2 expression with the clinicopathological features**

The associations between COX-2 expression and clinicopathological features are presented in Table 1. Sex, age, grade, or tumor location had no significant relationship with the expression of COX-2. However, the tumor stage and distant metastasis were significantly associated with COX-2 expression, with higher expression being more common in advanced tumors ( $P < 0.01$ ), and fewer distant metastases being found in tumors with negative expression ( $P < 0.02$ ). Of particular interest was the borderline association between lymph node (LN) involvement and expression of COX-2 ( $P < 0.07$ ); 63% of the tumors with LN involvement tested positive for COX-2, whereas only 37% of the cases with no COX-2 expression had LN involvement.

Similarly, COX-2 expression showed a clear association with DFS in that the patients who developed early recurrence (22 mo) had positive COX-2 expression, in



**Figure 3** Impact of cyclooxygenase-2 expression on survival in univariate (Kaplan-Meier) analysis. A: Disease-free survival ( $P = 0.026$ ); B: Disease-specific survival ( $P = 0.318$ ).

contrast to those with no expression of COX-2 who developed recurrence much later (32 mo) ( $P = 0.009$ ). The same trend was observed in DSS time, with patients with COX-2 negative tumors living significantly longer ( $P = 0.009$ ).

In Kaplan-Meier survival analysis, there was a significant ( $P = 0.02$ ) difference in DFS between patients with COX-2-negative tumors (longer DFS) and those with COX-2-positive tumors (Figure 3A). As to DSS, COX-2 did not show any predictive power in Kaplan-Meier analysis ( $P = 0.32$ ) (Figure 3B).

To assess the value of COX-2 as an independent predictor, a multivariate survival analysis was done, using the Cox proportional hazards regression model controlling for confounding by the following covariates: age, sex, tumor localization, T, grade, (for DFS), and recurrence as additional variable for DSS. In the final multivariate model, only tumor grade ( $P = 0.023$ ) and tumor stage ( $P = 0.0001$ ) remained significant predictors of DFS, decreasing with progressing dedifferentiation and increasing stage, as expected. In a similar model for DSS (including recurrence as a covariate), none of the included covariates were independent predictors of DSS.

## DISCUSSION

The aims of the present study were to cast further light on the issues related to prognostication of CRC, and assess the value of COX-2 expression profiles as predictive and prognostic markers. We focused on stage II-IV disease, in which molecular and other markers may help to pinpoint a subgroup of patients who would eventually benefit from the use of adjuvant therapy. This important therapeutic decision involves a careful weighing of the risks of drug toxicity and complications against the potential curability of the disease<sup>[18]</sup>. It is well established that early CRCs can be cured with radical surgical resection alone<sup>[19]</sup>. Unfortunately, however, some 30% of all patients who undergo curative resection subsequently

present with relapse and eventually die of their disease<sup>[20]</sup>. Prediction of disease outcome in individual patients after curative resection is still far from reliable<sup>[21]</sup>. However, there is some hope for improvement, and in fact, our results suggest that COX-2 expression assessed by immunohistochemistry might be helpful in this respect. In addition, more rational decisions can be done as soon as we learn more of the markers and other diagnostic tools for accurate prediction of disease outcome in individual patients<sup>[22,23]</sup>.

On the basis of the present results, we do believe that this detection can be improved using the assessment of COX-2 expression in the primary tumors. In this assessment, quantifying the COX-2 expression seems to be important. We compared different methods of grading this expression, and found the dichotomized negative/positive grading to provide the most consistent and meaningful correlations to the clinicopathological variables and outcome. This leads us to suggest that classifying CRCs as COX-2 positive/negative is the clinically most relevant approach.

In our series, 56% of the primary CRCs expressed COX-2, whereas the results in previous studies have shown that COX-2 was expressed in 85%-95% of CRCs<sup>[6,7]</sup>. This implies its important role in cancer progression. The discrepancy of these observations might be explained by differences in the technical aspects of recording COX-2 expression or by differences of interpretation of expression profiling. The possibility that these discrepancies may have a genetic basis is one favored hypothesis. However, the markedly different expression of COX-2 in normal and cancer tissues substantiates the view that paracrine effects in the crosstalk between normal and cancer cells are likely to be involved in the upregulation of COX-2 in the tumor tissue.

In the present cohort, several potentially important observations were made, all implying that the quantitatively measurable COX-2 expression in tumor cells could

provide significant prognostic information in CRC. First, positive expression of COX-2 was more common among advanced stage tumors than in early stage tumors. This is in agreement with a study by Lim *et al.*<sup>[24]</sup> who showed that COX-2 expression was correlated with the depth of invasion and advanced tumor stage. Similarly, we observed a significant correlation between COX-2 expression and distant metastasis, and tumors with more COX-2 expression revealed more distant metastasis than did those with no expression. This observation also substantiates the data of Tomozawa *et al.*<sup>[25]</sup>, who have reported that COX-2 overexpression was significantly associated with tumor recurrence, and especially with hematogenous metastasis. In contrast to these results, however, the study by Fujita *et al.*<sup>[26]</sup> failed to establish any correlation between COX-2 overexpression and metastasis.

A trend with borderline significance was observed between COX-2 and LN involvement; 63% of the tumors with LN involvement showed positive expression of COX-2, whereas only 37% of the cases not expressing COX-2 had LN involvement. Xiong *et al.*<sup>[27]</sup> have reported that among 45 cases of CRC with LN metastasis, COX-2 detection rate was 87% in the primary tumors, whereas diffuse cytoplasmic COX-2 staining in the cancer cells was detected in 100% of the LN metastatic lesions. The preferential expression of COX-2 in LN metastases is consistent with the view of clonal selection of tumor cells with COX-2 expression, conferring upon them a growth advantage with a higher potential of metastasis. This could be how COX-2 expression is related to progression of CRC. Altogether, these observations implicate COX-2 as a biological factor that might affect the behavior of tumor cell populations. Some studies have shown that higher expression of COX-2 is found in benign breast tissue adjacent to breast carcinoma<sup>[28]</sup>.

Obviously, one of the most important observations of the present study is that linking COX-2 expression with disease outcome, that is, disease recurrence and length of DFS. This is clinically relevant for several reasons. Some CRC patients at different stages are at high risk of recurrence, thus, it is of paramount importance to develop reliable markers that accurately predict those patients so that they may be considered for adjuvant therapy. In the present series, 37% of the patients eventually developed a recurrent disease within the median follow-up time of 15.6 mo (mean, 22 mo; range: 15.8-28.2 mo). This is a substantially high rate, particularly for a group of LN-negative (stage II) CRC patients. Importantly, our data showed that the patients who developed an early recurrence (mean DFS of 22 mo) had positive COX-2 expression, in contrast to patients with negative expression, who developed recurrence with a significantly longer delay (mean DFS of 32 mo).

As shown in Figure 3, at 5 years follow-up, only 40% of the patients with negative COX-2 expression had recurrence, as compared to 85% of those with COX-2-expressing tumors. This is in agreement with the study by Wan *et al.*<sup>[29]</sup>, who showed that the recurrence and metastasis rates were significantly higher in COX-2-posi-

tive patients than in their negative counterparts. Similarly, Tomozawa *et al.*<sup>[25]</sup> also have found that COX-2 overexpression was correlated with disease recurrence and hematogenous metastasis, with DFS time being significantly shorter for patients with high expression of COX-2 compared with low expression. Furthermore, COX-2 was the only independent predictor of survival time when adjusted for other prognostic factors, including age, histological type, and tumor size and stage. Therefore, COX-2 overexpression is considered to be correlated with recurrence and metastasis of CRC. Tsujii *et al.*<sup>[30]</sup> also have found that metastatic potential increases with COX-2 overexpression. These important results suggest that selective COX-2 inhibitors might be useful chemopreventive agents, not only in growth of the primary tumor but also for prevention of hematogenous metastasis of CRC. When adjusted for other potential predictors in multivariate Cox regression model, COX-2 expression lost its value as a significant independent predictor of DFS.

As to DSS, COX-2 expression was shown to be high (positive) more often among patients who eventually died of their disease as compared with those staying alive at the completion of the follow-up, although the difference was not significant ( $P = 0.30$ ). However, the difference in DSS time between COX-2-positive and -negative tumors was significant ( $P = 0.009$ ); being shorter for the former. However, this difference did not appear significant in univariate (Kaplan-Meier) or multivariate (Cox) survival analysis. These observations suggest that CRC tumors with positive COX-2 expression are at high risk for local or distant recurrence and, because of the strong adverse impact of the latter on survival (i.e., none of the patients without recurrence died of disease in our cohort), these patients are also more likely to eventually die of their disease. These patients should be appropriate candidates for close and frequent postoperative follow-up and eventual adjuvant therapy.

Taken together, COX-2 expression did not correlate with sex, age or tumor grade, as confirmed by other studies<sup>[27,31]</sup>. However, COX-2 expression showed significant associations with tumor stage and metastasis. Furthermore, positive COX-2 staining was related to a higher recurrence rate as compared to COX-2-negative tumors, suggesting a link towards development of a metastatic phenotype. Finally, positive COX-2 expression was associated with shorter DSS, as compared with COX-2-negative CRCs, implying some differences in the inherent malignancy of CRC that become manifest after a reasonable follow-up period. To elucidate this fully, however, we would need a larger cohort and a substantially longer follow-up time to provide the basis for clinical trials that evaluate the contribution of COX-2 inhibitors in therapy and prevention of CRC.

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## COMMENTS

**Background**

Phospholipase A2 (PLA2) is a key regulatory enzyme in arachidonic acid metabolism, which leads to synthesis of prostaglandins via the cyclo-oxygenase (COX)-1 and COX-2 pathways. Inhibition of COX-2 results in suppression of tumor development and growth, and it has been proposed that prostaglandins may have an important role in tumor growth in colorectal cancer (CRC). Prostaglandin E2 induces *bcl-2* gene expression and inhibits apoptosis in human colorectal cell lines, which may partially explain the COX-2-mediated tumor growth. Thus, in addition to COX, functional defects in PLA2 in tumor cells may interfere with the regulatory mechanisms of tumor growth. Therefore, COX-2 represents a potential molecular target in colorectal management and specific COX-2 inhibitors may be useful as chemopreventive as well as therapeutic agent in humans.

**Research frontiers**

It is mandatory to find new prognostic factors capable of identifying high-risk stage II CRC patients for better targeting of treatment options.

**Innovations and breakthroughs**

COX-2 expression did not correlate with sex, age or tumor grade, as confirmed by other studies. However, COX-2 expression showed significant associations with tumor stage and metastasis. Furthermore, positive COX-2 staining was related to a higher recurrence rate as compared to COX-2-negative tumors, suggesting a link towards development of a metastatic phenotype. Finally, positive COX-2 expression was associated with shorter disease-specific survival, as compared with COX-2-negative CRC, implying some differences in the inherent malignancy of CRC that become manifest after a reasonable follow-up period. To elucidate this fully, however, we would need a larger cohort and a substantially longer follow-up time to provide the basis for clinical trials that evaluate the contribution of COX-2 inhibitors in therapy and prevention of CRC.

**Applications**

Quantification of COX-2 expression seems to provide valuable prognostic information in CRC, particularly in selecting the patients at high risk for recurrent disease who might benefit from adjuvant therapy.

**Peer review**

Although this manuscript makes the point that COX-2 expression can predict disease outcomes in CRC, it is clear that COX-2 expression is not an independent predictor, and in fact, in the multivariate analysis, only tumor grade and stage were independent predictors.

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## Patient and physician perception of natural orifice transluminal endoscopic appendectomy

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### Abstract

**AIM:** To investigate perception of natural orifice transluminal endoscopic surgery (NOTES) as a potential technique for appendectomy.

**METHODS:** One hundred patients undergoing endoscopy and 100 physicians were given a questionnaire describing in detail the techniques of NOTES and laparoscopic appendectomy. They were asked about the reasons for their preference, choice of orifice, and extent of complication risk they were willing to accept.

**RESULTS:** Fifty patients (50%) and only 21 physicians (21%) preferred NOTES ( $P < 0.001$ ). Patients had previously heard of NOTES less frequently (7% vs

73%,  $P < 0.001$ ) and had undergone endoscopy more frequently (88% vs 36%,  $P < 0.001$ ) than physicians. Absence of hernia was the most common reason for NOTES preference in physicians (80% vs 44%,  $P = 0.003$ ), whereas reduced pain was the most common reason in patients (66% vs 52%). Physicians were more likely to refuse NOTES as a novel and unsure technique ( $P < 0.001$ ) and having an increased risk of infection ( $P < 0.001$ ). The preferred access site in both groups was colon followed by stomach, with vagina being rarely preferred. In multivariable modeling, those with high-school education [odds ratio (OR): 2.68, 95% confidence interval (CI): 1.23-5.83] and prior colonoscopy (OR: 2.10, 95% CI: 1.05-4.19) were more likely to prefer NOTES over laparoscopic appendectomy. There was a steep decline in NOTES preference with increased rate of procedural complications. Male patients were more likely to consent to their wives vaginal NOTES appendectomy than male physicians ( $P = 0.02$ ).

**CONCLUSION:** The preference of NOTES for appendectomy was greater in patients than physicians and was related to reduced pain and absence of hernia rather than lack of scarring.

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**Key words:** Natural orifice transluminal endoscopic surgery; Patient perception; Physician perception; Appendectomy

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## INTRODUCTION

Minimally invasive surgery has been challenging traditional open surgery. Laparoscopic surgery is less traumatic than open surgery, and is generally associated with fewer local and systemic complications, less postoperative pain, faster recovery and better cosmesis<sup>[1]</sup>. Natural orifice transluminal endoscopic surgery (NOTES) is a novel technique that takes advantages of natural orifices. It may be even less invasive than traditional laparoscopic surgery because it avoids abdominal incisions<sup>[2]</sup>. To date, a variety of surgical procedures using natural orifices have been performed in animals and humans<sup>[3,4]</sup>.

The advantages of NOTES have not yet been fully confirmed in randomized trials<sup>[4]</sup>, but are expected to include lack of scarring, a less profound systemic response, faster recovery, less pain and absence of hernia. Due to limited maneuverability and lack of efficient and safe closure of NOTES access sites, hybrid procedures involving additional laparoscopic instruments or vaginal access with hand-sutured closures have been used in most human procedures<sup>[5-7]</sup>. The overall complication rates reported in the first human studies have not exceeded those of laparoscopic surgery, but these findings remain to be proven in controlled trials<sup>[7]</sup>. Ultimately, the acceptance of NOTES procedures by both patients and physicians will be crucial in determining whether this new approach will become a part of our routine clinical practice.

Results of the few studies that have addressed the perception of NOTES have been variable. A very high patient preference of almost 80% for NOTES over laparoscopic cholecystectomy has been reported<sup>[8]</sup>. In contrast, the perception of NOTES among surgeons in another survey was receptive overall, but more cautious<sup>[9]</sup>. The aim of our study was to evaluate the perception of NOTES appendectomy among patients and physicians. Furthermore, we aimed to determine reasons for their preference, the preferred access site, and the extent of acceptable complication risk.

## MATERIALS AND METHODS

### Patients and physicians

A group of consecutive patients scheduled to undergo procedures in the endoscopy unit were given a questionnaire by a staff member and asked to complete it anonymously. Physicians in various departments of our hospital (cardiology, cardiac surgery, gastroenterology, general surgery, diabetology, nephrology and radiology) also participated in the study and were given the same questionnaire. One hundred questionnaires were completed by each group and were evaluated. Participation in the study was voluntary. Only subjects with an intact appendix were included. Prior to beginning the study, the questionnaire was evaluated for consistency and clarity in a group of 20 medical students.

### Questionnaire

The first page of the questionnaire described acute ap-

pendicitis and its management in detail. The technique of laparoscopic appendectomy with its benefits and risks was explained. Then, the concept of NOTES was described; stating clearly that it is still an experimental technique with its benefits and risk being only estimated. It was stated that the technique was not available in our hospital and that the response of the patients would in no way influence their further medical management.

This information was followed by a list of 12 questions. Age, sex, educational status, prior awareness of NOTES and prior experience with endoscopy or laparoscopy were queried first. Then, preference between NOTES and laparoscopic appendectomy was determined. Those who preferred laparoscopy were asked for reasons for their preference with options being safety and efficiency of the technique, willingness to tolerate scars and pain, and because NOTES is new, concerns about the technique and risk of infection. The options offered to those preferring NOTES were less pain, absence of scars and hernia, and a new unique technique. Patients who chose NOTES were asked to score their preference for access site (1, 2 or 3, mouth/stomach, anus/colon, vagina in women). Men were also asked if they would object to a vaginal NOTES procedure for their wives. Those who preferred NOTES were asked about their tolerance for procedure-related complications (less, equal to, 1.5 times or two times greater than that of laparoscopic appendectomy), given a complication rate of laparoscopic appendectomy of around 8%.

### Statistical analysis

We hypothesized a 56% preference of NOTES based on evidence available at the time of study design<sup>[10]</sup>. We expected physicians' preference to be 75% of that of patients. Thus, assuming a power of 0.8 and  $\alpha$  of 0.05, 99 patients and physicians needed to be included to show a statistically significant difference.

Data were analyzed for all participants overall and for both groups separately. The between-group differences were compared using a two-sample *t* test for continuous variables and Fisher's exact test for categorical variables. Odds ratio (OR) and 95% confidence interval (CI) were calculated. A two-tailed  $P < 0.05$  was required for statistical significance. To assess the effect of individual factors, multiple logistic regression was used. Analyses were performed using R software for statistical computing<sup>[11]</sup>.

## RESULTS

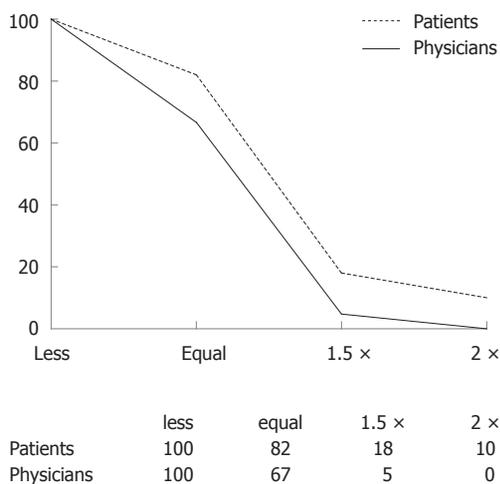
### Patients vs physicians

Questionnaires were obtained from a 100 patients and 100 physicians, and evaluated. Characteristics of the study subjects are summarized in Table 1. Fifty-six patients (56%) and 53 physicians (53%) were male. The mean age of patients was higher than that of physicians (52 years *vs* 41 years, respectively,  $P < 0.001$ ). Previously, 88 patients (88%) had an endoscopy; 67 (67%) had upper gastrointestinal endoscopy and 60 (60%) had colonoscopy. Only 36 phy-

**Table 1 Characteristics of survey participants *n* (%)**

	Patients	Physicians
Age (yr)	52 (mean)	41 (mean)
Men	56 (56)	53 (53)
Education		
Graduate	28 (28)	100 (100)
High school	58 (58)	0 (0)
Elementary school	14 (14)	0 (0)
Heard of NOTES	7 (7) <sup>a</sup>	73 (73)
Prior endoscopy	88 (88) <sup>a</sup>	36 (36)
Prior laparoscopy	20 (20)	9 (9)
NOTES preference	50 (50) <sup>a</sup>	21 (21)
Laparoscopy preference	50 (50) <sup>a</sup>	79 (79)
Reasons for preference		
Pain	33 (66)	11 (52)
Cosmesis	15 (30)	11 (52)
Absence of hernia	22 (44) <sup>a</sup>	17 (80)
Access site preference		
Colon	25 (50)	12 (57)
Stomach	19 (38)	8 (38)
Vagina	6 (12)	1 (5)
Approval of wife's NOTES	42/49 (86) <sup>a</sup>	29/46 (63)

<sup>a</sup>*P* < 0.05. NOTES: Natural orifice transluminal endoscopic surgery.



**Figure 1 Varying complication rates relative to laparoscopic appendectomy and preference for natural orifice transluminal endoscopic surgery.**

sicians (36%) had a previous endoscopy (*P* < 0.0001); 28 (28%) had upper gastrointestinal endoscopy (*P* < 0.0001) and 14 (14%) had colonoscopy (*P* < 0.0001). There was no statistically significant difference in prior laparoscopy experience (20 patients *vs* nine physicians, *P* > 0.05).

Fifty patients (50%) and only 21 physicians (21%) preferred NOTES over laparoscopic appendectomy (*P* < 0.01). For the patients, the OR for NOTES preference was 1 (95% CI: 0.676-1.480), and none of the queried characteristics influenced the preference for NOTES. For physicians, the OR for NOTES preference was low (OR: 0.152, 95% CI: 0.069-0.337). However, the OR for NOTES preference was significantly higher in female *vs* male physicians (OR: 2.875, 95% CI: 1.044-7.919).

The most common reasons for NOTES preference in patients was absence of pain [33 (66%)], followed by ab-

**Table 2 Association between participant characteristics and natural orifice transluminal endoscopic surgery preference**

	Odds ratio	<i>P</i> value	Confidence interval
Female	1.473	0.259	0.785-2.761
Age	0.985	0.587	0.960-1.011
High school education <sup>1</sup>	2.678	0.003	1.230-5.826
Elementary education <sup>1</sup>	3.222	0.003	0.907-11.444
Heard of NOTES	1.138	0.412	0.516-2.509
Prior colonoscopy	2.098	0.032	1.050-4.192
Prior laparoscopy	1.462	0.412	0.584-3.659

<sup>1</sup>University education serving as baseline. NOTES: Natural orifice transluminal endoscopic surgery.

sence of hernia [22 (44%)] and cosmetic issues [15 (30%)]. The most common reasons in physicians were absence of hernia [17 (80%)] followed by cosmetic issues and absence of pain [11 (52%) each]. Absence of hernia was given as a reason for NOTES preference by significantly more physicians than patients (80% *vs* 44%, *P* < 0.001).

In both groups, the most frequently chosen reasons for preference of laparoscopy were proven safety [33 (66%) *vs* 65 (82%)] followed by absence of cosmetic concerns [31 (62%) *vs* 43 (54%)] and proven efficiency [20 (40%) *vs* 43 (54%)]. Physicians were more likely to chose laparoscopy for the reason that NOTES was a novel, unproven technique [33 (42%) *vs* 6 (12%), *P* < 0.0001] and had a greater risk of infection [25 (32%) *vs* 2 (4%), *P* = 0.0001].

The preferred NOTES access site was the colon in both groups [25 (50%) *vs* 12 (57%)], followed by the stomach [19 (38%) *vs* 8 (38%)] with the vagina being rarely preferred [6 (27%) *vs* 1 (7%)]. Forty-two of 49 male patients (86%) but only 29 of 46 male physicians (63%) did not object to vaginal NOTES for their wives (*P* = 0.02). There was a steep decline in NOTES preference with an increased rate of procedural complications in both study groups (Figure 1). When the NOTES complication rate was lower than or equal to that of laparoscopic appendectomy ( $\leq$  8%), it was acceptable for a vast majority of those preferring NOTES. Once the complication rate increased to twofold that of laparoscopy, the NOTES preference rate dropped to 10%.

**Natural orifice transluminal endoscopic surgery vs laparoscopy**

When the study subjects were analyzed based on the overall NOTES preference *vs* laparoscopy, those with a university education [NOTES *vs* laparoscopy, 34 (27%) *vs* 94 (73%), *P* = 0.002], those who had heard of NOTES [20 (25%) *vs* 60 (75%), *P* = 0.02] and those who had not had prior colonoscopy [35 (28%) *vs* 91 (72%), *P* = 0.004] were more likely to prefer laparoscopic over NOTES appendectomy. In multivariable modeling, lower education status (high school) (OR: 2.678, 95% CI: 1.230-5.826) and prior colonoscopy (OR: 2.098, 95% CI: 1.050-4.192) were significantly associated with NOTES preference (Table 2).

## DISCUSSION

NOTES is an experimental surgical approach in which an endoscope is passed through a natural opening (e.g., mouth, anus or vagina) and then through an internal incision in the stomach, colon or vagina. So far, NOTES has been limited primarily to animal procedures. Although a variety of procedures are technically possible in animals and the first laparoscopically assisted procedures have been performed in humans, the efficiency and safety of NOTES as well as its expected benefits, such as less trauma, remain to be shown<sup>[12]</sup>. Wide adoption of NOTES will depend largely on public acceptance.

Public acceptance and demand have previously been shown to play a major role in adoption of a surgical technique. In the 1980s when a totally new technique of gallbladder surgery, laparoscopic cholecystectomy, was introduced, medical professionals expressed little interest in the new approach. The early reports did not even show much benefit of the new concept except for cosmesis<sup>[13]</sup>. However, public demand influenced in part by industry marketing spurred adoption of this minimally invasive technique despite unproven benefits and increased risks. In spite of the early difference in patient and physician perception of laparoscopy, the technique has gained significant importance in abdominal surgery with laparoscopy cholecystectomy now representing a gold standard procedure. In parallel to this interesting phenomenon, we aimed to determine the perception of NOTES among patients and physicians.

Our study results show a discrepancy between patient and physician perception of this new technique. Half the patients questioned would prefer to undergo NOTES appendectomy despite the fact that the vast majority had never heard of the technique before. In contrast, most physicians refused the novel technique in favor of laparoscopic appendectomy. A few differences between the groups may have contributed to this result. First, the medical profession is known for its general skepticism of a new technique with an unproven rate of complications; thus the sensitivity to potential NOTES-associated risks was expected from the physicians<sup>[14]</sup>. Second, the mean age of the patients was older than that of the physicians; however, older age has previously been associated with decreased NOTES preference. Third, a majority of patients had previously undergone an endoscopy, and a previous positive experience with endoscopy may have contributed to their preference for NOTES, being partly an endoscopic technique.

Patients' preference for NOTES in our study was lower than previously reported. Almost 80% of patients preferred NOTES over laparoscopy in a study performed by Varadarajulu *et al.*<sup>[8]</sup> in 2008. The preference decreased to 56% in a study reported in 2009 by Swanstrom *et al.*<sup>[10]</sup>. The observed decline may result from differences in the study design, chosen procedure or questioned populations. It may also reflect an actual trend of NOTES moving out of the public focus.

Physicians' negative perception of NOTES is not

completely new. In a recent study by Volckmann *et al.*<sup>[9]</sup>, surgeons were asked whether they would choose personally to undergo NOTES cholecystectomy. Only 26% of them opted for NOTES over laparoscopy, with most of the surgeons citing that NOTES was too new and was more risky. In another study by Thele *et al.*<sup>[15]</sup>, only 29% of gynecologists would recommend NOTES to their patients even if NOTES presented the same surgical risks as the laparoscopic approach. Interestingly however, female physicians in our study were almost three times more likely to choose NOTES than male physicians. The reason for this finding is unclear.

In patients, decreased postoperative pain was the major determinant for favoring NOTES. In physicians, the major determinant was absence of hernia. Surprisingly, cosmesis was considered important by only a third of patients and half of physicians. However, such a finding is consistent with a survey comparing NOTES and laparoscopy by Strickland *et al.*<sup>[16]</sup>, in which only 44% of women were concerned with scarring after laparoscopy. Even though we did not ask our subjects specifically for reasons for refusing NOTES, those who favored laparoscopic appendectomy most frequently reported proven safety of laparoscopy as their reason. The second most common reason was absence of cosmesis concerns, further supporting the above stated finding of cosmesis being a relatively infrequent reason for choosing NOTES. Thus, the most striking benefit of scarless surgery may not be the most important one for potential patients.

In our study, patients with high-school education and those with prior colonoscopy experience were significantly more likely to choose NOTES over laparoscopic appendectomy. However, this finding resulting from a multivariate analysis of the pooled data needs to be interpreted with caution. Our two groups of subjects differed a priori in their educational status, with all physicians having a university education. Furthermore, the frequency of prior colonoscopy was much lower in the physicians group. Thus, university education and low rate of prior colonoscopy experience were inherent attributes of the physicians group having a low preference for NOTES. Multivariate analysis of the patient group itself did not reveal any characteristics suggestive of NOTES preference.

Interestingly, in both groups, the preferred route of access was the colon, with the vagina preferred by only a minority of women. This finding was surprising given the very unpleasant bowel preparation required for transcolonic NOTES as opposed to transgastric and transvaginal NOTES, and is in contrast with some previously reported results<sup>[8]</sup>. It may be speculated that for some patients, removal of diseased organs may be better tolerated when performed via the anus as compared to the mouth. Another reason might be the high rate of prior colonoscopy experience in our subjects. The reserved attitude of women to transvaginal access is in contrast with previously published data<sup>[17]</sup>, and deserves attention because most of the current procedures are offered via

the transvaginal route due to uncertainty of transgastric or transcolonic closure.

The preference for NOTES decreased dramatically, even in those who preferred it, once the indicated rate of complications increased. This phenomenon was observed in both patients and physicians and has been reported previously by others<sup>[8]</sup>. However, in our study population, the NOTES preference was significantly lower than that reported by Swanstrom *et al*<sup>[10]</sup>. In their study group, almost 20% of patients would have still elected NOTES even if the complication risk was 10 times greater than that of laparoscopy. Only less than 5% of our patients tolerated only twice the risk of laparoscopic appendectomy.

Interestingly, physicians were less willing than patients to consent to their wives vaginal NOTES procedure. This finding may be explained in part by the negative perception of NOTES itself by physicians as well as their better awareness of the possible complications associated with transvaginal procedures such as dyspareunia, infection and infertility.

Several limitations of our study should be noted. Only patients undergoing an endoscopic procedure were questioned. Such a study population may not accurately represent the general public. Next, our patients were asked about their perception of NOTES appendectomy. Acceptance of NOTES may be disease related and may thus be different for another procedure. Furthermore, our patients did not suffer from complaints of acute appendicitis and their decision was being made only based on description of the disease. They may also have been biased in favor of NOTES due to attending an endoscopy clinic as opposed to a surgical clinic. Finally, only a theoretical description of the benefits and risks was provided, which may change as more data are available.

In conclusion, the survey results show that there is considerable public interest in a new and experimental technique of transluminal surgery. In contrast, physicians would be reluctant to undergo NOTES. Decreased pain and absence of hernia were the most frequently reported reasons for choosing NOTES in patients and physicians, with cosmesis being of minor importance. Physicians were much more concerned than patients about the risk of infection. The study indicates that NOTES should be seriously considered as a potential technique with considerable public demand. However, it also highlights the importance of further development of the technique and outcomes data reporting to enlighten physicians about NOTES.

## COMMENTS

### Background

Natural orifice transluminal endoscopic surgery (NOTES) is a novel surgical technique that takes advantages of natural orifices. It may be even less invasive than traditional laparoscopic surgery because it avoids abdominal incisions.

### Research frontiers

Wide adoption of NOTES will depend largely on public acceptance. Results of the few studies that have addressed perception of NOTES have been variable.

### Innovations and breakthroughs

The study shows that there is a considerable public interest for NOTES, even though it has declined over time. Physicians remain reluctant to undergo transluminal surgery.

### Applications

The study indicates that NOTES should be seriously considered as a potential technique, with considerable public demand. However, further development is needed to convince physicians about the potential benefits of NOTES.

### Terminology

NOTES is a minimally invasive surgical technique that eliminates abdominal incision. Flexible endoscopes are used to create a transvisceral opening via natural orifice access to enter the peritoneal cavity.

### Peer review

This is a survey evaluating the acceptance of NOTES among patients and physicians. The authors found considerable acceptance, which at some points contrasts with previous studies, and is important to report.

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## Differential expression of Rab27A/B correlates with clinical outcome in hepatocellular carcinoma

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### Abstract

**AIM:** To investigate the association of Rab27A and Rab27B expression with clinicopathological characteristics and prognosis of hepatocellular carcinoma (HCC).

**METHODS:** We used reverse transcription polymerase chain reaction (RT-PCR), real-time PCR, and western blotting to detect Rab27A and Rab27B mRNA and protein expression in 5 human HCC lines and the immortalized hepatic HL-7702 cell line. We further examined 148 primary HCC samples matched with adjacent normal tissue and 80 non-HCC specimens by immunohistochemistry to evaluate the correlation of Rab27A and Rab27B expression with clinicopathological features and prognosis.

**RESULTS:** Our data showed that Rab27A and Rab27B

were differentially expressed in cell lines and primary HCC tumors. Rab27A mRNA and protein were detected in 67% (4/6) of human cell lines and 80% (4/5) of HCC cell lines, while Rab27B was found in 50% (3/6) of human lines and 40% (2/5) of HCC lines. Rab27A expression was higher in primary HCC (46.2%, 66/143) than in matched adjacent tissue (24.3%, 33/136;  $P < 0.001$ ), whereas immunopositivity for Rab27B was lower in primary HCC (57.4%, 81/141) than in matched adjacent tissue (87.5%, 119/136;  $P < 0.001$ ). Analysis of clinicopathological characteristics of 148 HCC specimens revealed significant correlations between Rab27A and Rab27B expression and tumor tumor-node-metastasis (TNM) classification ( $P = 0.046$  and  $P = 0.027$ , respectively), and between strong Rab27A expression and tumor differentiation grade ( $P = 0.008$ ). Survival analyses revealed that patients with Rab27A<sup>+</sup> or Rab27B<sup>+</sup> tumors had significantly reduced overall survival compared with that of patients with Rab27A<sup>-</sup> or Rab27B<sup>-</sup> tumors ( $P = 0.015$  and  $P = 0.005$ , respectively). Risk analyses revealed that Rab27B<sup>+</sup> and TNM III-IV were independent poor prognosis factors associated with a 3.36- and 3.37-fold higher relative risk of death, respectively.

**CONCLUSION:** Rab27A and Rab27B expression were closely correlated with tumor progression and can be valuable prognostic indicators for HCC patients.

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**Key words:** Rab27A; Rab27B; Hepatocellular carcinoma; Immunohisto-chemistry; Prognosis

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## INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the top 10 most frequent tumor types globally, carrying a high mortality rate and accounting for more than 1 million deaths annually<sup>[1]</sup>. The identification of novel biomarkers correlating with HCC progression is critical in order to optimize treatment strategies. A large body of evidence indicates that vesicle trafficking and exocytosis are important in tumorigenesis, with many reports implicating the Rab family of proteins<sup>[2-4]</sup>. Rabs are a ubiquitously expressed family of small (20-29 kDa) monomeric Ras-like GTPases<sup>[5]</sup> composed of more than 60 mammalian members, each thought to localize to a distinct subcellular organelle. Rabs function as molecular switches, oscillating between GTP- or GDP-bound conformations, which enables them to reversibly recruit GTP-dependent effectors and elicit their regulatory functions at multiple stages of vesicular transport<sup>[6]</sup>. Rab27A and Rab27B constitute the Rab27 subfamily and share 71% identity<sup>[7]</sup>. Rab27A is expressed in a wide variety of secretory cell types, including exocrine, endocrine, ovarian, and hematopoietic cells, most of which function specifically in regulated exocytic pathways<sup>[8,9]</sup>. Loss-of-function mutations in the human *Rab27A* gene result in Griscelli syndrome, a rare autosomal disorder characterized by a combination of partial cutaneous albinism and severe immunodeficiency<sup>[10,11]</sup>. Its clinical picture appears to be the manifestation of defects in 2 specialized lysosome-related organelles: the failure to distribute melanosomes in melanocytes and the inability to release the contents of lytic granules in cytotoxic T lymphocytes<sup>[12]</sup>. In contrast to Rab27A, Rab27B expression is much more restricted, and is mainly expressed in platelets, the stomach, large intestine, pancreas, pituitary, and bladder<sup>[13-16]</sup>. Unlike Rab27A, Rab27B has not been well characterized, and no human disease or animal strain with mutations in the *Rab27B* gene has been identified<sup>[8]</sup>.

Several members of the Rab family have been well studied in cancer. Rab25, for example, has been shown to decrease apoptosis as well as increase the proliferation and aggressiveness of ovarian and breast cancer<sup>[17-19]</sup>. Increased expression of Rab25 has also been noted in prostate cancer<sup>[20]</sup>, transitional cell carcinoma of the bladder<sup>[21]</sup>, and in colon cancer cells<sup>[22]</sup>. Hou *et al.*<sup>[23]</sup> showed that the *Rab23* gene is over-expressed in gastric cancer, and has an important role in invasion. In addition, Rab11, Rab4, Rab14 and Rab35, among others, have been studied in various cancers<sup>[24]</sup>. However, research on Rab27 in cancer has been limited, and focused exclusively on breast cancer. These studies have shown that Rab27A, along with several other metastasis-associated genes, have vesicle trafficking roles, and were differentially expressed in murine xenograft models of breast cancer metastasis<sup>[25,26]</sup>.

Wang *et al.*<sup>[27]</sup> showed that Rab27A is associated with the invasive and metastatic potential of human breast cancer cells. Overexpression of Rab27A protein in breast cancer cells altered the cell cycle and increased the invasive and metastatic abilities both *in vitro* and *in vivo*. However, in another study, it was shown that Rab27B, not Rab27A, regulates invasive growth and metastasis in ER-positive breast cancer cell lines, with increased expression associated with poor prognosis in humans<sup>[28]</sup>. Despite the link between Rab27 and breast cancer, alterations in Rab27 expression have yet to be explored in other tumor types.

During a previous study of vesicle transport and metastasis in gastric cancer and colorectal cancer (unpublished data), we found a significant correlation between Rab27A and Rab27B expression and clinicopathological characteristics and prognosis. We then examined Rab27A and Rab27B expression in HCC to determine if a similar correlation was present. To accomplish this, we evaluated the expression of Rab27A and Rab27B mRNA and protein in 5 HCC cell lines and the human hepatic HL-7702 cell line. Additionally, we performed parallel immunohistochemical staining of Rab27A and Rab27B in 148 primary HCCs in order to analyze the association with clinicopathological characteristics and clarify the distinct roles of Rab27A and Rab27B in the progression of HCC.

## MATERIALS AND METHODS

### Patients and tissues

One hundred forty-eight HCC specimens and 80 non-HCC specimens were collected from 182 men and 46 women (age, 29-72 years; mean  $\pm$  SD, 51.6  $\pm$  8.9 years) who were inpatients at the PLA General Hospital, Beijing, China, from 2005 to 2009. Survival data were available for 120 patients; 52 of 120 patients (43.3%) died of cancer metastasis or local recurrence after surgery. Patient data are shown in Table 1. All patients underwent surgery, and no patients had received chemotherapy or radiation therapy. Tissue microarray blocks containing formalin-fixed and paraffin-embedded human tissues were constructed in our laboratory as described previously<sup>[29]</sup>. Tumor stage was classified according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification. The study was approved by the Research Ethics Boards of the hospitals, and informed consent was obtained from all patients.

### Cell lines and cell culture

The human HCC cell lines MHCC97L and MHCC97M3 were a kind gift from Professor Ye SL (FuDan University, Shanghai). Cell lines BEL-7402, Huh-7, SMMC-7721 and HL-7702 were routinely cultured in our laboratory. HL-7702 was cultured in Roswell Park Memorial Institute medium (RPMI 1640, Gibco, Grand Island, NY, United States), supplemented with 20% fetal bovine serum (FBS; Gibco). BEL-7402 and SMMC-7721 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The remaining cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco)

**Table 1 Rab27A status in relation to clinicopathological features in patients with hepatocellular carcinoma (%)**

	No. of cases	Positive	Negative	P value
<b>Rab27A clinicopathological features</b>				
All patients	148			
<b>Gender</b>				
Male	108 (75.5)	47 (43.5)	61 (56.5)	
Female	35 (24.5)	19 (54.3)	16 (45.7)	NS
<b>Age at diagnosis</b>				
< 60	116 (81.1)	51 (44)	65 (56)	
≥ 60	27 (18.9)	15 (56.6)	12 (43.5)	NS
<b>Carcinoma and adjacent tissue</b>				
Carcinoma tissue	143 (51)	66 (46.2)	77 (53.8)	
Adjacent tissue	136 (49)	33 (24.3)	103 (75.7)	< 0.001
<b>Hepatic and cirrhosis tissue</b>				
Normal hepatic tissue	40 (44.4)	10 (25)	30 (75)	
Hepatitis and cirrhosis tissue	50 (55.6)	13 (26)	37 (74)	NS
<b>Degree of differentiation</b>				
Well	39 (27.3)	2 (5.1)	37 (94.9)	
Moderate	91 (63.6)	9 (9.9)	82 (90.1)	
Poor	13 (9.1)	5 (38.5)	8 (61.5)	0.008 <sup>1</sup>
<b>TNM classification</b>				
Stage I / II	106 (74.1)	41 (38.7)	65 (61.3)	
Stage III / IV	37 (25.9)	25 (67.7)	12 (32.4)	0.002
<b>HBV</b>				
Negative	26 (19.4)	10 (38.5)	16 (61.5)	
Positive	108 (80.6)	53 (49.1)	55 (50.9)	NS
<b>AFP</b>				
Negative	42 (32.5)	15 (35.7)	27 (64.3)	
Positive	88 (67.7)	43 (48.9)	45 (51.1)	NS
<b>Type of hepatoma</b>				
Nodular	84 (66.7)	39 (46.4)	45 (53.6)	
Massive	33 (26.2)	12 (36.4)	21 (63.6)	
Diffuse	9 (7.1)	5 (55.6)	4 (44.4)	NS
<b>Rab27B clinicopathological features</b>				
All patients	148			
<b>Gender</b>				
Male	106 (75.2)	61 (57.5)	45 (42.5)	
Female	35 (24.8)	20 (57.1)	15 (42.9)	NS
<b>Age at diagnosis</b>				
< 60	114 (80.9)	65 (57)	49 (43)	
≥ 60	27 (19.1)	16 (59.3)	11 (40.7)	NS
<b>Carcinoma and adjacent tissue</b>				
Carcinoma tissue	141 (51)	81 (57.4)	60 (42.6)	
Adjacent tissue	136 (49)	119 (87.5)	17 (12.5)	< 0.001
<b>Hepatic and cirrhosis tissue</b>				
Normal hepatic tissue	40 (44)	29 (72.5)	11 (27.5)	
Hepatitis and cirrhosis tissue	50 (56)	38 (76)	12 (24)	NS
<b>Degree of differentiation</b>				
Well	39 (27.5)	26 (66.7)	13 (33.3)	
Moderate	89 (63.4)	49 (53.9)	41 (46.1)	
Poor	13 (9.2)	8 (61.5)	5 (38.5)	NS
<b>TNM classification</b>				
Stage I / II	103 (73)	51 (49.5)	52 (50.5)	
Stage III / IV	38 (27)	30 (78.9)	8 (21.1)	0.002
<b>HBV</b>				
Negative	27 (19.9)	17 (63)	10 (37)	
Positive	109 (80.1)	59 (54.1)	50 (45.9)	NS
<b>AFP</b>				
Negative	43 (33.6)	23 (53.5)	20 (46.5)	
Positive	85 (66.4)	49 (57.6)	36 (42.4)	NS
<b>Type of hepatoma</b>				
Nodular	84 (66.7)	40 (47.6)	44 (52.4)	
Massive	33 (26.2)	15 (45.5)	18 (54.5)	
Diffuse	9 (7.1)	6 (66.7)	3 (33.3)	NS

<sup>1</sup>Comparing the strong positive group with the other groups. TNM: Tumor-node-metastasis; NS: Not significant; HBV: Hepatitis B virus; AFP: Alpha fetoprotein.

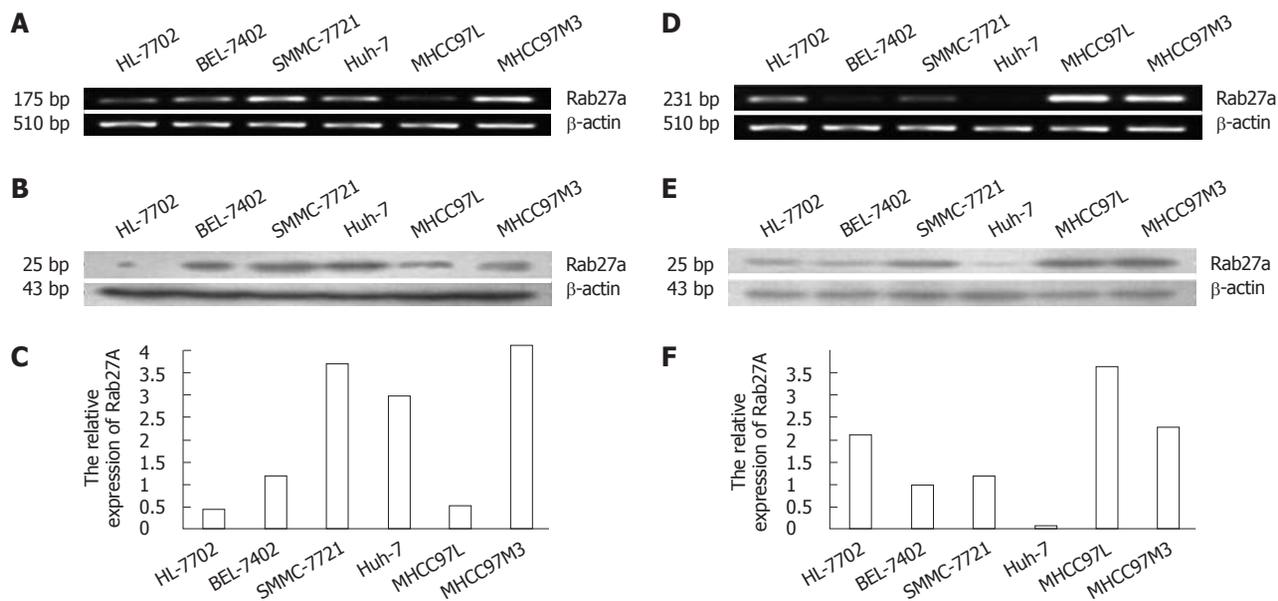
supplemented with 5% FBS. All media contained 100 units/mL penicillin and 100 µg/mL streptomycin. All cell lines were maintained at 37 °C in 5% CO<sub>2</sub>.

### Tissue microarray immunohistochemistry

Antigen retrieval was performed for 2.5 min in citrated buffers using a pressure cooker. A mouse monoclonal antibody specific to human Rab27A (diluted 1:50; cat. ab55667, Abcam, United Kingdom) was incubated on the sections for 36 h at 4 °C, followed by incubation with a rabbit anti-Rab27B polyclonal antibody (diluted 1:100; cat. 13412-1-AP, Proteintech Group, United States) overnight at 4 °C. The rest of the immunohistochemistry (IHC) procedure has been previously described<sup>[30]</sup>. Fewer than 5% specimens were missing from the tissue microarrays. Phosphate buffered saline was substituted for the primary antibodies for a general negative control. All sections were examined microscopically in a blinded fashion and scored by 2 independent pathologists. Rab27A and Rab27B IHC signal was scored on the following scale, taking into account both the proportion of cells stained and the intensity of staining in those cells: score 0: Weak or absent cytoplasmic staining, with less than 5% of cancer cells showing Rab27A or Rab27B localized on the plasma membrane; score 1: Cytoplasmic staining and between 5% and 30% of cancer cells showing Rab27A or Rab27B localized prominently on the plasma membrane; score 2: Cytoplasmic staining and more than 30% of the cancer cells containing Rab27A or Rab27B localized prominently on the membrane.

### Reverse transcription-polymerase chain reaction and real-time polymerase chain reaction

Total RNA was extracted from cell lines using TRIzol (Qiagen, United States). The prepared RNA (5 µg) was mixed with oligo-dT primers and reverse-transcribed with MMLV reverse transcriptase (Promega, United States) for 60 min at 37 °C, followed by polymerase chain reaction (PCR) amplification with specific primers for Rab27A (F,5'-GAAGCCATAGCACTCGCAGAG-3',R,5'-ATGACCATTGATCGCACCA-3') or Rab27B (F,5'-TGC-GGGACAAGAGCGGTTCCG-3',R,5'-GCCAGTTCGGAGCTTGCCGTT-3'). PCR amplification was performed in 20 µL using a thermocycler (Biometra, Germany) with the following PCR program: pre-denaturation for 3 min at 94 °C, denaturation for 45 s at 94 °C, annealing for 45 s at 56 °C, extension for 45 s at 72 °C, and a final elongation at 72 °C for 10 min. β-Actin served as an internal positive control. PCR was performed for 24-30 cycles (β-actin 24 cycles; Rab27A 28 cycles; Rab27B 30 cycles). PCR products were analyzed by electrophoresis on a 1.5% agarose gel, and band intensity was measured directly on an AlphaImager 2200 system (Alpha Innotech, San Leandro, CA). Real-time PCR reactions were carried out using Applied Biosystem's 7500 QPCR System (ABI, Foster, CA). Results were normalized to individual β-actin expression, and data were analyzed according to the relative standard curve. Melting curves for each PCR reaction were generated to ensure the purity of the amplified products.



**Figure 1** Detection of Rab27A and Rab27B mRNA and protein in 6 human cell lines. A and C: Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR, respectively, of Rab27A in 5 hepatocellular carcinoma cell lines and the human HL-7702 line; D and F: RT-PCR and real-time PCR, respectively, of Rab27B; B and E: Western blotting of Rab27A and Rab27B protein levels, respectively.  $\beta$ -actin served as an internal control.

### Western blotting

Equal amounts of protein were electrophoresed on a 12% sodium dodecylsulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane using standard techniques. Immunoreactivity was tested with anti-Rab27A (diluted 1:100; cat. ab55667, Abcam, United Kingdom) or anti-Rab27B (diluted 1:1200; cat. 13412-1-AP, Proteintech Group, United States) antibodies. Nonspecific binding was blocked by a 5% fat-free milk solution. Rab27A and Rab27B proteins were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

### Statistical analysis

Statistical analyses were performed using the SPSS software package (version 16.0; SPSS Inc., United States). The  $\chi^2$  test was used to evaluate relationships between clinicopathological variables and Rab27A and Rab27B expression. Kaplan-Meier survival analysis with the log-rank test was used to evaluate the prognosis of patients according to their levels of Rab27A and Rab27B expression. Multivariate analysis was performed with the Cox proportional hazards regression model to assess the effects of different variables on patient survival. Differences were considered significant at  $P < 0.05$ .

## RESULTS

### Expression of Rab27A and Rab27B in hepatocellular carcinoma cell lines

Rab27A and Rab27B mRNA and protein expression were examined in 5 HCC cell lines and the human hepatocyte line HL-7702. As shown in Figure 1, Rab27A mRNA and protein were detected in all 6 cell lines, though at very low intensities in some cell lines, whereas Rab27B was more differentially expressed. Rab27A pro-

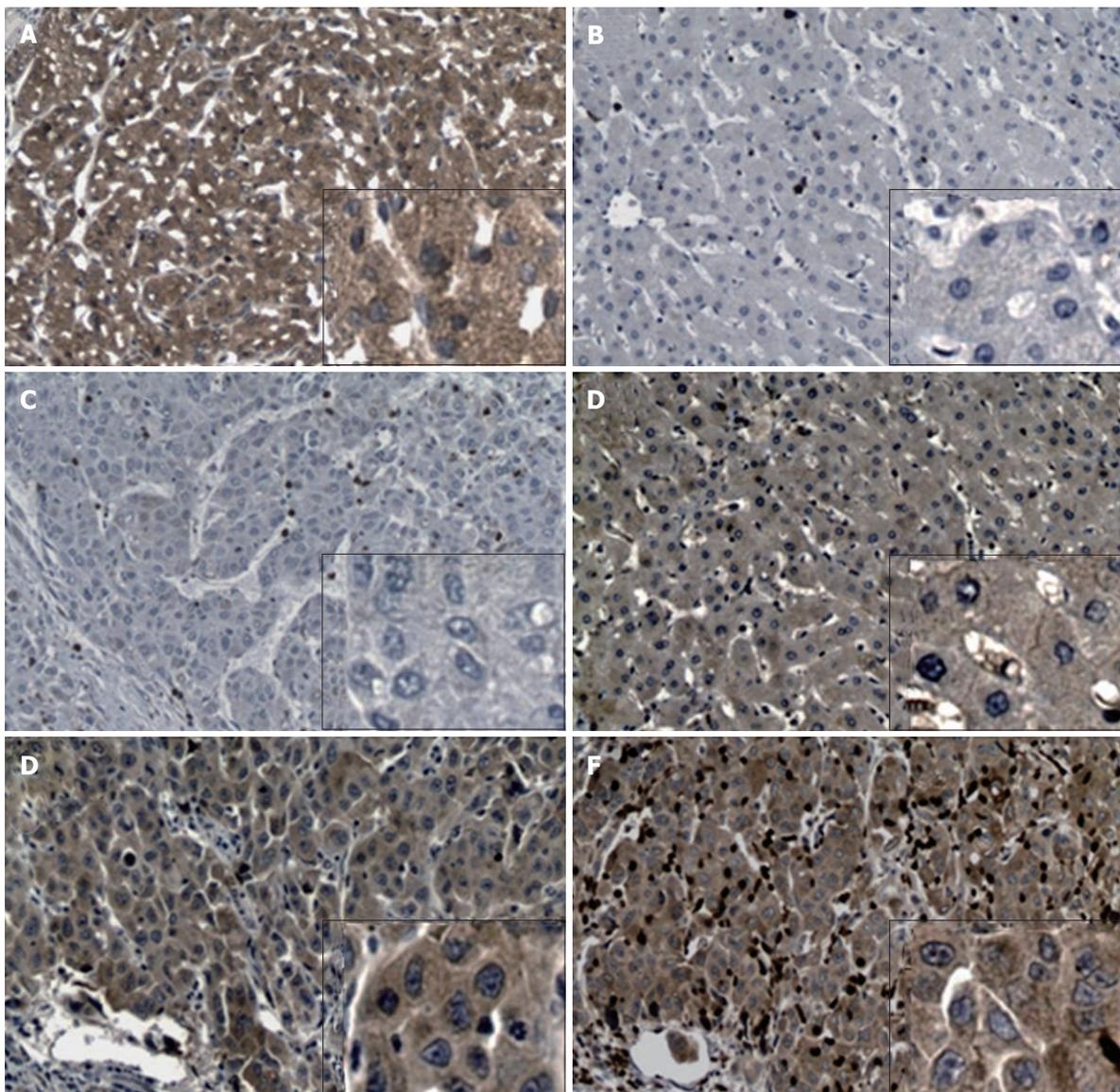
tein and mRNA were highly expressed in 67% (4/6) of all cell lines and 80% (4/5) of HCC cell lines; Rab27B protein and mRNA were highly expressed in 50% (3/6) of all cell lines and 40% (2/5) of HCC cell lines. Interestingly, Rab27A expression was weaker in the low metastatic cell line MHCC97L than in the high metastatic cell line MHCC97H, whereas Rab27B expression was higher in MHCC97L than in MHCC97H. In addition, Rab27A had only negligible expression in the immortal human hepatocyte line HL-7702, while Rab27B was moderately expressed.

### Rab27A and Rab27B expression in primary hepatocellular carcinoma specimens

To determine Rab27A and Rab27B expression in HCC specimens, IHC was performed on tumor tissue, tumor-adjacent normal tissue, unrelated normal hepatic tissue, and hepatitis or cirrhosis tissues. In primary HCC tumors, Rab27B and Rab27A were detected in 57.4% (81/141) and 46.2% (66/143) of specimens, respectively (Table 1, Figure 2). In adjacent tissue, Rab27A expression was less apparent, with significantly less positivity (24.3%, 33/136) than that in HCC specimens ( $P < 0.001$ ); however, Rab27B showed strong immunopositivity (87.5%, 119/136), with significantly higher expression than that in HCC specimens ( $P < 0.001$ ). In addition, we found no differences in immunostaining for Rab27A and Rab27B between normal ( $n = 40$ ) and hepatitis or cirrhosis tissues ( $n = 50$ ), excluding the possibility that alterations in Rab27A and Rab27B expression in primary HCC tumors were caused by hepatitis or liver cirrhosis (Table 1).

### Association of Rab27A and Rab27B expression in hepatocellular carcinoma specimens with clinicopathological features

Analysis of the clinicopathological characteristics of the



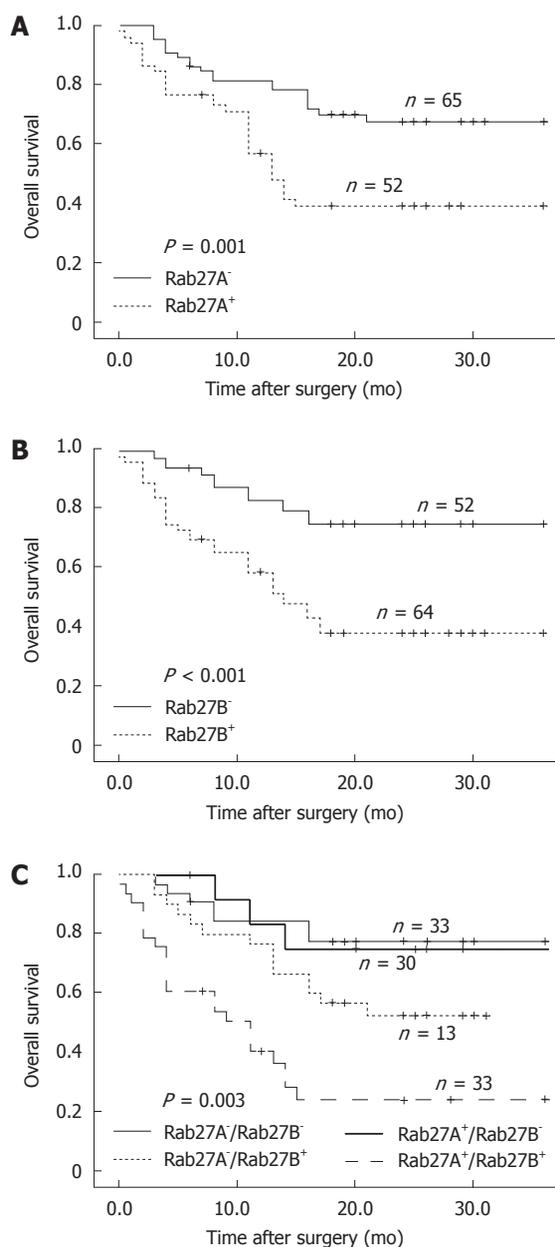
**Figure 2** Differential expression of Rab27A and Rab27B in hepatocellular carcinoma patients. A: Positive staining of Rab27A in hepatocellular carcinoma (HCC) tissue; B: Negative staining of Rab27A in adjacent tissue; C: Negative staining of Rab27B in HCC tissue; D: Positive staining of Rab27B in adjacent tissue; E and F: Positive staining of Rab27A and Rab27B, respectively, in the same HCC tissue. Both Rab27A and Rab27B were observed in the cytoplasm and plasma membrane of human cells. Original magnification  $\times 100$ ; the inset boxes are at original magnification  $\times 400$ .

148 HCC specimens revealed significant correlations between Rab27A and Rab27B expression and tumor TNM classification ( $P = 0.046$  and  $P = 0.027$ , respectively; Table 1), as well as strong Rab27A expression with tumor differentiation ( $P = 0.008$ , Table 1). Moreover, there was a statistically significant correlation between the expression of Rab27A and Rab27B in HCC ( $P = 0.017$ ;  $r = 0.192$ ). However, we found no relationship between Rab27A or Rab27B positivity and hepatitis B virus (HBV) status, alpha fetoprotein level, or type of hepatoma. Further subdivision of the HCC specimens using Rab27A and Rab27B expression as covariables (i.e., Rab27A<sup>-</sup>/Rab27B<sup>-</sup>, Rab27A<sup>-</sup>/Rab27B<sup>+</sup>, Rab27A<sup>+</sup>/Rab27B<sup>-</sup>, and Rab27A<sup>+</sup>/Rab27B<sup>+</sup> specimens) showed no significant differences between the 4 groups in relation to clinicopathological features (data not shown).

#### **Survival analyses of patients according to Rab27A and Rab27B expression status after surgery**

Survival data were available for 120 patients. The overall 1-year survival rate was 71%. Patients with Rab27A<sup>+</sup> or Rab27B<sup>+</sup> tumors had significantly reduced overall survival compared with that of patients with Rab27A<sup>-</sup> or Rab27B<sup>-</sup> tumors ( $P = 0.015$  and  $P = 0.005$ , respectively; Figure 3A and B). Subsequently, we analyzed the survival curves of the 4 groups according to the expression status of Rab27A and Rab27B. Patients with Rab27A<sup>-</sup>/Rab27B<sup>-</sup> tumors had the longest survival, with the Rab27A<sup>+</sup>/Rab27B<sup>-</sup> group showing a similar survival curve; the Rab27A<sup>-</sup>/Rab27B<sup>+</sup> group had lower survival, while the Rab27A<sup>+</sup>/Rab27B<sup>+</sup> group had the poorest survival ( $P = 0.003$ , Figure 3C).

To determine relative risk, we analyzed the data with



**Figure 3** Kaplan-Meier overall survival analysis of Rab27A and Rab27B in hepatocellular carcinoma patients. A: Survival analysis of hepatocellular carcinoma patients by expression of Rab27A; B: Survival analysis by expression of Rab27B; C: Survival analysis with expression of Rab27A and Rab27B as covariables.

a Cox proportional hazards model using tumor differentiation grade, TNM stage, HBV status, hepatoma type, and Rab27A and Rab27B expression status as covariates. The positive expression of Rab27B as well as TNM III-IV were independent poor prognosis factors associated with a 3.36- and 3.37-fold higher relative risk of death, respectively (95% CI, 1.33-8.5 and 1.391-8.186;  $P$  value, 0.01 and 0.007, respectively).

## DISCUSSION

To the best of our knowledge, this is the first study investigating the expression of Rab27A and Rab27B in a large

series of HCC patients. Utilizing RT-PCR, real-time PCR, Western blotting, and IHC, we found that Rab27A and Rab27B were strongly associated with tumor TNM stage and may serve as useful biomarkers to monitor the clinical course of patients with HCC. Although the expression of Rab27A and Rab27B was closely related, Rab27B seemed to play a key role in determining prognosis.

Our results revealed that Rab27A and Rab27B were differentially expressed in HCC cell lines and tissue specimens; this indicates that Rab27A and Rab27B may be related to malignant transformation in this carcinoma. In the present study, we found that Rab27A and Rab27B were expressed in the cytoplasm and plasma membrane of hepatic cells. Rab27A was expressed at higher levels in primary HCC than in matched adjacent tissue, whereas Rab27B expression was lower in primary HCC than in matched adjacent tissue. In accordance with these data, Rab27A and Rab27B mRNA and protein were differentially expressed in the 6 human cell lines. The results from the tissue specimens were mirrored in the immortal human hepatocyte line HL-7702, low metastatic cell line MHCC97L, and high metastatic cell line MHCC97H. In addition, Rab27A mRNA and protein were expressed in all 6 cell lines, albeit at different intensities, whereas Rab27B was differentially expressed; this finding is consistent with the findings of previous studies that Rab27A is more widely expressed than Rab27B.

We further analyzed the association between Rab27A and Rab27B expression and clinicopathologic variables utilizing IHC. In HCC specimens, positive expression of Rab27A and Rab27B was strongly correlated with stage III/IV, while negative expression was correlated with stage I/II. Furthermore, strong expression of Rab27A (score 2) also correlated with tumor differentiation. Our study is the first to identify that Rab27A and Rab27B strongly correlate with tumor progression, and provides evidence that Rab27A might function in cell differentiation. Previous research demonstrated that when Rab27A effectors are bound by another protein, Rab27B plays an important role in the regulation of many secretory mechanisms<sup>[31]</sup>. Another study showed that Rab27A and Rab27B play both redundant and distinct roles in regulating the secretion and released quantity of platelet dense granules<sup>[9]</sup>. In agreement with these studies, our results provide evidence that Rab27A and Rab27B may have redundant and distinct functions. In addition, numerous studies have demonstrated that tumor cells use exosomes (endosome-derived membrane vesicles) to communicate with surrounding tissues and immune cells, creating a suitably immunosuppressive microenvironment for tumor growth, invasion, and metastasis<sup>[32-35]</sup>. We hypothesize that Rab27A and Rab27B are associated with tumor progression because of their functions as transport vesicles.

Our study also showed that patients with Rab27A<sup>+</sup> or Rab27B<sup>+</sup> tumors had significantly reduced overall survival compared with that of patients with Rab27A<sup>-</sup> or Rab27B<sup>-</sup> tumors. Moreover, patients with Rab27A<sup>-</sup>/Rab27B<sup>-</sup> tumors had the longest survival, while those with Rab27A<sup>+</sup>/Rab27B<sup>+</sup> tumors had the poorest survival. An analysis of

the tumor variables as risk factors showed that Rab27B<sup>+</sup> and TNM III-IV were independent poor prognosis factors, conferring a 3.36-fold and 3.37-fold greater relative risk of death. These results provide further evidence that Rab27A and Rab27B can serve as useful biomarkers for determining prognosis as well as monitoring the clinical course of patients with HCC. Furthermore, Rab27B is more useful than Rab27A as an independent prognosis factor.

Although studies on Rab27 in breast cancer have provided contradictory results, previous findings are partially consistent with our results. Wang *et al.*<sup>[27]</sup> showed that overexpression of Rab27A is associated with invasive and metastatic potential in human breast cancer cells both *in vitro* and *in vivo*. However, they found no Rab27B expression in breast cancer. This discrepancy may be because they utilized cell lines and samples from nude mice, rather than human breast cancer tissue. Another study on Rab27 and breast cancer showed that Rab27B regulates invasive growth and metastasis in ER-positive breast cancer cell lines and that increased expression is associated with poor prognosis in humans<sup>[28]</sup>. However, they found no expression of Rab27A in normal tissue ( $n = 5$ ) or primary breast carcinoma ( $n = 20$ ), though this may have been due to the small sample size. Additionally, the results of our previous study on GC and CRC were not consistent with our HCC results. Expression of Rab27A and Rab27B was much lower in primary GC and CRC than in adjacent mucosal tissue. Among these patients, those with Rab27A<sup>+</sup>/Rab27B<sup>+</sup> tumors had the longest survival, while the Rab27A<sup>-</sup>/Rab27B<sup>-</sup> group had the poorest survival; a Cox proportional hazards model showed that Rab27A<sup>+</sup>/Rab27B<sup>+</sup> expression was a protective prognosis factor in both GC and CRC (unpublished data). These findings show that Rab27A and Rab27B may have different molecular mechanisms in HCC compared with those in GC and CRC.

In conclusion, our study demonstrated that Rab27A and, to a greater degree, Rab27B were correlated with tumor progression and may serve as valuable prognostic indicators for HCC patients. However, further investigation is required to determine the molecular mechanism of Rab27A and Rab27B in HCC.

## COMMENTS

### Background

Primary hepatocellular carcinoma (HCC) carries a high mortality rate and is one of the top 10 most frequent tumor types globally. The identification of novel biomarkers correlating with HCC progression is crucial to monitor clinical disease course and provide corresponding therapy strategies. A large body of evidence indicates that vesicle trafficking and exocytosis are important in tumorigenesis, with many reports implicating the Rab family of proteins.

### Research frontiers

Several members of the Rab family, such as Rab25, Rab23 and Rab11, have been well studied in cancer. However, research on Rab27 in cancer is both limited and contradictory, and has focused exclusively on breast cancer. Due to the high mortality rate and lack of efficacious therapy strategies, the identification of Rab27 expression on primary HCC as a novel biomarker of the disease may allow for the development of improved therapy strategies.

### Innovations and breakthroughs

This is the first study to investigate the expression of Rab27A and Rab27B in a large series of HCC patients. It was found that Rab27A and Rab27B expression were closely linked and both Rab27A and Rab27B were strongly associated with tumor progression, which can be valuable prognostic indicators for HCC patients.

### Applications

Rab27A and Rab27B were closely related with tumor progression and may serve as useful biomarkers to monitor the clinical course of patients with HCC. The relationship between the expression of Rab27A and Rab27B and tumor progression may be due to their functions as transport vesicles, which may provide potential therapeutic targets for HCC.

### Terminology

Rabs are a ubiquitously expressed family of small monomeric Ras-like GTPases. They function as molecular switches, oscillating between GTP- or GDP-bound conformations, which enables them to reversibly recruit GTP-dependent effectors and elicit their regulatory functions at multiple stages of vesicular transport; Rab27A is expressed in a wide variety of secretory cell types, most of which function specifically in regulated exocytic pathways. Loss-of-function mutations in the human Rab27A gene result in Griscelli syndrome; Rab27A and Rab27B constitute the Rab27 subfamily and share 71% identity. Rab27B expression is much more restricted than that of Rab27A and no human disease or animal strain with mutations in the *Rab27B* gene has been identified.

### Peer review

In the present study, the authors found that Rab27A and Rab27B were expressed differently in HCC. Rab27A was expressed at higher levels in primary HCC, whereas Rab27B expression was lower in primary HCC. In agreement with these findings, Rab27A and Rab27B mRNA and protein were also differentially expressed in 6 human cell lines. Survival analysis further showed that Rab27 expression may be used as an indicator for prognosis in HCC patients, providing important information for the treatment of patients.

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## Propofol vs midazolam plus fentanyl for upper gastrointestinal endomicroscopy: A randomized trial

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### Abstract

**AIM:** To compare the endomicroscopic image quality of integrated confocal laser endomicroscopy (iCLE) and sedation efficacy of propofol vs midazolam plus fentanyl (M/F).

**METHODS:** Consecutive outpatients undergoing iCLE were prospectively recruited and randomized to the propofol group (P group) or M/F group. The patient, performing endoscopist and endoscopic assistant were blinded to the randomization. The quality of endomicro-

scopic images and anesthetic efficacy outcomes were blindly evaluated after iCLE examination.

**RESULTS:** There were significantly more good quality endomicroscopic images in the propofol group than in the M/F group (72.75% vs 52.89%,  $P < 0.001$ ). The diagnostic accuracy for upper gastrointestinal mucosal lesions using confocal laser endomicroscopy favors the P group, although this did not reach statistical significance. Adverse events and patient assessment were not significantly different for M/F vs propofol except for more frequent intraprocedural recall with M/F. Procedure duration and sedation times were significantly longer in the M/F group, while the scores of endoscopist, anesthetist and assistant assessment were all significantly better in the P group.

**CONCLUSION:** Sedation with propofol might increase the proportion of good quality endomicroscopic images, and may result in improved procedural efficacy and diagnostic accuracy during iCLE examination.

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**Key words:** Confocal laser endomicroscopy; Conscious sedation; Randomized trial; Sensitivity and specificity; Image quality

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## INTRODUCTION

Confocal laser endomicroscopy (CLE) is a novel technique for gastrointestinal (GI) endoscopy. It enables high-resolution analysis of cellular structure during endoscopy. Clinical applications of CLE have been validated in various GI diseases, such as Barrett's esophagus, gastric cancer, colorectal cancer, ulcerative colitis and celiac disease<sup>[1-5]</sup>. Recent studies have expanded its application for *in vivo* molecular imaging of GI cancer<sup>[6]</sup>. However, integrated CLE (iCLE) is more cumbersome than a standard gastroscope because iCLE has a larger outer diameter (12.8 mm) and a longer rigid tip (43 mm) which contains the scanning head (tip angulations: up/down 130 degrees). In addition, since endomicroscopic imaging can only be achieved by placing the confocal imaging window directly onto the area of interest, patients may suffer from more discomfort, especially when the lesion is located at the pylorus or gastroesophageal junction. Moreover, motion artifacts, which are the most common cause of endomicroscopic image artifacts, can often be caused by patients' movement and unstable endoscope positions. Thus, compared with conventional esophago-gastroduodenoscopy (EGD), iCLE might require more patients' cooperation and better sedation to get images of good quality and make an accurate diagnosis.

Conscious sedation is routinely used during endoscopic examination because it can provide adequate anxiolysis, acceptance, and amnesia for most patients *vs* no sedation, and is safer than deep sedation<sup>[7,8]</sup>. The combined use of a benzodiazepine (e.g., midazolam) and narcotics (e.g., fentanyl) is the most widely applied sedative regimen for GI endoscopy<sup>[9]</sup>. Recent data suggest that the use of propofol for sedation is increasing<sup>[10]</sup>. In some endoscopic centers, benzodiazepines, narcotics or propofol have been administered during iCLE<sup>[11-13]</sup>. However, the most effective and satisfactory sedation agent for iCLE examination has not yet been investigated.

Recently, propofol has been advocated as an alternative to the commonly used combination of midazolam and narcotic regimen (fentanyl, meperidine)<sup>[7,14-19]</sup>. Compared with midazolam, propofol is a short-acting sedative-hypnotic agent with a faster recovery profile, and its application is associated with some additional advantages, such as being easy to maintain an appropriate sedation level and satisfactory amnestic effect<sup>[7,14,15,18,19]</sup>. Several studies have reported the effect of sedation of propofol *vs* midazolam on the quality of upper and lower GI endoscopy by randomized trials<sup>[16,17,20]</sup>, however, no investigation has compared propofol with midazolam plus fentanyl (M/F) as sedatives for iCLE. Therefore, the aim of the present study was to compare the quality of endomicroscopic images and sedation efficacy outcomes between propofol and M/F as sedatives for iCLE.

## MATERIALS AND METHODS

### Patients

Consecutive outpatients who underwent iCLE were re-

cruited prospectively from the endoscopy clinic of Qilu Hospital, Shandong University, from February to May 2010. The exclusion criteria: < 18 years of age, known or suspected strictures or stenosis, coagulopathy, acute upper digestive tract bleeding, pregnancy or breast feeding, allergy to propofol, fentanyl, midazolam or fluorescein sodium, contraindications to sedation, mental disorders or did not provide written informed consent. Informed consent was obtained from all patients who underwent endoscopic examination in this study. The study was approved by the Ethical committee of Qilu Hospital and was conducted in accordance with the revised Declaration of Helsinki (1989). This trial was registered at www.clinicaltrials.gov, ID number NCT01053871.

### Sample size calculation and randomization

The sample size was calculated to achieve a statistical power of 0.8 at an alpha value of 0.05. For patients sedated with midazolam and fentanyl, the rate of good quality endomicroscopic images was estimated to be 66% according to previously reported data<sup>[13]</sup>. We defined that sedative iCLE examination using propofol increases the rate of good quality endomicroscopic images by 21% as compared with the administration of midazolam and fentanyl. This resulted in a calculated sample size of 100 patients (50 per group). Therefore, we proposed recruiting 104 eligible patients to allow an attrition rate of 4%.

Patients were randomized at a 1:1 ratio into a propofol group (P group) or an M/F group using a computer-generated list. The respective randomization results were kept in sealed envelopes that were opened before the endoscopy by the anesthetist. Because the apparent difference in the color of the sedative agents in this study, the anesthetist was not blinded to the study agents. However, in order to maintain the patients, the endoscopist and the other investigators blinded about the study group, an opaque curtain was placed upon the patient's infusion arm during the following procedure.

### Confocal laser endomicroscopy

CLE is an advanced method which allows living tissue to be viewed *in situ*, providing real-time histology during endoscopy. The confocal microscope integrated into the distal tip of a conventional video endoscope can collect images with an adjustable depth of scanning ranging from 0 to 250  $\mu\text{m}$ , a field of view of 475  $\mu\text{m} \times 475 \mu\text{m}$ , an optical slice thickness of 7  $\mu\text{m}$ , and a lateral resolution of 0.7  $\mu\text{m}$ . The plane depth was controlled using two additional buttons on the back of the handpiece.

### Clinical procedure

After routine preparations for gastroscopy, intravenous access was established for both groups of patients. Patients in P group received a bolus of 0.8-1.0 mg/kg of 1% propofol before the start of endoscopy. Further bolus of 0.5 mg/kg of 1% propofol was evaluated by an anesthetist, and would be given if the sedation was judged as insufficient by the endoscopist. Patients in M/F group received a bolus of 0.05 mg fentanyl, followed by

3-4 mg midazolam before the start of endoscopy. Further bolus of 1-2 mg midazolam was administered by the anesthetist at certain intervals or when the sedation was judged as inadequate by the endoscopist. A reversal agent of midazolam (flumazenil) was administered after iCLE examination in the M/F group if needed. Endoscopic intubation commenced once the patient showed spontaneous eye closure, but responsive to name called.

All patients received supplemental oxygen (2-4 L/min) by nasal cannula. Their oxygen saturation, pulse rate and arterial blood pressure were continuously monitored and recorded every 5 min by pulse oxymetry and sphygmomanometry. Sedation was performed in accordance with the guidelines for conscious sedation and monitored by a professional anesthetist (Liu XP)<sup>[8]</sup>.

Patients in both groups received standard white-light endoscopic and endomicroscopic examinations using a Pentax EC-3870K confocal laser endomicroscope (Pentax, Tokyo, Japan). All endoscopic procedures were performed by one experienced endoscopist (Zuo XL), who had performed more than 300 iCLE procedures before the present study. After successful intubation of the endoscope into the duodenum, 5 mL fluorescein was administered intravenously to facilitate the endomicroscopic imaging. Endoscopic mucosal lesions (such as mucosal color changes, elevation, depression, ruggedness) and 9 standard locations (duodenal bulb, lesser and greater curvature of the antrum and gastric body, incisura angularis, fundus, gastric cardia and esophagus) were sequentially examined using iCLE. Serial endomicroscopic images were obtained from each examined area using the "movie mode" on the iCLE displaying screen and stored in separate files for further analysis of image quality. Image collection was started when the performing endoscopist activated the endomicroscopic scanning by pressing a control button on the handpiece of the endoscope, and it was stopped when the endoscopist pressed twice on the same button. Real-time endoscopic and endomicroscopic diagnoses were made during the procedure by the performing endoscopist and targeted biopsy specimens were obtained for histopathological assessment.

One endoscopic assistant (Zhen L) was responsible for the data collection, and not involved in patient selection or the randomization. The demographic data, history of alcohol or smoking, and the American Society of Anesthesiologists status were recorded for both groups of patients<sup>[21]</sup>.

### Outcome measures

**Assessment of endomicroscopic image quality:** Endomicroscopic images of each patient were reevaluated after the procedure by one investigator (Rui J), who was blinded to the patients' data and endoscopic findings. Good quality endomicroscopic images were defined as "no moving artifacts, and single cells can be differentiated". And the number of good quality endomicroscopic images was counted for each examined area<sup>[1]</sup>.

**Sedation-related outcomes:** The procedure duration

was recorded (from the first injection of the sedatives to the moment of the withdrawal of the endoscope), and the time required for sedation (start of the sedation to passage of the larynx). In addition, patient monitoring/complications, including oxygen de-saturation (< 90%), hypotension (SBP < 80 mmHg) and bradycardia (< 40 b/min) were also noted.

**Patient assessment:** After the endoscopic procedure, patients were transferred to a separate recovery area when vital signs were stable as judged by the anesthesiologist responsible for the sedation. As the patients awoke, a brief questionnaire was asked and collected by a blinded endoscopic assistant (Zhen L). Patient assessment of the procedure involved 4 parameters, including satisfaction (scores ranging from 1 to 10: 1 for "poor" and 10 for "excellent"), pain or discomfort (scores ranging from 0 to 10: 0 for "none" and 10 for "severe") and intraprocedure recall (scores ranging from 0 to 10: 0 for "none" and 10 for "complete"). Additionally, the patients were also asked whether they would prefer lighter, deeper or the same level sedation for their next EGD.

**Endoscopist assessment:** The endoscopist's assessment of the procedure had 4 parameters, including satisfaction with sedation (scores ranging from 1 to 10: 1 for "poor" and 10 for "excellent"), level of sedation (apparently inadequate, inadequate, adequate, oversedated), patient cooperation and quality of endoscopy (a scale ranging from 1 to 4: 1 for "very poor"; 2 for "poor"; 3 for "fair"; and 4 for "good").

In addition, the endoscopic assistant and anesthetist also scored their satisfaction of sedation at the end of each procedure independently using a 10-point scale: 1 (poor) to 10 (excellent).

### Statistical analysis

Continuous outcomes were compared using the independent sample *t* test for normally distributed data and the Mann-Whitney *U* test for nonparametric data. The  $\chi^2$  test and the Fisher exact test were applied for the comparison of categorical variables between the two groups. A *P* value less than 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 13.0 statistical software package (SPSS, Chicago, IL, United States). The study was reported in accordance with the Consolidated Standards of Reporting Trials<sup>[22]</sup>.

## RESULTS

Over the 3-mo study period, 156 subjects who required for sedated iCLE examination were screened for possible enrollment. In the end, 52 patients were excluded according to predefined exclusion criteria, including 15 cases of known or suspected strictures or stenosis, 4 cases of acute bleeding, 26 cases of contraindications to sedation, and 7 cases refused to participate. A total of 100 patients completed the study and were eligible for data analysis (49 in P group and 51 in M/F group) (Figure 1). The patient

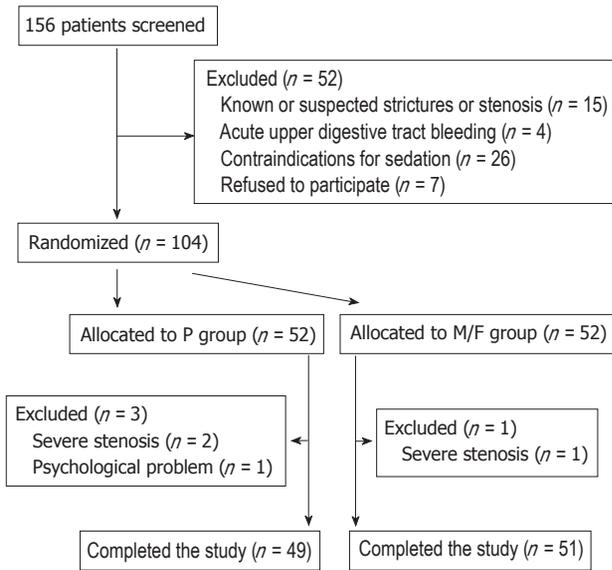


Figure 1 Study participants.

Patient characteristics	P group	M/F group	P value
Patients, <i>n</i>	49	51	
Gender (male/female), <i>n</i>	24/25	23/28	NS
Mean age, yr (range)	53 (27-77)	55 (32-78)	NS
Body weight, kg (mean ± SD)	64.14 ± 10.21	63.84 ± 9.48	NS
Habit, cases ( <i>n</i> )			
Alcohol consumption			NS
Daily drinker	8	5	
Social drinker	10	5	
None-drinker	31	41	
Tobacco			NS
≥ 1 PD	5	5	
< 1 PD	3	5	
Quit smoking	4	1	
None-smoker	37	40	
ASA I	34	37	NS
ASA II	15	14	NS

NS: Not significant; PD: Pack-day; ASA: American Society of Anesthesiologists; P group: Propofol group; M/F group: Midazolam plus fentanyl group.

characteristics for both groups are summarized in Table 1. The mean dosage of sedation used was 194 mg for propofol (range 50-380 mg) and 5.4 mg for midazolam (range 3-8 mg).

**Endoscopic image assessment**

Endoscopic mucosal lesions of the duodenum, stomach and esophagus were examined by iCLE. In addition, if multiple lesions, such as multiple polyps of the stomach, were detected, the endoscopic images obtained from lesions in the same anatomical compartment (e.g., antrum, incisura angularis, gastric body/fundus and cardia) were poorly analyzed for image quality. The proportion of good quality endoscopic images in each examined area is shown in Table 2. Propofol showed superiority to midazolam plus fentanyl in obtaining good

	P group	M/F group	P value
Duodenal bulb	72.17 (760/1053)	50.22 (577/1149)	< 0.001
Lesser curvature of antrum	66.44 (778/1171)	45.73 (562/1229)	< 0.001
Greater curvature of antrum	80.49 (916/1138)	64.99 (776/1194)	< 0.001
Incisura angularis	83.72 (581/694)	50.17 (438/873)	< 0.001
Lesser curvature of gastric body	71.41 (602/843)	48.07 (448/932)	< 0.001
Greater curvature of gastric body	81.85 (857/1047)	66.46 (757/1139)	< 0.001
Fundus	67.39 (217/322)	46.00 (236/513)	< 0.001
Cardia	71.83 (2068/2879)	49.84 (1395/2799)	< 0.001
Esophagus	67.94 (284/418)	56.04 (297/530)	< 0.001
Lesions	67.28 (1285/1910)	52.53 (1161/2210)	< 0.001
Total	72.75 (8348/11475)	52.89 (6647/12568)	< 0.001

P group: Propofol group; M/F group: Midazolam plus fentanyl group.

	P group	M/F group	P value
Number of lesions	36	38	NS
Locations			NS
Duodenum	1	2	
Antrum	15	14	
Incisure angularis	9	6	
Gastric body/fundus	3	3	
Cardia	3	5	
Esophagus	5	8	
Histopathology			NS
Inflammation	22	21	
Intestinal metaplasia	10	10	
Neoplasia	4	7	

NS: Not significant; P group: Propofol group; M/F group: Midazolam plus fentanyl group.

quality endoscopic images (72.75% vs 52.89%,  $P < 0.001$ ).  $\chi^2$  test revealed significant differences in the proportion of good quality endoscopic images between the two groups for each predefined area and endoscopic mucosal lesions ( $P < 0.001$ ).

There were no significant differences between the two groups for the number of endoscopic mucosal lesions, as well as their locations and corresponding histopathology (Table 3). According to prior published CLE diagnostic criteria<sup>[1,2,13,23-27]</sup>, the sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR) of the two groups were calculated respectively (Table 4). The PLR of the P group for diagnosing neoplasia was significantly higher than that of the M/F group. The NLR of the P group for diagnosing intestinal metaplasia

**Table 4** Diagnostic capacity of integrated confocal laser endomicroscopy for endoscopic mucosal lesions of the upper gastrointestinal tract (95% CI)

	Inflammation			Intestinal metaplasia			Neoplasia		
	P group	M/F group	P value	P group	M/F group	P value	P group	M/F group	P value
Sensitivity (%)	90.48 (71.09-97.35)	89.47 (68.61-97.06)	NS	90.00 (59.58-98.21)	80.00 (49.02-94.33)	NS	100 (51.01-1)	85.71 (48.69-97.43)	NS
Specificity (%)	92.86 (68.53-98.73)	94.12 (73.02-98.95)	NS	96.00 (80.46-99.29)	95.65 (79.01-99.23)	NS	96.77 (83.81-99.43)	89.66 (73.61-96.42)	NS
PLR	12.67	15.21	NS	22.50	18.40	NS	31	8.29	0.015
NLR	0.10	0.11	NS	0.10	0.21	0.014	0	0.16	< 0.001

PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; NS: Not significant; P group: Propofol group; M/F group: Midazolam plus fentanyl group.

**Table 5** Quality of sedation

	P group	M/F group	P value
Sedation time (min)	3.22 ± 1.70	4.47 ± 2.40	0.002
Procedure time (min)	25.00 ± 6.51	28.45 ± 8.04	0.028
Adverse events			0.339
Hypoxemia	0	0	
Hypotension	3	1	
Bradycardia	0	0	
Patient assessment			
Satisfaction	10 (10-10)	10 (9-10)	0.105
Pain or discomfort	0 (0-0)	0 (0-1)	0.145
Intraoperative recall	0 (0-0)	0 (0-1)	0.006
Willingness to repeat (n)			0.559
Lighter	5	4	
Deeper	4	7	
Same level	40	40	
Endoscopist assessment			
Satisfaction with sedation	10 (9-10)	9 (8-10)	0.003
Patient cooperation	4 (4-4)	4 (3-4)	0.002
Quality of endoscopy	4 (4-4)	4 (3-4)	0.018
Level of sedation			0.014
Apparently inadequate	0	3	
Inadequate	7	16	
Adequate	41	31	
Oversedated	1	1	
Assistant satisfaction	9 (9-10)	8 (7-10)	0.001
Anesthetist satisfaction	9 (9-10)	7 (5-8)	< 0.001

Continuous variables were given as the mean ± SD. Non-normally distributed variables were expressed as median (the 1st-3rd interquartile) and compared with the Mann-Whitney *U* test. P group: Propofol group; M/F group: Midazolam plus fentanyl group.

and neoplasia was significantly lower than that of the M/F group. The diagnostic sensitivity and specificity of the P group were higher than that of the M/F group, but the differences were not significant (Table 4). The assessment of intestinal metaplasia included only gastric mucosal lesions and the metaplastic esophageal mucosal lesions.

### Quality of sedation

Patients in M-group required significantly more time to achieve sedation (4.47 ± 2.40 min) than P group (3.22 ± 1.70 min). Procedure duration was also longer in M/F group (28.45 ± 8.04 min) than in P group (25.00 ± 6.51 min). Three patients in the P group and one patient in the M-group experienced a decrease in systolic blood pressure below 80 mmHg which were successfully recti-

fied by intravenous fluid administration. There was no case of de-saturation < 90% or bradycardia during or after the procedure.  $\chi^2$  analysis showed that there were no statistical differences between the two groups in terms of the above-mentioned parameters ( $P = 0.339$ ) (Table 5). In addition, the hemodynamic parameters, including the mean values of heart rate, hemoglobin oxygen saturation, and mean arterial pressure were all similar in both groups ( $P = 0.087$ ,  $P = 0.903$ ,  $P = 0.244$ ).

The results of patient assessment for the procedure are shown in Table 5. No significant differences were observed between the two groups in terms of patient satisfaction and pain or discomfort. However, the amnesic effect was significantly better in the P group than in M/F group ( $P = 0.006$ ). With regard to the patients' preference of sedation for their next EGD, some patients in the M/F group seemed to prefer deeper sedation and more patients in the P group preferred lighter sedation. The majority of the two groups (40 patients of each group) would like to receive the same level of sedation.

The endoscopists, based on the mean sedation score as judged by the performing endoscopist (Zuo XL), were significantly in favor of the P group *vs* the M/F group. In addition, the quality of endoscopy and patient cooperation were also rated as significantly superior in the P group. The level of sedation, as estimated by endoscopist immediately after the procedure, was significantly more adequate for the P group than for the M/F group ( $P = 0.014$  comparing "apparently inadequate and inadequate" *vs* "adequate"). Furthermore, the assistant and anesthetist scores for overall sedation also favored the P group receiving propofol as compared with the M/F group receiving midazolam plus fentanyl ( $P = 0.001$  and  $P < 0.001$ ) (Table 5).

## DISCUSSION

CLE is a new endoscopic device that can instantly validate tissue pathology via viewing endomicroscopic images during ongoing endoscopy. Good quality endomicroscopic images can be obtained by achieving full vertical contact of the confocal imaging window with the mucosa<sup>[28]</sup>. The main cause of reduced quality of endomicroscopic images is to the movement artifacts. Therefore, an adequate level of sedation is desirable to

make iCLE examination more tolerable to the patient and easier to perform for the endoscopist. So far, several sedative agents, such as midazolam and propofol, have been applied in iCLE examination to achieve conscious sedation. However, no investigation has yet compared the sedation efficacy of propofol with the regimen of benzodiazepines and narcotics during iCLE. Results of this prospective randomized study showed that the proportion of good quality endomicroscopic images increased by propofol (P group) as the sedative agent rather than midazolam plus fentanyl (M/F group).

Based on our results, the proportion of good quality endomicroscopic images is significantly influenced by the regimen of sedation. Propofol showed clear superiority, either for iCLE scanning of the 9 standard locations or endoscopic mucosal lesions of the upper GI tract. The diagnostic sensitivity, specificity, PLR and NLR were mostly better in patients receiving propofol, although these did not reach statistical significance except for PLR in diagnosing neoplasia and NLR in diagnosing intestinal metaplasia and neoplasia. In our opinion, the reason might be that patients under propofol sedation tolerated inflation of the stomach and the attachment of the iCLE onto the tissue to a greater extent than patients under midazolam and fentanyl sedation, who still tend to experience some retching and belching. The more frequent patient movement in the M/F group not only interferes in the full vertical contact of the confocal window on the interested area, but also disturbs the endoscopist's attention on making a definite judgment.

In addition, our findings suggest that propofol is more efficient compared to the regimen of midazolam plus fentanyl in the sedation of patients undergoing iCLE. The procedure duration and sedation time were all significantly longer in the M/F group. Since the number, endoscopic location and histological spectrum of mucosal lesions were well matched between the two groups, we therefore interpreted the prolonged procedure time in M/F sedation as being a consequence of the necessity for short-term interruptions of the endoscopic procedure due to the time interval required until repeated administrations of midazolam effectively resedated the patients. Adverse event and postprocedure patient assessment were not significantly different except for more frequent intraprocedural recall with midazolam and fentanyl. The endoscopist assessment, assistant satisfaction and anesthetist satisfaction all favor the use of propofol. These were in accordance with previously published data comparing the sedation effect of propofol *vs* midazolam-based regimen during endoscopy<sup>[7,17,18,29]</sup>. A prior study reported that propofol caused more pain on administration, thus leading to a lower acceptance rate by patients<sup>[30]</sup>. In this study, propofol was often mixed with lidocaine (50 mg of 2% lidocaine mixed with 200 mg of 1% propofol) at the time of injection, and no patient experienced pain or complained of pain.

Considering the extensive clinical application of the combined use of midazolam and fentanyl, we choose this regimen as a comparison arm to the increasingly advo-

cated anesthetic drug propofol in the present study. Thus the independent role of midazolam compared with propofol in sedative endomicroscopy may not be clear according to the present research. However, previous data showed that the addition of a narcotic to midazolam may result in better patients' cooperation, easier insertion of the gastroscope, and increased endoscopists' satisfaction with the procedure<sup>[31,32]</sup>. Nevertheless, further studies are warranted to explicit the independent role of midazolam in this procedure.

Our study has certain limitations. First, the difference of the proportion of good quality endomicroscopic images between the two groups did not reach the estimated value (19.86% *vs* 21%) with the current sample size (100 patients), which will certainly weaken the statistical power of this study. However, we did not expand patient recruitment because the predetermined study period has terminated. Anyway,  $\chi^2$  analyses demonstrated statistical significance either for total number of good quality endomicroscopic images or for each examined area between the two groups. Therefore, the results of this study need to be warranted in further researches with a larger sample size. Second, although the target level of sedation in this study was conscious sedation, it is possible that some patients may move to deeper sedation during the procedure since they were not continuously called or shaken in order to judge their sedation level when being examined. In addition, there have been reports comparing the sedation depth of propofol *vs* midazolam and meperidine, which demonstrated that propofol was more likely to produce a deeper level of sedation than midazolam and meperidine<sup>[17,19]</sup>. Given the narrow therapeutic window of propofol, the onset of sedation may be deeper at first, with effect moderating over time. Indeed, the anesthetic agents in both groups were titrated according to patient safety and comfort rather than sedation. Nevertheless, all patients in the present study were monitored with continuous pulse oxymetry and noninvasive arterial blood pressure measured at 5-min intervals, and no severe side effects were observed in either group of patients in this study.

In conclusion, propofol was superior to midazolam and fentanyl for conscious sedation in achieving good quality endomicroscopic images which an accurate endomicroscopic diagnosis is based on. The sedation related outcomes, such as procedure duration, sedation duration, amnesia, endoscopist satisfaction and patient cooperation, also favor the application of propofol. Therefore, conscious sedation using propofol rather than midazolam and fentanyl might be recommended for iCLE examinations. However, the results of the present study need to be further validated with a larger population in multiple centers.

## COMMENTS

### Background

Confocal laser endomicroscopy (CLE) is a novel endoscopic modality which enables real-time visualization of cellular and subcellular structures *in vivo*. Yet in-

egrated CLE (iCLE) examination might require more patients' cooperation and better sedation so as to get endomicroscopic images of good quality and make an accurate diagnosis. Although benzodiazepines, narcotics or propofol have been administered during iCLE procedures, the most effective and satisfactory sedation agent for iCLE examination has not yet been investigated.

### Research frontiers

The clinical applications of iCLE have been validated in various gastrointestinal (GI) diseases, including Barrett's esophagus, early esophageal and gastric cancer, ulcerative colitis, and colorectal neoplasia. The most widely used sedative combination for GI endoscopy is benzodiazepine and narcotics. Recent data suggest that the use of propofol for sedation is increasing.

### Innovations and breakthroughs

This study first validated that sedation with propofol could increase the proportion of good quality endomicroscopic images, and may result in improved procedural efficacy and diagnostic accuracy during iCLE examination.

### Applications

The results of the present study help make a preferable anesthetic regimen for sedative iCLE examination. Conscious sedation using propofol rather than midazolam and fentanyl might be recommended for iCLE examinations.

### Terminology

CLE is an outgrowth of conventional laboratory confocal microscope. Currently, there are 2 CLE imaging system available in clinical practice: one is the integrated CLE (iCLE) with a miniaturized confocal microscope integrated at the distal tape of a conventional endoscope, the other is a probe-based CLE (pCLE) which is ultrathin and can be passed through the working channel of standard endoscopes.

### Peer review

Sedation is a big issue in endoscopic procedures. The authors evaluated the quality of endomicroscopic images under anesthetic condition. For getting the good quality of endomicroscopic image, extremely sedative condition is required. Therefore, the authors used variable anesthetic medicines. However, the adverse effects of sedatives are sometimes very severe. In this study, the authors found similar side effects and good quality images in propofol group. That is an important study for the future application of sedative endomicroscopy.

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## Decompression of the small bowel by endoscopic long-tube placement

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**CONCLUSION:** For patients with adhesive small bowel obstruction, long-tube decompression is recommended and long-tube insertion by endoscopy was superior to fluoroscopic placement.

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**Key words:** Long-tube insertion; Small bowel obstruction; Decompression; Gastroscopy; Fluoroscopic guidance

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### Abstract

**AIM:** To investigate and compare the decompression effect on small bowel obstruction of a long tube inserted using either endoscopic or fluoroscopic placement.

**METHODS:** Seventy-eight patients with small bowel obstruction requiring decompression were enrolled in the study and divided into two groups. Intubation of a long tube was guided by fluoroscopy in one group and by endoscopy in the other. The duration of the procedure and the success rate for each group were evaluated.

**RESULTS:** A statistically significant difference in the mean duration of the procedure was found between the fluoroscopic group ( $32.6 \pm 14.6$  min) and the endoscopic group ( $16.5 \pm 7.8$  min) among the cases classified as successful ( $P < 0.05$ ). The success rate was significantly different between the groups: 88.6% in the fluoroscopic group and 100% in the endoscopic group ( $P < 0.05$ ).

### INTRODUCTION

Small bowel obstruction (SBO) is a major cause of morbidity and financial expenditure in hospitals worldwide. The etiology of SBO has changed in recent decades; whereas SBO was once predominantly due to hernias, it is now largely a result of adhesions<sup>[1-3]</sup>. It has been reported that about 50% to 80% of SBOs are caused by adhesions, mostly postoperative, with a minority being secondary to peritonitis<sup>[4-6]</sup>. Patients with partial adhesive SBO are usually given conservative management, including fasting, intravenous hydration, and decompression with a nasogastric tube<sup>[7]</sup>. Unfortunately, such treatments are successful in only 40% of cases<sup>[8]</sup>. Recently, clinical application of a long tube to decompress the obstructed intestine by aspirating the intestinal contents has achieved favorable outcomes<sup>[9]</sup>. However, because the procedure involves fluoroscopy, it is difficult to intubate a long tube

into the small bowel, which results in a protracted procedure, severe patient distress, increased x-ray exposure, and a low success rate. In this report, we describe our experience using an endoscopic technique to place a long tube into the small bowel, and evaluate the efficacy of the long tube to achieve decompression for treatment of SBO.

## MATERIALS AND METHODS

### Patients

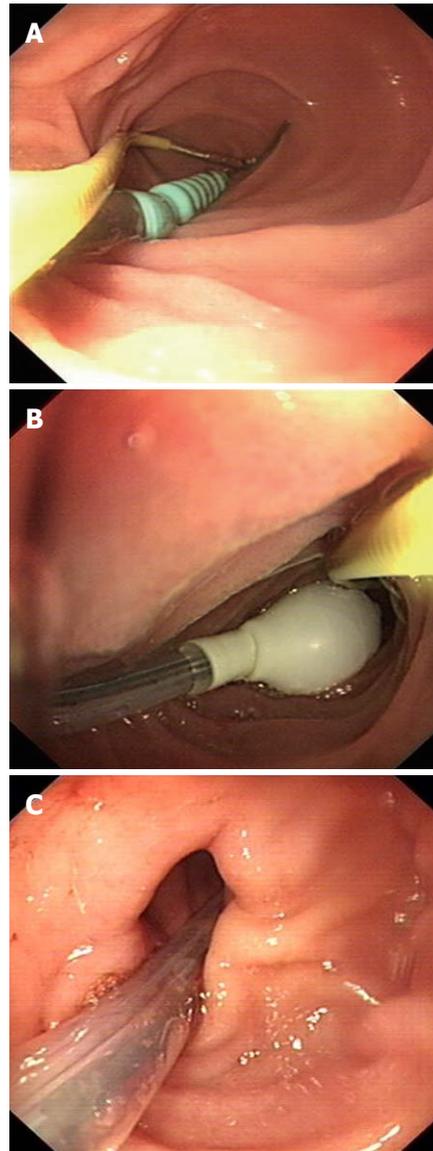
From April 2004 to August 2010, 78 patients with clinical and radiographic evidence of SBO were enrolled in this study (44 male and 34 female, age 20 to 94 years, average 58.6 years). None of the patients had contraindications for long-tube decompression, such as strangulation obstruction, incarcerated hernias, radiation enteritis, and peritonitis. The presenting manifestations were abdominal pain in 75 cases (96.2%), distension in 63 cases (80.8%), constipation in 47 cases (60.3%), and nausea and vomiting in 39 cases (50%). The study was conducted in compliance with the Helsinki Declaration and in accordance with local legislation, and was approved by the Ethics Committee of First Affiliated Hospital, Dalian Medical University. Written informed consent was obtained from all patients or their relatives before the study. The patients were divided into two groups: group A ( $n = 35$ ), in which the procedure was performed under fluoroscopy; group B ( $n = 43$ ), in which the procedure was performed with the assistance of gastroscopy.

### Instruments

A hydrophilic long tube (Create Medic, Tokyo, Japan) was used. It has an outer diameter of 16F, a working length of 3000 mm, an anterior balloon and a posterior balloon at its tip, a guidewire channel, and an injection channel with an anti-reflux valve. In addition to the tip hole, there are 8 side holes near the distal end of the tube. The guidewire was 1.24 mm in diameter and 3500 mm long (Create Medic). An endoscope (GIF Q260J; Olympus, Tokyo, Japan) was used in group B.

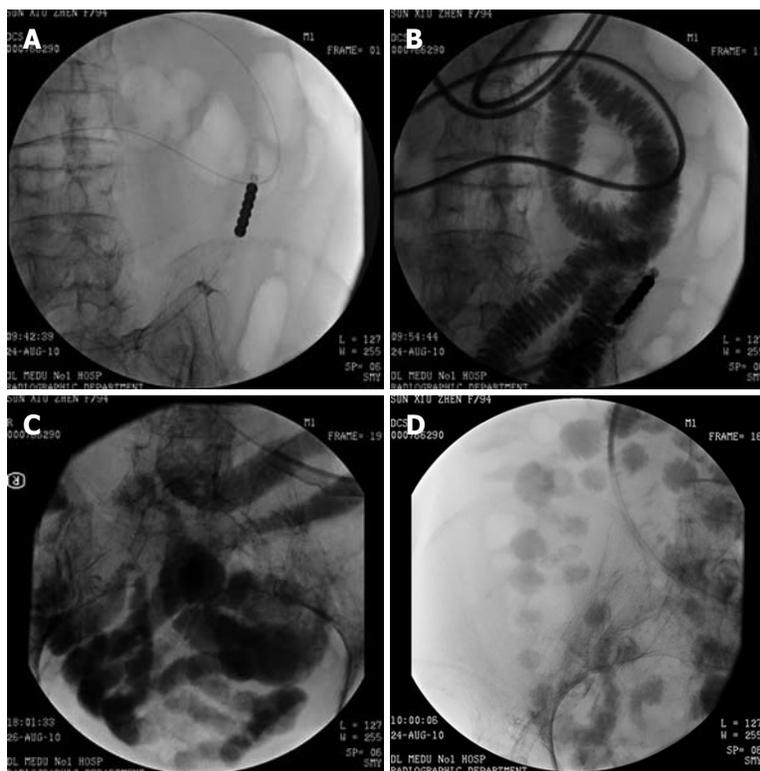
### Procedures

Tetracaine jelly was applied to the long tube to lessen both patient discomfort and the friction between the tube and the endoscope. In order to advance the long tube more easily, a guidewire was inserted into the tube to make it more rigid. The nasogastric tube was removed and a long tube was gently inserted through the nose and esophagus into the stomach. In group B, along with the long tube, an endoscope was also inserted through the mouth and esophagus into the stomach, with the patient in the left lateral decubitus position. The guidewire was placed 2 cm above the tip of the long tube so that it could be grasped easily by the biopsy forceps. The scope and tube were passed through the pylorus and advanced as far as possible (Figure 1A). Then the anterior balloon was fully inflated by injecting 20 ml of distilled water



**Figure 1** Endoscopic progress of a long-tube insertion. A: The guidewire was grasped with biopsy forceps and the scope and tube were passed through the pylorus to reach the duodenojejunal flexure; B: The anterior balloon was inflated to engage the wall of the bowel; C: The guidewire was released and the scope was withdrawn while maintaining the long tube in the small bowel.

to engage the wall of the bowel (Figure 1B), the biopsy forceps and the guidewire were released and the scope was withdrawn while maintaining the long tube in the small bowel (Figure 1C). The tube was advanced through the nose 5 cm per hour by gastrointestinal peristalsis. In group A, the long tube was inserted under fluoroscopic guidance. Postural change of the patients and transabdominal manipulation were frequently used to facilitate passage of the tube through the pyloric ring. After the long tube had reached the descending part of the duodenum, the anterior balloon was fully inflated to engage the wall of the bowel, as described above. Successful intubation was defined as insertion of the long tube into the descending part of the duodenum. The time required for the tube-insertion procedure was determined for the 2 groups. In group A, it was regarded as a failure for



**Figure 2** Abdominal flat plate images after long tube insertion. A: Location of the long tube; B: Jejunum after ingestion of contrast medium through the long tube; C: Ileum; D: Colon, showing complete relief of the small bowel obstruction after insertion of the long tube.

Table 1 Demographic characteristics of 78 patients with small-bowel obstruction		
	Group A (n = 35)	Group B (n = 43)
Gender: male to female	20:15	24:19
Age (yr)		
mean (SD)	55.6 (16.4)	56.5 (17.4)
Median (range)	57.4 (20-83)	59.3 (22-94)
Symptoms		
Abdominal pain	34	41
Distension	29	34
Constipation	22	25
Nausea/Vomiting	18	21

fluoroscopy-guided intubation if endoscopic assistance was required. During the procedure, vital signs and O<sub>2</sub> saturation were monitored if necessary. After insertion of the long tube, intermittent continuous suction was performed to reduce intraluminal pressure in the small bowel, and fluid and electrolyte deficits were corrected. Abdominal flat plate images were taken daily to evaluate the progress of the tube and the degree of decompression (Figure 2A). In some cases, water-soluble contrast medium was given through the long tube to determine the cause of the SBO, whether the obstruction was partial or complete, and whether it was completely relieved by this nonsurgical treatment (Figure 2B-D).

### Statistical analysis

Data analysis was performed using SPSS 10.0 software

(Chicago, IL, United States). Analysis of variance (ANOVA) or Wilcoxon statistical methods were used to determine statistical significance. All measurements in this study were expressed as mean ± SD. *P* < 0.05 was considered statistically significant.

## RESULTS

There was no statistically significant difference in the mean age, male-to-female ratio, and causes of bowel obstruction between the 2 groups (Table 1). The time required for placement of the long tube in the descending part of the duodenum in groups A and B was 32.6 ± 14.6 min and 16.5 ± 7.8 min, respectively (*P* < 0.05). The success rate of intubation was 88.6% in group A and 100% in group B (*P* < 0.05). Four intubations in group A failed under the guidance of fluoroscopy but were completed with the assistance of endoscopy; all 4 of these patients were male. No severe complications relevant to the procedure occurred in either group.

The obstructive symptoms of most patients were relieved within 3 d. Suction was discontinued and the balloon was aspirated when the patient had flatus. If the clinical and radiographic signs remained stable, oral intake was initiated. As the oral intake changed to full liquids, the tube was removed. Sixty-eight cases (87.2%) had complete remission. Patients who required operative intervention were defined as treatment failures. All 10 such cases (12.8%) in our study underwent laparotomy. Among them, 7 cases (9.0%) had neoplasms in the small

**Table 2** Characteristics of patients with small bowel obstruction who underwent surgery

	Group A ( <i>n</i> = 35)	Group B ( <i>n</i> = 43)
Etiology		
Postoperative adhesion	26	31
Neoplasm	3	4
Inflammatory bowel disease	2	3
Unknown	4	5
Surgery needed	5	5
Surgical method		
Bowel resection	3	4
Adhesiolysis	2	1

bowel and received bowel resections. The other 3 cases (3.8%) failed to respond to long-tube decompression because of the complete SBO and underwent adhesiolysis (Table 2).

## DISCUSSION

As one of the major causes of hospitalization and surgical consultation, SBO can come from many causes<sup>[10]</sup>. It used to be a fatal condition, with mortality as high as 50%. Since 1933, when Wangsteen used a long tube to decompress the obstructed intestine and achieved favorable results, this method has been widely used in clinical practice with improved technique<sup>[11-14]</sup>. Various long tubes have been developed for this purpose<sup>[15,16]</sup> which resulted in a remarkable reduction in mortality from bowel obstruction<sup>[9,17]</sup>. Studies<sup>[15,18]</sup> demonstrated that the decompression effect achieved with a long tube is superior to that of a nasogastric tube for the treatment of obstruction because a long tube can automatically pass into the deeper portion of the intestine by balloon transport, come closer to the obstruction and reduce the intraluminal pressure more effectively. However, because insertion of a long tube has traditionally been performed under the guidance of fluoroscopy, it is difficult to insert a long tube blindly into the small bowel, and this has many drawbacks such as prolonged procedural time, severe patient distress, and increased X-ray exposure. But direct observation by endoscopy makes it much easier and quicker to guide the tube through the pyloric ring<sup>[19]</sup>. However, the long tube is easily disturbed when the endoscope is withdrawn because of the strong friction between the tube and the endoscope. To avoid this in our studies, we fully inflated the anterior balloon to engage the wall of the bowel before withdrawing the endoscope. By using this method we improved both the success rate and the time required for tube placement. Because the entire procedure was performed by endoscopy, fluoroscopy was only used to confirm the position of the tube, thus improving the safety for both the medical staff and patients.

Postoperative adhesion is the major cause of SBO<sup>[2,3]</sup>, and adhesive SBO can be a complication of any abdominal surgery<sup>[20-24]</sup>. Long-tube decompression can aspirate

the intestinal contents, decrease edema of the bowel wall<sup>[25]</sup>, enhance bowel motility, and prevent bacterial translocation<sup>[26]</sup>. Long-tube decompression successfully relieves the obstructive symptoms in most patients with SBO<sup>[9]</sup>, especially adhesive obstructions, and may ultimately help to avoid abdominal operations in the majority of patients<sup>[2]</sup>.

Long-tube decompression achieved favorable outcomes, including reduced edema, improved circulation of the involved intestine, and correction of intestinal kinking, so that both normal size and function are restored in the distended loops of the bowel<sup>[9]</sup>. In our study, most patients with SBO were relieved of the obstruction within 72 h, and about 87.2% of the patients experienced full recovery following long-tube decompression and without the need for surgical intervention, which is consistent with other reports<sup>[9]</sup>. Moreover, no serious complications were found during the long-tube decompression treatment, which is also similar to other studies.

Although most SBOs can be resolved with tube decompression alone, surgical treatment may be required in some patients<sup>[27]</sup> because of neoplasm or strangulation. For adhesive SBO, if ileus persists more than 3 d after insertion of a long tube, or the drainage volume is still > 500 mL on day 3, surgery should be recommended to replace the conservative management<sup>[28-30]</sup>.

In our study, 10 cases underwent laparotomy. Among them, 7 cases received bowel resection because of neoplasms and 3 cases underwent adhesiolysis due to complete and multiple-site obstructions. However, even for those patients receiving laparotomy, long-tube decompression should be done before surgery to prevent the occurrence of perforation.

In conclusion, decompression with a long tube should be considered for all patients with clinical and radiographic evidence of SBO but without a strangulation obstruction or other contraindications. Long-tube insertion facilitated by endoscopy is superior to the conventional fluoroscopic method for SBO, as evidenced by the procedural success rate and time required.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Small bowel obstruction (SBO) is still one of the major causes of morbidity and financial expenditure in hospitals around the world. It has been reported that about 50%-80% of small bowel obstruction are caused by adhesions, mostly postoperative. Patients with partial adhesive small-bowel obstruction are usually given conservative management including fasting, intravenous hydration, and decompression with a nasogastric tube. But such treatment was successful only in 40% of all cases.

### Research frontiers

Recently, a long tube has been applied clinically to decompress the obstructed

intestine by aspirating the intestinal contents and achieved favorable outcomes. However, because the procedure used to be performed under fluoroscopy, it was difficult to incubate a long tube into the small bowel, which resulted in long procedure time, severe patient distress, increased X-ray exposure and low success rate.

### Innovations and breakthroughs

In this study, we introduced an easy technique to place a long tube assisted by endoscopy which was effective to relieve the small bowel obstruction. Compare with conventional method under fluoroscopy, the new method has advantage of less procedure time and high success rate.

### Applications

The study shows that decompression with a long tube should be considered for all patients with clinical and radiographic evidence of small bowel obstruction but without a strangulation obstruction or other contraindications. And long-tube insertion facilitated by endoscopy is superior to the conventional fluoroscopic method for small bowel obstruction according to procedural success and time required.

### Terminology

SBO: small bowel obstruction involves a partial or complete blockage of the bowel that results in the failure of the intestinal contents to pass through, mostly caused by adhesion. It can be divided into simple and strangulation obstruction according to whether the vascular supply to intestinal wall is compromised.

### Peer review

This is a good descriptive study in which authors describe their experience using an endoscopic technique to place a long tube into the small bowel, and evaluate the efficacy of the long tube to achieve decompression for treatment of SBO. The results suggest that long-tube decompression is recommended for patients with adhesive small bowel obstruction, and long-tube insertion by endoscopy was superior to fluoroscopic placement.

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## KISS-1 inhibits the proliferation and invasion of gastric carcinoma cells

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### Abstract

**AIM:** To investigate the function of the *KISS-1* gene in gastric carcinoma cells and to explore its potential mechanism.

**METHODS:** A *KISS-1* eukaryotic expression vector was constructed and transfected into BGC-823 cells. Resistant clones were obtained through G418 selection. reverse transcription-polymerase chain reaction and western blotting were used to detect *KISS-1* and matrix metalloproteinase-9 (MMP-9) expression in transfected cells. The growth of transfected cells was investigated by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) proliferation assays, and the cells' invasive potential was analyzed by basement membrane (Matrigel) invasion assays. The anti-tumor effects of *KISS-1* were tested *in vivo* using allografts in nude mice.

**RESULTS:** The expression level of *KISS-1* mRNA and

protein in BGC-823/*KISS-1* transfected cells were significantly higher than in BGC-823/pcDNA3.1 transfected cells ( $P < 0.05$ ) or the parental BGC-823 cell line ( $P < 0.05$ ). The expression level of MMP-9 mRNA and protein in BGC-823/*KISS-1* were significantly less than in BGC-823/pcDNA3.1 ( $P < 0.05$ ) or BGC-823 cells ( $P < 0.05$ ). MTT growth assays show the proliferation of BGC-823/*KISS-1* cells at 48 h ( $0.642 \pm 0.130$ ) and 72 h ( $0.530 \pm 0.164$ ) were significantly reduced compared to BGC-823/pcDNA3.1 ( $0.750 \pm 0.163$ ,  $0.645 \pm 0.140$ ) ( $P < 0.05$ ) and BGC-823 cells ( $0.782 \pm 0.137$ ,  $0.685 \pm 0.111$ ) ( $P < 0.05$ ). Invasion assays indicate the invasive potential of BGC-823/*KISS-1* cells ( $16.50 \pm 14.88$ ) is significantly reduced compared to BGC-823/pcDNA3.1 ( $20.22 \pm 14.87$ ) ( $P < 0.05$ ) and BGC-823 cells after 24 h ( $22.12 \pm 16.12$ ) ( $P < 0.05$ ). *In vivo* studies demonstrate the rate of pcDNA3.1-*KISS-1* tumor growth is significantly slower than pcDNA3.1 and control cell tumor growth in nude mice. Furthermore, tumor volume of pcDNA3.1-*KISS-1* tumors ( $939.38 \pm 82.08 \text{ mm}^3$ ) was significantly less than pcDNA3.1 ( $1250.46 \pm 44.36 \text{ mm}^3$ ) and control tumors ( $1284.36 \pm 55.26 \text{ mm}^3$ ) ( $P < 0.05$ ). Moreover, the tumor mass of pcDNA3.1-*KISS-1* tumors ( $0.494 \pm 0.84 \text{ g}$ ) was significantly less than pcDNA3.1 ( $0.668 \pm 0.55 \text{ g}$ ) and control tumors ( $0.682 \pm 0.38 \text{ g}$ ) ( $P < 0.05$ ).

**CONCLUSION:** *KISS-1* may inhibit the proliferation and invasion of gastric carcinoma cells *in vitro* and *in vivo* through the downregulation of MMP-9.

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**Key words:** *KISS-1*; Matrix metalloproteinase-9; BGC-823 cells; Proliferation; Metastasis; Nude mice

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Li N, Wang H, Zhang J, Ye Y, He G. KISS-1 inhibits the proliferation and invasion of gastric carcinoma cells. *World J Gastroenterol* 2012; 18(15): 1827-1833 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i15/1827.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i15.1827>

## INTRODUCTION

KISS-1 has been identified as a human melanoma metastasis suppressor gene using subtractive hybridization between the metastatic human melanoma cell line C8161 and non-metastatic variants generated after microcell-mediated transfer of chromosome 6 into C8161<sup>[1]</sup>. The *KISS-1* gene maps to chromosome 1 bands q32-q41 and encodes a largely hydrophobic 145-amino-acid protein<sup>[2]</sup>. The 54-amino-acid, C-terminally amidated fragment of the KISS-1 protein (amino acids 68-121) is termed metastin, while the full-length protein is called kisspeptin<sup>[2,3]</sup>. Expression of KISS-1 is detectable in normal heart, brain, liver and lung. In human tumors, KISS-1 expression is weak or undetectable. The *KISS-1* gene product functions as tumor metastasis suppressor and is reported to act after binding with hOT7T175, an orphan G-protein-coupled receptor. Previous studies demonstrated KISS-1 could suppress metastasis of human malignant melanoma and human breast carcinoma cells without affecting tumorigenicity<sup>[4,5]</sup>. Although the loss of KISS-1 expression has been associated with tumor progression and poor prognosis in various cancers, the mechanism underlying this activity is still unknown. Investigating the role of KISS-1 in other cancers and identifying its potential mechanism in suppressing tumor metastasis will require additional experiments.

## MATERIALS AND METHODS

### Construction of recombinant expression vectors

Two primers, K1(5'-CGAAGCTTATGAACTCACTGGTTTCT-3', carrying a *HinD* I site, underlined) and K2(5'-CTGGATCCTCACTGCCCCGCACCTG-3', carrying a *BamH* I site, underlined), were designed for the *KISS-1* gene's CDS (sequence coding for amino acids in protein) domain. This sequence was amplified by quantitative reverse transcription-polymerase chain reaction (RT-PCR) with primer K1 and K2 from 50 g of human gastric tissue derived from 5 cm of the edge of gastric carcinoma lesions. Quantitative RT-PCR conditions were as follows: denaturing at 94 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min. The PCR was run for 30 cycles. Final extension was performed at 72 °C for 10 min. The fragment carrying both the *HinD* I and *BamH* I site was acquired by quantitative RT-PCR. This PCR produced a product of 454 bp. The product was subjected to a double digestion with *HinD* I and *BamH* I enzymes, and the digested DNA product was ligated into a 5.4-kb fragment of pcDNA3.1 (Invitrogen, United States) that was digested with the same enzymes.

The ligated product was transformed into JM109 cells. Restriction endonuclease analysis, quantitative RT-PCR and plasmid sequencing were performed to validate the recombinant plasmid reading frame.

### Cell culture and transfection

The BGC-823 cells (a gift from Zheng Zhou University) were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum, 100 U/mL penicillin and 100 U/mL streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. NIH3T3 cells cultured in the same conditions were used as controls. Twelve hours before transfection, cells were seeded onto 24-well plates at a density of 1-2 × 10<sup>5</sup> cells per well. Cells were transfected when plate confluence was approximately 90%-95%. The cells were transfected with 1.25 μL/well of pcDNA3.1-KISS-1 vector using 1 μL Lipofectamine (Invitrogen). The culture medium was replaced with a selection medium containing G418 (at concentrations ranging from 100 μg/mL to 1000 μg/mL, Alexis Biochemicals) forty-eight hours later. When stably transfected cells were obtained, the cells were continuously maintained in 200 μg/mL of G418. BGC-823 cells were transfected with the empty pcDNA3.1 vector as a control.

### Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using Trizol purification (Gibco). After denaturing RNA at 94 °C for 5 min, 500 ng of RNA was transcribed into cDNA. Next, cDNA was amplified using the following primers: KISS-1 sense (5'-ATGAACTCACTGGTTTCTTGGCAG-3'), KISS-1 antisense (5'-TCACTGCCCCGCACCTG-3'); MMP-9 sense (5'-AGGAGCGGCTCTCCAAGAAG-3') and MMP-9 antisense (5'-GGGCACTGCAGGATGTCATAG-3'). Duplex amplification was performed using a thermocycler for 30 cycles according to the following program: 1 min at 94 °C, 30 s at 48 °C (KISS-1), 60 °C (MMP-9) and 1 min at 72 °C. PCR fragments were separated by electrophoresis on a 1.5% agarose gel, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (315 bp) as an internal standard. PCR products were quantitated using SYNGENE gel analysis software, and GAPDH was used to normalize the data.

### Western blotting

For Western blotting protein analysis, 40 μg of total protein was separated by 10%-15% gel gradient SDS-PAGE under reducing conditions. Proteins were subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated with blocking buffer (PBS containing 5% non fat milk) for 2 h at room temperature. Primary antibodies were either rabbit anti-KISS-1 (Boshide, China) or goat anti-MMP-9 (Santa Cruz, United States). Membranes were incubated overnight at 4 °C with gentle shaking. The membrane was washed twice with PBS for 5 min and incubated with secondary antibodies (Zhongshan, China) for 2 h at room temperature. After washing, KISS-1 and MMP-9 were detected using a chemiluminescence reaction. The results

were analyzed with TotalLab2.0 software. Protein levels were normalized to  $\beta$ -actin protein.

### Cell proliferation assay

Cells were seeded at  $5 \times 10^3$  cells per well in a 96-well plate and cultured in the presence of 10% fetal bovine serum for 24, 48 or 72 h. The cells were pulsed with 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, United States) 20  $\mu$ L/well [5 mg/mL in phosphate-buffered saline (PBS)] to measure cell growth. The purple-blue MTT formsazan precipitate was dissolved in 200  $\mu$ L of DMSO and swirled for 30 min. Absorbance (UA) was measured at 570 nm with a spectrophotometer. Experiments were repeated five times.

Proliferation inhibition rates (%) =  $(1 - \text{UAE}/\text{UAC}) \times 100\%$

UAE: Average UA value of experimental group; UAC: Average UA value of control group.

### Invasion assays

For invasion assays, transwell polycarbonate filters (8- $\mu$ m pore size, Millipore, United States) were coated with 100  $\mu$ L of matrigel (Sigma, United States) at a dilution of 1:20 in serum-free medium and were then air-dried for 24 h.  $5 \times 10^5$  cells in 400  $\mu$ L of complete medium were seeded into the upper chamber. Next, 200  $\mu$ L of medium was added to the lower chamber, and the plate was incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 24 h. Cells on the lower surface of the filter were stained with hexamethylpararosaniline and counted. The experiment was performed in triplicate for three different cell lines.

The invasion index is defined as the number of cells that migrated through the 8  $\mu$ m pores of the filter in the experimental group divided by the number of cells that migrated through the filter in the control group  $\times 100\%$ .

The cell invasion inhibition rate is equal to the number of cells that migrated through the filter in the control group minus the number of cells that migrated through the filter in the experimental group divided by the number of cells that migrated through the filter in the control group  $\times 100\%$ .

### Tumor development in nude mice

Fourteen 4-6 wk old athymic female nude BALB/c mice (weighing 18-22 g) were purchased from the Experimental Animal Center of Shanghai, Academia Sinica. These mice were bred in specific pathogen-free conditions and were randomly divided into three groups of 8 mice per group. Three different cell lines (pcDNA3.1-KISS-1, pcDNA3.1, BGC-823) were each suspended at a concentration of  $5 \times 10^7$  cells per 0.1 mL serum-free RPMI 1640. Cells were injected subcutaneously into the right forelimb. Tumor diameters were measured every 3 d. Tumor volume was calculated according to the following formula: volume (V) =  $0.5236AB^2$  (A and B represent the long and short tumor diameter respectively). The *in vivo* growth curve of cancer cells was drawn. The animals were sacrificed 45 d after inoculation, and tumors were weighed. Inhibition rate (%) =  $[\text{tumor volume (mass)}$

of control group-tumor volume (mass) of experiment group / tumor volume (mass) of control group]  $\times 100\%$ .

### Statistical analysis

Data were analyzed using the SPSS13.0 software. Analysis of variance was conducted followed by independent-sample *t*-tests. A *P* value less than 0.05 was considered to be statistically significant.

## RESULTS

### Construction and identification of recombinant plasmids

Full-length human KISS-1 CDS domain cDNA was inserted into a pcDNA3.1 vector at the *HinD* I / *Bam* H I site to form the recombinant plasmid pcDNA3.1-KISS-1. Recombinant pcDNA3.1-KISS-1 vectors containing the *KISS-1* gene were selected through  $\alpha$ -complementation. The expression vector was further analyzed by digestion with the restriction endonucleases *HinD* I / *Bam* H I. The resulting fragment sizes were consistent with the expected fragments predicted by the expression vector map.

### Transfection of the KISS-1 gene increased expression of KISS-1 and downregulated matrix metalloproteinase-9 expression in BGC-823 cells

Strong expression of KISS-1 was observed in pcDNA3.1-KISS-1 transfected BGC-823 cells by RT-PCR and Western blotting. BGC-823 cells transfected with pcDNA3.1-KISS-1 also showed a significant reduction in MMP-9 expression ( $P < 0.05$ ). The expression of KISS-1 in pcDNA3.1-transfected BGC-823 cells and control BGC-823 cells remained low ( $P < 0.05$ ) (Figure 1).

### Transfection of KISS-1 gene suppressed cell growth

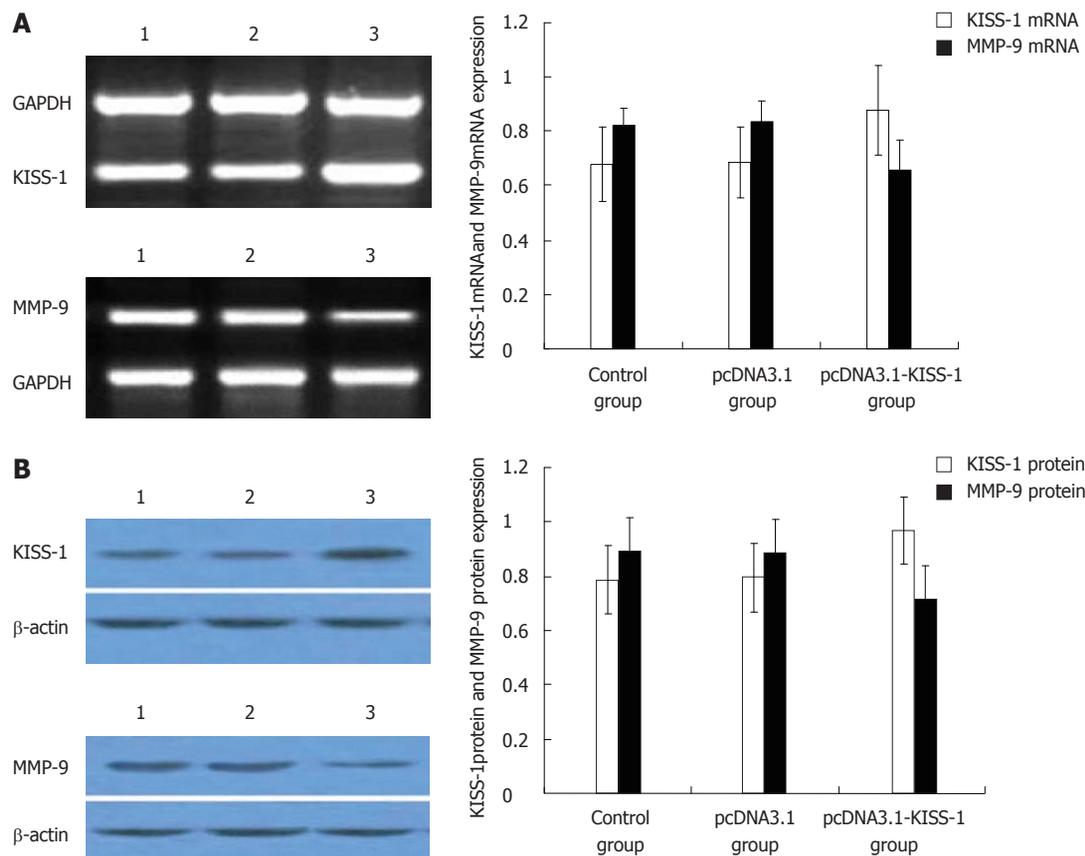
At 48 and 72 h after transfection, the absorbance values (570 nm) of pcDNA3.1-KISS-1-transfected BGC-823 cells were significantly different from those of the pcDNA3.1-transfected ( $P < 0.05$ ) and control BGC-823 cells ( $P < 0.05$ ). KISS-1 transfection decreased cell proliferation by 17.90% compared to pcDNA3.1-transfected cells and by 22.63% compared to control BGC-823 cells. These results indicate KISS-1 protein expression in BGC-823 cells strongly inhibits cell proliferation (Table 1).

### Effects of KISS-1 transfection on cell invasion

In order to invade surrounding tissue, epithelial cancer cells must degrade the underlying basement membrane. The numbers of cells that digested Matrigel and migrated through the 8- $\mu$ m pores in the filter were counted after 24 h (Figure 2). The total number of pcDNA3.1-KISS-1 transfected cells that migrated through the transwell polycarbonate filter was significantly less than the number of migrating cells in the pcDNA3.1 transfected group and control group ( $P < 0.05$ ). These data suggest that transfection with the KISS-1 expression vector results in a significant decrease in invasive capacity (Table 2).

### Tumor growth

Tumor volume was assessed 7 d after injection of tumor



**Figure 1** The expressions of KISS-1 and matrix metalloproteinase-9 mRNA and protein in BGC-823 cells. A: The expression level of KISS-1 mRNA was distinctly higher and the expression level of MMP-9 mRNA was distinctly lower in pcDNA3.1- KISS-1 group than that in pcDNA3.1 group ( $P < 0.05$ ) and control group ( $P < 0.05$ ); B: The expression level of KISS-1 protein was distinctly higher and the expression level of MMP-9 protein was distinctly lower in pcDNA3.1-KISS-1 group than that in pcDNA3.1 group ( $P < 0.05$ ) and control group ( $P < 0.05$ ). Lane 1: Control group; Lane 2: pcDNA3.1 group; Lane 3: pcDNA3.1- KISS-1group. MMP-9: Matrix metalloproteinase-9; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**Table 1** The influence on the proliferation ability of BGC-823 cells transfected with pcDNA3.1-KISS-1

Group	24 h		48 h		72 h	
	UA value	Inhibition rate (%)	UA value	Inhibitionv rate (%)	UA value	Inhibition rate (%)
Control group	0.744 ± 0.126	0	0.782 ± 0.137	0	0.685 ± 0.111	0
pcDNA3.1 group	0.713 ± 0.222	4.17	0.750 ± 0.163	4.09	0.645 ± 0.140	5.84
pcDNA3.1-KISS-1 group	0.691 ± 0.131	7.12	0.642 ± 0.130 <sup>a</sup>	17.90	0.530 ± 0.164 <sup>a</sup>	22.63

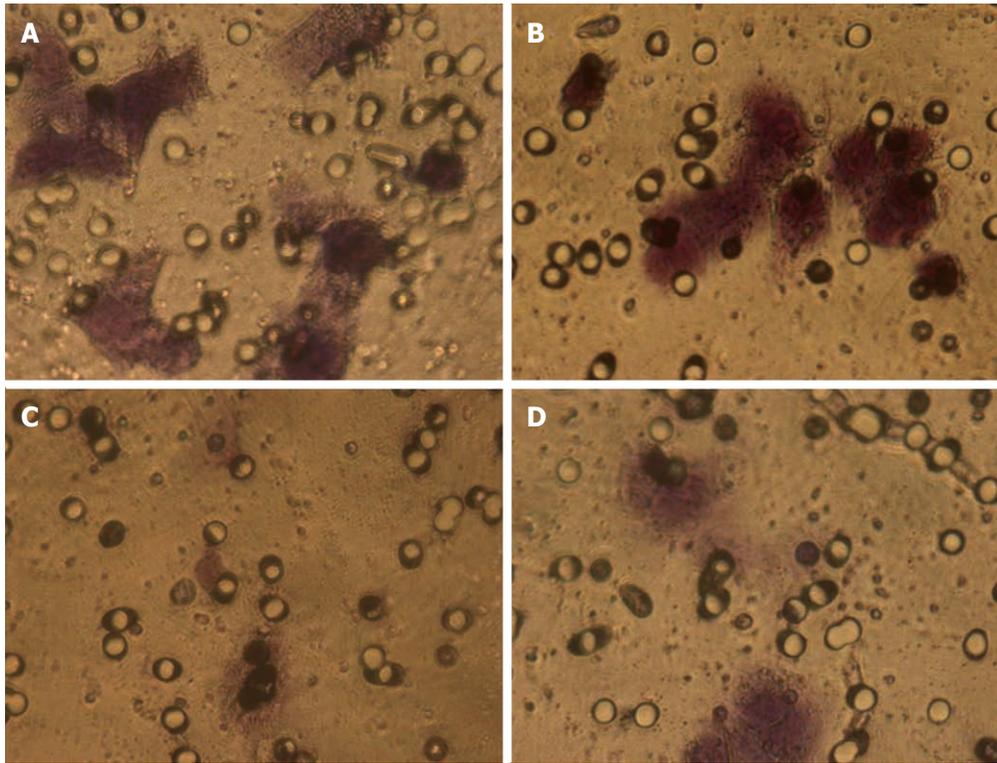
<sup>a</sup> $P < 0.05$  vs control group and pcDNA3.1 group.

cells and every 3 d thereafter until 45 d. Time curves show the percentage increase in tumor size (Figure 3). The tumor volume and mass in the pcDNA3.1-KISS-1 transfected group were both significantly less than the tumor volume and mass in the pcDNA3.1-transfected group and the control group ( $P < 0.05$ ). The tumor volume and mass inhibition rates for the pcDNA3.1-KISS-1 transfected group were 26.86% and 27.57%, respectively. No lung or liver metastatic nodules were observed. As a result of KISS-1 transfection, tumor growth was reduced (Table 3).

## DISCUSSION

The vast majority of gastric cancer deaths result from

complications caused by tumor cell metastasis rather than as a consequence of the original tumor growth. Metastasis is a multistep process involving complex interactions between tumor cells and host cells. To become metastatic, primary tumor cells must migrate from the tumor, dissociate from the tumor mass and travel to nearby or distant secondary sites. Single cells, homotypic clusters of cells, or heterotypic emboli subsequently arrest at a distant site with the use of both organ specific and nonspecific mechanisms. Next, these cells invade the surrounding tissue and respond to growth signals at the secondary site<sup>[6-9]</sup>. Interference at any one of these steps can block the metastatic cascade and prevent the formation of metastatic tumor growths. Consequently, there is increasing interest in researching the metastatic process to identify



**Figure 2** Suppression effects on invasion potency of BGC-823 by KISS-1 transfection. A: Control group; B: Group transfected with pcDNA3.1; C, D: Group transfected with pcDNA3.1-KISS-1.

**Table 2** The influence on the invasion potency of BGC-823 cells transfected by KISS-1

Group	24 h		
	Cells numbers	Invasion index (%)	Invasion ability inhibition rates (%)
Control group	22.12 ± 16.12	100.00	0.0
pcDNA3.1 group	20.22 ± 14.87	91.33 ± 12.14	8.75 ± 11.18
pcDNA3.1-KISS-1 group	16.50 ± 14.88 <sup>ac</sup>	74.66 ± 17.14 <sup>ac</sup>	25.38 ± 16.25 <sup>a</sup>

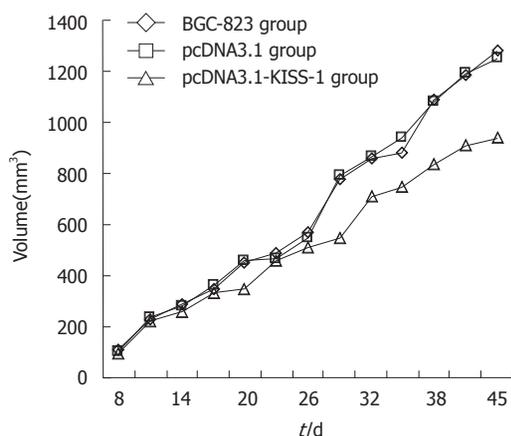
<sup>a</sup>*P* < 0.05 vs transfected with pcDNA3.1 group; <sup>c</sup>*P* < 0.05 vs control group.

possible ways to inhibit metastatic tumor progression.

Metastasis suppressor genes, which inhibit the spread of cancers to secondary sites, have become the target of mounting clinical and basic cancer research. Recently, the *KISS-1* gene was reported to be a novel metastasis suppressor gene in human melanoma and breast carcinoma cells<sup>[4,5]</sup>. *KISS-1* was found to reduce tumor cell invasive and migratory properties without affecting their tumorigenicity<sup>[5]</sup>. Kisspeptin, the product of the *KISS-1* gene, belongs to the RF-amide family of peptides that possess an Arg-Phe-amide sequence motif at the C terminus. This family of peptides is involved in the control of reproduction and tumor metastasis in mammals. In 2001, kisspeptin was first shown to be the endogenous ligand for the orphan G protein-coupled receptor GPR54 (or KISS1R). Kisspeptin is also called metastatin in consideration of its suppressive effects on tumor growth and tumor metastasis<sup>[2]</sup>. Blocked or reduced expression of

*KISS-1* has been found in a variety of tumor metastasis, including breast cancer, bladder cancer and pancreatic cancer<sup>[4,10,11]</sup>. These studies suggest that *KISS-1* is a human metastasis suppressor gene, and loss of *KISS-1* and its receptor may correlate with human tumor progression and metastasis. The mechanism of *KISS-1* suppression is still unknown. Recent publications suggest possible mechanisms for *KISS-1* metastasis suppression. Metastatin (this peptide is encoded from the *KISS-1* gene) induces Ca<sup>2+</sup> in receptor-transfected CHO cells<sup>[2]</sup>, as well as phosphorylation of ERK1/2 and weak phosphorylation of p38/MAPK but not of SAPK/JNK3. Metastatin inhibits motility, chemotaxis, and invasion *in vitro*<sup>[2,12]</sup>, possibly by repressing the transcription of MMP-9 via the induction of cytosolic IκBα<sup>[13]</sup>. Metastatin also induces excessive formation of focal adhesions and stress fibers in hOT7T175-transfected B16/BL6 and induces the phosphorylation of FAK and paxillin<sup>[13]</sup>, possibly through Rho<sup>[14]</sup>. Our previous work has shown that loss of *KISS-1* gene expression in tumors has a significant correlation with lymph node metastasis from gastric carcinoma. The purpose of this study was to determine whether *KISS-1* might function as a metastasis suppressor and to investigate a possible mechanism for this action in gastric carcinoma.

We constructed a eukaryotic vector containing the full-length human *KISS-1* cDNA and transfected it into BGC-823 cells. Selection with G418 enabled stably transfected cells expressing high levels of *KISS-1* protein to be obtained. In this study, we demonstrate a significant reduction in the growth of BGC-823 cells transfected



**Figure 3** Inhibition effects on growth of BGC-823 by KISS-1 transfection *in vivo*. The tumor volume in pcDNA3.1-KISS-1 transfected group was significantly lower compared with pcDNA3.1 transfected group and control group.

**Table 3** The comparison of average tumor volume and tumor weight and inhibition rate in groups

Group (n = 8)	Tumor volume (mm <sup>3</sup> )	Inhibitory rate (%)	Tumor weight (g)	Inhibitory rate (%)
BGC-823 group	1284.36 ± 55.26	-	0.682 ± 0.38	-
pcDNA3.1 group	1250.46 ± 44.36	2.64	0.668 ± 0.55	2.05
pcDNA3.1-KISS-1 group	939.38 ± 82.08 <sup>a</sup>	26.86	0.494 ± 0.84 <sup>a</sup>	27.57

<sup>a</sup>P < 0.05 vs BGC-823 group and pcDNA3.1 group.

with an exogenous *KISS-1* gene. Additionally, the colony-forming ability of KISS-1-transfected cells was reduced *in vitro*, and tumor growth in nude mice was inhibited. Using a reconstituted basement membrane assay (Matrigel), we showed a reduction in the number of invading KISS-1 transfected BGC-823 cells. This finding indicates that the propensity for local/regional invasion and distant metastasis of gastric cancer may be dependent on its ability to invade the basement membrane. These results are similar to those reported by Lee *et al.*<sup>[4]</sup> using a breast carcinoma cell line.

Cancer mortality is largely due to distant metastases and subsequent organ failure. Metastasis involves the degradation of the basement membrane and stromal extracellular matrix (ECM) and subsequent migration into the adjacent blood vessels. This phenomenon results in tumor growth in distant organs<sup>[8,15]</sup>. Degradation of the basement membrane and ECM involve the secretion of several proteases, such as one or more members of the MMP family<sup>[16-18]</sup>. MMP-9 can cleave ECM proteins to promote cell invasion and mobility and has been described as a negative prognostic marker of metastasis and disease-free interval<sup>[19,21]</sup>. We transfected KISS-1 cDNA into BGC-823 cells and subsequently monitored the expression of MMP-9 by western blotting and RT-PCR. Our results show *KISS-1* gene transfection decreases the expression of MMP-9 and suggests that KISS-1 could downregulate expression of MMP-9.

In summary, KISS-1 is able to be exogenously expressed in eukaryotic cells. Its transient expression significantly suppresses the growth and invasion of tumor cells. Importantly, our data suggests that the effect of KISS-1 on tumor growth and invasion might occur by decreasing expression of MMP-9. In the future, additional experiments will be required to determine a role for KISS-1 in other cancers and to explore the exact mechanism of KISS-1 function.

## COMMENTS

### Background

KISS-1 has been identified as a putative metastasis-suppressor gene in human melanomas and breast-cancer cell lines. However, the mechanism of how KISS-1 works and the effects of a synthesized truncated KISS-1 protein on the proliferation and invasive ability remain unknown. Our previous studies have shown that the loss of KISS-1 expression was correlated with lymph node metastasis in gastric carcinoma tissue.

### Research frontiers

This study investigated the function of KISS-1 in gastric carcinoma cells and explored its possible mechanism.

### Innovations and breakthroughs

This study explored KISS-1 inhibits the proliferation and invasion of gastric carcinoma cells *in vitro* and *in vivo* and concluded the mechanism is mediated through the downregulation of matrix metalloproteinase-9.

### Applications

By understanding the relationship between KISS-1 and tumor-cell proliferation and invasion in gastric carcinoma, this study may be useful in the clinical management of patients with gastric carcinoma.

### Peer review

This topic is an import topic for research and the manuscript is good.

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## Preoperative predictors of portal vein thrombosis after splenectomy with periesophagogastric devascularization

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### Abstract

**AIM:** To evaluate the predictive value of preoperative predictors for portal vein thrombosis (PVT) after splenectomy with periesophagogastric devascularization.

**METHODS:** In this prospective study, 69 continuous patients with portal hypertension caused by hepatitis B cirrhosis underwent splenectomy with periesophagogastric devascularization in West China Hospital of Sichuan University from January 2007 to August 2010. The portal vein flow velocity and the diameter of portal vein were measured by Doppler sonography. The hepatic congestion index and the ratio of velocity and diameter were calculated before operation. The prothrombin time (PT) and platelet (PLT) levels were measured before and after operation. The patients' spleens were weighed postoperatively.

**RESULTS:** The diameter of portal vein was negatively correlated with the portal vein flow velocity ( $P < 0.05$ ). Thirty-three cases (47.83%) suffered from postoperative PVT. There was no statistically significant difference in the Child-Pugh score, the spleen weights, the PT, or PLT levels between patients with PVT and without PVT. Receiver operating characteristic curves showed four variables (portal vein flow velocity, the ratio of velocity and diameter, hepatic congestion index and diameter of portal vein) could be used as preoperative predictors of postoperative portal vein thrombosis. The respective values of the area under the curve were 0.865, 0.893, 0.884 and 0.742, and the respective cut-off values (24.45 cm/s, 19.4333/s, 0.1138 cm/s<sup>-1</sup> and 13.5 mm) were of diagnostically efficient, generating sensitivity values of 87.9%, 93.9%, 87.9% and 81.8%, respectively, specificities of 75%, 77.8%, 86.1% and 63.9%, respectively.

**CONCLUSION:** The ratio of velocity and diameter was the most accurate preoperative predictor of portal vein thrombosis after splenectomy with periesophagogastric devascularization in hepatitis B cirrhosis-related portal hypertension.

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**Key words:** Hypertension; Portal; Thrombosis; Splenectomy; Diagnosis

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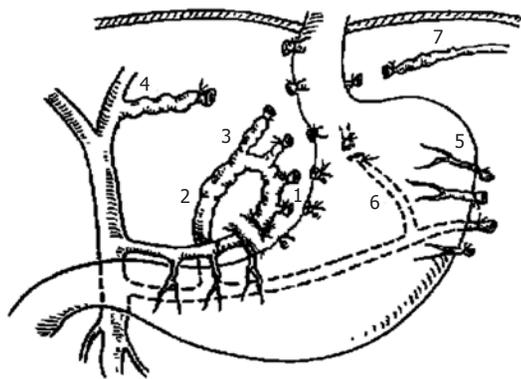
## INTRODUCTION

As a potentially fatal complication, portal vein thrombosis (PVT) can aggravate liver damage and increase the risk of gastrointestinal bleeding<sup>[1]</sup>. PVT can also increase the difficulty of future liver transplantation<sup>[2,3]</sup>. The incidence of PVT including splenic vein, superior mesenteric vein, or portal vein thrombosis after splenectomy with periesophagogastric devascularization in hepatitis B cirrhosis-related portal hypertension is 13.4%-43.5%. At present, most of patients with PVT undergo specific treatments after the diagnosis with color Doppler, computed tomography or magnetic resonance imaging. Alternatively, some patients undergo preventive measure, such as antiplatelet and anticoagulation therapy. However, it is not clear in PVT correlates with an increase in blood platelets count. The preventive effect of antiplatelet and anticoagulation therapy on PVT is also not conclusive. The prevention of PVT after splenectomy with periesophagogastric devascularization remains uncertain. A preoperative predictor of PVT is urgently required to guide clinical practice, to assist in the selection of an appropriate surgical procedure, and for considering the success of future liver transplantation.

## MATERIALS AND METHODS

### Subjects

From January 2007 to August, 2010, 69 patients with portal hypertension caused by hepatitis B cirrhosis underwent splenectomy with periesophagogastric devascularization in the same medical group in the West China Hospital of Sichuan University. Thirty-three (47.83%) cases suffered from postoperative PVT. The ages of the cases ranged from 35 to 68 years (mean  $37.3 \pm 10.7$  years). The inclusion criteria included clinically diagnosed portal hypertension caused by hepatitis B-induced cirrhosis in patients with a history of upper gastrointestinal hemorrhage or severe hypersplenism (white blood cell counts  $< 3 \times 10^3$ /dL or/and platelet (PLT)  $< 50 \times 10^3$ /dL). The values of the patients' platelet count ranged from  $9 \times 10^3$ /dL to  $85 \times 10^3$ /dL (mean  $33.2 \times 10^3 \pm 15.9 \times 10^3$ /dL). White blood cell counts ranged from  $0.9 \times 10^3$ /dL to  $5.7 \times 10^3$ /dL (mean  $2.4 \times 10^3 \pm 1.0 \times 10^3$ /dL). All 69 patients underwent routine preoperative endoscopic examination. The esophageal varices were evaluated by Dagradi classification<sup>[4,5]</sup>. All patients had endoscopically confirmed esophageal varices, with four mild cases, 15 moderate cases, and 50 severe cases. Sixteen cases were associated with the red-color sign. Thirty-nine cases had gastric fundus varices. Thirty patients had at least one previous instance of upper gastrointestinal bleeding. Before surgery, 33 cases were grade A according to the



**Figure 1** The anatomy of the lower part of the esophagus and periesophagogastric area after surgery. 1: Gastric branch of gastric coronary vein; 2: Esophageal branch of gastric coronary vein; 3: High esophageal branch of gastric coronary vein; 4: Aberrant high esophageal branch of gastric coronary vein; 5: Gastric short vein; 6: Gastric posterior vein; 7: Left subphrenic vein.

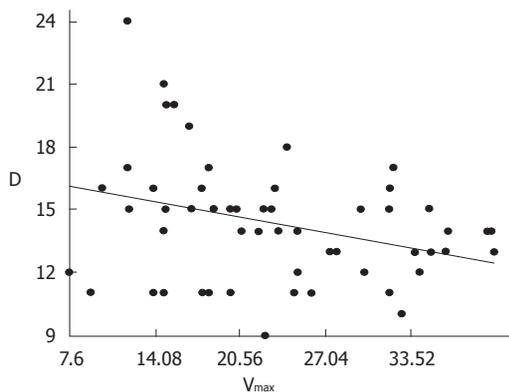
Child classification. Twenty-four cases were grade B and 12 cases were grade C. According to the Pugh-modified Child's score scale, the patients' scores ranged from 7 to 11 (mean  $7.59 \pm 1.22$ ). According to the model for end-stage liver disease score scale, the patients' score ranged from 5 to 12 (mean  $7.71 \pm 2.43$ ).

### Operation

Patients underwent surgery similar to that previously described in detail by Yang and Qiu<sup>[6]</sup>. In brief, an extended left subcostal incision or incision of the left upper abdomen was used for extreme splenomegaly. Routine splenectomy was an important part of periesophagogastric devascularization. The right gastric vein was disconnected near the gastric angular incisura. Then, the gastric branch of the right gastric vein and 5-8 small branches of the gastric coronary veins were disconnected. The esophageal branch was then disconnected and suture-ligated up to 7-9 cm of the esophageal inferior segment. The high esophageal branch went anteriorly and upward near the left-lateral hepatic lobe, and entered into the esophageal muscular layer at 4-6 cm above the cardia, and this branch should be disconnected. The gastric posterior veins and short gastric veins were ligated with sutures, and then the left subphrenic vein was also ligated. In addition, the arteries accompanied by the veins, including the left gastric artery, left gastroepiploic artery, gastric posterior artery, and left subphrenic artery, were disconnected. The net weight of the spleens were determined after surgery (Figure 1).

### Perioperative treatment

Preoperatively, patients underwent treatment to improve their functional hepatic reserve and blood clotting function [Vitk1 (20 mg), qd; 10% GS (500 mL) + 10% KCl (15 mL) + MgSO<sub>4</sub> (5 g) + RI (10 U), qd; BCAA (500 mL), qd]. On the day of surgery, Vitk1 (20 mg) and Reptilase (2000 U) were administered. On postoperative day (POD) 1, only Vitk1 (20 mg) was used. After POD 1, no hemostatic agent was administered. Postoperative patients



**Figure 2** The diameter of the portal vein is negatively correlated with the preoperative maximum portal vein flow velocity.  $V_{max}$ : The mean maximum portal blood flow velocity; D: Portal vein diameter.

underwent treatment to improve their functional hepatic reserve.

### Color Doppler ultrasound detection

Color Doppler ultrasound detection was performed by a color Doppler ultrasound system (Biosound AU 4, Esaote, Italy) on preoperative day 1 and postoperative day 7, respectively. For each measurement, at least three reproducible patterns were created to calculate the mean maximum portal blood flow velocity ( $V_{max}$ ) at the midposition of the main portal vein. The mean portal blood velocity ( $V_{mean}$ ) was calculated using the formula " $V_{mean} = 0.57 \times V_{max}$ " as described by Moriyasu *et al*<sup>[7]</sup>. The portal vein diameter was also measured at the midposition of the main portal vein. Hepatic congestion index (CI) and the ratio of velocity and diameter ( $V_{max}/D$ ) were calculated before surgery.

CI = portal vein cross sectional area/portal vein mean flow velocity =  $(\pi \times D^2/4) / V_{mean} = (\pi \times D^2/4) / 0.57 \times V_{max}$

Ratio of velocity and diameter = the maximum portal blood flow velocity/ the diameter of the portal vein =  $V_{max}/D$

All patients underwent routine PLT and prothrombin time (PT) tests on preoperative day 1 and postoperative day 7.

### Statistical analysis

Numeration data: patients were divided into two groups according the presence or absence of postoperative PVT, or were divided into two groups respectively according to the respective cut-off values of  $V_{max}$ ,  $V_{max}/D$ , CI and D. Numeration data was analyzed by  $\chi^2$  tests. Measurement data: results were expressed as mean  $\pm$  SD and were analyzed by paired-sample *t* test and by receiver operating characteristic (ROC) curves. All calculations were performed using the SPSS 12.0 statistical software. Results with *P* values < 0.05 (paired-tailed test) were considered statistically significant.

The Ethics Committee of our hospital approved the study, and all patients gave their informed consent prior to their inclusion into this investigation.

## RESULTS

### Postoperative complications

After surgery, 33 cases suffered from PVT, including splenic vein, superior mesenteric vein, or portal vein thrombosis. One case suffered from main portal vein complete obstruction, superior mesenteric vein, and splenic vein thrombosis and died on POD 7. The patient had suffered from hepatic encephalopathy and upper gastrointestinal hemorrhage before surgery, and had no opportunity to undergo liver transplantation. No patient suffered from hepatic encephalopathy after surgery. The remaining 68 patients have remained well postoperatively.

### Correlation between preoperative maximum portal blood flow velocity and the diameter of portal vein

The preoperative maximum portal blood flow velocity of the 69 cases ranged from 7.6 cm/s to 40.0 cm/s, and the mean value was  $(24.18 \pm 9.08)$  cm/s. The diameters of their portal veins ranged from 9mm to 24mm (mean value  $14.22 \pm 2.86$  mm). The diameter of portal vein was negatively correlated with the portal vein flow velocity, with the linear regression equation being  $Y = 1.6955 - 0.0113X$  ( $F = 9.88$ ,  $P < 0.05$ ) (Figure 2).

### Analytic results of the differences between the portal vein thrombosis and non-portal vein thrombosis groups

The preoperative maximum portal blood flow velocity of the group with PVT was  $18.06 \pm 5.97$  cm/s (7.6-32.3 cm/s), the preoperative maximum portal blood velocity of the group without PVT was  $29.79 \pm 7.75$  cm/s (14.0-40.0 cm/s). The diameter of portal vein of the group with PVT was  $15.39 \pm 2.97$  mm (11-24 mm), the diameter of portal vein of the group without PVT was  $13.17 \pm 2.31$  mm (9-21mm). The hepatic congestion index of the group with PVT was  $0.2126 \pm 0.1243$  cm/s<sup>-1</sup> (0.0641-0.6614 cm/s<sup>-1</sup>), hepatic congestion index of the group without PVT was  $0.0942 \pm 0.0702$  cm/s<sup>-1</sup> (0.041881-0.410575 cm/s<sup>-1</sup>). The ratio of velocity and diameter of the group with PVT was  $12.1774 \pm 4.7493/s$  (5-23.63636/s), the ratio of velocity and diameter of the group without PVT was  $23.3167 \pm 6.7956$  cm/s<sup>-1</sup> (7.047619-32.9/s). All of the above-mentioned four variables showed the statistically significant difference between the two groups ( $P < 0.05$ ). There was no statistically significant difference in the Child-Pugh score, the net weight of the patients' spleens, the value of PT and PLT count between the two groups (Table 1).

### Receiver operating characteristic curve analysis

The ROC curve (Figure 3) showed that the two variables  $V_{max}$  and  $V_{max}/D$  could be used as preoperative predictors of postoperative portal vein thrombosis. The respective values of the area under the curve were 0.865 (asymptotic 95% confidence interval: 0.780-0.950) and 0.893 (asymptotic 95% confidence interval: 0.815-0.970), and the respective cut-off values (24.45 cm/s and 19.4333 /s) were diagnostically efficient, with sensitivities of 87.9% and 93.9%, respectively, specificities of 75% and 77.8%, respectively (Figure 3).

Table 1 Differences between patients with and without portal vein thrombosis

Group	Group with PVT ( <i>n</i> = 33)	Group without PVT ( <i>n</i> = 36)	Tort' value	<i>P</i> value
Preoperative maximum portal blood velocity (cm/s)	18.06 ± 5.97	29.79 ± 7.75	6.9978	< 0.05
The diameter of portal vein (mm)	15.39 ± 2.97	13.17 ± 2.31	3.4935	< 0.05
Hepatic congestion index (cm/s <sup>-1</sup> )	0.2126 ± 0.1243	0.0942 ± 0.0702	4.812	< 0.05
The ratio of velocity and diameter (/s)	12.1774 ± 4.7493	23.3167 ± 6.7956	7.9439	< 0.05
The net weight of spleen (g)	619.4 ± 132.6	636.4 ± 235.3	0.3736	> 0.05
Preoperative PLT count (× 10 <sup>9</sup> /L)	31.5 ± 14.3	34.8 ± 17.2	0.8622	> 0.05
Postoperative PLT count (× 10 <sup>9</sup> /L)	237.8 ± 84.4	267.7 ± 137.6	1.0978	> 0.05
Preoperative PT value (s)	15.6 ± 1.4	15.9 ± 1.3	0.923	> 0.05
Postoperative PT value (s)	16.0 ± 1.8	16.4 ± 1.6	0.9772	> 0.05
Child-Pugh score	7.54 ± 1.24	7.63 ± 1.22	0.3037	> 0.05

PVT: Portal vein thrombosis; PLT: Platelet; PT: Prothrombin time.

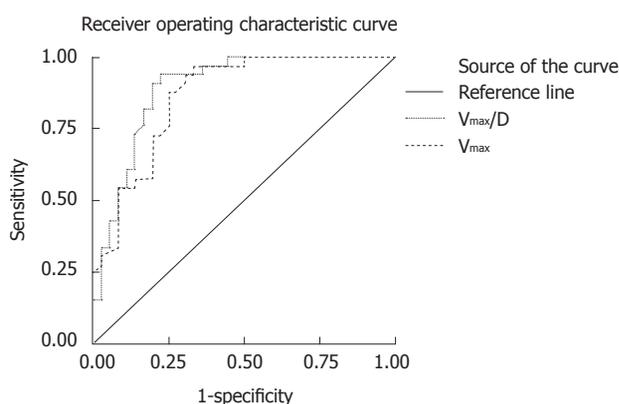


Figure 3 Receiver operating characteristic curve of the mean maximum portal blood flow velocity and  $V_{\max}$ /portal vein diameter.  $V_{\max}$ : The mean maximum portal blood flow velocity; D: Portal vein diameter.

#### Analytic results for the difference between cases with $V_{\max}$ under 24.45 cm/s ( $V_{\max} \leq 24.45$ cm/s) and cases with $V_{\max}$ above 24.45 cm/s ( $V_{\max} > 24.45$ cm/s)

The mean preoperative maximum portal blood velocity of the group with  $V_{\max}$  under 24.45 cm/s ( $n = 38$ ) was  $17.10 \pm 4.60$  cm/s (7.6-24.2 cm/s). The mean preoperative maximum portal blood velocity of the group with  $V_{\max}$  above 24.45 cm/s ( $n = 31$ ) was  $32.85 \pm 4.46$  cm/s (24.7-40.0 cm/s). Twenty-nine cases suffered from PVT in the group with  $V_{\max}$  under 24.45 cm/s (29/38, 76.32%); only four cases suffered from PVT in the group with  $V_{\max}$  above 24.45 cm/s (4/31, 12.90%). The incidence of PVT in the cases with  $V_{\max}$  under 24.45 cm/s was significantly higher than in the cases with  $V_{\max}$  above 24.45 cm/s ( $\chi^2 = 27.51$ ,  $P < 0.05$ ) (Table 2).

#### Analytic results for the differences between cases with $V_{\max}/D$ under 19.43/s ( $V_{\max}/D \leq 19.43$ /s) and cases with $V_{\max}/D$ above 19.43/s ( $V_{\max}/D > 19.43$ /s)

The mean value of cases with  $V_{\max}/D$  under 19.43/s ( $n = 39$ ) was  $(11.79 \pm 3.99)$ /s (5.00-19.00/s), the mean value of cases with  $V_{\max}/D$  above 19.43/s ( $n = 30$ ) was  $(26.05 \pm 3.82)$ /s (19.87-32.90/s). Thirty-one cases suffered from PVT in the group with  $V_{\max}/D$  under 19.43/s (31/39, 79.49%); only two cases suffered from PVT in the group with  $V_{\max}/D$  above 19.43/s (2/30, 6.67%). The incidence

of PVT in the cases with  $V_{\max}/D$  under 19.43/s was significantly higher than in the cases with  $V_{\max}/D$  above 19.43/s ( $\chi^2 = 36.04$ ,  $P < 0.05$ ) (Table 2).

#### Receiver operating characteristic curve analysis of variable congestion index and D

This ROC curve (Figure 4) showed that the two variables (CI and D) could also be used as preoperative predictors of postoperative portal vein thrombosis. The respective values of the area under the curve were 0.884 (asymptotic 95% confidence interval: 0.799-0.970) and 0.742 (asymptotic 95% confidence interval: 0.624-0.861), and the respective cut-off values (0.1138 cm/s<sup>-1</sup> and 13.5 mm) were diagnostically efficient, with sensitivities of 87.9% and 81.8%, respectively, and specificities of 86.1% and 63.9%, respectively (Figure 4).

#### Analytic results for the differences between cases with congestion index under 0.1138 cm/s<sup>-1</sup> and cases with congestion index above 0.1138 cm/s<sup>-1</sup>

The mean CI of cases with CI under 0.1138 cm/s<sup>-1</sup> ( $n = 35$ ) was  $0.0733 \pm 0.0190$  cm/s<sup>-1</sup> (0.041881-0.112652 cm/s<sup>-1</sup>). The mean CI of cases with CI above 0.1138 cm/s<sup>-1</sup> was  $0.2306 \pm 0.1193$  cm/s<sup>-1</sup> (0.114922-0.661389 cm/s<sup>-1</sup>). Twenty-nine cases suffered from PVT in the group with CI above 0.1138 cm/s<sup>-1</sup> (29/34, 85.29%); four cases suffered from PVT in the group with CI under 0.1138 cm/s<sup>-1</sup> (4/35, 11.43%). The incidence PVT in the cases with CI above 0.1138 cm/s<sup>-1</sup> was significantly higher than in the cases with CI under 0.1138 cm/s<sup>-1</sup> ( $\chi^2 = 37.71$ ,  $P < 0.05$ ) (Table 2).

#### Analytic results for the differences between cases with D under 13.5 mm and cases with D above 13.5 mm

The mean diameter of cases with D under 13.5 mm ( $n = 40$ ) was  $11.79 \pm 1.21$  mm (9-13 mm). The mean diameter of cases with D above 13.5mm was  $16.00 \pm 2.35$  mm (14-24 mm). Twenty-seven cases suffered from PVT in the group with  $V_{\max}/D$  above 13.5 mm (27/40, 67.5%). Six cases suffered from PVT in the group with D under 13.5 mm (6/29, 20.69%). The incidence of PVT in the cases with D above 13.5mm was significantly higher than in the cases with D under 13.5 mm ( $\chi^2 = 14.76$ ,  $P < 0.05$ ) (Table 2).

Table 2 Analysis of respective variables

Variable(s)	Area under the curve	Cut-off value	Sensitivity (%)	Specificity (%)	Cases above the value	Cases with PVT	Cases under the value	Cases with PVT	$\chi^2$ value	P value
$V_{\max}$	0.865	24.45 cm/s	87.9	75	31	4	38	29	27.51	< 0.05
$V_{\max}/D$	0.893	19.4333/s	93.9	77.8	30	2	39	31	36.04	< 0.05
CI	0.884	0.1138 cm/s <sup>-1</sup>	87.9	86.1	34	29	35	4	37.71	< 0.05
D	0.742	13.5 mm	81.8	63.9	40	27	29	6	14.76	< 0.05

PVT: Portal vein thrombosis; CI: Congestion index.

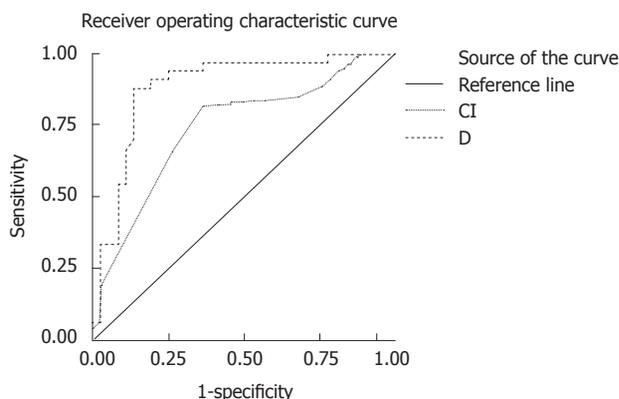


Figure 4 Receiver operating characteristic curve of hepatic congestion index and portal vein diameter. CI: Congestion index; D: Portal vein diameter.

## DISCUSSION

The causes of PVT after splenectomy with periesophago-gastric devascularization are disputed. Extrahepatic portal vein thrombosis frequently results from multiple concurrent factors, including procoagulant states and underlying myeloproliferative disorders (MPDs). The JAK2 V617F mutation is a point mutation in the Janus kinase 2 (JAK2) tyrosine kinase that is variably present in MPDs. The role of screening for the JAK2 V617F mutation in patients presenting with thrombosis without overt MPD is unclear, but appears justified in cases of idiopathic splanchnic vein thrombosis<sup>[8]</sup>. Silvia's research showed that pre-operative splenic vein diameter is a risk factor for portal-splenic vein thrombosis after laparoscopic splenectomy<sup>[9]</sup>. Shetty's research showed that among the acquired thrombophilias, MPD are the most frequent cause, while antiphospholipid antibodies and hyperhomocysteinemia have not shown very strong association with PVT<sup>[10]</sup>. Many scholars think that the rebound in PLT count post splenectomy and the hypercoagulable state cause postoperative PVTs in hepatitis B cirrhosis-related portal hypertension. Similar to Roberto's research<sup>[11]</sup>, our research showed that there was no statistically significant difference in the Child-Pugh score, the net weight of the patients' spleens, the value of PT and the PLT count between the group with PVT and group without a PVT. However, our research did show a statistically significant difference in the preoperative maximum portal blood velocity and the diameter of the portal vein between the two groups. This indicated that the rebound in PLT

count was not the main cause of postoperative PVTs. The occurrence of postoperative PVTs also did not show any correlation with the Child-Pugh score or the net weight of spleens.

Our research showed that preoperative maximum portal vein flow velocity in patients with postoperative PVT was significantly lower than in patients without postoperative PVT. The diameter of the portal vein in patients with a PVT was significantly wider than in patients without a PVT. Thus, the preoperative portal vein flow velocity and the diameter of portal vein were the important factors influencing the incidence of postoperative PVT. Our study showed that the diameter of the portal vein was negatively correlated with the preoperative maximum portal vein flow velocity. Considering to that result, the hepatic CI and the ratio of velocity and diameter ( $V_{\max}/D$ ) were both calculated before surgery.

ROC curves showed that four variables ( $V_{\max}$ ,  $V_{\max}/D$ , CI and D) could be used as preoperative predictors of postoperative portal vein thrombosis. The area under the curve of  $V_{\max}/D$  was the largest (0.893); therefore,  $V_{\max}/D$  was the most accurate preoperative predictor of portal vein thrombosis after splenectomy with periesophago-gastric devascularization in hepatitis B cirrhosis-related portal hypertension.

After surgery, four (21.90%) cases suffered from PVT in the group with  $V_{\max}$  above 24.45 cm/s and only two (6.67%) cases suffered from PVT in the group with  $V_{\max}/D$  above 19.43/s. Four cases (11.43%) suffered from PVT in the group with CI under 0.1138 cm/s<sup>-1</sup> and six cases (20.69%) suffered from PVT in the group with D under 13.5 mm. Okuda *et al.*<sup>[12]</sup> reported that the natural incidence of PVT was 6.6% in patients with hepatitis B cirrhosis-related portal hypertension that have not undergone surgery. We also showed that the incidence of PVT was very low in the patients with  $V_{\max}$  above 24.45 cm/s,  $V_{\max}/D$  above 19.43/s, CI under 0.1138 cm/s<sup>-1</sup>, or D under 13.5 mm.

Reports concerning the incidence of portal vein thrombosis splenectomy with periesophago-gastric devascularization are very uniform. Our study showed that postoperative portal vein thrombosis is mainly due to the change of portal vein blood flow dynamics, rather than a change in the value of PT or the PLT count. The incidence of postoperative PVT did not show any correlation with the Child-Pugh score or the net weight of the patients' spleens. The change of portal vein blood flow dynamics in patients with portal hypertension included

decreased portal vein velocity and increased portal vein diameter<sup>[13,14]</sup>. The decreased blood flow velocity can lead to the development of thrombus, and even the formation of eddy currents; increased portal vein diameter would lead to a vortex, causing venous intimal damage and “atherosclerosis-like” changes. In part, endothelial cells’ detachment and collagen exposure would lead to blood cell adhesion and thus thrombosis. When  $V_{\max}/D$  was above 19.43/s, postoperative thrombosis very unlikely to occur. According to the report of Deng *et al.*<sup>[15]</sup>, thrombosis mainly occurs in the perioperative period (within about one month after surgery). Thus, these patients with  $V_{\max}/D$  above 19.43/s should have a relatively good prognosis, but it still require long-term follow-up. When the  $V_{\max}/D$  is under 19.43/s, it is necessary to pay special attention to the prevention of a potential PVT after splenectomy with periesophagogastric devascularization. Further study is required to determine whether such patients require liver transplantation, but not splenectomy with periesophagogastric devascularization.

## COMMENTS

### Background

As a potentially fatal complication, portal vein thrombosis (PVT) can aggravate liver damage and increase the risk of gastrointestinal bleeding. PVT can also increase the difficulty of the future liver transplantation. The incidence of PVT, including splenic vein, superior mesenteric vein or portal vein thrombosis after splenectomy with periesophagogastric devascularization in hepatitis B cirrhosis-related portal hypertension, is 13.4%-43.5%.

### Research frontiers

The preventive effect of antiplatelet and anticoagulation therapy on PVT is not conclusive. It is still unknown as to how to prevent PVT after splenectomy with periesophagogastric devascularization. A preoperative predictor of PVT is urgently required to guide clinical practice.

### Innovations and breakthroughs

In this prospective study, 69 patients with portal hypertension caused by hepatitis B cirrhosis underwent splenectomy with periesophagogastric devascularization in West China Hospital of Sichuan University from January 2007 to August 2010. The portal vein flow velocity and diameter of portal vein were measured by Doppler sonography. Hepatic congestion index and the ratio of velocity and diameter were calculated before surgery, and the prothrombin time values and platelet levels were detected before and after surgery. The patients’ spleens were weighed after surgery.

### Applications

The ratio of velocity and diameter was most accurate as a preoperative predictor of portal vein thrombosis after splenectomy with periesophagogastric devascularization in hepatitis B cirrhosis-related portal hypertension.

### Peer review

This is a prospective study of preoperative predictors for the risk of portal vein thrombosis after splenectomy with periesophagogastric devascularization.

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## Gastric angiodysplasia in a hereditary hemorrhagic telangiectasia type 2 patient

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### Abstract

Hereditary hemorrhagic telangiectasia (HHT) is a rare autosomal-dominantly inherited disease that occurs in approximately one in 5000 to 8000 people. Clinical diagnosis of HHT is made when a person presents three of the following four criteria: family history, recurrent nosebleeds, mucocutaneous telangiectasis, and arteriovenous malformations (AVM) in the brain, lung, liver and gastrointestinal (GI) tract. Although epistaxis is the most common presenting symptom, AVMs affecting

the lungs, brain and GI tract provoke a more serious outcome. Heterozygous mutations in endoglin, activin receptor-like kinase 1 (ACVRL1; ALK1), and SMAD4, the genes involved in the transforming growth factor- $\beta$  family signaling cascade, cause HHT. We report here the case of a 63 year-old male patient who presented melena and GI bleeding episodes, proven to be caused by bleeding from multiple gastric angiodysplasia. Esophagogastroduodenoscopy revealed multiple angiodysplasia throughout the stomach. Endoscopic argon plasma coagulation was performed to control bleeding from a gastric angiodysplasia. The patient has been admitted several times with episodes of hemoptysis and hematochezia. One year ago, the patient was hospitalized due to right-sided weakness, which was caused by left basal ganglia hemorrhage as the part of HHT presentation. In family history, the patient's mother and elder sister had died, due to intracranial hemorrhage, and his eldest son has been suffered from recurrent epistaxis for 20 years. A genetic study revealed a mutation in exon 3 of ALK1 (c.199C > T; p.Arg67Trp) in the proband and his eldest son presenting epistaxis.

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**Key words:** Hereditary hemorrhagic telangiectasia; Angiodysplasia; Intracranial hemorrhage; Epistaxis; [Activin receptor-like kinase 1](#)

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Ha M, Kim YJ, Kwon KA, Hahm KB, Kim MJ, Kim DK, Lee YJ, Oh SP. Gastric angiodysplasia in a hereditary hemorrhagic telangiectasia type 2 patient. *World J Gastroenterol* 2012; 18(15): 1840-1844 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i15/1840.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i15.1840>

## INTRODUCTION

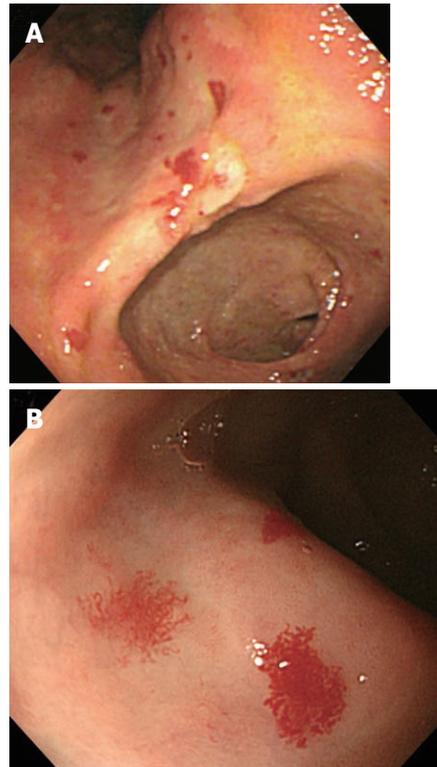
Hereditary hemorrhagic telangiectasia (HHT), also called Osler-Weber-Rendu syndrome, is a rare autosomal dominant genetic disorder occurring in about one in 5000 to 8000 people worldwide<sup>[1-3]</sup>. HHT is characterized by family history, recurrent nosebleeds, mucocutaneous telangiectasis, and arteriovenous malformations (AVMs) in the brain, lung, liver and gastrointestinal (GI) tract<sup>[4,5]</sup>. Clinical diagnosis of HHT is made when a person presents with three of these four symptoms<sup>[6]</sup>. However, due to the highly variable onset and penetrance of these clinical symptoms, HHT is often misdiagnosed. Epistaxis and skin telangiectasis are the most common presenting symptoms that appear in over 90% of HHT patients aged over 60 years<sup>[7]</sup>. Cerebral and pulmonary AVMs occur in around 10%-20% and 50% of HHT patients, respectively, and are associated with high mortality and morbidity due to stroke or brain abscess<sup>[8]</sup>. AVMs in the GI tract can lead to melena, brisk GI bleeding, and hematochezia. Most elderly HHT patients suffer from anemia due to epistaxis and GI bleeding.

Genetic studies have shown that heterozygous mutations in endoglin (*ENG*) or activin receptor-like kinase 1 (*ALK1*; *ACVRL1*) cause HHT<sup>[9-12]</sup>. HHT by *ENG* or *ALK1* mutations is designated as HHT1 or HHT2, respectively, although they are clinically indistinguishable. Some juvenile polyposis (JP) patients with *SMAD4* heterozygous mutations have shown to present HHT symptoms and are designated as JP-HHT<sup>[13,14]</sup>. Two additional genetic loci in chromosomes 5 (HHT3) and 7 (HHT4) have been identified<sup>[15,16]</sup>. HHT1 and HHT2 account for over 80% of HHT worldwide<sup>[17]</sup>.

In this report, we describe a case of a patient presenting with multiple gastric angiodysplastic lesions, intracranial hemorrhage (ICH), and a family history of ICH and epistaxis. Through genetic testing, we found a known mutation in *ALK1* (c.199 C > T; p.Arg67Trp) in the proband<sup>[18-23]</sup>. Additionally, the patient's son had been suffering from epistaxis. To our knowledge, this is the first case in Korea of genetic confirmation of HHT2 with multiple gastric angiodysplasia.

## CASE REPORT

A 63-year-old male was admitted due to melena. Examination revealed stable vital signs with normal blood pressure and heart rate, but routine laboratory tests revealed mild anemia with a hemoglobin level of 9.6 g/dL. Esophagogastroduodenoscopy (EGD) revealed multiple angiodysplastic lesions throughout the stomach (Figure 1A and B). Conservative treatment, including proton pump inhibitors, led to a stable clinical condition. The patient was subsequently hospitalized again due to hemoptysis, and then once more due to hematochezia; all were managed by conservative treatment regimens. We carefully investigated the patient's medical and family history. The patient's mother and older sister died from ICH. His eldest son had been suffering from recurrent epistaxis

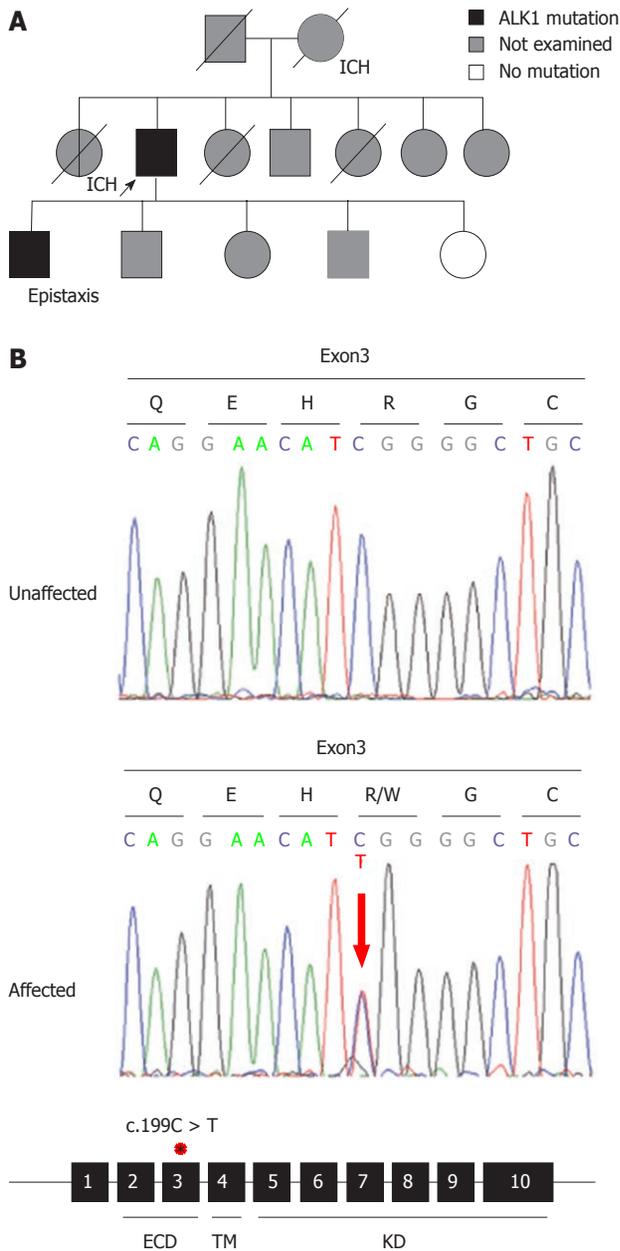


**Figure 1** Multiple angiodysplastic lesions were noted in the entire stomach upon endoscopic examination. A: A view of the lesser curvature of the angle showing multiple scattered angiodysplastic lesions; B: A view of the anterior wall of the antrum, magnified view of angiodysplastic lesions showing vessel-pulled appearance.

for 20 years (Figure 2A). Therefore, due to the presumptive diagnosis of HHT, we carried out genetic screening for the *ENG* and *ALK1* genes. A heterozygous mutation in *ALK1* was detected from the proband of both the patient and his eldest son, but not from a daughter who did not show any apparent HHT-related symptoms (Figure 2B). A single nucleotide substitution from “C” to “T” at the 199th coding nucleotide (c.199 C > T) in exon 3 changed the 67th amino acid, arginine, to tryptophan (p.Arg67Trp), resulting in loss of *ALK1* function (Figure 2B)<sup>[18-23]</sup>. Abdomino-pelvic computed tomography (CT) scan of the proband, performed for evaluation of a gallbladder stone, revealed multiple intrahepatic AV shunts (Figure 3A). Later, the proband received peritoneoscopic cholecystectomy, and then the patient was hospitalized again due to the sudden onset of right-sided weakness. Brain CT revealed ICH at the left basal ganglion, and he was transferred to a local rehabilitation facility (Figure 3B). The patient was recently admitted for percutaneous endoscopic gastrostomy insertion.

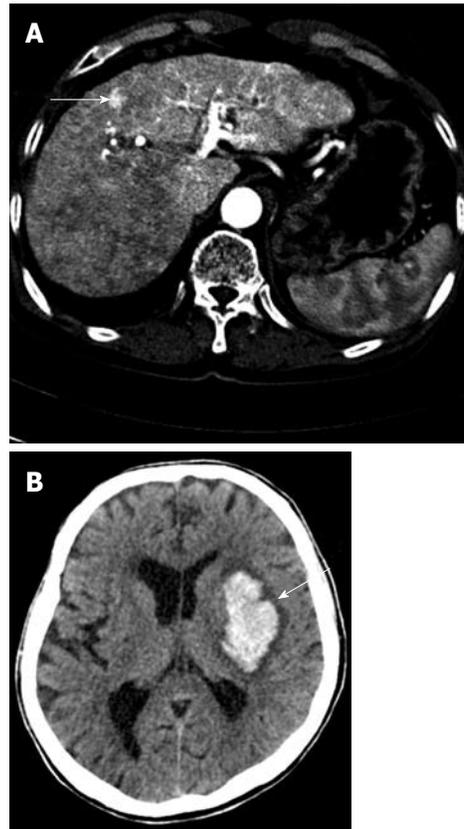
## DISCUSSION

In the present case, we report a patient with multiple angioplatic lesions in the stomach, chronic hemorrhages in the GI tract, and stroke by ICH. Presence of ICH and epistaxis in his immediate family members (mother, sister and son) led us to diagnose HHT. Genetic screen-



**Figure 2** Pedigree and genetic analysis of an hereditary hemorrhagic telangiectasia family. A: Pedigree of a family with genetic mutations and/or symptoms of hereditary hemorrhagic telangiectasia (HHT) and intracranial hemorrhage (ICH). The proband is indicated by an arrow. A divided symbol represents the individual with ICH. Deceased individuals are indicated by a slash; B: Genetic studies of unaffected and affected family members. The affected member had a heterozygous *ALK1* mutation (c.199C > T; p.Arg67Trp). The amino acid translation is shown above each codon. The mutation (a) was found in exon 3. Protein domains of *ALK1* are indicated under the exons: extracellular domain (ECD), transmembrane domain (TM), and kinase domain (KD).

ing of two HHT genes, *ALK1* and *ENG*, revealed a substitution mutation at the 199th coding nucleotide of the *ALK1* gene, specifically in the proband and the son presenting as epistaxis, but not in the daughter who had no apparent symptoms<sup>[18-23]</sup>. These data confirmed the clinical diagnosis and showed that the patient carries the HHT2 subtype. HHT1 and HHT2 are clinically similar in presentation, but genotype-phenotype correlation studies have shown that occurrence of pulmonary AVMs is sig-



**Figure 3** Hepatic arterio-venous shunt and cerebral hemorrhage. A: Abdominal computed tomography (CT) showing an intra-hepatic arterio-venous shunt; B: Cerebral hemorrhage was noted in the left basal ganglia on brain CT.

nificantly higher in HHT1, whereas liver AVM tend to be more common in HHT2<sup>[20,21,24-28]</sup>. Cerebral involvement and spinal AVMs are reported in 10%-20% and 1%-2% of HHT1 and HHT2 cases, respectively<sup>[18,27]</sup>. Whether genetic or environmental factors contributed to this observation in this family or the wider Korean HHT population remains to be determined. The mutation found in this family (c.199 C > T; p.Arg67Trp) has previously been reported by five other groups<sup>[18-23]</sup>. The 67th amino acid is located in the ligand binding domain of *ALK1*. Previous biochemical analysis of the Arg67Gln substitution resulted in a deficiency in signal transduction, suggesting a null mutation<sup>[29]</sup>. Life-threatening cerebral and pulmonary AVMs that form during development and the neonatal period are often asymptomatic for a prolonged period. Manifestations of epistaxis, skin telangiectasia, and GI AVMs are generally absent at birth, but develop over puberty and progressively worsen with age<sup>[7,30,31]</sup>. Genetic screening of asymptomatic family members would allow detection of deadly forms of vascular lesions in advance, before complications arise.

GI bleeding associated with HHT occurs in about 15%-30% of patients, and GI telangiectasia, including angiodysplasia, is a relatively common manifestation of HHT<sup>[7,32,33]</sup>. However, reports of this syndrome in the form of angiodysplasia on EGD in the GI tract, including the stomach, are rare.

Together with epistaxis, GI bleeding is a serious is-

sue for elderly HHT patients. Recent studies indicate that environmental factors such as injury and inflammation, in addition to genetic predisposition (*ALK1* or *ENG* mutations), are critical for development of AVMs in adults<sup>[34,35]</sup>. Multiple anti-inflammatory, anti-angiogenic, and anti-oxidants drugs such as thalidomide, bevacizumab and N-acetyl-cysteine have been tested as potential therapies for ameliorating epistaxis and GI bleeding<sup>[7,36]</sup>. We report here that the proband had multiple angiodysplastic lesions in the stomach and also hematochezia, indicating that vascular lesions had spread to the lower GI tract. In addition, close monitoring for GI bleeding is needed. Capsule endoscopy and conventional endoscopy may provide important information on whether these drugs can induce regression of existing vascular lesions.

In summary, we report the case of a patient in whom we found multiple gastric angiodysplastic lesions. With both clinical evaluation and genetic screening, we confirmed that the patient was suffering from HHT2. Family history and other HHT symptoms should be carefully evaluated when a patient shows multiple angiodysplastic lesions. Genetic screening has tremendous benefit not only for confirming the diagnosis but also in preventing asymptomatic family members developing life-threatening complications.

## ACKNOWLEDGMENTS

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## Solitary gastric Peutz-Jeghers type stomach polyp mimicking a malignant gastric tumor

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### Abstract

Most cases of Peutz-Jeghers type polyps of the stomach are associated with mucocutaneous pigmentation and multiple intestinal polyposis. A solitary Peutz-Jeghers type polyp of the stomach is rare. We here report a case of a 71-year-old woman with a solitary Peutz-Jeghers type polyp of the stomach who presented with intolerable epigastric pain and weight loss of 5 kg over the prior two months. During the hospital treatment course for this patient, endoscopic examination revealed a bulging lesion with a central hole, mucosal ulceration, an asymmetrical wall thickness and a narrowing of the gastric lumen. A gastric biopsy further revealed ulceration with moderate dysplasia. The patient received endoscopic ultrasonography which showed a second subepithelial lesion that measured 4 cm × 3 cm. Computed tomography of the abdomen subsequently showed a thickened gastric wall with three visibly enlarged lymph nodes, all greater than 1 cm. The suspected diagnosis was malignant gastric cancer with lymph node metastases. The other lesion,

which measured 2 cm × 2 cm × 1 cm was noted in the submucosa of the jejunum during surgery. The patient was treated using a subtotal gastrectomy and partial resection of the jejunal tumor. The final pathological report indicated a gastric Peutz-Jeghers type polyp with proliferation of smooth muscle bundles in the submucosal layer, and hyperplastic glands in the mucosal layer and ectopic pancreas of the jejunum. This is the first reported clinical case of a solitary Peutz-Jeghers type polyp of the stomach accompanying a lymph node enlargement and ectopic pancreas in the jejunum that simulates stomach cancer with lymph node metastases.

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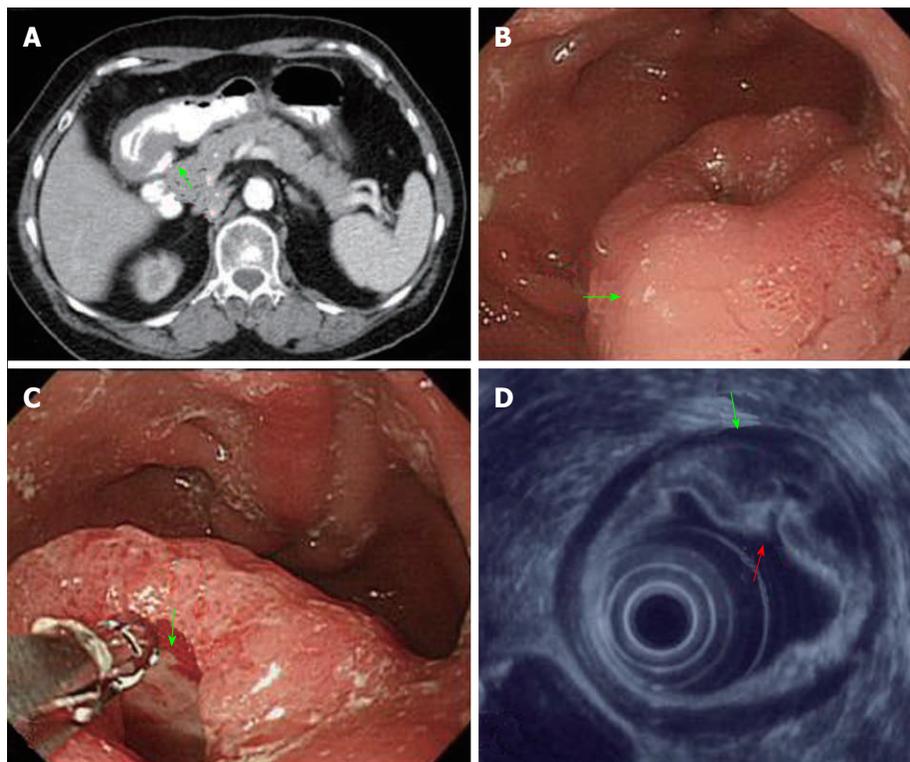
**Key words:** Peutz-Jeghers type polyp; Submucosa lesion; Stomach cancer; Ectopic pancreas; Endoscopic ultrasonography

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### INTRODUCTION

A solitary Peutz-Jeghers type polyp of the stomach is rare<sup>[1-4]</sup> and most cases are associated with mucocutaneous pigmentation and multiple intestinal polyposis<sup>[5,6]</sup>. Peutz-Jeghers type polyposes occur mainly in the small intestine, colon, and stomach, and these hamartomatous polyps are characterized by hyperplastic epithelia and by the proliferation of smooth muscle bundles around the mucosal glands<sup>[5]</sup>. We here report a case of a woman who had a rare solitary gastric Peutz-Jeghers type polyp of the stomach that mimicked gastric cancer with lymph node metastases.



**Figure 1** Image findings of gastric Peutz-Jeghers type stomach polyp. A: Abdominal computed tomography scans of the patient revealing a thickened gastric wall over the antrum of the stomach (arrow); B: Gastroendoscopy revealing a bulging lesion (4 cm × 3 cm) of the antrum (arrow); C: The bulging lesion displayed a central hole (arrow); D: Endoscopic ultrasonography demonstrating that the appearance of a lesion was due to a thickened subepithelial layer (second layer lesion), submucosal layer thickened (third layer, green arrow), and corresponding hole (red arrow) over the antrum of the stomach.

## CASE REPORT

A 71-year-old woman presented at our hospital with an extremely rare case of a solitary gastric Peutz-Jeghers type polyp of the stomach. This patient had experienced intolerable epigastric pain over the previous two months and also 5 kg of weight loss during this time. She attended the emergency room and was subsequently admitted to the hospital to receive treatment for pain. During her hospitalization, emergency computed tomography (CT) scans of the abdomen were performed and revealed a thickened gastric wall in the antrum (Figure 1A). Three enlarged lymph nodes greater than 1 cm were also detected. The CT report thus indicated a diagnosis of gastric cancer with lymph node metastases.

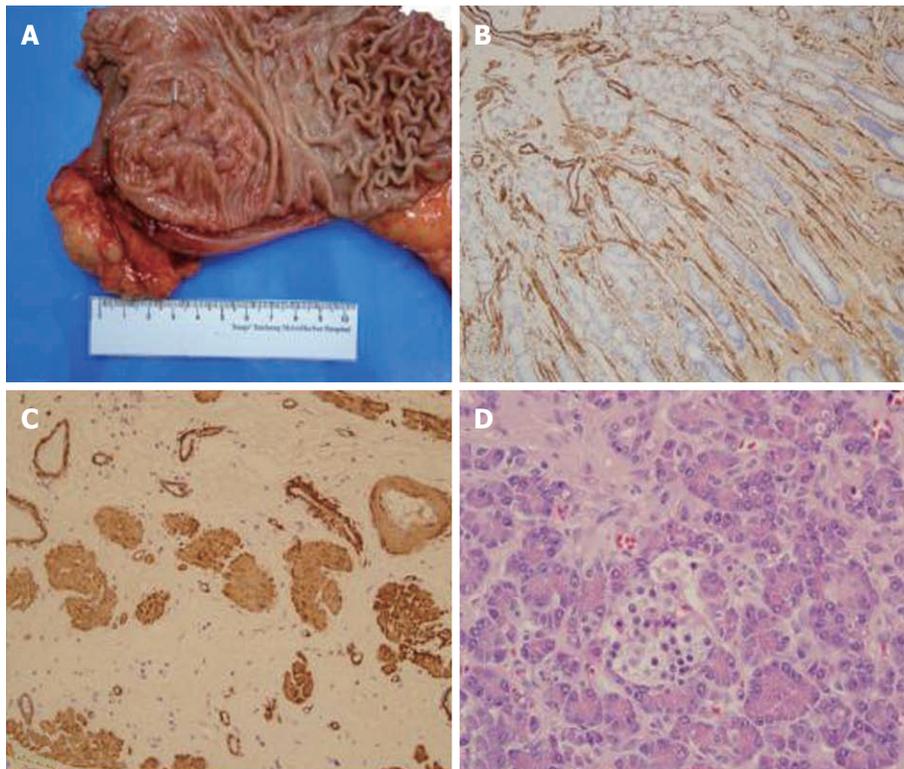
A gastroduodenoscopy was also performed and showed a bulging gastric lesion of 4 cm × 3 cm with a central hole (Figure 1B and C), in addition to an asymmetrical wall thickness and narrowing of gastric lumen in the antrum. A gastric biopsy revealed ulceration with moderate dysplasia of the mucosal glands. The patient's pain was not relieved by antacids. Further studies *via* endoscopic ultrasonography showed the presence of a subepithelial lesion of 4 cm × 3 cm with a central hole (Figure 1D).

The patient additionally underwent a diagnostic endoscopic mucosectomy (EMR), and a gastric specimen measuring 1.3 cm × 0.9 cm × 0.2 cm was thereby obtained. The subsequent pathology report identified this specimen as a hamartomatous polyp with proliferation of smooth

muscle bundles in the submucosal layer and hyperplastic glands in the mucosal layer. Because the results of the clinical examination and pathology were conflicting, a clinical diagnosis of malignant gastric cancer with lymph node metastases was made. The patient then underwent a subtotal gastrectomy. An additional lesion measuring 2 cm × 2 cm × 1 cm was noted in the submucosa of the jejunum during this surgery. Partial resection of the lesion in the jejunum was thus performed and the specimens were sent for pathological examination.

Gross examination of the lesion revealed a 4 cm × 3 cm polypoid tumor, with a central ulceration over the antrum of the stomach after diagnostic endoscopic mucosectomy (Figure 2A). Seventeen lymph nodes were dissected from the perigastric region measuring up to 1.2 cm in diameter. A submucosal lesion measuring 2 cm × 2 cm × 1 cm was also noted in the jejunum region. Histopathological examination of the lesion that had been diagnosed as a hamartomatous polyp of the stomach revealed a characteristic cell growth that increased the thickness of the mucosal glands to a maximum of about 9 mm and of the muscularis propria to 11 mm (Table 1). Smooth muscle bundles were found to be interspersed around the glands (Figure 2B), and smooth muscle bundles were observed to have proliferated over the submucosal layer (Figure 2C).

The 17 dissected lymph nodes showed reactive hyperplasia without tumor metastasis. The jejunum lesion contained ectopic pancreatic tissue with ducts, acini and



**Figure 2** Pathological findings of gastric Peutz-Jeghers type stomach polyp. A: Gross examination of a stomach specimen from the patient showed a polyp-like lesion (4 cm × 3 cm) over the antrum following endoscopic mucosectomy; B: Immunohistochemical staining of smooth muscle actin in the mucosal layer revealed a proliferation of smooth muscle bundles (× 200); C: Submucosal layer of the lesion revealed a proliferation of smooth muscle bundles (× 200); D: Photomicrogram demonstrating the presence of an ectopic pancreas in the submucosa of the jejunum and of pancreatic ducts, acini and islet cells (hematoxylin and eosine staining, original magnification × 200).

	Patient	Normal (n = 3)
Mucosa layer (mm)	5-9	3-5
Smooth muscle bundles	Diffuse increase	Focal few
Muscularis mucosa (μm)	10-30	10-30
Submucosal layer (mm)	3-5	3-5
Diffuse increase	Smooth muscle bundles	none
Muscle propria (mm)	5-11	5-7

islet cells, with interspersed smooth muscle bundles (Figure 2D). Immunohistochemical staining revealed strong cytoplasmic signals for smooth muscle actin between the mucosal glands (Figure 2B) and the submucosal layer (Figure 2C). A gastrointestinal stromal tumor was ruled out by negative staining for c-Kit (c-Kit pharmDx™; Dako Corporation, Glostrup, Denmark). The final pathological determination was a gastric Peutz-Jeghers type polyp and jejunal ectopic pancreas. This is therefore the first reported case of a solitary Peutz-Jeghers type polyp of the stomach that presented with clinical signs mimicking a stomach cancer with lymph node metastases.

## DISCUSSION

A solitary Peutz-Jeghers type polyp of the stomach is found in fewer than 0.1% of all endoscopic examina-

tions<sup>[5]</sup>. Bartholomew *et al*<sup>[5]</sup> have previously observed that polyps are present in the stomach in only 24% of cases of Peutz-Jeghers syndrome associated with intestinal polyposis, mainly in the small intestine. Moreover, only 27 cases of gastric solitary hamartomatous polyps have been reported thus far in the literature. The predominant components of hamartomas are hyperplastic mucosal glands and smooth muscle bundles in the mucosal layer<sup>[1-4]</sup>. Some authors have concluded that solitary Peutz-Jeghers type polyps are histologically identical to those in Peutz-Jeghers syndrome<sup>[4]</sup>. In contrast, other investigators have suggested that solitary Peutz-Jeghers type polyps of the stomach have less branching of the muscularis mucosae than those of the familial form<sup>[3]</sup>. Our current case demonstrated extensive branching of the muscularis mucosae to the mucosal layer that also extended to the submucosal layers. This distinct finding of a proliferation of smooth muscle bundles in the submucosal layer has not been previously reported.

Gastric hamartomatous polyps are usually asymptomatic, and most cases are found incidentally during a routine gastric endoscopy. Few previous cases have presented with intestinal obstruction or anemia due to chronic blood loss, or were found to be accompanied by a transformation to adenocarcinoma<sup>[6-9]</sup>. Our patient presented with intolerable epigastric pain and weight loss of 5 kg during the two months prior to admission to our hospital. CT scans of this patient's abdomen revealed three

enlarged lymph nodes, all of which measured more than 1 cm. In addition, a submucosal lesion over the jejunum was noted. Our patient showed extensive proliferation of smooth muscle bundles in the submucosal layer, which gave the gastric lesion the appearance of a submucosal tumor. These characteristics thus need to be differentiated from those of a submucosal gastrointestinal stromal tumor (GIST). A diagnosis of gastric GIST was ruled out in our current case by negative c-Kit (CD117) staining.

In conclusion, this is the first reported case of a solitary Peutz-Jeghers type polyp that was associated with symptoms mimicking a gastric tumor with lymph node metastases. In addition, the association of an ectopic pancreas with solitary Peutz-Jeghers type polyp of stomach has also not been reported previously.

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## What is the optimal treatment for appendiceal mass formed after acute perforated appendicitis?

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### Abstract

We read with great interest the editorial article by Meshikhes AWN published in issue 25 of *World J Gastroenterol* 2011. The article described the advantages of emergency laparoscopic appendectomy compared with interval appendectomy as a new safe treatment modality for the appendiceal mass. The author concluded that the emergency laparoscopic appendectomy was a safe treatment modality for the appendiceal mass, and might prove to be more cost-effective than conservative treatment, with no need for interval appendectomy. However, we would like to highlight certain issues regarding the possibility of percutaneous catheter drainage to successfully treat the appendiceal mass, with no need for appendectomy, too.

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**Key words:** Appendiceal mass; Percutaneous drainage; Antibiotic therapy; Interval appendectomy; Laparoscopic appendectomy

### TO THE EDITOR

We commend Meshikhes AWN<sup>[1]</sup> for an interesting editorial article about the advantage of emergency laparoscopic appendectomy (LA) compared with interval appendectomy (IA) as a new and safe treatment modality for the appendiceal mass. The author concluded that the emergency LA was a safe treatment modality for the appendiceal mass, and might prove to be more cost-effective than conservative treatment, with no need for IA. LA is associated with a much shorter hospital stay and it obviates the need for long-term intravenous antibiotic treatment. If emergency LA becomes the standard of care, IA will certainly become 'something' of the past.

However, on the basis of our long-term experience<sup>[2]</sup>, we would like to highlight certain issues regarding the possibility of percutaneous catheter drainage (PCD) as the treatment modality which can successfully solve the appendiceal mass formed after acute perforated appendicitis, thereby obviating the need for an emergency appendectomy. LA is less aggressive than open surgery, but it is a much more aggressive procedure as compared with PCD performed under ultrasound or computerized

tomography. The latter can be performed without general anesthesia and with less tissue dramatization. We strongly believe that PCD followed by vigorous irrigation can achieve good results in the majority of patients with appendiceal mass.

A few years ago, we conducted a randomized controlled trial<sup>[2]</sup> in order to evaluate whether PCD could successfully and completely treat the appendiceal mass, thereby avoiding the need for appendectomy. The conclusion of our study was that PCD with antibiotics administration was a safe and effective treatment protocol for acute perforated appendicitis. The appendicitis recurrence rate was rather low and very often interval appendectomy was not required. For patients with appendiceal mass  $\geq 3$  cm in diameter, antibiotic treatment alone was insufficient and the recurrence rate was high.

Our hypothesis was based on the estimation that obstruction of proximal appendiceal lumen by fecaliths, foreign bodies, tumors, parasites, and lymphoid hyperplasia accounted for approximately 70% of the causes of appendicitis<sup>[2]</sup>. Obstruction of appendiceal lumen is followed by mucus secretion, bacterial overgrowth, increasing intraluminal pressure and wall tension, vascular congestion, gangrene, perforation, and disintegration of appendix distal to the site of obstruction. Our idea was derived from the fact that appendiceal lumen obliteration

with subsequent fibrosis of the rest of perforated appendix after drainage of necrotic content and pus so that appendicitis could be resolved without appendectomy<sup>[2]</sup>.

We agree that the advantage of emergency laparoscopic appendectomy lies in the detection of hidden pathologies. However, hidden pathology can be encountered occasionally and failure to detect it can be avoided by performing appropriate follow-up investigations to exclude its presence.

In summary, we believe that PCD is technically feasible in the majority of patients with appendiceal mass. Therefore, PCD with continuous drainage and followed by vigorous irrigation and proper administration of antibiotics should be considered the therapy of choice for some patients with appendiceal mass formed after acute perforated appendicitis.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
 San Antonio, TX 78249,  
 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
 2012 Joint International

Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

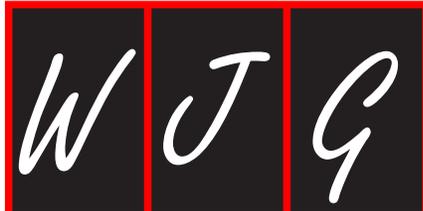
October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

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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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- 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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- 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 dis-

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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

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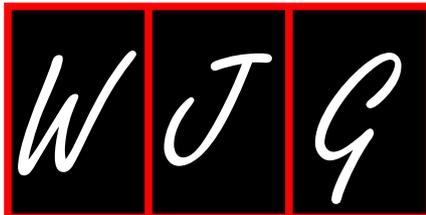
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## Gastrointestinal and hepatic complications of hematopoietic stem cell transplantation

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### Abstract

Recognition and management of gastrointestinal and hepatic complications of hematopoietic stem cell transplantation has gained increasing importance as indications and techniques of transplantation have expanded in the last few years. The transplant recipient is at risk for several complications including conditioning chemotherapy related toxicities, infections, bleeding, sinusoidal obstruction syndrome, acute and chronic graft-versus-host disease (GVHD) as well as other long-term problems. The severity and the incidence of many complications have improved in the past several years as the intensity of conditioning regimens has diminished and better supportive care and GVHD prevention strategies have been implemented. Transplant clinicians,

however, continue to be challenged with problems arising from human leukocyte antigen-mismatched and unrelated donor transplants, expanding transplant indications and age-limit. This review describes the most commonly seen transplant related complications, focusing on their pathogenesis, differential diagnosis and management.

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**Key words:** Stem cell transplantation; Graft-versus-host disease; Sinusoidal obstruction syndrome; Complications

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### INTRODUCTION

The indications of both autologous and allogeneic hematopoietic stem cell transplantation have expanded over the past decade including for malignant and nonmalignant disorders<sup>[1,2]</sup>. Transplant clinicians routinely encounter gastrointestinal and hepatic disorders and complications before, during and after the transplant. The outcome of transplant is often closely related to how well these complications are managed. This review provides a current overview of these disorders including pathogenesis, clinical diagnosis and management.

**Table 1** Emetogenic potential of chemotherapeutic agents used in stem cell transplantation

	Chemotherapeutic agent
High (90%) emetogenic risk	Carmustine > 250 mg/m <sup>2</sup> Cyclophosphamide > 1500 mg/m <sup>2</sup>
Moderate (30%-90%) emetogenic risk	Busulfan Cytarabine > 200 mg/m <sup>2</sup>
Low (10%-30%) emetogenic risk	Melphalan Etoposide
Minimal (< 10%) emetogenic risk	Fludarabine Rituximab

## EVALUATION OF TRANSPLANT CANDIDATE

Patients referred for hematopoietic stem cell transplantation undergo a detailed evaluation including history and physical examination which encompasses the history of disease requiring transplant as well as pre-existing conditions including dental problems, vaccination, travel, blood transfusion and infectious disease exposure history<sup>[3,4]</sup>. Imaging studies including computerized tomography (CT) with/without positron emission tomography scan or magnetic resonance imaging (MRI) can be performed to localize and restage the malignancy. Infectious disease markers including human immunodeficiency virus (HIV)-1 and (HIV)-2, human T-cell leukemia virus (HTLV)-1 and (HTLV)-2, hepatitis B virus (HBV) and hepatitis C virus serologies, cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV)-1 and (HSV)-2, varicella zoster virus (VZV) and rubella titers are checked within 30 d prior to transplant admission. In addition to routine blood count, liver function and coagulation studies are obtained. Donors for allogeneic transplant also undergo the same serologic testing within 30 d of stem cell collection. Donors with viral hepatitis B and hepatitis C pose the risk of disease transmission to the recipient up to 30% and 100% respectively<sup>[5,6]</sup>. The risk of fatal HBV infection in recipients who become HBsAg positive is about 12%<sup>[5]</sup>. By contrast, HCV transmission during transplant does not usually pose an increased short or mid-term clinical risk to the recipient yet does increase the long-term risk of cirrhosis<sup>[7]</sup>. Therefore donors should be treated with anti-viral agents; pegylated interferon  $\alpha$  (IFN- $\alpha$ ), famciclovir or lamivudine for hepatitis B and IFN- $\alpha$  plus ribavirin for hepatitis C before stem cell collection if time permits<sup>[8,9]</sup>. Of note, IFN- $\alpha$  should be stopped at least 1 wk before stem cell collection to avoid engraftment problems.

Patients with existing nausea, heartburn, dysphagia, abdominal pain, diarrhea, Crohn's disease or ulcerative colitis should be investigated with endoscopy prior to transplant to rule out mucosal ulcers and infections as the risk of bleeding is increased during the periods of thrombocytopenia.

Abnormal liver enzymes and organomegaly should be investigated with ultrasound, CT or MRI. Liver biopsy is

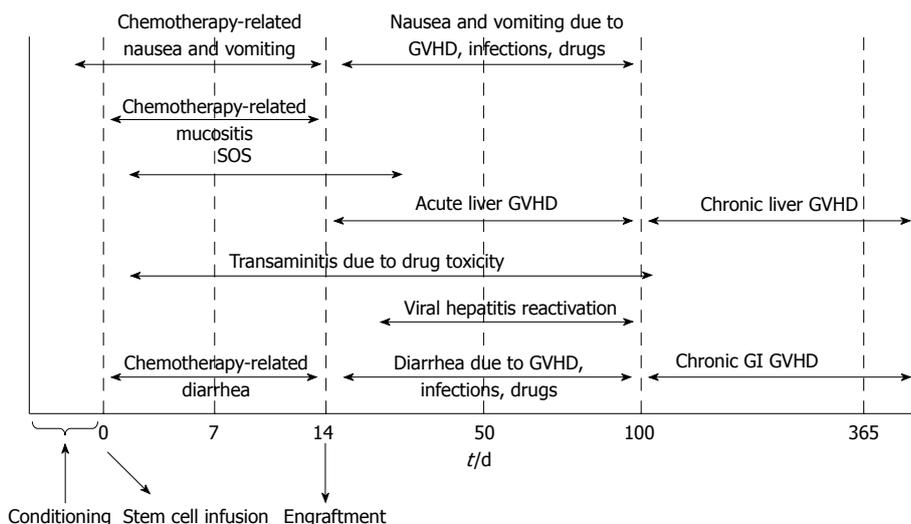
indicated in patients with positive hepatitis B surface antigen or hepatitis C antibody to rule out hepatic fibrosis or cirrhosis which increases the risk of fatal sinusoidal obstruction syndrome (SOS)<sup>[10]</sup>. Evidence of advanced liver fibrosis is a contraindication to proceed to stem cell transplantation because of excess transplant-related mortality. Pre-existing liver dysfunction can be secondary to viral hepatitis as well as alcoholic or non-alcoholic steatohepatitis, iron overload, fungal infection, chemotherapy-induced cholestatic injury or hepatocyte damage, fibrosis, extramedullary hematopoiesis or prior liver irradiation<sup>[11]</sup>. Myeloablative conditioning regimens, recent exposure to alkylating agents (especially cyclophosphamide) and exposure to newer drugs, such as imatinib and gemtuzumab ozogamicin, are known associations with increased risk of SOS<sup>[12-14]</sup>.

Hepatitis B infected transplant candidates are at risk for hepatitis flare and fulminant hepatitis can occur in up to 50% of transplant recipients in the absence of antiviral prophylaxis<sup>[15,16]</sup>. In the presence of isolated HBV core antibody, observation or prophylaxis are both acceptable approaches. If HBV surface antigen is detected, it is usually recommended that prophylaxis with oral nucleoside therapy is initiated prior to transplant and that HBV DNA levels are monitored frequently during the post-transplant period<sup>[17]</sup>. Even in the absence of cirrhosis, HCV infected patients have an increased risk of SOS especially if pre-transplant aspartate aminotransferase is elevated<sup>[18]</sup>. There is no effective prophylaxis or treatment of hepatitis C for transplant recipients as pegylated IFN- $\alpha$  is contraindicated due to myelosuppression and has the potential to exacerbate graft-versus-host disease (GVHD). Ribavirin alone can be tried while patient is on immunosuppressive therapy.

Patients with conditions causing transfusion dependency such as myelodysplastic syndrome, leukemia, lymphoma and aplastic anemia should be screened for iron overload as excess iron can impair Kupffer cell function and increase the risk for mold infections<sup>[19]</sup>. The excess iron can be demonstrated either by quantification of iron in liver biopsy tissue or MRI of the liver. Patients with severe iron overload can benefit from chelation pre-transplant. However the urgent need for the transplant may preclude this option. The relationship between iron overload and transplant-related toxicity has not been well established and in most cases chelation can be postponed after the transplant.

## NAUSEA AND VOMITING

Many chemotherapeutic agents, with or without total body irradiation used in the conditioning regimens, have significant emetogenic potential (Table 1). The pathogenesis includes stimulation of the chemotherapy trigger zone in the brainstem which activates the vomiting center by increasing efferent output to target organs in the gastrointestinal tract, resulting in subsequent emesis. Chemotherapy also causes cell damage in the gastroin-



**Figure 1** Timeline summary of hepatic and gastrointestinal complications of stem cell transplantation. GVHD: Graft-versus-host disease; SOS: Sinusoidal obstruction syndrome; GI: Gastrointestinal.

testinal (GI) tract, resulting in the release of neuroactive agents and vagal stimulation, increasing afferent input to the chemotherapy trigger zone and the vomiting center in the brainstem.

Patients usually experience the chemotherapy related side effects during the early post-transplant (15 d) period before engraftment (Figure 1). Nausea and vomiting in later phases may be due to other potential etiologies including upper GI acute GVHD and infections such as HSV, VZV, CMV, adenovirus, fungus and *Helicobacter pylori*. Persistent or recurrent nausea not responsive to routine anti-emetic regimens should be investigated further for GVHD with upper GI endoscopy which may show mucosal edema and erythema and biopsy findings consistent with local lymphocytic infiltrates and epithelial apoptosis<sup>[20,21]</sup>. Specimens should also be studied for bacterial or fungal cultures, HSV and CMV infections as viral infections can be detected in the GI tract without the presence of virus DNA in serum. Other common medical etiologies such as medication intolerance, gastroparesis, intestinal obstruction, intraabdominal infections, neurologic and metabolic causes should also be considered.

Prevention is the key to success in managing nausea and vomiting during the peri-transplant period<sup>[22]</sup>. Acute emesis prevention (up to 24 h after chemotherapy) can be achieved with a combination of corticosteroids (dexamethasone 10-20 mg iv/po daily or methylprednisolone 40-125 mg iv/po daily) and 5-hydroxytryptamine-3 receptor antagonists (ondansetron 16-24 mg iv/po daily or granisetron 1-2 mg daily). Delayed emesis (up to 5 d after treatment) can usually be prevented with corticosteroids. Aprepitant neurokinin-1 antagonist is an effective agent for this purpose; however it may interact with several post-transplant immunosuppressive agents and therefore is sparingly and cautiously used, especially in the allogeneic stem cell transplant setting.

Treatment options for breakthrough nausea and vomit-

ing include phenothiazines (prochlorperazine, promethazine), metoclopramide, lorazepam, haloperidol, dronabinol and corticosteroids<sup>[23]</sup>.

## OROPHARYNGEAL MUCOSITIS AND DYSPHAGIA

Breakdown of mucosal barrier presenting as mucositis is a common complication during the early post-transplant period. It affects up to 80% of transplant recipients, especially with radiation-based myeloablative regimens<sup>[24]</sup>. Chemotherapeutic agents commonly causing mucositis include busulfan, etoposide, melphalan and methotrexate. Pre-existing periodontal disease and prior radiation to the head and neck area increase the risk of post-transplant complications. Mucositis can result in significant oral pain and dysphagia, decreased oral caloric intake as well as bleeding, infection, upper airway edema and obstruction. Clinically apparent mucositis usually starts 5-10 d after initiation of the conditioning regimen (Figure 1). Initial erythema and atrophy is followed by ulceration and healing phases. It may take up to two weeks for healing of chemotherapy-induced mucositis.

Infectious causes of mucositis include CMV, HSV, VZV, varicella zoster virus, *Candida* species and bacterial pathogens. The incidence of viral and fungal infections has been significantly lower since the standardization of antiviral and antifungal prophylaxis regimens. Other noninfectious causes of dysphagia should be included in the differential diagnosis such as acid-reflux disease, pill esophagitis and acute and chronic GVHD with esophageal strictures.

Prevention and early treatment is critical to minimize the duration and severity of symptoms. Frequent mouth rinsing with topical agents and oral cryotherapy with ice chips are started with chemotherapy and continued until

Table 2 Differential diagnosis of post-transplant diarrhea	
<b>Conditioning regimen-related</b>	
Acute GVHD	
Drug toxicity	
Antibiotic-related	
Opioid withdrawal	
Mycophenolate mofetil toxicity	
Tacrolimus (thrombotic microangiopathy)	
Proton pump inhibitors	
Promotility agents	
Magnesium salts	
Metoclopramide	
Infectious	
Clostridium difficile	
CMV	
Rotavirus	
Adenovirus	
EBV	
HSV	
Astrovirus	
Norovirus	
Bacterial infections including ESBL	
Fungal infections	
Parasitic infections ( <i>Cryptosporidium</i> , <i>Microsporidia</i> , <i>Giardia</i> )	
Mycobacterial infections	
Others	
Lactose intolerance	
Malabsorption	
Pancreatic insufficiency	

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HSV: Herpes simplex virus; GVHD: Graft-versus-host disease; ESBL: Extended spectrum β lactamase.

engraftment. Keratinocyte growth factor (palifermin) has been shown to decrease the incidence of mucositis by 40% in patients receiving autologous stem cell transplant with aggressive total body irradiation (TBI)-based regimens<sup>[25,26]</sup>. It is administered iv for 3 d before and after cytotoxic therapy. Other supportive measures include saline and bicarbonate rinses, mucosal coating agents (such as aluminum hydroxide), topical anesthetics such as lidocaine rinse and/or narcotic analgesia, topical nystatin for signs of candidiasis and proton-pump inhibitor prophylaxis. Total parenteral nutrition should be considered for patients who are unable to tolerate oral supplementation for more than 7 d.

## DIARRHEA

Diarrhea occurs in almost half of patients receiving high-dose chemotherapy conditioning and radiotherapy. It is most commonly associated with toxicity of conditioning regimens within the first 2 wk after transplant (Figure 1). Alkylating agents, busulfan and combination regimens are frequent etiologies and cause diarrhea due to mucosal inflammation. Several other etiologies should be considered in patients having diarrhea in the post-transplant period (Table 2). Acute GVHD is the most common reason for diarrhea after engraftment (> 15 d) in allogeneic transplants<sup>[27]</sup>; persistent or new diarrhea beyond 3 wk of transplant should be investigated for GVHD. The diarrhea

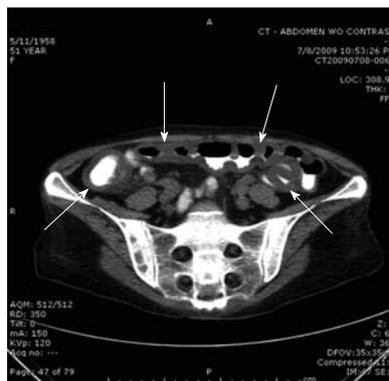


Figure 2 Bowel wall edema in a patient with gastrointestinal graft-versus-host disease.

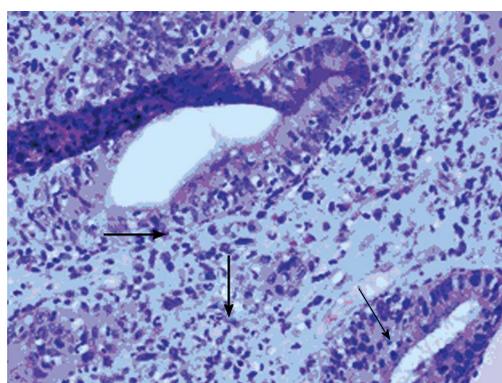


Figure 3 Histologic findings of acute graft-versus-host disease of the colon (hematoxylin and eosin stain, x 400). Thin arrow marks apoptotic bodies; thick arrow marks pericryptal acute inflammation.

with GVHD can be watery, mucoid and in large volumes; can be accompanied by vomiting, gastrointestinal bleeding and severe abdominal pain<sup>[28]</sup>. Infectious etiologies account for only 10%-15% of cases, yet diarrhea at any time after transplant should still prompt obtaining stool studies for *Clostridium difficile* toxin<sup>[29,30]</sup> as well as bacterial, viral and parasitic cultures if indicated. Abdominal imaging with CT may show bowel wall edema and/or pneumatosis intestinalis which may be associated with either GVHD or CMV infection (Figure 2). If cultures are negative, patients are usually treated with loperamide 4 mg po once followed by 2 mg/24 h as needed up to 24 mg/24 h. If diarrhea persists, strategies include scheduling loperamide every 4-6 h, adding atropine and diphenoxylate or tincture of opium. Octreotide starting at 150 mg iv every 8 h can be considered for protracted cases and can be titrated to response<sup>[31]</sup>. Other critical measures include maintaining adequate hydration and electrolyte supplementation, treating infections, discontinuation of medications causing diarrhea and assessment of nutritional status. Persistent symptoms despite the above measures and/or new diarrhea presenting after engraftment should be investigated with endoscopy and biopsy.

Visual findings of acute GVHD may include mucosal

**Table 3** Staging of acute graft-versus-host disease (modified Keystone criteria)

Stage	Intestinal tract	Liver	Skin
0	Diarrhea $\leq$ 500 mL/d	Bilirubin < 2.0 mg/dL	No rash
1	Diarrhea 501-1000 mL/d or nausea ( $\pm$ vomiting)	Bilirubin 2.0-3.0 mg/dL	Maculopapular rash < 25% of body surface
2	Diarrhea 1001-1500 mL/d	Bilirubin 3.1-6.0 mg/dL	Maculopapular rash 25%-50% of body surface
3	Diarrhea > 1501 mL/d	Bilirubin 6.1-15 mg/dL	Generalized erythroderma
4	Severe abdominal pain +/- ileus	Bilirubin > 15 mg/dL	Generalized erythroderma with blister/bullous formation and desquamation

**Table 4** Grading of acute graft-versus-host disease (modified Keystone criteria)

Grade	Gut	Liver	Skin
0 (none)	0	0	0
I (mild)	0	0	1-2
II (moderate)	1	1 or	3 or
III (severe)	2-4	2-3 or	0-3
IV (life-threatening)		4	4 or

edema/erythema and ulceration/bleeding. The diagnostic yield is the best when biopsies are obtained either from the stomach and distal colon or from the colon and ileum<sup>[32]</sup>. Histologic findings include crypt epithelial cell apoptosis and dropout, crypt destruction (Figure 3), pericapillary hemorrhage and variable lymphocytic and eosinophilic infiltration of the epithelium and lamina propria<sup>[33]</sup>. It is also important to study the biopsy specimens for CMV involvement which is the only common infectious cause of enteritis after transplant that requires biopsy for diagnosis. The negative predictive value of other infectious studies in stools is high and therefore usually does not necessitate endoscopy.

The severity of acute GVHD is clinically determined by the amount of diarrhea which helps defining the staging and grading (Tables 3 and 4). In addition to the general management measures described above, patients with high clinical suspicion for  $\geq$  grade II acute GVHD or biopsy proven acute GVHD should be promptly started on high dose steroids<sup>[34]</sup>; methylprednisolone 0.5-2.0 mg/kg per day iv in addition to their existing GVHD prophylaxis regimens. Half of the patients respond to steroid treatment<sup>[35]</sup> which can be tapered starting after approximately 1 wk. Patients who do not respond to steroids tend to have a poor prognosis; several second line immunosuppressive agents have been tried with variable success<sup>[34]</sup>. Other supportive measures consist of addition of oral non-absorbable steroids (budesonide)<sup>[36]</sup> and cholestyramine as well as dietary adjustments (bowel rest and start-

**Table 5** Differential diagnosis for liver function abnormalities after hematopoietic stem cell transplantation

First 3 wk post-transplant
Drug toxicity
Conditioning regimens (cyclophosphamide, total body irradiation, bis-chloroethylnitrosourea, busulfan)
Calcineurin inhibitors
Azole antifungals
SOS
Sepsis, candidiasis
Ischemic liver disease
From 3 wk to 3 mo post-transplant
Acute GVHD
Drug toxicity
SOS
Hepatitis (fulminant, acute or chronic):
Viral (HBV, HCV, HSV, VZV, adenovirus) reactivation
Bacterial or fungal Infection
Fungal abscess
Gall bladder disease/cholecystitis
Hyperalimentation
Post-transplant lymphoproliferative disorder (EBV-related)
After 3 mo post-transplant
Chronic GVHD
Iron overload
Chronic viral hepatitis
Drug toxicity
Liver fibrosis or cirrhosis:
SOS
Viral infections
Hemosiderosis
Disease recurrence or new malignancy including hepatocellular carcinoma, lymphoproliferative disorder
Nodular regenerative hyperplasia
Gallbladder disease

GVHD: Graft-versus-host disease; SOS: Sinusoidal obstruction syndrome; EBV: Epstein-Barr virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

ing total parenteral nutrition) particularly for moderate to severe diarrhea.

## GASTROINTESTINAL BLEEDING

The incidence of bleeding has been significantly reduced (1%-2%) as post-transplant care has improved with routine anti-viral, anti-fungal and GVHD prophylaxis, yet it remains one of the major causes of transplant related mortality, particularly among patients with GVHD<sup>[37,38]</sup>. Common infectious etiologies include CMV, VZV, adenovirus, fungal and clostridial infections. Mucosal necrosis from conditioning therapy, acute and chronic GVHD, peptic ulcer disease, mycophenolate-related ulcerations and gastric antral vascular ectasia (GAVE) are the common known etiologies of noninfectious causes of bleeding. Treatment is mainly focused on supportive care (platelet transfusion and continuous octreotide infusion) as well as implementing prophylaxis for viral infections and GVHD. Treating the underlying cause is important as the benefit of endoscopic methods alone is limited to focal lesions.

**Table 6 Risk factors for sinusoidal obstruction syndrome**

Existing liver disease:
Chronic viral hepatitis
Alcohol related hepatitis
Steatohepatitis
Cirrhosis, lobular fibrosis
Cholestatic disorders
Extramedullary hematopoiesis with sinusoidal fibrosis
Prior history of:
SOS
Extensive chemotherapy and stem cell transplantation
Hepatic radiation
Drugs
Recent gemtuzumab ozogamicin use
Conditioning agents:
High dose TBI (> 14 Gy)
Cyclophosphamide metabolite: acrolein
Busulfan
Melphalan
Concomitant use of sirolimus during conditioning

SOS: Sinusoidal obstruction syndrome; TBI: Total body irradiation.

## LIVER FUNCTION ABNORMALITIES AND JAUNDICE

Severe liver dysfunction after transplant (total serum bilirubin > 4 mg/dL) may be an indicator of poor outcome as there is often no curative treatment<sup>[39]</sup>. Therefore, efforts are usually geared towards identifying the transplant candidates at risk as well as routine implementation of prophylactic measures such as ursodiol<sup>[40]</sup> (through 80 d post transplant), viral and fungal prophylaxis and careful selection of conditioning regimens to minimize hepatotoxicity<sup>[41]</sup>. The incidence of liver-related complications has declined significantly over the past decade with the preventive measures integrated into standard care. Some common etiologies for liver function abnormalities after transplant are summarized in Table 5. Drug toxicity, sepsis, GVHD and SOS are the most common etiologies for liver dysfunction. Calcineurin inhibitors (cyclosporine and less commonly tacrolimus, sirolimus), azole antifungal agents, trimethoprim-sulfamethoxazole, ribavirin, busulfan and bis-chloroethylnitrosourea are commonly associated with cholestasis. A declining incidence of acute GVHD is observed in 20%-25% of allogeneic transplant recipients due to widespread use of prophylactic immunosuppressive drugs, with peak incidence after engraftment (day 15) until the first 100 d of transplant<sup>[42]</sup>. It typically follows skin and/or GI GVHD and manifests by progressive parallel elevations of serum bilirubin and alkaline phosphatase; serum aminotransferase enzymes are elevated up to 10 times the upper limit of normal (Tables 3 and 4). Hepatic-variant of GVHD has also been described where serum aminotransferase enzymes are elevated more than 10 times the upper limit of normal and clinical presentation resembles acute viral hepatitis<sup>[43]</sup>. The diagnosis is usually made by transjugular liver biopsy which typically reveals lymphocytic infiltration of small bile ducts with epithelial cell apoptosis<sup>[44]</sup>. Routine ursodiol prophylaxis is

usually continued through day 80 of allogeneic transplants and reduces the incidence of GVHD<sup>[45]</sup>. The initial treatment of acute hepatic GVHD is similar to cutaneous and GI GVHD with high dose steroids as described above. However, only 30%-50% of patients respond to initial treatment and half of patients develop chronic GVHD.

## SINUSOIDAL OBSTRUCTION SYNDROME

SOS (AKA veno-occlusive disease or VOD) is a clinical entity characterized by tender hepatomegaly, elevated serum bilirubin levels and weight gain which typically complicates myeloablative hematopoietic stem cell transplantation (HSCT) and was first described in 1979<sup>[46]</sup>. SOS is a well-recognized conditioning-related toxicity. The incidence is quite variable, ranging from less than 5% to as high as 70% in different reports<sup>[47,48]</sup>.

Endothelial injury appears to be the initiating event triggering the hepatic changes and clinical manifestation of SOS. The prevailing hypothesis centers on damage to the hepatic venular and sinusoidal endothelium as an initial trigger inducing a hypercoagulable state by activation of the coagulation cascade, favoring clot formation over natural anticoagulation<sup>[49,50]</sup>. As a result, the venular and sinusoidal lumen is reduced due to an edematous concentric subendothelial zone containing fragmented red cells and fibrillar material, inducing partial to complete fibrotic obliteration of the venular lamina.

There are several risk factors for SOS and patients may present with elevated transaminase levels prior to the conditioning regimen due to various pre-existing conditions (Table 6). Previous cumulative exposure to high doses of cytotoxic agents may contribute to these risks including a second HSCT. Infection with hepatitis B is not considered a risk factor alone for SOS unless it is complicated with cirrhosis. The relationship between HCV infection and SOS is somewhat controversial. One report supports the increased risk even in the absence of cirrhosis<sup>[18]</sup> while another cohort did not confirm the association although there was an increased long term risk of non-relapse mortality for transplant patients who had chronic hepatitis C<sup>[51]</sup>. Other risk factors include certain conditioning regimens such as cyclophosphamide, busulfan, and/or total body irradiation<sup>[52-54]</sup>.

Cyclophosphamide is a common conditioning agent with the highest incidence of SOS, which becomes a particular concern in regimens combined with TBI and busulfan. The hepatotoxicity is usually dependent on the toxic metabolite acrolein and the exposure to toxic metabolites can be minimized by metabolism-based dosing. The incidence of SOS seems to be higher in transplant recipients who receive TBI doses over 14 Gy<sup>[53]</sup>. Various fractionated schedules of TBI have been associated with decreased incidence. Increasing the interval between TBI and cytotoxic therapy also may decrease the risk. Busulfan exposure, on the other hand, is not proven to be directly related to SOS although it potentiates the toxicity of cyclophosphamide especially when it is administered

after this drug<sup>[54]</sup>. Oral busulfan has a variable and unpredictable absorption and studies have shown that the risk of SOS increases when the area under the curve for busulfan is greater than 1500  $\mu\text{mol}/\text{min}$ . When busulfan is adjusted to normal drug levels by close monitoring, a decreased incidence has been reported<sup>[54]</sup>.

Gemtuzumab ozogamicin may induce sinusoidal injury<sup>[55]</sup> (15%-40% risk) especially if it is administered preceding a cyclophosphamide-based conditioning regimen.

Most cases are observed within the first 3 wk after transplantation. Usually, an unexplained weight gain is the first symptom. This weight gain, attributable to water and sodium retention by the kidney, appears within 6 d to 8 d following the transplant in 95% of patients. This is often followed by varying degrees of hyperbilirubinemia and elevation in aspartate aminotransferase and alkaline phosphatase levels. Most patients develop ascites and pain in the upper right quadrant, and clinical examination usually reveals a firm and painful hepatomegaly. Platelet refractoriness is a common occurrence<sup>[56]</sup>. Renal insufficiency in the form of hepatorenal syndrome is also present in 50% of patients developing SOS (mainly patients with severe form) and 25% of them will require hemodialysis. Finally, patients with advanced disease can display severe encephalopathy and/or multiorgan failure.

Doppler ultrasound of the liver usually shows reversal of portal and/or hepatic venous flow in severe cases. Most patients are diagnosed on clinical basis, given the risk of liver biopsy in the setting of coagulopathy and platelet refractoriness. Nevertheless where feasible, the clinical suspicion should be confirmed by transjugular liver biopsy which is the gold standard for diagnosis. Hepatic venous pressure gradients are often measured at the time of biopsy; a gradient greater than 10 mmHg is highly specific for SOS<sup>[57]</sup> and correlates with worse prognosis.

Given the very high mortality rate in patients with severe SOS, it is critical to implement preventive measures such as ursodeoxycholic acid which has been shown to reduce the incidence of SOS<sup>[40]</sup>. The efficacy of low-dose heparin has not been confirmed and is usually not part of standard management.

Up to 70% of patients with SOS will recover spontaneously and the focus of treatment is supportive care such as maintaining intravascular volume and renal perfusion without causing fluid overload by optimizing sodium restriction and diuretics and transfusions to keep hematocrit levels higher than 40%. The role of albumin or other colloids is unclear but could be considered in patients with severe hypoalbuminemia and large third space fluid accumulations. Low-dose dopamine has been used in patients with renal insufficiency because the mechanism of renal dysfunction appears to be hepatorenal in origin. Avoidance of other hepatotoxic drugs is important in these patients, and infections should be identified and treated promptly. Therapeutic paracentesis can help relieve symptoms in patients with large, tense ascites and may help improve renal function. Also, use of hemodialysis or continuous venous hemofiltration is reported to

help with fluid overload in patients with a poor response to diuretics.

There are no effective established treatments for patients with severe SOS characterized by rapidly increasing serum bilirubin and transaminase levels, portal vein thrombosis and multiorgan failure. Several antithrombotic agents have been tested with mixed results. Defibrotide which is an antithrombotic agent without significant systemic effects and with a manageable side effect profile, has been reported to improve signs and symptoms of SOS in 42% of patients<sup>[58]</sup>. Its mechanism of action is poorly understood. Other tested agents include prostaglandin E1 and tissue plasminogen activator with or without concurrent heparin, intravenous N-acetylcysteine, human antithrombin III concentrate, activated protein C and prednisone. None of these approaches are considered a part of standard management.

## LONG-TERM COMPLICATIONS

### *Chronic GVHD*

Long-term survivors of stem cell transplantation are at increased risk of several serious complications related to chronic GVHD which may affect liver, GI tract, skin, mucosal surfaces, lungs, joints, eyes and bone marrow; these patients should be followed regularly. Complications may occur in up to 50% of transplant recipients. Liver GVHD usually manifests with progressive or sudden elevation of alkaline phosphatase and gamma glutamyl transpeptidase. Hyperbilirubinemia is usually a late manifestation that coincides with development of cirrhosis and findings of small bile destruction in biopsy<sup>[44]</sup>. Viral etiologies should be excluded and liver biopsy should be performed to establish the diagnosis, followed by initiation of immunosuppressive therapy which usually includes steroids with or without a calcineurin inhibitor. An improvement in liver function studies is usually observed within four weeks of treatment and 50%-80% of patients respond to the initial therapy with improvement in histopathologic findings. Addition of ursodeoxycholic acid should also be considered and this is usually well tolerated. The prognosis of patients who do not respond to immunosuppressive regimens is poor and correlates with shortened survival<sup>[59]</sup>.

Chronic GVHD may affect several parts of the GI tract, causing esophageal webs and strictures leading to dysphagia, failure to thrive and chronic aspiration. Early lesions can be reversible with immunosuppression, proton pump inhibitors and dilatation. Patients may also experience chronic intermittent diarrhea, clinically and histologically similar to acute GVHD, which may respond to non-absorbable steroids. Chronic malabsorption is rare and is usually a result of long term inadequate treatment.

### *Chronic viral hepatitis and cirrhosis*

Longstanding viral hepatitis C and B can lead to end-stage liver disease in transplant survivors. Rate of progression to cirrhosis in patients with chronic hepatitis

B is comparable to non-transplant patients whereas the incidence of cirrhosis in transplant patients with chronic hepatitis C infection seems to be higher than controls and it can be as high as 24% after 20 years<sup>[60]</sup>. Otherwise these patients are also at risk of developing hepatocellular carcinoma (HCC) (2%-8% per year) and lymphoproliferative disorders. Patients with chronic hepatitis C should therefore be routinely monitored for viral load and considered for combination therapy with ribavirin and pegylated IFN- $\alpha$ . They should also be screened for HCC with  $\alpha$ -feto-protein and ultrasound every 6 mo. Close monitoring is essential for treatment complications such as neutropenia and thrombocytopenia secondary to pegylated IFN- $\alpha$  or exacerbation of coexisting chronic GVHD. Screening and treatment of iron overload may augment the success of anti-viral therapy. Liver transplantation for end-stage liver disease or HCC can be considered, especially from the original stem cell donor<sup>[61]</sup>.

Patients with chronic hepatitis B may have atypical serologic course due to immunosuppression. They may benefit from clearance of antigenemia particularly if the donor has natural HBV immunity. Patients should be monitored for HBV DNA levels and alanine transaminase levels and considered for antiviral treatment at times of tapering of immunosuppressive therapy as well as initiation of new chemotherapy, as they are at risk of flares of hepatitis<sup>[62]</sup>.

### Iron overload

The etiology of iron overload in transplant survivors is usually multifactorial, including transfusion dependency and abnormal iron transport by the intestine due to bone marrow dysfunction. It can be an important contributor to chronic liver disease and should be considered in the differential diagnosis<sup>[63]</sup>. It affects cardiac, endocrine and pancreatic function as well as increasing the risk of opportunistic infections. HFE gene testing should be considered when patients have unexpectedly high levels of iron stores. Clinically significant iron overload usually occurs when serum ferritin exceeds 1000  $\mu\text{g}/\text{dL}$ <sup>[64]</sup>. In the presence of other inflammatory conditions such as chronic GVHD, serum ferritin levels may be falsely elevated. Liver biopsy or noninvasive methods such as liver MRI or FerriScan can be utilized to document the severity of iron overload. Patients with severe iron overload may benefit from mobilization with improved hepatic and cardiac function<sup>[65]</sup>. If liver iron content is greater than 15 000  $\mu\text{g}/\text{g}$  dry weight, both phlebotomy and chelation should be offered. The liver iron content of 7000-15 000  $\mu\text{g}/\text{g}$  dry weight should be treated with phlebotomy only and if it is less than 7000  $\mu\text{g}/\text{g}$  dry weight, treatment is needed only if there is liver disease<sup>[66]</sup>.

### Acute hepatocellular injury

Long term transplant survivors may present with acute elevations of transaminases. The differential diagnosis should include flares of chronic viral hepatitis, hepatitis presentation of chronic GVHD, VZV, HSV infection or drug-induced (antihypertensives, statins, hypoglycemic

agents, antibiotics) liver injury.

### Malignancies

The risk of new malignancies among transplant survivors increases significantly after 10 years. Patients with chronic hepatitis C have accelerated incidence of HCC<sup>[67]</sup> and lymphomas<sup>[68]</sup>.

## CONCLUSION

Gastrointestinal and hepatic complications count for a significant part of the morbidity during and after hematopoietic stem cell transplant. Recent advances in transplant approaches have changed the outcome and the post-transplant course of many patients<sup>[69]</sup>. As most transplant survivors are affected by multiple complications, it is imperative that they should receive long-term and systematic follow-up without compromising from individualized care.

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## Surgical treatment of ulcerative colitis in the biologic therapy era

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### Abstract

Recently introduced in the treatment algorithms and guidelines for the treatment of ulcerative colitis, biological therapy is an effective treatment option for patients with an acute severe flare not responsive to conventional treatments and for patients with steroid dependent disease. The reduction in hospitalization and surgical intervention for patients affected by ulcerative colitis after the introduction of biologic treatment remains to be proven. Furthermore, these agents seem to be associated with increase in cost of treatment and risk for serious postoperative complications. Restorative proctocolectomy with ileal pouch-anal anastomosis is the surgical treatment of choice in ulcerative colitis patients. Surgery is traditionally recommended as salvage therapy when medical management fails, and, despite advances in medical therapy, colectomy rates

remain unchanged between 20% and 30%. To overcome the reported increase in postoperative complications in patients on biologic therapies, several surgical strategies have been developed to maintain long-term pouch failure rate around 10%, as previously reported. Surgical staging along with the development of minimally invasive surgery are among the most promising advances in this field.

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**Key words:** Ulcerative colitis; Inflammatory bowel disease; Infliximab; Surgery; Laparoscopy; Single incision laparoscopy; Total abdominal colectomy; Ileal pouch anal anastomosis; Restorative proctocolectomy

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### INTRODUCTION

Ulcerative colitis (UC) is a mucosal inflammatory process affecting the rectum and the colon. It is characterized by contiguous inflammation starting in the rectum and progressing for variable distance proximally<sup>[1]</sup>. Intermittent exacerbations are typical, with symptoms characterized by bloody diarrhea associated with urgency and tenesmus<sup>[2]</sup>. The activity of disease can range from complete remission to fulminant symptoms along with systemic toxic effects<sup>[3]</sup>.

Although the exact pathogenesis of UC remains poorly

understood, the most credited model states that the intestinal flora triggers and drives an aberrant intestinal immune response and subsequent inflammation in a genetically susceptible host<sup>[4]</sup>. Medical therapy aims at the control of symptoms and the resolution of the underlying inflammatory process, classically by a variety of agents in combination, such as 5-aminosalicylates, corticosteroids, and immunosuppressants, including purine antimetabolites and cyclosporine<sup>[5]</sup>. Treatment schemes are based on disease severity, (defined as mild, moderate or severe based on clinical and laboratory parameters) and on the extent of the disease (pancolitis, left-sided colitis, rectosigmoiditis or proctitis)<sup>[6]</sup>. However, about a quarter of patients with UC end up needing a colectomy because of failure of medical therapy, onset of unacceptable side effects of chronic therapy, occurrence of acute complication of UC (fulminant colitis, severe bleeding, toxic megacolon, perforation), or development of malignancy<sup>[7]</sup>.

For all of these patients, the removal of the colon and rectum represents a definitive cure for their disease, with cessation of symptoms, withdrawal of morbid medical therapy, and avoidance of the risk of developing a malignancy associated with the persistence of inflammation<sup>[8]</sup>.

However, surgery is not without risks and can significantly affect patients' lifestyle, therefore, is traditionally deemed as a salvage treatment when medical therapy is ineffective<sup>[1]</sup>.

During the last three decades astounding progress has been accomplished both in medical and surgical treatments, which might lead to substantial changes in the traditional principles for the management of UC patients. Medical therapy of UC has recently entered the era of biologic treatments with the approval by Food and Drug Administration (FDA) in 2005 of Infliximab, a monoclonal antibody directed against tumor necrosis factor- $\alpha$ . The initial enthusiasm raised by the promise to reduce the colectomy rate in acute presentations, was subsequently partially dampened by conflicting reports regarding Infliximab's safety and impact on the need for surgery in urgent/emergent setting<sup>[9-13]</sup>.

As the number of available medications increases, more and more often patients are referred for surgery severely malnourished, immunocompromised, and experiencing the side effects of corticosteroids, immunomodulators, and biological agents. Whether they are referred for colectomy in an acute or chronic setting, these patients represent a unique challenge for colorectal surgeons, given the compromised general conditions and poor nutritional status in the former, and the side effects of long term corticosteroid use in the latter<sup>[14-16]</sup>.

Together with the advances in medical therapy, the surgical treatment and techniques in UC has evolved as well. Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is today considered the gold standard and, in experienced hands, can now be performed safely for UC with a low postoperative complication rate and a long-term pouch failure rate reported less than 10%<sup>[17-19]</sup>. Moreover, the introduction of minimally invasive tech-

niques might further decrease postoperative morbidity and improve patients' satisfaction, with reduced impact on body image and better cosmesis<sup>[20-22]</sup>.

The purpose of this report is to discuss the recent advances in medical and surgical treatment of UC patients addressing surgical concerns in the era of biologic therapy.

## BIOLOGIC THERAPY IN UC: THE GASTROENTEROLOGIST'S VIEW

The primary goals of medical therapy in the treatment of UC are to induce remission of symptoms and maintain it on a long-term basis: by reducing the number of relapses, which occurs in 67% of patients and, at least, once over a 10-year period<sup>[23]</sup>, medical therapy lowers the risk of long-term complications and improves patients' quality of life.

The majority of UC patients present with moderate-to severe disease (80%) rather than mild disease (20%)<sup>[24]</sup> and, during their illness, nearly 20% of patients afflicted with UC will experience a severe acute episode that requires hospitalization<sup>[25]</sup>.

Despite the progress accomplished in medical therapy, which broadened the horizon of possible treatments after failure of corticosteroids<sup>[26,27]</sup>, the need for surgery in this patient population seems to be unchanged or slightly decreased over time. Reported colectomy rates are steadily ranging between 20% and 30% in most of the epidemiological studies with additional risk for needing a resection as the extent and severity of the disease increase<sup>[8,28-31]</sup>. Beside a 10% who have surgery for cancer or pre-neoplastic degeneration, the vast majority of patients need an operation for acute colitis with severe complications not responsive to medical therapy<sup>[32,33]</sup>. The advantage of prolonged medical therapy *vs* surgery in patients with acute severe colitis failing initial high dose corticosteroids is still debated. About one third of these patients undergo a colectomy within one year, most likely in an emergency setting, and even if second-line medical therapy may reduce the need for immediate colectomy, most of them will require colectomy by 10 years<sup>[32,34]</sup>. In this setting, early subtotal colectomy and ileostomy combined with a late reconstructive surgery remains a safe alternative<sup>[19]</sup> since second-line medical therapy carries with it a not negligible mortality risk<sup>[35]</sup>.

Additionally, about 20% of patients with UC have a persistent active disease often requiring several courses of systemic steroids, but followed by relapse of symptoms during steroid tapering or soon after their discontinuation, a condition known as steroid-dependency. Steroid dependency is associated with serious complications, which, for a significant proportion of patients, become an indication for surgery<sup>[36]</sup>.

Although surgery is curative of the underlying inflammation and restorative proctocolectomy with IPAA preserves the normal anatomic route for defecation, the procedure may lead to new symptoms, such as diarrhea,

incontinence, nocturnal leakage, and in some patients does not obviate the need for medication. In several surgical series that follow patients a minimum of 5 years, up to 60% of patients are still having more than 8 bowel movements daily, with 55% of patients experiencing incontinence, and 50% nocturnal leakage<sup>[37-39]</sup>. Even if surgical techniques have dramatically evolved, surgery is still associated with significant early and late postoperative complications, e.g. anastomotic leak, pelvic sepsis, small bowel obstruction, pouchitis, sexual dysfunction, reduced fecundity in women and pouch failure<sup>[40,41]</sup>. Repeated surgery is sometimes necessary. A population-based study reported that approximately 20% of patients who had undergone IPAA required at least one additional surgery, and 15% of patients required at least two additional surgeries<sup>[42]</sup>. Pouch leak and the associated pelvic sepsis rate in large series have been reported to range from 5% to 15%<sup>[43]</sup>; incidence of late small-bowel resection after IPAA ranges from 12% to 35%. Pouchitis is the most frequent long-term complication of the IPAA<sup>[1]</sup>. It has been reported in 12% to 50% of patients postoperatively, and some patients (5%-19%) require chronic therapy. Finally, the risk of long-term pouch loss has been reported to range from 1% to 20% in different studies with an overall rate of pouch loss less than 10%, needing diverting ileostomy, pouch excision and end ileostomy, or pouch revision<sup>[17-19]</sup>.

### Acute severe ulcerative colitis

According to current treatment algorithms, in case of acute colitis, unless toxic megacolon, perforation or severe bleeding-which are absolute indication for surgery-occur, patients are started on high-dose iv steroids<sup>[44]</sup>. Response to treatment is assessed by objective measures (e.g., Oxford index or Sweden index) on day 3-4. Two different strategies have been developed in the attempt of avoiding surgery when a first course of steroids fails to control an acute flare. The standard approach in the '80s was to prolong the administration of steroids for other 7-10 d, which did not show any reduction in colectomy rates<sup>[45-47]</sup>. Ten years later, cyclosporine was found to be effective in patients with acute severe UC non responsive to steroids, and has been used as rescue therapy<sup>[44,48-51]</sup>. In a randomized controlled trial (RCT) 82% of patients on cyclosporine improved, while no patient improved in the placebo group<sup>[52]</sup>. However, as many as 50% of patients that responded to cyclosporine, required colectomy in subsequent studies with longer follow-up<sup>[35,53]</sup>. Moreover, the management of patients under cyclosporine can represent a real challenge, given the risk of severe and potentially fatal toxicities, which greatly limit the use of this medication.

Infliximab, an anti-tumor necrosis factor (TNF) antibody, has been approved recently by the United States FDA for the treatment of UC to reduce signs and symptoms, to induce clinical remission and healing of the intestinal mucosa, and to eliminate the use of corticosteroids in patients presenting with moderately-to-severely

active UC without adequate response or who are intolerant or have medical contraindications to therapy with corticosteroids or immune modulators<sup>[54]</sup>.

Response to infliximab has been assessed in RCTs with various endpoints such as clinical response, remission and colectomy rates. In patients with severe, steroid-refractory UC, the initial small trials demonstrated modest efficacy after single infusions when early clinical response was determined. The first published trial by Sands *et al*<sup>[55]</sup> in 2001 randomized 11 patients with steroid refractory UC to a single infliximab infusion or placebo, and noted a 50% (4/8) clinical response rate with infliximab at a week 2 evaluation. Subsequent studies by Probert *et al*<sup>[56]</sup> and Järnerot *et al*<sup>[51]</sup> also enrolled patients with steroid-refractory disease. The first study failed to show any significant difference between placebo and 2 infusions of infliximab 5 mg/kg in inducing remission as measured by endoscopy or clinical score. However, Järnerot *et al*<sup>[51]</sup> demonstrated in patients with moderate and severe steroid-refractory UC that only 7/24 (29%) patients who received a single infliximab infusion underwent colectomy within 90 d, compared with 14/21 (67%) who received placebo. The superiority of infliximab was only statistically significant in patients with moderate to severe disease, but not in those with more severe disease on the fulminant colitis score, although the study was not powered to detect differences between these two last groups. Even though a later report showed that at 2 years follow-up, the colectomy rate in patients who received infliximab had increased to 46%<sup>[57]</sup>, these studies positioned infliximab as a therapeutic option for patients with steroid-refractory disease. The first controlled trial<sup>[58]</sup> involving patients who had moderate to severe disease that were neither steroid-dependent nor steroid-refractory, reported superior clinical response rates compared to those seen in steroid-refractory populations. These trials reported high response rates (100% and 83%, respectively), but follow-up was short (9.7 and 3 mo, respectively). The active ulcerative colitis trial (ACT) 1 and ACT 2 trials<sup>[59]</sup> each randomized 364 patients with moderate to severe UC who were failing conventional therapy (but did not require hospitalization) to either placebo or induction/maintenance infliximab 5 mg/kg or 10 mg/kg. Both in ACT 1 and ACT 2, eligible patients had moderate to severe UC despite concurrent treatment with corticosteroids, alone or in combination with azathioprine or mercaptopurine, but ACT 2 also required that the patient had failed 5-aminosalicylic acid (5-ASA) therapy. In ACT 1, both doses of infliximab (5 mg/kg and 10 mg/kg) resulted in a statistically significant clinical response at week 8 (68.4% and 61.5% respectively,  $P < 0.01$ , compared to a placebo response of 37.2%). This was similar in ACT 2, with clinical response at week 8 in 64.5% of patients in the infliximab 5 mg/kg group and 69.2% in the infliximab 10 mg/kg group, compared to a 29.3% response rate in the placebo group ( $P < 0.001$ ). Clinical remission rates in the infliximab arms at week 8 ranged from 27.5% to 38.8% across both studies compared to

placebo-induced remission rates of 14.9% (ACT 1) and 5.7% (ACT 2). Mucosal healing and steroid-free remission rates were also superior in the infliximab arms of these studies. Sandborn *et al*<sup>[60]</sup> reported colectomy rates in ACT 1 and ACT 2 patients in a follow-up study. The cumulative colectomy rate at 54 wk was 10% in patients treated with infliximab, compared with 17% in those treated with placebo. These colectomy rates were not unexpected since the enrolled patients had moderate to severe disease, however in 13% of the enrolled patients the colectomy follow-up data was unavailable. The ACT 1 and ACT 2 studies were well-designed, large studies, with comprehensive assessment of clinical and secondary endpoints. They provided important data to support the use of infliximab in patients with moderate to severe UC who have failed other therapies such as steroids, immunomodulators and mesalamine. However, infliximab is not a panacea for all; the proportion of patients who started the study on steroids and were able to come off and remain in remission, was low (20%)<sup>[59]</sup>.

In a recent study by Colombel *et al*<sup>[61]</sup>, the association between early mucosal healing (defined as Mayo endoscopy subscore at week 8 endoscopy) and clinical outcomes in ACT-1 and ACT-2 patients was investigated. The authors observed that a low week 8 endoscopy subscore was significantly associated with a lower rate of colectomy at 54 wk follow-up ( $P = 0.0004$ ; placebo  $P = 0.47$ ) and better outcomes in terms of symptoms and need for steroids at weeks 30 and 54 ( $P < 0.0001$ , infliximab;  $P < 0.01$ , placebo), especially for those patients who did not achieve clinical remission at week 8<sup>[61]</sup>.

A Cochrane meta-analysis of RCTs concluded that, when compared to placebo, treatment with infliximab is three-fold as effective in inducing clinical remission [relative risk (RR) 3.22; 95% CI: 2.18-4.76] and nearly twice as effective in inducing clinical response (RR: 1.99; 95% CI: 1.65-2.41) or endoscopic remission (RR: 1.88; 95% CI: 1.54-2.28) at week 8 in patients presenting with moderate-to-severe UC refractory to conventional treatment with corticosteroids and/or immune modulators<sup>[10]</sup>.

### **Steroid dependent ulcerative colitis**

Another specific pattern of UC disease is represented by steroid-dependent patients, in whom a response can be obtained with systemic steroids, but the relapse will occur as the dose is tapered or a few weeks or months after discontinuation, making it necessary to increase the dosage again or resume treatment to achieve control of symptoms<sup>[6]</sup>. As UC patients become dependent-upon or refractory to corticosteroids, the range of action from a medical standpoint become limited and a colectomy becomes a treatment option as the disease is deemed as refractory to medical treatment, or because of the occurrence of complications either related to the disease or associated with side effects of medications<sup>[1]</sup>.

Often, immunomodulator therapies, such as azathioprine or mercaptopurine (6-mercaptopurine) are considered

in these patients before surgery as a steroid-sparing treatment. However, the efficacy of azathioprine or mercaptopurine in UC is still debated<sup>[62]</sup>. Thiopurines are an effective maintenance therapy for patients who require repeated courses of steroids, however the quality of available data is quite poor, as stated in a recent Cochrane review<sup>[63]</sup>. Currently, the recommendation for using thiopurines in UC is based on the evidence shown by only one RCT of Ardizzone *et al*<sup>[64]</sup> which found steroid-free, clinical and endoscopic remission in 53% patients on azathioprine compared with 21% given only 5-ASA [odds ratio (OR) on intention to treat analysis 4.78, 95% CI: 1.57-14.5]. Azathioprine maintenance treatment of UC is beneficial for at least 2 years if patients have achieved remission while taking the drug, but not in those with chronic activity despite the drug<sup>[65]</sup>.

When a steroid-dependent patient fails to benefit from thiopurines or shows intolerance to them, there are very few alternatives to conventional drugs, which lack of current definitive evidence of efficacy. Methotrexate has been tested and, although some uncontrolled studies suggested some benefit with its use<sup>[66-68]</sup>, the only double-blind, placebo-controlled trial, showed no therapeutic benefit<sup>[69]</sup>. Therefore, current guidelines do not consider methotrexate as an evidence-based therapy in steroid-dependent UC.

After the demonstration of clinical efficacy of infliximab in the treatment of moderate-severe resistant UC, few small series have included steroid dependent patients. Only one study from Italy<sup>[70]</sup> specifically evaluated steroid dependent UC in an open-label study on 20 patients randomized to infliximab or methylprednisolone. This was the first RCT to implement a regimen of a triple infliximab infusion for induction followed by infusions to maintain remission. Even if this study was statistically underpowered, it demonstrated the benefit of infliximab therapy for responders, who were able to taper and then discontinue steroids during the maintenance phase (9 of 10 patients), as compared with the methylprednisolone group (8 of 10 patients), where responders required continued steroid therapy.

## **BIOLOGIC THERAPY IN UC: THE SURGEON'S VIEW**

Biologic therapy has shown the ability to induce and maintain remission, but, as we stated above, its introduction in the therapeutic algorithm did not substantially affect the overall rate of colectomies, suggesting that it is effective only in delaying but not in avoiding surgery for a subgroup of patients who at some point will require an operation<sup>[54,59,60,71]</sup>. The clinical efficacy of infliximab in UC still remains unpredictable. Induction therapy is not always effective, and, to date, clinical and/or molecular predictors of response have not been identified. No RCT has been conducted comparing infliximab and cyclosporine in severe UC. Most of the current knowledge comes from the ACT 1 and ACT 2 trials. Those results are in part influenced by the heterogeneity of the sample (in-

Table 1 Literature-based comparison of postoperative complication risk associated with preoperative use of infliximab

Ref.	Year	Non-IFX/IFX patients	Infectious complication			Non-infectious complication		
			IFX group	Non-IFX group	OR (95% CI)	IFX group	Non-IFX group	OR (95% CI)
Selvasekar <i>et al</i> <sup>[13]</sup>	2007	254/47	13 (28%)	25 (10%)	3.50 (1.64-7.5)	16 (34%)	99 (39%)	0.81 (0.4-1.55)
Schluender <i>et al</i> <sup>[12]</sup>	2007	134/17	3 (18%)	11 (8%)	2.40 (0.6-9.63)	3 (18%)	26 (19%)	0.89 (0.24-3.33)
Kunitake <i>et al</i> <sup>[9]</sup>	2008	312/101	6 (6%)	32 (10%)	0.55 (0.22-1.36)	11 (11%)	17 (5%)	2.12 (0.96-4.69)
Mor <i>et al</i> <sup>[85]</sup>	2008	46/46	10 (22%)	1 (2%)	13.8 (1.82-105)	6 (13%)	6 (13%)	1.00 (0.3-3.37)
Ferrante <i>et al</i> <sup>[83]</sup>	2009	119/22	2 (9%)	29 (24%)	0.31 (0.07-1.141)	NR	NR	NR
Coquet-Reinier <i>et al</i> <sup>[84]</sup>	2010	13/13	NR	NR	NR	3 (23%)	4 (38%)	NR
Gainsbury <i>et al</i> <sup>[86]</sup>	2011	52/29	5 (17%)	14 (27%)	1.87 (0.46-7.57)	12 (41%)	16 (31%)	0.59 (0.19-1.87)

IFX: Infliximab; OR: Odd ratio; NR: Not reported; CI: Confidence interval.

cluding both steroid-dependent and/or immunomodulator-dependent and steroid responsive and/or immunomodulator-responsive patients). More studies are needed to assess the role of concomitant administration of immunosuppressants and infliximab<sup>[59]</sup>. Furthermore, data on maintenance therapy with infliximab in UC are scant and the benefits of continued maintenance therapy, as well as its long-term safety, are poorly known. The results of the ACT-1 and ACT-2 extension studies conducted on the 229 patients who achieved improvements with infliximab during the trials, showed that the benefits observed in the main studies are basically maintained up to 3 additional years, however an high drop-off rate was observed, due to adverse events (10.5%), lack of efficacy (4.8%), need for surgery (0.4%), or other reasons (14.8%)<sup>[72]</sup>. Furthermore, it is not clear to what extent postponing surgery by the means of a quite morbid medical therapy represents a safe and effective strategy.

Because of the early onset and chronic nature of inflammatory bowel diseases, patients can be expected to utilize considerable health care resources. Costs analysis are complicated, because they must calculate the impact that such therapies have on the direct costs of health care and the indirect costs for both the patient and their families and the health care system<sup>[73]</sup>. Surgeries and hospitalizations account for the majority of health care direct costs in inflammatory bowel disease (IBD), and medication costs, on the other hand, accounted for a quarter of total direct medical costs. Moreover, the cost data are right-skewed, with 25% of patients accounting for 80% of total costs<sup>[74]</sup>. This division of health care costs implies that the most effective cost-containment measure would be the one that reduces the number of hospitalizations and surgeries. With the improved response and remission rates seen with the use of infliximab for induction and maintenance treatment in IBD patients, the clinical benefits may likely translate into economic benefits as well<sup>[75]</sup>. Surprisingly, many of the cost-effectiveness and utility analyses suggested that infliximab use was associated with rather high incremental cost per quality adjusted year life<sup>[73]</sup> and the expanding use of infliximab has not significantly impacted the use of surgical procedures for patients with either UC or Crohn's disease, and rates of nonsurgical hospitalizations have actually increased<sup>[76,77]</sup>. This belief is supported by

the observation that in the United States the hospitalization rates for IBD increased between 1998 and 2004, leading to a concurrent rise in the economic burden, with medical hospitalizations accounting for the largest proportion (58%) of inpatient services costs and biologic agents representing the most costly medications<sup>[78,79]</sup>. Further pharmaco-economic analyses are needed to accurately assess the impact of infliximab treatment on the costs associated with the treatment of UC.

#### **Surgery in the biologic era: Treatment in evolution**

The concept of pushing conservative treatment until surgery is strictly required may be risky, as it has been shown that mortality three years after elective colectomy for UC (3.7%) is significantly lower than that after admission without surgery (13.6%) or when an emergency operation is performed (13.2%)<sup>[80]</sup>. Moreover, a British study recently reported a significantly higher risk to develop major complications at a 5 year follow up for patients who received a longer course of medical therapy for acute severe UC before surgery, suggesting that the threshold for elective surgery may be too high in current practice<sup>[81]</sup>.

While it's well known that high-dose systemic corticosteroid therapy (> 40 mg/d prednisolone-equivalent) is a widely recognized risk factor for pouch-related septic complications after restorative surgery<sup>[82]</sup>, whether or not the preoperative administration of infliximab may increase the rates of septic complications is still controversial (Table 1)<sup>[9,12,13,83-86]</sup>. Nevertheless, the group from the Cleveland Clinic has found a covariate-adjusted risk of early complication for patients treated with infliximab 3.54 times higher, with the rate of sepsis increased by 13.8 folds, despite a significantly higher rate of three-stage procedures in the infliximab group<sup>[85]</sup>. Similar results have been shown in a paper by Mayo Clinic, where patients treated with infliximab prior to pouch surgery had a significantly higher incidence of anastomotic leaks, pouch specific and infectious complications, with the administration of anti-TNF-alpha as the only factor independently associated with septic complications (OR 3.5)<sup>[13]</sup>. In another study, a synergic interaction in increasing surgical morbidity was found between infliximab and cyclosporine A when administered together in the preoperative time<sup>[12]</sup>. These concerns are supported by a recent meta-analysis conducted including 5 studies and 706 patients, which revealed an

increased risk of short-term post-operative complications (OR 1.80, 95% CI: 1.12-2.87) associated with preoperative infliximab use, along with a trend towards increased post-operative infection<sup>[87]</sup>.

Given the concern of increased rate of complications in patients on aggressive medical management, several different surgical approaches have been proposed. First described by Parks and Nichols in 1978, restorative proctocolectomy with IPAA has progressively gained acceptance to become the gold standard in the surgical treatment of UC for the last 25 years<sup>[88,89]</sup>. The introduction of this technique—most often fashioned as a J pouch created with the terminal ileum and anastomosed to the anal canal—was a real breakthrough, offering a curative treatment to these patients without the need for a permanent stoma, thus preserving their body image, achieving a quality of life comparable to that of the general population<sup>[38,90]</sup>. However, the procedure is technically demanding and is associated with a significant morbidity rate (around 30%), and an incidence of postoperative pelvic sepsis ranging between 5%-24%<sup>[91]</sup>. Since it has been shown that the occurrence of a pelvic infection can dramatically affect the functional outcome of the pouch, and considering that long-term steroid use and malnutrition are recognized risk factors for pelvic sepsis, surgical strategies have been developed in order to minimize the occurrence of infectious complications, especially in this subset of patients<sup>[92,93]</sup>. A total abdominal colectomy with end ileostomy is the operation of choice as first step of a restorative procedure, as it can be performed safely and quickly in the hands of an experienced colorectal surgeon, allowing the patient to overcome the colitis, wean off the medications, and return to an optimal health and nutritional status<sup>[94,95]</sup>. Moreover, as it is well known that a postoperative diagnosis of indeterminate colitis or Crohn's disease is not rare after colectomy in these patients<sup>[96]</sup>, a multistep surgical procedure allows for selecting the most appropriate reconstructive surgery on the basis of the pathological findings of the colectomy specimen<sup>[19,94,97]</sup>.

The removal of the rectum and the restoration of the bowel continuity with IPAA are performed as a second step when the patient has fully recovered, and the creation of a temporary ileostomy, although adding the need for one more operation, can further reduce the risk of local sepsis secondary to anastomotic leaks<sup>[98,99]</sup>. Albeit restorative surgery is not free from long term complications, such as incontinence and soiling (10%-60% of patients, depending on series and entity), pouchitis (about 50% of patients), and sexual dysfunction (20%-25% of cases), with a rate of pouch failure requiring excision ranging between 5%-15%, the majority of these conditions are manageable with medical and physical therapy, which explains the overall satisfaction in patients after IPAA, which exceeds the 90% in most series<sup>[40,98,100-105]</sup>.

Indeed, most recent researches have shown that social and sexual function as well as overall quality of life is significantly improved after restorative surgery, when compared to the period with active UC or diverting ileostomy<sup>[106-109]</sup>.

The application of minimally invasive techniques to the surgical treatment of UC at the beginning of the 1990s contributed in significantly improving the acceptance and tolerability of the procedure<sup>[110]</sup>. Numerous case series and, finally, two meta-analyses have been published since then, demonstrating the feasibility and safety of the laparoscopic approach, at the cost of longer operative times<sup>[110-117]</sup>. A subsequent RCT showed that operative time could be significantly reduced with the adoption of a hand-assisted technique, which at the same time allows for preserving the advantages of a minimal invasive approach<sup>[118]</sup>. Scant data is available so far regarding long-term outcomes, however the few series with adequate follow-up report laparoscopy pouch function results as good as the ones achieved with open surgery<sup>[21,119]</sup>. Laparoscopy has also been adopted with good results in the emergency setting<sup>[120,121]</sup>, and similarly as for open surgery, a staged approach to a minimally invasive restorative procedure should be preferred which is as effective in significantly reducing the rate of postoperative pelvic sepsis<sup>[121-123]</sup>. Furthermore, when a staged procedure is planned, laparoscopy has been shown to decrease postoperative adhesion formation with less intraoperative adhesiolysis required during subsequent completion proctectomy and IPAA<sup>[124]</sup>. Similarly, a study by Indar and colleagues on 34 patients who underwent laparoscopic IPAA, where a laparoscopic exploration of the abdominal cavity was performed during the ileostomy closure, found that no patient had dense adhesion and only a minority of patients had filmy avascular adhesion to the abdominal wall (32%) and to the adnexa (29%), which represents a significant improvement compared to the rates reported for open surgery (as high as 90%)<sup>[125]</sup>.

Despite the lack of strong evidence about the benefits attainable with laparoscopy in terms of short-term outcomes<sup>[21,126]</sup>, it has been proven that patients treated laparoscopically are more satisfied with the cosmetic results and perceive a better body image—anything but negligible in this usually young and socially active patient population—especially in the women's subset, as confirmed by the results of a RCT with a median follow-up of 2.7 years<sup>[21,119]</sup>. More recently, the quest for further minimizing surgical trauma and extent of incisions, has led to the development of single incision laparoscopy (SIL), which has already been applied in the field of colorectal diseases with proven benefits in terms of short-term outcomes over standard laparoscopy<sup>[127-131]</sup>. To date only few cases of SIL for UC has been reported, but preliminary results show that particularly for the total abdominal colectomy, this “no scar” approach have the potential for improving not only the cosmesis, but also the postoperative course, with less pain and reduced need for narcotics, which may translate in shorter hospital stay and faster return to normal activities<sup>[132-135]</sup>. Considering the excellent outcome of restorative surgery, heightened by the potentials of minimal invasive techniques, surgery should not be considered the last resort when everything has failed, but rather a valid alternative to an expensive and risky medical therapy<sup>[136]</sup>.

## CONCLUSION

Medical therapy in UC is rapidly evolving and the introduction of modern biological drugs has led to substantial changes in the traditional principles of management. Infliximab, the first biological agent used as rescue therapy after failure of steroids in UC, appears to be effective in reducing the need for urgent colectomy, although its efficacy in the long-term is not proven. In addition, concerns have been raised regarding the economic burden related to this drugs and the risk for serious postoperative complications.

Surgery continues to play an important role in UC treatment and its evolution keeps pace with the advance in medical therapy and the risk associated with it. Restorative proctocolectomy with IPAA, staged procedures, and minimally invasive surgery are important treatment tools to limit postoperative morbidity and achieve excellent long-term outcomes in these patients.

In an attempt at avoiding surgery, aggressive medical therapy is not without complications. A complex decision making process in a multidisciplinary fashion should take into consideration the excellent results of modern surgical therapies to avoid unnecessary morbidity.

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## Colitis associated with biological agents

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### Abstract

In the past, there has been considerable focus on a host of drugs and chemicals that may produce colonic toxicity. Now, a variety of new biological monoclonal antibody agents, usually administered by infusion, have appeared in the clinical realm over the last decade or so to treat different chronic inflammatory or malignant disorders. For some of these agents, adverse effects have been documented, including apparently new forms of immune-mediated inflammatory bowel disease. In some, only limited symptoms have been recorded, but in others, severe colitis with serious complications, such as bowel perforation has been recorded. In others, adverse effects may have a direct vascular or ischemic basis, while other intestinal effects may be related to a superimposed infection. Some new onset cases of ulcerative colitis or Crohn's disease may also be attributed to the same agents used to treat these diseases, or be responsible for disease exacerbation. Dramatic and well documented side effects have been observed with ipilimumab, a humanized monoclonal antibody developed to reduce and overcome cytotoxic T-lymphocyte antigen 4, a key negative feedback regulator of the T-cell anti-tumor response. This agent has frequently been used in the treatment of different malignancies, notably, malignant melanoma. Side effects with this agent occur in up to 40% and these are believed to be largely immune-mediated. One of these is a form of enterocolitis that may be severe, and occa-

sionally, fatal. Other agents include rituximab (an anti-CD20 monoclonal antibody), bevacizumab (a monoclonal antibody against the vascular endothelial growth factor) and anti-tumor necrosis factor agents, including infliximab, adalimumab and etanercept.

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**Key words:** Biological agents; Colitis; Crohn's disease; Inflammatory bowel disease; Infliximab; Ipilimumab; Rituximab; Ulcerative colitis

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### INTRODUCTION

Different patterns of inflammatory disease involving the small and large intestine have been historically recognized over the past century or so, and their features are well detailed in clinical textbooks especially focused on inflammatory bowel diseases. Crohn's disease, for example, is recognized as a pattern of inflammatory disease that may involve any site along the length of the gastrointestinal tract, usually in a segmental or focal distribution, typically with transmural involvement, and frequently, granulomas may be demonstrated. Ulcerative colitis has been traditionally defined by its colonic distribution, a more continuous pattern of mucosal involvement extending proximally within the colon from the rectum for variable distances. In reality, however, clinical and pathological overlap may occur frequently, and in many patients, precise differentiation of these different forms of chronic

inflammatory disease based on these descriptive parameters may not be so precise.

In the past, a variety of drugs have been used to control the inflammatory process in these disorders and improve quality of life. In addition, a host of biological agents have also emerged in recent years to treat a number of chronic inflammatory disorders, including Crohn's disease and ulcerative colitis, as well as a lengthening list of malignant disorders. Some of these biological agents have also been associated with the appearance of novel forms of colonic inflammatory disease, often severe and potentially fatal, as well as apparent paradoxical intestinal complications, including the *de novo* appearance or worsening of an underlying or unrecognized intestinal inflammatory disorder that may, in themselves, lead to serious complications.

Although a number of administered drugs and chemicals causing colonic toxicity have been enumerated elsewhere and reviewed in detail during the past 3 decades<sup>[1-3]</sup>, this review focuses on newer agents, largely administered by the parenteral route, that interfere with key regulatory biological molecules. These include ipilimumab, rituximab, bevacizumab and a number of anti-tumor necrosis factor agents.

## IPILIMUMAB-INDUCED COLITIS

A relatively novel strategy has emerged in cancer treatment in recent years to induce tumor regression and prolong patient survival involving control and reduction of the effect of specific immune regulatory molecules, such as the cytotoxic T-lymphocyte antigen 4 (CTLA-4). Ipilimumab is a fully human monoclonal antibody that has been developed to reduce and overcome cytotoxic CTLA-4, a key negative regulator of the T-cell anti-tumor immune response. In recent years, evidence has appeared showing tumor regression with prolonged time to progression in melanoma patients treated with CTLA-4 antibodies<sup>[4,5]</sup>. Ipilimumab plus dacarbazine showed improved survival in malignant melanoma compared to dacarbazine alone, a drug most frequently compared with new agents in randomized treatment trials on melanoma<sup>[5]</sup>. In addition to melanoma, prolonged effects with ipilimumab have been noted in other malignancies including ovarian cancer<sup>[6]</sup>, prostate cancer<sup>[7]</sup> and renal cell cancer<sup>[8]</sup>. Inhibition of CTLA-4 with this antibody is also associated with characteristic side effects in an estimated 40%<sup>[4]</sup>. These are believed to be largely immune-mediated and include an ever-lengthening list of adverse effects such as dermatitis, endocrinopathies, particularly hypophysitis, uveitis, nephritis, inflammatory myopathies, hepatitis, and diarrhea or colitis<sup>[9,10]</sup>. Similar immune-related adverse events may result from another monoclonal CTLA-4 antibody, tremelimumab, used for the treatment of metastatic melanoma<sup>[11]</sup>.

Colonic toxicity has been recorded in about 20% and appears to occur relatively rapidly after administration of ipilimumab, sometimes within days marked by the onset of abdominal cramping pain and profuse diarrhea, often bloody<sup>[9,12]</sup>. In others with few or mild symptoms, colitis could still be present since only those with more severe

symptoms were recorded<sup>[12]</sup>. Up to 5% of patients may suffer a fatal outcome attributed to a significant complication, a protracted clinical course or failure of prompt treatment, sometimes related to limited compliance<sup>[12]</sup>. Colonoscopy and ileoscopy as well as upper endoscopy with duodenal biopsies have documented both small bowel and colonic inflammatory changes. In some, a diffuse, but non-specific colitis may occur, in the absence of any detectable infectious agent, while in others, the inflammatory process may be patchy or segmental in distribution. The appearances may not be distinguishable by endoscopy from other forms of inflammatory bowel disease. Endoscopic biopsies may show a non-specific acute and chronic inflammatory infiltrate, including cryptitis as well as crypt abscess formation. Colon biopsy samples show a colitis that has an abundant T-cell infiltrate<sup>[13]</sup>. Granulomatous inflammation has not been recorded.

Treatment for this enterocolitis largely based upon supportive measures, specifically, fluid and electrolyte replenishment and, sometimes, parenteral nutrition. In addition, the colitis has often been treated with intravenous high dose steroids (or oral budesonide) and, if the response to steroids fails or has been limited, infusions of infliximab have been used<sup>[14,15]</sup>. If no response for the colitis is evident, diverting ileostomy or partial/complete colectomy has been recommended. The incidence of life-threatening colon perforation has been recorded at 4 in 700 cases with doses of ipilimumab of 3 mg/kg or more (i.e., less than 1%). Even during treatment with steroids or infliximab for the colitis, the anti-tumor response for metastatic melanoma still appears to be sustained. In a recent study of ipilimumab with dacarbazine for previously untreated metastatic melanoma, rates of intestinal adverse events were reported to be lower, while the rates of altered liver chemistry test changes were higher<sup>[5]</sup>.

## RITUXIMAB-ASSOCIATED COLITIS

Rituximab is an anti-CD20 monoclonal antibody that has been used in the management of nephrotic syndrome in children and adults<sup>[16-18]</sup> as well as a form of B-cell targeted therapy in rheumatoid arthritis<sup>[19,20]</sup>. It appears to result in depletion of systemic as well as intestinal B cell populations. Although the agent appears to be efficacious, adverse effects have been noted in about 27% of children treated with rituximab for refractory nephrotic syndrome<sup>[21]</sup>. Some of the reported adverse effects have included fever and chills, mucocutaneous reactions, fatal infusion reactions, progressive multifocal leukoencephalopathy, and bowel perforation<sup>[18,22]</sup>. New onset ulcerative colitis<sup>[23]</sup> and an exacerbation of previously documented colitis have been recorded<sup>[24]</sup>. In a later report, it was hypothesized that a severe colitis that developed after rituximab therapy may have been related to an infectious torovirus agent<sup>[22]</sup>.

## BEVACIZUMAB-ASSOCIATED COLONIC ULCERATION

Bevacizumab is a humanized monoclonal antibody against

the vascular endothelial growth factor receptor. This antibody has shown promise in the treatment of recurrent and metastatic colorectal cancer as well as metastatic non-small cell lung cancer. The agent has also been used to treat other malignancies, including ovarian cancer. Several mechanisms of action have been proposed, including an ability to restrict or deprive tumors of their neovascularity required to permit tumor progression and growth.

A number of cases have now been reported describing bowel perforation following bevacizumab treatment<sup>[25-29]</sup>. There also appears to be an especially increased risk of leakage at anastomotic suture sites following surgery for either ulcerative colitis or colorectal cancer<sup>[26]</sup>. In some, delayed anastomotic complications have been observed, some more than 1 year after surgery. Some hypothesized risk factors included anastomotic leakage during the original operative procedure or prior pre-operative pelvic irradiation<sup>[27]</sup>. Other mechanisms that have been recorded include an ischemic pathogenesis with anastomotic perforation after a partial colectomy<sup>[28]</sup> or more diffuse perforation associated with histological evidence of ischemia in a patient with non-small cell lung cancer<sup>[29]</sup>.

## ANTI-TUMOR NECROSIS FACTOR ADVERSE EVENTS

Most intriguing is the recent increased recognition of paradoxical adverse events following therapy with anti-tumor necrosis factor. In selected patients with inflammatory bowel disease, improved symptoms and mucosal changes may result. Similar treatment effects have been recorded for different agents including the chimeric monoclonal agent, infliximab, along with more humanized forms, such as adalimumab. Interestingly, in some treated with these agents for other disorders, in particular spondyloarthropathies or rheumatoid arthritis, so-called “paradoxical” adverse effects have been recorded, including flares or new onset inflammatory bowel disease<sup>[30]</sup>. Initially, in this early evaluation, intestinal effects appeared to occur more often with etanercept than either of the monoclonal antibody agents<sup>[30]</sup>. Later, however, other effects appeared to develop during arthritis treatment, particularly the skin disorder, *pyoderma gangrenosum*<sup>[31,32]</sup>. Later, new onset ulcerative colitis was initially recorded during infliximab treatment<sup>[33]</sup> as well as adalimumab<sup>[34]</sup>. Similar cases of new onset inflammatory bowel disease, specifically, Crohn’s disease have also been recorded usually after etanercept therapy administered for spondyloarthropathy (as opposed to Crohn’s disease where etanercept has not been effective)<sup>[35-39]</sup>. Some have suggested that the *de novo* appearance of inflammatory bowel disease following anti-tumor necrosis factor therapy may simply be related to “unmasking” of an underlying inflammatory disease process<sup>[40]</sup>. Others have documented a superimposed infectious agent<sup>[41,42]</sup>. A large retrospective study concluded that paradoxical adverse events of anti-tumor necrosis factor therapy may occur, but none were agent specific<sup>[43]</sup>.

## CONCLUSION

Several intestinal, particularly colonic complications have been recorded with the emerging armamentarium of monoclonal antibody agents used in the management of different inflammatory or malignant disorders. For some, immune-mediated adverse events may occur regularly, while for others, a complication may be rare and the mechanism not so evident. The precise frequency of colonic complications after treatment with these agents has been difficult to determine. In large part, this has been related to the clinical focus being largely directed to the most severely symptomatic cases. Although selected patients treated for either ulcerative colitis or Crohn’s disease with these biological agents has increased over the past decade, in retrospect, it may be that some labeled as “refractory” or not responsive to these agents may simply have been made worse. Published clinical trials may not always detail a failed therapeutic event as an adverse event. Future awareness of the possible adverse intestinal effects of monoclonal agents may be important.

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## Probiotic metabolites from *Bacillus coagulans* GanedenBC30™ support maturation of antigen-presenting cells *in vitro*

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### Abstract

**AIM:** To study the effects of probiotic metabolites on maturation stage of antigen-presenting immune cells.

**METHODS:** Ganeden *Bacillus coagulans* 30 (GBC30) bacterial cultures in log phase were used to isolate the secreted metabolite (MET) fraction. A second fraction was made to generate a crude cell-wall-enriched fraction, by centrifugation and lysis, followed by washing. A preparation of MET was subjected to size exclusion centrifugation, generating three fractions: < 3 kDa, 3-30 kDa,

and 30-200 kDa and activities were tested in comparison to crude MET and cell wall in primary cultures of human peripheral blood mononuclear cell (PBMC) as a source of antigen-presenting mononuclear phagocytes. The maturation status of mononuclear phagocytes was evaluated by staining with monoclonal antibodies towards CD14, CD16, CD80 and CD86 and analyzed by flow cytometry.

**RESULTS:** Treatment of PBMC with MET supported maturation of mononuclear phagocytes toward both macrophage and dendritic cell phenotypes. The biological activity unique to the metabolites included a reduction of CD14<sup>+</sup> CD16<sup>+</sup> pro-inflammatory cells, and this property was associated with the high molecular weight metabolite fraction. Changes were also seen for the dendritic cell maturation markers CD80 and CD86. On CD14<sup>dim</sup> cells, an increase in both CD80 and CD86 expression was seen, in contrast to a selective increase in CD86 expression on CD14<sup>bright</sup> cells. The co-expression of CD80 and CD86 indicates effective antigen presentation to T cells and support of T helper cell differentiation. The selective expression of CD86 in the absence of CD80 points to a role in generating T regulatory cells.

**CONCLUSION:** The data show that a primary mechanism of action of GBC30 metabolites involves support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

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**Key words:** Mononuclear phagocytes; Dendritic cell maturation; Co-stimulatory molecules; Antigen-presentation; Probiotics; Metabolites

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## INTRODUCTION

Bacteria are ubiquitous in the environment, having colonized every extreme of nature. This includes the human body where they outnumber human cells by an order of magnitude. The biggest reservoir of these symbiotic bacteria on the human body is the lower gastrointestinal tract<sup>[1]</sup> where large numbers of coexisting (commensal) bacteria participate in nutrient assimilation including the breakdown of indigestible carbohydrates. They also produce amino acids and vitamins for their host and play a key role in healthy immune system development.

The immune system recognizes both pathogenic and commensal bacteria through a family of pattern recognition receptors known as the toll-like receptor (TLR) family<sup>[2]</sup>. These receptors interact with molecules present on the exterior surface of bacteria and include lipopolysaccharide (LPS), flagellin, lipoteichoic acid and lipoproteins as well as bacterial DNA. Toll-like receptors are present on cells participating in both innate and adaptive immunity such as monocytes/macrophages and dendritic cells (DC) as well as epithelial cells of the intestinal mucosa.

The emerging picture is that commensal bacteria have an enormous impact on health. While a healthy microbiota can aid the host by increasing nutrient absorption and training the immune system to not respond to self, conversely an unhealthy (i.e., unbalanced) microbiota can lead to malabsorption, inflammation and disease<sup>[3-5]</sup>. A growing body of evidence suggests that these effects, both positive and negative, of the microbiota on the host are mediated by the immune system. Probiotics are defined as microorganisms that when ingested in a sufficient amount confer a health benefit upon the host and are known to interact with the immune system. Probiotic microorganisms have a long history of human consumption in the form of fermented foods and have shown health benefits in treating dysbiosis, irritable bowel syndrome, and eczema<sup>[6]</sup>. GaneddenBC30™ (*Bacillus coagulans* GBI-30, 6086) (GBC30) is a proprietary strain of the gram positive, lactic acid producing spore-forming bacteria known as *Bacillus coagulans*. This strain of *B. coagulans* can survive extremes of heat and pressure in manufacturing as well as the harsh, acidic environment of the human gastrointestinal tract, leading to a very high survival rate and germination in the lower intestinal tract. The safety of consumption of this strain was documented in acute and sub-chronic studies in rats<sup>[7]</sup>.

One way in which commensal bacteria modulate the immune response is by the secretion of certain bioactive compounds. This suggests that metabolites of commensal bacteria have effects of their own and that there may be unique health benefits to be derived from the consumption of live probiotic cultures or probiotic metabolite preparations. Recent studies on the bacterial compound polysaccharide A from *Bacteroides fragilis* have shown the ability of this molecule to prevent intestinal inflammation caused by *Helicobacter pylori* infection and to correct the symptoms of encephalomyelitis in mice, an animal model for human multiple sclerosis<sup>[8-10]</sup>. The recent sequencing data from 178 commensal microbial genomes has identified over 30 thousand potential protein-coding sequences of which 97% are unique<sup>[11]</sup>. This suggests a vast untapped reservoir of novel genes including those coding for potential secreted compounds.

The work presented here build on a previous study that showed both enhancement of innate immune responses as well as anti-inflammatory effects of GBC30 *in vitro*<sup>[12]</sup>. In particular, the data presented here has aimed at investigating the differences between a crude preparation versus the metabolite fraction in more detail with a particular focus on modulation of key regulatory immune cells by specific size-selected fractions of GBC30 metabolite (MET) compounds.

## MATERIALS AND METHODS

### Reagents

The following buffers and reagents were obtained from Sigma-Aldrich (St. Louis, MO): Histopaque 1077 and 1119, phosphate-buffered saline (PBS), RPMI-1640 culture medium, fetal calf serum, L-glutamine 200 mmol/L, penicillin-streptomycin 100X solution, and bovine serum albumin. CD80-FITC, CD86-PE, CD16-PE and CD14-PerCP were obtained from BD Biosciences (San Jose, CA). Sodium Azide (NaN<sub>3</sub>) was obtained from LabChem Inc. (Pittsburgh, PA). Low-binding 100 μm zirconium beads were obtained from OPS Diagnostics (Lebanon, NJ) and 0.2 μm cellulose acetate filters from Whatman (Florham Park, NJ). The *Bacillus coagulans* strain (GaneddenBC30™) was obtained from Ganedden Biotech Inc. (Mayfield Height, OH).

### Preparation of *Bacillus coagulans* metabolite fractions

Using sterile technique, two separate samples of 2.0 g of GaneddenBC30™ spores were each placed into 25 mL PBS and heated at 70 °C for 30 min. Spores were then centrifuged at 2400 rpm for 5 min, PBS was removed and each tube of spores re-suspended and placed in culture flasks containing 25 mL of RPMI-1640 culture medium. The cultures were incubated at 37 °C for 24 h at which time an additional 20 mL of RPMI-1640 was added and the cultures incubated for an additional 24 h. Following 48 h of incubation, the bacterial cultures contained 5 × 10<sup>7</sup> bacteria/mL.

Preparation of GaneddenBC30™ culture supernatant as

a source of metabolites (MET): Cultures were transferred to 50 mL centrifuge tubes and initially spun at 1000 rpm for 2 min to remove any remaining spores. The liquid containing the bacteria and metabolites was decanted into new tubes and centrifuged at 3500 rpm for 20 min. The supernatant was decanted from the large bacterial pellets and combined in a single tube followed by filtration twice through a 0.2  $\mu\text{m}$  cellulose acetate syringe filter. This filtrate was either frozen directly as 250  $\mu\text{L}$  aliquots (crude metabolite fraction) or spun through Amicon Ultra protein size separation columns from Millipore (Bedford, MA) followed by aliquot preparation and storage at  $-20\text{ }^{\circ}\text{C}$ . Separation of the metabolites into different molecular weight fractions was performed in the following manner: for the fraction that is  $< 3\text{ kDa}$ , the crude metabolite preparation was placed onto a 3 kDa molecular weight cutoff centrifuge column and spun at 2500 rpm for 10 min. The filtrate that passed through the filter contained material that was less than 3 kDa. Aliquots were made from this material and frozen (metabolites  $< 3\text{ kDa}$  fraction). The material that did not pass through the column was then placed into a tube containing a 30 kDa molecular weight cutoff filter and spun at 2500 rpm for 10 min. The filtrate that passed through this filter contained material that was 3-30 kDa. Aliquots were made from this material and frozen (metabolites 3-30 kDa fraction). The remaining metabolite material that did not pass through the 30 kDa molecular weight filter was also aliquoted and frozen and this fraction was called metabolites 30-200 kDa fraction. It is important to note that these size separations are not exact and that while large molecules are excluded from the fractions containing the smaller molecules, some small molecules may still remain in the fractions containing the larger molecules.

Preparation of GanedenBC30<sup>TM</sup> crude cell wall (CW): The two bacterial pellets were each processed separately and then combined after the final bead milling step. Following centrifugation of the bacterial culture at 3500 rpm for 20 min and decanting of the supernatant, the bacterial pellets were washed twice in 45 mL of PBS with subsequent pelleting by centrifugation at 2500 rpm for 10 min. The washed pellets went through one freeze/thaw cycle followed by multiple bead milling cycles. In brief, the pellets were resuspended in 4 mL of PBS and then 4 mL of 100  $\mu\text{m}$  low-binding zirconium beads were added. One cycle of bead milling consisted of 60 one-second pulses of the bacteria/bead mixture on a vortex mixer. Ten of these cycles were performed. The bacteria/bead mixture was placed on ice in between bead milling cycles. At the end of the 10 bead-milling cycles, the beads were allowed to settle in the tubes and the liquid removed from the two tubes and combined. The liquid containing the fragmented bacteria was spun at 3500 rpm for 20 min followed by transfer of the liquid to Eppendorf tubes and centrifugation at 14 000 rpm for 5 min. The high speed centrifugation was necessary to remove any large fragments of bacteria that were not disrupted by the bead milling. The final solution was brought up to 45 mL with PBS and fil-

tered through a 0.2  $\mu\text{m}$  cellulose acetate filter and stored directly at  $-20\text{ }^{\circ}\text{C}$  as 250  $\mu\text{L}$  aliquots (crude cell wall). It is important to note that the cell wall preparation will also contain some GBC30 cellular contents in addition to cell wall components.

### **Purification of peripheral blood mononuclear cells**

Healthy human volunteers between the age of 20 years and 60 years served as blood donors after obtaining written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board. Isolation of peripheral blood mononuclear cell (PBMC) was performed as previously described<sup>[13]</sup>.

### **Cell surface staining of CD14 positive mononuclear phagocytes**

Complete cell culture media used for the culture of PBMC consisted of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin. Peripheral blood mononuclear cells were cultured for 3 d in the presence of serial dilutions of BC30 metabolite fractions or crude cell wall followed by cell surface immunostaining with CD14, CD80, CD86 and CD16 monoclonal antibodies. Processing of cells for immunostaining was performed as previously described<sup>[13]</sup> with the following modifications: optimal amounts of monoclonal antibodies per sample were 3  $\mu\text{L}$  for CD14-PerCP and 4  $\mu\text{L}$  for CD80-FITC, CD86-PE and CD16-PE. Experiments were performed three times using PBMC isolated from three different blood donors. Each test condition was performed in duplicate and untreated and LPS-treated controls were tested in quadruplicate and triplicate, respectively.

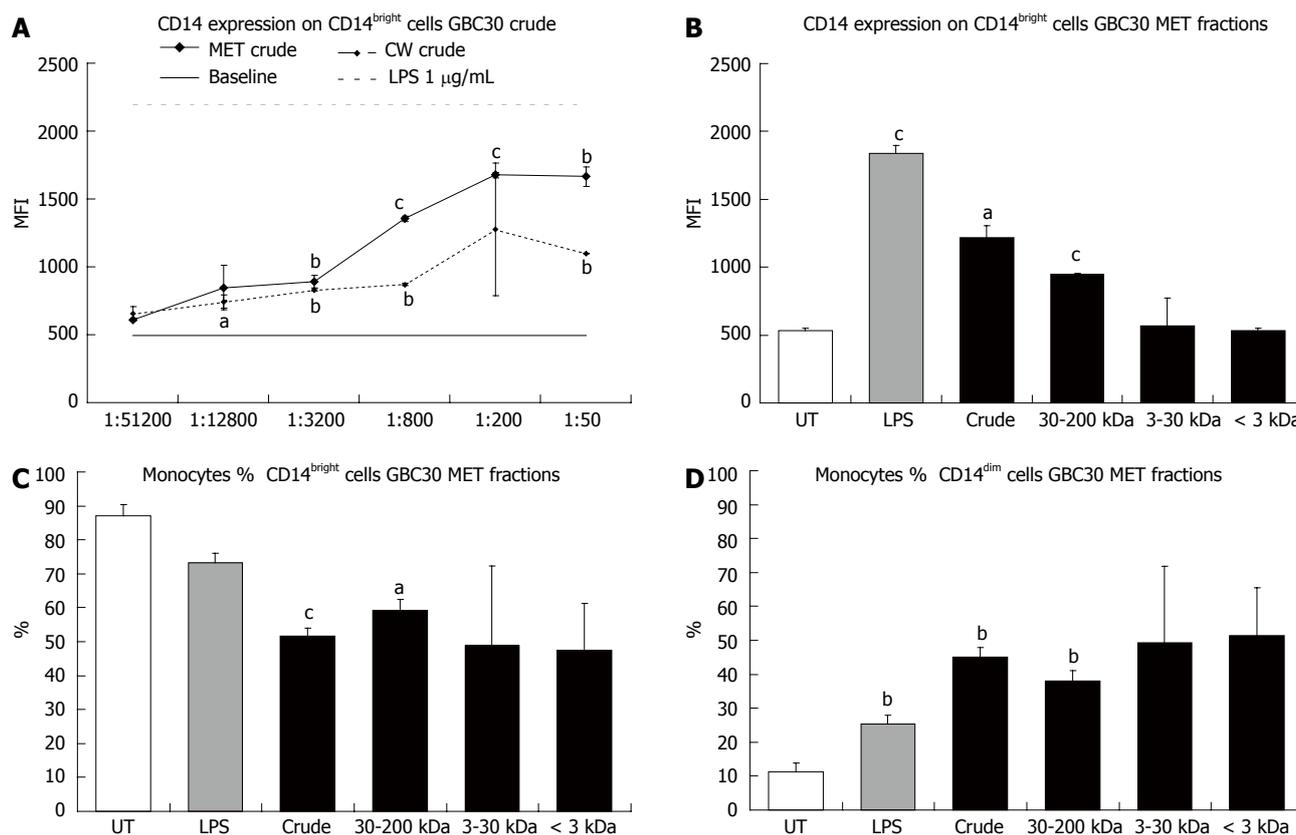
### **Statistical analysis**

Statistical significance was tested using Student's *t*-test performed with Microsoft Excel. All *P* values were two-sided and were considered significant when  $P < 0.05$  and highly significant when  $P < 0.01$ . Only statistically significant *P* values are reported.

## **RESULTS**

### **GBC30 effects on mononuclear phagocyte differentiation**

Mononuclear phagocyte differentiation in 3-d primary PBMC cultures was examined by cell surface staining for proteins expressed by monocytes/macrophages and dendritic cells. These included CD14 (Figure 1) and CD80 and CD86 (Figures 2 and 3). CD14 expression on CD14<sup>bright</sup> cells was increased following exposure of cells to GBC30 crude MET and CW fractions (Figure 1A). Treatment of cells with crude MET showed a strong dose-dependent response with statistically significant increases occurring with the 4 highest doses. As expected, LPS treatment of cells greatly increased CD14 expression<sup>[14]</sup>. Crude CW also led to statistically significant increases in CD14 expression. When the effects of crude and size-selected MET fractions of GBC30 at a 1:200 dilution



**Figure 1 CD14 expression on mononuclear phagocytes.** Mononuclear phagocytes present in 3-d peripheral blood mononuclear cell cultures exposed to either the Ganeden *Bacillus coagulans* 30 (GBC30) metabolites (MET), cell wall-enriched (CW), or MET fractions, were identified using electronic gating of the flow cytometry data by gating on forward scatter/side scatter followed by gating for CD14 positivity. A comparison was made between cells that were untreated (UT), exposed to lipopolysaccharide (LPS) or to the different GBC30 fractions. A: Comparison of CD14 mean fluorescence intensity showed a dose-dependent increase in CD14 expression in cells treated with crude MET. A milder increase was seen for cells treated with crude CW. The baseline indicates CD14 expression on untreated cells; B: The increase in CD14 expression was primarily caused by high molecular weight compounds present in MET; C: The percent of CD14<sup>bright</sup> cells in the mononuclear phagocyte population was decreased by all fractions of MET; D: The percent of CD14<sup>dim</sup> cells in the mononuclear phagocyte population was increased by treatment of cells with all MET fractions. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 and <sup>c</sup>P < 0.001. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity.

were compared (Figure 1B), high molecular weight fractions (crude and 30-200 kDa) increased CD14 expression while PBMC treated with either the 3-30 kDa or < 3 kDa fractions showed CD14 expression levels similar to untreated cultures.

**Reduction of CD14<sup>bright</sup> cells**

Because CD14 expression on mononuclear phagocytic cells varies and expression levels have been correlated with different cell populations, the percent of CD14<sup>bright</sup> versus CD14<sup>dim</sup> cells was determined for PBMC cultures exposed to different GBC30 MET fractions. All fractions of MET at a 1:200 dilution led to decreased numbers of CD14<sup>bright</sup> cells (Figure 1C) while an inverse pattern of response was seen regarding changes in CD14<sup>dim</sup> cell numbers (Figure 1D). In this case all fractions of MET led to increases in the percent of CD14<sup>dim</sup> cells.

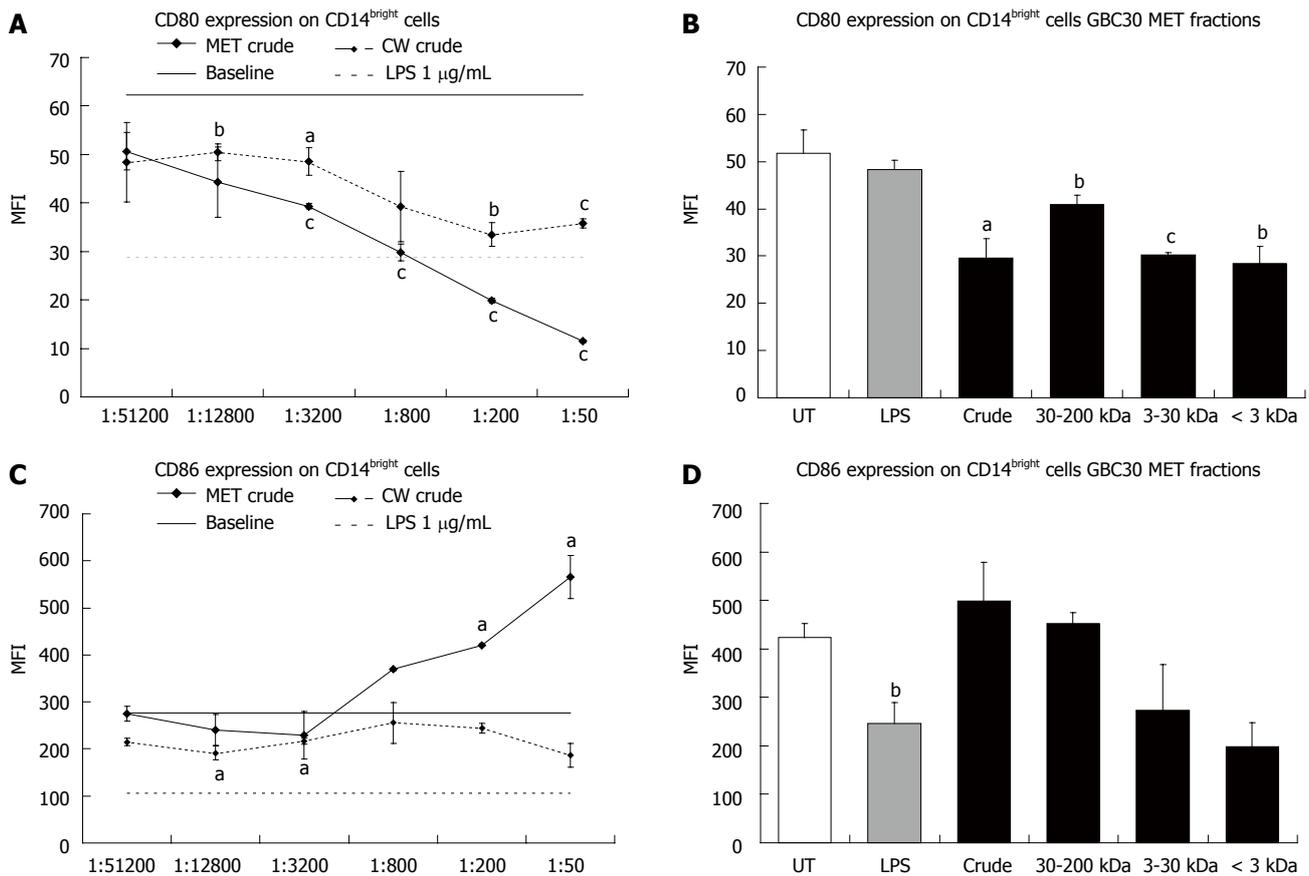
**CD14<sup>bright</sup> cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression**

Next, CD80 and CD86 expression was determined for the CD14<sup>bright</sup> cell population. Both MET and CW crude

fractions led to statistically significant decreases in CD80 expression on CD14<sup>bright</sup> cells (Figure 2A) while only MET crude increased CD86 expression (Figure 2C). When crude and size selected fractions of MET were compared at the 1:200 dilution all fractions of MET led to similar statistically significant decreases in CD80 expression (Figure 2B). A comparison of the effect of MET fractions on CD86 expression showed that the 3-30 kDa and < 3 kDa fractions led to a decrease but these changes were not statistically significant (Figure 2D).

**CD14<sup>dim</sup> cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression**

When expression of the co-stimulatory molecules CD80 and CD86 was determined for the CD14<sup>dim</sup> cell population, both MET and CW crude increased CD80 (Figure 3A) and CD86 (Figure 3C) expression with MET crude having the biggest effect, particularly on CD86 expression. When crude and size selected fractions of MET were compared at the 1:200 dilution, only the crude and 30-200 kDa fractions led to statistically significant increases in CD80 expression (Figure 3B). A comparison



**Figure 2** Expression of the co-stimulatory molecules CD80 and CD86 on CD14<sup>bright</sup> mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14<sup>bright</sup> cells showed dose-dependent decreases in CD80 expression. Both MET and CW reduced CD80 expression to levels similar to those seen with Lipopolysaccharide (LPS) treatment; B: A comparison of the effects of size-fractionated MET on CD80 expression on CD14<sup>bright</sup> cells shows that all MET fractions reduce expression; C: Comparison between the effects of serial dilutions of crude MET or CW on CD86 expression on CD14<sup>bright</sup> cells showed dose-dependent increases in CD86 expression when cells were exposed to the three most concentrated dilutions of MET. Treatment of cells with CW resulted in a uniform modest decrease in CD86 expression; D: The effect on increased CD86 expression is present only in the crude preparation of MET. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 and <sup>c</sup>P < 0.001. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated.

of the effect of MET fractions on CD86 expression showed that crude MET increased CD86 expression while the 3-30 kDa and < 3 kDa fractions decreased expression (Figure 3D).

**Reduction in CD14+ CD16+ cells**

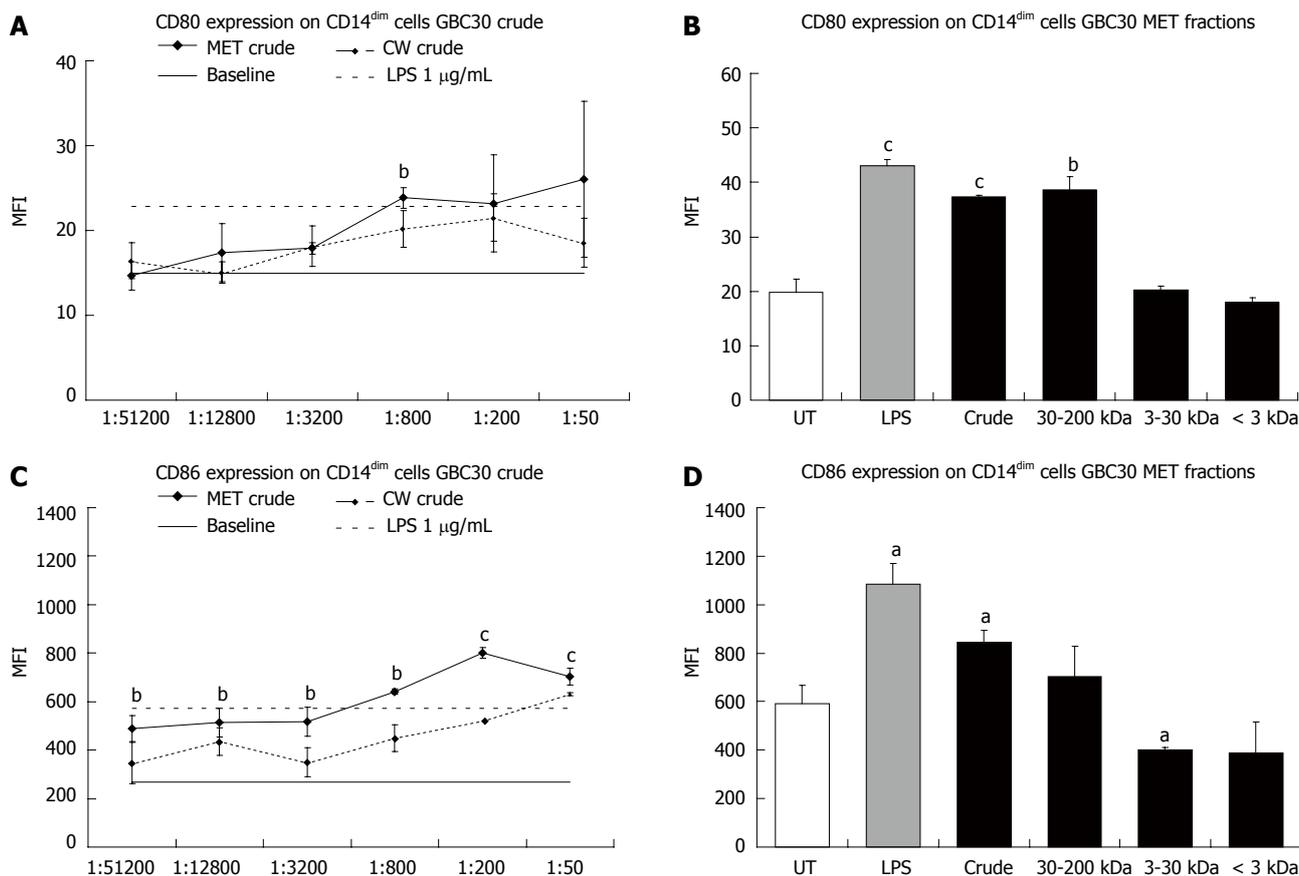
Mononuclear phagocytes have also been classified according to expression of the cell surface protein CD16 with CD14+ CD16+ cell subsets considered to be pro-inflammatory<sup>[15]</sup>. The effect of crude MET and CW fractions on the percent of CD14+ CD16+ and CD14+ CD16- cells in 3-d PBMC cultures was investigated. Crude MET treatment of cells resulted in a dose dependent decrease in CD14+ CD16+ cells (Figure 4A) and an increase in CD14+ CD16- cells (Figure 4C). Crude CW had a much milder effect that mirrored that of crude MET. When cells were exposed to the crude or size fractionated preparations of MET at a 1:200 dilution, it was the fractions with the largest compounds (crude and 30-200 kDa) that showed the greatest effect on CD14+ CD16+ (Figure 4B) and CD14+ CD16- (Figure 4D) cell numbers although

the 3-30 kDa and < 3 kDa fractions also produced statistically significant reductions in the number of CD14+ CD16+ cells.

**DISCUSSION**

The work presented here investigated the effects of MET and CW fractions of the GBC30 probiotic strain on mononuclear phagocyte phenotypes in primary PBMC cultures. The cellular model for examining the immune effects was carefully chosen, and primary PBMC cultures were used because this allows the simultaneous interaction of multiple cell types and has been shown to support the survival of blood dendritic cells without the addition of exogenous cytokines<sup>[16]</sup>. One of the main findings was the biological activities of the metabolites and the data showed that a primary mechanism of action of BC30 metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

Compounds present in the MET crude fraction con-



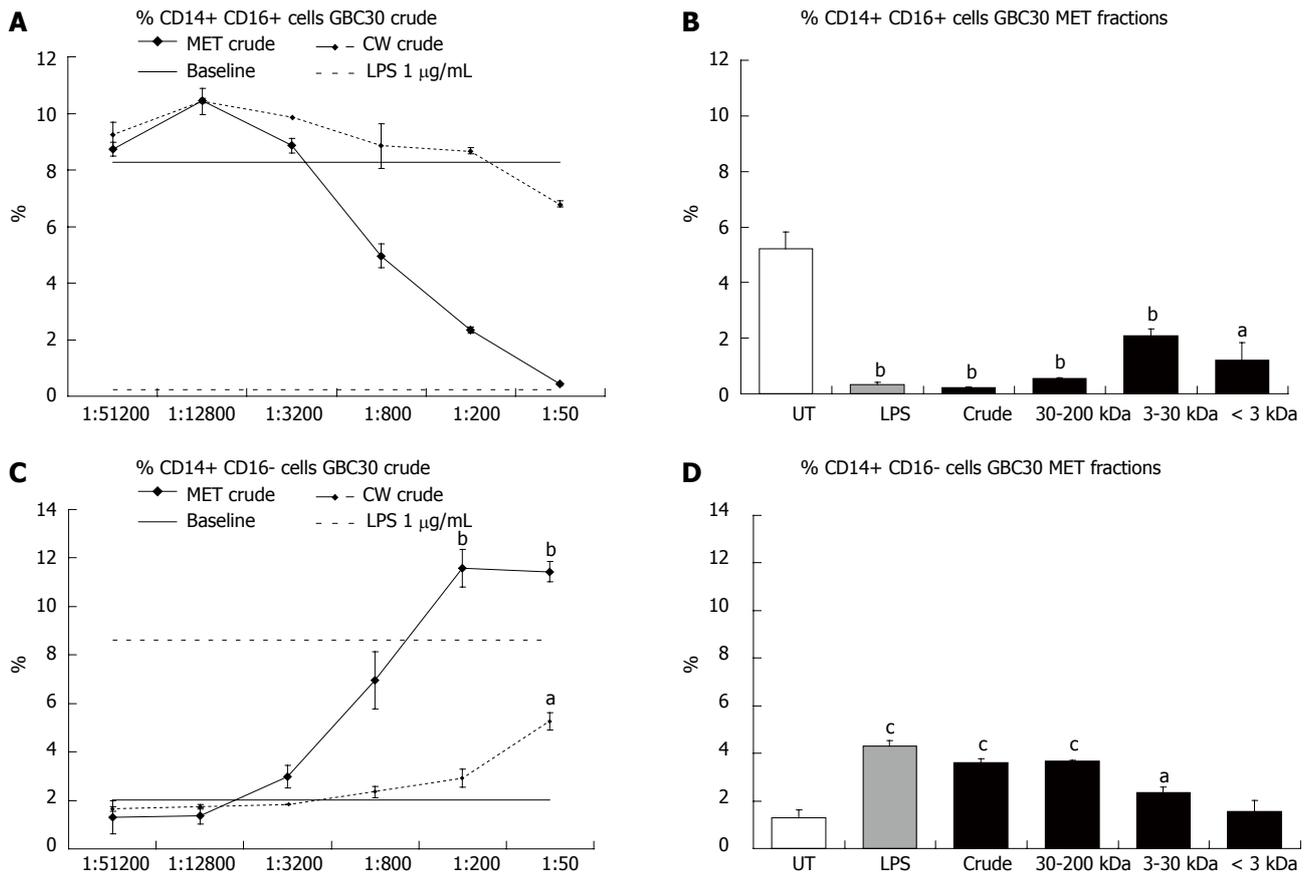
**Figure 3** Expression of the co-stimulatory molecules CD80 and CD86 on CD14<sup>dim</sup> mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14<sup>dim</sup> cells showed that both MET and CW led to increased expression; B: The increase in CD80 expression following treatment of cells with MET was due to high molecular weight compounds; C: Comparison of CD86 expression on CD14<sup>dim</sup> cells exposed to crude fractions of MET or CW resulted in increased CD86 expression; D: Size-selected fractions of MET did not have uniform effects on CD86 expression on CD14<sup>dim</sup> cells. Crude MET increased expression while 3-30 kDa and < 3 kDa fractions decreased expression. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 µg/mL). <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 and <sup>c</sup>*P* < 0.001. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated; LPS: Lipopolysaccharide.

sisted entirely of compounds that were secreted by GBC30 into the culture media. The CW crude fraction was isolated from whole bacteria and may contain some compounds present in the MET preparation in addition to compounds unique to the cell wall. Size fractionation of crude MET was used to evaluate immune modulating compounds based on MW and their association with one or more fractions.

Probiotic organisms support mucosal immunity and similar to commensal bacteria in the human gut, they interact with mononuclear phagocytic cells such as dendritic cells and macrophages<sup>[17-19]</sup>. The expression levels of CD80 and CD86 co-stimulatory molecules can be used to indicate the differentiation of mononuclear phagocytes to that of antigen presenting cells such as dendritic cells. While CD14 is still present on some subsets of dendritic cells, typically when mononuclear phagocytes adopt a dendritic cell identity, CD14 expression is down regulated with the concurrent up regulation of CD80 and CD86<sup>[20]</sup>. The differential roles of the co-stimulatory molecules CD80 and CD86 suggests that co-expression of both molecules on dendritic cells leads to T helper cell differentiation, whereas the predominant expression of CD86 support T regulatory cells, and supports an anti-inflammatory cyto-

kine profile by decreasing Interferon-gamma production and increasing interleukin (IL)-4 production<sup>[21]</sup>. Since the current literature suggests that mononuclear phagocytes present in the circulation are already committed in their developmental path<sup>[22]</sup>, the changes seen in CD14 expression suggest that MET and CW simultaneously enhance the maturation of two separate subpopulations of mononuclear phagocytic cells (CD14<sup>bright</sup> and CD14<sup>dim</sup>) towards their corresponding macrophage and dendritic cell phenotypes. The effect of GBC30 on putative DC maturation in PBMC cultures, suggests that DC may be responsible for the IL-6 production that was previously shown *in vitro*<sup>[12]</sup>, and this increased IL-6 production may reflect normal physiological interactions between DC and commensal bacteria in the human gut<sup>[17,23]</sup>.

The data suggest that live GBC30 in the gut lumen would provide metabolites from GBC30, different from the immune modulating compounds associated with the cell wall enriched fraction, and support the interpretation that the live metabolically active GBC30 has stronger immune modulating activity than accounted for by its cell wall alone. Immune modulating activity has been identified from the supernatant of the probiotic strains *Lactoba-*



**Figure 4** Changes in the percent of CD14+ CD16+ and CD14+ CD16- cell populations following exposure of 3-d peripheral blood mononuclear cell cultures to Ganeden *Bacillus coagulans* 30. A: Exposure of cells to serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) led to a strong dose-dependent decrease in CD14+ CD16+ double positive cells while exposure to cell wall enriched (CW) did not reduce this cell population; B: Treatment of cells with size-selected MET fractions show that all fractions of MET reduce the number of CD14+ CD16+ cells; C: The percent of CD14+ CD16- cells in peripheral blood mononuclear cell cultures increased in cultures treated with crude MET and CW. Treatment with MET resulted in a very strong dose-dependent increase while CW treatment produced a milder increase at the two highest concentrations; D: Treatment of cells with size-selected MET fractions show that only fractions containing high molecular weight compounds increase the number of CD14+ CD16- cells. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 µg/mL). <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 and <sup>c</sup>*P* < 0.001. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. UT: Untreated; LPS: Lipopolysaccharide.

*Lactobacillus casei* Shirota<sup>[24]</sup> and *Bifidobacterium breve*<sup>[25]</sup>, the probiotic yeast *Saccharomyces boulardii*<sup>[26]</sup>, the commensal bacterium *Faecalibacterium prausnitzii*<sup>[27]</sup> and gut-derived lactobacilli and bifidobacteria<sup>[28]</sup>. In the case of *Faecalibacterium prausnitzii*, injection of the supernatant completely protected mice from trinitrobenzenesulphonic acid induced colitis while live bacteria provided only partial protection<sup>[27]</sup>. Most of these studies focused on cytokine production in monocyte-derived dendritic cell cultures<sup>[26,27]</sup> and have determined this to occur through a TLR2 dependent mechanism. In one study, it was determined that the active component in the supernatant from *Lactobacillus casei* was a polysaccharide peptidoglycan complex<sup>[24]</sup> while another study has suggested that the immune boosting effect of common botanical extracts is through effects of bacterial lipoproteins and lipopolysaccharides (derived from endophytes, the resident bacteria present in all plants) on macrophage activation<sup>[29]</sup>.

Thus, due to direct effects on mononuclear phagocyte differentiation, GBC30 metabolites lend support to two important cell types responsible for antigen recognition, presentation to cells within the adaptive immune system,

and execution of regulatory functions, including immunological memory. The effect of dried/reconstituted material was tested in three different bioassays previously reported to show bioactivity<sup>[12]</sup>, including anti-inflammatory effects (data not shown), and no significant difference was seen between this and frozen/thawed material. The stability of the bioactive compounds in the metabolite fraction holds promise for development of a consumable product.

Results from the GBC30 MET fractions suggest that the metabolic activity of this probiotic organism is an integral part of its immune modulating functions, and that multiple different compounds act in synergy to support key aspects of mucosal immune protection. These results suggest specific mechanisms of action and may give insight into some aspects of previous clinical studies showing reduced symptoms from irritable bowel syndrome<sup>[30]</sup>. We suggest that further studies include *ex vivo* evaluation of mononuclear cells isolated from lamina propria and Peyer's patches, in terms of antigen presentation, dendritic cell and B lymphocyte maturation, and IgA production. Further clinical work is warranted, not only

in populations with inflammatory syndromes, but also in populations with reduced mucosal immune protection, and should include assessment of inflammatory markers in serum, as well as secretion of IgA.

In conclusion, the biological activities reported here for the metabolites point to a unified mechanism of action directed at the differentiation and maturation state of antigen-presenting cells such as the macrophage/dendritic cells. In terms of immune regulation, this plays a pivotal role in decision-making, for example in whether T lymphocytes are induced into immunological anergy (unresponsiveness, tolerance) or whether they are triggered into proliferation, cytokine production, and other mechanism of inter-cellular communication. It is conceivable that metabolites are absorbed into the mucosal immune tissue along the intestinal track and help direct more efficient antigen-recognition, while reducing immune reactivity towards harmless food-borne antigens. This may provide a mechanism to explain the improved immune protection, while also seeing a reduction in food allergies and associated inflammatory reactions with consumption of certain probiotic strains.

## COMMENTS

### Background

The mucosal surface of the human gastrointestinal tract is an interface between the external and internal environments, separated by a single epithelial cell layer. On the one side are food antigens, commensal bacteria and potential pathogens while cells of the immune system reside on the other. Oral tolerance refers to the ability of the immune system to not react towards food and commensal bacterial antigens while still evoking a robust immune response towards pathogens. Probiotic bacteria interact with the host immune system and elicit beneficial immune modulating effects that include a reduction in inflammation in inflammatory bowel disease, amelioration of antibiotic-induced diarrhea, and protection from pathogen infection.

### Research frontiers

Recent evidence suggest that the interaction of commensal bacteria and probiotics with the immune system is more than a mechanical engagement of bacterial cell wall components with immune cell receptors and includes an active cross-talk between live bacteria and the host through secreted substances (metabolites). This is an active area of research and data from microbiome genomic sequencing suggests that the majority of predicted genes encode proteins with unknown functions.

### Innovations and breakthroughs

Most of the published work on probiotics interacting with the immune system has focused on the bacterial cell wall activating the immune system through engagement of the Toll-like receptor (TLR) family, in particular TLR2 and TLR4. Much less research has focused on secreted metabolites and very little is known about what these secreted compounds are. The data presented here showed that a primary mechanism of action of Ganeden *Bacillus coagulans* 30 (GBC30) metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making. An immature antigen presenting cell may fail in triggering an appropriate immune defense reaction, while either inducing immunological unresponsiveness (anergy) towards the antigen, or induce an allergic reaction to the antigen.

### Applications

The support of antigen-presenting cells *in vitro* by GBC30 metabolites suggests that consumption of GBC30 may lead to *in vivo* effects of improved decision-making in the gut-associated lymphoid tissue (GALT), translating into clinical observations of improved immunity against infections, and reduced immunological anergy and allergy.

### Terminology

Cluster of differentiation 14 is a monocyte marker and functions as a co-

receptor for bacterial lipopolysaccharide recognition. It is highly expressed on the cell surface of monocytes and macrophages; GALT is a mucosa-associated lymphoid tissue lining the gastrointestinal tract from the esophagus to the colon. It contains immune cells and plays an important part in preventing the immune system from reacting to the resident microflora as well as defence from pathogens; Anergy refers to the absence of a normal immune response to a specific antigen or allergen.

### Peer review

The authors present a paper that aimed to investigate any differences between the effects of a crude preparation of GBC30 bacterial culture metabolites compared to the fractionated preparations on the maturation of peripheral blood mononuclear cell. It demonstrates that probiotic bacteria produce metabolites that activate cells of the immune system, beyond what is expected from simple bacterial cell wall components.

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## Antithrombin III injection *via* the portal vein suppresses liver damage

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### Abstract

**AIM:** To investigate the effects of antithrombin III (AT III) injection *via* the portal vein in acute liver failure.

**METHODS:** Thirty rats were intraperitoneally challenged with lipopolysaccharide (LPS) and D-galactosamine (GalN) and divided into three groups: a control group; a group injected with AT III *via* the tail vein; and a group injected with AT III *via* the portal vein. AT III (50 U/kg body weight) was administered 1 h after challenge with LPS and GalN. Serum levels of inflammatory cytokines and fibrin degradation products, hepatic fibrin deposition, and hepatic mRNA expression of hypoxia-

related genes were analyzed.

**RESULTS:** Serum levels of alanine aminotransferase, tumor necrosis factor- $\alpha$  and interleukin-6 decreased significantly following portal vein AT III injection compared with tail vein injection, and control rats. Portal vein AT III injection reduced liver cell destruction and decreased hepatic fibrin deposition. This treatment also significantly reduced hepatic mRNA expression of lactate dehydrogenase and heme oxygenase-1.

**CONCLUSION:** A clinically acceptable dose of AT III injection into the portal vein suppressed liver damage, probably through its enhanced anticoagulant and anti-inflammatory activities.

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**Key words:** Antithrombin III; Acute liver failure; Intravascular coagulation; Portal vein

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### INTRODUCTION

In some patients with acute liver injury (ALI), the liver disease proceeds to acute liver failure (ALF); a severe condition associated with a high mortality rate. Liver transplantation is an effective treatment for patients with

severe ALF<sup>[1]</sup>, whereas artificial liver support systems such as plasma exchange and hemodiafiltration are less effective<sup>[2,3]</sup>. The difficulties associated with the development of effective treatments for ALF may be attributed to the incomplete understanding of the mechanisms involved in disease progression.

ALF is pathologically characterized by massive hepatocellular necrosis, therefore, intrahepatic microcirculatory disturbances are involved in the pathogenesis and progression of liver disease. Observation of sinusoidal fibrin deposition, increased fibrinogen catabolism and decreased platelet counts suggest that the intrahepatic coagulation system may be activated in ALF, and the following microcirculatory disturbances may play a role in the formation of massive hepatocellular necrosis<sup>[4]</sup>. The hypothesis that activation of the intrahepatic coagulation system is a fundamental pathogenic factor underlying the development of ALF has prompted researchers to develop new treatments using anticoagulants in experimental animal models and in clinical trials. Intravenous injection of antithrombin III (AT III), which inhibits serine proteases involved in the coagulation cascade, has been reported to attenuate the progression of liver disease in animal models of ALF induced by concanavalin A, dimethylnitrosamine (DMN) and endotoxin<sup>[5-8]</sup>. However, the need for high doses of AT III (200-400 U/kg body weight) to suppress liver damage makes it difficult to apply this treatment in clinical practice. Therefore, the development of regimens using clinically acceptable doses of AT III is necessary.

It has been shown that direct drug delivery into the target organs is more efficient than systemic administration. Direct delivery of 5-fluorouracil and cisplatin into the hepatic artery has been reported to control tumor progression and to extend the median survival time of patients with unresectable hepatocellular carcinoma, which is resistant to systemic chemotherapy<sup>[9]</sup>. Similarly, we have reported that the progression of severe ALI toward fulminant liver failure is inhibited by transcatheter arterial steroid injection, in which methylprednisolone is directly delivered into the diseased liver *via* the hepatic artery<sup>[10]</sup>. The effectiveness of direct steroid delivery into the liver has been confirmed in an experimental animal model. Injection of steroids *via* the portal vein in rats with lipopolysaccharide (LPS)- and D-galactosamine (GalN)-induced ALF more effectively suppresses hepatic inflammation and improves survival than injection *via* the tail vein<sup>[11]</sup>. These observations suggest that the direct delivery of AT III into the liver, *via* the hepatic artery or portal vein, may improve liver damage more effectively than peripheral injection of AT III.

In this study, we administered a clinically acceptable dose of AT III *via* the portal vein or a peripheral vein (tail vein) in rats with LPS/GalN-induced ALF. The suppressive effects of AT III on hepatic inflammation were estimated based on the serum levels of transaminase and inflammatory cytokines, and hepatic histology. The extent of damage to the intrahepatic coagulation system was estimated by determining sinusoidal fibrin deposition.

Hypoxia in the diseased liver, which is caused by hepatic microcirculatory disturbances, was estimated by analyzing the hepatic mRNA expression of hypoxia-related genes. These parameters were compared among three groups: a control group; rats injected with AT III *via* the tail vein; and rats injected *via* the portal vein. Our observations suggest that the injection of AT III *via* the portal vein suppresses liver damage more effectively than *via* the tail vein, because of its enhanced anticoagulant and anti-inflammatory activities.

## MATERIALS AND METHODS

### Chemicals

Human concentrated AT III (Anthrabin P500) was purchased from CSL Bering (King of Prussia, PA, United States). LPS (*Escherichia coli*, 055:B5), GalN and other chemicals were purchased from Sigma (St. Louis, MO, United States). All experiments were performed using the same lot of LPS.

### Animals

Eight-week-old male Wistar rats weighing 200 g were purchased from Japan SLC (Hamamatsu, Japan). Rats were maintained under controlled conditions with free access to standard chow and water. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Animal Care Committee of Kyushu University.

### Animal treatment

LPS (5 µg/kg body weight) and GalN (500 mg/kg body weight) dissolved in 500 µL phosphate buffered solution (PBS) were injected intraperitoneally into rats. One hour after the injections, the animals were anesthetized with pentobarbital sodium, and AT III (50 U/kg body weight) dissolved in 200 µL of PBS was injected into the portal or tail vein. Control animals underwent sham injections. Each group consisted of 10 rats.

### Transaminase and cytokine assays

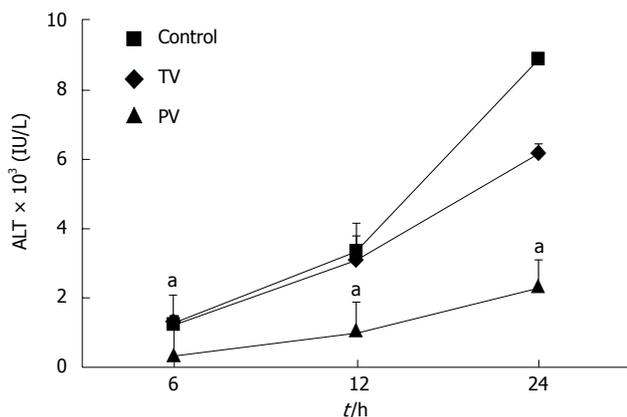
Blood samples were taken from the tail vein at 6 h, 12 h and 24 h after injection of LPS and GalN. The serum levels of alanine aminotransferase (ALT) were estimated by Transaminase C-test (Wako Pure Chemical Industry, Osaka, Japan). Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-6 (IL-6) were measured using enzyme linked immunosorbent assay (ELISA) kits (Endogen, Rockford, IL, United States).

### Assay of serum fibrin degradation products

Blood samples were taken from the tail vein 24 h after injection of LPS and GalN. Serum fibrin degradation products (FDPs) levels were measured using an ELISA kit (Cusabio Biotech, Barksdale, DE, United States).

### Histology

Liver tissue samples were collected 24 h after injecting LPS and GalN, fixed in 10% formalin, and embedded in



**Figure 1** Effects of antithrombin III on serum alanine aminotransferase levels in rats with acute liver failure. Lipopolysaccharide (LPS) and D-galactosamine (GalN) were injected intraperitoneally into 8-wk-old Wistar rats. One hour after the challenge, antithrombin (AT) III (50 U/kg body weight) was injected into the portal or tail vein. Serum alanine aminotransferase (ALT) levels were measured at 6 h, 12 h and 24 h after injection of LPS and GalN. Control: Untreated; TV: AT III injection *via* the tail vein; PV: AT III injection *via* the portal vein. Values are mean  $\pm$  SD ( $n = 10$  rats/group). <sup>a</sup> $P < 0.01$  vs the control group.

paraffin. The sections were stained with hematoxylin and eosin to assess hepatic damage. To determine intrasinusoidal fibrin deposition, the sections were stained with phosphotungstic acid-hematoxylin<sup>[12]</sup>.

### Reverse transcription polymerase chain reaction

Total RNA from liver tissue was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and cDNA was synthesized from 1.0  $\mu$ g RNA by GeneAmp RNA polymerase chain reaction (PCR) (Applied Biosystems, Branchburg, NJ, United States) using random hexamers. Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche, Basel, Switzerland). The reaction mixture (20  $\mu$ L) contained Master SYBR Green I, 4 mmol MgCl<sub>2</sub>, 0.5  $\mu$ mol upstream and downstream PCR primers, and 2  $\mu$ L first-strand cDNA as a template. To control variations in reactions, all PCR data were normalized against glyceraldehyde 3-phosphate dehydrogenase expression. The forward and reverse PCR primers were 5'-ACTTTCAGAAGGGT-CAGGTGTCC-3' and 5'-TTGAGCAGGAAGGCG-GTCTTAG-3', respectively, for heme oxygenase-1 (HO-1) and 5'-AGACTGCCGTCCCGAACAAC-3' and 5'-ACATCCACCAGGGCAAGCTC-3', respectively, for lactate dehydrogenase (LDH), respectively.

### Statistical analysis

All results are expressed as means  $\pm$  SD. Significant differences between two groups were assessed using Wilcoxon's rank-sum test. A value of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Portal vein AT III injection reduced liver cell destruction more effectively than tail vein injection

In the control group, the serum levels of ALT increased

over time, reaching  $1262 \pm 240$ ,  $3381 \pm 808$  and  $8906 \pm 766$  U/L (Figure 1) at 6, 12 and 24 h, respectively. Injection of AT III into the tail vein did not affect ALT levels at 6 h or 12 h after the injection of LPS and GalN. However, at 24 h, the ALT levels in the tail vein injection group were significantly lower than those in the control group ( $8906 \pm 766$  U/L *vs*  $6181 \pm 823$  U/L,  $P < 0.01$ ). This suggests that the suppressive effects of AT III injected *via* the tail vein may be limited to the late stage of liver disease. In contrast, in rats injected with AT III *via* the portal vein, ALT levels were reduced during the early stage (i.e., 6 h,  $369 \pm 141$  U/L), which was maintained at all time-points. At 24 h, the ALT levels in this group were significantly lower than those in the control group ( $2352 \pm 760$  U/L *vs*  $8906 \pm 766$  U/L,  $P < 0.01$ ).

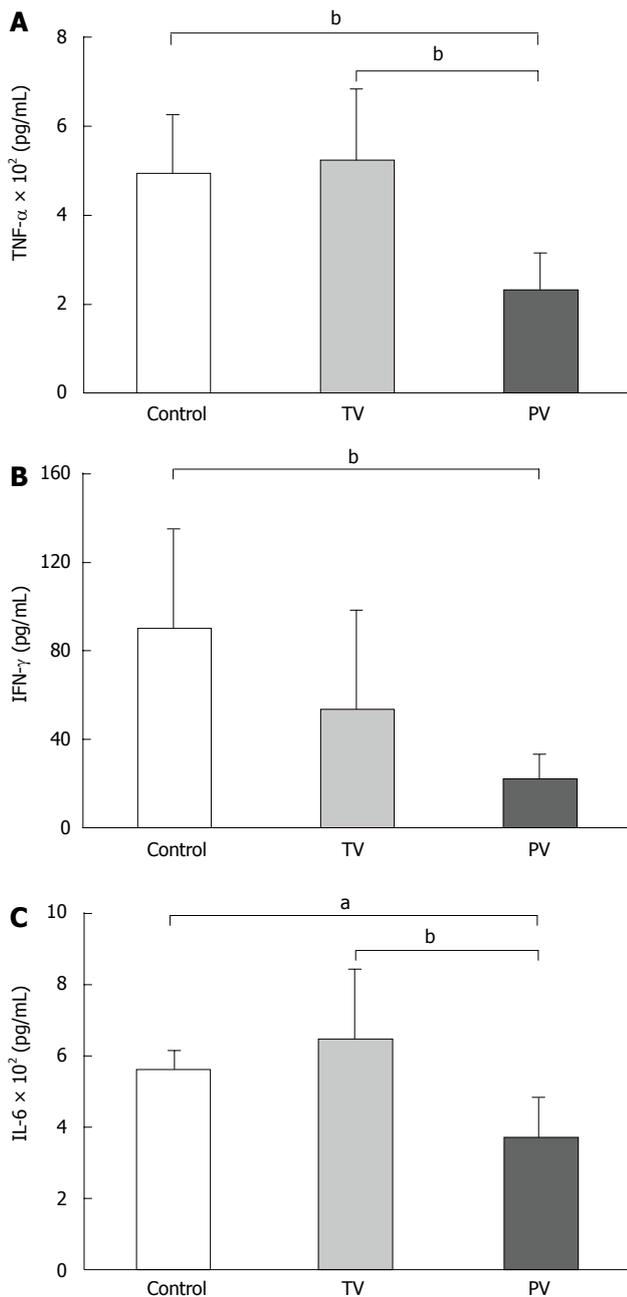
To support the effects of these treatments on the suppression of liver damage, the serum levels of inflammatory cytokines were measured. The cytokine levels demonstrate the greater anti-inflammatory effects of AT III injected *via* the portal vein. TNF- $\alpha$  levels in the tail vein injection group were similar to those in the control group. In contrast, TNF- $\alpha$  levels in the portal vein injection group were significantly lower than those in the control group ( $235 \pm 79$  pg/mL *vs*  $500 \pm 127$  pg/mL,  $P < 0.01$ , Figure 2A). AT III injection *via* the tail vein reduced the IFN- $\gamma$  levels compared with the controls, but the difference was not significant. As with other cytokines, IFN- $\gamma$  was significantly reduced in the portal vein group compared with the control group ( $21 \pm 12$  pg/mL *vs*  $89 \pm 45$  pg/mL,  $P < 0.05$ , Figure 2B). The IL-6 levels showed similar trends to those observed for TNF- $\alpha$ . AT III injection *via* the tail vein did not suppress IL-6 levels, whereas its injection *via* the portal vein significantly reduced IL-6 levels compared with those in the control group ( $368 \pm 120$  pg/mL *vs*  $572 \pm 47$  pg/mL,  $P < 0.01$ , Figure 2C).

### Effects of AT III on liver damage analyzed by liver histology

Histological examination showed extensive hepatocellular necrosis and hemorrhaging in the control liver (Figure 3A). In tail-vein-injected rats, extensive hepatocellular necrosis was not found but areas with confluent necrosis were scattered throughout the liver (Figure 3B). Consistent with the suppression of ALT and inflammatory cytokine levels, prominent histological improvement was noted in the liver of rats injected with AT III *via* the portal vein, because relatively few, scattered areas of necrosis with disordered hepatic cords were observed (Figure 3C).

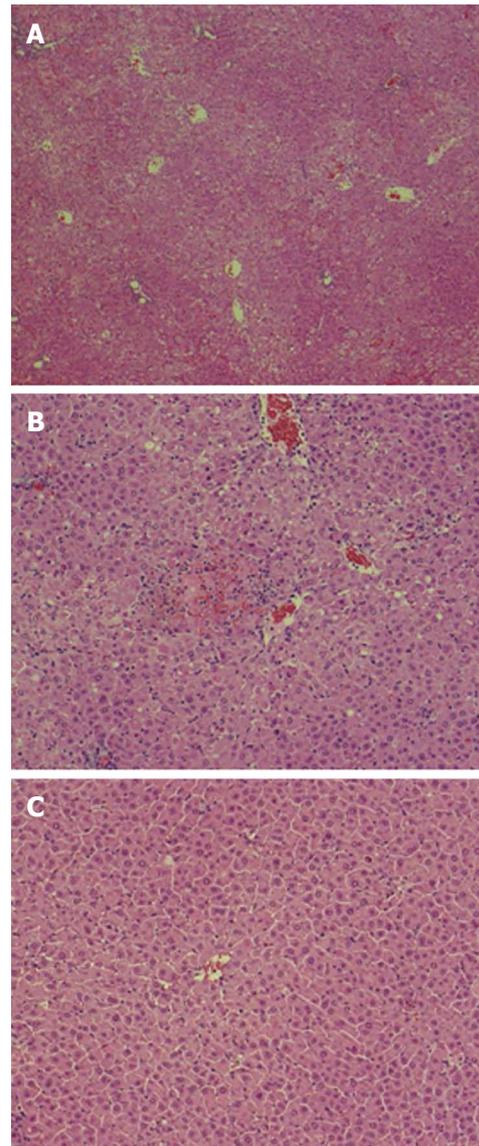
### Effects of AT III on hepatic mRNA expression of hypoxia-related genes in ALF

To evaluate the extent of hepatic hypoxia, as induced by microcirculatory disturbances, we determined the hepatic expression of hypoxia-related genes such as LDH and HO-1. LDH is an essential enzyme for anaerobic respiration, and its expression increases in cells exposed to hypoxia<sup>[13-15]</sup>. The transcriptional expression of HO-1 is also increased in hypoxia, resulting in increased produc-



**Figure 2** Effects of antithrombin III on serum inflammatory cytokine levels. The serum levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (A), interferon- $\gamma$  (IFN- $\gamma$ ) (B) and interleukin (IL)-6 (C) were determined 24 h after injection of lipopolysaccharide and D-galactosamine. Control: Untreated; TV: Antithrombin (AT) III injection *via* the tail vein; PV: AT III injection *via* the portal vein. Values are means  $\pm$  SD ( $n = 10$  rats/group). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group.

tion of carbon monoxide, a vasodilator, and bilirubin, an antioxidant<sup>[16,17]</sup>. Increased serum LDH levels and hepatic expression of HO-1 have been reported in patients with ALF and might be useful to predict prognosis<sup>[18,19]</sup>. Therefore, we speculated that the expression of these genes could reflect the extent of hypoxia in the diseased liver. Tail vein AT III injection did not affect the expression of LDH, suggesting that peripheral administration of AT III does not improve hepatic hypoxia. In contrast, LDH expression was significantly reduced in rats injected with

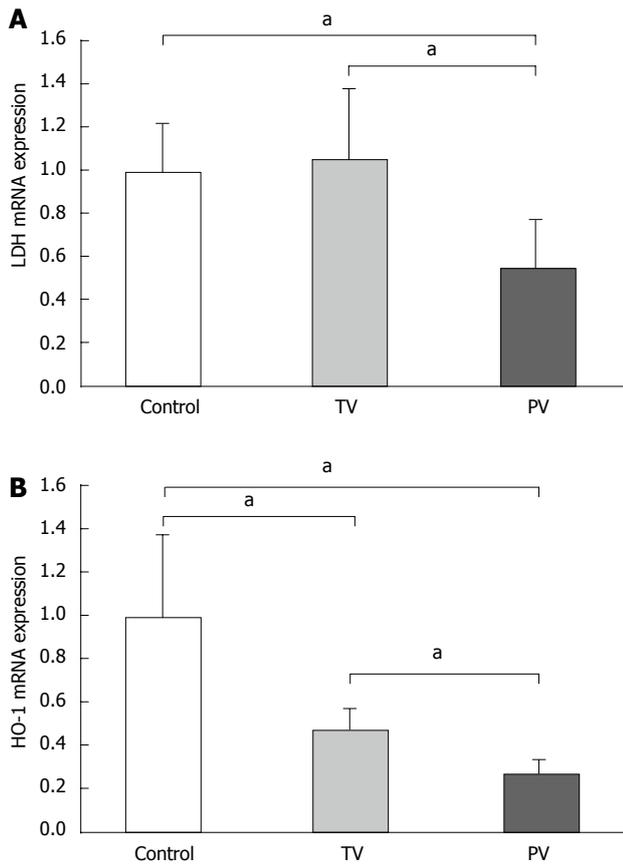


**Figure 3** Effects of antithrombin III on liver histology. Liver samples were obtained 24 h after lipopolysaccharide and D-galactosamine injection and stained with hematoxylin and eosin (magnification,  $\times 200$ ). A: Control; B: Antithrombin (AT) III injected *via* the tail vein; C: AT III injected *via* the portal vein.

AT III *via* the portal vein compared with the tail vein, and the control group (Figure 4A). The expression patterns of HO-1 differed from those of LDH. AT III injection *via* the tail vein significantly reduced HO-1 expression levels compared with those in the control group, and its expression was further reduced by AT III injection *via* the portal vein (Figure 4B). These observations suggest that AT III injection *via* the portal vein reduces intrahepatic hypoxia, probably by controlling the microcirculatory disturbances.

#### **Portal vein injection of AT III injection improved activity of the deteriorated coagulation system**

Serum levels of FDPs are a marker for the extent of deterioration in the coagulation system in ALF<sup>[20,21]</sup>. Injection of AT III *via* the tail vein did not change FDP levels, whereas injection of AT III *via* the portal vein significant-

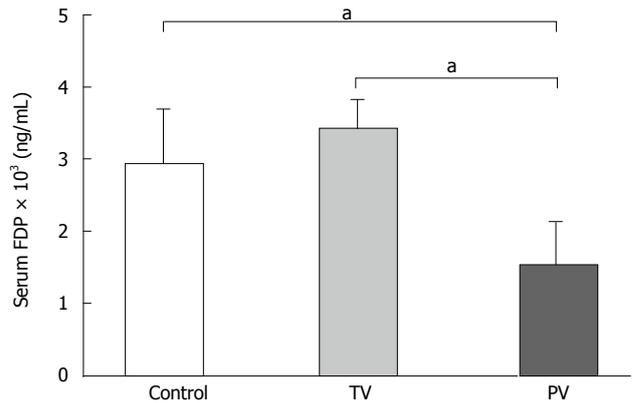


**Figure 4** Effects of antithrombin III on hepatic mRNA expression of lactate dehydrogenase and heme oxygenase-1. Hepatic mRNA expression of lactate dehydrogenase (LDH) (A) and heme oxygenase (HO)-1 (B) was determined by real-time polymerase chain reaction. Reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase expression and the relative expression in the untreated liver was used as a control. Control: Untreated; TV: Antithrombin (AT) III injection *via* the tail vein; PV: AT III injection *via* the portal vein. Values are means  $\pm$  SD ( $n = 10$  rats/group). <sup>a</sup> $P < 0.05$  vs the tail vein group and the control group.

ly reduced FDP levels compared with those in the control and tail vein groups (Figure 5). Taken together, improvements in the coagulation system were only achieved by injecting AT III directly into the diseased liver.

**Effects of AT III on intrahepatic fibrin deposition**

Fibrin deposition in hepatic sinusoids has been observed in ALF. It is recognized as a manifestation of disturbances in the intrahepatic coagulation system and is mainly caused by sinusoidal endothelial cell injury<sup>[22]</sup>. In our study, phosphotungstic acid-hematoxylin staining revealed that fibrin was diffusely deposited in the sinusoids in the control liver, suggesting intrahepatic coagulation (Figure 6A). In rats treated with AT III *via* the tail vein, hepatic fibrin deposition was reduced but it was still sparsely distributed (Figure 6B). Meanwhile, injection of AT III *via* the portal vein diminished fibrin deposition in the liver parenchyma, which suggests that it may affect the maintenance of the intrahepatic coagulation system (Figure 6C).



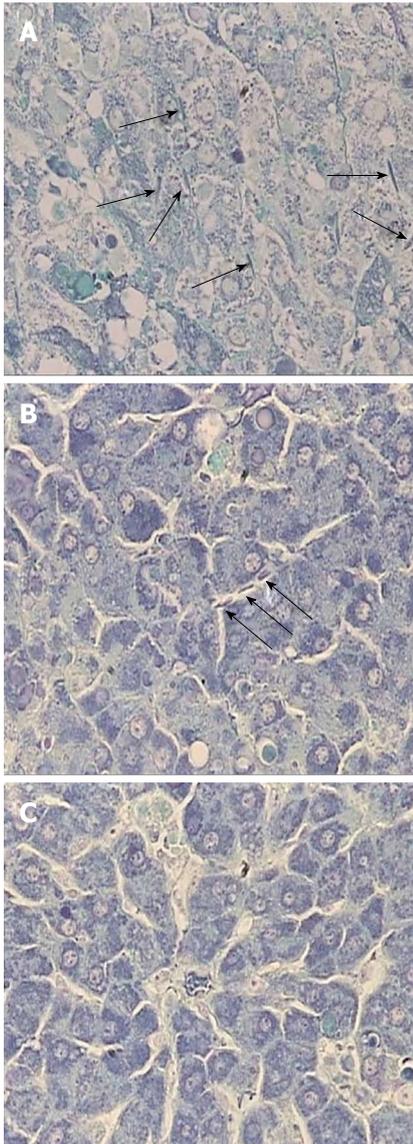
**Figure 5** Effects of antithrombin III on serum fibrin degradation product levels. The serum fibrin degradation product (FDP) levels were determined 24 h after injection of lipopolysaccharide and D-galactosamine. Control: Untreated; TV: Antithrombin (AT) III injection *via* the tail vein; PV: AT III injection *via* the portal vein. Values are means  $\pm$  SD ( $n = 10$  rats/group). <sup>a</sup> $P < 0.05$  vs control and tail vein groups.

**DISCUSSION**

We demonstrated that directly injecting AT III into the portal vein improved liver damage in a rat model of ALF induced by LPS and GalN. AT III injection *via* the portal vein suppressed the increases in serum ALT and inflammatory cytokine levels, and intrahepatic fibrin deposition, and reduced the mRNA expression of hypoxia-related genes associated with ALF. These effects were accomplished by administering a relatively low dose of AT III that should be acceptable in clinical practice.

Microcirculatory disturbances are involved in the pathogenesis of ALF. Activation of the inflammatory cascade may affect the coagulation system and the resulting intrahepatic coagulation may worsen sinusoidal blood flow, leading to massive liver necrosis; a histological feature of ALF<sup>[23]</sup>. Activation of macrophages, as represented by the elevated serum levels of CD163 and osteopontin in ALF patients, seems to be a trigger for this inflammatory process<sup>[24]</sup>. Following the onset of inflammation, disturbances in the intrahepatic coagulation system may enhance the destruction of liver cells. In patients with ALF, electron microscopy has revealed disorders in sinusoidal endothelial cells (SECs)<sup>[25]</sup>. Moreover, reduced mRNA expression of anticoagulants such as tissue factor pathway inhibitor and thrombomodulin are observed in SECs. Following the activation of tissue factor, intrahepatic coagulation causes sinusoidal fibrin deposition and thus restricts blood circulation in the diseased liver<sup>[26-28]</sup>.

Based on these observations, AT III treatment has been tested in animal models of ALF because of its anti-coagulant activity. Systemic injection of AT III (500 U/kg body weight) prevented Con-A-induced liver injury by inhibiting macrophage inflammatory protein-2 release and endothelial cell production of prostacyclin<sup>[7]</sup>. Meanwhile, a similar dose of AT III (400 U/kg body weight) reduced



**Figure 6** Effects of antithrombin III on hepatic phosphotungstic acid-hematoxylin staining. To estimate the extent of intrasinusoidal coagulation, fibrin deposition was analyzed by phosphotungstic acid-hematoxylin staining (magnification,  $\times 400$ ). Fibrin deposition was observed as a dense rod-like structure (arrow). A: Control; B: Antithrombin (AT) III injected *via* the tail vein; C: AT III injected *via* the portal vein.

liver damage in an ALF model with coagulopathy induced by DMN, which was characterized by marked intrasinusoidal fibrin deposition and elevated serum fibrin monomer complexes<sup>[5]</sup>. However, large doses of AT III, which are unacceptable in clinical practice, were used to suppress liver injury in these experimental models.

The effects of AT III for the treatment of ALF patients seem to be limited. Fujiwara *et al.*<sup>[29]</sup> treated 26 patients with fulminant hepatic failure with daily infusion of 3000 U AT III. Notably, survival time was longer in patients with plasma AT III levels within the normal range compared with levels beyond the normal range. However, the survival rates were not significantly different between patients treated with AT III and control patients. Another research group treated 13 ALF patients

with 3000 U AT III, followed by a further 1000 U every 6 h. However, survival time was not improved by AT III and the extent of intravascular coagulation was similar between AT-III-treated and control patients<sup>[30]</sup>. These different outcomes of clinical trials and animal experiments might be due to the insufficient concentration of AT III in the patients' liver. Indeed, the relative doses of AT III per body weight used in patients were  $< 10\%$  of those used in animals. Moreover, even though the plasma concentrations of AT III were maintained within the normal ranges, the survival rate was not improved, which suggests that the intrahepatic concentration of AT III did not reach the levels needed to maintain the integrity of the coagulation system in the diseased liver because of impaired sinusoidal circulation.

From our previous experience of treating ALF rats with methylprednisolone, we have demonstrated that direct delivery of steroid into the liver suppresses liver damage more effectively than does systemic injection. We found that injecting methylprednisolone *via* the portal vein significantly increased the survival rate, reduced serum cytokine levels, and decreased the number of apoptotic liver cells compared with tail vein injection<sup>[11]</sup>. Therefore, we speculated that the anticoagulant activity of AT III would be more effective when injected *via* the portal vein than *via* a peripheral vein. In our study, significant reductions in the serum FDP levels and fibrin deposition were only observed in rats injected with AT III *via* the portal vein, suggesting that direct drug delivery is necessary to achieve therapeutic concentrations of AT III in the diseased liver. Of particular interest is that, using this method, we reduced the dose of AT III to levels acceptable for clinical practice.

Anti-inflammatory activities of AT III have been reported in addition to its anticoagulant activity. In septic patients, AT III improves lung injury by suppressing the production of inflammatory cytokines, and prevents liver and kidney failure<sup>[31,32]</sup>. The mechanisms involved in the anti-inflammatory activities of AT III have been analyzed in rats treated with endotoxin<sup>[33]</sup>. AT III prevents pulmonary vascular injury by inhibiting leukocyte activation mediated by the enhanced release of prostacyclin from endothelial cells. Additionally, AT III has been reported to inhibit the activation of inflammatory signaling cascades in several cell types, including the activation of nuclear factor (NF)- $\kappa$ B in human monocytes and vascular endothelial cells<sup>[34]</sup>; the production of TNF- $\alpha$  and IL-6 in LPS-stimulated murine macrophages<sup>[35]</sup>; and human neutrophil migration<sup>[36]</sup>. In our study, portal vein injection of AT III significantly reduced serum TNF- $\alpha$ , IFN- $\gamma$  and IL-6 levels compared with tail vein injection, and the control group. These results support two possible actions of AT III injected *via* the portal vein to suppress inflammation: (1) the anti-inflammatory activity of AT III was enabled because the tissue concentration reached effective levels following direct drug delivery; and (2) the reduced liver cell destruction mediated by the anticoagulant activity of AT III suppressed the activation of surrounding inflammatory cells. We are currently unable to postulate

which action of AT III might be dominant; however, it seems reasonable to suggest that the anticoagulant and anti-inflammatory activities of AT III may act together to suppress tissue inflammation.

In patients with ALF, it has been reported that micro-circulatory disturbances induce hypoxia in the liver<sup>[18,19]</sup>. Increased serum LDH levels and hepatic HO-1 expression are markers for the extent of hypoxia, reflecting the damage to the hepatic microcirculation. In this study, serum LDH levels were significantly reduced in rats injected with AT III *via* the portal vein, whereas hepatic HO-1 expression was decreased in both groups of rats injected with AT III compared with the control group. Expression of LDH and HO-1 are induced by hypoxia-inducible factor-1, therefore, the different expression patterns of these genes are unlikely to be due to hypoxia-mediated transcriptional regulation<sup>[37]</sup>. In contrast, the expression of HO-1 is transactivated by activator protein-1 and NF- $\kappa$ B, which are transcriptional factors that can activate various inflammatory signals<sup>[38]</sup>. In this context, we speculate that the reduced HO-1 expression in rats injected with AT III *via* the tail vein may partly reflect decreased hepatic inflammation; however, the hypoxia may only be improved by injecting AT III *via* the portal vein.

In conclusion, we demonstrated that injecting AT III *via* the portal vein suppressed liver damage in a rat model of ALF. The increased concentration of AT III in the diseased liver following direct drug delivery might enhance its anticoagulant and anti-inflammatory activities. Furthermore, the dose of AT III used in this method was < 10% of that used in previous studies where AT III was injected *via* peripheral veins. We believe that further studies are needed to establish this method as an effective treatment for ALF.

## COMMENTS

### Background

Acute liver damage occasionally progresses to acute liver failure (ALF) with extremely high mortality. Liver transplantation is the only effective treatment for patients with ALF. Plasma exchange and hemodiafiltration have been used as artificial liver support systems for affected patients but are only partially effective.

### Research frontiers

Intravascular coagulation is thought to be involved in the pathogenesis of ALF. Anticoagulation therapy using antithrombin (AT) III effectively suppresses liver damage in experimental models of ALF; however, extremely high doses of AT III (200-500 U/kg body weight) are necessary. In this study, the authors demonstrated that injection of AT III into the portal vein may help to improve the efficiency of AT III compared with injected into peripheral vein.

### Innovations and breakthroughs

The authors found that injection of AT III *via* the portal vein showed superior effects to those achieved by tail vein injection in terms of lowering the serum levels of transaminase and inflammatory cytokines, reducing damage to the intrahepatic coagulation system, and improving hypoxia in the diseased liver. A clinically acceptable dose of AT III injected *via* the portal vein suppressed liver damage, therefore, direct delivery of AT III into the diseased liver could enhance the anticoagulant and anti-inflammatory activities of AT III.

### Applications

The development of injection of AT III *via* the portal vein might be useful to enhance the effects of AT III and ultimately improve the outcomes of patients with ALF, such as increasing the survival rate and reducing the number of patients

who need liver transplantation.

### Terminology

AT III inhibits serine proteases involved in the coagulation cascade. Additionally, AT III is reported to inhibit activation of inflammatory signaling cascades, including the activation of nuclear factor- $\kappa$ B, production of tumor necrosis factor- $\alpha$  and interleukin-6 in various cell types.

### Peer review

The manuscript is clearly written and the authors discuss their findings in an adequate way. Moreover, the reduction of the dose of AT III *via* injection in the portal vein has clinical implications.

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## Over-expression of uPA increases risk of liver injury in pAAV-HBV transfected mice

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### Abstract

**AIM:** To investigate the relationship between over-expression of urokinase plasminogen activator (uPA) and hepatitis B virus (HBV) related liver diseases in a transgenic mouse model.

**METHODS:** Albumin-tetracycline reverse transcriptional activator and tetO-uPA transgenic mice were generated respectively through pronuclear injection and crossed to produce the double transgenic in-alb-uPA mice, for which doxycycline (Dox)-inducible and liver-specific over-expression of uPA can be achieved. Hydrodynamic transfection of plasmid adeno-associated virus (AAV)-1.3HBV was performed through the tail veins of the Dox-induced in-alb-uPA mice. Expression of uPA and HBV antigens were analyzed through double-staining immunohistochemical assay. Cytokine production was detected by enzyme linked immunosorbent assay and  $\alpha$ -fetoprotein (AFP) mRNA level was evaluated through real-time quantitative polymerase chain reaction.

**RESULTS:** Plasmid AAV-1.3HBV hydrodynamic trans-

fection in Dox-induced transgenic mice not only resulted in severe liver injury with hepatocarcinoma-like histological changes and hepatic AFP production, but also showed an increased serum level of HBV antigens and cytokines like interleukin-6 and tumor necrosis factor- $\alpha$ , compared with the control group.

**CONCLUSION:** Over-expression of uPA plays a synergistic role in the development of liver injury, inflammation and regeneration during acute HBV infection.

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**Key words:** Tet-on system; Albumin promoter; Urokinase-type plasminogen activator; Hydrodynamic transfection; Liver injury

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### INTRODUCTION

Hepatitis B virus (HBV) infection causes a necroinflammatory liver disease of variable duration and severity, with a high risk of developing cirrhosis and hepatocellular carcinoma. The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection<sup>[1]</sup>. However,

the roles of urokinase plasminogen activator (uPA)/uPA's receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China<sup>[2]</sup>. Clinical studies almost focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology<sup>[3]</sup>. In cancer cells, the effects of uPA and uPAR were thought to be related to cell migration<sup>[4]</sup>, metastasis<sup>[5]</sup>, and a more recent role of uPA in cancer growth has emerged<sup>[6]</sup>. The levels of uPA and uPAR have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis, such as in patients with colon adenocarcinoma<sup>[7]</sup>, lymphomas and leukemia<sup>[8]</sup>.

The tetracycline (Tet)-inducible expression system is one of the most prominent and widely-used systems, which allows relatively stringent, reversible, and quantitative regulation of transgene expression in a wide range of cells in culture as well as in transgenic animals<sup>[9,10]</sup>. It consists of two parts: the ligand-dependent transactivator tetracycline reverse transcriptional activator (rtTA) as the effector and a tetO-cytomegalovirus (CMV) minimal promoter cassette regulating the expression of the transgene as the responder<sup>[11]</sup>. When doxycycline (Dox) is present, rtTA binds to the tetO-sequence and induces expression of the target gene<sup>[12]</sup>. Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

In this study, an effective inducible and liver specific uPA expression mouse model was constructed in which the murine uPA expression was controlled by rtTA which is regulated by murine albumin enhancer/promoter. Through administration of Dox, the inducible expression of uPA specifically in mouse liver can be achieved with lower mortality. Then hydrodynamic injection of pAAV-1.3HBV, which contained inverted terminal repeat elements of adeno-associated virus (AAV) and 1.3 copies of HBV genome (ayw subtype), was performed to mimic the acute HBV infection. The mouse liver showed specific and inducible expression of uPA. Plasmid AAV-1.3HBV transfection in Dox-induced transgenic mice resulted in severer liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the over-expression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection.

## MATERIALS AND METHODS

### Plasmid construction

For liver-specific expression of rtTA, the transgenic construct albumin-rtTA was generated, which has rtTA gene

under the control of the liver-specific albumin promoter and was based the plasmid pTet-on (Clontech Lab, Inc). To introduce appropriate restriction sites in pTet-on, linker sequences were designed as follows, Tet-on-linker-F: 5'-CTAGGATATCACTAGTGGTACCGGGCCCGC-GGCCGCG-3' and Tet-on-linker-R: 5'-AATTTCGCG-GCCGCGGGCCCGGTACCACTAGTGATATC-3'. The linkers were annealed at 95 °C for 10 min and then were digested with *EcoR* I and *Spe* I and ligated to pTet-on digested with the same restriction enzymes, and the construct was named pTet-on-link. The albumin promoter fragment and enhancer fragment (Genbank accession no. AC140220.4) were separately amplified by polymerase chain reaction (PCR) using genomic DNA extracted from C57BL/6 mouse liver as the template. The primers for albumin enhancer were Alb-En-FP: 5'-GCCGAGCTCCTGCCGGCTAGCTTCCTTAGCATG-3' and Alb-En-RP: 5'-GGGTTAAGGATCCCAAG CTGGAG-3'. The primers for albumin promoter were Alb-Pro-FP: 5'-CGGGATCCACAGCTCCAGATGGCAAACATAC-3' and Alb-Pro-RP: 5'-TTTGC-CAGAGGCTAGTGGG GTTG-3'.

The albumin enhancer PCR product was digested with *BamH* I and cloned into pGEM-7ZF, then the albumin promoter sequence was inserted behind the enhancer at the site of *BamH* I, and the plasmid was named p7ZF-Albumin, which was confirmed by restriction enzyme digestion analysis and DNA sequence analysis. Then p7ZF-Albumin was digested by *Sac* I and *Kpn* I, and the released 2233bp fragment was ligated to pTet-on-link digested by *EcoR* V and *Kpn* I, to yield the recombinant construct named pTet-on-Albumin.

For rtTA responsive expression of uPA, the transgenic construct pTRE<sub>2</sub>-uPA was generated which is based on the plasmid pTRE<sub>2</sub> containing tetO. The uPA cDNA and uPA exon 11 was amplified by reverse transcription polymerase chain reaction (RT-PCR) and PCR from the total RNA and genomic DNA extracted from the kidney of C57BL/6 mouse, respectively. For uPA DNA, the primers were uPA-cDNA-F: 5'-CGGGATCC ATGAAAGTCTGGCTGGCGAGCCTG-3' and uPA-cDNA-R: 5'-CGGTCCGACCATCAGAAGGCC-CAGACCTTCTCTTC-3'. The RT-PCR product was ligated to pMD18T and the construct pMD18T-uPA cDNA was confirmed by sequence analysis. The uPA exon 11 was amplified by PCR from the genomic DNA template, the primers were uPA-3'-F: 5'-CGGTCCGAC-GCCCTCAGGTAGCTGAGGGAAG-3' and uPA-3'-R: 5'-CGGTCCGACGTGAAACCGACATTTAGT-GCTAGTC-3'. The PCR product was ligated to the *Sal* I site of pMD18T-uPA cDNA to yield the construct pMD18T-uPA and sequence was confirmed. Then the uPA (cDNA + exon 11) fragment was subcloned into the *Pvu* II and *Xba* I sites of pTRE<sub>2</sub> to yield pTRE<sub>2</sub>-uPA.

### Generation and PCR analysis of the albumin-rtTA and tetO-uPA transgenic mice

The albumin-rtTA and tetO-uPA transgenic mice were generated in C57BL/6 × CBA F1 zygotes using standard

pronuclear injection, which was performed by Shanghai Research Center for Biomedicine. For microinjection, the 6034 bp fragment of transgene albumin-rtTA and the 5739 bp fragment of transgene tetO-uPA were excised from the vector backbone of pTet-on-albumin by *Xho* I digestion and pTRE2-uPA by *Pvu* I digestion, respectively, isolated and purified using QIA quick gel extraction kit (Qiagen), and then microinjected into the pronuclei of one cell-stage fertilized embryos. The DNA injected fertilized eggs were implanted into the oviducts of 12 pseudopregnant recipient mice. All together 9 positive albumin-rtTA transgenic mice and 5 tetO-uPA positive ones were confirmed by PCR. One upstream pair and one downstream pair of primers, which were designed to amplify the sequences between vector and inserted fragment were designed for albumin-rtTA as follows, 1-up-F: 5'-GTGCAGCTTGGCTTGAACCTCGTTC-3'; 1-up-R: 5'-GAGTATGGTGCCTATCTAACATCTC-3'; 1-down-F: 5'-GACGCGCTAGACGATTTTCGATCTG-3'; 1-down-R: 5'-ACCTTGCACAGATAGCGTGGTC-3'. By the same way, one upstream pair and one downstream pair of primers were designed for tetO-uPA as follows 2-up-F: 5'-GTTTAGTGAACCGTCAGATCGCCTG-3'; 2-up-R: 5'-CTAGGCTAATAGCATCAGGTCTG-3'; 2-down-F: 5'-GGTAGCTTAGGAGTAGAGACACT-3'; 2-down-R: 5'-GACAATGTTGTCAACAGAGTAG-3'. The PCR conditions were the same for each of the primer pairs: 34 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s. Genomic DNA from wild-type mice was amplified as negative control. The PCR positive mice were the transgenic founders.

#### Mouse propagation and PCR analysis

At 6-8 wk of age, founder mice were backcrossed with wild-type C57BL/6J mice to generate F1. Genomic DNA were isolated from tail biopsy samples of F1 mice at 4 wk and analyzed by PCR, for which the protocols were mentioned above.

#### rtTA expression in different tissues of albumin-rtTA F1 transgenic mice

The isolation of total RNA from different tissues of 6-8 wk old F1 PCR-positive and negative offsprings of the founders was performed using the RNeasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40 µL DNA-free water. 400 ng of total RNA reverse transcribed with the Takara RNA LA PCR Kit (AMV)Ver1.1 (TaKaRa), the reaction condition was 30 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, 5 °C for 5 min. The oligonucleotide primers used for RT-PCR were rtTA-F: 5'-GACGCGCTAGACGATTTTCGATCTG-3'; rtTA-R: 5'-ACCTTGCACAGATAGCGTGGTC-3', the PCR reaction condition was 34 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control, the primers were GAPDH-F: 5'-TTCACCACCATG-GAGAAGGC-3' and GAPDH-R: 5'-CCTCAGTGTAGCCCAAGATGC-3', PCR reaction condition was 34 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s.

The total protein was isolated from different tissues of 6-8 wk old F1 PCR-positive and negative offsprings of the founders by using the Tissue Lysis Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate) supplemented with aprotinin (2 µg/mL), pepstatin A (0.7 µg/mL), leupeptin (0.5 µg/mL), phenylmethanesulfonyl fluoride (PMSF) (1 mmol/L). Aprotinin, Pepstatin A, Leupeptin, PMSF were purchased from Amresco. For Western blotting analysis, 25 µg of the total protein was used for each loading; the primary antibody for rtTA was TetR monoclonal antibody (Clontech) (used in 1:1000 dilution), and the primary antibody for GAPDH was an anti-GAPDH polyclonal antibody (Sigma) (1:10000 dilution); and the second antibodies were HRP-labeled goat anti-mouse IgG and goat anti-rabbit IgG (both in 1:5000 dilution), respectively. For imaging results, the SuperSignal WestDura Trial Kit (Pierce) was used following the instructions.

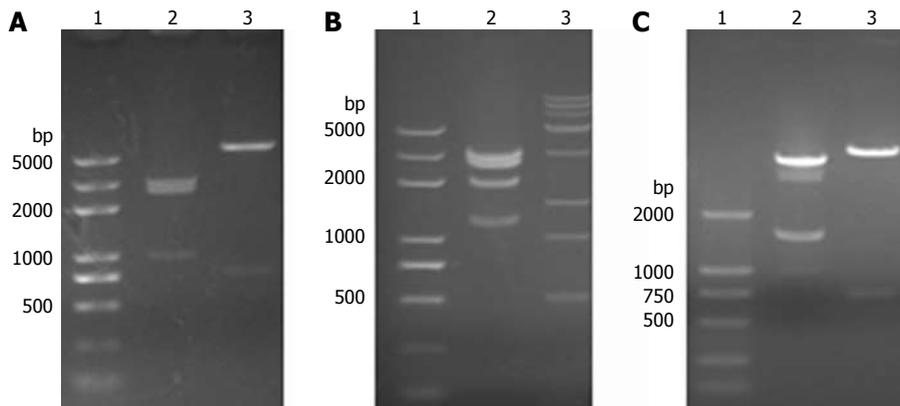
#### Generation of double transgenic mice in-alb-uPA and Doxycycline administration

Double transgenic in-alb-uPA and wild type female offspring were generated from a cross between the albumin-rtTA F1 transgenic positive mice and the tetO-uPA F1 positive mice. 20 d after born, these mice were given two intramuscular injection of 2 mg Dox in 0.2 mL 0.9% NaCl-solution each week for a period of 3 wk. Another group of each type of mice was maintained off doxycycline administration.

#### Hydrodynamic transfection of AAV-HBV and histological analysis

After 3 weeks' induction, a 20 µg pAAV-HBV1.3 DNA was injected hydrodynamically into the tail veins of the in-alb-uPA mice within 5 seconds. A control group of in-alb-uPA mice was injected with pAAV-internal ribosome entry site (IRES). At 20 d post transfection, mice were sacrificed and the livers were fixed with 4% (v/v) phosphate-buffered formalin, and paraffin-embedded liver sections were prepared and stained with hematoxylin and eosin. Semi-quantitative assessment of liver injury in each group was evaluated by the area of liver necrosis on the whole slide in each group. NP for no necrosis; P1 for < 10% area of necrosis; P2 for 10%-30% area of necrosis; P3 for > 30% area of necrosis. All the evaluation of liver damage was conducted by two independent observers. The average score of three mice in each group was taken as score for that group.

For uPA and HBV antigens detection, the expression of uPA protein and hepatitis B core antigen (HBcAg) were identified by double-staining with a polyclonal rabbit anti-rodent urokinase (uPA) antibody (American diagnostica Inc) and monoclonal anti-HBcAg antibody (Thermo Scientific). Diamino-benzidine and alkaline phosphatase substance (ZhongShan Goldenbridge biotech, Beijing, China) were used to visualize the uPA and HBV antigens.



**Figure 1** Identification of pTet-on-link, pTet-on-Albumin and pTRE<sub>2</sub>-urokinase plasminogen activator by restriction endonucleases. A1 and B1: 2K plus DNA Marker; A2: pTet-on-link/*EcoR* V + *Sal* I; A3: pTet-on-link/*EcoR* V + *Bam*H I; B2: pTet-on-Albumin/*Bam*H I; B3: 15K DNA marker; C1: 2K DNA marker; C2: pTRE<sub>2</sub>-urokinase plasminogen activator (uPA)/*pvu* II; C3: pTRE<sub>2</sub>-uPA/*Sal* I.

### Enzyme linked immunosorbent assay for HBV antigens and cytokine production

At 10 d and 20 d post transfection, mouse serum samples from different groups were harvested. The HBeAg and HBsAg enzyme linked immunosorbent assay (ELISA) kit (Wantai Biotech, Beijing) were used for the detection of the serum HBV antigens respectively. And interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  ELISA kit (Dakewei Biotech, Beijing) were used for the quantitation of the serum cytokines. Serum ALT were measured with an Olympus Model 640 automated analyzer.

### qRT-PCR analysis of $\alpha$ -fetoprotein mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice

The isolation of total RNA from livers of the AAV-HBV transfected in-alb-uPA mice was performed using the RNeasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40  $\mu$ L DNA-free water and 400 ng of total RNA in a 10  $\mu$ L reaction mixture was reverse transcribed with the Takara RNA LA PCR Kit (AMV) Ver1.1 (TaKaRa). Relative quantization of the  $\alpha$ -fetoprotein (AFP) mRNA level was performed using RNA Master SYBR Green1 (Roche Diagnostics) by Eppendorf Replex. The primers used for amplification were AFP-real-F: 5'-TCT-GCTGGCACGCAAGAAG-3' and AFP-real-R: 5'-TCG-GCAGGTTCTGGAAACTG-3'. GAPDH serves as a control and the primers were GAPDH-real-F: 5'-TCAC-CACCATGGAGAAGGC-3' and GAPDH-real-R: 5'-GC-TAAGCAGTGTGGTGGTGCA-3'. The amplification conditions included initial denaturation at 95  $^{\circ}$ C for 2 min, followed by 40 cycles of denaturation at 95  $^{\circ}$ C for 15 s, annealing at 55  $^{\circ}$ C for 15 s, extension at 68  $^{\circ}$ C for 30 s.

### Statistical analysis

Results are expressed as mean  $\pm$  SE. Statistical analysis was performed using Student's *t* test.

## RESULTS

### Construction and identification of pTet-on-albumin and pTRE<sub>2</sub>-uPA

For inserting the albumin enhancer and promoter se-

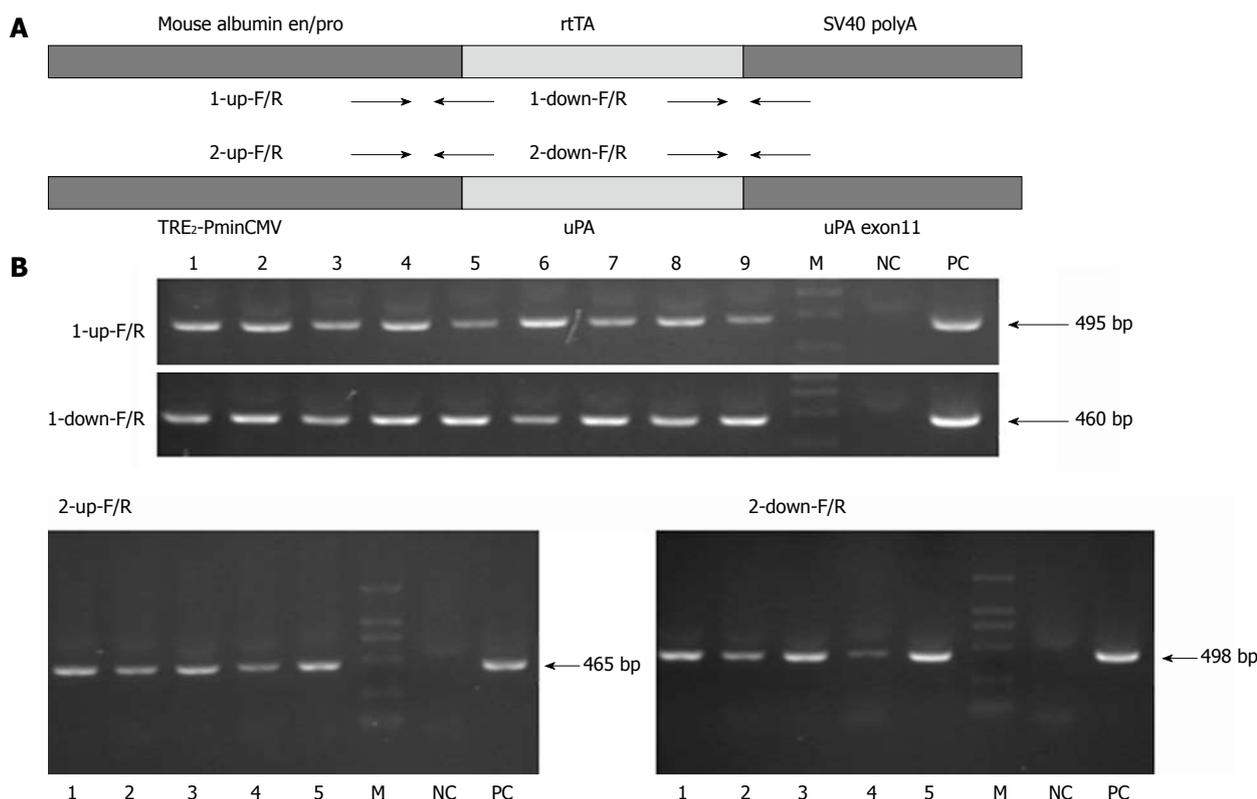
quence into pTet-on in place of the CMV promoter, a linker as designed and the following restriction sites were introduced: *Spe* I, *EcoR* V, *Spe* I, *Kpn* I, *Apa* I, *Not* I, *EcoR* I. pTet-on-link was identified by *EcoR* V/*Sal* I and *EcoR* V/*Bam*H I double digestion respectively (Figure 1A), and results showed that the linker was introduced into pTet-on. pTet-on-Albumin was identified by *Bam*H I digestion (Figure 1B), the expected 5022 bp, 2689 bp and 1284 bp fragment can be observed. pTRE<sub>2</sub>-uPA was identified by *pvu* II and *Sal* I respectively (Figure 1C). In addition, DNA sequence analysis of the albumin enhance/promoter and uPA sequence shows complete accordance with those in the National Center for Biotechnology Information database (data not shown).

### Generation of the albumin-rtTA and tetO-uPA transgenic mice and PCR analysis

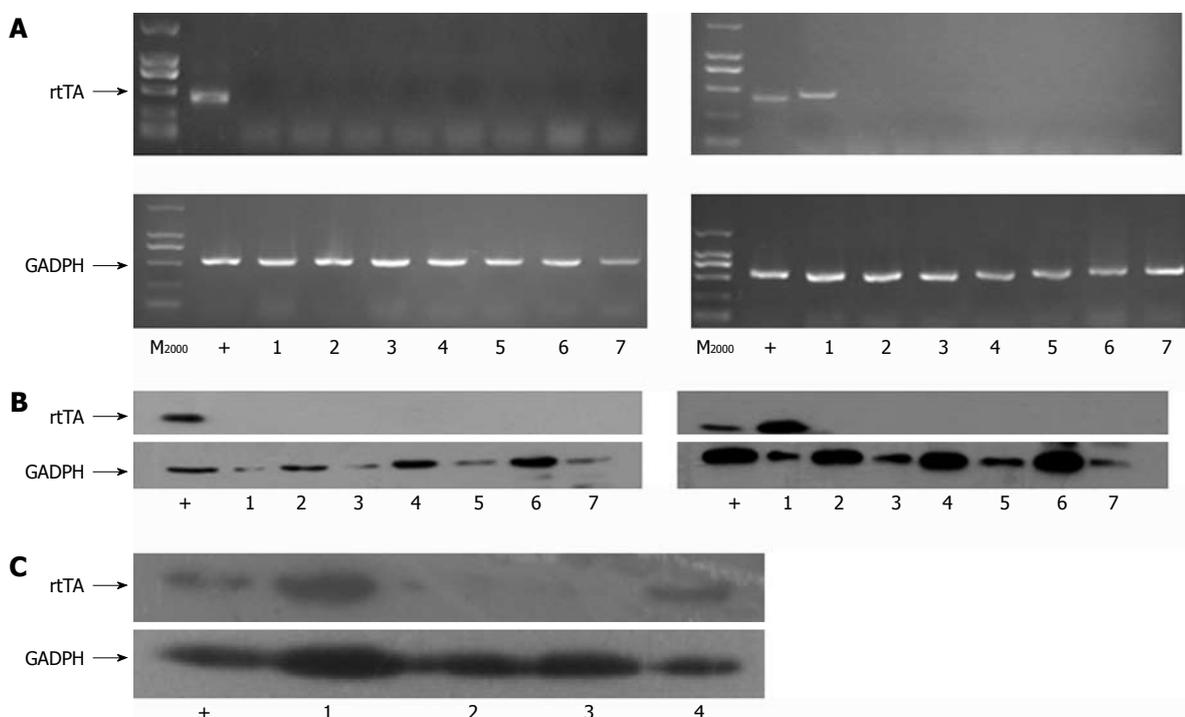
The albumin-rtTA expression unit contains the mouse albumin enhancer/promoter, rtTA coding sequence and SV40 polyA, and the tetO-uPA expression unit contains the TRE<sub>2</sub>-PminCMV, uPA cDNA and uPA exon11 (Figure 2A). In the end, 9 albumin-rtTA transgenic founder mice and 5 tetO-uPA transgenic founder mice were confirmed positive by PCR for both the upstream and downstream primers (Figure 2B).

### Specific expression of rtTA in the livers of albumin-rtTA and in-alb-uPA transgenic mice

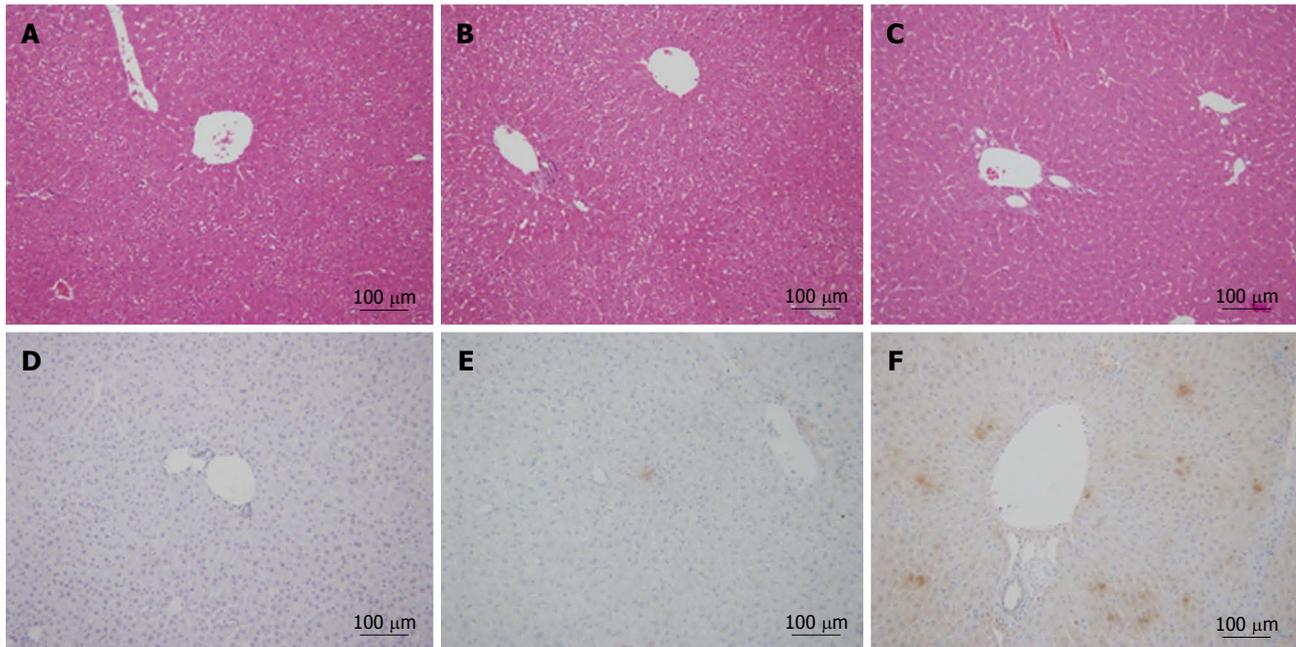
To identify the liver-specific expression of uPA in the livers of transgenic mice, RT-PCR and Western blotting analysis was performed. The data showed that rtTA mRNA expressed specifically in the livers of F1 albumin-rtTA transgenic positive mice (Figure 3A, right image), while there was no rtTA mRNA expression in all the tissues of the albumin-rtTA transgenic negative mouse (Figure 3A, left image). GAPDH mRNA was expressed equally in different tissues of these mice (Figure 3A). Results from Western blotting analysis (Figure 3B) were in accordance with those from RT-PCR analysis. By Western blotting analysis, rtTA expression was also confirmed specifically in the livers of in-alb-uPA transgenic mice and albumin-rtTA transgenic mice (Figure 3C). The cell



**Figure 2** Establishment of albumin-tetracycline reverse transcriptional activator and tetO-urokinase plasminogen activator transgenic mice. A: The albumin-tetracycline reverse transcriptional activator (rtTA) unit contains the mouse albumin enhancer/promoter, rtTA coding sequence, and SV40 polyA. The tetO-urokinase plasminogen activator (uPA) unit contains the TRE<sub>2</sub>-PminCMV, uPA cDNA, uPA exon11. Arrowheads depict the positions and directions of the polymerase chain reaction (PCR) primers; B: PCR identification of the transgenic founders. 1-9, PCR identification for the nine albumin-rtTA transgenic founder mice; 1-5, PCR identification for the five tetO-uPA transgenic founder mice. CMV: Cytomegalovirus; M: Marker; NC: Negative control; PC: Positive control.



**Figure 3** The specific expression of tetracycline reverse transcriptional activator in the livers of albumin-tetracycline reverse transcriptional activator transgenic mice and in-alb-urokinase plasminogen activator transgenic mice. A, B: Reverse transcription polymerase chain reaction (A) and Western blotting (B) analysis of tetracycline reverse transcriptional activator (rtTA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in different tissues of the 6-8 wk old F1 albumin-rtTA PCR-negative (left for A, B) or positive (right for A, B) transgenic mice. 1: Liver; 2: Brain; 3: Thymus; 4: Heart; 5: Lung; 6: Kidney; 7: Spleen; C: Western blotting analysis of rtTA and GAPDH expression in the liver extracts of mice with different genotypes. 1: In-alb-urokinase plasminogen activator (uPA) mice group; 2: Liver extracts of wild type mice group; 3: tetO-uPA mice group; 4: Albumin-rtTA mice group. pTet-on transfected Huh7 cell extracts were used as positive control (+).



**Figure 4** The expression of urokinase plasminogen activator in the livers of in-alb-urokinase plasminogen activator transgenic mice. A: Histology of livers from wild type (WT) mice; B: Histology of livers from in-alb-urokinase plasminogen activator (uPA) mice without doxycycline (Dox) induction; C: Histology of livers from in-alb-uPA mice with Dox induction; D: uPA expression in hepatocytes from WT mice; E: uPA expression in hepatocytes from in-alb-uPA mice without Dox induction; F: uPA expression in hepatocytes from in-alb-uPA mice with Dox induction. Magnification,  $\times 20$ .

extract from Huh7 transfected with pTet-on was used as positive control while tetO-uPA and WT mice served as negative control.

#### **Histological change of liver in in-alb-uPA transgenic mice after Dox induced uPA expression**

To confirm the expression of uPA in liver and its role on the hepatocytes, uPA expression and the histological changes of liver was analyzed with immunohistochemistry and HE staining respectively. The results showed light degeneration of hepatocytes and mild inflammation in the livers with in-alb-uPA double transgenic mice after 3 wk of Dox induction when compared to that of double transgenic mice without Dox or the control group mice (Figure 4A-C), which was coincident with that of uPA expression with immunohistochemistry in the livers of in-alb-uPA double transgenic mice after Dox induction while almost no expression of uPA detected in double transgenic mice without Dox. The data showed that the specific expression of uPA after Dox induction induced slight histological changes in the liver of this in-alb-uPA double transgenic mice.

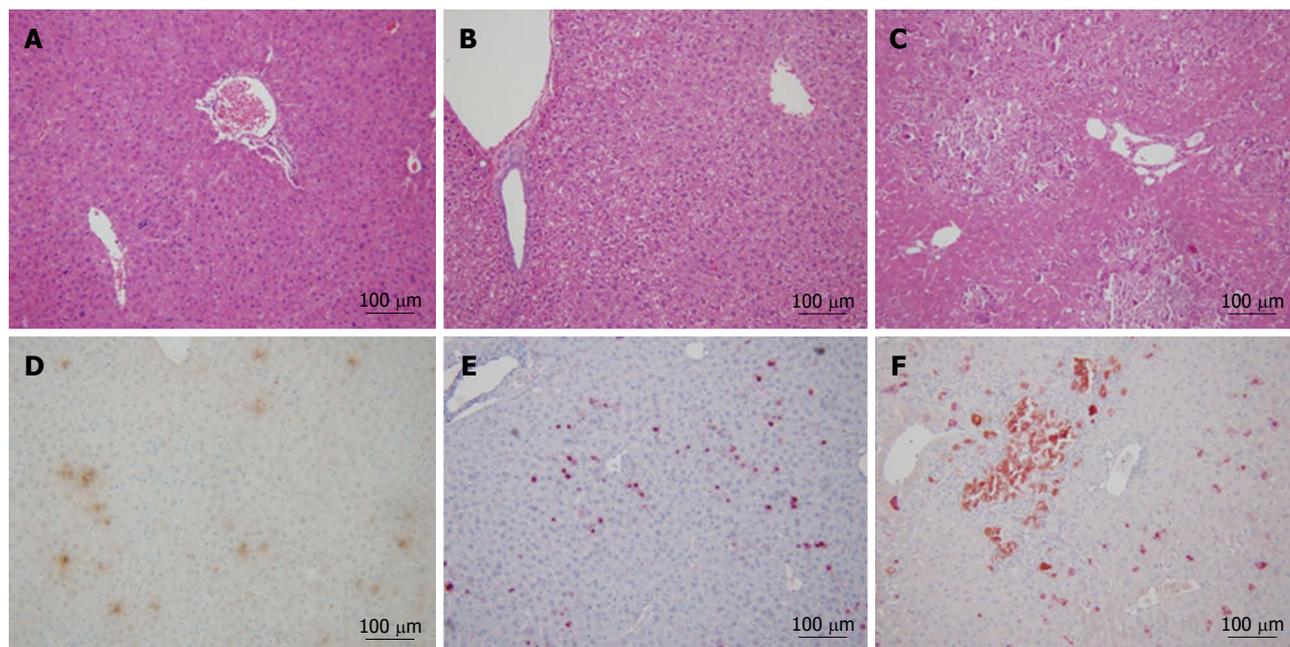
#### **Synergistic liver injury in in-alb-uPA transgenic mice after AAV-1.3HBV transfection**

Although uPA plays critical role in hepatic repair via proteolysis of matrix elements and clearance of cellular debris from the field of injury, clinical data showed that the levels of uPA and uPAR in patients with acute and chronic hepatitis B significantly higher than that in healthy controls, which indicated that uPA level was closely related to the degree and period of inflammation and liver injury<sup>[13]</sup>. To confirm if the coexisting uPA ex-

pression and HBV replication induced serious acute liver injury, the in-alb-uPA transgenic mice were transfected with pAAV-1.3HBV, a plasmid which could mediated the production of replicative HBV virus<sup>[14]</sup> *in vivo*. Large area necrosis was observed 20 d later in the liver of Dox-induced in-alb-uPA double transgenic mice that were transfected with pAAV-1.3HBV (Figure 5C), compared with that of non-induced in-alb-uPA mice transfected with pAAV-1.3HBV (Figure 5B) or with the control plasmid pAAV-IRES (Figure 5A). Double-staining immunohistochemical analysis confirmed both uPA expression (in brown) and HBcAg expression (in red) in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 5F). Interestingly, the coexpression of uPA and HBcAg exist in the most of the necrosis areas and the hepatocytes with HBcAg expression alone were morphological intact. The severe liver damage in the mice after HBV transfection indicated that the expression of uPA accelerated the liver injury. The result was confirmed by a statistics analysis that about 86.7% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver pathogenic changes compared with the 20% AAV-HBV transfected non-induced in-alb-uPA mice, which could be explained by the leaky expression of uPA due to the tet-on inducible system. And about 40% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver injury (Table 1).

#### **Comparison of the serum HBV antigens and cytokines produced in mice from different groups**

Previous reports have shown that HBV infection is associated with the production of a broad range of pro-inflammatory cytokines and chemokines such as IL-1 $\beta$ ,



**Figure 5 Synergistic injury of liver in in-alb-urokinase plasminogen activator transgenic mice after adeno-associated virus-1.3hepatitis B virus transfection.** Histological and immunohistochemical staining for hepatitis B core antigen in the livers of different groups of mice 20 d later after adeno-associated virus (AAV)-1.3hepatitis B virus (HBV) transfection. A: Histology of the AAV-internal ribosome entry site (IRES) transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice; B: Histology of the AAV-HBV transfected non-induced in-alb-uPA mice; C: Histology of the AAV-HBV transfected Dox-induced in-alb-uPA mice; D: Double-staining immunohistochemical analysis of the AAV-IRES transfected Dox-induced in-alb-uPA mice; E: Double-staining immunohistochemical analysis of the AAV-HBV transfected non-induced in-alb-uPA mice; F: Double-staining immunohistochemical analysis of the AAV-HBV transfected Dox-induced in-alb-uPA mice. Magnification, × 20.

**Table 1 Statistical analysis of the liver pathogenic rates for mice in different groups (%)**

Group	1	2	3
NP	8 (100)	8 (80)	2 (13.3)
P1	0	2 (20)	2 (13.3)
P2	0	0	5 (33.3)
P3	0	0	6 (40)
Total	8	10	15

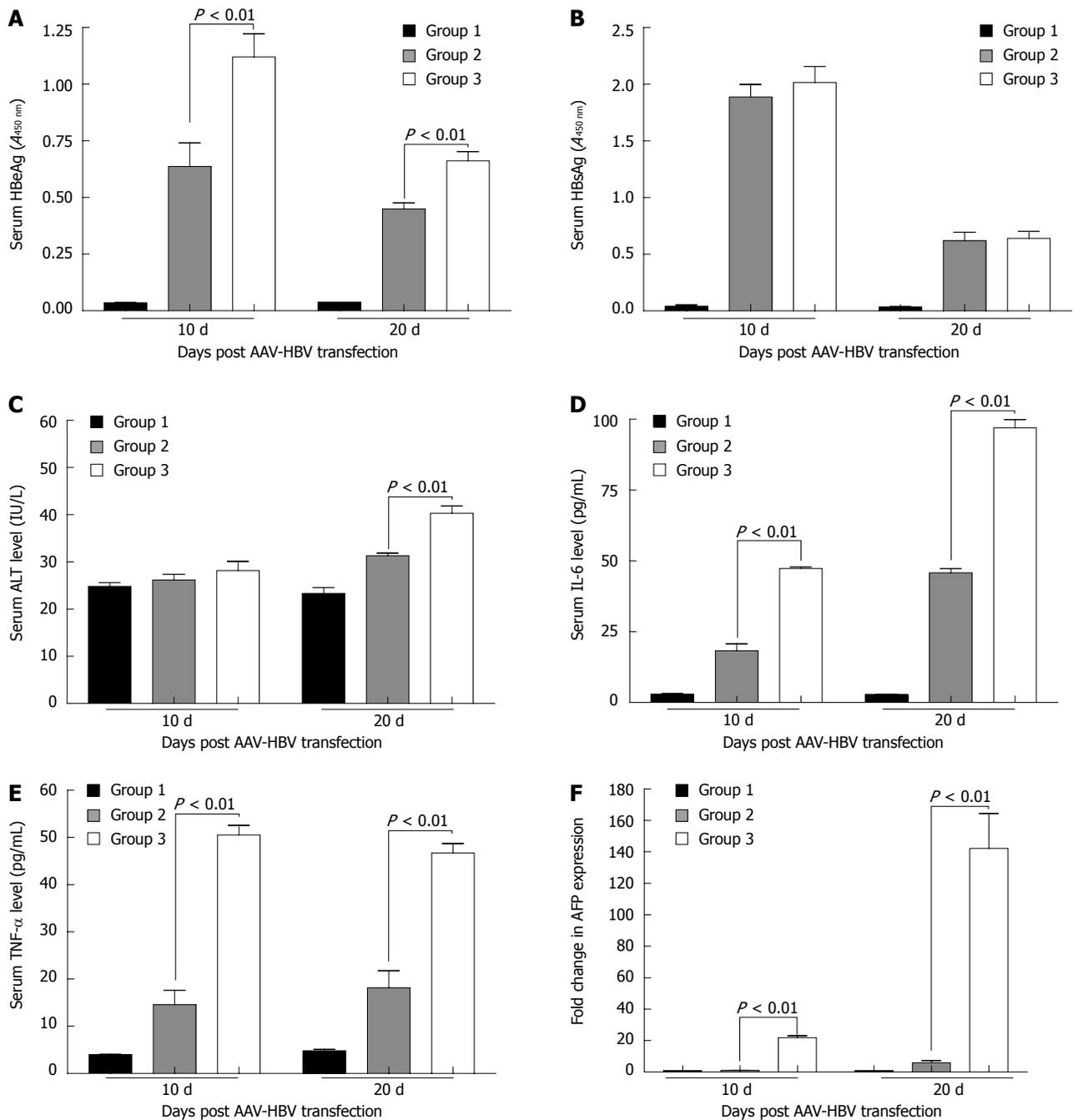
Group 1: Adeno-associated virus (AAV)-internal ribosome entry site transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice; Group 2: AAV-hepatitis B virus (HBV) transfected non-induced in-alb-uPA mice; Group 3: AAV-HBV transfected Dox-induced in-alb-uPA mice. NP stands for number of the non-pathogenic mice; P1 stands for number of mice in which the liver pathogenic area is below 10%; P2 stands for number of mice in which the liver pathogenic area ranges between 10% and 30%; P3 stands for number of mice in which the liver pathogenic area is above 30%.

IL-6, IL-8, IL-12, TNF- $\alpha$  and IFN- $\gamma$ <sup>[15-17]</sup>, among which IL-6 and TNF- $\alpha$  are important components of the early signaling pathway that lead to liver regeneration<sup>[18]</sup>. In this study, results also confirmed the elevation of serum IL-6 and TNF- $\alpha$  levels in the AAV-HBV transfected in-alb-uPA mice (Figure 6). 10 d and 20 d after AAV-HBV transfection, the serum IL-6 level for the Dox-induced in-alb-uPA mice was  $47.28 \pm 0.57$  and  $96.97 \pm 2.91$  (pg/mL), while the level for the non-induced in-alb-uPA mice was  $18.32 \pm 2.38$  (pg/mL) and  $45.83 \pm 1.50$  (pg/mL) ( $P < 0.01$ ) (Figure 6D). The serum TNF- $\alpha$  level for the Dox-induced in-alb-uPA mice was  $50.55 \pm 2.01$  (pg/mL)

and  $46.72 \pm 2.01$  (pg/mL), while the level for the non-induced in-alb-uPA mice was  $14.58 \pm 3.05$  (pg/mL) and  $18.17 \pm 3.63$  (pg/mL) ( $P < 0.01$ ) (Figure 6E). Compared with the non-induced in-alb-uPA mice, the average serum HBeAg level of the Dox-induced in-alb-uPA mice was significantly higher both at 10 d and 20 d after AAV-HBV transfection ( $P < 0.01$ ) (Figure 6A), while there was no significant difference between the average serum HBeAg level of the Dox-induced and non-induced mice (Figure 6B). Compared with the non-induced in-alb-uPA mice, the average serum ALT level of the Dox-induced in-alb-uPA mice was slightly higher at 20 d after AAV-HBV transfection ( $P < 0.01$ ) (Figure 6C).

**Relative quantitative analysis of AFP mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice**

It has been reported that AFP level *in vivo* decreases abruptly soon after birth and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration<sup>[19]</sup>. In this study, we found that, compared with the non-induced in-alb-uPA mice that were transfected by AAV-HBV, the AFP mRNA level for the Dox-induced in-alb-uPA mice that were transfected by AAV-HBV was greatly higher. 10 d after AAV-HBV transfection, the average level of the AFP mRNA for those induced in-alb-uPA mice increased about 21.8 times, while the fold change further increased to 142.1 times at 20 d after AAV-HBV transfection (Figure 6F). The data further confirmed our hypothesis that uPA expression and HBV



**Figure 6** Comparison of the serum hepatitis B e antigen, hepatitis B surface antigen, interleukin-6, tumor necrosis factor- $\alpha$ , alanine aminotransferase levels and hepatic  $\alpha$ -fetoprotein mRNA levels between mice from different groups. Group 1: Adeno-associated virus (AAV)-internal ribosome entry site transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice ( $n = 6$ ); Group 2: AAV-hepatitis B virus (HBV) transfected non-induced in-alb-uPA mice ( $n = 6$ ); Group 3: AAV-HBV transfected Dox-induced in-alb-uPA mice ( $n = 6$ ). HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; IL: Interleukin; TNF: Tumor necrosis factor; AFP:  $\alpha$ -fetoprotein.

infection have close relations and HBV infection further accelerated liver injury and regeneration when uPA was overexpressed.

## DISCUSSION

Tet-inducible expression system is one of the most suitable inducible systems which could be used to investigate the function of a given gene *in vivo*, which including the

tTA (Tet-off) system<sup>[20]</sup> and rtTA (Tet-on) system<sup>[21]</sup>, and facilitates not only the understanding of gene function in development and pathogenesis, but also in transgenic mouse modeling<sup>[22-24]</sup>. On the other hand, tissue-specific expression of a target gene relies on tissue-specific promoters. Tissue-specific expression is vital for gene function research in organism development and can reduce immunological response and side effects in gene therapy applications. Many liver-specific promoters have been

identified so far, such as the AFP promoter<sup>[25]</sup>, the albumin promoter, mouse major urinary protein promoter<sup>[26]</sup>, hSAP promoter (human serum amyloid P component promoter)<sup>[27]</sup>, and apoE promoter (human apolipoprotein E promoter)<sup>[28]</sup>, which have been applied in liver-specific expression of target genes. In addition, elements like enhancers influence the transcriptional activity of the these tissue-specific promoters<sup>[29]</sup>.

The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology<sup>[3]</sup>. Previous studies showed uPA diverse functions in tissue remodeling, angiogenesis, wound healing and protective effects in liver diseases<sup>[30]</sup>. The levels of uPA have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased<sup>[2]</sup>. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients<sup>[13]</sup>. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

In 1990, an Alb-uPA transgenic mouse which carried the mouse uPA gene under the control of the mouse albumin enhancer/promoter, was developed by Dr. Brinster's team to study the pathophysiology of plasminogen hyperactivation<sup>[31]</sup>. The over-expression of the uPA gene in the liver resulted in high plasma uPA levels and hypofibrinogenemia, which led to severe and sometimes abdominal bleeding soon after birth. And the high mortality also increases the difficulty for the generation of human liver chimeric mice<sup>[32,33]</sup> and the study of hepatitis C virus infections *in vivo*<sup>[34-36]</sup>. In this study, we established an uPA inducible double transgenic mouse in-alb-uPA, in which uPA can be expressed specifically in the liver only after Dox induction. Hypofibrinogenemia and neonatal hemorrhaging were not observed in the Dox-induced in-alb-uPA mice, which greatly brought down the mortality rate. Also the inducible expression of uPA makes it possible for us to study and illuminate the relations of uPA over-expression and HBV infection clinically.

Hydrodynamic transfection method was suitable for the AAV-mediated delivery of HBV genome *in vivo*<sup>[37]</sup>. To investigate the risk of HBV induced liver injury in the case of uPA over-expression, the hydrodynamic transfection of pAAV-HBV1.3, which could mediated the production of replicative HBV virus *in vivo*, was performed. In the Dox-induced in-alb-uPA mice that were hydrodynamically transfected by AAV-1.3HBV, severe liver histological changes were observed in the liver (Figure 5). Also uPA over-expression in the liver resulted in higher HBV antigen expression, higher IL-6 and TNF- $\alpha$  produc-

tion and slight elevation of serum ALT level (Figure 6). Our results also found a significant increase of the AFP mRNA level in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 6). Produced by the embryonic yolk sac and fetal liver, the AFP level decreases abruptly soon after birth and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration<sup>[19]</sup>. As IL-6 and TNF- $\alpha$  are proinflammatory cytokines that lead to liver regeneration, we came to the conclusion that the uPA over-expression in AAV-HBV transfected mice increased the liver necrosis injury, inflammation and liver regeneration, which reflects a process that may eventually lead to hepatocellular carcinoma.

It is generally considered that cell-mediated immunity and inflammation are the main mediators of the hepatic pathology induced by HBV infection. In this study, we found that HBV infection further accelerated liver injury and regeneration when uPA was overexpressed, indicating a close relation between uPA expression and HBV infection. Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection causes a high risk of developing liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection. The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology. However, the roles of uPA/uPA's receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China. Clinical studies almost focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

### Research frontiers

Various researchers have found the levels of uPA to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

### Innovations and breakthroughs

In this study, an inducible liver-specific uPA transgenic mice model was developed. Plasmid adeno-associated virus-1.3HBV transfection in doxycycline (Dox)-induced transgenic mice resulted in severer liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the over-expression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection. Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

### Applications

This study deepens our knowledge of uPA function in HBV-induced liver diseases, which may not only facilitate the elucidation of the molecular mechanism

of HBV pathogenesis, but also provide a basis for the uPA-targeted anti-HBV therapies.

### Terminology

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology. Tetracycline (Tet)-inducible expression system consists of two parts: the ligand-dependent transactivator rTA as the effector and a tetO-CMV minimal promoter cassette regulating the expression of the transgene as the responder. When Dox is present, rTA binds to the tetO-sequence and induces expression of the target gene. Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

### Peer review

The authors studied the role of uPA and found that the over-expression of uPA may have a synergistic role in the development of liver injury, inflammation and liver regeneration during acute HBV infection. They added as such new information to the field on the knowledge about uPA function in HBV-induced liver diseases.

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## siRNA targeting of Cdx2 inhibits growth of human gastric cancer MGC-803 cells

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### Abstract

**AIM:** To investigate the effects of small interference RNA (siRNA) targeting of Cdx2 on human gastric cancer MGC-803 cells *in vitro* and *in vivo*.

**METHODS:** The recombinant pSilencer 4.1-Cdx2 siRNA plasmids were constructed and transfected into gastric cancer MGC-803 cells *in vitro*. The stable transfectants were selected. The effects of Cdx2 siRNA on growth, proliferation, cell cycle, apoptosis, migration and invasiveness of human gastric cancer MGC-803 cells were evaluated and the expression of phosphatase and tensin homolog (PTEN), caspase-9 and caspase-3 was observed *in vitro* by reverse transcription polymerase

chain reaction (RT-PCR) and Western blotting analysis. We also investigated the effect of Cdx2 siRNA on growth of MGC-803 cells in nude mice *in vivo*.

**RESULTS:** Cdx2 siRNA led to inhibition of endogenous Cdx2 mRNA and protein expression as determined by RT-PCR and Western blotting analysis. Cdx2 siRNA significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. Cdx2 siRNA also increased PTEN expression, and activated caspase-9 and caspase-3 in MGC-803 cells *in vitro*. In addition, siRNA targeting of Cdx2 inhibited the growth of MGC-803 cells and promoted tumor cell apoptosis *in vivo* in nude mice tumor models.

**CONCLUSION:** Cdx2 was involved in regulating progression of human gastric cancer cells MGC-803. Manipulation of Cdx2 expression may be a potential therapeutic strategy for gastric cancer.

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**Key words:** Cdx2; Gastric cancer; Growth; Small interference RNA

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### INTRODUCTION

The transcription factor, Cdx2, is a member of the caudal-related homeobox gene family, and is mainly expressed in

the intestine. Cdx2 plays important roles in early differentiation, proliferation, and maintenance of intestinal epithelial cells, and in the transcription of genes such as multidrug resistance 1<sup>[1,2]</sup>. Overexpression of Cdx2 in the small intestine is associated with reduced postnatal growth, early epithelial maturation, alterations in the development of a differentiated phenotype in crypt base organization, and changes in paneth and goblet cell lineages<sup>[3]</sup>.

Initially, Cdx2 was reported to be a tumor suppressor. Several investigators reported that low levels of Cdx2 is a characteristic feature of human colon and squamous esophageal cancer<sup>[4,5]</sup>, and overexpression of Cdx2 could decrease mobility and antagonize metastasis of colon cancer cells<sup>[6]</sup>. However, other studies showed that strong and robust expression of Cdx2 was found in > 80% of colorectal cancers and non-small cell lung cancer<sup>[7,8]</sup>. In addition, Cdx2 was found to enhance proliferation and have tumorigenic potential in the human colon cancer cell lines, LoVo and SW48<sup>[9]</sup>. These studies suggested that Cdx2 also had oncogenic property. Together, these conflicting findings point to a complex role for Cdx2 in the regulation of cell proliferation.

Gastric cancer is the third most common cancer in China, and is one of the most frequent causes of cancer-related mortality in China, with an incidence of 0.4 million new cases and 0.3 million deaths annually<sup>[10]</sup>. Intestinal metaplasia has been shown to be a precursor of intestinal-type gastric adenocarcinoma. Since intestinal metaplasia cannot be eradicated, it is important to determine how to reduce the morbidity from intestinal metaplasia to gastric cancer. However, the histogenesis of intestinal metaplasia and factors in the metaplastic epithelium that lead to its development into carcinoma is still in dispute<sup>[11,12]</sup>. In adult humans, Cdx2 has been reported to be associated with intestinal metaplasia in the stomach in which ectopic expression of Cdx2 is speculated to cause the gastric epithelial cells to trans-differentiate and take the intestinal phenotype<sup>[13]</sup>. In addition, Cdx2 transgenic mice have been shown to induce intestinal metaplasia and have a high incidence of gastric carcinoma<sup>[14,15]</sup>. This indicates a direct relationship between Cdx2-induced intestinal metaplasia and gastric carcinogenesis.

In the present study, we constructed small interference RNA (siRNA) sequences targeting of Cdx2, transfected them into the human gastric cancer cell line MGC-803, selected the stable transfectants, and explored changes in growth, proliferation, cell cycle, apoptosis, metastasis and invasiveness. We also observed the effect of Cdx2 siRNA on the expression of phosphatase and tensin homolog (PTEN), caspase-9, and caspase-3. Moreover, we investigated the effects of Cdx2 downregulation on the growth and apoptosis of MGC-803 cells in nude mice.

## MATERIALS AND METHODS

### Cell culture

The human gastric carcinoma cell line, MGC-803, was supplied by the Cell Bank of Shanghai Institute of Cell

Biology, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, United States). All media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were cultured in an incubator with 5% CO<sub>2</sub> at 37 °C with medium changes every 3 d.

### Antibodies

Anti-Cdx2, anti-β-actin and secondary antibody were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States. Antibodies specific for PTEN, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology, Beverly, MA, United States.

### Plasmid construction and transfection

Double strand siRNA oligonucleotides were obtained from Gima Biotechnology Company (China). There were two reversed repeated sequences with 21 inserted sequences (GACAAATATCGAGTGGTGTAC, TA-ACCCGCGATCTGTTCTGCA) in the complementary sequence, with *Bam*H I and *Hind*III sites for ligation into the pSilencer 4.1 vector, which contained a neomycin resistance marker for the selection of stable transfectants in the presence of G418. The siRNA targeting site of the transcribed product was nucleotides 115-818 of Cdx2 mRNA (GeneBank No. NM-001265). The negative control was the siRNA sequence with no homology to any human gene sequence.

After ligation, the plasmid was transformed into *Escherichia coli* TOP10 cells, and then cultured on solid LB medium (LB solid medium containing 50 ng/L ampicillin and 2% agarose gel) at 37 °C for 16 h. Positive clones were identified by DNA sequence analysis (Majorbio Biotech Co., Ltd), and the resulting plasmid was named pSilencer 4.1-Cdx2(+) or pSilencer 4.1-Cdx2(-). Six-well plates were inoculated with MGC-803 cells (1 × 10<sup>5</sup>), and cells were transfected with pSilencer 4.1-Cdx2(+) recombinant plasmids. For selection of stable transfectants, G418 (Life Technologies) was added to the cells 48 h after transfection. The concentration of G418 for selection was gradually decreased as follows: 1 mg/mL for 4 d; 750 µg/mL for 4 d; 500 µg/mL for 4 d; and 250 µg/mL as a sustaining dose. At day 20 after transfection, G418-resistant clones were isolated. The selected cell colonies were transferred from 10-mm dishes to 96-well plates, and then from 96-well plates to 24-well plates. The transformants selected by G418 were analyzed by measuring the expression of Cdx2 mRNA and protein. The negative control cells were transfected with vector pSilencer 4.1-Cdx2(-) alone, and maintained under identical conditions. In the case of cells that were selected in medium containing G418, antibiotics were routinely included in their growth medium until 1 to 2 d before experiments were carried out. The cells were divided into 3 groups: MGC-803/Cdx2 siRNA, MGC-803/Cdx2 negative control and MGC-803 group.

### Semi-quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the positive cell clone using TRIzol Reagent (Invitrogen). Neo gene segments were amplified and verified by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Complementary deoxyribonucleic acids (cDNAs) were reverse-transcribed from 2  $\mu\text{g}$  of total RNA. Primers used in this study were as follows: Cdx2 forward primer (5'-CGGCAGCCAAGTGAAC-3') and reverse primer (5'-GATGGTGATGTAGCGACTGTAGTG-3'), PCR product: 100 base pairs (bp);  $\beta$ -actin forward primer (5'-AACTCCATCATGAAGTGTGA-3') and reverse primer (5'-ACTCCTGCTTGCTGATCCAC-3'), PCR product: 247 bp. The PCR products were checked by agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of  $\beta$ -actin mRNA.

### Western blotting analysis

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-Cl (pH 7.6), 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 1  $\mu\text{g}/\text{mL}$  aprotinin, 100  $\mu\text{g}/\text{mL}$  phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowry protein assay, equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three buffer system. The membrane was blocked with 5% bovine serum albumin in phosphate buffer solution Tween-20 (PBST) (PBS, pH 7.5, containing 0.1% Tween-20), and incubated with a 1:500 dilution of primary antibody (anti-Cdx2) overnight at 4  $^{\circ}\text{C}$ . The membrane was then washed with PBST and incubated with a peroxidase-conjugated secondary antibody (1:1000) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, United States), according to the manufacturer's recommendations. Western blotting film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, United States). After development, the membrane was stripped and reprobed with antibody against  $\beta$ -actin (1:1000) to confirm equal sample loading.

### Cell growth and proliferation assay

The growth of MGC-803 cells was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using a CellTiter 96 AQueous assay system (Promega, Madison, WI, United States), according to the manufacturer's instructions. This assay measures dehydrogenase enzyme activity in metabolically active tumor cells, as reflected by the conversion of MTT to formazan, which is soluble in tissue culture medium and is detected by absorbance ( $A$ ) at 490 nm. The production of formazan is proportional to the number of living cells, with the intensity of the produced color serving as an indicator of cell viability. Briefly, MGC-803

cells were plated at a density of  $5 \times 10^3$  cells/well in 96-well plates, and cultured for 72 h. The percentage of cell survival was calculated using the background-corrected absorbance: % proliferation rate =  $100 \times A$  of experimental well/ $A$  of untreated control well. All experiments were performed at least three times.

### Colony formation assay

Cell suspensions from each group were diluted in DMEM with 10% FBS, and immediately re-plated in 6-well plates at a density of 20 cells/ $\text{cm}^2$ . The plates were incubated until cells in control wells formed sufficiently large colonies. After that, the colonies were fixed in 6% glutaraldehyde and stained with 0.5% crystal violet. The plates were photographed and their digital images were analyzed manually to determine colony number.

### Cell cycle analysis by flow cytometry

For cell cycle analysis, MGC-803 cells ( $1 \times 10^6$ ) were washed twice with ice-cold PBS, treated with trypsin, and then fixed in 70% cold ethanol at 4  $^{\circ}\text{C}$  for 30 min. The cell pellet was incubated in a solution containing 50 ng/mL propidium iodide, 0.2 mg/mL RNase, and 0.1% Triton X-100 at room temperature for 30 min, and then analyzed by flow cytometry using a FACscan (Becton Dickinson, Mountain View, CA, United States). Data were analyzed with the MultiCycle for Windows (Phoenix Flow Systems, San Diego, United States).

### Apoptosis assay by flow cytometry

Apoptotic cells were determined using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Jingmei Biotech Co., Shenzhen, China) and an EPICS XL-MCL flow cytometer (Becton Dickinson) according to the manufacturer's instructions. Briefly,  $1 \times 10^6$  cells were stained with Annexin V/FITC for 30 min at 4  $^{\circ}\text{C}$  in the dark and then stained with propidium iodide for 10 min before flow cytometric analysis.

### Wound healing assay

The cells were cultured to confluence in 6-well plates, and were then treated with mitomycin C to inhibit cell proliferation. A central linear wound was made with a 200  $\mu\text{L}$  sterile pipet tip. Media were changed gently to remove any floating cells. Phase micrographs of the wound cultures were taken at 0 and 36 h. The photographs were analyzed by measuring the distance from the wound edge of the cell sheet to the original wound site. Migratory activity was calculated as the mean distance between edges of three points in 12 fields per well. Relative motility = (mean original distance - mean distance at a time point)/mean original distance  $\times 100\%$ . Each test group was assayed in triplicate.

### Cell invasion assay

Cell invasion was assessed using Transwell chambers (6.5 mm; Corning, New York, United States) with 50  $\mu\text{L}$  serum-free DMEM containing 1  $\mu\text{g}/\text{mL}$  Matrigel (De-

partment of Biology, Beijing University, China) in the upper chamber. The lower chamber was filled with 50  $\mu$ L DMEM containing 0.1  $\mu$ g/mL fibronectin (Beijing University). Cells ( $1 \times 10^5$ ) were suspended in 100  $\mu$ L DMEM with 1% fetal calf serum and plated into the upper chamber. PBS (5%) 500  $\mu$ L was added in the lower chamber. After a 24 h incubation with 5% CO<sub>2</sub> at 37 °C, the number of cells with Giemsa staining on the under-surface of the polycarbonate membranes was scored visually in five random fields at a 400  $\times$  magnification by light microscopy.

#### Analyses of PTEN, caspase-9 and caspase-3 expression

Semi-quantitative RT-PCR was performed as previously described. Primers used in this study were as follows: (1) PTEN forward primer (5'-CTGGAAAGGGAC-GAACTG-3') and reverse primer (5'-AGGTAACG-GCTGAGGGA-3'), PCR product: 368 bp; (2) Caspase-9 forward primer (5'-GGCTGTCTACGGCACAGAT-GGA-3') and reverse primer (5'-CTGGCTCGGGGT-TACTGCCAG-3'), PCR product: 200 bp; (3) Caspase-3 forward primer (5'-AAGCGAATCAATGGACTC-3') and reverse primer (5'-TTCCTGACTTCATATTTCAA-3'), PCR product: 192 bp; (4) GAPDH (a) forward primer (5'-ACAGCAACAGGGTGGTGGAC-3') and reverse primer (5'-TTTGAGGGTGCAGCGAACTT-3'), PCR product: 252 bp; and (5) GAPDH (b) forward primer (5'-ACCACAGTCCATGCCATCAC-3') and reverse primer (5'-TCACCACCCTGTTGCTGTA-3'), PCR product: 450 bp. Western blotting analysis was carried out as previously described.

#### Animal studies

BALB/c male nude mice at 5 wk old were obtained from Guangxi Animal Center, China. All animals were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. MGC-803/Cdx2 siRNA cells, MGC-803/Cdx2 negative control cells and MGC-803 cells were used for tumor implantation. There were six mice in each group. Approximately  $2 \times 10^6$  tumor cells were implanted subcutaneously into the flanks of the nude mice. Tumor sizes were measured every 4 d with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula:  $TV = W^2 \times L/2$ , where L was the length and W was the width of the tumor. The relative tumor volume (RTV) was calculated by the formula:  $RTV = V_t/V_0$  ( $V_0$  is the TV at the day when the chemicals were given, and  $V_t$  is the TV of subsequent measurement). After mice were killed, total RNA and protein were extracted from tumor tissues. The expression of Cdx2 mRNA and protein were detected by semi-quantitative RT-PCR and Western blotting analysis, respectively. Tumor cells were assessed for apoptosis using *in situ* terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling assays (TUNEL). Apoptosis was evaluated

by counting the positive cells (brown-stained cells) as well as the total number of cells in 10 arbitrarily selected fields at 400  $\times$  magnifications in a double-blinded manner. The apoptotic index (per 400  $\times$  microscopic field) was calculated as the number of apoptotic cells  $\times$  100/total number of cells. Brown-stained nuclei immediately at the edge of a tissue section were excluded from cell counts to minimize false positives.

#### Statistical analysis

Data are expressed as mean  $\pm$  SE. Statistical significance was determined using  $\chi^2$  test, student's *t* test, or one-way analysis of variance. Statistical analysis were carried out using SPSS, version 13.0 (SPSS Inc., Chicago, IL, United States) or Origin 7.5 software programs (OriginLab Co., Northampton, MA, United States). A value of  $P < 0.05$  was considered as statistically significant.

## RESULTS

#### pSilencer 4.1-Cdx2(+) inhibits Cdx2 mRNA and protein expression

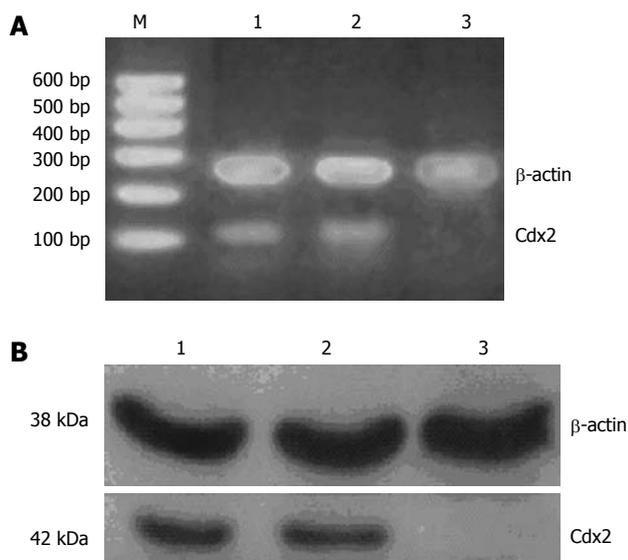
Recombinant pSilencer 4.1-Cdx2(+) and pSilencer 4.1-Cdx2(-) sequences were verified by DNA sequenced analysis (data not shown) which demonstrated that the inserted siRNA coding frames and frame sequences were correct. This confirmed that the construction of Cdx2 siRNA expression plasmid was successful.

The transfection of pSilencer 4.1-Cdx2(+) plasmid into MGC-803 cells led to remarkable inhibition of Cdx2 mRNA and protein expression. Densitometric analysis showed that Cdx2 mRNA and protein in MGC-803/Cdx2 siRNA cells were about 11- and 7-fold lower, respectively, than those in the two control groups ( $P < 0.05$ ), while no differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells (Figure 1).

#### Cdx2 siRNA inhibits cell growth and proliferation in gastric cancer MGC-803 cells

Next, we determined the *in vitro* survival rates of gastric tumor cells stably transfected with pSilencer 4.1-Cdx2(+) plasmids, using the gastric carcinoma cell line, MGC-803, as a model for gastric cancer. As shown in Figure 2, Cdx2 siRNA significantly reduced cell survival, as assessed by the MTT assay. We observed that MGC-803/Cdx2 siRNA cells obviously grew slower than MGC-803/Cdx2 negative control cells and MGC-803 cells ( $P < 0.05$ ), which was consistent with the decreased levels of Cdx2 in MGC-803/Cdx2 siRNA cells. Additionally, MGC-803/Cdx2 negative control cells and MGC-803 cells exhibited about 3-fold higher mean proliferation rates than MGC-803/Cdx2 siRNA cells ( $P < 0.05$ ). These results indicate a suppressive effect of Cdx2 siRNA on MGC-803 cell growth and survival.

To confirm the inhibitory effect of Cdx2 siRNA on the growth of MGC-803 cells, we performed colony formation assays to measure the capability of the cells to grow in an anchorage-independent environment by

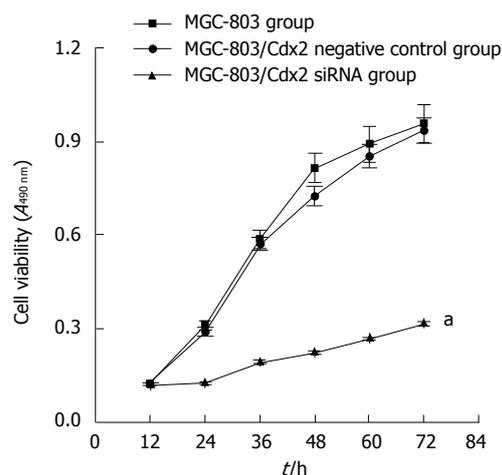


**Figure 1** Cdx2 small interference RNA significantly reduced Cdx2 mRNA and protein expression in MGC-803 cells. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The RNA samples (2  $\mu$ g in each) extracted from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were subjected to RT-PCR for Cdx2 and  $\beta$ -actin mRNAs. RT-PCR for  $\beta$ -actin was performed in parallel to show an equal amount of total RNA in the sample; B: Western blotting analysis. Whole protein extracts (100  $\mu$ g in each) were prepared from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 siRNA cells. The expression of Cdx2 protein was determined by Western blotting with an anti-Cdx2 antibody. The  $\beta$ -actin expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; M: 600 bp marker.

culturing the cells in soft agarose. As shown in Figure 3, three cell lines were able to form colonies in soft agarose, but the number of colony formation in MGC-803/Cdx2 siRNA cells after 3 wk was  $51.4 \pm 3.2$ , with a 60.1% and 57.6% decrease, compared to the two control groups, respectively ( $P < 0.05$ ). Together, these data suggest that Cdx2 siRNA inhibits cell growth and proliferation in gastric cancer cells.

#### Effect of Cdx2 siRNA on cell cycle control in gastric cancer MGC-803 cells

We used flow cytometry to determine whether the inhibitory effect of Cdx2 siRNA on MGC-803 cell proliferation was mediated, at least in part, through affecting cell cycle progression. We found that MGC-803/Cdx2 siRNA cells were 73.1% in G0/G1 phase and 18.2% in S phase, with a 13.8% and 16.2% increase in the G0/G1 phase cell population, and a 17% and 18% decrease in the S phase cell population, compared to MGC-803 cells and MGC-803/Cdx2 negative control cells ( $P < 0.05$ ) (Figure 4). These data indicate that cell growth inhibition by Cdx2 siRNA is associated with significant cell cycle arrest in G0/G1 phase, and suggest that siRNA directed against the Cdx2 gene suppresses cell proliferation by controlling the G1 and S checkpoints and inducing a specific block in cell cycle progression.



**Figure 2** Cdx2 small interference RNA inhibits cell proliferation in MGC-803 cells. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at days 1-3. The cell viability in each group was presented. Each time point represented the mean of cell viability for each group. <sup>a</sup> $P < 0.05$  for MGC-803/Cdx2 siRNA group vs MGC-803 and MGC-803/Cdx2 negative control group.

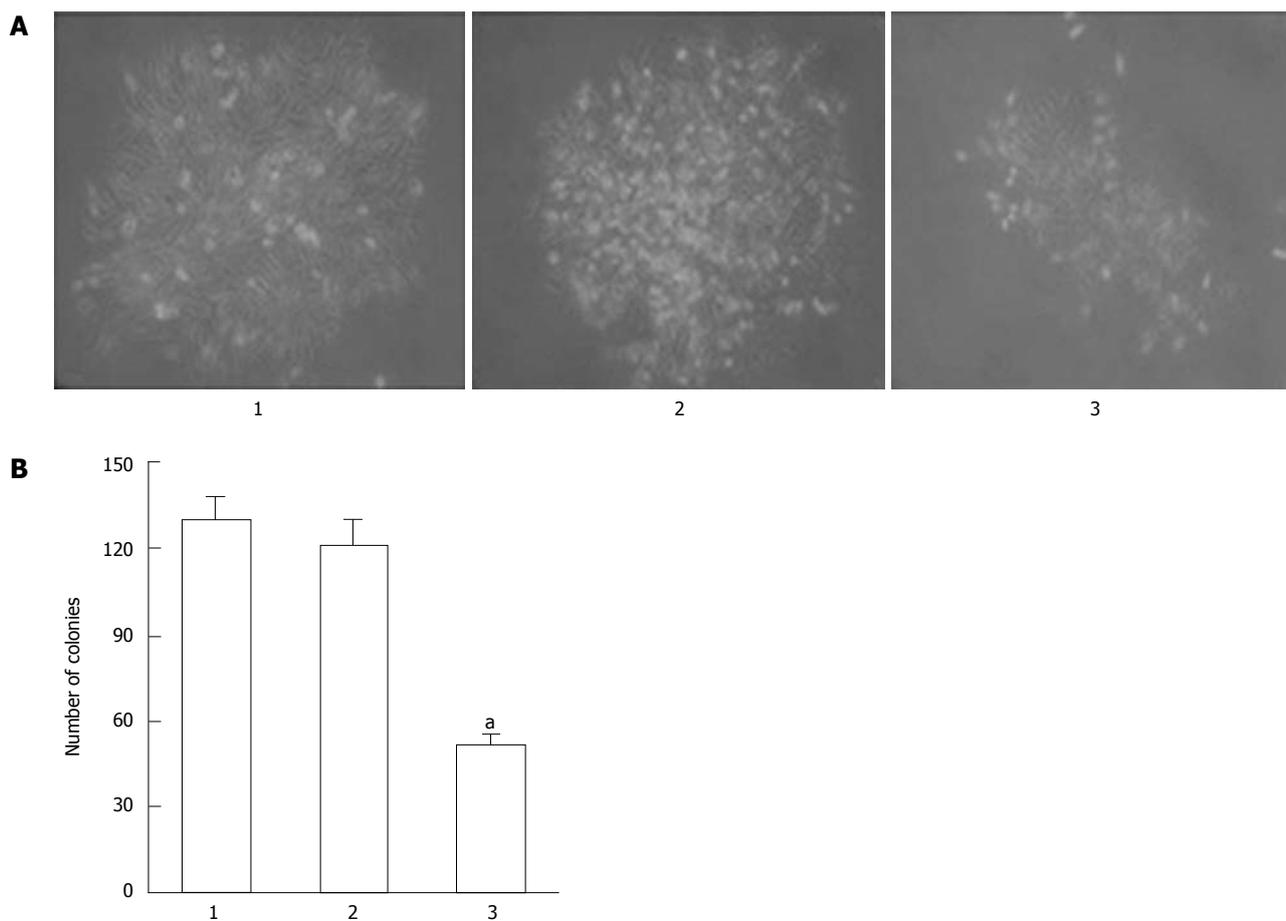
#### Cdx2 siRNA induces cellular apoptosis

To further study the effect of Cdx2 siRNA on MGC-803 cell apoptosis, cells were stained with Annexin V-FITC and propidium iodide, and then subsequently analyzed by flow cytometry. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in Figure 5, the apoptotic percentage of MGC-803/Cdx2 siRNA cells was  $11.7\% \pm 2.2\%$ , which was significantly higher than that of MGC-803/Cdx2 negative control ( $5.3\% \pm 1.3\%$ ) and MGC-803 cells ( $5.6\% \pm 1.1\%$ ) ( $P < 0.05$ ). This implies that inhibition of Cdx2 is able to induce apoptosis in gastric cancer MGC-803 cells.

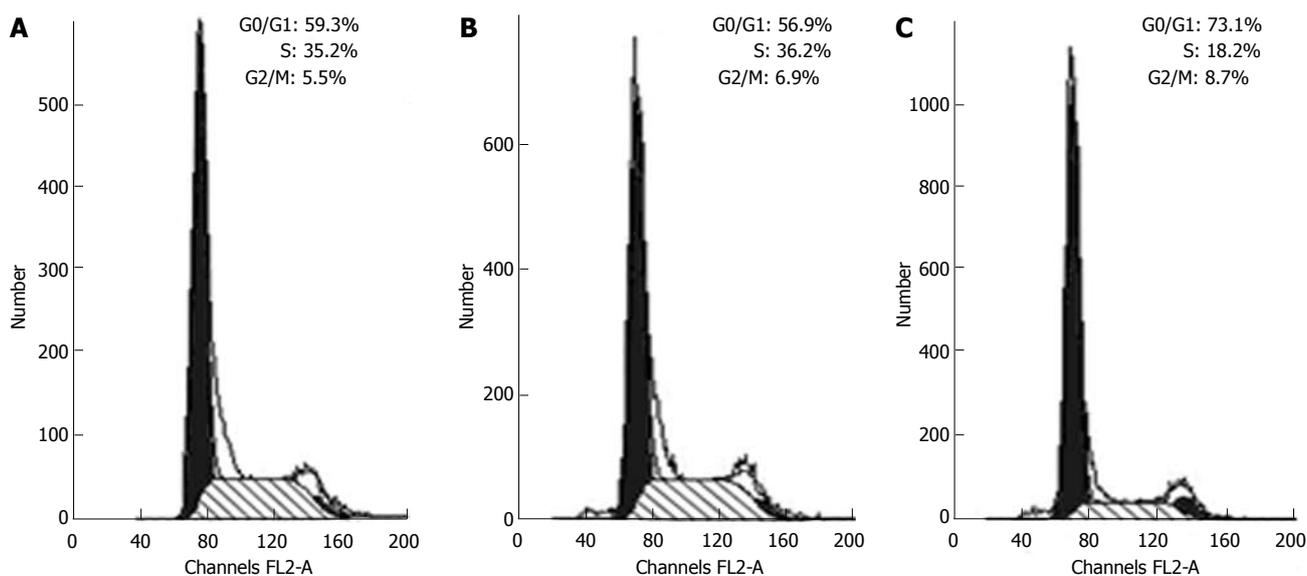
#### Cdx2 siRNA decreases migration and invasion of gastric cancer cells

We measured the migratory ability of three cell groups using the wound healing assay by scratching the single-layer cells. As shown in Figure 6, the distance between the wound edges was determined at 0 and 36 h and the healing rate was calculated in the three groups. MGC-803/Cdx2 siRNA cells showed a lower migratory ability at 36 h than MGC-803/Cdx2 negative control and MGC-803 cells. The healing rate of MGC-803/Cdx2 siRNA cells after 36 h was  $53.7\% \pm 7.2\%$ , with a 39.9% and 40.8% decrease, as compared to MGC-803/Cdx2 negative control cells and MGC-803 cells ( $P < 0.05$ ).

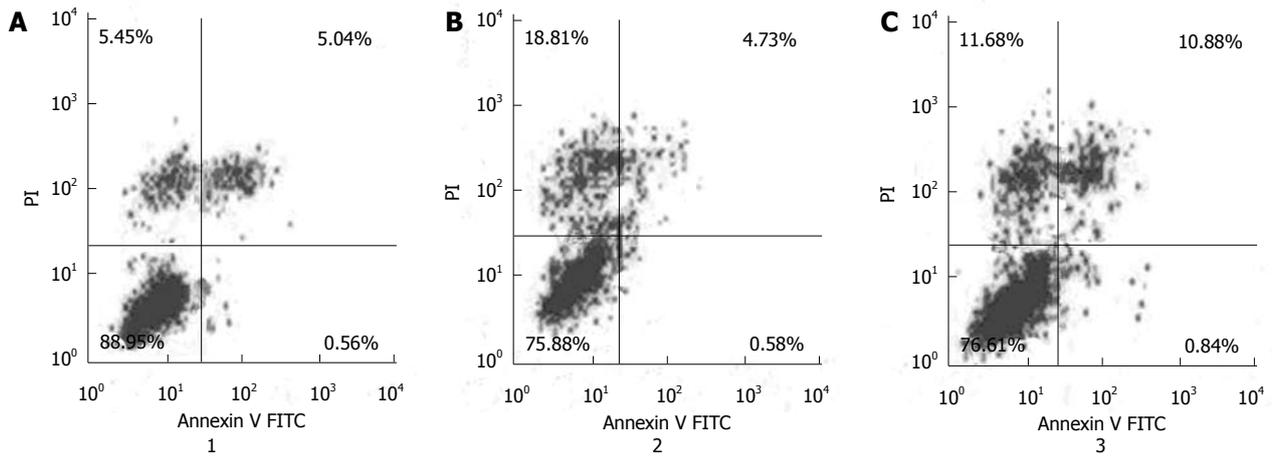
Since siRNA targeting of Cdx2 inhibited the expression of Cdx2 gene in gastric cancer cells, we assessed its ability to inhibit cell invasion. After incubation for 24 h in the invasion assay, the numbers of MGC-803/Cdx2 negative control and MGC-803 cells invaded through the membrane of Matrigel chamber were 2.9- and 3.0-fold greater than that of MGC-803/Cdx2 siRNA cells, re-



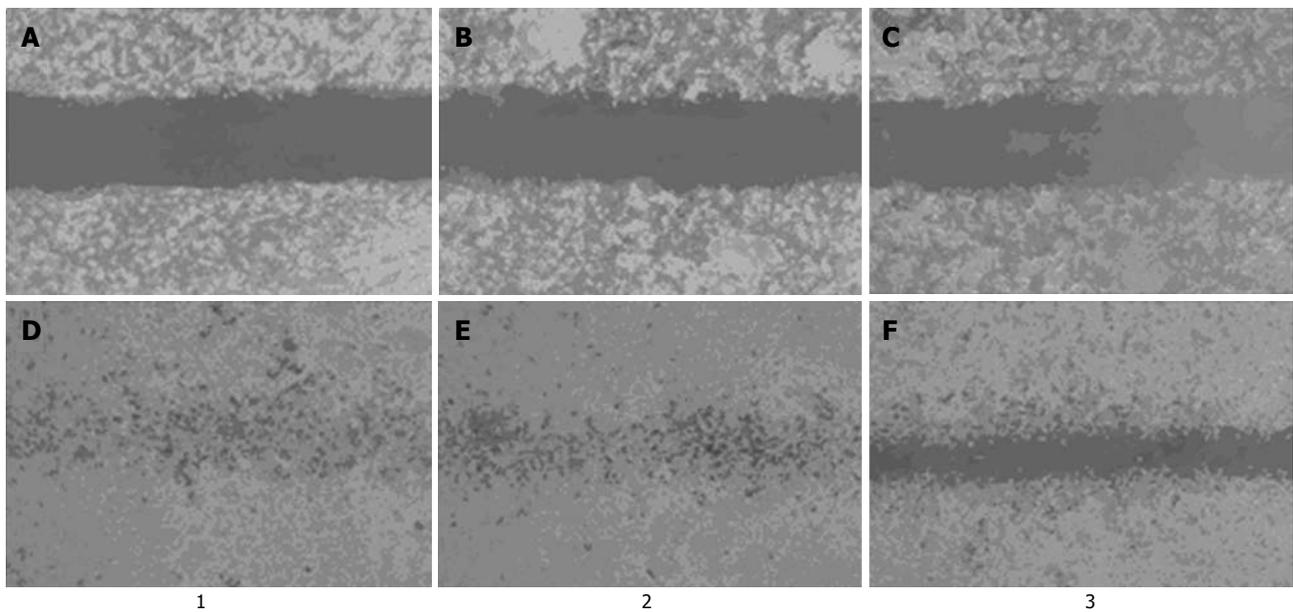
**Figure 3** MGC-803/Cdx2 small interference RNA cells exhibited fewer colonies than MGC-803/Cdx2 negative control cells or MGC-803 cells. A: MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were plated in 6-well plates at a density of 20 cells/cm<sup>2</sup>, and the colonies were observed under optical microscope at 13 d (×100); B: The surviving fraction of cells (visible colonies) was stained with gentian violet, and counted manually. MGC-803/Cdx2 siRNA cells exhibited fewer colonies than MGC-803/Cdx2 negative control cells or MGC-803 cells. Each column presents as mean ± SE from 3 independent experiments. <sup>a</sup>*P* < 0.05 for MGC-803/Cdx2 siRNA group vs MGC-803 and MGC-803/Cdx2 negative control group. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.



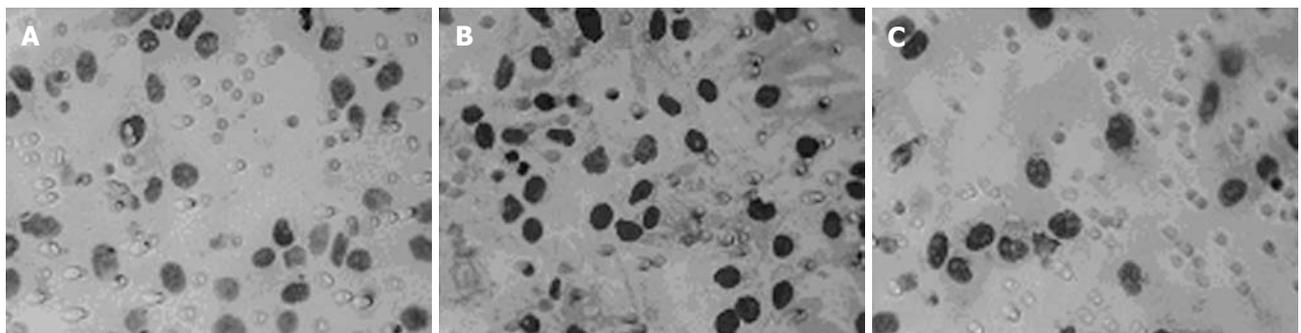
**Figure 4** Cdx2 small interference RNA caused cell cycle arrest in the G0/G1 phase. Cell cycle was analyzed by flow cytometry in MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells. The data were representative of 3 independent experiments. A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 siRNA group.



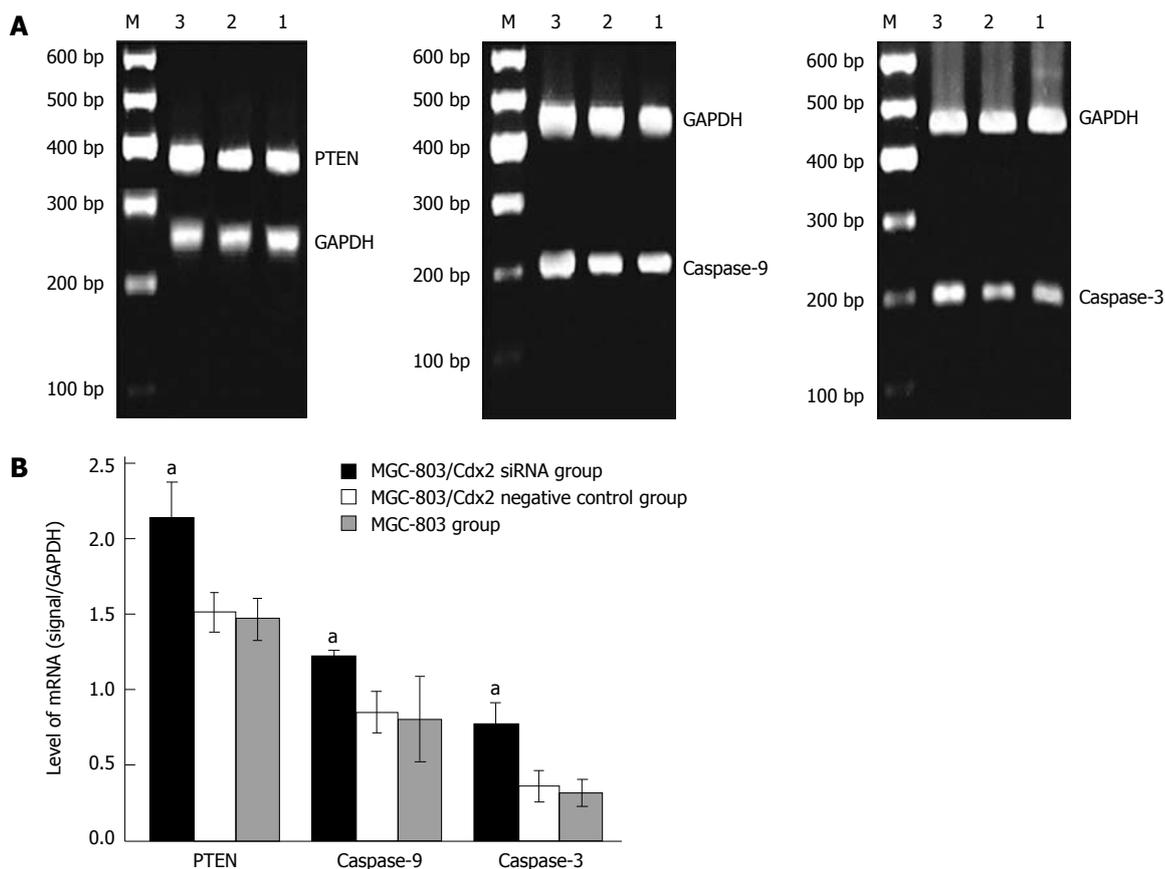
**Figure 5** The mean apoptotic rate in MGC-803/Cdx2 small interference RNA cells was significantly higher than that in MGC-803/Cdx2 negative control or MGC-803 cells. Percentages of apoptotic cells analyzed by flow cytometry. Numbers in the quadrants reflected the percentage of cells. A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 small interference RNA group. PI: Propidium iodide; FITC: Fluorescein isothiocyanate.



**Figure 6** Cdx2 small interference RNA decreased migration of MGC-803 cells in wound healing assay. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were cultured to confluence on 6-well plates, a central linear wound was made with a 200  $\mu$ L sterile pipet tip. The central linear was photographed at different intervals ( $\times 100$ ). A: MGC-803 cells at 0 h; B: MGC-803/Cdx2 negative control cells at 0 h; C: MGC-803/Cdx2 siRNA cells at 0 h; D: MGC-803 cells at 36 h; E: MGC-803/Cdx2 negative control cells at 36 h; F: MGC-803/Cdx2 siRNA cells at 36 h. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.



**Figure 7** Cdx2 small interference RNA decreased invasion of MGC-803 cells. MGC-803 cells (A), MGC-803/Cdx2 negative control cells (B) and MGC-803/Cdx2 small interference RNA cells (C) were loaded onto Matrigel-coated upper chambers of Transwell plates. Filtrated cells on the undersurface of the polycarbonate membranes were stained and counted under a optical microscope at 24 h ( $\times 200$ ).



**Figure 8** Cdx2 small interference RNA upregulated phosphatase and tensin homolog, caspase-9 and caspase-3 mRNA expression. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The RNA samples (2 μg in each) extracted from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were subjected to RT-PCR for phosphatase and tensin homolog (PTEN), caspase-9, caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. RT-PCR for GAPDH was performed in parallel to show an equal amount of total RNA in the sample. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; M: 600 bp marker; B: PTEN, caspase-9 and caspase-3 mRNA levels were measured at three groups, normalized to those of GAPDH and presented as mean ± SE. <sup>a</sup>*P* < 0.05 for MGC-803/Cdx2 siRNA group vs MGC-803 and MGC-803/Cdx2 negative control group.

spectively (*P* < 0.05) (Figure 7). The results indicate that Cdx2 siRNA reduces the migratory and invasion ability of gastric cancer MGC-803 cells.

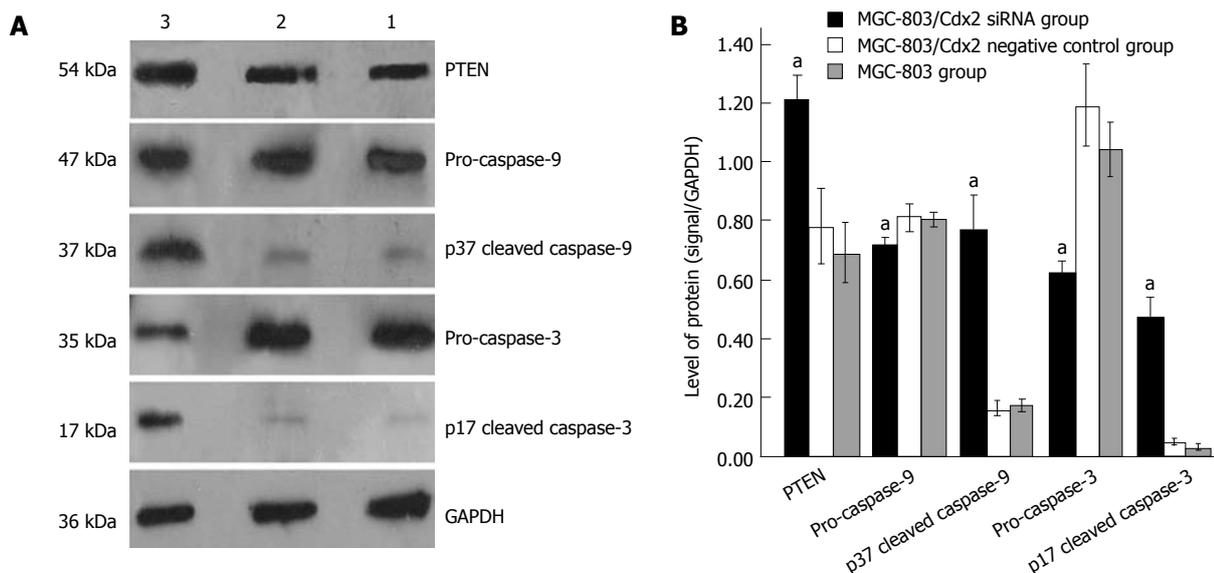
**Cdx2 siRNA increases PTEN expression, and activates caspase-9 and caspase-3**

To investigate the mechanism by which Cdx2 siRNA induces apoptosis in MGC-803 cells, we detected expression levels of several apoptotic family members including PTEN, caspase-9, and caspase-3 by semi-quantitative RT-PCR and Western blotting analysis. As shown in Figure 8, densitometric analysis showed that PTEN, caspase-9, and caspase-3 mRNA of MGC-803/Cdx2 siRNA cells were higher than that in MGC-803 cells and MGC-803/Cdx2 negative control cells (*P* < 0.05), while no differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells. As shown in Figure 9, Cdx2 siRNA led to the cleavage of pro-caspase-9 (47 kDa) and pro-caspase-3 (35 kDa) into other multiple, cleaved, maturation products (data not shown), but only the 37-kDa form of cleaved caspase-9 and the 17-kDa form of cleaved caspase-3 were observed. Den-

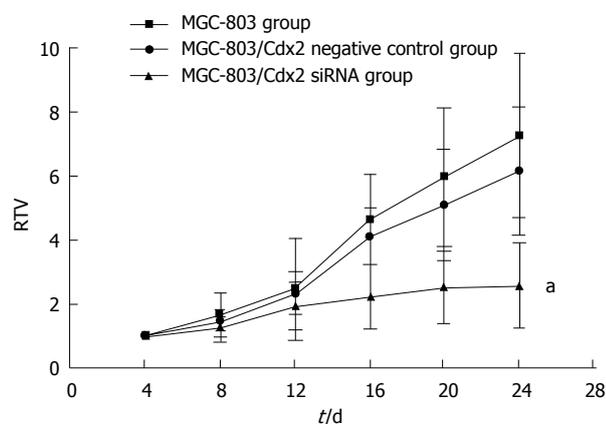
sitometric analysis showed that PTEN, p37 cleaved caspase-9, and p17 cleaved caspase-3 protein of MGC-803/Cdx2 siRNA cells were higher, while pro-caspase-9 and pro-caspase-3 were lower than that in MGC-803 cells and MGC-803/Cdx2 negative control cells (*P* < 0.05). No differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells.

**Inhibitory effect of Cdx2 siRNA in vivo**

We also examined the effect of Cdx2 siRNA on growth of MGC-803 cells *in vivo* by implanting MGC-803/Cdx2 siRNA cells subcutaneously into the flanks of BALB/c nude mice. Four weeks after implantation, tumor weight from MGC-803/Cdx2 siRNA cells was 0.773 ± 0.054 g, which was significantly less than 2.334 ± 0.087 g from MGC-803 cells, and 2.356 ± 0.092 g from MGC-803/Cdx2 negative control cells (*P* < 0.05). As shown in Figure 10, the tumor growth curves indicate the significant growth inhibition in MGC-803/Cdx2 siRNA cells (*P* < 0.05). Densitometric analysis showed that Cdx2 mRNA expression in MGC-803/Cdx2 siRNA cells (0.305 ± 0.053) was lower than that in MGC-803 cells (1.524 ±

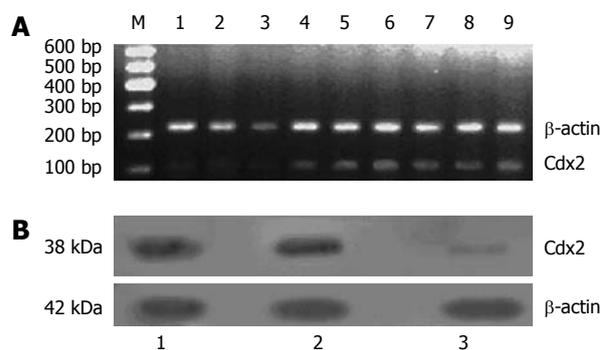


**Figure 9** Cdx2 small interference RNA significantly increased phosphatase and tensin homolog, cleaved caspase-9 and cleaved caspase-3 protein concentrations while pro-caspase-9 and pro-caspase-3 are decreased. **A:** Western blotting analysis. Whole protein extracts (100  $\mu$ g in each) were prepared from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells. The expression of phosphatase and tensin homolog (PTEN), pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3, and p17 cleaved caspase-3 was determined by Western blotting with an anti-PTEN, pro-caspase-9, cleaved caspase-9, pro-caspase-3 and cleaved caspase-3 antibody. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; **B:** PTEN, pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3 and p17 cleaved caspase-3 protein levels were measured at three groups, normalized to those of GAPDH and presented as mean  $\pm$  SE. \* $P < 0.05$  for MGC-803/Cdx2 siRNA group vs MGC-803 and MGC-803/Cdx2 negative control group.



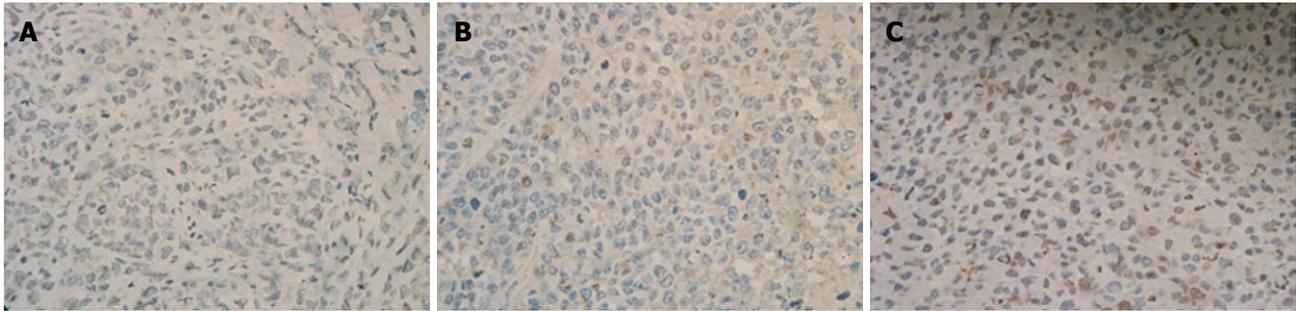
**Figure 10** Tumor growth curve showed a significant growth tendency in MGC-803 cells and in MGC-803/Cdx2 negative control cells, while the tumor growth in MGC-803/Cdx2 small interference RNA cells was obviously inhibited. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were implanted subcutaneously into the flanks of the nude mice. The relative tumor volume (RTV) of nude mice in each group were presented. Each time point represented the mean of RTV for each group; \* $P < 0.05$  for MGC-803/Cdx2 siRNA group vs MGC-803 and MGC-803/Cdx2 negative control group.

0.323) and MGC-803/Cdx2 negative control cells (1.441  $\pm$  0.269), as determined by semi-quantitative RT-PCR ( $P < 0.05$ ) (Figure 11A). In addition, the relative protein expression of Cdx2 in MGC-803/Cdx2 siRNA cells (0.134  $\pm$  0.087) also significantly decreased ( $P < 0.05$ ), when compared to MGC-803 cells (0.634  $\pm$  0.156) and MGC-803/Cdx2 negative control cells (0.569  $\pm$  0.167),



**Figure 11** Cdx2 mRNA and protein expression was suppressed in MGC-803/Cdx2 small interference RNA tumor tissue. **A:** Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNAs (2  $\mu$ g in each) extracted from tumor tissue were subjected to RT-PCR for Cdx2 and  $\beta$ -actin mRNAs. RT-PCR for  $\beta$ -actin was performed in parallel to show an equal amount of total RNA in the sample; Lanes 1-3: MGC-803/Cdx2 small interference RNA (siRNA) group; Lanes 4-6: MGC-803/Cdx2 negative control group; Lanes 7-9: MGC-803 group; M: 600 bp marker; **B:** Western blotting analysis. Equal amounts of protein extracts (100  $\mu$ g in each) were prepared from tumor tissue. The expression of Cdx2 protein was determined by Western blotting with an anti-Cdx2 antibody. The  $\beta$ -actin expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.

as determined by Western blotting analysis (Figure 11B). As shown in Figure 12, the percent of apoptotic tumor cells in MGC-803/Cdx2 siRNA cells was 16.7%  $\pm$  5.6%, which was more than 10.5%  $\pm$  4.1% in MGC-803/Cdx2 negative control cells and 11.2%  $\pm$  4.3% in MGC-803 cells, as determined by the TUNEL method.



**Figure 12 Cdx2 small interference RNA promoted tumor cells apoptosis.** Tumor cells were assessed for apoptosis using terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling assay. The apoptotic cells were brown-stained and counted under a optical microscope ( $\times 400$ ). A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 small interference RNA group.

## DISCUSSION

The Cdx2 homeobox gene, which is homologous to the *Drosophila* gene caudal, has an essential role during early development<sup>[4]</sup>. In adults, Cdx2 expression is restricted to intestinal epithelial cells. Overexpression of Cdx2 in human colon cancer cells induces a less malignant phenotype, inhibiting proliferation, invasion, and migration<sup>[16]</sup>, and Cdx2 expression is progressively reduced in gastric cancer<sup>[17]</sup>. Moreover, heterozygous-null Cdx2 mice are more sensitive to azoxymethane-induced colonic adenocarcinomas<sup>[18]</sup>, and mice that are compound heterozygotes for Cdx2 and the tumor suppressor Adenomatous Polyposis Coli (Apc) developed more adenomatous polyps in the colon than their heterozygous Apc littermates<sup>[19]</sup>. These studies suggested that Cdx2 is a putative tumor suppressor.

However, other reports have shown that Cdx2 plays a pivotal role in the development of intestinal metaplasia<sup>[20,21]</sup>. The implication of Cdx2 in intestinal metaplasia has been demonstrated in intestinal metaplasia of the stomach where Cdx2 was ectopically overexpressed, suggesting that it could play a major role during intestinal metaplasia formation in the stomach<sup>[21]</sup>. Intestinal metaplasia is a precursor of intestinal-type gastric adenocarcinoma. Long-term intestinal metaplasia induced gastric adenocarcinoma in the Cdx2-transgenic mouse stomach, and no significant changes were noted in wild-type littermates<sup>[14]</sup>. The tumor incidence was 100% at 100 wk after birth<sup>[15]</sup>. It can thus be concluded that Cdx2-induced intestinal metaplasia itself is a precancerous lesion leading to gastric carcinoma. Furthermore, Cdx2 is overexpressed in most colorectal tumors compared to matched normal mucosa in adults<sup>[7]</sup>. Dang *et al.*<sup>[22]</sup> showed that Cdx2 does not suppress tumorigenicity in the human gastric cancer cell line, MKN45. It can be concluded that, in contrast to the prevailing paradigm, Cdx2 does not serve as a tumor suppressor in the development of most sporadic colorectal tumors. Rather, in the context of earlier observations of its role in promoting the neoplastic phenotype in some cells and tissues, many observations suggest the intriguing possibility that Cdx2 could serve as an oncogene in the gastrointestinal

tract<sup>[9,23]</sup>. This suggests that the level of Cdx2 expression may contribute to its function<sup>[9]</sup>, thereby raising the possibility that intervening with Cdx2 expression in gastric cancer cells with RNA interference may control their growth rate.

Our study indicated that Cdx2 siRNA led to remarkable inhibition of Cdx2 mRNA and protein expression in MGC-803 cells, inhibited cell growth, caused cell cycle arrest in the G0/G1 phase, and induced cell apoptosis. Furthermore, RNAi-directed targeting of Cdx2 in MGC-803 cells reduced the capability of cell motion, invasion, and colony formation. Moreover, a strong anti-tumor effect of Cdx2-siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when Cdx2 mRNA and protein was silenced by Cdx2 siRNA. These findings suggest that Cdx2 has tumorigenic potential in the human gastric cancer cell lines MGC-803.

However, our previous study showed that Cdx2 overexpression in human gastric cancer MGC-803 cells produce similar results as Cdx2 siRNA<sup>[24]</sup>. Moreover, Cdx2 overexpression was associated with cell cycle arrest in the G0/G1 phase which was the same as Cdx2 siRNA. This suggests that Cdx2 plays a double role in the regulation of MGC-803 cell growth and death. Thus, we can only speculate on potential explanations for these observed contrasts. First, appropriate activity and expression levels of Cdx2 are necessary for the normal cell cycle, even in promoting tumor proliferation and regression. Just like E2F-1, both the upregulation and downregulation of E2F-1 can suppress human gastric cancer MGC-803 cell growth *in vitro*<sup>[25,26]</sup>. Second, these two conflicting results may involve different mechanisms. Our previous data showed that overexpression of Cdx2 inhibits MGC-803 cell progression *via* the Wnt signaling pathway (unpublished data). In this result, PTEN, caspase-9 and caspase-3 expression were all increment when Cdx2 was downregulated. The PTEN protein product is a lipid phosphatase that antagonizes PI3K function and consequently inhibits downstream signaling transduction through Akt<sup>[27]</sup>. Caspase-9, a member of the protease family, is intimately associated with the initiation of apoptosis, and is thought to be activated while

Akt is inhibited<sup>[28]</sup>. Activated caspase-9 is able to cleave caspase-3 *in vitro*, leading to apoptosis<sup>[29]</sup>. Therefore, in the present study, inhibition of Cdx2 expression may increase PTEN expression directly or indirectly, leading to activation of caspase-9 and caspase-3 *via* the PI3K/Akt signaling pathway, which is responsible for inhibition of MGC-803 cell growth *in vitro* and *in vivo*. Further studies are needed to confirm our results.

Gastric cancer is a worldwide problem. Besides the undetermined etiological factors, there are also limitations in surgery, chemotherapy and radiotherapy, which to date, are the major therapies for gastric cancer<sup>[30]</sup>. Many patients lose the chance of surgery because of their systemic condition, while many cannot tolerate the side effects of chemotherapy or radiotherapy. It is important to find a new way to effectively inhibit cancer cell growth and avoid the side effects of drugs or radioactive rays. Gene target therapies have proved to be a promising way to achieve this goal<sup>[26]</sup>. In this study, we showed that Cdx2 plays a critical role in gastric cancer cell proliferation, invasion, and apoptosis. The down-regulation of Cdx2 using RNAi successfully reduced the progression of gastric cancer MGC-803 cells *in vitro* and *in vivo*. In conclusion, this study lays the foundation for treatment of gastric cancer through manipulation of Cdx2 expression.

## COMMENTS

### Background

Gastric cancer is a worldwide problem. Besides the undetermined etiological factors, there are also limitations in surgery, chemotherapy and radiotherapy, which to date, are the major therapies for gastric cancer. It is important to find a new way to effectively inhibit cancer cell growth and avoid the side effects of drugs or radioactive rays. Gene target therapies have proved to be a promising way to achieve this goal. The caudal-type homeobox gene, Cdx2, plays an important role in intestinal metaplasia, and is a precursor of intestinal-type gastric carcinoma. However, the effect of Cdx2 in gastric cancer is still not very clear.

### Research frontiers

Cdx2 plays important roles in early differentiation, proliferation and maintenance of intestinal epithelial cells. The role of Cdx2 as an oncogene or a tumor suppressor gene is still in dispute at the present time. The Cdx2 research hotspot is how it affect the progression of human cancer.

### Innovations and breakthroughs

This study for the first time demonstrated that Cdx2 small interference RNA (siRNA) significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. Cdx2 siRNA also increased phosphatase and tensin homolog expression, and activated caspase-9 and caspase-3 in MGC-803 cells *in vitro* as determined by reverse transcription polymerase chain reaction and Western blotting analysis. In addition, siRNA targeting of Cdx2 inhibited the growth of MGC-803 cells and promoted tumor cell apoptosis *in vivo* in nude mice tumor models.

### Applications

This study lays the foundation for treatment of gastric cancer through manipulation of Cdx2 expression.

### Terminology

The transcription factor, Cdx2, is a member of the caudal-related homeobox gene family, and is mainly expressed in the intestine. It is also known to be a key factor in the development of intestinal metaplasia.

### Peer review

In this study, the authors constructed recombinant pSilencer 4.1-Cdx2 siRNA

plasmids and transfected them into human gastric cancer MGC-803 cells *in vitro*. The authors demonstrated that Cdx2 siRNA led to inhibition of endogenous Cdx2 mRNA and protein expression and Cdx2 siRNA significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. The authors conclude that Cdx2 is involved in the regulation of tumor growth, proliferation, apoptosis and invasion of gastric cancer cells. Overall this is a well-conducted pilot study.

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## Caspase-cleaved cytokeratin-18 and tumour regression in gastro-oesophageal adenocarcinomas treated with neoadjuvant chemotherapy

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cancers were constructed into tissue microarrays. The first set consisted of 122 gastric/gastro-oesophageal cancer cases not exposed to neoadjuvant chemotherapy and the second set consisted of 97 gastric/gastro-oesophageal cancer cases exposed to pre-operative platinum-based chemotherapy. Expression of CK-18 and caspase-cleaved CK-18 was investigated using immunohistochemistry.

**RESULTS:** CK18 was commonly expressed in gastro-oesophageal tumours (92.6%). Fifty-six point seven percent of tumours previously exposed to neoadjuvant chemotherapy were positive for caspase-cleaved CK-18 expression compared to only 24.6% of tumours not previously exposed to neoadjuvant chemotherapy ( $P = 0.009$ ). In patients who received neoadjuvant chemotherapy, caspase-cleaved cytokeratin-18 expression correlated with favourable TRG response (TRG 1, 2 or 3,  $P = 0.043$ ).

**CONCLUSION:** This is the largest study to date of CK-18 and caspase-cleaved CK-18 expression in gastro-oesophageal tumours. We provide the first evidence that caspase-cleaved CK-18 predicts tumour regression with neoadjuvant chemotherapy.

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**Key words:** Tumour regression grade; Gastro-oesophageal cancers; Chemotherapy; Full length cytokeratin-18; Caspase-cleaved cytokeratin-18

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Fareed KR, Soomro IN, Hameed K, Arora A, Lobo DN, Parsons SL, Madhusudan S. Caspase-cleaved cytokeratin-18 and tumour regression in gastro-oesophageal adenocarcinomas treated

### Abstract

**AIM:** To examine cytokeratin-18 (CK-18) and caspase-cleaved CK-18 expression in tumours and correlate with clinicopathological outcomes including tumour regression grade (TRG) response.

**METHODS:** Formalin-fixed human gastro-oesophageal

with neoadjuvant chemotherapy. *World J Gastroenterol* 2012; 18(16): 1915-1920 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1915.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1915>

## INTRODUCTION

Neoadjuvant platinum-based chemotherapy followed by surgery is the standard of care for patients with gastro-oesophageal adenocarcinoma<sup>[1,2]</sup>. However, there is an urgent need to develop predictive markers to individualize patient therapy<sup>[3]</sup>. We have recently shown that tumour regression grade (TRG) is a marker of histopathological response and tumour down-staging in tumours receiving neoadjuvant chemotherapy<sup>[4]</sup>. TRG was defined as per Mandard's criteria<sup>[5]</sup>. TRG1 (complete regression) showed absence of residual cancer and fibrosis extending through the different layers of the oesophageal wall; TRG2 was characterised by the presence of rare residual cancer cells scattered through the fibrosis; TRG3 was characterised by an increase in the number of residual cancer cells but fibrosis predominated; TRG4 showed residual cancer outgrowing fibrosis; and TRG5 was characterised by the absence of regressive changes<sup>[5]</sup>. In patients who received neoadjuvant chemotherapy (CS group), 46.7% of gastric/gastro-oesophageal junction adenocarcinomas and 45.5% of lower third oesophageal adenocarcinomas had TRG 1, 2 or 3 compared to 13.7% in patients who did not receive neoadjuvant chemotherapy but proceeded to primary surgery. In the CS group, responders (TRG 1, 2 or 3) showed significant tumour down-staging [early ypT-stage disease ( $P = 0.002$ )]. In gastric cancers specifically, additional associations were seen with negative nodal disease ( $P = 0.044$ ) and absence of vascular invasion ( $P = 0.027$ )<sup>[4]</sup>. More recently, we have also demonstrated that favourable tumour regression predicts better clinical outcomes in patients receiving neoadjuvant chemotherapy in gastro-oesophageal adenocarcinomas<sup>[6]</sup>.

The anticancer activity of chemotherapeutic agents is directly related to the induction of apoptosis in tumours. Whilst the apoptotic pathway is complex, the intrinsic mitochondrial pathway is the predominant apoptotic pathway in cancer cells. In the intrinsic pathway, the mitochondrial release of cytochrome c activates caspase-9, which in turn activates caspase-3 and caspase-7<sup>[7,8]</sup>. Among the several cellular substrates of the capsases, members of the cytokeratin family, including cytokeratin-18 (CK-18), contribute to cellular collapse and apoptosis. Caspase-cleaved CK-18 is a specific marker of epithelial cell death and correlates with apoptosis in gastrointestinal epithelial cancers<sup>[9-11]</sup>.

In the current study we have evaluated full length CK-18 and caspase-cleaved CK-18 protein expression using immunohistochemistry. We show for the first time that caspase-cleaved CK-18 expression in tumours correlates with histopathological tumour regression in early stage gastro-oesophageal adenocarcinomas.

## MATERIALS AND METHODS

### Patients

We identified patients referred to our centre with resectable gastric, gastro-oesophageal junction (GOJ) and lower third oesophageal adenocarcinomas between January 2001 and May 2008. GOJ tumours were defined as per Siewert's classification<sup>[12]</sup>. The Union for International Cancer Control TNM staging system for oesophageal and gastric cancer was used in this study. The study was approved by the Ethics Committee of Nottingham University Hospitals.

### Construction of tissue micro-array

Tissue micro-arrays (TMAs) were constructed. In brief, HE-stained slides (5  $\mu\text{m}$ ) were used to identify and mark out representative areas of viable tumour tissue. Then 0.6 mm-diameter needle core biopsies from the relevant areas of corresponding paraffin-embedded blocks were placed at defined coordinates in the recipient paraffin array blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). Array blocks were constructed at a density of 80-150 cores per array. Two broad sets of TMA blocks were constructed. An array set of 97 patient cores to include gastric and gastro-oesophageal tumours that had received neoadjuvant chemotherapy and an array set of 122 cores of patients who had received no neoadjuvant chemotherapy were constructed. These TMA blocks were constructed in triplicate, each containing one sample from a different region of the tumour.

### Immunohistochemistry

A standard streptavidin-biotin complex technique was used. In brief, 5  $\mu\text{m}$  TMA sections were deparaffinised with xylene and rehydrated through graded alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Antigen retrieval was carried out by microwave treatment of the slides in sodium citrate buffer (pH 6) for 10 min at 750 W followed by 10 min at 300 W. The slides were rinsed in phosphate buffer saline (PBS) and incubated with Vectastain blocking serum diluted in PBS to block non-specific absorption. The slides were incubated for 30 min with the primary antibody M30 to detect caspase-cleaved CK-18 (Peviva, Bromma, Sweden) at a dilution of 1:75 and primary antibody M6 to detect full length CK-18 (Peviva, Bromma, Sweden) at a dilution of 1:150 at room temperature. After washing with PBS, sections were incubated with secondary antibody (Vectastain) for 30 min followed by avidin-biotin complex for a further 30 min. 3-3' Diaminobenzidine tetrahydrochloride was used as a chromogen. All sections were counterstained with Gill's haematoxylin, dehydrated and mounted using DPX (a mixture of disterene, plasticizer, and xylene; Sigma).

### Evaluation of staining

Evaluation of staining was performed with the observer blinded to the corresponding clinicopathological data. For

Table 1 Patient demographics *n* (%)

	Neoadjuvant chemotherapy group	Primary surgery group
Total number of patients	97	122
Median age (yr)	64	74.5
Sex		
Male	74 (76)	92 (75.4)
Female	23 (24)	30 (24.6)
T stage		
T1	6 (6.1)	12 (9.8)
T2	29 (29.8)	41 (33.6)
T3	50 (51.5)	65 (53.2)
T4	8 (8.2)	4 (3.2)
TX	1 (1)	
N stage		
N0	31 (31.9)	32 (26.2)
≥ N1	66 (68.1)	90 (73.8)
M stage		
M0	97 (100)	122 (100)
M1	-	-
Tumour type		
Adenocarcinoma	83 (85.5)	122 (100)
Squamous cell carcinoma	12 (12.3)	-
Adenosquamous	2 (2)	-
Site of tumour		
Gastric	20 (20.6)	122 (100)
GOJ	47 (48.4)	-
Lower third of oesophagus	36 (37.1)	-
Surgery		
Total gastrectomy	22	70
Partial gastrectomy	5	39
Oesophagectomy/	70	13
Oesophago-gastrectomy		
Survival status		
Alive	47 (48.4)	47 (38)
Dead	50 (51.6)	75 (62)

GOJ: Gastro-oesophageal junction.

full length CK-18 expression, cytoplasmic expression in cancer cells was considered positive. For caspase-cleaved CK-18 expression, TMA cores from tumour showing any positively stained apoptotic cells were considered positive. Caspase-cleaved CK-18 positive apoptotic cells were counted in TMA cores and the total number of positive cells from each tumour was taken as the number of positive cells.

### Statistical analysis

All statistical analyses were carried out using SPSS package (version 15 for Windows, SPSS, Inc.). Associations between categorical variables were examined using cross-tabulation and the Pearson  $\chi^2$  test. Kaplan Meier curves were derived to assess disease-specific survival, and the significance of differences in disease-specific survival between groups was calculated using the log-rank test. Patients whose death related to their oesophago-gastric cancer were considered in the disease-specific survival calculations. This was determined by death certification entries. Deaths resulting from non-oesophago-gastric cancer-related causes were censored. Survival rates were calculated from the date of diagnosis until the 13th January 2009, when any remaining survivors were censored

and Kaplan Meier curves were plotted. In all cases,  $P < 0.05$  was considered statistically significant.

## RESULTS

### Patient demographics

There were 2 groups of patients: those who received neoadjuvant chemotherapy (neoadjuvant group) and those who underwent primary surgery only (primary group). There were 97 patients in the neoadjuvant group with a median age of 64 years; 76% ( $n = 74$ ) were male and 51.5% ( $n = 50$ ) of cases were T3 tumours. There were 122 cases in the primary group with a median age of 74.5 years; 75.4% ( $n = 92$ ) were male and 53.2% ( $n = 65$ ) had T3 tumours. Patients in the primary surgery group did not receive any adjuvant chemotherapy after surgery. In the neoadjuvant group, 78% of patients had received all the planned three cycles of neoadjuvant ECF/ECX chemotherapy (adenocarcinomas) and 96.4% had received all the planned two cycles of neoadjuvant CF chemotherapy (squamous cell carcinomas). Of the patients who received all three cycles of ECF/ECX chemotherapy, 42% went on to receive a further three cycles of ECF/ECX chemotherapy. There was no significant difference between the primary surgery group and the perioperative chemotherapy group (gastric/GOJ) with regards to T stage [T2 (33.6% *vs* 29.8%), T3 (53.2% *vs* 51.5%)] and N stage [N0 (26.2% *vs* 31.9%),  $> N0$  (73.8% *vs* 68.1%)]. Only adenocarcinomas were included in the immunohistochemical and survival analyses in this study (Table 1).

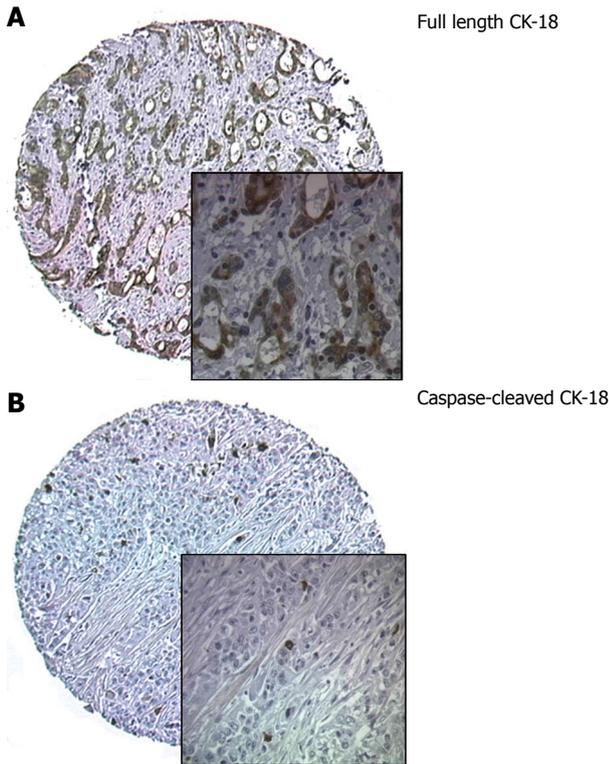
### Full length cytokeratin-18 expression

All 122 tumours in the primary surgery group were available for CK-18 analyses. One hundred and thirteen tumours stained positive for CK-18 (92.6%) (Figure 1A) and 9 cores were negative for CK-18 expression. There was no statistically significant correlation between tumour differentiation, T stage, N stage, vascular/perineural invasion, resection margin involvement and full length CK-18 expression in tumours.

### Caspase-cleaved cytokeratin-18 expression

All tumours were suitable for caspase-cleaved CK-18 expression analyses (Figure 1B). In tumours previously exposed to neoadjuvant chemotherapy (neoadjuvant TMA,  $n = 97$ ), 56.7% of tumours (55/97) were positive compared to 24.6% (30/122) of tumours not previously exposed to neoadjuvant chemotherapy (primary TMA). This was statistically significant ( $P = 0.009$ ). The mean total number of caspase-cleaved CK-18 positive cancer cells per tumour was 4.16 in the neoadjuvant group (range: 1-92) compared to 2.7 in the primary surgery group (range: 1-51).

We have previously demonstrated that TRG as assessed using Mandard's criteria is a useful tool to assess response to neoadjuvant chemotherapy in gastro-oesophageal adenocarcinomas; favourable TRG correlated with tumour down-staging in that study<sup>[4]</sup>. In the current study we



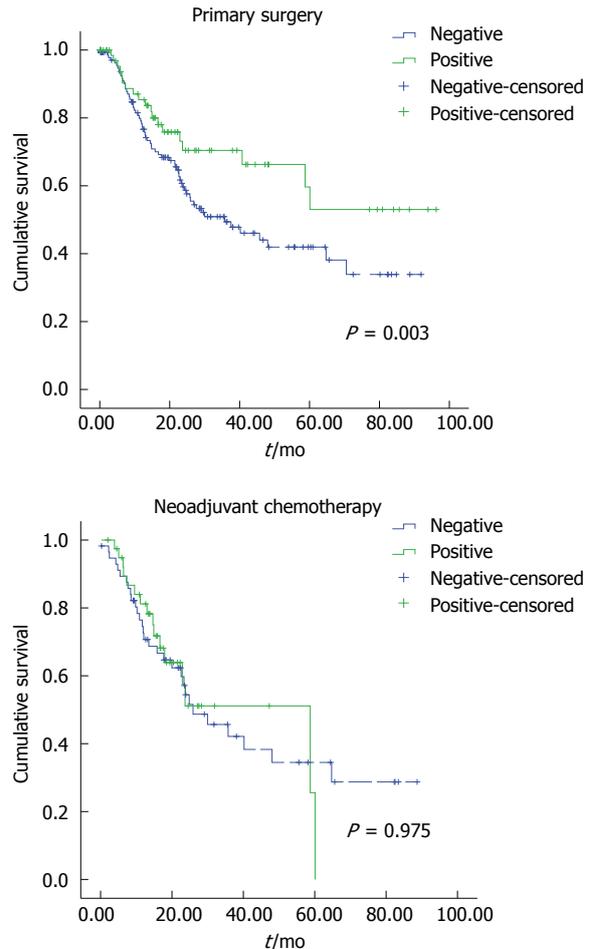
**Figure 1** Immunohistochemical staining of full length cyokeratin-18 and caspase-cleaved cyokeratin-18. A: Immunohistochemical staining of full length cyokeratin-18 (CK-18) showing strong cytoplasmic staining; B: Immunohistochemical staining for caspase-cleaved CK-18. Cores from tumour showing positively-stained apoptotic cells. Original magnification  $\times 100$ ; insets  $\times 400$ .

Caspase-cleaved CK-18	TRG1, TRG2, TRG3	TRG4, TRG5	Total
Negative	10 (23.8)	32 (76.2)	42 (100)
Positive	24 (43.6)	31 (56.4)	55 (100)
Total	34 (35.1)	63 (64.9)	97 (100)

CK-18: Cyokeratin-18; TRG: Tumour regression grade.

evaluated whether caspase-cleaved CK-18 expression correlated with TRG. We found that 43.6% of tumours that were positive for caspase-cleaved CK18 also had a favourable tumour response (TRG 1-3) compared to 23.8% that were negative for caspase-cleaved CK-18 expression (Table 2). This was statistically significant ( $P = 0.043$ ). There was no statistically significant correlation between tumour differentiation, T stage, N stage, vascular/perineural invasion, resection margin involvement and caspase-cleaved CK-18 positivity.

Clinicopathological correlations were also observed in tumours not exposed to neoadjuvant chemotherapy. Well-differentiated tumours were more likely to be caspase-cleaved CK-18 positive (62.5%) compared to poor- and moderately-differentiated tumours which were only positive in 23.6% and 19% of tumours respectively ( $P = 0.031$ ). However, no differences between T, N stage, vascular/perineural invasion, resection margin involvement



**Figure 2** Kaplan Meier curves representing the relationship between caspase-cleaved cyokeratin-18 and disease-specific survival. A: In months from time of diagnosis in patients who received primary surgery only; B: In patients who received neoadjuvant chemotherapy.

and caspase-cleaved CK-18 positivity were observed. Regarding patients who had not received neoadjuvant chemotherapy, Kaplan Meier plot showed that in patients whose tumours stained positive for caspase-cleaved CK-18, a longer disease-specific survival was observed compared to patients whose tumours were negative (mean 84 mo *vs* 51 mo,  $P = 0.003$ ) (Figure 2). However, in patients who had received neoadjuvant chemotherapy, no statistically significant differences were observed (mean 37.3 mo *vs* 41.6 mo,  $P = 0.975$ ) (Figure 2).

Those factors found to be significant in univariate analyses were also included in a multivariate logistic regression analysis to estimate the independent effect of each factor after adjusting for the contributions of other factors. There was no one factor in the cohort of patients studied that showed significance on multivariate analysis (Table 3).

## DISCUSSION

The ability to predict response to chemotherapy and individualize patient treatment is a high priority in gastro-oesophageal adenocarcinomas. Whilst the role of multi-

**Table 3** Univariate and multivariate analyses showing predictive factors for disease-specific and overall survival in neoadjuvant and surgery only groups

	Univariate analysis		Multivariate analysis
	DSS	OS	OS
<b>Neoadjuvant group</b>			
Caspase-cleaved CK-18 positivity	0.975	0.865	1.32
TRG 1-3 vs 4-5	0.038	0.136	0.109
Tumour diff. (well/mod vs poor)	0.087	0.101	0.32
T stage (T1, 2 and 3, 4)	0.07	0.09	0.101
N stage (N0 and > N1)	0.106	0.124	0.23
M stage (M0 and > M0)	0.23	0.14	1.72
Vascular invasion	0.87	0.76	0.86
Perineural invasion	1.32	1.455	1.76
Resection margin involvement	2.34	1.98	0.24
<b>Surgery only group</b>			
Caspase-cleaved CK-18 positivity	0.003	0.668	0.10
TRG 1-3 vs 4-5	0.87	0.003	0.23
Tumour diff. (well/mod vs poor)	0.39	0.34	0.21
T stage (T1, 2 and 3, 4)	0.04	0.08	0.19
N stage (N0 and > N1)	0.16	0.10	0.43
M stage (M0 and > M0)	1.27	1.64	1.99
Vascular invasion	1.98	1.67	1.34
Perineural invasion	1.56	1.99	2.43
Resection margin involvement	0.34	0.25	0.34

DSS: Disease-specific survival; OS: Overall survival; CK-18: Cytokeratin-18; TRG: Tumour regression grade; diff.: Differentiation.

modality therapy in improving patient outcomes is well established, these treatments are toxic and have a considerable impact on patient morbidity. In the United Kingdom, neoadjuvant chemotherapy followed by surgery is routinely offered to patients who are fit with no significant co-morbidities. Accordingly, these patients generally tend to be young and can tolerate toxic chemotherapy. Patients considered not suitable for chemotherapy are usually elderly with significant co-morbidities. Moreover, until 2006 in the United Kingdom, the standard treatment for patients with early stage gastro-oesophageal adenocarcinoma was surgery only and patients were not routinely offered adjuvant chemotherapy. With the publication of results from a large United Kingdom trial of perioperative chemotherapy<sup>[1]</sup>, neoadjuvant chemotherapy was established as a standard treatment option for patients. This is reflected in the differences in mean age between the two groups in our study.

In the current study we have evaluated the potential role of full length CK-18 and caspase-cleaved CK-18 as biomarkers in gastro-oesophageal adenocarcinomas. To evaluate the prognostic significance of full length CK-18 in gastro-oesophageal adenocarcinomas, we first evaluated CK-18 expression in tumours not exposed to neoadjuvant chemotherapy ( $n = 122$ ). We found that CK-18 was commonly expressed in tumours. This is consistent with a previously reported study<sup>[13]</sup>. Although Xu *et al*<sup>[13]</sup> demonstrated that CK18 mRNA expression correlated with lymph node metastasis and tumour differentiation in gastric cancer, we were unable to demonstrate any positive clinicopathological correlations in our study.

Caspase-cleaved CK-18 has recently emerged as a promising marker of apoptosis in gastrointestinal cancers<sup>[11]</sup>. We therefore evaluated caspase-cleaved CK-18 expression in gastro-oesophageal adenocarcinomas. Fifty-six point seven percent of tumours previously exposed to neoadjuvant chemotherapy were positive for caspase-cleaved CK-18 expression, compared to only 24.6% of tumours not previously exposed to neoadjuvant chemotherapy ( $P = 0.009$ ). This provides direct evidence that chemotherapy exposure leads to apoptosis-induced increased caspase-cleaved CK-18 expression in gastro-oesophageal tumours. We then demonstrated, for the first time, that the caspase-cleaved CK-18 expression correlated well with favourable tumour regression in patients receiving neoadjuvant chemotherapy ( $P = 0.043$ ). Factors found to be significant in univariate analyses were not significant in a multivariate logistic regression analysis. This may be because the current study is a small retrospective study and a larger prospective study is required to confirm our observations. However, our study provides evidence that caspase-cleaved CK-18 may be a promising predictive biomarker in gastro-oesophageal adenocarcinomas. Moreover, our study also supports a rational hypothesis for evaluating serial blood caspase-cleaved CK18 secretion, using a recently developed assay<sup>[11,14,15]</sup>, as a promising non-invasive biomarker in operable gastro-oesophageal adenocarcinomas where patients routinely receive three cycles of platinum-based neoadjuvant chemotherapy<sup>[1,2]</sup>. In a recent study of advanced colorectal cancer, serum levels of caspase-cleaved CK18 were significantly higher in patients who responded to chemotherapy compared to those who did not<sup>[11]</sup>. Similar results were also reported in advanced colorectal, oesophageal and gastric adenocarcinoma patients receiving palliative chemotherapy<sup>[16]</sup>. Although these results are promising, whether tumour tissue and serum caspase CK-18 levels are related to each other is not clear. Moreover, whether similar results could be achieved in early stage gastro-oesophageal adenocarcinoma patients receiving neoadjuvant chemotherapy is an area of ongoing investigation in our laboratory.

We also made interesting clinicopathological observations in tumours not exposed to chemotherapy. Caspase-cleaved CK18 expression correlated with better differentiation and improved survival in this group. Although the reason for this unexpected finding is not clear, whether host immune factors such as lymphocytic infiltration could contribute to cancer cell death is currently unknown and is an area of ongoing investigation.

In summary, we have conducted a study of full length CK-18 and caspase-cleaved CK-18 expression in gastro-oesophageal cancer. We provide evidence that caspase-cleaved CK-18 is a promising predictive biomarker in patients who receive platinum-based neoadjuvant chemotherapy.

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## COMMENTS

### Background

Neoadjuvant chemotherapy followed by surgery is a standard treatment option in patients with early stage gastro-oesophageal adenocarcinomas. However, only 40% of patients respond to chemotherapy. There is an urgent need to develop predictive biomarkers.

### Research frontiers

Development of a serum biomarker test that can predict response to chemotherapy is highly desirable in gastro-oesophageal adenocarcinomas.

### Innovations and breakthroughs

Cytokeratin-18 (CK-18) is commonly expressed in epithelial tumours. Caspase-cleaved CK-18 is expressed in cancer cells undergoing apoptosis following chemotherapy. Caspase-cleaved CK-18 is also secreted by tumour cells and can be evaluated using a blood test. In early stage gastrooesophageal cancers, the authors show that caspase-cleaved CK-18 is prevalent in tumour tissue following chemotherapy and that this correlates with tumour regression. The study suggests that serum testing of caspase-cleaved CK-18 may be feasible to predict response in early stage gastro-oesophageal adenocarcinomas.

### Applications

A prospective study of serial serum testing of caspase-cleaved CK-18 elevation and correlation with tumour response to chemotherapy is required to evaluate CK-18 as a promising predictive biomarker in early stage gastro-oesophageal adenocarcinomas.

### Terminology

CK-18 is widely expressed in epithelial cancers. Caspase-cleaved CK-18: in epithelial cells undergoing apoptosis, caspase-cleaved CK-18 is expressed and can be detected by immunohistochemistry.

### Peer review

This is a study to evaluate whether caspase-cleaved CK-18 can predict response to chemotherapy in gastro-oesophageal adenocarcinomas receiving neoadjuvant chemotherapy. Data presented here support a rational hypothesis to test if secretion of caspase-cleaved CK-18 in blood can be used as a marker of response to chemotherapy in patients.

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## Helpfulness of the combination of acetic acid and FICE in the detection of Barrett's epithelium and Barrett's associated neoplasias

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### Abstract

**AIM:** To investigate the mucosal morphology in Barrett's oesophagus by chromo and magnifying endoscopy.

**METHODS:** A prospective pilot study at a tertiary medical centre was conducted to evaluate the use of acetic acid pulverisation combined with virtual chromoendoscopy using Fujinon intelligent chromoendoscopy (FICE) for semiological characterization of the mucosal morphology in Barrett's oesophagus and its neoplastic complications. Upper endoscopy using high definition white

light, 2% acid acetic pulverisation and FICE with high definition videoendoscopy were performed in 20 patients including 18 patients who presented with aspects of Barrett's oesophagus at endoscopy examination. Two patients used as controls had normal endoscopy and histological results. Prospectively, videos were watched blind from histological results by three trained FICE technique endoscopists.

**RESULTS:** The videos of patients with high-grade dysplasia showed an irregular mucosal pattern in 14% using high definition white light endoscopy and in 100% using acid acetic-FICE combined. Videos did not identify irregular vascular patterns using high definition white light endoscopy, while acid acetic-FICE combined visualised one in 86% of cases.

**CONCLUSION:** Combined acetic acid and FICE is a promising method for screening high-grade dysplasia and early cancer in Barrett's oesophagus.

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**Key words:** Acetic acid; Barrett's metaplasia; Chromoendoscopy; Fujinon intelligent chromoendoscopy

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## INTRODUCTION

Barrett's oesophagus is a premalignant lesion of oesophageal adenocarcinoma and endoscopic surveillance has been proposed for the diagnosis of this condition<sup>[1,2]</sup>. A stepwise four quadrant biopsy protocol is considered the gold standard procedure<sup>[3]</sup>. Current guidelines advise that biopsies should be obtained from any visible abnormality and that four random quadrant biopsies every 2 cm should be taken to detect inconspicuous dysplasia during endoscopic surveillance<sup>[4,5]</sup>. In theory, a high sensitivity endoscopic technique for the detection of high-grade dysplasia or early carcinoma is warranted and targeting biopsies and a four quadrant biopsy protocol will be unnecessary to improve Barrett's oesophagus cancer detection.

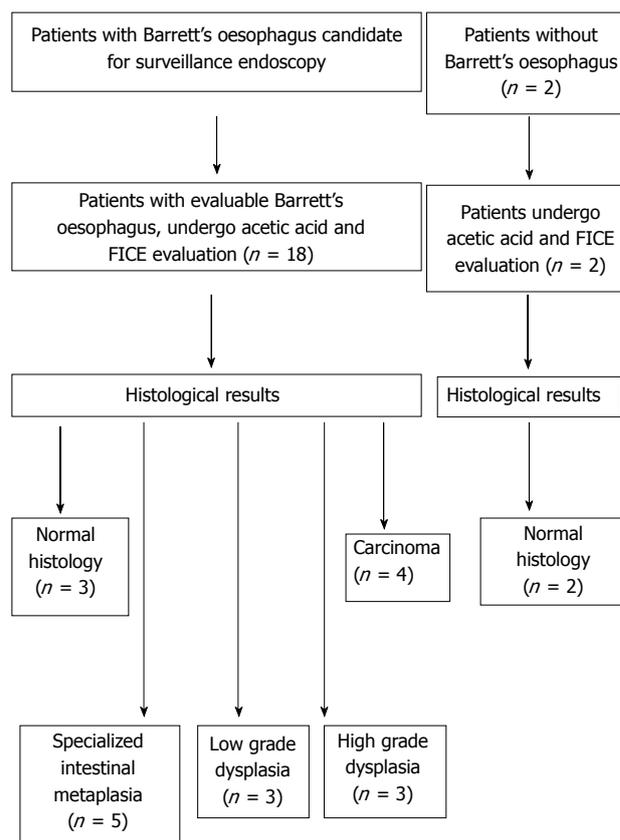
Chromoendoscopy with methylene blue, acetic acid, or virtual chromoendoscopy have been proposed to improve the detection of preneoplastic lesions of Barrett's oesophagus. Although acid acetic pulverisation improves visibility of the mucosal pattern by removing the superficial mucus and enhancing the pit pattern, virtual chromoendoscopy was developed to identify abnormalities from superficial mucosal or vascular patterns, and, therefore, facilitate diagnosis of Barrett's associated neoplasias<sup>[6,7]</sup>. Virtual chromoendoscopy using narrow band imaging (NBI) was first studied in this indication and abnormalities of pit and vascular patterns have been described<sup>[6]</sup>. Improvements in endoscopic material allows functional imaging to be incorporated, which, in turn, permits visualisation of more detail in mucosal and vascular patterns and may complement high-resolution endoscopy to increase the sensitivity of the endoscopic detection of early neoplasia in Barrett's oesophagus. Kim *et al*<sup>[8]</sup> showed that NBI was not reproducible and had a sensitivity of 89% in detecting preneoplastic lesions of Barrett's oesophagus. Virtual chromoendoscopy using Fujinon intelligent chromoendoscopy (FICE) has been shown to be a useful tool in identifying gastric lesions<sup>[9]</sup>. Pohl *et al*<sup>[10]</sup> failed to show, in a single prospective study, significant differences between FICE and acetic acid combined with conventional chromoendoscopy for the detection of high-grade dysplasia or early cancer in patients with Barrett's oesophagus. No study has shown any benefit with the combination of acetic acid and FICE. These two techniques could be complementary, since acetic acid enhances visualisation of the pit pattern and FICE allows detection of vascular abnormalities in Barrett's oesophagus. With the Pohl *et al*<sup>[10]</sup> study in mind, we conducted a pilot study to evaluate the combination of 2% acetic acid pulverisation and FICE for semiological characterization of the mucosal morphology in Barrett's oesophagus.

These two techniques could be complementary, since acetic acid enhances visualisation of the pit pattern<sup>[11]</sup> and FICE allows detection of vascular abnormalities in Barrett's oesophagus.

## MATERIALS AND METHODS

### Ethics

All patients enrolled in this study gave written informed



**Figure 1** Patient selection for the present study. FICE: Fujinon intelligent chromoendoscopy.

consent. The study was in accordance with the Declaration of Helsinki and the Institutional review board (Centre de protection des personnes d'Ile de France III) approved the study (ref: CPP: AT102).

### Patients

The study population consisted of patients with Barrett's oesophagus, as confirmed by pathological analysis. None of the patients had received previous therapy for Barrett's oesophagus. The eligibility criteria also included a four-quadrant biopsy protocol, a standardized procedure as described above and a video record of the overall procedure. Patients were required to have at least one dysplastic lesion that could be evaluated. Data were collected retrospectively from a review of the electronic medical records and endoscopy database of our institution from November 2006 to September 2009. Eighteen patients had endoscopic Barrett's oesophagus (Figure 1). Of these patients, fifteen had specialized intestinal metaplasia, dysplasia or carcinoma and three patients had normal histology results. In addition, two patients with no history of Barrett's oesophagus or endoscopic lesions, and normal biopsies were included as controls.

### Endoscopy procedure

All explorations were performed using a high definition Fujinon 1.3-million-pixel EG 490 ZW5 gastroscope zoom with optical magnification up to 100 times equipped with

**Table 1** Clinical characteristics of patients undergoing combined acetic acid and Fujinon intelligent chromoendoscopy endoscopy

	Normal mucosa	SIM or LGD	HGD or carcinoma	All patients
Number of patients (%)	5 (25)	8 (40)	7 (35)	20
Age (yr) (mean $\pm$ SD)	56.4 $\pm$ 21.7	65 $\pm$ 10.5	71 $\pm$ 13.8	65 $\pm$ 15
Sex (men/women)	5/0	7/1	7/0	19/1
Concomitant therapy PPI (%)	60	100	100	90
Median Barrett's oesophagus Length (cm) $\pm$ IQR	1 (0-1)	2.3 (2-3.3)	3.5 (2.5-3.6)	2 $\pm$ 1.3

SIM: Specialized intestinal metaplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; PPI: Proton-pump inhibitor; IQR: Interquartile range.

a short soft transparent hood by one expert endoscopist and were video recorded. In each case, videos of the oesophagus were taken before biopsies. Endoscopies were carried out following total intravenous anaesthesia of the patient using Propofol. The endoscopic technique was standardized as follows: first the oesophagus was examined with high definition white light endoscopy, followed by 6-10 mL of 2% acetic acid pulverisation, and, finally, FICE was activated. Acetic acid pulverisation was performed with a spray catheter PW-1V-1 (Olympus Optical Co., Ltd., Japan) and the endoscopist gently sucked up excess acetic acid from the oesophageal lumen before inspection. In the case of macroscopic abnormalities in colour or pit-pattern, the zoom was used with a magnification of 10-15. Videos were made with high definition white light and with the combination of acetic acid and FICE. FICE channels 4, 7 and 0 were used following previous studies using FICE in upper endoscopy<sup>[9,10]</sup>. In the case of macroscopic abnormalities, separate biopsy samples were performed and then systematic biopsies were used in "normal macroscopic areas" of Barrett's oesophagus using Radial Jaw® Single-Use Biopsy Forceps (Boston Scientific, Fremont, CA, United States) with jumbo capacity.

### Histological analysis

All biopsies were evaluated by two pathologists with extensive experience in Barrett's neoplasia, and reviewed by a third pathologist in cases of dysplasia. Histological results were classified according to the revised Vienna classification<sup>[12]</sup>. The highest grade of dysplasia obtained from any biopsy sample was used to determine the diagnosis in each patient.

### Evaluation of Barrett's oesophagus

Retrospectively, videos were watched blind from histological results by three FICE trained endoscopists. For each patient, the most severe lesion was selected for evaluation. Experts noted the characteristics of Barrett's oesophagus and any other abnormalities with and without combined

acetic acid-FICE: macroscopic appearance of the lesion using the Paris classification<sup>[12]</sup>, type of mucosal pattern (regular: ridged/villous, circular, irregular), vascular pattern characteristics (regular, irregular) using the Sharma classification<sup>[13]</sup>, raised lesion, ulcerous lesion, pigmented lesion; and spontaneous bleeding lesion. The last four items were considered on clinical findings.

### Statistical analysis

Means and standard deviations were used to summarize continuous variables with an apparently Gaussian distribution, whereas the median and the interquartile range (IQR) were used to summarize variables with a skewed distribution. Kappa statistics with their 95% confidence intervals (CI) were used to test for inter-observer agreement of the 3-step classification system using arbitrary interpretation by Landis and Koch (0, poor agreement; 0.00-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.80-1.00, almost perfect agreement)<sup>[14]</sup>. Because Kappa statistics can only be calculated with pair-wise observations, Kappa values were calculated for all pair-wise combinations obtained by the observers.

## RESULTS

### Patient characteristics

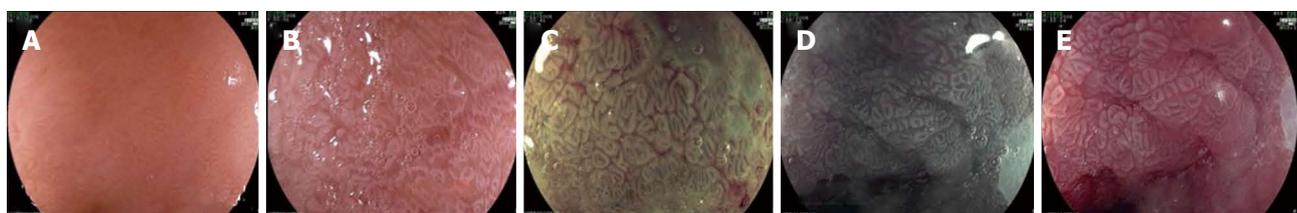
Twenty patients were included. The patients' characteristics are shown in Table 1. Histological results were normal histology, specialized intestinal metaplasia, low-grade dysplasia, high-grade dysplasia, and carcinoma in 25%, 25%, 15%, 15%, and 20%, respectively. With high definition white light endoscopy, abnormalities in mucosal or vascular pattern were detected in one patient (6%) out of the 18 patients with suspected Barrett's oesophagus. With combined acetic acid-FICE, seven patients (39%) out of the 18 with suspected Barrett's oesophagus had a visible irregular mucosal pattern (Table 2). All of these patients had high-grade dysplasia or carcinoma (sensitivity: 100%). With combined acetic acid-FICE, six patients (33%) out of the 18 with suspected Barrett's oesophagus had a visible irregular vascular pattern. All had high-grade dysplasia or carcinoma (sensitivity: 100%). No patient with metaplasia or low-grade dysplasia had visible irregular mucosal vascular patterns, raised lesions, or spontaneous bleeding (Figure 1).

An irregular mucosal pattern was identified in patients with high-grade dysplasia or carcinoma using high definition white light endoscopy or the combination of acetic acid-FICE in 14% and 100%, respectively. An irregular vascular pattern was identified in patients with high-grade dysplasia or carcinoma using high definition white light endoscopy or the combination of acetic acid-FICE in 0% and 86%, respectively (Figures 2 and 3). An irregular mucosal or vascular pattern was not identified in patients without high-grade dysplasia or carcinoma using the combination of acetic acid and FICE (specificity 100%).

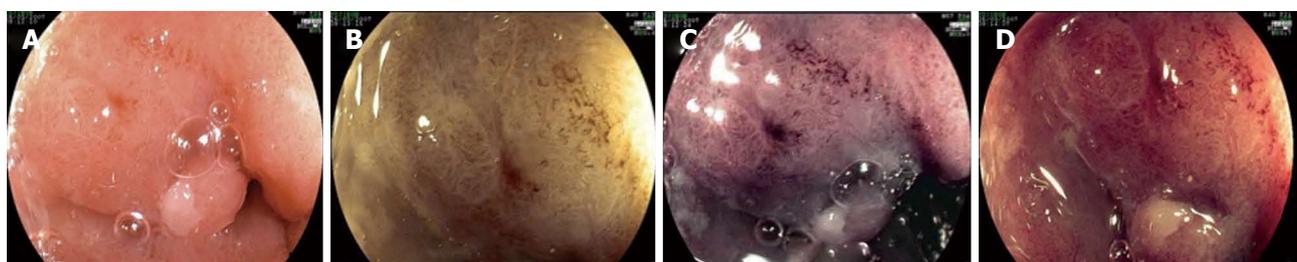
**Table 2** Correlation of the predominant mucosal and vascular patterns with histological results during high resolution white light endoscopy or after 2% acetic acid pulverisation and Fujinon intelligent chromoendoscopy 0, 4 and 7

	High resolution white light			Acetic acid pulverisation and FICE		
	Normal histology	SIM or LGD	HGD or carcinoma	Normal histology	SIM or LGD	HGD or carcinoma
Number of patients (%)	5	8	7	5	8	7
Regular or not visualized mucosal pattern <sup>1</sup> (%)	100	100	86	100	100	0
Irregular mucosal pattern (%)	0	0	14	0	0	100
Regular or not visualized vascular pattern <sup>1</sup> (%)	100	100	100	100	100	14
Abnormal blood vessels (%)	0	0	14	0	0	86
Raised lesion (%)	0	0	14	0	0	71
Pigmented lesion (%)	0	0	0	0	37.5	0
Bleeding lesion (%)	0	0	14	0	0	57
Ulcerous lesion (%)	0	14	14	0	14	14

FICE: Fujinon intelligent chromoendoscopy; SIM: Specialized intestinal metaplasia; HGD: High-grade dysplasia; LGD: Indicate Low grade dysplasia. <sup>1</sup>Endoscopists were not able to exactly classify the mucosal pattern according to Sharma's classification of mucosal pattern due to lack of visibility, but they judged that the mucosa was regular.



**Figure 2** Acetic acid and Fujinon intelligent chromoendoscopy image of the oesophagus. Specialized intestinal metaplasia using high definition white light (A), 2% acetic acid (B), and the combination of acetic acid with Fujinon intelligent chromoendoscopy (FICE) 4 (C), FICE 0 (D) and FICE 7 (E).



**Figure 3** Acetic acid and Fujinon intelligent chromoendoscopy image of an oesophageal carcinoma. Irregular pit pattern and abnormal vascularisation is shown with 2% acetic acid (A), or following the combination of acetic acid and Fujinon intelligent chromoendoscopy (FICE) 4 (B), FICE 0 (C) and FICE 7 (D).

Among the 7 patients with high-grade dysplasia, 4 patients (57%) presented with early carcinoma on biopsy. The sizes of the lesions were 1 cm, 0.5 cm and 0.4 cm. The lesion size was not recorded in one patient. All patients underwent a mucosectomy within 2 mo of the primary endoscopy.

Definitive histological staging was intramucosal carcinoma (pT1m) for 3 of 4 patients following mucosectomy histological analysis, and resection margins were healthy. Only high-grade dysplasia was found in the fourth patient.

**Interobserver agreement for mucosal morphology**

When the evaluations by the experts were grouped together, the interobserver agreement for mucosal morphology assessed on high definition white light images was substantial on 5 items (mucosal pattern:  $\kappa = 0.97$ , raised lesion:  $\kappa = 1.00$ , pigmented lesion:  $\kappa = 1.00$ , bleeding lesion:  $\kappa = 1.00$ , and ulcerous lesion:  $\kappa = 1.00$ ) and

moderate on 2 items (vascular pattern:  $\kappa = 0.73$ , abnormal blood vessels:  $\kappa = 0.76$ ). There was no difference in interobserver agreement between high definition white light images and combined acetic acid-FICE images, except for 3 evaluations (abnormal blood vessels, vascular pattern and pigmented lesion). In the evaluation of abnormal blood vessels using high definition white light images, the interobserver agreement ( $\kappa = 0.76$ ; 95% CI: 0.64-1.00) was lower than that of combined acetic acid-FICE images ( $\kappa = 0.91$ ; 95% CI: 0.86-1.00). In the evaluation of vascular pattern using high definition white light images, the interobserver agreement ( $\kappa = 0.73$ ; 95% CI: 0.64-0.91) was lower than that of combined acetic acid-FICE images ( $\kappa = 0.83$ ; 95% CI: 0.76-0.88). In the evaluation of pigmented lesion using high definition white light images, the interobserver agreement ( $\kappa = 1.00$ ; 95% CI: 0.64-1.00) was better than that of combined acetic acid-FICE images ( $\kappa = 0.37$ ; 95% CI: 0.28-0.54).

## COMMENTS

### Background

Barrett's oesophagus is a pre-neoplastic lesion with an estimated rate of transformation to carcinoma of approximately 0.3%-0.6% each year. Guidelines from the American College of Gastroenterology proposed a surveillance programme to detect high-grade dysplasia or carcinoma in Barrett's oesophagus. This screening programme recommends performing 4 quadrant biopsies every 2 cm at 1 year in low-grade dysplasia and every 3 mo in high-grade dysplasia.

### Research frontiers

Acetic acid pulverisation is a simple and inexpensive method of improving visibility of the pit pattern, but does not allow appreciation of the vascular pattern. Acetic acid instillation increased the detection of cancer compared to white light endoscopy with or without high resolution endoscopy. Whereas acetic acid instillation, indigo carmine chromoendoscopy, narrow-band imaging and chromoendoscopy by Fujinon intelligent chromoendoscopy (FICE) seem to be interesting new techniques, each technique alone seems to be insufficient to warrant abandonment of the Seattle protocol of multiple blind sample biopsies. The authors showed significant differences between FICE and chromoendoscopy with acetic acid for the detection of high-grade dysplasia or early cancer in patients with Barrett's oesophagus. Both the acetic acid and FICE techniques showed separate per-lesion sensitivity of up to 87% for the detection of high-grade neoplasia and early cancer in patients with Barrett's oesophagus. The authors conducted a video study to evaluate the combined acetic acid and FICE technique.

### Innovations and breakthroughs

Virtual chromoendoscopy has begun to receive greater attention as a potential technique in the diagnosis of Barrett's oesophagus. In the series, the study highlight the positive effect of the combination of acetic acid and virtual chromoendoscopy. The study confirms the usefulness of FICE technique combined with acetic acid using video reviews. However, the generalization of the results is made difficult by the author's small population size. Therefore, a larger study is warranted to confirm the author's results. The data suggest a high sensitivity and specificity with this combination, however, data are lacking on a real-time basis rather than relying on subsequent video reviews. The report, for the first time, on the benefit of the combination of acetic acid and FICE in identifying high-grade dysplasia or early oesophageal neoplasia.

### Applications

By using the presented procedure of acetic acid and FICE, this may represent a future strategy for the diagnosis of Barrett's oesophagus lesions. In the study, taking into account two criteria (irregular mucosal and/or vascular patterns), 100% of patients with high-grade dysplasia or carcinoma were identified by endoscopy, with no errors in the diagnosis of high-grade dysplasia or carcinoma. The study showed that combined acetic acid and FICE had a positive benefit in the identification of high-grade dysplasia or carcinoma. By combining the two techniques of acetic acid and FICE, the results showed improvement in the quality of endoscopic images obtained and visualisation of both mucosal and vascular patterns or irregularities. The endoscopic detection of high-grade dysplasia or carcinoma was enhanced by this combination method as compared to using high definition white light imaging alone. In order to further verify the high sensitivity and specificity findings in this study, future prospective multi-centre studies of patients presenting for endoscopic evaluation of Barrett's oesophagus with all levels of early neoplasia are required to definitively compare the combination imaging algorithm of acetic acid and FICE to Seattle Protocol random biopsies.

### Peer review

This is an interesting study, which adds information to how to tackle detection and surveillance of Barrett's esophagus.

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## Factors associated with the overall survival of elderly patients with hepatocellular carcinoma

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### Abstract

**AIM:** To identify the factors associated with overall survival of elderly patients with hepatocellular carcinoma (HCC).

**METHODS:** A total of 286 patients with HCC (male/female: 178/108, age: 46-100 years), who were diagnosed and treated by appropriate therapeutic procedures between January 2000 and December 2010, were enrolled in this study. Patients were stratified into two groups on the basis of age: Elderly ( $\geq 75$  years old) and non-elderly ( $< 75$  years old). Baseline clinical characteristics as well as cumulative survival rates were then compared between the two groups. Univariate and

multivariate analyses were used to identify the factors associated with prolonged overall survival of patients in each group. Cumulative survival rates in the two groups were calculated separately for each modified Japan Integrated Stage score (mJIS score) category by the Kaplan-Meier method. In addition, we compared the cumulative survival rates of elderly and non-elderly patients with good hepatic reserve capacity ( $\leq 2$  points as per mJIS).

**RESULTS:** In the elderly group, the proportion of female patients, patients with absence of hepatitis B or hepatitis C viral infection, and patients with coexisting extrahepatic comorbid illness was higher (56.8% vs 31.1%,  $P < 0.001$ ; 27.0% vs 16.0%,  $P = 0.038$ ; 33.8% vs 22.2%,  $P = 0.047$ ; respectively) than that in the non-elderly group. In the non-elderly group, the proportion of hepatitis B virus (HBV)-infected patients was higher than that in the elderly group (9.4% vs 0%,  $P = 0.006$ ). The cumulative survival rates in the elderly group were 53.7% at 3 years and 32.9% at 5 years, which were equivalent to those in the non-elderly group (55.9% and 39.4%, respectively), as shown by a log-rank test ( $P = 0.601$ ). In multivariate analysis, prolonged survival was significantly associated with the extent of liver damage and stage ( $P < 0.001$  and  $P < 0.001$ , respectively), but was not associated with patient age. However, on individual evaluation of factors in both groups, stage was significantly ( $P < 0.001$ ) associated with prolonged survival. Regarding mJIS scores of  $\leq 2$ , the rate of female patients with this score was higher in the elderly group when compared to that in the non-elderly group ( $P = 0.012$ ) and patients  $\geq 80$  years of age tended to demonstrate shortened survival.

**CONCLUSION:** Survival of elderly HCC patients was associated with liver damage and stage, but not age, except for patients  $\geq 80$  years with mJIS score  $\leq 2$ .

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**Key words:** Age; Hepatocellular carcinoma; Liver damage; Stage; Survival

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## INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common causes of mortality worldwide, usually occurs in a cirrhotic liver<sup>[1-3]</sup>. Moreover, the number of elderly HCC patients is increasing, and the average age of patients with hepatitis C is increasing in Japan<sup>[4-8]</sup>. This trend indicates the need to investigate and identify the optimal treatment of HCC in elderly patients. Elderly patients have a high incidence of comorbid illnesses and the risks of major surgery are usually higher in these patients when compared to those in younger patients. Therefore, radical surgical resection of HCC is less feasible in elderly patients than in younger patients.

Some studies have indicated that treatment outcomes in elderly patients are essentially similar to those in non-elderly patients; moreover, there are several studies comparing different treatment procedures<sup>[9-16]</sup>. However, the prognostic factors for survival in elderly patients remain obscure. This article aims to review a retrospective cohort of elderly ( $\geq 75$  years) and non-elderly ( $< 75$  years) patients in order to clarify the characteristics of elderly HCC patients and reveal the factors associated with prolonged survival of these patients.

## MATERIALS AND METHODS

### Study population

A total of 286 patients with HCC treated at Aiseikai-Yamashina Hospital between January 2000 and December 2010 were enrolled in this study. A follow up study of patient survival was performed until the end of December 2010. This study was conducted in accordance with the Declaration of Helsinki. We explained our therapeutic strategy and other regimens to each patient prior to every treatment procedure and obtained written informed consent from all patients.

The 286 patients who fulfilled the inclusion criteria were categorized into two groups as elderly ( $\geq 75$  years) and non-elderly ( $< 75$  years old). The breakpoint of 75 years old was chosen because it enabled comparison with other relevant reports. Moreover, in Japan, patients  $\geq 75$  years of age are covered by a health insurance system

which is different from that of patients  $< 75$  years.

### Etiology of liver disease

The etiology of liver disease was classified as follows: (1) hepatitis B virus (HBV), if patients were hepatitis B surface antigen (HBsAg) positive; (2) HCV, if patients were anti-HCV positive; (3) HCV and HBV, if patients were both HBsAg and anti-HCV positive; and (4) non-B non-C, if patients were negative for both HBsAg and anti-HCV.

### Diagnosis of hepatic reserve capacity

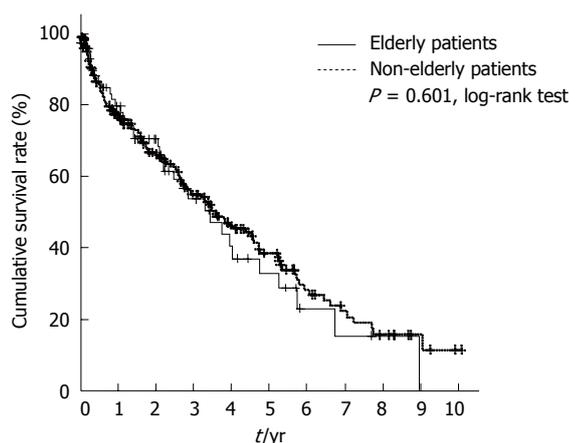
The severity of liver damage was scored according to the Liver Damage Classification scheme proposed by the Liver Cancer Study Group of Japan (LCSGJ)<sup>[17]</sup>.

### Diagnosis and staging of hepatocellular carcinoma

The diagnosis of HCC was based on histopathology and/or imaging studies such as ultrasonography (US), computed tomography (CT) scans, angiography, CT angiography, and magnetic resonance imaging. The final diagnosis was confirmed when at least two diagnostic modalities identified the presence of HCC. The tumor stage was defined on the basis of the LCSGJ. To compare treatment outcomes with those of other institutions, the modified Japan Integrated Stage score (mJIS score) was selected as the integrated staging system for HCC<sup>[17]</sup>.

### Treatment

After diagnosis of HCC, the most appropriate therapeutic procedure was selected according to the tumor status and underlying hepatic reserve capacity of each patient. As a general rule, we treated all patients except for those with uncontrollable ascites or hepatic encephalopathy and those who rejected any treatment. Hepatic resection (HR) was particularly considered in patients with localized HCC and preserved hepatic reserve capacity. Nonsurgical treatments, such as transcatheterarterial chemoembolization (TACE), transcatheterarterial infusion chemotherapy (TAI), percutaneous ethanol injection therapy (PEIT), radiofrequency ablation therapy (RFA), and hepatic arterial infusion chemotherapy (HAIC) were considered when the patients refused surgical treatment or HR was not feasible. TACE was performed using doxorubicin hydrochloride or cisplatin with iodized oil (Lipiodol Ultra Fluide; Laboratoire Guerbet, Roissy, France) and gelatin sponge particles. TAI was performed using doxorubicin hydrochloride or cisplatin. Locoregional ablative therapies such as PEIT and RFA were considered in patients with one to three tumor nodules that were devoid of vascular invasion and not associated with extrahepatic metastases. All locoregional ablative therapies were CT- or US-assisted. HAIC was performed using intra-arterial hepatic injections with low-dose 5-fluorouracil/cisplatin<sup>[18]</sup>. In many cases, patients were treated by a combination of several procedures. These therapies were repeated when HCC relapsed until patients reached maximum tolerability. The best supportive care was considered when the pa-



**Figure 1** Cumulative survival rates by Kaplan-Meier method in elderly (*n* = 74, thin line) and non-elderly groups (*n* = 212, thick line).

tient had compromised hepatic reserve capacity or when he/she refused any treatment for HCC.

**Comorbid illnesses**

The presence of malignant tumors other than HCC, cardiovascular diseases, renal diseases, pulmonary diseases, and neurological diseases, all of which could have potential impact on the prognosis, were recorded.

**Statistical analysis**

To evaluate the differences in clinical features of the patients and tumor characteristics, the Mann-Whitney *U* and Pearson  $\chi^2$  tests were used for continuous and discrete data, respectively. The analytical data were first collected on the day of initial HCC diagnosis. The patients were followed-up as long as they lived or, in some cases, until their last visit to the hospital. The primary outcome was overall survival. Cumulative survival rates were calculated using the Kaplan-Meier method and compared using the logrank test. Patient survival was followed up to 31 December, 2010. For the analysis of predictors of survival, a Cox proportional hazards model was used, in which the following parameters were evaluated: age ( $\geq 75$  years or  $< 75$  years), gender, presence of comorbid illnesses, HCV positivity, serum alpha-fetoprotein  $\geq 200$  ng/mL, serum des-gamma-carboxyprothrombin  $\geq 200$  mAU/mL, and liver damage classification and stage. A *P* value of  $\leq 0.05$  was considered statistically significant. All statistical analyses were performed using the SPSS 19.0 statistical package (SPSS Incorporated, Chicago, Illinois, United States).

**RESULTS**

**Clinical features of patients**

The clinical profiles of 286 patients, divided into elderly ( $\geq 75$  years) and non-elderly ( $< 75$  years) groups, are shown in Table 1. In the elderly group, the number of female patients, patients with comorbid illness, and patients with absence of HBV and HCV infection (non-B non-C) were significantly higher (*P* < 0.001, *P* = 0.047, *P* = 0.038,

**Table 1** Clinical characteristics of the patients

	Elderly patients ( <i>n</i> = 74 )	Non-elderly patients ( <i>n</i> = 212 )	<i>P</i> value
Age (yr) (range)	80.5 (75.4-100.0)	65.8 (46.0-74.8)	-
Male/female (%)	32/42 (43.2/56.8)	146/66 (68.9/31.1)	<i>P</i> < 0.001
Extrahepaticcomorbidity (%)	25 (33.8)	47 (22.2)	<i>P</i> < 0.05
Malignant tumors except HCC	6	12	
Cardiovascular	10	15	
Renal	3	7	
Pulmonary	3	4	
Neurological	3	9	
Cause of liver dysfunction			
HBV (%)	0	20 (9.4)	<i>P</i> < 0.01
HCV (%)	54 (73.0)	155 (73.2)	NS
HBV HCV (%)	0	3 (1.4)	NS
Non-B Non-C (%)	20 (27.0)	34 (16.0)	<i>P</i> < 0.05
Liver damage A/B/C	39/31/4	97/86/29	NS
Stage I / II / III/IV	13/24/23/14	45/71/51/45	NS
mJIS score	9/16/22/18/8/1	24/53/59/36/27/13	NS
0/1/2/3/4/5			
Death except hepatic disease/total death	10/35	18/113	NS

NS: Not significant; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; mJIS: Modified Japan Integrated Stage.

respectively) than those in the non-elderly group. In the non-elderly group, the proportion of patients with HBV infection was significantly (*P* = 0.006) higher than that in the elderly group.

**Overall survival rates**

Over a median follow-up of 1.8 years, 148 patients died, of whom 35 were elderly and 113 were non-elderly patients. The median survival period was comparable in the two groups [elderly: 3.46 years, 95% confidence interval (CI), 2.26-4.66; non-elderly: 3.56 years, 95% CI, 2.58-4.55; *P* = 0.167] (Figure 1). The survival rates at one, three, five, and 10 years were 79.7%, 53.7%, 32.9%, and 0.0% in the elderly group, and 77.9%, 55.9%, 39.4%, and 12.4% in the non-elderly group, respectively. There were no significant differences in survival rates between the two groups (*P* = 0.601).

With regard to the cause of mortality, 10 patients (28.6%) in the elderly group and 18 patients (16.8%) in the non-elderly group died from causes other than hepatic diseases (tumor progression, hepatic failure, variceal bleeding, or other complications of cirrhosis), and there were no significant differences between the two groups (*P* = 0.095, Table 1). In addition, we performed an analysis of survival rates after excluding patients who died from causes other than hepatic diseases. As a result, the survival rates at one, three, five, and 10 years were 86.8%, 64.0%, 42.3%, and 0.0% in the elderly group, and 80.8%, 60.7%, 44.9%, and 18.6% in the non-elderly group, respectively. There were no significant differences in survival rates between the two groups (*P* = 0.779, data not shown).

**Table 2 Univariate and multivariate analyses of the relative risks for overall survival**

	Univariate analysis relative risk	P value	Multivariate analysis relative risk	P value
Age ≥ 75 yr	1.107	NS	1.161	NS
Gender (male)	1.155	NS	1.135	NS
Comorbid illness	1.335	NS	1.144	NS
HCV+	0.636	< 0.05	1.036	NS
AFP (ng/mL) ≥ 200	2.098	< 0.001	1.229	NS
DCP (mAU/mL) ≥ 200	1.763	< 0.05	1.229	NS
Liver damage				
A/B	2.345	< 0.001	2.506	< 0.001
B/C	7.674	< 0.001	10.463	< 0.001
Stage				
I / II	3.168	< 0.001	3.126	< 0.001
II / III	3.818	< 0.001	6.323	< 0.001
III / IV	20.064	< 0.001	35.498	< 0.001

NS: Not significant; HCV: Hepatitis C virus; AFP: α-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

**Table 3 Univariate and multivariate analyses for overall survival in elderly and non-elderly patients**

	Elderly		Non-Elderly	
	Univariate analysis P value	Multivariate analysis P value	Univariate analysis P value	Multivariate analysis P value
Gender (male)	NS	NS	NS	NS
Comorbid illness	NS	NS	NS	NS
HCV+	NS	NS	< 0.05	NS
AFP (ng/mL) ≥ 200	NS	NS	< 0.001	NS
DCP (mAU/mL) ≥ 200	NS	NS	< 0.05	NS
Liver damage				
A/B	NS	NS	< 0.001	< 0.001
B/C	< 0.001	NS	< 0.001	< 0.001
Stage				
I / II	NS	< 0.001	< 0.05	< 0.05
II / III	NS	< 0.001	< 0.05	< 0.001
III / IV	NS	< 0.001	< 0.001	< 0.001

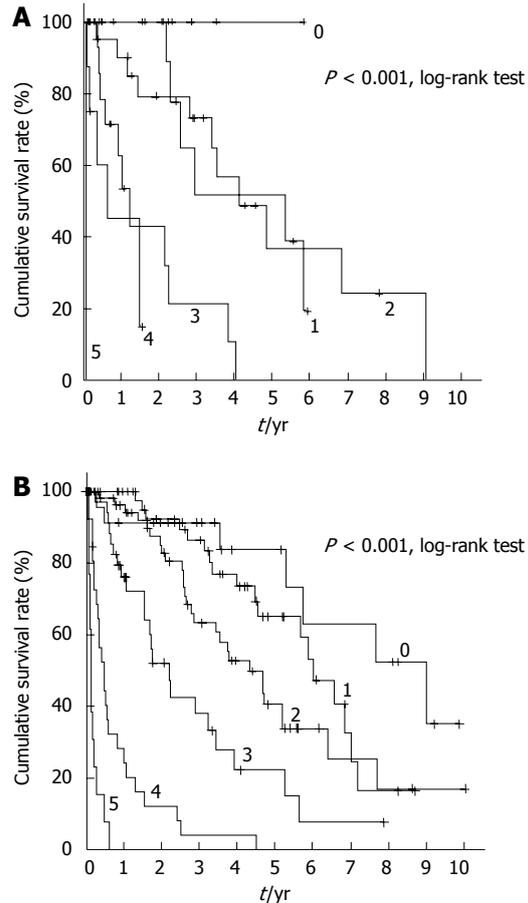
NS: Not significant; HCV: Hepatitis C virus; AFP: α-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

**Factors affecting survival**

The factors affecting survival in all patients were calculated by multivariate analysis, and liver damage and stage were selected as the significant factors (Table 2). In elderly patients, multivariate analysis demonstrated that stage was independently associated with survival. In non-elderly patients, multivariate analysis demonstrated that liver damage and stage were independently associated with survival (Table 3). Gender, comorbid illness, HCV positivity, and tumor markers were not associated with survival in both groups.

**Survival curve according to the mJIS score**

Because survival was influenced by both liver damage and stage, we applied mJIS scores to both patient groups, and reached the conclusion that there was no clear association between these scores and survival in elderly patients (Fig-



**Figure 2** Cumulative survival rates by Kaplan-Meier method in each modified Japan Integrated Stage score group in elderly and non-elderly patients are shown. A: Elderly patients; B: Non-elderly patients.

ure 2A), whereas there was a clear association between the two in non-elderly patients (Figure 2B).

Furthermore, we analyzed the clinical profile of patients with mJIS scores of ≤ 2 in both groups (Table 4). The 5-year survival rate of these patients was expected to be ≥ 50%. In the elderly group, the proportion of females with this score was higher than that in the non-elderly group. In the non-elderly group, the proportion of patients with HBV infection was higher than that in the elderly group. Other clinical factors were not statistically different. There was no significant difference in survival rates between patients with this score in both groups ( $P = 0.386$ , Figure 3A). Furthermore, we analyzed the survival rate of elderly patients with mJIS scores ≤ 2 by dividing them into two subgroups, one comprising patients between 75 and 80 years of age and the other comprising patients ≥ 80 years of age. The survival rates tended to deteriorate in the latter group of patients, although the difference was not significantly different ( $P = 0.335$ , Figure 3B).

**DISCUSSION**

In this study, we reviewed HCC cases by dividing them into two groups and demonstrated that the survival rates of elderly patients ≥ 75 years of age were generally

**Table 4** Clinical characteristics of the patients whose modified Japan Integrated Stage score were two or less

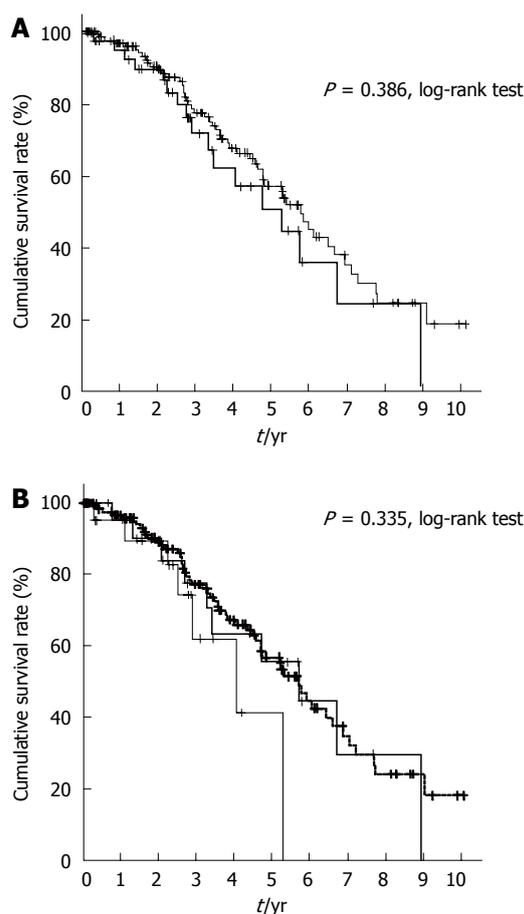
	Elderly patients (n = 47)	Non-elderly patients (n = 136)	P value
Age (yr) (range)	80.3 (75.4-87.7)	65.8 (46.0-74.8)	-
Male/female (%)	21/26 (43.2/56.8)	89/47 (68.9/31.1)	P < 0.05
Extrahepatic comorbidity (%)	16 (34.0)	33 (24.3)	NS
Malignant tumors except HCC	5	9	
Cardiovascular	5	8	
Renal	2	4	
Pulmonary	2	4	
Neurological	2	8	
Cause of liver dysfunction			
HBV (%)	0	11 (8.1)	P < 0.05
HCV (%)	37 (78.7)	109 (80.1)	NS
HBV HCV (%)	0	2 (1.5)	NS
non-B non-C (%)	10 (21.3)	14 (10.3)	NS
Liver damage A/B/C	32/14/1	87/45/4	NS
Stage I / II / III / IV	13/24/10	45/64/27	NS

NS: Not significant; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

equivalent to those of non-elderly patients < 75 years of age. There have been many previous studies reporting the efficiency and safety of each treatment modality for HCC in elderly patients, and most reports have shown similar survival rates and safety when compared with those of non-elderly patients<sup>[9-16]</sup>. However, in clinical practice, HCC is treated with several modalities in Japan. A previous paper, which evaluated the total survival rates of older HCC patients in comparison with those of younger HCC patients<sup>[19-21]</sup>, reported cumulative survival rates similar to those reported in our present study.

Our elderly and non-elderly HCC patients differed in several clinical characteristics. Elderly patients were more likely to be female and negative for both HBV and HCV. This was expected since all these factors are known to influence the age of HCC development. The peak age of HCC occurrence in females is 5 years later than that in males<sup>[22]</sup>. HCV infection is generally acquired during adult life<sup>[23]</sup>, while HBV infection frequently occurs in childhood<sup>[24]</sup>. A multifactorial etiology accelerates the progression of chronic liver disease, hence anticipating the appearance of HCC<sup>[22,25]</sup>.

Despite a higher prevalence of comorbid illnesses and a difference of 14.7 years in the mean age, elderly patients demonstrated an overall 5-year survival similar to that of their younger counterparts. This unexpected result could be because of the low survival rate of both groups (overall survival of approximately < 40% at 5 years). The Liver Cancer Study Group of Japan has reported the 5-year overall survival rate after initial HCC diagnosis as 35.4%<sup>[5]</sup>. There may be many specific factors that influence the treatment strategy for elderly patients. Aggressive and risky treatments in these patients may be avoided due to comorbid illnesses. However, the impact of HCC occurrence on life expectancy outweighs that



**Figure 3** The analysis of the survival rate with modified Japan Integrated Stage score two points or less. A: Cumulative survival rates by Kaplan-Meier method in elderly (n = 47, thick line) and non-elderly groups (n = 136, thin line) whose modified Japan Integrated Stage (mJIS) score is two points or less; B: Cumulative survival rates by Kaplan-Meier method in 75-80 year-olds (n = 25, thick line), 80 years old or more (n = 22, thin line) and non-elderly groups (n = 136, dotted line) whose mJIS score is two points or less are shown.

of both comorbid illnesses and age. We suggest that comorbid conditions and therapeutic procedures (including hepatectomy) had little effect on the survival of the patients especially the elderly ones.

Before analyzing the prognostic factors in each group, it was expected that the presence of comorbid illnesses may be a poor prognostic factor for elderly patients. However, this factor was not statistically associated with survival rates in either the elderly or non-elderly patient groups. The analysis of prognostic factors revealed that liver damage and stage were significantly associated with survival rates in both groups. Furthermore, stage was a very strong factor in elderly patients. Patients who have a sufficient hepatic reserve capacity may survive long enough for HCC to develop. The association of other factors with survival in elderly HCC patients seems insignificant in comparison with that of stage.

We also analyzed patients with a good hepatic reserve capacity ( $\leq 2$  points as per mJIS), whose 5-year survival rate was expected to exceed 50% (Figure 3A). However, there was no statistical difference between survival rates of patients  $\geq 75$  and < 75 years of age. Interestingly,

when divided into subgroups, the survival of patients  $\geq 80$  years of age was shorter than that of patients in the other subgroup (Figure 3B). In other words, extreme old age influenced the survival rate even in patients with good hepatic reserve capacity.

With regard to the cause of death, there was no difference between elderly and non-elderly patients (Table 1). Similar to non-elderly patients, elderly patients also died from liver-associated diseases. If the HCC patients  $\geq 80$  years of age were divided into two subgroups (treated and untreated), there were obvious differences in the prognosis (data not shown). Further studies with a larger sample of patients will clarify this issue in future.

In conclusion, survival of elderly HCC patients ( $\geq 75$  years old) was associated with liver damage and stage. The effectiveness of treatment for HCC was equivalent in elderly and non-elderly patients. Survival was unaffected by age; however, when individually evaluated for patients with a mJIS score of  $\leq 2$ , those  $\geq 80$  years of age tended to demonstrate shortened survival.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC), one of the most common causes of mortality worldwide, usually occurs in a cirrhotic liver. At present, the number of elderly HCC patients is increasing, as is the average age of hepatitis C is increasing in Japan. However, the factors associated with overall survival of patients with HCC especially the elderly ( $\geq 75$  years old) are not adequately investigated.

### Research frontiers

Some studies have indicated that treatment outcomes in elderly patients are essentially similar to those in non-elderly patients. In addition, there are several studies comparing different treatment procedures. The research hotspot is to clarify general prognostic factors for survival in elderly HCC patients in Japan.

### Innovations and breakthroughs

In the elderly HCC patients of Japan, the proportion of female patients, patients with absence of hepatitis B or hepatitis C viral infection, and patients with co-existing extrahepatic comorbid illness was higher than that in the non-elderly group. The cumulative survival rates in the elderly group were 53.7% at 3 years and 32.9% at 5 years, which were equivalent to those in the non-elderly group, as shown by a log-rank test. In multivariate analysis, prolonged survival was significantly associated with the extent of liver damage and stage, but was not associated with patient age. Patients  $\geq 80$  years of age tended to demonstrate shortened survival.

### Applications

In the present study, the authors have reached the conclusion that survival of the HCC patients treated by appropriate procedures depends on liver damage and stage, but not on patient age. Only patients  $\geq 80$  years of age tended to demonstrate shortened survival.

### Terminology

The modified Japan Integrated Stage score (mJIS score) is the integrated staging system which combined the degree of liver damage and the degree of tumor stage.

### Peer review

This is a good descriptive study in which authors analyze the factors associated with overall survival of patients with HCC especially the elderly ( $\geq 75$  years old). The results are interesting and give a useful information to the hepatologists in the world.

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## Is hepatic arterial infusion chemotherapy effective treatment for advanced hepatocellular carcinoma resistant to transarterial chemoembolization?

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### Abstract

**AIM:** To evaluate the effectiveness of hepatic arterial infusion chemotherapy (HAIC) for advanced hepatocellular carcinoma (HCC) resistant to transarterial chemoembolization (TACE).

**METHODS:** This study was conducted on 42 patients who received HAIC for advanced HCC between 2001

and 2010 at our hospital. 5-fluorouracil (5-FU) was administered continuously for 24 h from day 1 to day 5 every 2-4 wk *via* an injection reservoir. Intra-arterial cisplatin or subcutaneous interferon was administered in combination with the 5-FU. The patients enrolled in this retrospective study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese); one group of patients who did not fulfill the criteria for TACE resistance (group A,  $n = 23$ ), and another group who fulfilled the criteria for TACE resistance (group B,  $n = 19$ ). We compared the outcomes in terms of the response and survival rates between the two groups.

**RESULTS:** Both the response rate and tumor suppression rate following HAIC were significantly superior in group A than in group B (response rate: 48% *vs* 16%,  $P = 0.028$ , tumor suppression rate: 87% *vs* 53%,  $P = 0.014$ ). Furthermore, both the progression-free survival rate and survival time were significantly superior in group A than in group B (3-, 6-, 12-, and 24-mo = 83%, 70%, 29% and 20% *vs* 63%, 42%, 16% and 0%, respectively,  $P = 0.040$ , and 9.8 mo *vs* 6.2 mo,  $P = 0.040$ ). A multivariate analysis (Cox proportional hazards regression model) showed that resistance to TACE was an independent predictor of poor survival ( $P = 0.007$ ).

**CONCLUSION:** HAIC administering 5-FU was not effective against advanced HCC resistant to TACE. Other tools for treatment, i.e., molecular-targeting agents may be considered for these cases.

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**Key words:** Hepatocellular carcinoma; Hepatic arterial infusion chemotherapy; 5-fluorouracil; Transarterial chemoembolization

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases around the world, and the number of HCC-related deaths has been increasing worldwide<sup>[1-5]</sup>. HCC has a poor prognosis due to its rapidly-infiltrating growth characteristic and occurrence in a background of liver cirrhosis (LC). Surgical treatment is only indicated in a small proportion of patients, due to the frequently large tumor size, presence of multiple tumors, and poor hepatic function<sup>[6,7]</sup>. Regional interventional therapies have led to major breakthroughs in the management of HCC; transarterial chemoembolization (TACE) has been reported as an effective treatment modality for patients with advanced HCC, especially those with multiple nodules<sup>[8-15]</sup>, therefore, it is often repeated several times for the treatment of recurrent HCC. Furthermore, advances in implantable drug delivery systems have made it possible to administer repeated arterial infusions of anticancer agents, and recent studies, including our previous reports, have shown the effectiveness of combined therapy with intra-arterial 5-fluorouracil (5-FU) plus cisplatin or subcutaneous interferon (IFN) therapy in patients with advanced HCC<sup>[16-24]</sup>. We previously reported a case of unresectable advanced HCC with portal vein tumor thrombosis (PVTT) who was treated successfully by combined intra-arterial 5-FU plus subcutaneous pegylated interferon- $\alpha$ 2b (PEG-IFN- $\alpha$ 2b) therapy<sup>[23]</sup>, and also a retrospective cohort study of this combined hepatic arterial infusion chemotherapy (HAIC)<sup>[24]</sup>. However, the precise efficacy of HAIC in patients with advanced HCC resistant to TACE still remains unclear.

In the present cohort study, we evaluated the effectiveness and outcomes, in terms of the overall survival rate, median survival time and response to therapy, of HAIC in patients with unresectable advanced HCC with and without a resistance to TACE.

## MATERIALS AND METHODS

### Patients and eligibility

The subjects of this study were 42 patients with HCC in

**Table 1** Criteria for transarterial chemoembolization resistance

The evaluation was performed on the day of TACE and 1 mo after the TACE; the following were observed at least two times
Staining with the injected agent (lipiodol-anticancer agent emulsion) was considered insufficient with evaluation CT [the occupation rate was less than 50% of lesion(s)]
Appearance of multiple new recurrent lesions on the evaluation CT
Appearance of vessel invasion after TACE
Appearance of distal metastasis after TACE
Persistent elevation of tumor marker(s) regardless of TACE

TACE: Transarterial chemoembolization; CT: Computed tomography.

whom the diagnosis was made on the basis of the pathological or radiological findings between January 2001 and December 2010 at Yokohama City University Hospital, Kanagawa, Japan. Of the 42 patients, 5 had not received any treatment before enrollment in this study, 27 had been treated by TACE, 8 had undergone hepatic resection, and 2 had been treated by local ablation therapy before enrollment in this study. All the patients satisfied the following criteria: Child-Pugh class A or B, white blood cell > 2000/ $\mu$ L, neutrophil count > 1000/ $\mu$ L, Plt > 50 000/ $\mu$ L, total bilirubin < 3.0 mg/dL, serum creatinine < 1.5 mg/dL, unresectable or unsuitable for local ablation therapy, 4 or more lesions throughout the liver or presence of vessel invasion, Eastern Cooperative Oncology Group Performance Status, 0-2<sup>[25]</sup>, absence of extra-hepatic metastases, and absence of past history of treatment with 5-FU. The PVTT grade and tumor stage were determined according to the criteria of the Liver Cancer Study Group of Japan<sup>[26]</sup>. All patients gave written informed consent for participation in this study, and the study was conducted with the approval of the Ethics Committee of Yokohama City University Graduate School of Medicine. The patients enrolled in this retrospective study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese) (Table 1); one group of patients who did not fulfill the criteria (group A,  $n = 23$ ), and another group of patients who fulfilled the criteria for TACE resistance (group B,  $n = 19$ ). We compared the outcomes in terms of the response and survival rates between the two groups. A comparison of the patient characteristics between the two groups before the start of HAIC is shown in Table 2. The duration of treatment from the first detection of HCC to the time of the HAIC (i.e., to enrollment in this study) was significantly longer in group B than in group A (36.2 mo *vs* 16.3 mo,  $P = 0.004$ ). The liver function parameters did not differ significantly between the two groups.

### Arterial catheterization

The arterial catheter was inserted into the right or left femoral artery by the Seldinger method. A heparin-coated catheter (Clinical Supply, Gifu, Japan) was inserted into the femoral artery and its tip was advanced to the

**Table 2** Comparison of the patient characteristics in the two groups prior to hepatic arterial infusion chemotherapy *n* (%)

	Group A	Group B	P value
Patients	23	19	
Age (yr)	66.6 ± 6.9	65.5 ± 7.3	NS ( <i>P</i> = 0.635)
Gender			
Male/female	20 (87)/3 (13)	15 (79)/4 (21)	NS ( <i>P</i> = 0.488)
Etiology of LC			
HCV	13 (57)	11 (58)	NS ( <i>P</i> = 0.070)
HBV	2 (9)	6 (32)	
HCV + HBV	0 (0)	1 (5)	
Alcohol	4 (17)	0 (0)	
NonB-nonC	4 (17)	1 (5)	
Albumin (g/dL)	3.6 ± 0.6	3.5 ± 0.6	NS ( <i>P</i> = 0.503)
Total bilirubin (mg/dL)	1.1 ± 0.7	1.3 ± 0.5	NS ( <i>P</i> = 0.397)
PT (INR)	1.19 ± 0.13	1.17 ± 0.10	NS ( <i>P</i> = 0.607)
AST (U/L)	64 ± 33	79 ± 51	NS ( <i>P</i> = 0.256)
ALT (U/L)	47 ± 30	53 ± 38	NS ( <i>P</i> = 0.569)
GGT (U/L)	155 ± 169	76 ± 76	NS ( <i>P</i> = 0.067)
WBC (/μL)	4600 ± 1400	4400 ± 900	NS ( <i>P</i> = 0.431)
Hb (g/dL)	13.1 ± 2.0	12.8 ± 1.0	NS ( <i>P</i> = 0.521)
Plt (× 10 <sup>4</sup> /μL)	14.3 ± 6.5	12.1 ± 5.8	NS ( <i>P</i> = 0.262)
AFP (median, ng/mL)	7550	3116	NS ( <i>P</i> = 0.434)
DCP (median, mAU/mL)	12314	3363	NS ( <i>P</i> = 0.159)
Child-Pugh			
A/B	12 (52)/11 (48)	6 (32)/13 (68)	NS ( <i>P</i> = 0.219)
Child-Pugh score	6.8 ± 1.7	7.1 ± 1.4	NS ( <i>P</i> = 0.582)
Number of tumor (s)			
≤ 5/6-10/> 10	5 (22)/7 (30) /11 (48)	5 (26)/8 (42) /6 (32)	NS ( <i>P</i> = 0.515)
Size of the largest tumor (cm)	7.3 ± 5.2	3.8 ± 1.3	<i>P</i> = 0.008
Vessel invasion			
presence/absence	12 (52)/11 (48)	7 (37)/12 (63)	NS ( <i>P</i> = 0.320)
Clinical stage			
I / II / III / IVA	0 (0)/0 (0)/ 11 (48)/12 (52)	0 (0)/0 (0)/ 13 (68)/6 (32)	NS ( <i>P</i> = 0.180)
Duration of treatment received prior to HAIC (mo)	16.3 ± 20.7	36.2 ± 21.5	<i>P</i> = 0.004
Previous number of TACE session (s)	0.9 ± 0.6	4.5 ± 1.8	<i>P</i> < 0.0001
HAIC regimens			
5-FU, cisplatin	8 (35)	7 (37)	NS ( <i>P</i> = 0.923)
5-FU, natural IFN-α	4 (17)	4 (21)	
5-FU, PEG-IFN-α2b	11 (48)	8 (42)	

HCV: Hepatitis C virus; HBV: Hepatitis B virus; LC: Liver cirrhosis; PT: Prothrombin time; INR: International ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT:  $\gamma$  glutamyl transferase; AFP:  $\alpha$ -fetoprotein; WBC: White blood cell; DCP: Des- $\gamma$ -carboxyprothrombin; HAIC: Hepatic arterial infusion chemotherapy; TACE: Transarterial chemoembolization; 5-FU: 5-fluorouracil; IFN: Interferon; PEG-IFN- $\alpha$ 2b: Pegylated interferon- $\alpha$ 2b.

common hepatic artery or proper hepatic artery. The other end of the catheter was connected to the injection reservoir, already implanted into a subcutaneous pocket created in the right or left lower quadrant of the abdomen. The gastroduodenal and right gastric arteries were occluded with coils to prevent potential gastroduodenal injury by the anticancer agents.

### Treatment protocol

Patients received arterial infusions of the anticancer agents

*via* the injection reservoir. Each chemotherapy cycle lasted 2-4 wk. 5-FU (300 mg/m<sup>2</sup> per day, Kyowa Hakko, Tokyo, Japan) was administered continuously for 24 h *via* the infusion pump on days 1 to 5 of each of the two weeks. PEG-IFN- $\alpha$ 2b (PEG-INTRON, MSD KK, Tokyo, Japan) on Day 1 of every week or natural IFN- $\alpha$  (OIF, Otsuka Pharmaceuticals, Tokyo, Japan) on Days 1, 3, 5 of every week was administered by the subcutaneous route. The administered dose of PEG-IFN- $\alpha$ 2b was adjusted by the weight of each patient (50  $\mu$ g-100  $\mu$ g), and the dose of natural IFN- $\alpha$  was fixed at 5.0 × 10<sup>6</sup> unit. In another HAIC regimen, cisplatin (10 mg/body per day, Nihon-Kayaku Pharmaceuticals, Tokyo, Japan) was combined with 5-FU (250 mg/body per day) administered continuously for 24 h *via* the infusion pump on days 1 to 5 of each of the four weeks. Each of the HAIC therapy regimens was repeated for a total of at least 2 cycles until the response changed to progressive disease (PD) or a severe adverse reaction appeared.

### Evaluation

The duration of the progression-free survival was measured from the date of start of HAIC to the date on which the response was judged to have changed to PD. The response to the HAIC was evaluated by contrast-enhanced computed tomography (CT) after every 2 cycles of treatment. The response criteria of the Response Evaluation Criteria in Solid Tumors were used<sup>[27]</sup>. The duration of the response was measured from the date of start of treatment to the date of documented progression. Adverse reactions were assessed every week during therapy based on the United States National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 3.0)<sup>[28]</sup>.

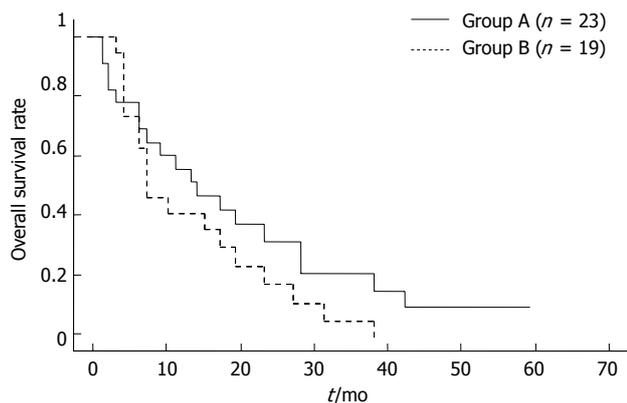
### Statistical analysis

The statistical analysis was performed using the StatView software, version 5.0 (SAS, Cary, NC). Group comparisons were performed by the chi-square test for independence or by Fisher's exact test for comparison of more than two independent groups. The overall survival rate of each group was evaluated by the Kaplan-Meier method and the logrank test from the start of HAIC until the patient's death, and the progression-free survival rate was evaluated until the effect of the HAIC changed to PD. *P* values of < 0.05 were considered to denote significance in all the statistical tests. The closing date of the study was May 31, 2011.

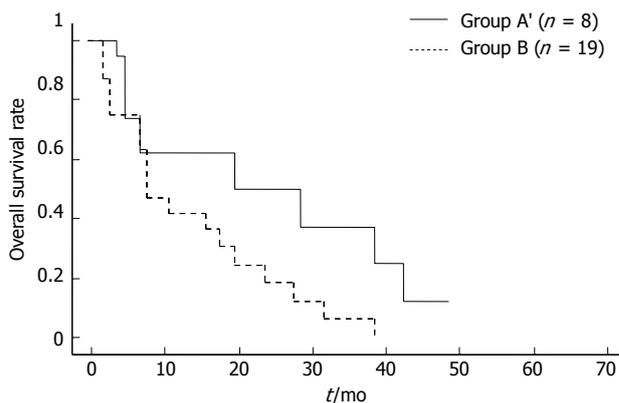
## RESULTS

### Response to the HAIC

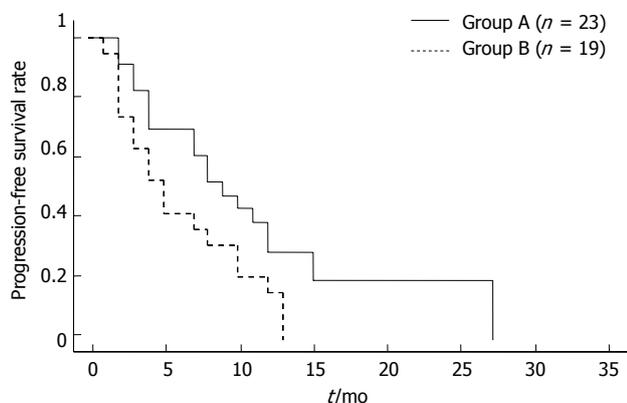
In group A, 2 patients (8.7%) showed complete response (CR), 9 patients (39.1%) showed partial response (PR), 9 patients (39.1%) showed stable disease (SD), and the remaining 3 patients (13.1%) showed PD. On the other hand, in group B, none of the patients (0%) showed CR, 3 patients (15.8%) showed PR, 7 patients (36.8%) showed SD, and the remaining 9 patients (47.4%) showed PD.



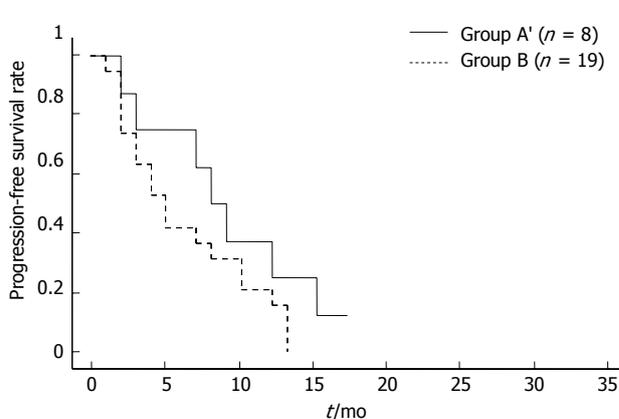
**Figure 1** The overall survival rate tended to be superior in group A (a solid line) than in group B (a dotted line) (3-, 6-, 12-, 24-, and 36 mo = 82.6%, 78.3%, 56.5%, 32.8% and 21.9% vs 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively,  $P = 0.203$ ).



**Figure 3** The overall survival rate and survival time tended to be superior in group A' (a solid line) than in group B (a dotted line) (3-, 6-, 12-, 24-, and 36 mo = 75.0%, 75.0%, 62.5%, 50.0% and 37.5% vs 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively,  $P = 0.095$ ).



**Figure 2** The progression-free survival rate was significantly superior in group A (a solid line) than in group B (a dotted line) (3-, 6-, 12-, and 24 mo = 82.6%, 69.6%, 29.3%, and 19.6% vs 63.2%, 42.1%, 15.8% and 0%, respectively,  $P = 0.040$ ).



**Figure 4** The progression-free survival rate and time tended to be superior in group A' (a solid line) than in group B (a dotted line) (3-, 6-, 12-, and 24 mo = 75.0%, 75.0%, 25.0%, and 0% vs 63.2%, 42.1%, 15.8% and 0%, respectively,  $P = 0.192$ ).

Both the response rate [CR and PR patients/all patients  $\times 100(\%)$ ] and the tumor suppression rate [CR, PR, and SD patients/all patients  $\times 100(\%)$ ] following HAIC were significantly superior in group A than in group B (response rate: 47.8% vs 15.8%,  $P = 0.028$ , tumor suppression rate: 86.9% vs 52.6%,  $P = 0.014$ ).

**Survival**

The overall survival rate and survival time tended to be superior in group A than in group B (3-, 6-, 12-, 24-, and 36 mo = 82.6%, 78.3%, 56.5%, 32.8% and 21.9% vs 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively,  $P = 0.203$  (Figure 1), and 18.8 mo vs 14.0 mo,  $P = 0.267$ ). Furthermore, the progression-free survival rate and time were significantly superior in group A than in group B (3-, 6-, 12-, and 24 mo = 82.6%, 69.6%, 29.3%, and 19.6% vs 63.2%, 42.1%, 15.8% and 0%, respectively,  $P = 0.040$  (Figure 2), and 9.8 mo vs 6.2 mo,  $P = 0.040$ ).

**Subgroup analysis**

In group A, both the patients who received TACE once or twice ( $n = 8$ ) and who did not receive TACE ( $n = 15$ ) were

included. Therefore, to evaluate the effectiveness of HAIC after TACE, we performed subgroup analysis compared the patients who received TACE in group A (group A',  $n = 8$ ) to group B. In group A', 1 patient (12.5%) showed CR, 3 patients (37.5%) showed PR, 3 patients (37.5%) showed SD, and the remaining 1 patient (12.5%) showed PD. Both the response rate and the tumor suppression rate following HAIC tended to be superior in group A' than in group B (response rate: 50.0% vs 15.8%,  $P = 0.064$ , tumor suppression rate: 87.5% vs 52.6%,  $P = 0.087$ ).

The overall survival rate and survival time tended to be superior in group A' than in group B (3-, 6-, 12-, 24-, and 36 mo = 75.0%, 75.0%, 62.5%, 50.0% and 37.5% vs 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively,  $P = 0.095$  (Figure 3), and 24.0 mo vs 14.0 mo,  $P = 0.086$ ). Furthermore, the progression-free survival rate and time also tended to be superior in group A' than in group B (3-, 6-, 12-, and 24 mo = 75.0%, 75.0%, 25.0%, and 0% vs 63.2%, 42.1%, 15.8% and 0%, respectively,  $P = 0.192$  (Figure 4), and 9.1 mo vs 6.2 mo,  $P = 0.143$ ). These results of comparison between group A' and group B was similar to that between group A and group B.

**Table 3** Multivariate analysis (Cox proportional hazards regression model) to identify factors influencing the survival

	Odds ratio	95% CI	P value
Age > 66 (yr)	0.284	0.077-1.044	NS ( $P = 0.058$ )
Gender: female	3.995	0.704-22.662	NS ( $P = 0.118$ )
Resistance to TACE	8.264	1.770-38.461	$P = 0.007$
AFP > 200 (ng/mL)	0.385	0.121-1.230	NS ( $P = 0.107$ )
DCP > 200 (mAU/mL)	1.181	0.218-6.390	NS ( $P = 0.847$ )
Albumin > 3.5 (g/dL)	0.012	0.001-0.181	$P = 0.001$
Total bilirubin > 1.0 (mg/dL)	4.000	1.004-15.933	$P = 0.049$
PT (INR) > 1.20	0.490	0.155-1.551	NS ( $P = 0.225$ )
ALT > 50 (U/L)	1.229	0.378-3.999	NS ( $P = 0.732$ )
Plt > 15.0 ( $\times 10^4/\mu\text{L}$ )	1.251	0.330-4.736	NS ( $P = 0.742$ )
Number of tumors > 6	0.403	0.090-1.794	NS ( $P = 0.233$ )
Size of the largest tumor > 5.0 cm	0.913	0.215-3.884	NS ( $P = 0.902$ )
Clinical stage: IVA	13.800	1.638-116.257	$P = 0.016$
Response to HAIC: CR, PR	0.024	0.004-0.160	$P = 0.0001$
Child-Pugh: B	0.251	0.019-3.307	NS ( $P = 0.293$ )
Hepatic encephalopathy: presence	0.643	0.123-3.347	NS ( $P = 0.599$ )
Ascites: presence	3.471	0.835-14.419	NS ( $P = 0.087$ )

TACE: Transarterial chemoembolization; AFP:  $\alpha$ -fetoprotein; DCP: Des- $\gamma$ -carboxyprothrombin; PT: Prothrombin time; INR: International ratio; ALT: Alanine aminotransferase; HAIC: Hepatic arterial infusion chemotherapy; CR: Complete response; PR: Partial response; CI: Confidence interval.

### Multivariate analysis to identify factors influencing the survival

A multivariate analysis (Cox proportional hazards regression model) was performed to identify factors that might influence the survival following HAIC, which identified resistance to TACE [odds ratio (OR): 8.264,  $P = 0.007$ ], serum albumin > 3.5 g/dL (OR: 0.012,  $P = 0.001$ ), serum total bilirubin > 1.0 mg/dL (OR: 4.000,  $P = 0.049$ ), clinical stage IVA (OR: 13.800,  $P = 0.016$ ), and CR, PR to HAIC (OR: 0.024,  $P = 0.0001$ ) as significant independent predictors influencing of the survival (Table 3).

### Adverse reactions

The common systemic adverse reactions were fever, loss of appetite and general fatigue, however, none exceeded Grade 1 to 2 in severity. Furthermore, no case of serious leukopenia or thrombocytopenia was observed, with the severity of these adverse reactions not exceeding Grade 1 to 2 in any of the cases; none of the patients required administration of granulocyte-colony-stimulating factor or blood transfusion. On the other hand, among the 42 patients, there were 3 patients who developed Grade 2 generalized skin rash, 3 patients who developed obstruction of hepatic artery, and 2 patients who developed infection of reservoir. There were no cases of adverse event-related death.

## DISCUSSION

According to the treatment algorithm for hepatocellular carcinoma in the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan<sup>[15]</sup>, TACE and HAIC are recommended when the number of HCCs is four or more,

with preserved liver function. In a large prospective cohort study of 8510 patients with a long follow-up period of 8 years, Takayasu *et al.*<sup>[14]</sup> reported that TACE using an anticancer agent-lipiodol emulsion with or without gelatin sponge particles improved the survival of patients with advanced HCC, with overall 1-, 3-, 5-, and 7-year survival rates of 82%, 47%, 26%, and 16%, respectively, and a median survival duration of 34 mo. We also reported the superior effectiveness of TACE using a cisplatin- or epirubicin-lipiodol emulsion as compared with that of palliative treatment in a recent study of patients with advanced HCC<sup>[13]</sup>; both the overall survival rate and median survival time in patients who received TACE were significantly superior to those in patients who received only palliative treatment (1-, 2-, 5-, and 8-year survival rates of 98%, 90%, 56% and 16% *vs* 47%, 39%, 23% and 0%, respectively; median survival duration, 25 mo *vs* 10 mo). However, repeat sessions of TACE were often required which can potentially result in deterioration of the liver function<sup>[29]</sup>. Another group reported that selective TACE using conventional doses of anticancer drugs can cause persistent, serious worsening of the liver function<sup>[50]</sup>.

Several recent studies have reported the effectiveness and survival benefit of combined therapy with intra-arterial 5-FU plus cisplatin or systemic various IFN in patients with unresectable advanced HCC<sup>[16-24]</sup>. Ando *et al.*<sup>[21]</sup> investigated the outcomes of HAIC using a combination 5-FU plus cisplatin for HCC patients with complicating PVTT ( $n = 48$ ), and reported a response rate of 48%, median survival time of 31.6 mo, and 1-, 2-, 3- and 5-year survival rates of 45%, 31%, 25% and 11%, respectively. Obi *et al.*<sup>[18]</sup> reported an objective response rate of 52.6% (61/116 patients) in 116 patients with advanced HCC and Vp 3 or 4 treated with a combination of 5-FU plus natural IFN- $\alpha$ . A recent study conducted by us demonstrated the effectiveness of combined therapy with 5-FU plus subcutaneous PEG-IFN- $\alpha$ 2b for unresectable advanced HCC ( $n = 18$ ); the response rate was 33.3%, the median survival time was 17.7 mo, and the 6-, 12-, 24- and 36-mo survival rates were 89%, 71%, 39% and 29%, respectively<sup>[24]</sup>. However, few reports have investigated the effectiveness of HAIC in patients with advanced HCC resistant to TACE. This study revealed that HAIC yielded an unsatisfactory survival rate and survival time in patients with HCC resistant to TACE, and a multivariate analysis identified resistance to TACE as one of the independent predictors of poor survival in these patients.

Recently, a multikinase inhibitor, sorafenib, was approved as the first molecular targeted agent for advanced HCC, and two global phase III trials<sup>[31,32]</sup> showed survival benefit with this drug administered orally for advanced HCC patients with preserved liver function. The SHARP Study was a randomized double-blind placebo-controlled multicenter study conducted in western countries, which showed that both the overall survival and the time to progression were significantly superior in the sorafenib group ( $n = 299$ ) than in the placebo group ( $n = 303$ ) (10.7 mo *vs* 7.9 mo, and 5.5 mo *vs* 2.8 mo, respectively). Interestingly, 86 patients (29% of sorafenib group) and 90 patients (30%

of placebo group) who had previously received TACE were included in the SHARP Study. Galle *et al.*<sup>[33]</sup> reported that among 176 patients after TACE, the overall survival and the time to progression were superior in the sorafenib group ( $n = 86$ ) than in the placebo group ( $n = 90$ ) (11.9 mo *vs* 9.9 mo, and 5.8 mo *vs* 4.0 mo, respectively) in sub-analysis of the SHARP Study. These results suggest that sorafenib may be an effective treatment agent for patients with advanced HCC resistant to TACE. Furthermore, the Asia-Pacific Study, performed in eastern Asian countries, also showed, similar to the SHARP study, significant survival prolongation in the sorafenib group as compared with that in the placebo group. Therefore, in Japan, sorafenib has recently been recommended for the treatment of patients with advanced HCC and extra-hepatic metastasis or major vessel invasion with preserved liver function, e.g., Child-Pugh class A<sup>[34,35]</sup>.

In conclusion, although the evaluation needs to be conducted in a larger number of patients and the study was a retrospective cohort study, the results of this study revealed that HAIC administered with 5-FU exerted insufficient effect against advanced HCC resistant to TACE. Molecular-targeting agents may need to be considered in the future for patients with HCC resistant to TACE.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases around the world, and interventional therapies such as transarterial chemoembolization (TACE) or hepatic arterial infusion chemotherapy (HAIC) has been performed for patients with advanced HCC, especially those with multiple nodules, therefore, it is often repeated several times for the treatment of recurrent HCC. However, the precise efficacy of HAIC in patients with advanced HCC resistant to TACE still remains unclear.

### Research frontiers

Advances in implantable drug delivery systems have made it possible to administer repeated arterial infusions of anticancer agents, and recent studies, including our previous reports, have shown the effectiveness of combined therapy with intra-arterial 5-fluorouracil (5-FU) plus cisplatin or subcutaneous interferon (IFN) therapy in patients with advanced HCC which have multiple intra-hepatic lesions or portal vein tumor thrombosis.

### Innovations and breakthroughs

The study was considered the first report which investigated the effectiveness of HAIC administering 5-FU for advanced HCC resistant to TACE. The patients enrolled in their study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese) (Table 1); one group of patients who did not fulfill the criteria for TACE resistance (group A,  $n = 23$ ), and another group who fulfilled the criteria for TACE resistance (group B,  $n = 19$ ). They compared the outcomes in terms of the response and survival rates between the two groups. Both the response rate and tumor suppression rate following HAIC were significantly superior in group A than in group B. Furthermore, both the progression-free survival rate and survival time were significantly superior in group A than in group B. A multivariate analysis (Cox proportional hazards regression model) showed that resistance to TACE was an independent predictor of poor survival.

### Applications

The results of this study revealed that HAIC administered with 5-FU exerted insufficient effect against advanced HCC resistant to TACE.

### Terminology

HAIC administering 5-FU was not effective against advanced HCC resistant to

TACE, and our study showed the limitation of interventional therapies to prolong the survival for advanced HCC and consideration of new strategy including other tools for treatment, i.e., molecular-targeting agents.

### Peer review

In this study, the authors report that patients with HCC resistant to TACE exhibit a poorer response to HAIC. This paper is clearly written and the topic material is important.

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## Preoperative microcoil embolization of the common hepatic artery for pancreatic body cancer

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### Abstract

**AIM:** To evaluate safety and feasibility of microcoil embolization of the common hepatic artery under proper or distal balloon inflation in preoperative preparation for en bloc celiac axis resection for pancreatic body cancer.

**METHODS:** Fifteen patients (11 males, 4 females; median age, 67 years) with pancreatic body cancer involving the nerve plexus surrounding the celiac artery underwent microcoil embolization. To alter the total hepatic blood flow from superior mesenteric artery (SMA), microcoil embolization of the common hepatic artery (CHA) was conducted in 2 cases under balloon inflation at the proximal end of the CHA and in 13 cases under distal microballoon inflation at the distal end of the CHA.

**RESULTS:** Of the first two cases of microcoil embolization with proximal balloon inflation, the first was successful, but there was microcoil migration to the proper hepatic artery in the second. The migrated microcoil

was withdrawn to the CHA by an inflated microballoon catheter. Microcoil embolization was successful in the other 13 cases with distal microballoon inflation, with no microcoil migration. Compact microcoil embolization under distal microballoon inflation created sufficient resistance against the vascular wall to prevent migration. Distal balloon inflation achieved the requisite 1 cm patency at the CHA end for vascular clamping. All patients underwent en bloc celiac axis resection without arterial reconstruction or liver ischemia.

**CONCLUSION:** To impede microcoil migration to the proper hepatic artery during CHA microcoil embolization, distal microballoon inflation is preferable to proximal balloon inflation.

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**Key words:** Embolization; Microcoil; Balloon inflation; En bloc celiac axis resection; Pancreas body cancer

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Takasaka I, Kawai N, Sato M, Tanihata H, Sonomura T, Minamiguchi H, Nakai M, Ikoma A, Nakata K, Sanda H. Preoperative microcoil embolization of the common hepatic artery for pancreatic body cancer. *World J Gastroenterol* 2012; 18(16): 1940-1945 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1940.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1940>

### INTRODUCTION

The overall 5-year survival rate after surgical resection for pancreatic cancer is extremely poor (< 10%)<sup>[1-3]</sup>. There is an ongoing effort toward treating this difficult disease and improving survival. Surgery plays a main role for a complete

cure of pancreatic body cancer. En bloc celiac axis resection (modified Appleby operation) has been introduced to expand the surgical treatment for pancreatic body cancer with celiac axis involvement<sup>[4,5]</sup>, and Hirano *et al*<sup>[6]</sup> report a promising estimated 5-year survival rate of 42% for locally advanced pancreatic body cancer. This surgical procedure has the aim of en bloc lymphadenectomy together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and common hepatic artery (CHA). The safety of this operation is based on the rationale that hepatic arterial blood is supplied from the superior mesenteric artery *via* the pancreaticoduodenal arcades following ligation of the CHA. However, weak pulsation of the proper hepatic artery was observed in some patients during surgery, immediately after surgical ligation of the CHA<sup>[7-9]</sup>. When poor pulsation of the proper hepatic artery is observed after clamping of the CHA, arterial reconstruction is necessary because liver necrosis is fatal once it occurs<sup>[10-13]</sup>. To avoid this complicated procedure, Kondo *et al*<sup>[14]</sup> reported the preparatory technique of enlarging the collateral pathways from the SMA before surgery, by preoperative embolization of the CHA using interlocking detachable coils. Following this report, surgeons first asked interventional radiologists to embolize the CHA in preoperative management to enlarge the collateral pathways from the superior mesenteric artery (SMA). However, exact microcoil embolization of the short segment of the CHA is not easy, even for experienced interventional radiologists, because of its rapid arterial flow and, of particular concern, the possibility of coil migration to the proper hepatic artery. In our case, the surgeon also asked that we retain vascular lumen patency 1 cm from the distal end of the common hepatic artery and the proximal celiac artery trunk, to enable clamping of these vessels. In response to these requirements, we conducted microcoil embolization of the CHA under either proximal or distal microballoon inflation. The purpose of this clinical study is to describe these techniques and to evaluate their safety and feasibility.

## MATERIALS AND METHODS

### Patients

Approval of the Institutional Ethics Committee of our institution was obtained for this clinical trial prior to initiation of the study. All patients were fully informed of the extent of their diseases and of the risks and benefits associated with preoperative CHA embolization and en bloc resection.

In cases of right or common hepatic artery branching from the SMA, it was not necessary to conduct microcoil embolization of the CHA. Between May 2007 and January 2010, 15 patients with pancreatic body cancer involving the nerve plexus surrounding the celiac artery were scheduled for surgical radical pancreatectomy and underwent microcoil embolization preoperatively. Tumor stage was T4 in 14 patients and T3 in 1 patient according to the tumor, node and metastasis classification of the Union for

International Cancer Control (UICC)<sup>[15]</sup>. Eleven patients were male and 4 were female; age ranged from 46 to 79 years (median, 67 years) (Table 1). All patients suffered from severe back pain and/or abdominal pain. Enhanced computed tomography using contrast medium revealed tumors sized 10-76 mm located in the body to the tail of the pancreas and involving the celiac, splenic, and/or common hepatic arteries, but with no evidence of liver metastases or invasion to the superior mesenteric artery. Microcoil embolization of the common hepatic artery was performed 7 d to 14 d before surgery. Two interventional radiologists, each with more than 7 years experience in transcatheter arterial embolization, conducted the following procedure.

### Interventional procedure

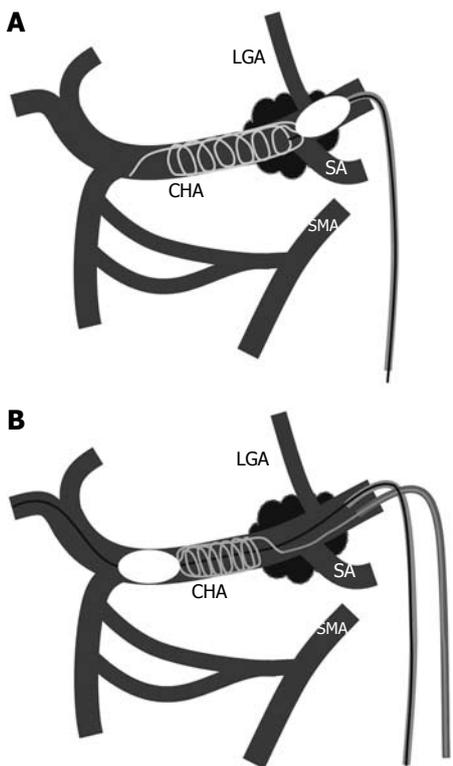
**Microcoil embolization under proximal balloon inflation:** A 5F balloon catheter (balloon diameter 10 mm; Seilecon MP, Catheter Rosch II; Terumo, Tokyo, Japan) was inserted into the celiac artery through a 6F long sheath (Terumo) *via* the right femoral artery. After celiac arteriography, a 5F balloon catheter was advanced to the CHA using a guide wire (0.035, angle type; Radiofocus, Terumo). Under balloon inflation at the proximal end of the CHA, a microcatheter with two markers for detachable coil embolization (Rapidtransit, Johnson and Johnson, New Brunswick, NJ) was inserted coaxially and advanced to the distal CHA, 1 cm before the branching of the gastroduodenal artery. Detachable microcoils (interlocking detachable coil, Boston Scientific, Boston, MA) of diameter at least 1 mm greater than that of the CHA were deployed by making a lengthwise and/or sidewise frame (Figure 1A).

**Microcoil embolization under distal balloon inflation:** 6F and 4F long sheaths (Terumo) were inserted *via* the right and left femoral arteries, respectively. A 6F guiding catheter (Elway, Terumo Clinical Supply, Tochigi, Japan) was advanced through a 6F sheath to the celiac artery. After celiac arteriography, a 3.3F microballoon catheter (8 mm in maximum inflated diameter, 1 cm in length; Iiguman, Fuji System, Tokyo, Japan) was inserted into the common hepatic artery through a 6F guiding catheter and placed at the distal end of the CHA (Figure 1B). The microballoon was inflated, taking care not to interrupt blood flow in the gastroduodenal artery (Figure 2A). A 4F catheter (Rosch celiac type, Medikit, Tokyo, Japan) was advanced through a 4F sheath to catheterize the celiac artery, and a microcatheter with two markers was then advanced to the proximal end of the balloon inflation (Figure 2A). Detachable coils of diameter greater than that of the CHA were used initially, and fiber coils (Tornado, Boston Scientific) were added to fill the space if necessary. Microcoils were placed in the CHA from the proximal end of the microballoon inflation to the inlet of the left gastric artery (LGA) branching from the celiac trunk artery (Figure 2B). In fluoroscopic guidance, the tube angle that enabled the best visualization of the CHA or LGA was used. After microcoil embolization, the microballoon catheter was deflated and withdrawn.

**Table 1** Backgrounds of patients with pancreatic body cancer for common hepatic artery microcoil embolization

N	Age (yr)	Sex	Tumor		Balloon catheter inflation proximal/distal	Coil migration	CHA diameter (mm)	Coil used	
			Major axis (mm)	Stage (UICC TNM ver.6)				Detachable coil diameter (mm)/length (cm) × N	Fiber coil proximal/distal diameters (mm) × N
1	51	F	10	T4	+/-	-	7.0	8/20	
2	70	M	35	T4	+/-	+	6.7	10/10	
3	67	M	40	T4	+/+	-	6.1	8/20	4/8 × 3
4	56	M	46	T4	-/+	-	5.6	7/10	4/8 × 2
5	64	M	30	T4	-/+	-	7.4	10/20, 7/20, 6/10	
6	71	M	61	T4	-/+	-	6.4	9/20, 7/20, 6/10	
7	71	M	35	T4	-/+	-	7.7	10/10	7/3 × 2
8	54	M	31	T4	-/+	-	4.5	6/10 × 2, 5/10	6/2 × 2
9	69	F	76	T3	-/+	-	5.1	7/10	7/3 × 2, 8/4 × 3
10	46	M	35	T4	-/+	-	4.4	6/10	6/2 × 4
11	78	F	42	T4	-/+	-	6.4	8/10, 6/10	6/2
12	64	M	45	T4	-/+	-	2.1	3/10	3/2
13	74	F	37	T4	-/+	-	3.6	5/10	6/2, 5/2
14	62	M	34	T4	-/+	-	4.4	6/10	7/3 × 3
15	79	M	45	T4	-/+	-	5.5	7/10	8/4 × 3

N: Number; F: female; M: Male; UICC: Union for International Cancer Control; CHA: Common hepatic artery; TNM: Tumor, node and metastasis.



**Figure 1** Schematic drawing of microcoil embolization under proximal balloon inflation (A) and distal balloon inflation (B) in the common hepatic artery. LGA: Left gastric artery; CHA: Common hepatic artery; SA: Splenic artery; SMA: Superior mesenteric artery.

**Microcoil embolization under distal balloon inflation and proximal balloon inflation at the time of withdrawal:** After microcoil embolization of the CHA under distal balloon inflation, as described above, a 5F balloon catheter (Selecon MP, Catheter Rosch II, Terumo) was inserted *via* the femoral artery to the celiac artery through a 6F long sheath. This 5F balloon catheter was used to prevent microcoil migration from the CHA to the celiac

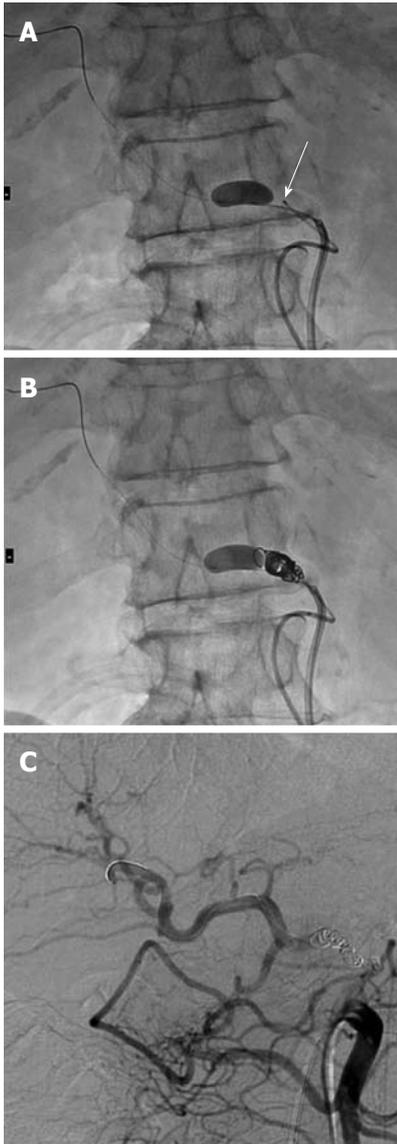
trunk artery. Specifically, when the distal balloon catheter was deflated and withdrawn, the 5F balloon catheter was inflated at the CHA proximal end or celiac trunk artery. After confirming no proximal coil migration, the proximal balloon catheter was deflated, and both balloon catheters were removed.

After coil embolization in each procedure, superior mesenteric arteriography was conducted to confirm the alteration of blood flow from the SMA to the hepatic artery (Figure 2C). We aimed for total hepatic arterial blood flow to be supplied from the superior mesenteric artery. When the left hepatic artery branched from the LGA, microcoil embolization of left gastric artery was also performed.

## RESULTS

In the present series, the first two patients underwent microcoil embolization of the CHA under proximal balloon inflation. Successful microcoil embolization of the CHA was completed in the first case. However, in the second case, distal migration of the microcoils occurred from the CHA to the proper hepatic artery after deflation of the proximal balloon catheter following microcoil placement. When this occurred, we immediately inserted the deflated microballoon catheter into the proper hepatic artery, advanced the catheter beyond the migrated microcoil, inflated the balloon catheter, and successfully withdrew the migrated coil to the common hepatic artery (Figure 3).

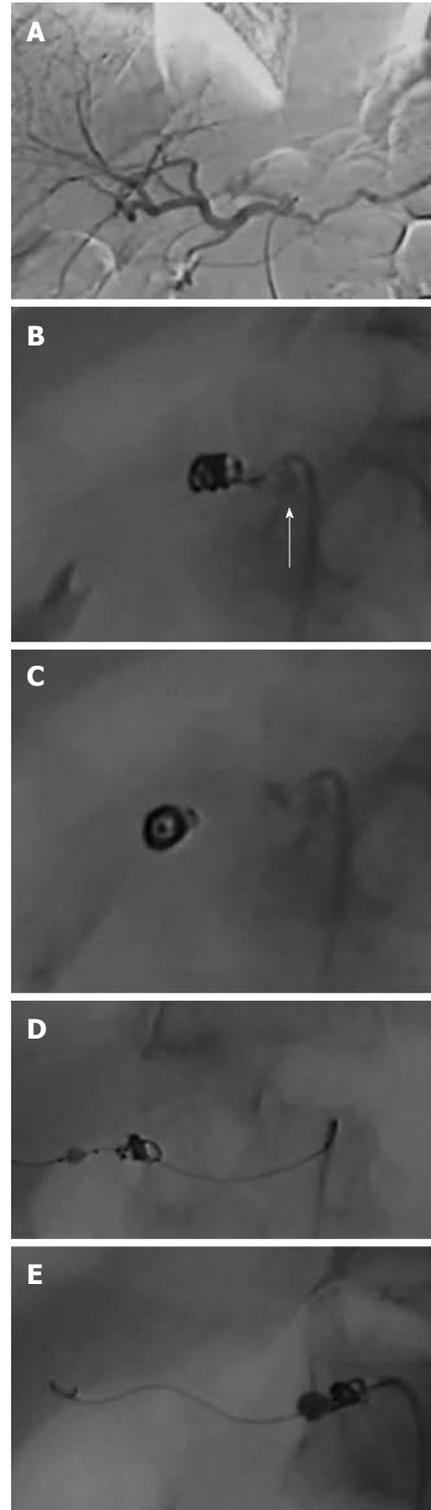
The third case underwent microcoil embolization under distal microballoon inflation. At the time of withdrawing the distal deflated balloon catheter located at the distal end of the CHA, we inflated the proximal balloon at the proximal end of the CHA to prevent microcoil migration from the CHA to the celiac trunk artery. However, we realized that the second (proximal) balloon catheter was not necessary because of the rigid fixation of the microcoils against the vascular wall. Thereafter, the distal



**Figure 2 Microcoil embolization under distal balloon inflation.** A: Radiograph during catheterization shows microcatheter (arrow) insertion to the common hepatic artery (CHA) via the celiac artery under distal microballoon inflation in the CHA; B: Radiograph during microcoil embolization shows a tight widthwise frame; C: Superior mesenteric arteriography after microcoil embolization shows blood flow from the superior mesenteric artery to the proper hepatic artery via the pancreaticoduodenal arcades.

deflated microballoon catheter was withdrawn without the assistance of proximal balloon inflation. There was no microcoil migration in the following 12 cases under distal microballoon inflation (Table 1). Accordingly, distal balloon inflation achieved the 1 cm patency at the end of the CHA required for vascular clamping.

In three cases having the variation of left hepatic artery branching from the LGA, we performed additional embolization of the LGA trunk using microcoils. In two of these three cases, total hepatic arterial blood flow was confirmed in superior mesenteric arteriography to come from the SMA via intrahepatic communication between the right and left hepatic arteries. In the remaining case, there was communication between the LGA and the



**Figure 3 Distal migration of the microcoils and the successful withdraw.** A: Following celiac arteriography; B: Microcoil embolization under proximal balloon inflation (arrow) was performed; C: Microcoil migration from the common hepatic artery (CHA) to the proper hepatic artery occurred after deflation of the proximal balloon catheter; D: Under fluoroscopic guidance using the tube angle that enabled the best visualization of the CHA and with the assistance of a microwire, a microballoon catheter was then inserted through the migrated coil and inflated; E: The migrated coils were withdrawn to their original position in the CHA by the inflated balloon catheter.

short gastric artery and left gastroepiploic artery, which

branched from the splenic artery. In this case, LGA and the splenic artery were embolized, aiming to achieve total hepatic blood flow from the SMA. However, following these embolizations, hepato-petal blood flow did not come from the SMA but from the inferior phrenic artery. No evidence of liver ischemia was observed during surgery, enabling radical pancreatectomy to be performed.

Superior mesenteric arteriography after embolization showed good hepato-petal blood flow from the SMA to the proper hepatic artery in all cases except that described above. All patients successfully underwent radical pancreatectomy without liver or gastric ischemia, and experienced no problems or complications related to the microcoil embolization.

## DISCUSSION

In the present series, microcoil embolization under distal balloon inflation was superior to that under proximal balloon inflation in terms of impeding distal embolization. White<sup>[16]</sup> described two techniques of coil placement in the pulmonary artery: an anchor technique in which the microcoil tip was hooked into the small branch, and a scaffold technique in which a long frame was created lengthwise to increase friction against the vessel wall and fill the feeding artery. There are no small branch arteries from the CHA; therefore, using the scaffold technique we made a lengthwise and sidewise frame, by pushing and pulling the coils. If the microcoils continued to push out under proximal balloon inflation, then they tended to move to a more peripheral site than intended; in this case, the microcoils needed to be pulled back. In the second case of the present series, under proximal balloon inflation the microcoils migrated to the proper hepatic artery despite the microcoils having a diameter 3 mm greater than that of the CHA; this probably occurred because the loose frame of placed microcoils did not create sufficient friction against the vascular wall. The migrated microcoils were pulled back by the distal inflated balloon catheter to the original CHA site. We found that microcoils placed under distal balloon inflation became more compact than those under proximal balloon inflation, creating enough friction against the vascular wall to prevent migration.

We anticipated that the microcoils could migrate from the CHA to the celiac trunk artery or abdominal aorta after retrieval of the deflated distal microballoon catheter following microcoil embolization. For this reason, in the third case of this series, an additional balloon catheter was inserted and inflated in the celiac trunk artery, with the aim of blocking proximal coil migration from the CHA to the celiac trunk. However, this precaution proved unnecessary. The rigid friction of the microcoils against the vessel wall resulted in no proximal migration when the distal deflated microballoon catheter was retrieved. The use of detachable coils of diameter 1-2 mm greater than that of the CHA was sufficient to create increased friction in the subsequent 12 cases under distal microballoon inflation.

As an additional positive outcome of the described technique, the long dimension of the inflated microballoon (10 mm) enables patency to be maintained at the distal CHA end. Accordingly, the distal microballoon inflation method fulfilled the surgeon's requirement to retain 1 cm patency at the distal CHA end for vascular clamping.

It is a weakness of the distal microballoon catheter method that performing the procedure is somewhat complicated. However, the dual femoral artery approach is minimally invasive, and maneuvering the microballoon catheter does not have a steep learning curve.

In conclusion, the distal microballoon inflation method in CHA microcoil embolization was preferable to the proximal balloon inflation method, in terms of creating a compact microcoil frame that caused no coil migration to the proper hepatic artery, and of supplying a sufficient length of CHA patency to enable vascular clamping.

## COMMENTS

### Background

In preoperative management to avoid liver ischemia during surgery for pancreatic body cancer, the surgeons requested the interventional radiologists to embolize the common hepatic artery, to enlarge the collateral pathways from the superior mesenteric artery.

### Research frontiers

This surgical procedure has the aim of en bloc lymphadenectomy together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and common hepatic artery (CHA). The safety of this operation is based on the rationale that hepatic arterial blood is supplied from the superior mesenteric artery via the pancreatico-duodenal arcades following ligation of the CHA.

### Innovations and breakthroughs

The previous method using microcoils was conducted to occlude CHA without a balloon catheter. The present study occluded the CHA using microcoil embolization with the assistance of a microballoon catheter.

### Applications

As the actual application for CHA occlusion, the distal microballoon inflation method in CHA microcoil embolization was preferable to the proximal balloon inflation method, in terms of creating a compact microcoil frame that caused no coil migration to the proper hepatic artery, and of supplying a sufficient length of CHA patency to enable vascular clamping.

### Terminology

For surgical treatment for pancreas body cancer, en bloc lymphadenectomy is performed together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and CHA. Because of the risk of liver ischemia following ligation of the CHA during surgery, preoperative occlusion of the CHA using microcoil embolization is necessary.

### Peer review

This study describes an interesting variant of interventional radiology that allows to minimize ischemic hepatic damage in Appleby operation. All technical steps are well and clearly described. Results are encouraging and convincing. Figure are clear and pertaining. The study is important for surgeons and interventional radiologists, even if only 15 patients have been enrolled.

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## Hepatocellular carcinoma in Budd-Chiari syndrome: A single center experience with long-term follow-up in South Korea

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### Abstract

**AIM:** To evaluate long-term clinical course of Budd-Chiari syndrome (BCS) and predictive factors associated with the development of hepatocellular carcinoma (HCC) and survival.

**METHODS:** We analyzed 67 patients with BCS between June 1988 and May 2008. The diagnosis of BCS was confirmed by hepatic venous outflow obstruction shown on abdominal ultrasound sonography, computed tomography, magnetic resonance imaging, or venogra-

phy. The median follow-up period was  $103 \pm 156$  [interquartile range (IQR)] mo.

**RESULTS:** The median age of the patients was  $47 \pm 16$  (IQR) years. At diagnosis, 54 patients had cirrhosis, 25 (37.3%) Child-Pugh class A, 23 (34.3%) Child-Pugh class B, and six (9.0%) patients Child-Pugh class C. During the follow-up period, HCC was developed in 17 patients, and the annual incidence of HCC in patients with BCS was 2.8%. Patients in HCC group ( $n = 17$ ) had higher hepatic venous pressure gradient (HVPG) than those in non-HCC group ( $n = 50$ ) ( $21 \pm 12$  mmHg vs  $14 \pm 7$  mmHg,  $P = 0.019$ ). The survival rate of BCS patients was 86.2% for 5 years, 73.8% for 10 years, and 61.2% for 15 years. In patients with BCS and HCC, survival was 79% for 5 years, 43.1% for 10 years, and 21.5% for 15 years.

**CONCLUSION:** The incidence of HCC in patients with BCS was similar to that in patients with other etiologic cirrhosis in South Korea. The HVPG is expected to provide additional information for predicting HCC development in BCS patients.

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**Key words:** Budd-Chiari syndrome; Hepatocellular carcinoma; Prognosis

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Park H, Yoon JY, Park KH, Kim DY, Ahn SH, Han KH, Chon CY, Park JY. Hepatocellular carcinoma in Budd-Chiari syndrome: A single center experience with long-term follow-up in South Korea. *World J Gastroenterol* 2012; 18(16): 1946-1952 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1946.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1946>

## INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare hepatic disease caused by occlusion of the hepatic venous outflow. Thrombogenic conditions among the known causes of BCS are well documented, such as coagulopathy, chronic intake of contraceptives, myeloproliferative diseases, autoimmune diseases, and others<sup>[1]</sup>. Hepatic venous outflow block can also be caused by malignant tumors<sup>[2]</sup>. BCS was initially defined as a symptomatic occlusion of the hepatic veins, but with increasing reports on various obstructive cases in the hepatic portion of the inferior vena cava (IVC), it became to include obstructive IVC lesions as well as the major hepatic veins. BCS induces chronic liver congestion so that it causes hepatomegaly, ascites, leg edema, collateral venous dilatation in the body trunk and portal hypertension<sup>[3]</sup>. Several studies have suggested that hepatic congestion caused by obstruction of hepatic venous outflow can lead to cirrhosis and hepatocellular carcinoma (HCC)<sup>[4,5]</sup>. The incidence of HCC in patients with BCS has varied according to regions and investigators<sup>[3,6-9]</sup>. Japan and South Africa showed relatively higher incidences compared to those of United States and France: 6.4%-47.5% vs 4%-20%<sup>[5,7,9,10]</sup>. Prognosis of HCC in BCS has varied as well<sup>[5,6,11]</sup>.

We followed BCS patients treated at our hospital for more than 20 years, and our long term follow-up data can be helpful to understand the prognosis of BCS patients and natural course of the disease. Thus, we (1) evaluated the incidence and cumulative annual risk of HCC in BCS patients; (2) analyzed the characteristics associated with the development of HCC in BCS patients; and (3) investigated the prognosis of BCS and HCC in BCS patients.

## MATERIALS AND METHODS

### Patients

From June 1988 to May 2008, 95 consecutive patients who were diagnosed with BCS at Severance Hospital were studied retrospectively. Among them, 28 patients were excluded based on the criteria as follows: patients with secondary BCS [occlusion of the hepatic venous outflow by an outside structure (HCC, a Klatskin tumor, and renal cell carcinoma)], hematologic diseases which could result in venous obstructive disease, hepatic septic emboli of colon cancer, or post-operative complication of HCC. Finally 67 patients who were diagnosed with primary BCS were investigated. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the institutional review board of our institute.

### Methods

We retrospectively reviewed patients' age, gender, and presenting symptoms of decompensate liver cirrhosis such as ascites, encephalopathy and variceal bleeding. We also investigated their history of medication which can induce thrombosis such as oral contraceptive, herbal

medication, and steroid. Patients were assessed for other risk factors of cirrhosis and HCC such as alcohol, hepatitis B virus and hepatitis C virus (HCV) infections.

Laboratory tests included complete blood count, prothrombin time, alanine aminotransferase (ALT), bilirubin, albumin, creatinine, viral markers such as hepatitis B virus surface antigen (HBsAg) and anti-HCV antibody. Based on laboratory and physical examination results, Model for End-Stage Liver Disease (MELD) score and Child Pugh score were calculated to evaluate liver function. Every 3-6 mo, imaging studies such as computed tomography (CT), ultrasonography or magnetic resonance imaging (MRI) were performed, and alpha-fetoprotein (AFP) level was checked for HCC surveillance.

### Diagnosis of BCS and HCC

The diagnosis of BCS was confirmed by hepatic venous outflow obstruction shown on ultrasound sonography (US), contrast-enhanced CT, MRI, or venography. Patients were confirmed to have HCC according to the American Association for the Study of Liver Disease practice guidelines for the HCC diagnosis<sup>[12]</sup>. Briefly, patients were diagnosed with HCC if they had a tumor with a maximum diameter of > 2 cm and the typical features of HCC on dynamic CT (defined as enhancement in the arterial phase and early washout in the portal phase), and an AFP > 200 ng/mL<sup>[12]</sup>. If the maximum diameter of the tumor was 1 cm to 2 cm, dynamic CT and MRI were performed. HCC was diagnosed if coincidental typical features of HCC were noted. If the tumor did not satisfy above criteria, a biopsy was performed. During hospital stay, abdominal ultrasonography was carried out to evaluate the presence of cirrhosis and hepatocellular carcinoma.

### Measurement of hepatic venous pressure gradient

The hepatic venous pressure gradient (HVPG) was measured through catheterization during venography or angioplasty. Forty-five patients underwent venography or angioplasty when diagnosed with BCS or during follow up period. Patients were diagnosed with clinical liver cirrhosis if they satisfied one or more of the following 3 conditions: (1) a platelet count < 100 000/ $\mu$ L and ultrasonographic findings suggestive of cirrhosis including a blunted, nodular liver edge accompanied by splenomegaly (> 12 cm)<sup>[13,14]</sup>; (2) the presence of esophageal or gastric varices; and (3) overt complications of liver cirrhosis, including ascites, variceal bleeding, and hepatic encephalopathy.

### Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 17.0). Descriptive statistics were computed for all variables, including median  $\pm$  interquartile range (IQR) and percentiles for continuous variables and frequencies for categorical factors. Mann-Whitney *U* test and  $\chi^2$ -test were used to compare HCC and non-HCC groups. Kaplan-Meier analysis was performed to estimate the cumulative incidence

**Table 1** Baseline clinical characteristics of patients with Budd-Chiari syndrome (*n* = 67)

Characteristics	Budd-Chiari syndrome
Age (yr)	47 ± 16
Gender (male)	34 (50.7)
Obstruction site	
IVC	56 (83.6)
Hepatic vein	5 (7.5)
Combined	6 (9.0)
Alcohol consumption	
None/social/heavy (> 80 g/d)	32 (47.8)/15 (22.3)/20 (29.9)
Positive viral marker	
HBsAg/anti-HCV	3 (4.5)/0 (0)
Liver cirrhosis at diagnosis	54 (80.6)
Child-Pugh A/B/C	25/23/6 (37.3/34.3/9.0)
MELD score	11 ± 6
Decompensate LC symptoms	23 (34.3)
Length of obstruction (cm)	2.0 ± 4.0
HVPG (mmHg)	15 ± 10
Laboratory data	
ALT (IU/L)	19 ± 17
Bilirubin (mg/dL)	1.4 ± 1.3
Albumin (g/dL)	3.8 ± 0.7
Hemoglobin (g/dL)	12.3 ± 2.6
Platelet count (k/ $\mu$ L)	109 ± 78
Creatinine (mg/dL)	0.9 ± 0.2
PT (%)	73 ± 27
Treatment modality	
Angioplasty	27 (40.3)
Shunt operation	4 (5.9)
TIPS	3 (4.5)
Thrombolysis	1 (1.5)
Symptomatic medical treatment	32 (47.7)
Median follow up period (mo)	103 ± 156

Data were expressed as median ± IQR or *n* (%). IVC: Inferior vena cava; HCV: Hepatitis C virus; HbsAg: Hepatitis B surface antigen; MELD: Model for End-Stage Liver Disease; LC: Liver cirrhosis; HVPG: Hepatic venous pressure gradient; ALT: Alanine aminotransferase; PT: Prothrombin time; TIPS: Transjugular intrahepatic portosystemic shunt; IQR: Interquartile range.

of HCC from the time of BCS diagnosis and survival of all BCS patients and those who developed HCC. To evaluate the factors associated with the development of HCC in patients with BCS, multivariate Cox regression analysis was used. A two-sided *P* value < 0.05 was considered to indicate a significant difference.

## RESULTS

### Baseline clinical characteristics of the patients

The baseline characteristics of the 67 patients are shown in Table 1. The site of obstruction was IVC in 56 patients (83.6%), hepatic vein in five patients (7.4%), and both IVC and hepatic vein sites in six patients who were classified as “combined”. There was no patient with history of chronic use of medication such as oral contraceptive, herbal medication, and steroid. Patients with heavy alcohol consumption defined as over 80 g/d were 20 (29.9%). HBsAg was positive in 3 patients (4.5%) and there was no patient with positive for anti-HCV antibody. Fifty-

**Table 2** Characteristics of the patients with hepatocellular carcinoma at the time point of diagnosis (*n* = 17)

Variables	HCC
Age (yr)	53 ± 12
Time period from BCS to HCC (mo)	51 ± 115
Child-Pugh class	
A/B/C	6 (35.3)/8 (47.1)/3 (17.6)
Tumor stage (AJCC 6th) <sup>1</sup>	
I / II / III / IV	8 (47.1)/6 (35.3)/3 (17.6)/0 (0)
Treatment modality	
TACE/TACI	9 (52.9)
Intra-arterial chemotherapy	3 (17.6)
Conservative management	3 (17.6)
Operation	2 (11.9)
Prognosis	
Alive	12 (70.5)
Death	3 (17.6)
F/U loss	2 (11.9)

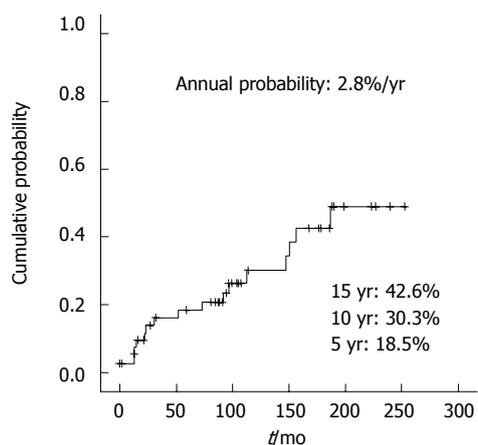
Data were expressed as median ± IQR or *n* (%). BCS: Budd-Chiari syndrome; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; TACI: Transarterial chemo-infusion; F/U: Follow up; IQR: Interquartile range. <sup>1</sup>AJCC 6th, American Joint Committee on Cancer staging system, 6th edition.

four patients had underlying liver cirrhosis at the time of BCS diagnosis. Twenty-five of them (37.3%) were classified as Child-Pugh class A, 23 (34.3%) as class B, and six (9.0%) as class C. The median MELD score was 11 ± 6 (IQR). Twenty three patients (34.3%) had decompensated liver cirrhosis symptoms such as variceal bleeding, uncontrolled ascites or hepatic encephalopathy higher than grade 3. Twenty-seven (40.3%) patients were treated with percutaneous angioplasty, four (5.9%) with shunt operation, three (4.5%) with transjugular intrahepatic portosystemic shunt (TIPS), and one (1.5%) with thrombolysis. Thirty-two (47.7%) patients received only symptomatic medical treatment. The median follow up period was 103 ± 156 (IQR) mo.

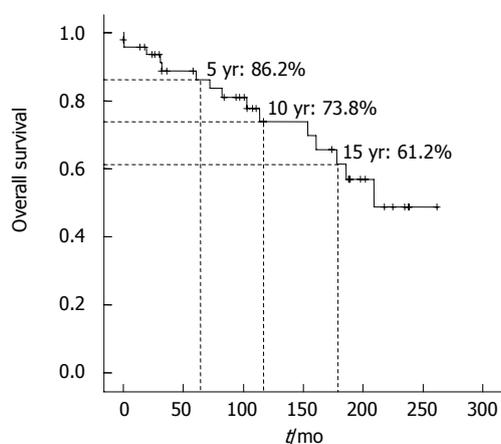
### Development of HCC in the patients with BCS

During follow-up periods, HCC was occurred in 17 patients. At the time of diagnosis of HCC, the median age of the patients was 53 ± 12 (IQR) years, and time period between diagnoses of BCS and HCC was 51 ± 115 (IQR) mo (Table 2). HCC was histologically confirmed in 2 patients with hepatic resection. According to the Kaplan-Meier analysis, as shown in Figure 1, the cumulative probability was 18.5% at 5 years, 30.3% at 10 years, and 42.6% at 15 years. The annual occurrence of HCC in BCS patients was 2.8%.

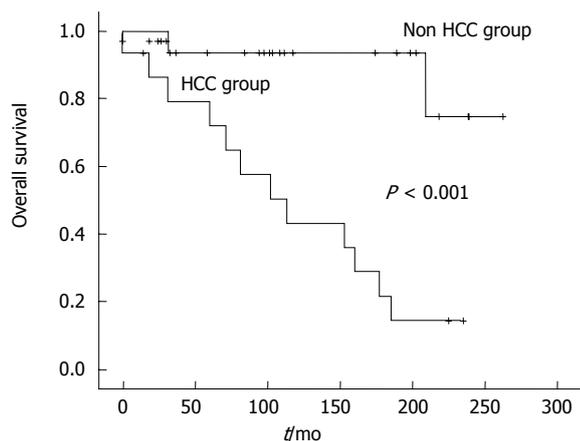
We compared the baseline characteristics (at the time of diagnosis with BCS) of HCC group (*n* = 17) with non-HCC group (*n* = 50). The differences between the two groups are shown in Table 3. There were no significant differences in the comparison of age, gender, obstruction site, alcohol consumption and presence of viral markers between two groups. HVPG was significantly higher in HCC group [21 ± 12 (IQR) mmHg] than in non-HCC



**Figure 1** Cumulative probability of hepatocellular carcinoma in patients with Budd-Chiari syndrome ( $n = 17$ ).



**Figure 2** Overall survival of patients with Budd-Chiari syndrome.



**Figure 3** Comparison of survival between hepatocellular carcinoma Group ( $n = 17$ ) and non-hepatocellular carcinoma group ( $n = 50$ ). HCC: Hepatocellular carcinoma.

group [ $14 \pm 7$  (IQR) mmHg] ( $P = 0.019$ ). However, in the multivariate Cox regression analysis, HVPg showed no significant difference between in HCC group and in non-HCC group ( $P = 0.452$ ).

**Table 3** Comparison of baseline clinical characteristics between hepatocellular carcinoma group and non hepatocellular carcinoma group

Variables	HCC ( $n = 17$ )	Non HCC ( $n = 50$ )	P value
Age (yr)	47 ± 11	47 ± 18	0.863
Gender (male)	10 (58.8)	24 (48)	0.441
Obstructive site			0.264
IVC	15 (88.2)	41 (82)	
Hepatic vein	2 (11.8)	3 (6)	
Combined	0 (0)	6 (12)	
Alcohol consumption			0.329
None	6 (35.3)	26 (52.0)	
Social	4 (23.5)	11 (22.0)	
Heavy (> 80 g/d)	7 (41.2)	13 (26.0)	
Positive for HBsAg	0 (0)	3 (6.0)	0.554
LC at diagnosis	14 (82.4)	40 (80.0)	0.832
Decompensate LC symptoms	8 (47.1)	15 (28.0)	0.148
Child Pugh A/B/C	5/7/2	20/16/4	0.647
MELD score	11 ± 6	11 ± 6	0.778
Follow up period (mo)	103 ± 146	103 ± 160	0.648
Length of obstruction (cm)	1.0 ± 2.5	2.0 ± 4.4	0.144
HVPg (mmHg)	21 ± 12	14 ± 7	0.019
Laboratory data			
ALT (IU/L)	21 ± 21	18 ± 14	0.160
Bilirubin (mg/dL)	1.5 ± 1.9	1.3 ± 1.3	0.521
Albumin (g/dL)	3.6 ± 0.85	3.8 ± 0.72	0.245
Hemoglobin (g/dL)	12.5 ± 4.1	12.3 ± 2.3	0.897
Platelet count (k/ $\mu$ L)	97 ± 59.5	113 ± 80.7	0.559
Creatinine (mg/dL)	0.9 ± 0.14	0.85 ± 0.24	0.798
PT (%)	71 ± 31	74 ± 22	0.756
Survival (%)			< 0.001
5 yr	79	93.4	
10 yr	43.1	74.7	
15 yr	21.5	74.7	

Data were expressed as median ± IQR or  $n$  (%). HCC: Hepatocellular carcinoma; IVC: Inferior vena cava; LC: Liver cirrhosis; MELD: Model for End-Stage Liver Disease; HVPg: Hepatic venous pressure gradient; ALT: Alanine aminotransferase; PT: Prothrombin time; IQR: Interquartile range.

**Prognosis and survival of patients with BCS and patients who were diagnosed with HCC**

We estimated the overall survival rates of all 67 BCS patients using the Kaplan-Mayer method. The overall survival rate was 86.2% at 5 years, 73.8% at 10 years, and 61.2% at 15 years (Figure 2). During the follow-up periods, three patients among 17 who were diagnosed with HCC died, and two of them died from hepatic failure and the other from massive variceal bleeding. In HCC group, the 5-year survival rate was 79%, 10-year rate 43.1%, and 15-year rate 21.5% (Figure 3). Meanwhile, non-HCC group ( $n = 50$ ) showed significantly higher survival rates than HCC group: 93.4% for 5 years and 74.7% for 15 years ( $P < 0.001$ ).

**DISCUSSION**

BCS is caused by obstruction of hepatic venous outflow at any level from the small hepatic veins to the junction of the IVC with the right atrium. There are two forms of BCS according to the obstruction site: primary hepatic vein obstruction (classical BCS) and obstruction of the

Table 4 Prevalence and characteristics of hepatocellular carcinoma in patients with Budd-Chiari syndrome from the literature

Ref.	Matsui <i>et al.</i> <sup>[3]</sup>	Shin <i>et al.</i> <sup>[24]</sup>	Moucarri <i>et al.</i> <sup>[7]</sup>	Gwon <i>et al.</i> <sup>[6]</sup>
Year, country	2000, Japan	2004, South Korea	2008, France	2010, South Korea
Study design	Retrospective	Retrospective	Retrospective	Retrospective
Study period	Apr 1968-Feb 1999	Mar 1989-Aug 2001	1987-2005	Mar 1990-Nov 2008
No. of patient with BCS	12	73	97	98
HCC (%)	3 (25)	15 (20.5)	11 (11.3)	23 (23)
Cumulative incidence of HCC	-	-	4 yr 3% 7 yr 6% 14 yr 12%	1 yr 7.3% 5 yr 13.5% 10 yr 31.8%
Tx. for HCC	Resection (1) TAE (1) iA chemotherapy (1)	TACE (11) Resection (2) Conservative tx. (2)	TACE (7) LT (3) Conservative tx. (1)	TACE (20) TACE + LT (3)
Survival rate of HCC in patients with BCS				
Median survival period (mo)	-	58 (range, 3-59)	-	-
Cumulative survival	-	1 yr 93% 2 yr 84% 3 yr 72%	-	1 yr 90% 2 yr 85% 3 yr 61% 4 yr 61% 5 yr 46%
Risk factors for HCC in patients with BCS				
Risk factors	Chronic congestion in the liver, caused by an outflow block of hepatic veins	-	Male gender, Coagulopathy <sup>1</sup> , IVC obstruction	Female gender <sup>2</sup>
Analysis method	No statistical analysis (mere presumption)	-	Univariate analysis	Multivariate analysis

HCC: Hepatocellular carcinoma; BCS: Budd-Chiari syndrome; Tx.: Treatment; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; iA: Intra-arterial; LT: Liver transplantation. <sup>1</sup>Coagulopathy harbored factor V Leiden; <sup>2</sup>Female gender showed an odds ratio of 6.02 with  $P < 0.001$  in Cox regression analysis; IVC: Inferior vena cava.

hepatic portion of the inferior vena cava (IVCO). The IVCO form is common in Asia and Africa but rarely reported in Western countries<sup>[9,15]</sup>. Most of our patients (83.6%) had the IVCO form, which is similar to previous studies which reported the predominance of IVCO form in Asia. The major difference between classical BCS and IVCO is that the former is rarely associated with HCC, while the latter is frequently complicated by HCC<sup>[3,8]</sup>. In our study, HCC was developed in 17 of 67 patients, and the annual incidence was 2.8%, similar to the incidence in patients with other etiologic cirrhosis in South Korea<sup>[16,17]</sup>.

Until now, the accurate pathogenesis of HCC in BCS has not been elucidated yet. Gwon *et al.*<sup>[6]</sup> suggested that chronic liver injuries and congestion caused by obstruction of hepatic venous outflow might contribute to a fibrotic process and development of nodular type of HCC. Prolonged congestion can lead to hepatocyte necrosis, and its replacement with fibrous tissue results in fibrosis, which is assumed to be the mechanism of cirrhosis and HCC development<sup>[18-20]</sup>. This hypothesis is supported by frequent findings of liver parenchymal cirrhotic change adjacent to HCC in BCS context<sup>[7]</sup>.

The HVPG was significantly higher in HCC group than non-HCC group in our study. HVPG has been accepted as the gold standard for assessing the severity of portal hypertension<sup>[21]</sup>. With the pathogenesis proposed above, the higher pressure gradient means a greater degree of portal hypertension and hepatic congestion; this might have contributed to a higher pressure gradient in HCC group in our study. Our study that showed significantly

high HVPG in HCC group is worthy, which supports the hypothesis of development of HCC in patients with BCS. Until now, there were several published reports to analyze the risk factors for HCC in patients with BCS. Although Moucarri *et al.*<sup>[7]</sup> showed that BCS patients with HCC compared with those without HCC presented with IVC obstruction more frequently, there was no report that showed direct differences of pressure gradient as our data presented. For comparison, other published data concerning the prevalence and characteristics of HCC in patients with BCS were reviewed in Table 4.

Varying survival results of BCS have been reported, and the 5-year survival rate ranges from 69% to 87%<sup>[22,23]</sup>. In our data, the overall survival rate was 86.2% for 5 years, 73.8% for 10 years, and 61.2% for 15 years. In patients with BCS and HCC, the survival rate was 79% for 5 years, 43.1% for 10 years, and 21.5% for 15 years in our study. This is comparable with the results of other published reports<sup>[6,24]</sup>. Improvement in availability and techniques of diagnostic tools and development of treatment modalities may allow earlier diagnosis of BCS patients and better prognosis<sup>[18-20]</sup>.

Although our study has an advantage of long-term follow up data of patients with BCS, there are still some limitations. First, this was retrospective and therefore has the limitations of such an investigational design. Another limitation was that the values of HPVG obtained during venography were not checked in all patients. Because of invasiveness of venography, it could not be performed on all patients for HVPG measurement. These days,

Doppler US is used to non-invasively assess the HPVG with portal vein velocity. Considering the low incidence of BCS, a multicenter study should be performed to overcome the limitations of patient number and insufficient information. Despite these limitations, this study is worthy to analyze the incidence and prognosis of HCC in BCS with long-term follow-up periods in South Korea.

In conclusion, the annual incidence of HCC in patients with BCS was similar to the incidence in patients with other etiologic cirrhosis in South Korea. Furthermore, the HVPVG can be a possible predictive factor of BCS-associated HCC development. Thus, BCS patients who are expected to have high pressure gradient should be actively managed as a high risk group for HCC development. An intervention to decrease the pressure gradient in BCS patients may be helpful to reduce the incidence of HCC. A large-scale study will be necessary to further investigate whether the treatment of congestion decreases the incidence of HCC.

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## COMMENTS

### Background

Budd-Chiari syndrome (BCS) is a rare disease caused by obstruction of the hepatic venous outflow. BCS induces chronic liver congestion so that it causes hepatomegaly, ascites, leg edema, collateral venous dilatation in the body trunk, and portal hypertension. Several studies have suggested that hepatic congestion caused by obstruction of hepatic venous outflow can lead to cirrhosis and hepatocellular carcinoma (HCC). However, the incidence of HCC in patients with BCS has varied according to regions and investigators and there has been a lack of reports for long-term prognosis of HCC in patients with BCS.

### Research frontiers

Although BCS is a relatively rare disease as contrasted with other viral liver disease that can lead to advanced liver disease such as liver cirrhosis or HCC, several studies have reported the association between BCS and HCC. Long-term follow-up data of BCS may help to understand not only the prognosis of BCS but also the process of HCC in patients with BCS.

### Innovations and breakthroughs

This study showed that the hepatic venous pressure gradient (HVPVG) was significantly higher in HCC group than non-HCC group. Although the accurate pathogenesis of HCC in BCS has not been elucidated, there were several suggestions that chronic liver injuries and congestion caused by obstruction of hepatic venous outflow might contribute to a fibrotic process and development of HCC. The study is worthy because it supports this hypothesis about the development of HCC in patients with BCS.

### Applications

From the study, it was suggested that the HVPVG can be a possible predictive factor of BCS-associated HCC development. Thus, BCS patients who are expected to have a high pressure gradient should be actively managed as a high risk group for HCC development. An intervention to decrease the pressure gradient in BCS patients may be helpful to reduce the incidence of HCC.

### Peer review

This study provides basic and essential data for the clinical care of the patients with BCS.

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## Transparent-cap-fitted colonoscopy shows higher performance with cecal intubation time in difficult cases

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**CONCLUSION:** CFC facilitated shortening of the cecal intubation time in difficult cases, and was more sensitive for detecting adenomas than was NCF.

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**Key words:** Colonoscopy; Cap-fitted colonoscopy; Cecal intubation

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Kim HH, Park SJ, Park MI, Moon W, Kim SE. Transparent-cap-fitted colonoscopy shows higher performance with cecal intubation time in difficult cases. *World J Gastroenterol* 2012; 18(16): 1953-1958 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1953.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1953>

### Abstract

**AIM:** To investigate the efficacy of cap-fitted colonoscopy (CFC) with regard to cecal intubation time.

**METHODS:** Two hundred and ninety-five patients undergoing screening colonoscopy at Gospel Hospital, Kosin University College of Medicine were enrolled in this randomized controlled trial between January and December 2010. Colonoscopies were conducted by a single endoscopist. Patient characteristics including age, sex, body mass index, history of abdominal surgery, quality of preparation, and the presence of diverticulosis were recorded.

**RESULTS:** One hundred and fifty patients were allocated into a CFC group and 145 into a non-CFC (NCF) group. Cecal intubations were achieved in all patients. Cecal intubation time in the CFC group was significantly shorter than in the NCF group for specific conditions: age  $\geq$  60 years, prior abdominal surgery, and poor bowel preparation. The number of detected adenomas was higher in the CFC group than in the NCF group ( $P = 0.040$ ).

### INTRODUCTION

Colorectal cancer is a major cause of cancer-related mortality and morbidity, and it is evident that this fatality rate has led to an increase in colonoscopy preventative treatment<sup>[1-3]</sup>. A multinational and multicenter survey performed in Asia has shown that the overall prevalence of advanced colorectal neoplasm in asymptomatic individuals is comparable with that in the West<sup>[4]</sup>. Removal of colonic adenomas by screening colonoscopy could reduce colorectal cancer incidence and mortality rate.

Colonoscopy, however, can be a complicated procedure and requires a skillful endoscopist<sup>[5-7]</sup>. The anatomical factors of difficult cecal intubation can usually be categorized into one of two problems: (1) an angulated and/or narrowed sigmoid colon; and (2) a redundant colon<sup>[8]</sup>. These anatomical difficulties are commonly observed in specific cases, such as female patients, older age, previous gynecologic surgery, and the presence of diver-

ticulosis<sup>[9-17]</sup>. Published studies have suggested the use of a narrower instrument shaft or one with both a narrower shaft and a shorter bending section for use in angulated or narrowed sigmoid colons, and a stiffened shaft with simultaneous application of abdominal pressure for overcoming the problems associated with redundant colons<sup>[8,18,19]</sup>. However, these maneuvers are not always successful and may require the endoscopist to change instruments during the procedure.

Several studies have evaluated the efficacy of transparent cap-fitted colonoscopy (CFC) compared to that of non CFC (NCF), and have found no difference in cecal intubation time between CFC and NCF<sup>[20-22]</sup>. The one established advantage of CFC is that it is more sensitive to polyp detection than is NCF<sup>[20,21]</sup>. However, these results are not consistent with our daily experience, in that CFC requires a shorter time for cecal intubation than does NCF.

Short cecal intubation time is important for several reasons: less anesthetic medication is required; colonic inflation results in less discomfort; and sufficient withdrawal time for accurate examination. The purpose of this study was to evaluate whether CFC could result in shorter cecal intubation time compared with NCF. Additionally, we compared the detection rate of colonic adenomas in this study.

## MATERIALS AND METHODS

### Patients

From January to December 2010, 300 consecutive patients scheduled for their first ever colonoscopy as a routine health check at Gospel Hospital, Kosin University College of Medicine were included in the study. Exclusion criteria were as follows: age < 18 years; hospitalization due to other diseases undergoing colonoscopy investigation; evidence of acute or chronic renal failure; cardiovascular diseases including recent myocardial infarction, congestive heart failure, unstable angina, and cardiac arrhythmias; ascites; electrolyte imbalance; active inflammatory bowel disease, ileus and/or suspected bowel obstruction; pregnant or breast feeding; or child-bearing potential without adequate contraception. Patient medical history, demographic data, and body weight were recorded. For all patients, clinical hemodynamic, hematological, and biochemical measurements, including whole blood count, blood sugar, blood urea nitrogen, creatinine, and serum electrolyte (sodium, potassium, chloride, phosphorus, ionized calcium and magnesium) levels were measured. After initial evaluations, patients who had no exclusion criteria were randomized to receive CFC or NCF by one physician who was blinded to the results of previous colonoscopies. This study was approved by the Institutional Review Board of Kosin University College of Medicine, Busan, South Korea.

### CFC and NCF

After providing informed consent, patients in both groups

were encouraged to adhere to a clear liquid diet from 06:00 h to midnight on the day before colonoscopy, and further oral intake was not allowed after midnight. All patients drank 4 L of polyethylene glycol electrolyte lavage solution, starting 7 h before colonoscopy at a rate of 250 mL every 15 min until all of the solution had been consumed, as recommended by the manufacturer (Olympus Optical Corp, Tokyo, Japan). Before colonoscopy, a physical examination and clinical hemodynamic measurements were repeated. The transparent plastic cap (D-14304; Olympus Optical Corp., Tokyo, Japan) used for CFC was 14 mm in outer diameter, 10 mm in length, and had a 1 mm wall thickness. This cap can be fitted and fixed to the tip of the colonoscope (CIF H260; Olympus Optical Corp.). To ensure consistency in the evaluations, all colonoscopies were performed by the same attending endoscopist using the standard technique of negotiating the colon with as little air insufflation as possible. The principal examination was carried out during withdrawal. Ileal intubation was attempted when it was relevant.

### Variables

For evaluating the efficacy of CFC against NCF, the duration time of insertion up to the cecum was compared between the CFC and NCF groups. Additionally, the number of adenomas detected during colonoscopy was calculated. Factors presumed to influence cecal intubation time were sex, age, body mass index, and history of abdominal surgery; all of which were evaluated before colonoscopy. The quality of preparation was classified as follows: grade 0, percentage of visible mucosa > 90%, excellent visibility (small volume of clear liquid requiring minimal suctioning for adequate visualization), and no intestinal bubbles; grade 1, percentage of visible mucosa > 90%, good visibility (large volume of clear liquid or small amount of fecal residue, not preventing a reliable examination), and small number of intestinal bubbles; grade 2, percentage of visible mucosa > 90%, fair visibility (some semi-solid stool that could be suctioned or washed away, preventing a reliable examination), and moderate number of intestinal bubbles; and grade 3, percentage of visible mucosa < 90%, poor visibility (large amount of semi-solid stool that could not be suctioned or washed away, not allowing a complete examination to be done), and large number of intestinal bubbles. The number of adenomas was calculated during colonoscopy. Discomfort of each patient was recorded using a four-point scale (1: easy; 2: tolerable; 3: some pain; 4: severe pain).

### Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL, United States). For normally distributed continuous variables, Student's *t* test was used to assess differences between the two groups. For categorical variables, Fisher's exact test was used. Cox multivariate regression analysis was performed to produce statistically significant variables in the present study. Two-sided *P* values < 0.05 were considered statistically significant.

Table 1 Baseline characteristics *n* (%)

	CFC ( <i>n</i> = 150)	NCF ( <i>n</i> = 145)	<i>P</i> value
Gender			
Male	94 (62.7)	87 (62.6)	1.000
Female	56 (37.3)	58 (37.4)	
Age (yr) mean ± SD	65.4 ± 15.3	66.1 ± 14.8	0.736
Body mass index (kg/m <sup>2</sup> ), mean ± SD	26.2 ± 10.6	27.4 ± 9.6	0.489
History of abdominal surgery	43 (28.6)	32 (22.1)	0.181
Cesarean section	20 (13.3)	15 (10.3)	0.474
Appendectomy	16 (10.6)	10 (6.9)	0.306
Distal gastrectomy due to peptic ulcer	7 (4.7)	7 (4.8)	1.000
Diverticulosis	51 (34.0)	42 (30.0)	0.382
Preparation score 2 or 3	30 (20.0)	31 (21.3)	0.776

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

Table 2 Comparison of two groups with regard to cecal intubation time and number of detected colonic adenomas (mean ± SD)

	CFC ( <i>n</i> = 150)	NCF ( <i>n</i> = 145)	<i>P</i> value
Cecal intubation time (s)	262 ± 154	281 ± 138	0.057
Number of detected adenomas	2.0 ± 2.5	1.2 ± 1.6	0.040
Size of adenoma (cm)	2.0 ± 3.1	2.6 ± 2.9	0.061
Sessile type, <i>n</i>	1.8 ± 1.9	1.0 ± 0.9	0.039
Pedunculated type, <i>n</i>	0.4 ± 0.8	0.3 ± 0.7	0.557
Patient discomfort, scores	2.3 ± 1.0	2.3 ± 0.8	0.741

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

## RESULTS

Initially, 150 patients volunteered for each group. However, five patients in the conventional endoscopy group withdrew their consent after finishing all examinations; therefore, 150 patients for cap-assisted colonoscopy and 145 patients for conventional colonoscopy were enrolled. There were no significant differences in the backgrounds between the group with CFC and that with NCF (Table 1).

Cecal intubation was achieved in all cases regardless of method. The average time for insertion from anus to cecum was shorter in the CFC group than in the NCF group, but the difference was not statistically significant (262 ± 154 s *vs* 281 ± 138 s, *P* = 0.057). CFC showed greater adenoma detection than did NCF (2.0 ± 2.5 *vs* 1.2 ± 1.6; *P* = 0.040), especially for sessile adenomas (1.8 ± 1.9 *vs* 1.0 ± 0.9; *P* = 0.039). There was no significant difference between the two groups regarding patient discomfort scores during colonoscopy (2.3 ± 1.0 *vs* 2.3 ± 0.8, *P* = 0.741). These results are shown in Table 2.

Multivariate analyses revealed that cecal intubation time was significantly longer in patients aged > 60 years, with a history of abdominal surgery, and two or three points in quality of bowel preparation as described in Table 3.

Multivariate analyses revealed that CFC required a significantly shorter cecal intubation time than did NCF in specific patients, including older patients (244 ± 123 s

Table 3 Cecal intubation time in all patients (mean ± SD)

	Cecal intubation time	<i>P</i> value
Gender		0.881
Male	254 ± 145	
Female	257 ± 135	
Age (yr)		0.012
< 60	244 ± 114	
≥ 60	322 ± 113	
Body mass index (kg/m <sup>2</sup> )		0.047
< 23	243 ± 114	
≥ 23	277 ± 125	
History of abdominal surgery		0.044
Yes	387 ± 173	
No	221 ± 117	
Diverticulosis		0.747
Yes	251 ± 146	
No	256 ± 142	
Quality of preparation		0.861
0 or 1	251 ± 147	
2 or 3	302 ± 176	0.006

Table 4 Multivariate analysis for influencing factors on cecal intubation time(s) between cap-fitted colonoscopy and non-cap-fitted colonoscopy (mean ± SD)

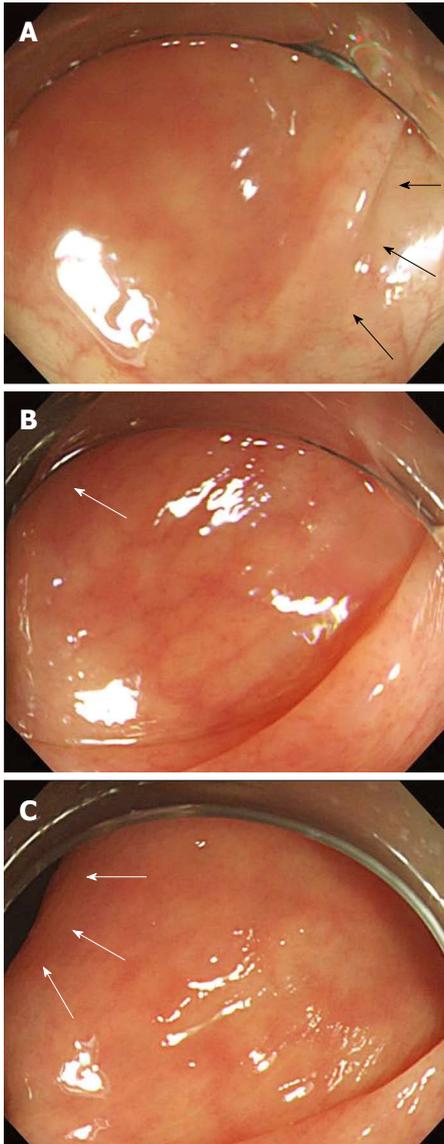
	CFC	NCF	<i>P</i> value
Gender			
Male	234 ± 109	257 ± 137	0.053
Female	276 ± 173	257 ± 134	0.997
Age (yr)			
< 60	249 ± 130	233 ± 106	0.784
≥ 60	244 ± 123	330 ± 213	0.009
Body mass index (kg/m <sup>2</sup> )			
< 23	246 ± 125	240 ± 118	0.067
≥ 23	296 ± 170	248 ± 148	0.674
History of abdominal surgery	240 ± 106	351 ± 219	0.012
Diverticulosis	218 ± 66	279 ± 189	0.169
Quality of preparation			
0 or 1	255 ± 133	251 ± 147	0.861
2 or 3	224 ± 96	302 ± 176	0.006

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

*vs* 330 ± 213 s; *P* = 0.009), those with history of abdominal surgery (240 ± 106 s *vs* 351 ± 219 s; *P* = 0.012), and those with bowel preparation score 2 or 3 (224 ± 96 s *vs* 302 ± 176 s; *P* = 0.006), as shown in Table 4.

## DISCUSSION

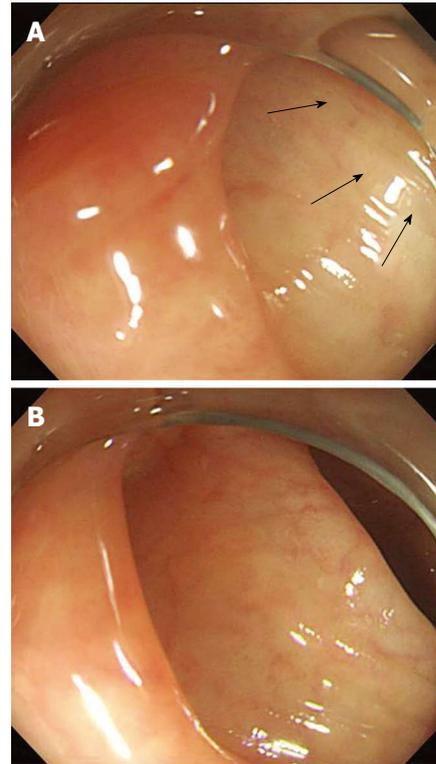
Colonoscopy is a common endoscopic procedure. It is widely used for the investigation of lower gastrointestinal tract disorders and screening for colorectal adenomas<sup>[23]</sup>. However, failure to reach the cecum occurs in up to 10% of cases<sup>[10,24]</sup>. A transparent cap was initially designed for mucosectomy and was later used during colonoscopy to enhance colonic polyp detection<sup>[22]</sup>. CFC is an effective rescue method for patients who fail to achieve cecal intubation<sup>[25]</sup>. This benefit is more apparent for inexperienced colonoscopists<sup>[26]</sup>. Moreover, it has been shown that such a device can shorten cecal intubation time among experienced colonoscopists<sup>[27]</sup>. However, the present study



**Figure 1 Advantage of cap-fitted colonoscopy for preventing red-out.** A: Although the precise direction could not be judged, colonoscopy showed a slight fold (white arrows) without red-out; B: A subtle movement showed a dark area at the 11 o'clock position (white arrow); C: Following the dark area at the 11 o'clock position enabled the colonoscopist to find the direction of insertion (black arrows).

showed that there was no significant difference between CFC and NCF in cecal intubation time, although the average cecal intubation time of CFC was shorter than that for NCF.

Although there was no significant difference in cecal intubation time between the two groups, CFC showed a shorter time than did NCF in several specific situations: age  $\geq 60$  years, history of abdominal surgery, and poor bowel preparation. In previous studies, predictive factors for incomplete colonoscopy were female sex, older age, previous gynecologic surgery, and the presence of diverticulosis<sup>[9-12]</sup>. In addition, female sex and older age are well known factors responsible for longer cecal intubation times<sup>[13-17]</sup>. Consistent with these previous results, CFC in the current study displayed a shorter cecal intubation

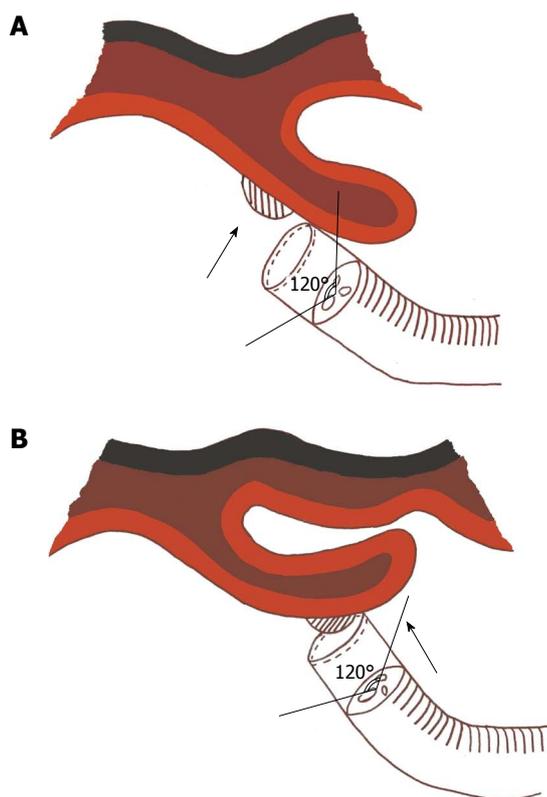


**Figure 2 Advantage of cap-fitted colonoscopy for observing lateral side.** A: The colonoscopist easily noticed the route of insertion because the transparent cap showed the small dark lumen at the 1 o'clock position through its lateral wall (black arrows); B: Following the route shown through the lateral wall of the transparent cap, a wide lumen was found easily.

time in difficult cases, such as in older patients, and those with a history of abdominal surgery, and poor bowel preparation. In our study, a single experienced endoscopist performed all procedures; this might be the reason why there was no difference between the two groups in cecal intubation time.

A possible explanation for this difference is less air insufflation during CFC than with NCF. A recent study revealed that the limited use of low-air insufflation in the rectum and sigmoid colon shortened the cecal intubation time and decreased post-procedural abdominal bloating<sup>[28]</sup>. Low-air insufflation causes less bowel inflation and produces less angulations of the bowel, thus enhancing cecal intubation. The use of CFC requires extremely low air insufflations. Experts in CFC can advance a cap-fitted colonoscope by pushing and pulling using meticulous lever manipulation without air insufflation, especially in the rectum and sigmoid colon. Extremely low air insufflation can be achieved in CFC because the cap prevents the mucosa from touching the lens directly and enables continuous lumen observation (Figure 1)<sup>[21]</sup>. Another important CFC characteristic is that the lateral side can be observed through the transparent wall of the cap (Figure 2)<sup>[21]</sup>. In the hepatic and splenic flexures, observing the lateral side through the transparent wall of the cap can help endoscopists determine the next step of the colonoscopy.

Another advantage of CFC in cecal intubation was that more adenomas were observed in the CFC group



**Figure 3** The opposite, blind side of the folds could be observed with fewer problems during colonoscopy. A: Compressing the tip of a fold straightened the entire fold and improved the view; B: Bending the tip of the endoscope allowed a front view of the lesion at the blind side of the fold.

during withdrawal compared to those in the NCF group. Cap usage greatly facilitates the identification of small adenomas. During insertion and withdrawal of the cap-fitted colonoscope, the lumen of the colon can always be seen clearly because the mucosa never directly touches the lens<sup>[21]</sup>. The opposite, blind side of the folds can easily be observed and treated with fewer problems in CFC because they can be straightened to improve the view (Figure 3)<sup>[20]</sup>, and the lateral side can be observed through the transparent wall of the cap<sup>[21]</sup>. Fecal matter may stick to the inside of the cap in cases of poor bowel preparation, thereby impairing the view. Using the water insufflation button or simple flushing through the biopsy channel can lead to ineffective cap clearing. The cap, however, was easily cleaned in CFC by pressing the whole circumference of the cap against the mucosal surface and then flushing the biopsy channel<sup>[20]</sup>. Moreover, CFC enhanced cecal intubation compared to that of NCF in cases of poor bowel preparation (scores of 2 and 3).

In conclusion, CFC has advantages in overcoming the problems associated with angulated and/or narrowed sigmoid and redundant colon, thereby resulting in significantly higher performance in cecal intubation time in difficult cases such as old age, prior abdominal operation, and poor bowel preparation. Furthermore, CFC displayed a higher sensitivity in detecting colonic adenomas than did NCF.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Colorectal cancer is a major cause of cancer-related mortality and morbidity, and it is evident that this fatality rate has led to an increase in colonoscopy preventative treatment. Colonoscopy, however, can be a complicated procedure and requires a skillful endoscopist. The anatomical factors of difficult cecal intubation can usually be categorized into one of two problems: (1) an angulated and/or narrowed sigmoid colon; and (2) a redundant colon. These anatomical difficulties are commonly observed in specific cases, such as female patients, older age, previous gynecological surgery, and the presence of diverticulosis. Published studies have suggested the use of a narrower instrument shaft or one with both a narrower shaft and a shorter bending section for use in angulated or narrowed sigmoid colons, and a stiffened shaft with simultaneous application of abdominal pressure for overcoming the problems associated with redundant colons.

### Research frontiers

Several studies have evaluated the efficacy of a transparent cap-fitted colonoscopy (CFC) compared to that of non-CFC (NCF) and found that there was no difference in cecal intubation time between CFC and NCF. The one established advantage of CFC is that it is more sensitive to polyp detection than is NCF.

### Innovations and breakthroughs

The transparent plastic cap is made by Olympus Optical Corp., Tokyo, Japan. It is 17 mm in outer diameter, with a 2 mm wall thickness and 10 mm in length, and can be fitted and fixed to the tip of the colonoscope. This can cause less air insufflation during CFC than with NCF. Low-air insufflation causes less bowel inflation and produces less angulations of the bowel, thus enhancing cecal intubation. The present study aimed to evaluate whether CFC could result in shorter cecal intubation time compared with NCF. Additionally, the study compared the detection rate of colonic adenomas.

### Applications

CFC has advantages in overcoming the problems associated with angulated and/or narrowed sigmoid and redundant colon, thereby resulting in significantly higher performance in cecal intubation time in difficult cases, such as elderly patients, and those with prior abdominal operation, and poor bowel preparation. Furthermore, CFC displayed a higher sensitivity in detecting colonic adenomas than did NCF.

### Terminology

Transparent CFC: a colonoscopic procedure with a transparent cap at the front view of the colonoscope.

### Peer review

The present study showed that CFC had shorter cecal intubation time in difficult cases. This is an interesting and good study.

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## Laparoscopic distal pancreatectomy is as safe and feasible as open procedure: A meta-analysis

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### Abstract

**AIM:** To evaluate the feasibility and safety of laparoscopic distal pancreatectomy (LDP) compared with open distal pancreatectomy (ODP).

**METHODS:** Meta-analysis was performed using the databases, including PubMed, the Cochrane Central Register of Controlled Trials, Web of Science and BIOSIS Previews. Articles should contain quantitative data of the comparison of LDP and ODP. Each article was reviewed by two authors. Indices of operative time, spleen-preserving rate, time to fluid intake, ratio of malignant tumors, postoperative hospital stay, incidence rate of pancreatic fistula and overall morbidity rate were analyzed.

**RESULTS:** Nine articles with 1341 patients who underwent pancreatectomy met the inclusion criteria. LDP was performed in 501 (37.4%) patients, while ODP was performed in 840 (62.6%) patients. There were significant differences in the operative time, time to fluid in-

take, postoperative hospital stay and spleen-preserving rate between LDP and ODP. There was no difference between the two groups in pancreatic fistula rate [random effects model, risk ratio (RR) 0.996 (0.663, 1.494),  $P = 0.983$ ,  $I^2 = 28.4\%$ ] and overall morbidity rate [random effects model, RR 0.81 (0.596, 1.101),  $P = 0.178$ ,  $I^2 = 55.6\%$ ].

**CONCLUSION:** LDP has the advantages of shorter hospital stay and operative time, more rapid recovery and higher spleen-preserving rate as compared with ODP.

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**Key words:** Laparoscopy; Distal pancreatectomy; Pancreatic fistula; Spleen-preserving; Morbidity

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Xie K, Zhu YP, Xu XW, Chen K, Yan JF, Mou YP. Laparoscopic distal pancreatectomy is as safe and feasible as open procedure: A meta-analysis. *World J Gastroenterol* 2012; 18(16): 1959-1967 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1959.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1959>

### INTRODUCTION

With improvement of advanced surgical techniques and endoscopic instrument, laparoscopic distal pancreatectomy (LDP) is becoming a primary modality for the treatment of benign or borderline tumors of distal pancreas<sup>[1-3]</sup>.

Recently, several researches have shown the advantages of LDP of shorter hospital stay and operative time and less intraoperative blood loss<sup>[4-5]</sup>. But the efficacy of LDP compared with open distal pancreatectomy (ODP) required further assessment, especially the incidence of pancreatic fistula (PF) which may lead to further com-

plications such as an intra-abdominal abscess, sepsis or lethal bleeding<sup>[6]</sup>. With a better understanding of the anatomy and immune function of spleen, especially the increased risks of overwhelming post splenectomy infection (OPSI) and long-term lung thrombosis<sup>[7]</sup>, laparoscopic spleen preserving distal pancreatectomy (LSPDP) was performed first by Kimura *et al*<sup>[8]</sup> and Warshaw<sup>[9]</sup>. However, the role of “laparoscopy” in the spleen preservation is still unclear.

All the published studies we retrieved were based on a small number of patients and no randomized trials were available. Therefore, we strictly established the inclusion criteria and conducted a comprehensive meta-analysis to evaluate more systematically the feasibility and safety of LDP.

## MATERIALS AND METHODS

### Search strategy

We searched databases of PubMed, The Cochrane Central Register of Controlled Trials, Web of Science and BIOSIS Previews for the literatures comparing LDP and ODP published between January 1995 and June 2011. The language of the publications was confined to English. Two investigators reviewed the titles and abstracts, and assessed the full text to establish the eligibility. The search strategies were as follows (Table 1).

### Inclusion criteria

All clinical studies should meet the following criteria for the meta-analysis: (1) published in English with data comparing ODP and LDP; (2) with clear case selection criteria, containing at least the following information: the number of cases, surgical methods and perioperative data; (3) continuous variables (e.g., operative time and hospital stay) expressed in mean  $\pm$  SD. Dichotomous variables (e.g., incidence of PF and number of death) such as odds ratio (OR) and 95% confidence interval (CI); and (4) if there was overlap between authors, centers, or patient cohorts, the higher quality or recent literatures were selected.

### Exclusion criteria

The papers containing any of the followings were excluded: (1) intra-operative conversion of LDP to an open laparotomy, which was classified into the laparoscopic group; (2) single surgical procedure; and (3) laparoscopy-assisted DP or hand-assisted LDP.

### Data extraction and quality assessment

Two authors independently extracted the data using a unified datasheet, and decided the controversial issues through discussion. Extracted data included: first author, study period, the number of cases, operative time, spleen preservation, hospital stay, cases of malignant tumors, incidence of post-operative complications, and PF. Selected documents were rated according to the Grading of the Centre of Evidence-Based Medicine (Oxford, United Kingdom; www.cebm.net).

Table 1 Database and search strategy

Database	Search strategy
PubMed	"laparoscopy" (MeSH terms) or "laparoscopy" (all fields) or "laparoscopic" (all fields) or (minimally (all fields) and invasive (all fields) and ("pancreas" (MeSH terms) or "pancreas" (all fields) or "pancreatic" (all fields) and "humans" (MeSH terms) and English (lang) and "1995/1/1" (PDAT): "2011/06/30" (PDAT)
Web of Science	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011)
Cochrane Library	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011)
BIOSIS Previews	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011) (related term and limited English and human and year: 1995-2011)

PDAT: Publication date; MeSH: Medical subject headings.

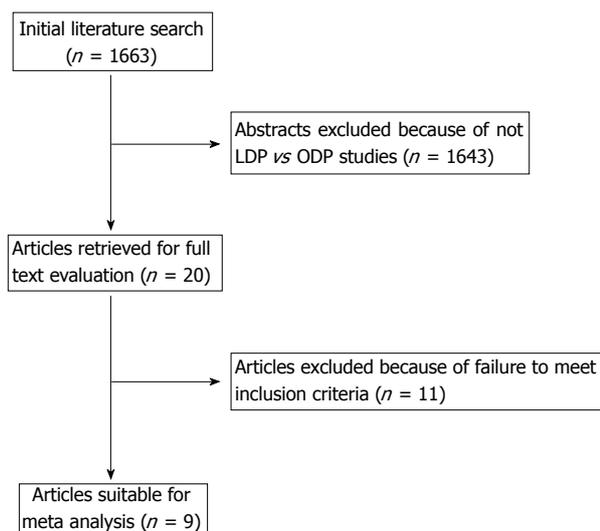
### Statistical analysis

This meta-analysis was performed according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) and the Quality of Reporting of Meta-analyses (QUORUM) as a guideline<sup>[10,11]</sup>. Weighted mean differences (WMD) were used for continuous variables, and relative risk for dichotomous variables. *P* values < 0.05 indicated statistically significant difference between the two groups. When heterogeneity test showed no significant differences (*P* > 0.05), we used fixed effects model to calculate the summary statistics. When the heterogeneity test showed statistically significant differences (*P* < 0.05), we used random effects model based on DerSimonian and Laird method. If the heterogeneity was high or extracted data were less than three sets, we performed descriptive analysis. The potential publication bias was determined by the Begg's test and funnel plots based on the dichotomous variables. All data were analyzed using Stata SE11.0 software.

## RESULTS

We retrieved 1663 papers in English. After the titles and abstracts were reviewed, papers without comparison of LDP and ODP were excluded. As a result, a total of 20 studies<sup>[12-31]</sup> were collected, of which 11 studies were excluded because of intraoperative conversion and using “assisted” approach. However, we preserved them for the analysis as “conversion to open”. Finally, 9 studies<sup>[23-31]</sup> were included and extracted for detailed data. A flow chart of search strategies is illustrated in Figure 1.

Totally, 1341 patients (sample sizes ranging from 44 to 310) entered into this meta-analysis, including 501 (37.4%) cases of LDP and 840 (62.6%) cases of ODP. The detailed study design and surgical techniques in 10 trials are summarized in Table 2.



**Figure 1** A flow chart of search strategies. The initial search strategy retrieved 1663 papers in English. Finally 9 studies were included and extracted for detailed data. LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

### Intraoperative effects

The operative time was reported in four articles<sup>[23,24,26,28]</sup>. Meta-analysis of the pooled data showed that the operative time of ODP was significantly shorter than LDP [random effects model, WMD 44.947 (13.857, 76.037),  $P = 0.005$ ] (Figure 2A).

In the included articles, five studies<sup>[23,25,27,29,30]</sup> covered the spleen-preserving DP, 95 cases (29.6%) of LSPDP were conducted among 321 cases of LDP as compared with 76 cases (13.3%) of SPDP among 571 cases of ODP. The pooled data showed that the spleen-preserving rate in LDP was significantly higher than in ODP [random effects model, RR 2.380 (1.177, 4.812),  $P = 0.016$ ,  $I^2 = 73.2\%$ ], (Figure 3A). Although there was moderate heterogeneity, spleen-preservation occurred more often in LDP. Most authors tended to use the “Kimura method”, and “Warshaw method” was used in a few LDPs and in cases with severe adhesion or vessels involved in tumors. Technical details of spleen-preservation are listed in Table 3.

Among 714 cases of LDP, 100 cases (14.0%) converted to open surgery and 6 cases converted to the hand-assisted approach as shown in 20 articles<sup>[12-31]</sup> because of severe bleeding, abdominal adhesions, large tumor, organ injury, and difficult anatomy.

The techniques of pancreatic stump closure in the included studies are summarized in Table 4. Because the pooled data was derived from 9 institutions, no single technique was used for both procedures, but similar principles were applied. In LDP, the gland was divided by staplers and in ODP, stapler or scalpel + suture was used. In some cases, bio-sealant was attached to the stump reported by Baker, DiNorcia, Kim and Aly<sup>[23,24,27,30]</sup>.

### Postoperative outcome

Three studies<sup>[23,26,30]</sup> contained information about time to post-operative fluid intake. Meta-analysis of the pooled

data showed that time to fluid intake was shorter in LDP than in ODP [random effects model, WMD -0.948 (-1.863, 0.032),  $P = 0.042$ ] (Figure 2B). Another three studies<sup>[23,26,28]</sup> reported the postoperative hospital stay. The pooled data showed that postoperative hospital stay was significantly shorter in LDP than in ODP [random effects model, WMD -2.713 (-3.799, 1.628),  $P = 0.00$ ] (Figure 3B).

The proportion of malignant tumors reported by four articles<sup>[24,26,28,31]</sup> was 20% (36/180) in LDP and 20.1% (54/269) in ODP, and most of them were adenocarcinomas. The proportion of malignant tumors in LDP was not significantly different as compared with ODP [fixed effects model, RR 1.036 (0.708, 1.516),  $P = 0.000$ ,  $I^2 = 0\%$ ] (Figure 3C). There was no difference in patient selection between the two groups.

All studies illustrated the criteria for PF. Seven articles followed the criteria by International Study Group for Pancreatic Fistula (ISGPF): any measurable volume of fluid on or after postoperative day 3 with an amylase level > 3 times that of normal serum amylase level. Eom *et al.*<sup>[28]</sup> used the following PF criteria: drainage exceeding 30 mL with an amylase level > 600 U/dL on or after postoperative week 1. Kim *et al.*<sup>[30]</sup> chose the PF criteria: a level of drain amylase five times greater than the serum level and drainage of more than 30 mL 5 d or longer after the operation.

One study was excluded<sup>[31]</sup> due to no available data, 8 studies reported 50 (12.5%) PF cases in 401 LDP (12.5%) and 99 (13.4%) PF cases in ODP. The pooled data showed no significant difference between the two groups [random effects model, RR 0.996 (0.663, 1.494),  $P = 0.983$ ,  $I^2 = 28.4\%$ ] (Figure 2C) and no publication bias was found by Begg's test (Figure 4). Similarly, there was no significant difference in the overall morbidity between LDP and ODP [random effects model, RR 0.81 (0.596, 1.101),  $P = 0.178$ ,  $I^2 = 55.6\%$ ] (Figure 5), although there was moderate heterogeneity.

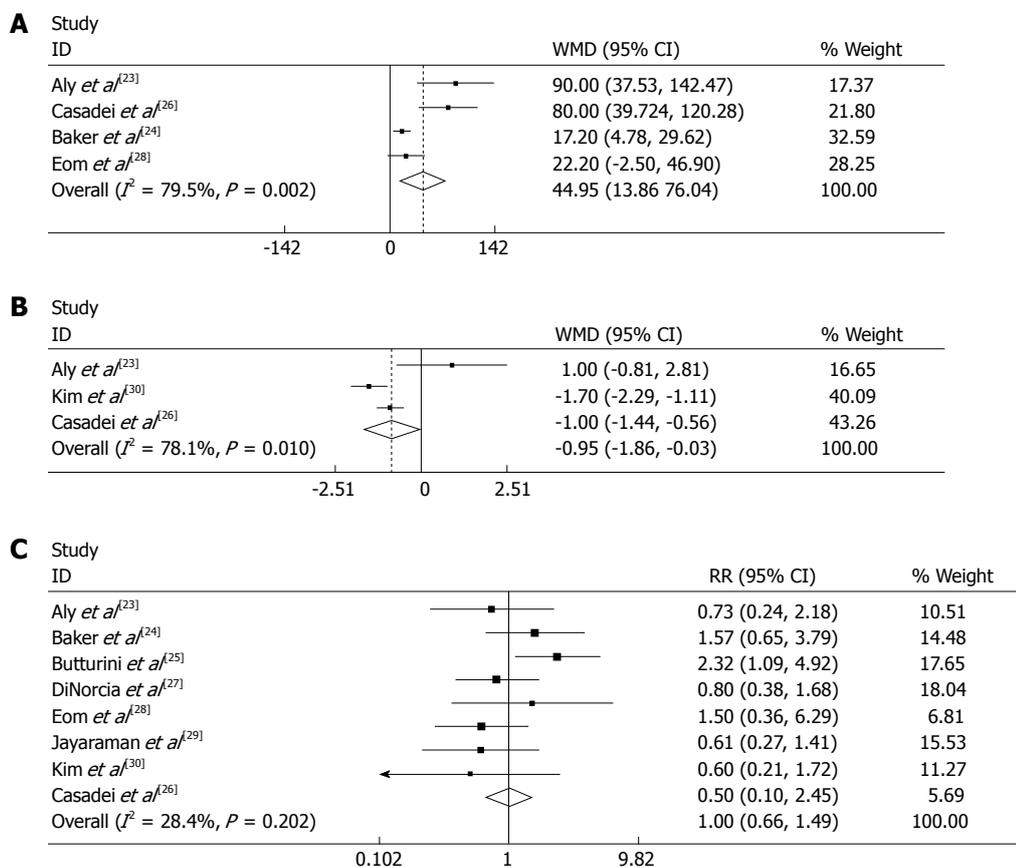
## DISCUSSION

Since Cuschieri and Gagner<sup>[32,33]</sup> documented the earliest attempts at LDP in humans, there have been an increasing number of reports indicating the advantages of LDP of minimal trauma, rapid recovery, and shorter hospital stay. But due to the high postoperative morbidity and a high level of laparoscopic technical requirements and extensive experiences in open pancreatic surgery, the progression of LDP was considerably restricted. In particular, the randomized clinical trials (RCT), which are the ideal objects of meta-analysis, have been extremely difficult to achieve. The published articles comparing LDP and ODP were all retrospective studies with common defects such as long-term research, small number of cases and incomplete data. With the development of surgical technology, potential bias and inappropriate results could be produced from the recent literature analyzed with the earlier clinical data. Furthermore, due to a relatively low incidence of left pancreas diseases, there are few LDP

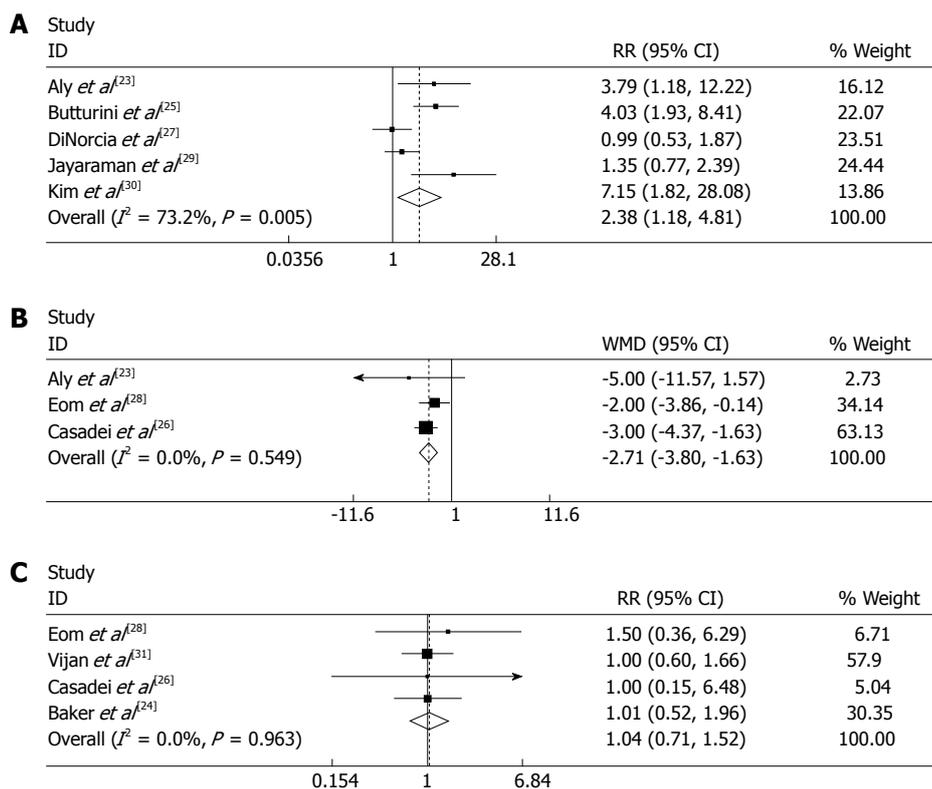
**Table 2 Characteristics of the literatures**

Ref.	Study year	Nation	Case number		Study type	Pancreatic transection		Spleen preservation		Total morbidity		PF		Mortality %		Level of evidence
			LDP	ODP		LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	
Aly <i>et al</i> <sup>[23]</sup>	1998-2009	Japan	40	35	Retro	Stapler	Stapler/ scalpel + suture	13	3	8	11	5	6	0	0	4
Baker <i>et al</i> <sup>[24]</sup>	2003-2008	USA	27	85	Pros	Stapler/ scalpel + micro sealer device	Scalpel + suture	NA		10	30	6	12	0	0	2b
Butturini <i>et al</i> <sup>[25]</sup>	1999-2006	Italy	43	73	Retro	Stapler	Scalpel + suture	19	8	21	33	12	10	0	0	2b
Casadei <i>et al</i> <sup>[26]</sup>	2000-2010	Italy	22	22	Case control	Stapler	Stapler	NA		6	6	2	4	0	0	2b
DiNordia <i>et al</i> <sup>[27]</sup>	1991-2009	USA	71	192	Retro	Stapler	Stapler/ Scalpel + suture	11	30	20	84	8	27	0	2	4
Eom <i>et al</i> <sup>[28]</sup>	1995-2006	Korea	31	62	Retro	Stapler	Scalpel + suture	13	NA	11	15	3	4	0	0	4
Jayaraman <i>et al</i> <sup>[29]</sup>	2003-2009	USA	74	236	Retro	Stapler/ scalpel + suture	Stapler/ scalpel + suture	14	33	11	94	6	31	NA		4
Kim <i>et al</i> <sup>[30]</sup>	NA	Korea	93	35	Retro	Stapler	Stapler/ Scalpel + suture	38	2	23	11	8	5	NA		4
Vijan <i>et al</i> <sup>[31]</sup>	2004-2009	USA	100	100	Retro	Stapler	NA	25	NA	34	29	17	17	3	1	4

Retro: Retrospective observational study; Pros: Prospective observational study; LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; PF: Pancreatic fistula; NA: Not available.



**Figure 2 Meta-analysis of the pooled data.** A: operative time was significantly shorter in open distal pancreatectomy (ODP) than in laparoscopic distal pancreatectomy (LDP) [random effects model, WMD 44.947 (13.857, 76.037),  $P = 0.005$ ]; B: Time for fluid intake was shorter in LDP than in ODP [random effects model, WMD -0.948 (-1.863, 0.032),  $P = 0.042$ ]; C: Pancreatic fistula occurrence has no significant difference between LDP and ODP [random effects model, RR 0.996 (0.663, 1.494),  $P = 0.983$ ,  $I^2 = 28.4\%$ ]. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio; WMD: Weighted mean differences.



**Figure 3** The pooled data. A: The spleen-preserving rate of laparoscopic distal pancreatectomy (LDP) was significantly higher than open distal pancreatectomy (ODP) [random effects model, RR 2.380 (1.177, 4.812),  $P = 0.016$ ,  $I^2 = 73.2\%$ ]; B: Postoperative hospital stay was significantly shorter in LDP than in ODP [random effects model, WMD -2.713 (-3.799, 1.628),  $P = 0.00$ ]; C: The proportion of malignant tumors showed no significant difference between LDP and ODP [fixed effects model, RR 1.036 (0.708, 1.516),  $P = 0.000$ ,  $I^2 = 0\%$ ]. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio; WMD: Weighted mean differences.

Ref.	Spleen preserving %		Technical details
	LDP	ODP	
Aly <i>et al</i> <sup>[23]</sup>	32.5	8.6	Both procedures, spleen vessel ligation were performed, leaving the short gastric vessels to supply the spleen (Warshaw)
Baker <i>et al</i> <sup>[24]</sup>	NA	NA	In ODP, the benign and premalignant pathology, the spleen was routinely saved by means of the splenic vein and artery preserved In LDP, splenic salvage by means of Warshaw: ligating the splenic artery and vein but preserve the short gastric vessel
Butturini <i>et al</i> <sup>[25]</sup>	44.2	11.0	Both procedures, exposing the splenic vein up to the splenic hilum; the distal pancreas was detached from the splenic artery in the opposite direction by tractioning the parenchyma
Casadei <i>et al</i> <sup>[26]</sup>	NA	NA	Mobilization of the distal pancreas from retroperitoneum and splenic vessels
DiNorcia <i>et al</i> <sup>[27]</sup>	15.5	15.6	For spleen preserving distal pancreatectomy, an attempt to spare the splenic artery and vein was made in all patients
Eom <i>et al</i> <sup>[28]</sup>	41.9	NA	For spleen preserving distal pancreatectomy, both the splenic artery and vein were preserved
Jayaraman <i>et al</i> <sup>[29]</sup>	18.9	14.0	When splenic preservation was performed, the splenic vein and artery were isolated
Kim <i>et al</i> <sup>[30]</sup>	40.9	5.70	In spleen preserving distal pancreatectomy, both the splenic artery and vein were preserved. In one case, the splenic artery was ligated with preservation of splenic vein. In the other case, both the splenic artery and vein were ligated, with preservation of short gastric vessels (Warshaw)
Vijan <i>et al</i> <sup>[31]</sup>	25	NA	If splenic preservation is indicated, the pancreas is dissected off the splenic vessels

LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; NA: Not available.

studies with large sample sizes. Therefore, with strictly defined inclusion and exclusion criteria, we performed a comprehensive analysis to assess the current status of LDP *vs* ODP.

The proportions of malignant tumors were 20% in both LDP and ODP, which showed no difference in the

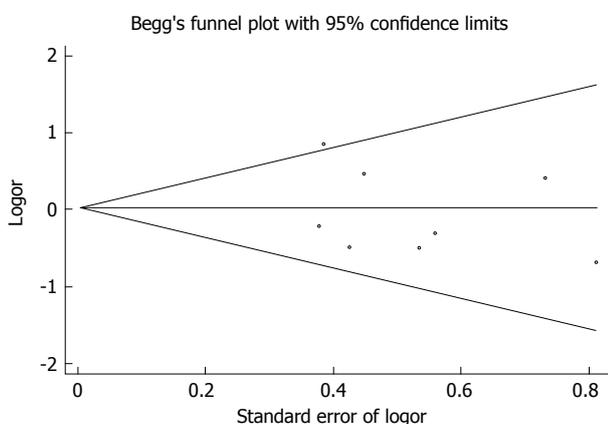
patient selection between the two groups. DP was most frequently used in the treatment of benign or borderline tumors, which was in agreement with previous studies<sup>[34,35]</sup> using either “laparoscopy” or “open”.

The most important indicators to represent the operative effect were shorter operative time, time to fluid

**Table 4** Technique of pancreatic stump closure

Ref.	Technique description	
Aly <i>et al</i> <sup>[23]</sup>	LDP	The pancreatic parenchyma was transected using a laparoscopic linear stapler
	ODP	The pancreatic parenchyma was transected using a scalpel, and the main pancreatic duct was ligated using nonabsorbable sutures. The pancreatic stump was closed with fish-mouth sutures. A linear stapler was used to transect the pancreatic parenchyma
Baker <i>et al</i> <sup>[24]</sup>	LDP	The gland was divided by one of 3 mechanisms: vascular stapler, harmonic scalpel, or harmonic scalpel following ablation at the pancreatic resection margin with the Habib 4*3 microsealer device
	ODP	Directly ligate the pancreatic duct when visible with a monofilament absorbable suture. The neck of the gland was oversewn with nonabsorbable monofilament suture
Butturini <i>et al</i> <sup>[25]</sup>	LDP	The pancreatic body was transected by a linear endostapler
	ODP	Pancreatic parenchyma was sharply transected. The main pancreatic duct was closed with nonabsorbable sutures (polypropylene 4/0). Subsequently the pancreatic stump was oversewn with interrupted mattress nonabsorbable sutures or closed using a linear stapler
Casadei <i>et al</i> <sup>[26]</sup>	LDP	The pancreas was divided at the neck using an endo-GIA instrument
	ODP	The pancreas was divided using GIA 55
DiNorcia <i>et al</i> <sup>[27]</sup>	LDP	Sutures, staples, sutures and staples combined, or staples with bioabsorbable staple-line reinforcement
	ODP	
Eom <i>et al</i> <sup>[28]</sup>	LDP	The pancreas was transected using the 48- or 35-mm vascular endoscopic linear stapler
	ODP	The pancreatic parenchyma was divided using a blade and electrocautery. The main pancreatic duct was ligated with nonabsorbable sutures, and the transected pancreas was occluded with interlocking interrupted mattress sutures of 4-0 black silk and reinforced with 4-0 polypropylene
Jayaraman <i>et al</i> <sup>[29]</sup>	LDP	The pancreas was stapled using a vascular stapler with or without a Seamguard attachment
	ODP	Ligate pancreas with staples, or via suture ligation, or a combination of techniques
Kim <i>et al</i> <sup>[30]</sup>	LDP	For pancreatic transaction, straight endoscopic linear staplers of various sizes (staple height, 3.5-4.2 mm) were used according to the thickness or hardness of the pancreas. Four or five small titanium clips were applied along the stapling line
	ODP	The pancreatic stump underwent main duct ligation, multiple suture ligation of the branch duct exposed at the resection margin, and reinforcement of the mattress suture to the pancreas stump
Vijan <i>et al</i> <sup>[31]</sup>	LDP	The pancreatic parenchyma is divided with the harmonic scalpel (preferred) or with an Endo GIA stapler
	ODP	NA

LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; NA: Not available; GIA: Gastrointestinal incision anastomose.

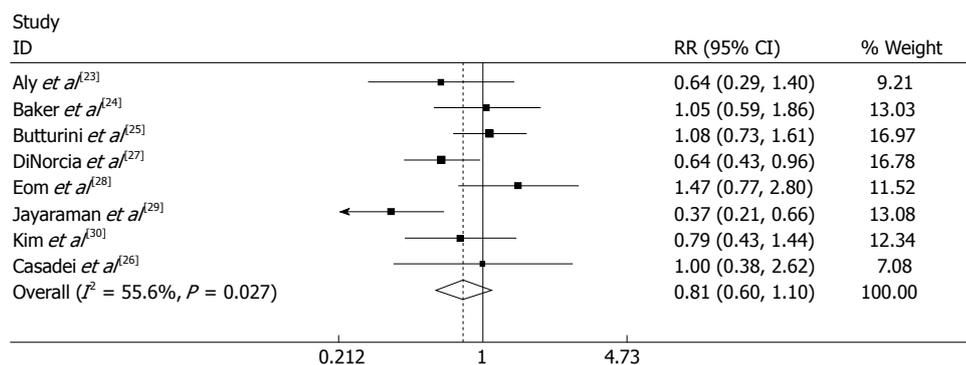


**Figure 4** Begg's test showing no publication bias of pancreatic fistula occurrence.

intake and hospital stay, and less blood loss, low conversion rate and high spleen-preserving rate. These indicators have demonstrated the safety and feasibility of the laparoscopic procedures. In this meta-analysis, the operative time of LDP was longer than ODP (WMD 44.947,  $P = 0.005$ ), but a recent research showed that operative time is becoming shorter with the improved expertise of surgeons<sup>[17]</sup>. And the time to fluid intake and post-operative hospital stay were also shorter in LDP than in ODP (WMD -0.948,  $P = 0.042$ ) and (WMD -2.713,  $P = 0.00$ ). Blood loss estimate was not conducted in this

study because of different numeric types. The results of the included articles showed that blood loss was less in LDP than in ODP, which were similar with other literatures<sup>[18,19]</sup>. In addition, conversion rate of LDP from the pooled data showed a low level of 14% in 714 LDPs because of severe bleeding and abdominal adhesion<sup>[14]</sup>.

The rate of spleen-preservation ranged from 15.5% to 44.2% in LDP and from 5.7% to 15.6% in ODP as shown in Table 3. Kimura method was more frequently used as compared with Warshaw method used when intraoperative bleeding, adhesion, and blood vessels embedded by the tumors occurred. Effects of the two surgical methods have long been a concern. Rodriguez *et al*<sup>[36]</sup> retrospectively reported Kimura method used in 185 cases compared with Warshaw method in 74 cases of LDP from 1994 to 2004; the two groups had no statistically significant difference in occurrence of ascites (9% *vs* 8%), intra-abdominal abscess (14% *vs* 8%), pancreatic leakage (33% *vs* 36%) and incision complications (10% *vs* 8%). Although Warshaw method was proved to be sufficiently safe<sup>[37,38]</sup>, due to individual differences of the short gastric vessels, spleen relied entirely on the short gastric blood vessels which inevitably brought some uncertainties. In the event of severe splenic infarction, reoperation was often required. So spleen-preservation by Kimura method was widely accepted in LDP, but under some special conditions, such as bleeding, adherent tumor and difficult anatomy, Warshaw method could elevate the spleen-preserving rate. In this meta-



**Figure 5** There was no significant difference in overall morbidity between laparoscopic distal pancreatectomy and open distal pancreatectomy [random effects model, risk ratio 0.81 (0.596, 1.101),  $P = 0.178$ ,  $I^2 = 55.6\%$ ] and there was moderate heterogeneity. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio.

analysis, five articles described the spleen-preserving DP, the pooled data showed that the spleen-preserving rate of LDP was significantly higher than that of ODP (RR 2.380,  $P = 0.016$ ,  $I^2 = 73.2\%$ ). Although there was moderate heterogeneity, spleen-preservation occurred more often in LDP. The reasons for the high spleen-preserving rate in LDP may be as follows: (1) surgeons in different stages of learning curve may achieve different clinical outcomes. In the early period, because of the immature LDP technique, especially laparoscopic vascular treatment, fewer cases of LSPVP were performed; and (2) many cases of ODP without spleen-preservation were included in each study, leading to a low spleen-preservation rate of ODP.

PF was the most important complications after DP which resulted in serious consequences such as extended hospital stay, poor quality of life, even intra-abdominal bleeding, and infection. Although at some high-volume centers, PF after DP has declined over the past decade, the incidence of PF still kept from 5% to 30%<sup>[39-41]</sup>. In this study, a large variation in the PF rate was recorded, ranging from 8.1% to 27.9% in LDP and 6.5% to 18.2% in ODP. The major reason for the variability may be lacking uniform criteria for PF. The diagnostic criteria of PF were generally based on clinical signs and laboratory indicators, including the occurrence time, the daily amount of leakage, leakage amylase, the duration, *etc.* The ISGPF criterion<sup>[42]</sup> was most frequently used, but it failed to explain whether the drainage amount was related to the diagnosis of PF. Because of the lack of different quantitative indicators, other criteria were also questioned<sup>[43,44]</sup>.

The original disease, pancreatic transection, pancreas texture, blood supply, and stump closure are factors affecting the incidence of PF. Recently, body mass index  $> 25 \text{ kg/m}^2$  was also reported contributing to the increased incidence of PF after DP<sup>[45]</sup>. However, the treatment of pancreatic stump is a unique controllable factor for preventing PF. In order to reduce the incidence of PF, a variety of stump closure techniques were applied or used in combination, but the coexistence of methods may reflect the lacking of a widely accepted and effective method. In this study, stapler was used in LDP while both stapler/scalpel + suture were used in ODP. The surgeons

could choose different staplers according to the pancreatic texture and size in LDP. And in some groups, small titanium clips were applied along the stapling line<sup>[50]</sup> and fibrin glue was splashed over the pancreatic stump in an attempt to prevent PF and postoperative bleeding<sup>[23,24,27,30]</sup>. Subset analysis could not be accomplished as no detailed data was available. A published meta-review analyzed 16 articles with 2286 patients who underwent DP and compared the preventive effect for PF between 671 cases with stapler closure and 1615 cases with suture closure. The results showed no significant differences between suture and stapler closure of the pancreatic remnant with respect to the PF or intra-abdominal abscess<sup>[46]</sup>. Likewise, the pooled data of this study showed no significant difference both in the incidence of PF (RR 0.996,  $P = 0.983$ ) and overall morbidity between LDP and ODP (RR 0.81,  $P = 0.178$ ).

The authentication strength of this study may be affected by the following factors: (1) publication bias: some gray literatures which contained negative results were difficult to obtain because most authors tended to show positive results; (2) grouping bias: notwithstanding the literatures dealing with significantly different diseases and surgical methods have been excluded in this study, in practice, patients should be grouped inevitably according to the disease condition and surgeons' choices; and (3) observation bias: due to the varied measurement methods used by different authors, significantly different results were almost inevitable in the non-RCT or non-blind RCT studies.

In summary, LDP has shown the advantages of intra-operative effects, rapid recovery and spleen-preservation for benign and borderline tumors. But the superiority has not been displayed in preventing the overall morbidity and occurrence of PF. Thus, the RCT studies with a large sample size should be conducted and new surgical techniques should be introduced in future studies.

## COMMENTS

### Background

Laparoscopic distal pancreatectomy (LDP) is becoming a primary treatment

modality for benign or borderline tumors of distal pancreas. But due to the high postoperative morbidity and a high level of laparoscopic technical requirements and extensive experiences in open pancreatic surgery, the progression of LDP was considerably restricted.

### Research frontiers

Recently, several studies have shown shorter hospital stay and operative time and less intraoperative blood loss in LDP. But the efficacy of LDP compared with open distal pancreatectomy (ODP) required further assessment.

### Innovations and breakthroughs

Pancreatic fistula (PF) and spleen-preservation in LDP have been the major concern after the surgery. What the role of "laparoscopy" in the spleen preservation and PF prevention is unclear. In this meta-analysis, the authors pointed out that LDP has the advantages of shorter hospital stay and operative time, more rapid recovery and higher spleen-preserving rate compared with ODP.

### Applications

Due to a relatively low incidence of left pancreas diseases, fewer LDP studies with a large sample size have been published. Especially the RCT clinical study, which is the ideal object of meta-analysis, is extremely difficult to accomplish. This meta-analysis assessed the safety and feasibility of LDP compared with ODP based on the review of the literature published over the past 15 years.

### Peer review

This paper is a systematic review of the laparoscopic distal pancreatectomy. The summary of LDP experiences and results is interesting.

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## A prospective randomized trial of transnasal ileus tube vs nasogastric tube for adhesive small bowel obstruction

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### Abstract

**AIM:** To study the therapeutic efficacy of a new transnasal ileus tube advanced endoscopically for adhesive small bowel obstruction.

**METHODS:** A total of 186 patients with adhesive small bowel obstruction treated from September 2007 to February 2011 were enrolled into this prospective randomized controlled study. The endoscopically advanced new ileus tube was used for gastrointestinal decompression in 96 patients and ordinary nasogastric tube (NGT) was used in 90 patients. The therapeutic efficacy was compared between the two groups.

**RESULTS:** Compared with the NGT group, the ileus tube group experienced significantly shorter time for relief of clinical symptoms and improvement in the findings of abdominal radiograph ( $4.1 \pm 2.3$  d vs  $8.5 \pm 5.0$  d) and laboratory tests ( $P < 0.01$ ). The overall effectiveness rate was up to 89.6% in the ileus tube group and 46.7% in the NGT group ( $P < 0.01$ ). And 10.4% of the patients in the ileus tube group and 53.3% of the NGT group underwent surgery. For recurrent adhesive bowel obstruction, ileus tube was also significantly more effective than NGT ( $95.8\%$  vs  $31.6\%$ ). In the ileus tube group, the drainage output on the first day and the length of hospital stay were significantly different depending on the treatment success or failure ( $P < 0.05$ ). The abdominal radiographic improvement was correlated with whether or not the patient underwent surgery.

**CONCLUSION:** Ileus tube can be used for adhesive small bowel obstruction. Endoscopic placement of the ileus tube is convenient and worthy to be promoted despite the potential risks.

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**Key words:** Adhesive; Small bowel obstruction; Ileus tube; Nasogastric intubation; Gastrointestinal decompression

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## INTRODUCTION

Gastrointestinal decompression is the most effective approach to treat the patients with acute bowel obstruction without any indications of strangulation<sup>[1,2]</sup>. The traditional nasogastric tube (NGT) is not long enough for suctioning the distal intestine and its decompression efficacy is relatively low. Since the 1930s, the concept of nasointestinal decompression and use of nasointestinal tubes have been developed and applied in clinical practice. Some studies have verified the efficacy of long nasointestinal tubes in treating adhesive small bowel obstructions (SBO)<sup>[3-7]</sup>. However, a prospective randomized trial demonstrated no significant differences with regard to the decompression achieved, the success of non-surgical treatment, or the morbidity rate after surgical intervention as compared with the use of short NGT<sup>[8]</sup>. In 2003, a new hydrophilic silicon triple-lumen ileus tube was first introduced and used in Japan for nasointestinal decompression. It could be advanced through the gastroscope in shorter time with a higher tolerance<sup>[9]</sup>. Up till now, there has been no randomized controlled study about the efficacy of the ileus tube. This randomized controlled trial attempted to investigate and compare the decompression efficacy between the new ileus tube and the traditional NGT for patients with adhesive SBO.

## MATERIALS AND METHODS

### Patients

Approved by the hospital's ethics committee, a total of 186 patients with acute adhesive SBO who were admitted to the Gastroenterology and Colorectal Surgery wards of the First Affiliated Hospital, College of Medicine, Zhejiang University and its Ningbo Branch Hospital from September 2007 to February 2011 were enrolled into this study. The entry criteria were as follows: (1) clinical symptoms and physical signs arising from acute bowel obstruction; (2) a diagnosis of adhesive SBO based on abdominal plain films and computed tomography (CT) scans confirmed by at least two attending radiologists; and (3) admission to the hospital within 12 h after bowel obstruction onset. All patients who presented with symptoms of fever, vomiting or hematemesis, hematochezia, severe or sudden abdominal pain, and the signs of tachycardia, leukocytosis, abdominal tenderness, peritoneal irritation, asymmetric abdominal distension or isolated swelling bowel loops and even shock, should be suspected of strangulation obstruction, which needed immediate operation. Besides, patients who had contraindications for endoscopy, or with postoperative adynamic obstruction or malignancy, or who had been treated in other hospitals before admission were excluded. Patients were randomized into two groups by the sealed envelope method: the ileus tube group and the NGT group; an opaque box containing an equal number of envelopes that indicated either ileus tube or NGT, was used for randomization. Written informed consents were obtained

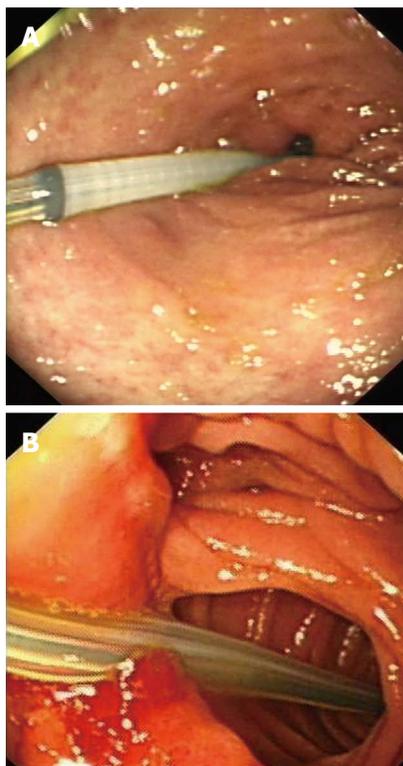
from all the patients before enrollment. This trial conformed to the provisions of the World Medical Association Declaration of Helsinki. Ileus tube was used in 96 patients (56 men and 40 women) for gastrointestinal decompression; their ages ranged from 21 to 86 years (mean, 58 years). Among the 96 patients, 25 had a history of prior adhesion, and 89 patients received prior abdominal surgery. Ninety patients (56 men and 34 women) treated with NGT served as the control group; their ages ranged from 19 to 86 years (mean, 54 years). Among the 90 patients, 38 had a history of prior adhesion, and 86 had a history of abdominal surgery.

### Patient preparation

On admission, all patients underwent abdominal plain film radiography and CT scanning to confirm acute bowel obstruction. Patients requiring emergency surgery were excluded. Performed by the same technicians, the ileus tube was advanced endoscopically and the traditional NGT was inserted for gastrointestinal decompression at a similar negative pressure level for constant suction. For all patients, routine laboratory blood tests were performed, and C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) were determined.

### Instrument and procedures

The CLINY Ileus Tube suite (Create Medic, Tokyo, Japan) and the ordinary NGT (Terumo Medic, Hangzhou, China) were used. The ileus tube is 300 cm in length and 16 Fr with three channels (suction channel, injection channel and balloon channel) and two balloons (anterior balloon and posterior balloon). Beside the tip hole, there are side holes in the distal end of the tube. Compared with other long tubes, this tube has weighted tip which consists of several metal balls for easier advancing. The posterior balloon is intended for contrast radiography. Water and contrast medium can be injected into the tube for lavage and imaging. Under some instances, the tube can directly remove the obstruction by its weighted tip. The guidewire is 350 cm long and 1.24 mm in diameter. The NGT is 110 cm in length and 16 Fr. All patients received gastrointestinal decompression within 12 h after admission. In the ileus tube group, the tube was pre-set through the nasal cavity to the stomach. The guidewire was inserted into the main channel to the tips. After endoscopic suction of stomach contents, the wire was moved into the descending duodenum by forceps, and the tube was inserted into the duodenum while the wire was kept fixed (Figure 1). Then the anterior balloon was inflated with 20 mL distilled water. The gastroscope was withdrawn after the long tube was fixed to the cheek. The tube was propelled by bowel peristalsis and its weighted tip, and the outside terminal of the tube was connected to a spontaneous negative pressure bag. Intermittent lavage (twice a day) through the long tube was performed from the second day after intubation, and the length of the advanced tube was carefully measured. In the control group, the NGT was inserted from nose to stomach to



**Figure 1** Endoscopic placement of ileus tube. A: An ileus tube is passed through the pylorus under gastroscopy in a patient with postoperative adhesive small bowel obstruction; B: An ileus tube led by a guidewire is endoscopically advanced into the efferent loop in a patient who had distal partial gastrectomy.

a depth of 45-55 cm. All patients were supported with total parenteral nutrition and received nothing by mouth. Emergency surgery was performed when the patient was suspected to have developed bowel ischemia. The potential risks for endoscopic placement of the ileus tube were throat injury, upper gastrointestinal perforation and bleeding, aspiration pneumonia and cardiovascular events.

### Outcome measurement

We compared the clinical and laboratory variables before intubation, including age, sex, type of prior surgery, symptoms and physical signs, and laboratory indexes on admission between the two groups. Physical examination (every 2 h), laboratory test (once a day) and abdominal imaging (days 2-7) were conducted frequently after intubation. The clinical and laboratory findings included: the time for relief of abdominal symptoms; time for abdominal radiographic improvement and recovery of white blood cell (WBC) counts, CRP level and ESR; drainage volume on the first day; surgery rate, the overall efficacy and the different therapeutic responses to the two kinds of tubes. The therapeutic effectiveness in the ileus tube group was defined as clinical or radiological improvement, relief of abdominal symptoms, decreased drainage volume, disappearance of air-fluid levels or reduced gas and fluid in bowel loops. Oral feeding was then administered gradually and the tube was removed. If the patient

presented no improvement 72 h after decompression, or even progressed into strangulation, surgery is recommended<sup>[4]</sup>. In the NGT group, if a fairly prompt response occurs within 48 h, especially within the first 8-12 h after nasogastric decompression and resuscitation, the obstruction will probably be resolved without surgery<sup>[10,11]</sup>. Surgery was recommended if patients showed no response 72 h after non-surgical treatment, which was defined as treatment failure<sup>[12]</sup>, otherwise, conservative treatment was continued.

### Sample size calculation

Determination of the sample size was based on the previous studies<sup>[4,9]</sup>. The effectiveness rate was 51% in the short tube decompression while 85.7% in the long nasointestinal tube. If the effectiveness rate of the NGT and the ileus tube was defined as 50% and 80%, respectively, a study with 48 patients per group would have a 90% power to detect a difference at a two-sided significance level of 0.05. We extended our sample size to account for potential dropouts

### Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc, Chicago). Results were expressed as mean  $\pm$  SD. The  $\chi^2$  test was used to identify differences in the effectiveness rate between the two groups. The Student's *t* test was used for unpaired data to determine differences in means between the two groups. Odds ratios (ORs) were determined by logistic regression analysis. Two-tailed *P* value of  $< 0.05$  was considered statistically significant.

### Study limitations

This study was designed as a randomized controlled trial (RCT), but it was not double-blinded. No standard criteria are available for the treatment success by this long tube in the literature. The two kinds of tubes were placed by different methods, while the patients in ileus tube group suffered more during intubation.

## RESULTS

### Patient clinical characteristics

Of the 186 patients, 96 were treated with ileus tube, and 90 were randomized into NGT group. There was no significant difference between the two groups with regard to clinical characteristics and laboratory variables documented on admission, including age, sex, abdominal symptoms, and laboratory indexes such as WBC counts, CRP, and ESR ( $P > 0.05$ ). The type of prior surgery and obstruction also did not differ significantly ( $P > 0.05$ ). In this study, the ileus tube or NGT was successfully placed in all the patients, without any obvious complications. Patient characteristics of the two groups are shown in Table 1.

### Therapeutic efficacies

The time for improvement in abdominal symptoms, ra-

**Table 1** Clinical characteristics, difference decompression responses and therapeutic efficacies of ileus tube group and nasogastric tube group

Clinical characteristics	Ileus tube group ( <i>n</i> = 96)	NGT group ( <i>n</i> = 90)	<i>P</i> value	OR (95% CI)
Mean age (yr)	58	54	0.07	-
Male/female	56/40	56/34	0.59	-
Past laparotomies ( <i>n</i> )	89	86	0.41	0.59 (0.17–2.09)
Surgery type				
Colorectal surgery	35	28	0.35	1.34 (0.72–2.50)
Small-bowel resection	15	14	0.92	1.04 (0.47–2.31)
Gastroduodenal surgery	8	12	0.3	0.61 (0.24–1.57)
Appendectomy	8	6	0.62	1.32 (0.44–3.97)
Gallbladder and pancreas surgery	4	4	1	0.97 (0.23–3.99)
Splenectomy	3	4	0.96	0.72 (0.16–3.29)
Bladder or kidney surgery	2	2	1	0.97 (0.13–7.01)
Gynecologic surgery	14	16	0.61	0.82 (0.37–1.80)
Symptoms on admission ( <i>n</i> )				-
Abdominal pain	85	82	0.56	0.75 (0.29–1.97)
Distention	93	85	0.65	1.82 (0.42–7.86)
Nausea or vomiting	58	65	0.09	0.59 (0.32–1.09)
Disappearance of flatus and defecation	84	72	0.17	1.75 (0.29–3.88)
Laboratory data before intubation ( <i>n</i> )				
Elevated WBC count	70	67	0.81	0.92 (0.48–1.78)
Elevated CRP level	37	42	0.26	0.72 (0.40–1.29)
Elevated ESR level	31	27	0.74	1.11 (0.60–2.07)
Therapeutic efficacies (%)				
Rate of abdominal pain or distention relieved within 48 h	95.8 (92/96)	46.7 (42/90)	< 0.01 ( $\chi^2 = 55.75$ )	26.29 (8.90–77.66)
Surgery rate	10.4 (10/96)	53.3 (48/90)	< 0.01 ( $\chi^2 = 39.87$ )	0.10 (0.05–0.22)
Effectiveness rate for recurrent adhesive small bowel obstruction	95.8 (24/25)	31.6 (12/38)	< 0.01 ( $\chi^2 = 25.55$ )	52.00 (6.28–430.67)
Total effectiveness rate	89.6 (86/96)	46.7 (42/90)	< 0.01 ( $\chi^2 = 39.87$ )	9.83 (4.53–21.33)
Differences in decompression responses by ileus tube and NGT (mean $\pm$ SD)				<i>t</i>
Time for relief of abdominal pain or distention (h)	23.8 $\pm$ 10.9	59.1 $\pm$ 30.1	< 0.01	-10.4
Appearance of flatus and defecation (d)	2.4 $\pm$ 1.7	6.5 $\pm$ 3.2	< 0.01	-10.4
Time to abdominal radiographic improvement (d)	4.1 $\pm$ 2.3	8.5 $\pm$ 5.0	< 0.01	-6.9
WBC recovery (d)	4.0 $\pm$ 2.4	7.0 $\pm$ 4.8	< 0.01	-4.6
CRP recovery (d)	5.5 $\pm$ 2.5	8.8 $\pm$ 3.9	< 0.01	-4.4
ESR recovery (d)	5.8 $\pm$ 2.4	8.7 $\pm$ 3.9	< 0.01	-4.1
Drainage volume on the first day (mL)	698 $\pm$ 428	280 $\pm$ 167	< 0.01	8.9

CI: Confidence interval; CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; NGT: Nasogastric tube; OR: Odds ratio; SD: Standard deviation; WBC: White blood cells.

diographic findings, and laboratory variables was significantly shorter ( $P < 0.01$ ) in the ileus tube group as compared with the NGT group. In addition, more patients had relief from abdominal pain or distention within 48 h in the ileus tube group ( $P < 0.01$ ). The drainage volume on the first day after intubation was  $698 \pm 428$  mL in the ileus tube group and  $280 \pm 167$  mL in the NGT group, with a significant difference ( $P < 0.01$ ,  $t = 8.9$ ). After ileus tube decompression, 86 patients presented clinical or radiographic relief (Figure 2), the tube was removed one week after oral feeding was started, with an effectiveness rate up to 89.6% (86/96). The other 10 patients defined as treatment failure by gastroenterography underwent operation to determine the site of the obstruction (Figure 3). In follow-up study, 6 patients still had recurrent adhesive SBO confirmed by surgery, the intervals varied from one month to seven months. In the control group, the total effectiveness rate was 46.7% (42/90); the other 48 patients defined as treatment failure were managed with surgery. However, 16 patients had recurrent adhesive SBO after successful treatment, the recurrence peak occurred between 3 mo and 5 mo (Table 1).

### Therapeutic outcome in ileus tube group treated with or without surgery

In the ileus tube group, 10 patients without initial relief underwent surgery to remove the obstruction. Significant differences were found in the drainage output on the first day and the length of hospital stay ( $P < 0.05$ ). Besides, no patient showed abdominal radiographic improvement within 72 h in decompression in the surgical group as compared to 51.2% (44/86) in the non-surgical group. These results are summarized in Table 2.

## DISCUSSION

Conservative treatment is usually administered to the patients with acute bowel obstruction when ischemic bowel is excluded. Surgeons are inclined to choose conservative treatment for adhesive bowel obstruction because of the risk of recurrence along with surgery<sup>[13]</sup>. Since the 1930s, various types of tubes have been devised and used for nasointestinal decompression<sup>[14,15]</sup>. A tube was designed specifically for endoscopic placement and the ileus tube has been developed with three channels and two bal-



**Figure 2 Radiographs of ileus tube decompression.** Plain abdominal radiographs (A) and (B) reviewed 3 d after ileus tube decompression compared with scans on admission in a patient with postoperative adhesive small bowel obstruction. A: The diffuse distended loops of small bowel that was filled with gas and fluid before intubation; air-fluid levels were seen in the enteric cavity; B: Reviewed 3 d after intubation; the previous gas-filled or fluid-filled small bowel loops showed no evidence of distention, the air-fluid levels disappeared, and the long tube had moved downward while the tip reached the distal jejunum.



**Figure 3 Diagnostic radiographic enteroclysis.** Gastroenterography displayed on the 5th day of ileus tube decompression in a patient with postoperative adhesive small bowel obstruction. The tip of the tube had reached to the distal jejunum. After the anterior balloon was inflated with gas, angiografin was injected into the tube for gastrointestinal imaging to locate the lesion or stenosis in the bowel. Stenosis was found (arrow) in the small intestine with a filling defect, but none was developed in the distal bowel.

loons. A study confirmed an efficacy rate of ileus tube of up to 85.7% for intraluminal decompression in the bowel<sup>[9]</sup>. Intubation methods then changed from fluoroscopy to direct placement under endoscopy and afforded safety and high success rates<sup>[16-21]</sup>. However, if there is

**Table 2 Therapeutic outcomes in ileus tube group treated with or without surgery (mean ± SD)**

Variables	Surgical treatment	Non-surgical treatment	P value	t
Cases (n)	10	86	-	-
Time for relief of distention (h)	27.6 ± 16.9	23.4 ± 10.2	0.51	0.69
Appearance of flatus and defecation (d)	3.2 ± 2.4	2.4 ± 1.7	0.27	1.12
Radiographic improvement within 72 h (n)	0	44	-	-
WBC recovery (d)	5.2 ± 1.9	4.0 ± 2.4	0.26	1.14
CRP recovery (d)	8.3 ± 5.0	5.3 ± 2.1	0.40	1.04
ESR recovery (d)	5.0 ± 2.8	5.9 ± 2.3	0.61	-0.51
Drainage volume on the first day (mL)	390 ± 287	734 ± 428	0.02 <sup>a</sup>	-2.47
Length of hospital stay (d)	33.0 ± 13.7	21.5 ± 10.4	< 0.01 <sup>a</sup>	3.19

<sup>a</sup>P < 0.05 vs therapeutic outcomes in ileus tube group treated with or without surgery. CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; SD: Standard deviation; WBC: White blood cells.

any side effect along with the endoscopic placement procedure, the tube should be pulled out and endoscopic treatment for gastrointestinal bleeding or perforation should be given if possible. If patients have aspiration pneumonia, antibiotics and mechanical ventilation should be considered. Intensive care and emergent therapy are needed for any cardiovascular event. Our clinical practice testified the safety and flexibility of the endoscopic placement of the ileus tube, and the procedure and the instruments we used are available in most hospitals. However, the long tube and endoscopy cost more than ¥4000 RMB, that is 20 times more than an ordinary NGT, even though it is much lower than surgery. The cost as well as the discomfort caused by endoscopy may limit the promotion of the use of ileus tube.

From the data of this study, we found a great comparability between our two groups with regard to sex, age, past laparotomies, symptoms, and radiographic signs as well as laboratory findings on admission. It is known that delayed visit to hospital leads to a higher failure in conservative measures, so we selected the patients admitted within 12 h after obstruction onset to enforce the rigidity of the study. With bowel peristalsis and the weighted tip, the ileus tube passes downward to the small bowel and decompression can be achieved. As the tip can reach to the site of obstruction, thorough suctioning can be performed, leading to a rapid relief of the symptoms, as shown in our results. In addition, the recovery time for laboratory variables of inflammatory markers was shorter in ileus tube group, probably because of the improvement in the blood supply to the bowel wall, which can reduce the local inflammatory response and bacterial multiplication. A previous study demonstrated no significant differences in therapeutic efficacy between the long and short tube decompression<sup>[8]</sup>. However, we found that in the ileus tube group the effectiveness rate was significantly higher and the surgery rate was lower than that in the control group. We attribute it to the ad-

vanced technique of the tube and the endoscopic placement method that can avoid the delay by passing beyond the pylorus. Another study confirmed that a previous episode of adhesive bowel obstruction and the duration of the tips not advancing (> 72 h) were highly correlated with a recurrence of obstruction. If patients fail to respond 3 d after decompression or have indications of ischemic bowel or the drainage volume is > 500 mL on the third day, surgery is recommended<sup>[22,23]</sup>. For NGT decompression, after 48 h of non-operative management, the risk of complications increases substantially, and the probability for resolving the obstruction diminishes. Surgery is required if a patient's condition has deteriorated or has not significantly improved within 72 h. In the ileus tube group, intermittent lavage was performed from the second day after intubation so that we could record the drainage volume on the first 24 h to compare the decompression responses with the NGT group.

We admitted relatively a large number of patients with adhesive SBO for this study. Our results showed that for adhesive SBO, the ileus tube had the decompression efficacy that was significantly superior to the NGT, especially for patients with recurrent adhesive SBO. Although a tendency toward recurrence can not be avoided after successful treatment using the ileus tube in patients with past episodes, it is still superior to the traditional NGT treatment and should be therefore recommended in clinical practice. The ileus tube has many advantages in addition to thorough decompression. It can remove the kinks in the obstructed bowel loops when the tip progresses downward, and the long tube itself can perform through a straddle mechanism to arrange the bowel and reduce the adhesion recurrence rate. Diagnostic radiographic enteroclysis studies are facilitated, which are helpful to surgeons for preoperative preparation<sup>[24,25]</sup>.

Previous studies have confirmed the efficacy of nasointestinal decompression through a long tube for SBO, especially for adhesive SBO<sup>[3-7]</sup>. The approach of endoscopic placement of the long tube was also advised<sup>[15,26]</sup>. According to our clinical application, the ileus tube has a prospective therapeutic efficacy for adhesive SBO. However, surgical intervention can easily be undertaken when NGT decompression failed, because it is thought to be the most immediate modality for remission. Another focus is that water-soluble contrast agent (WSCA) is helpful in the diagnosis and treatment of adhesive SBO according to a recent meta-analysis<sup>[27]</sup>, appearance of contrast in the colon within 4-24 h after administration had a sensitivity of 96% and a specificity of 98% in predicting resolution of SBO. The WSCA can draw fluid from intravascular and extracellular spaces into bowel lumen because of its high osmolarity, thus promoting proximal bowel distension and peristalsis, and avoiding the operative interference. However, as there are potential risks of renal failure and anaphylaxis, these agents still can not take the place of gastrointestinal decompression, which is thought to be the key to the treatment of bowel obstruction. Based on our results, we highly recommend

this triple-lumen tube for patients with adhesive SBO. The endoscopic placement of the tube is convenient, and with close monitoring and intermittent lavage, surgeries can be avoided.

We also tried to find certain indications for surgical interventions in the patients treated with ileus tube. Compared with patients who underwent surgery in the ileus tube group, those who were successfully managed without surgery had a significantly shorter hospital stay and a larger drainage output on the first day after intubation. Up to 51.2% of the patients showed abdominal radiographic improvement within the first 3 d in the non-surgical group while no patient achieved relief in the surgical group. This indicates that the drainage output on the first day and radiographic improvement could be two independent factors for evaluating the therapeutic efficacy of nasointestinal decompression. They may also be indications for surgery. Further studies should be performed to identify the clinical value of the ileus tube within the parameters of indications for surgery.

In summary, we believe that endoscopic placement of transnasal ileus tube is safe, effective, and convenient and is worth being promoted in clinical practice. The ileus tube can quickly relieve the clinical symptoms and reduce the rate of surgical indications. It is greatly superior to the NGT in treating patients with adhesive SBO. However, the potential risks and extra costs should be taken into consideration when selecting patients. For patients with recurrent adhesive SBO, the use of triple-lumen ileus tube is the optimal choice. Close observation of drainage output and abdominal radiographic changes during decompression can help provide some clues for indications of surgery.

## COMMENTS

### Background

Adhesive small bowel obstruction is a worldwide problem characterized by a high incidence rate and repeated episodes. Gastrointestinal decompression is one of the important approaches in conservative therapy. However, surgical intervention can easily be undertaken when nasogastric tube decompression failed. Long intestinal tube for nasointestinal decompression is a new method for adhesive small bowel obstruction and has been successfully applied in clinical practice and the therapeutic efficacies were satisfactory.

### Research frontiers

A new long tube named ileus tube was first introduced in Japan in 2003, and later studies have confirmed its therapeutic value in adhesive small bowel obstruction. However, there had been no randomized controlled study about the efficacy of the ileus tube. This randomized controlled trial attempted to investigate and compare the decompression efficacy between the new ileus tube and the traditional nasogastric tube (NGT) for patients with adhesive small bowel obstruction.

### Innovations and breakthroughs

The previous studies of long intestinal tube for gastrointestinal decompression were mainly described retrospectively. This research compared the therapeutic efficacies between the new long tube and the ordinary NGT in a large number of patients. The authors confirmed the superiority of the new ileus tube to the ordinary NGT through a series of statistical analysis. The authors also introduced the detailed procedure of the endoscopic placement of this long tube.

### Applications

The study indicated the therapeutic value of the new ileus tube. The application

of the ileus tube can significantly reduce the surgery rate and the hospital-stay cost, and the endoscopic placement method introduced by the authors can be applied in almost all the hospitals. Thus, the use of ileus tube is worthy to be promoted.

### Peer review

This is an important area and the work represents a significant advance in clinical therapy. As it is possible not only to increase efficacy on a single intervention but also reduce the need for subsequent intervention, the technology provides the possibility to lower costs and reduce intervention to the patient.

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## Analysis of infections in the first 3-month after living donor liver transplantation

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### Abstract

**AIM:** To identify factors related to serious postoperative bacterial and fungal infections in the first 3 mo after living donor liver transplantation (LDLT).

**METHODS:** In the present study, the data of 207 patients from 2004 to 2011 were reviewed. The pre-, intra- and post-operative factors were statistically analyzed. All transplantations were approved by the ethics committee of West China Hospital, Sichuan University. Patients with definitely preoperative infections and infections within 48 h after transplantation were excluded from current study. All potential risk factors were analyzed using univariate analyses. Factors significant at a  $P < 0.10$  in the univariate analyses were involved in the multivariate analyses. The diagnostic accuracy of the identified risk factors was evaluated using receiver operating curve.

**RESULTS:** The serious bacterial and fungal infection rates were 14.01% and 4.35% respectively. *Enterococcus faecium* was the predominant bacterial pathogen, whereas *Candida albicans* was the most common fun-

gal pathogen. Lung was the most common infection site for both bacterial and fungal infections. Recipient age older than 45 years, preoperative hyponatremia, intensive care unit stay longer than 9 d, postoperative bile leak and severe hyperglycemia were independent risk factors for postoperative bacterial infection. Massive red blood cells transfusion and postoperative bacterial infection may be related to postoperative fungal infection.

**CONCLUSION:** Predictive risk factors for bacterial and fungal infections were indentified in current study. Pre-, intra- and post-operative factors can cause postoperative bacterial and fungal infections after LDLT.

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**Key words:** Bacterial infection; Fungal infection; Living donor liver transplantation

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### INTRODUCTION

Despite the major advances in immunosuppressant regimens, perioperative management and medical care have contributed to improvements in the survival rate of solid organ transplant recipient, infection continues to be a leading cause of postoperative mortality and morbidity resulting from the poor preoperative condition, immunosuppressive therapy and exposure to nosocomial pathogens<sup>[1,2]</sup>. Liver transplantation has one of the highest rates

of postoperative infection among all solid organ transplant procedures<sup>[3]</sup>. It has been reported that the postoperative bacterial infection rate may up to more than 60% and accounted for an in-hospital mortality rate of 30%-50%<sup>[4]</sup>. Previous studies have reported that the incidence of postoperative fungal infection ranged from 5% to 40%, and the mortality associated with fungal infection was between 25% and 69%<sup>[5]</sup>. Moreover, the mortality of patient with *aspergillus* has been found to approach 100% if untreated<sup>[6]</sup>. Accordingly, to identify which factors may cause postoperative bacterial and fungal infections is important to transplant surgeon. However, this issue is still not well established until now. In current study, we used a large cohort to identify the pattern and risk factors associated with postoperative bacterial and fungal infections that occurred within the first 3 postoperative months after living donor liver transplantation.

## MATERIALS AND METHODS

### Study group

Patients who received adult-to-adult living donor liver transplantation (LDLT) from 2004 to 2011 at our center were considered in present study. All transplantations were approved by the ethics committee of West China Hospital, Sichuan University. Patients with definitely preoperative infections and infections within 48 h after transplantation were excluded from current study.

### Donor selection

Donors must be healthy close relatives with compatible ABO blood types. Serological testing for viral hepatitis and human immunodeficiency virus antibodies as well as testing for other acute or chronic diseases was negative. Volumetric computed tomography with contrast was performed to evaluate the hepatic volume of the donors. Right hepatic lobe without middle hepatic vein of the donors need to be at least 0.8% of the recipient's standard weight and the remnants must be at least 40% of the donor's liver volume. Magnetic resonance cholangiopancreatography was performed to assess the anatomy of the biliary tree<sup>[7,8]</sup>.

### Immunosuppression

The postoperative immunosuppression consisted of tacrolimus, mycophenolate mofetil and steroids. Steroids were withdrawn as soon as possible. Acute rejection episodes were confirmed by pathology. Steroid pulse therapy was conducted to patient with rejection<sup>[9]</sup>. OKT3 monoclonal antibody was administrated to patients with persistent rejection or steroid-resistant rejection. When necessary, these treatments were repeated<sup>[10]</sup>.

### Infection prophylaxis

Antimicrobial prophylaxis consisted of Cefoperazone and Sulbactam for three to five days. Fluconazole was administrated to patients with risk factors of fungal infection for one to two weeks after liver transplantation.

### Definitions

Serious infection was defined as the culture-positive bacterial or fungal infection in blood, sputum, urine, or ascetic fluid which was obtained on the basis of clinical suspicion of an infection<sup>[11,12]</sup>. Preoperative renal dysfunction was defined as the level of serum creatinine greater than 1.5 mg/dL<sup>[13]</sup>. Bile leak was defined as bilirubin concentration in the drainage greater than the plasma level<sup>[14]</sup>. Model for end-stage liver disease (MELD) scores were calculated according to the formula: MELD score =  $9.57 \times \text{Ln creatinine (mg/dL)} + 11.2 \times (\text{Ln INR}) + 3.78 \times \text{Ln bilirubin (mg/dL)} + 6.43$ <sup>[15]</sup>. Massive red blood cells (RBCs) transfusion was defined as transfusion not less than 6 packed RBCs in the first 24 h of surgery<sup>[16]</sup>. Severe hyperglycemia was defined as glucose concentrations more than or equal to 20 mg/dL<sup>[17]</sup>. Hyponatremia was defined as a serum sodium concentration of less than 130 mEq/L<sup>[18]</sup>.

### Statistical analysis

All continuous variables were presented as mean  $\pm$  SD and compared using one way analysis of variance.  $\chi^2$  test or Fisher's exact test was used for comparing categorical variables. Independent risk factors were identified by Cox regression. Factors significant at a  $P < 0.10$  in the univariate analyses were involved in the multivariate analyses. The diagnostic accuracy of the identified risk factors was evaluated using receiver operating curve (ROC). All analyses were performed using SPSS 16.0. We considered a  $P$  value of less than 0.05 to be significant.

## RESULTS

### Patient characteristics

A total of 207 patients were included in current study. The mean age was  $42.93 \pm 8.77$  years for the recipients, whereas the mean age was  $34.83 \pm 9.99$  years for the donors. Twenty-seven patients were female, whereas eighty-one donors were female. Nine patients had pre-transplant diabetes mellitus. Sixteen patients suffered from preoperative renal dysfunction. The mean MELD score was  $16.40 \pm 9.84$ . The mean graft to recipient weight ratio (GRWR) was  $0.94\% \pm 0.18\%$ . The causes for transplantation were hepatitis B ( $n = 116$ ), hepatocellular carcinoma ( $n = 75$ ), hepatolithiasis ( $n = 3$ ), hepatitis C ( $n = 3$ ), alcoholic cirrhosis ( $n = 3$ ), polycystic liver ( $n = 1$ ), primary biliary cirrhosis ( $n = 2$ ), hepatic hydatidosis ( $n = 1$ ), huge hepatic hemangioma ( $n = 1$ ), trauma ( $n = 1$ ), autoimmune hepatitis ( $n = 1$ ).

### Pattern of infection

During the first 3 postoperative months, serious bacterial infection was observed in 29 recipients, whereas serious fungal infection was found in 9 patients. Among the 9 patients with fungal infection, 6 patients were combined with or secondary to bacterial infection. Only 3 patients infected fungal infection alone. Four patients had two kinds of bacterial infection. One patient suffered from three kinds of bacterial infection. *Enterococcus faecium* ( $n = 8$ ) was the most common pathogen in patients with bacterial infec-

**Table 1** Univariate analysis for risk factors for postoperative bacterial infection

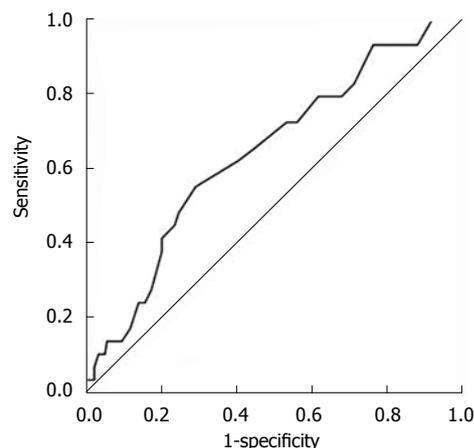
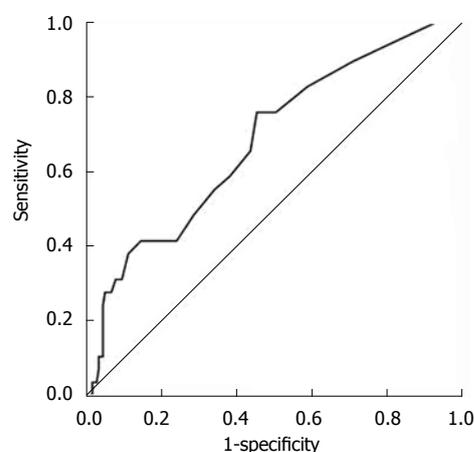
Variables	Infected	No infected	P value
Donor variables			
Age (yr)	34.45 ± 8.78	34.89 ± 10.19	0.825
Gender (female)	10	71	0.580
BMI (kg/m <sup>2</sup> )	22.80 ± 2.69	22.99 ± 2.53	0.711
Recipient variables			
Age (yr)	46.72 ± 8.27	42.31 ± 8.63	0.012
Gender (female)	2	25	0.384
BMI (kg/m <sup>2</sup> )	21.77 ± 4.21	22.63 ± 2.93	0.172
MELD score	17.24 ± 7.40	16.26 ± 10.18	0.621
Renal dysfunction	1	15	0.705
Diabetes mellitus	1	8	0.798
Starting albumin level < 2.8 g/dL	10	32	0.040
Starting TB level ≥ 20 mg/dL	3	24	0.774
Starting INR level	1.62 ± 0.54	1.71 ± 1.50	0.744
Hyponatremia	7	12	0.008
Graft variables			
GRWR (%)	0.99 ± 0.23	0.93 ± 0.17	0.111
Intraoperative variables			
Massive RBCs transfusion	8	35	0.335
FFP transfusion > 10 units	7	42	0.949
Postoperative variables			
Bile leak	10	6	0.000
Rejection	2	3	0.090
Hyperglycemia	5	10	0.042
ICU stay	17.38 ± 11.11	12.28 ± 12.48	0.040

BMI: Body mass index; MELD: Model for end-stage liver disease; TB: Total bilirubin; INR: International normalized ratio; GRWR: Graft to recipient weight ratio; RBC: Red blood cell; FFP: Fresh frozen plasma; ICU: Intensive care unit.

tion, followed by *Escherichia coli* ( $n = 6$ ), *Staphylococcus aureus* ( $n = 4$ ), *Bowman acinetobacter* ( $n = 4$ ), *Hemolytic streptococcus* ( $n = 3$ ), *Pseudomonas aeruginosa* ( $n = 2$ ), *Burkholderia cepacia* ( $n = 1$ ), *Acinetobacter lwoffii* ( $n = 1$ ), *Xanthomonas maltophilia* ( $n = 1$ ), *Haemophilus influenzae* ( $n = 1$ ), *Klebsiella pneumoniae* ( $n = 1$ ), *Enterobacter cloacae* ( $n = 1$ ), *Leuconostoc pseudomesenteroides* ( $n = 1$ ). The most common bacterial infective site was lung ( $n = 15$ ), followed by abdominal cavity ( $n = 12$ ), blood ( $n = 3$ ). *Candida albicans* ( $n = 6$ ) was the most common pathogen in patients with fungal infection, follow by *Saccharomyces* ( $n = 2$ ), *Aspergillus* ( $n = 1$ ). Fungal infections were observed in lung in 7 patients and in gastrointestinal tract in 2 recipients.

### Risk factors related to bacterial infection

As shown in Table 1, recipient age, starting albumin level < 2.8 g/dL, preoperative hyponatremia, postoperative bile leak, severe hyperglycemia, rejection and longer intensive care unit (ICU) stay were potential risk factors in univariate analysis. ROC curve analysis showed the best cut-off values for recipient age and ICU stay were 45 years and 9 d respectively (Figures 1 and 2). The corresponding are under the ROC were 0.641 and 0.680 respectively (Figures 1 and 2). When we analyzed the potential risk factors using Cox regression, only recipient age > 45 years, preoperative hyponatremia, postoperative bile leak, severe hyperglycemia and length of ICU stay > 9 d were independent risk factors for bacterial infection (Table 2).

**Figure 1** Receiver operating curve curve for recipient age.**Figure 2** Receiver operating curve curve for the length of intensive care unit stay.

### Risk factors related to fungal infection

As listed in Table 3, we studied the factors may be related to fungal infection using univariate analysis. Correlation testing showed preoperative hyponatremia, massive intraoperative RBCs transfusion and postoperative bacterial infection may be contributed to postoperative fungal infection. These potential risk factors were further examined with Cox regression analysis. Only bacterial infection and massive intraoperative RBCs transfusion showed prognostic power in multivariate analysis (Table 4).

## DISCUSSION

Postoperative infection is one of the most common complications in liver transplant recipients. In our current study, the incidences of bacterial and fungal infections were 14.01% and 4.35%, which were lower than previous reports<sup>[19-21]</sup>. We suggested this difference may be related to the different definition of infection. In current study, only culture-positive infections were included. Consistent with previous studies, gram-negative bacteria, especially *Enterococcus faecium* and *Escherichia coli*, were the predomi-

**Table 2** Multivariate analysis for risk factors for postoperative bacterial infection

Variables	B	SE	Wald	P value	Exp (B)	95% CI	
						Lower	Upper
Bile leak	1.890	0.421	20.156	0.000	6.622	2.901	15.116
Hyponatremia	1.512	0.487	9.649	0.002	4.535	1.747	11.770
Hyperglycemia	1.171	0.508	5.308	0.021	3.226	1.191	8.737
ICU stay > 9 d	0.932	0.458	4.145	0.042	2.540	1.035	6.230
Recipient age > 45 yr	1.253	0.408	9.440	0.002	3.501	1.574	7.785

SE: Standard error; CI: Confidence interval; ICU: Intensive care unit.

**Table 3** Univariate analysis for risk factors for postoperative fungal infection

Variables	Infected	No infected	P value
Donor variables			
Age (yr)	37.00 ± 7.76	34.73 ± 10.08	0.507
Gender (female)	4	74	0.739
BMI (kg/m <sup>2</sup> )	21.64 ± 2.27	23.02 ± 2.54	0.110
Recipient variables			
Age > 45 yr	5	62	0.153
Gender (female)	1	26	0.860
BMI (kg/m <sup>2</sup> )	21.44 ± 2.80	22.56 ± 3.16	0.295
MELD score	20.11 ± 9.35	16.23 ± 9.85	0.248
Renal dysfunction	1	15	0.698
Diabetes mellitus	0	9	0.513
Starting albumin level < 2.8 g/dL	3	39	0.391
Starting total bilirubin level	2	25	0.332
Starting INR level	1.91 ± 0.55	1.69 ± 1.43	0.650
Hyponatremia	3	16	0.010
Graft variables			
GRWR (%)	0.93 ± 0.21	0.94 ± 0.18	0.886
Intraoperative variables			
Massive RBCs transfusion	6	37	0.003
FFP transfusion > 10 units	4	45	0.220
Postoperative variables			
Bile leak	1	15	0.698
Rejection	0	5	0.629
Hyperglycemia	2	13	0.076
ICU stay > 9 d	5	98	0.748
Postoperative bacterial infection	6	23	0.000

BMI: Body mass index; MELD: Model for end-stage liver disease; INR: International normalized ratio; GRWR: Graft to recipient weight ratio; RBC: Red blood cell; FFP: Fresh frozen plasma; ICU: Intensive care unit.

nant bacterial pathogens, whereas *Candida albicans* was the most common fungal pathogen<sup>[22]</sup>.

Postoperative bile leak was an independent risk factor for bacterial infection of LDLT in current study. This risk factor was not considered in some studies following deceased donor liver transplantation (DDLTL)<sup>[23]</sup>. This difference was related to the low incidence of postoperative bile leak in patients underwent DDLTL. However, bile leak was one of the most common complications in LDLT recipients. This factor should not be ignored in LDLT. Patients with postoperative bile leak suffered from longer abdominal drainage which may increase the risk of intraabdominal and wound infection<sup>[24]</sup>. Additionally, bile leak can cause biloma that often progress to an infected abscess<sup>[25]</sup>.

**Table 4** Multivariate analysis for risk factors for postoperative fungal infection

Variables	B	SE	Wald	P value	Exp (B)	95% CI	
						Lower	Upper
Massive RBCs transfusion	1.887	0.710	7.062	0.008	6.599	1.641	26.542
Bacterial infection	2.429	0.711	11.686	0.001	11.346	2.819	45.673

SE: Standard error; CI: Confidence interval; RBC: Red blood cell.

It was interesting that patient more than 45 years old was a risk factor related to postoperative bacterial infection. We acknowledge the cut-off value of recipient age was so young in our study. The mean recipient age in current study was 42.93 ± 8.77 years. This was a potential explanation. Similar to our results, Nayaranan *et al*<sup>[26]</sup> suggested patient's age greater than 42 years old was significantly associated with a poor long-term survival. This finding suggested the incidence of postoperative bacterial infection may be increased with the increasing of recipient age.

Preoperative diabetes mellitus didn't increase the risk of postoperative infection in our study. However, John *et al*<sup>[27]</sup> suggested pretransplant diabetes was associated with increased postoperative morbidity and mortality. Recently, Ling *et al*<sup>[28]</sup> confirmed preexisting diabetes was not a contraindication for liver transplantation. Well controlled pretransplant diabetes will not increase the risk of postoperative complication. In our practice, the nine patients with pretransplant diabetes had normal blood sugar level at the time of transplantation. Contrary to pretransplant diabetes mellitus, severe postoperative hyperglycemia was an independent risk factor for bacterial infection in current study. However, after transplantation, the administration of immunosuppressive agents, including cyclosporine, steroids and tacrolimus, may cause postoperative hyperglycemia. Ata *et al*<sup>[29]</sup> confirmed postoperative hyperglycemia was the most important risk factor for surgical site infection in general surgery patients. Rueda *et al*<sup>[30]</sup> reported hyperglycemia will increase the risk for and severity of pneumonia among non-diabetic patients.

It was easy to understand prolonged ICU stay and hyponatremia were associated with postoperative bacterial infection. Mnatzaganian *et al*<sup>[31]</sup> confirmed the incidences of bloodstream and urinary infections of patients in ICU were higher than those in regular ward. Suljagic *et al*<sup>[32]</sup> confirmed the incidence of nosocomial bloodstream infection of ICU patients was higher than non-ICU patients. Stormont *et al*<sup>[33]</sup> confirmed hyponatremia was associated with pneumonia. Zilberberg *et al*<sup>[34]</sup> suggested hyponatremia was associated with worsened clinical outcomes among patients with pneumonia.

Although the relationship between massive RBCs transfusion and bacterial infection was well established in previous studies, there were little information of the correlation of massive RBCs transfusion and fungal in-

fection. Current study suggested massive RBCs transfusion will increase the risk of fungal infection after liver transplantation. Blood transfusion can cause transfusion-related immunomodulation which will suppress the recipient's immune function<sup>[35]</sup>. However, it remains unclear why massive transfusion was not a risk factor for bacterial infection in current study.

Postoperative bacterial infection showed significantly prognostic power for fungal infection in current study. Antibiotics, especially broad-spectrum antibiotics, were administered to patients with bacterial infection in the case of lacking culture results. Broad-spectrum antibiotics might lead to dysbacteriosis and increase fungal infection<sup>[36]</sup>. However, a recent study which was performed by Nafady-Hego *et al.*<sup>[37]</sup> suggested bacterial infection was not a risk factor for fungal infection after pediatric LDLT. Younger recipient age, lower dosages of immunosuppressive agents and different infection prophylaxis might be the potential explanation for this difference.

In conclusion, preoperative hyponatremia, recipient age > 45 years, longer ICU stay, postoperative bile leak and severe hyperglycemia may be related to postoperative bacterial infection, whereas massive intraoperative RBCs transfusion and postoperative bacterial infection may lead to postoperative fungal infection. Current finding suggested postoperative bacterial and fungal infections were associated with pre-, intra- and post-operative factors.

## COMMENTS

### Background

Infection is a leading cause of postoperative mortality and morbidity after liver transplantation. To identify the pattern and risk factors related to postoperative bacterial and fungal infections is important to transplant surgeon.

### Research frontiers

The pattern and risk factors of postoperative infections following living donor liver transplantation are not well established. This study was performed to identify the pattern and risk factors related to bacterial and fungal infections in the first 3-mo after living donor liver transplantation.

### Innovations and breakthroughs

This study outlines a comprehensive experience of 207 patients over an eight year period of infections in the first 3-mo following living donor liver transplantation. It documents the site of infection, organisms involved and the predictive risk factors.

### Applications

This study could guide the clinical management of early bacterial and fungal infections after living donor liver transplantation.

### Terminology

Serious infection was defined as the culture-positive bacterial or fungal infection in the blood, sputum, urine, or ascetic fluid which was obtained on the basis of clinical suspicion of an infection.

### Peer review

The article is well written. The analysis of the data is sound and the conclusions reached appear valid. Overall, it significantly contributes to a relevant issue in this field and should be considered for publication.

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## Individualized peri-operative fluid therapy facilitating early-phase recovery after liver transplantation

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### Abstract

**AIM:** To investigate the correlation between peri-operative fluid therapy and early-phase recovery after liver transplantation (LT) by retrospectively reviewing 102 consecutive recipients.

**METHODS:** Based on whether or not the patients had pulmonary complications, the patients were categorized into non-pulmonary and pulmonary groups. Twenty-eight peri-operative variables were analyzed in both groups to screen for the factors related to the occurrence of early pulmonary complications.

**RESULTS:** The starting hemoglobin (Hb) value, an intra-operative transfusion > 100 mL/kg, and a fluid balance  $\leq$  -14 mL/kg on the first day and the second or third day post-operatively were significant factors for

early pulmonary complications. The extubation time, time to initial passage of flatus, or intensive care unit length of stay were significantly prolonged in patients who had not received an intra-operative transfusion  $\leq$  100 mL/kg or a fluid balance  $\leq$  -14 mL/kg on the first day and the second or the third day post-operatively. Moreover, these patients had poorer results in arterial blood gas analysis.

**CONCLUSION:** It is important to offer a precise and individualized fluid therapy during the peri-operative period to the patients undergoing LT for cirrhosis-associated hepatocellular carcinoma.

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**Key words:** Fluid therapy; Liver transplantation; Early-phase recovery; Pulmonary complications; Hemoglobin

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### INTRODUCTION

Liver transplantation (LT) is the optimal therapy for end-stage liver diseases. Although LT has undergone a rapid progress, early pulmonary complications are common and known to contribute significantly to the morbidity and mortality of the patients<sup>[1-4]</sup>. Post-operative pulmonary complications may be caused by many factors during the patient's recovery, and fluid therapy is an important

factor<sup>[5,6]</sup>. Individualized fluid therapy during the peri-operative period may be a significant strategy to achieve a better early-phase recovery after LT. In this study, early-phase refers to the first month after LT.

In a previous study, we investigated and assessed the use of fluid therapy in all the patients undergoing LT<sup>[7]</sup>. This study focused on the patients with cirrhosis-associated hepatocellular carcinoma (HCC), and fluid transfusion was administered based on the body weight of the patients per kg.

The purpose of this study was to investigate the clinical significance of the correlation between peri-operative fluid therapy and early-phase recovery after LT in an attempt to establish a precise and individualized fluid therapeutic strategy in the peri-operative period of LT.

## MATERIALS AND METHODS

The medical records of all consecutive patients with cirrhosis-associated HCC who underwent orthotopic LT at the First Affiliated Hospital of Guangxi Medical University between July 1996 and July 2009 were retrospectively reviewed. Patients aged from 23-72 years with a mean  $\pm$  SD of  $45.26 \pm 9.54$  years. This series represents the first 102 consecutive LT recipients with cirrhosis-associated HCC in our cohort study.

All LT procedures were performed using the piggy-back technique without venovenous bypass. In addition to fluid transfusion given intra-operatively, small amounts of vasopressors (dopamine and noradrenaline) and shallow anesthesia were used to maintain hemodynamic stability. All patients were admitted to the intensive care unit (ICU) immediately after surgery and extubated as soon as they met standard criteria for termination of mechanical ventilation, such as the presence of adequate gas exchange function, hemodynamic stability, and ability to protect the airway. Antibiotics and anti-fungals for infection were used prophylactically.

Radiographic analysis was standardized by assessment of eight separate observations designed to determine the presence of pulmonary edema<sup>[8]</sup>. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) were defined according to the American-European Consensus Conference<sup>[9]</sup>, and pneumonia was defined according to a Joint Committee of the American Thoracic Society and Infectious Diseases Society of America<sup>[10]</sup>.

### All variables

Twenty-five peri-operative variables affecting post-operative early-phase recovery were as follows: age; body weight index; hemoglobin (Hb); hematocrit (HCT); serum creatinine (CRE); blood urea nitrogen (BUN); serum uric acid (UA); American Society of Anesthesiologists (ASA) grade; Child-Pugh score; acute physiology and chronic health evaluation (APACHE) II score; model for end-stage liver disease (MELD) score; warm ischemia time; cold ischemia time; anhepatic phase; operative time; diabetes; lung function; volume of intra-operative blood transfusion;

volume of intra-operative packed red blood cell (RBC) transfusion; volume of intra-operative plasma transfusion; volume of intra-operative fluid transfusion; volume of intra-operative bleeding; intra-operative fluid balance; transfused volume and fluid balance on the first post-operative day. Bivariate correlation analysis for the relationship between these intra-operative variables and occurrence of pulmonary complications showed the following significant variables: blood transfusion  $> 30$  mL/kg ( $P = 0.046$ ); packed RBC transfusion  $> 0.05$  U/kg ( $P = 0.041$ ); plasma transfusion  $> 25$  mL/kg ( $P = 0.042$ ); fluid transfusion  $> 100$  mL/kg ( $P = 0.014$ ); bleeding  $> 10$  mL/kg ( $P = 0.018$ ); fluid balance  $> 64$  mL/kg ( $P = 0.037$ ); fluid transfusion volume on the first post-operative day  $\leq 44$  mL/kg ( $P = 0.010$ ); and fluid balance on the first post-operative day  $\leq -14$  mL/kg ( $P = 0.018$ ). The threshold values of these variables were obtained by bivariate correlation analysis between the volume of intra-operative transfusion and post-operative pulmonary complications. The statistically significant threshold value was 100 mL/kg. If no statistically significant threshold value was obtained, the lowest  $P$  value was recorded. The critical value of other fluid variables was determined by the same analysis.

Because of the special importance of the first 3 post-operative days for patients' recovery, the following three variables were analyzed: fluid balance  $\leq -14$  mL/kg on the first day and the second or the third day after operation ( $P = 0.010$ ); fluid balance  $\leq -14$  mL/kg on post-operative  $\geq 1$  d ( $P = 0.612$ ); and fluid balance  $\leq -14$  mL/kg on post-operative  $\geq 2$  d ( $P = 0.014$ ).

Other variables analyzed included the worst outcome of arterial blood gas analysis in the first 7 post-operative days. The outcome variables reflecting post-operative recovery included: extubation time; time to initial passage of flatus; and ICU length of stay.

In this study, fluid balance in the surgery did not include the "third space" loss, evaporative loss, and insensible loss. So far, no method has provided estimated losses for LT.

### Statistical analysis

Data were presented as the mean  $\pm$  SD, median/range, or percentage (%). Group means were compared using Student's  $t$  test or the Mann-Whitney  $U$  test as appropriate. The  $\chi^2$  test was used to compare percentages. Bivariate correlation was used to determine the significant threshold value of peri-operative fluid variables. Multivariate regression analysis was performed with stepwise elimination of non-significant variables. A  $P < 0.050$  was considered significant. All analyses were performed with SPSS 12.0 software (SPSS, Chicago, IL, United States).

## RESULTS

Of the 102 patients (89 males and 13 females), 47 patients (46.08%) had pulmonary complications after LT. No hypoxia was found preoperatively. Pulmonary edema (PE;  $n = 8$ , 17.02%), acute lung injury (ALI;  $n = 12$ ,

**Table 1 Comparison of 25 variables between the groups with and without pulmonary complications**

Variables	Non-pulmonary complication group (n = 55)	Pulmonary complication group (n = 47)	P value
Age (yr)	46.29 ± 9.14	44.06 ± 9.95	0.242
Weight index (kg/m <sup>2</sup> )	22.78 ± 3.35	22.36 ± 3.29	0.531
Hb (g/L)	122.79 ± 21.12	109.54 ± 25.25	0.005
HCT (%)	36.21 ± 6.42	32.53 ± 7.53	0.009
CRE (mmol/L)	83.75 ± 21.35	85.96 ± 23.88	0.623
BUN (mmol/L)	4.70 ± 1.92	5.22 ± 3.09	0.323
UA (mmol/L)	303.76 ± 113.27	272.43 ± 117.94	0.175
ASA grade	2.76 ± 0.61	3.0 ± 0.75	0.082
Child-Pugh score	7.29 ± 2.40	8.38 ± 2.88	0.039
APACHE II score	3.95 ± 2.47	4.79 ± 3.53	0.174
MELD score	14.22 ± 7.34	16.68 ± 9.39	0.140
Warm ischemia time (min)	5.80 ± 1.52	5.53 ± 1.63	0.392
Cold ischemia time (min)	710.13 ± 184.99	654.21 ± 208.25	0.154
Anhepatic phase (min)	123.04 ± 34.46	126.00 ± 47.00	0.715
Operative time (min)	423.22 ± 84.92	489.38 ± 150.00	0.009
Diabetes (%)	9.09	8.51	1.000
Lung dysfunction (%)	12.73	19.15	0.374
Volume of intra-operative blood transfusion > 30 mL/kg (%)	65.45	82.98	0.046
Volume of intra-operative packed RBC transfusion > 0.05 U/kg (%)	76.36	91.49	0.041
Volume of intra-operative plasma transfusion > 25 mL/kg (%)	43.64	63.83	0.042
Volume of intra-operative fluid transfusion > 100 mL/kg (%)	58.18	80.85	0.014
Volume of intra-operative bleeding > 10 mL/kg (%)	67.27	87.23	0.018
Intra-operative fluid balance > 64 mL/kg (%)	32.73	53.19	0.037
a (%)	21.82	4.26	0.010
b (%)	38.18	17.02	0.018
c (%)	25.45	6.38	0.010
d (%)	72.73	68.09	0.612
e (%)	36.36	14.89	0.014

Hb: Hemoglobin; HCT: Hematocrit; CRE: Creatinine; BUN: Blood urea nitrogen; UA: Serum uric acid; ASA: American Society of Anesthesiologists; APACHE: Acute physiology and chronic health evaluation; MELD: Model for end-stage liver disease. a, transfused volume on the first post-operative day ≤ 44 mL/kg; b, fluid balance on the first post-operative day ≤ -14 mL/kg; c, fluid balance ≤ -14 mL/kg on the first day and the second or the third day after operation; d, fluid balance ≤ -14 mL/kg on ≥ 1 d of the first 3 d after operation; e, fluid balance ≤ -14 mL/kg on ≥ 2 d after operation. ASA grade I was assigned a score of 1, grade II was assigned a score of 2, grade III was assigned a score of 3, and grade IV was assigned a score of 4. Mean ± SD was used for continuous variables, otherwise percentage was used (%).

25.53%), adult respiratory distress syndrome (ARDS; n = 6, 12.77%), and pneumonia (n = 21, 44.68%) occurred after surgery. Four patients survived no more than one month after LT.

Table 1 shows the comparison of 28 variables between the non-pulmonary and pulmonary complication groups. The following variables were found significant: Hb; HCT; Child-Pugh score; operative time; intra-operative blood

**Table 2 Summary of the logistic regression model and odds ratios**

Variables	Beta	SE	Sig	Exp (B)	95% CI
Hb	-0.025	0.010	0.11	0.975	0.956-0.994
Volume of intra-operative transfusion	1.097	0.496	0.27	2.995	1.132-7.922
c	2.037	0.722	0.05	7.670	1.862-31.603

SE: Standard error; Sig: Statistical significance; CI: Confidence interval; Hb: Hemoglobin. c, fluid balance ≤ -14 mL/kg on the first day and the second or the third after operation. When the volume of intra-operative transfusion ≤ 100 mL/kg, the variable was assigned a score of 0, and when the variable > 100 g/L, the variable was assigned a score of 1.

**Table 3 Comparison of outcome variables reflecting post-operative recovery between groups A and B**

	Group A (n = 32)	Group B (n = 70)	Z or t	P value
Extubation time (h)	12/8-99	16.5/5-504	-2.779	0.005
Time to initial passage of flatus (h)	78.66 ± 21.05	90.51 ± 45.28	-1.411	0.161
ICU length of stay (h)	36.5/8-144	62/14-600	-4.173	0.000

Values are given as the mean ± SD (median/range). ICU: Intensive care unit.

transfusion > 30 mL/kg; intra-operative packed RBC transfusion > 0.05 U/kg; intra-operative plasma transfusion > 25 mL/kg; intra-operative fluid transfusion > 100 mL/kg; intra-operative bleeding > 10 mL/kg; intra-operative fluid balance > 64 mL/kg; fluid transfusion volume on the first post-operative day ≤ 44 mL/kg; fluid balance ≤ -14 mL/kg on the first post-operative day; fluid balance ≤ -14 mL/kg on the first day and the second or the third day after operation; and fluid balance ≤ -14 mL/kg ≥ 2 d after operation.

The statistically significant variables were regarded as independent variables, and post-operative pulmonary complications were regarded as dependent variables. Multivariate regression analysis was performed to screen out the variables related to early pulmonary complications. Table 2 shows the statistically significant variables: Hb; intra-operative transfusion > 100 mL/kg; and fluid balance ≤ -14 mL/kg on the first day and the second or the third day after operation.

The 32 patients who received an intra-operative transfusion ≤ 100 mL/kg were referred to as group A and the other 70 patients who received an intra-operative transfusion > 100 mL/kg were as group B. Table 3 shows the comparison of outcome variables reflecting post-operative recovery between the two groups. As expected, both the extubation time and ICU length of stay in group A were shorter than in group B (P < 0.01). A comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between the two groups showed that both PaO<sub>2</sub> and arterial partial pressure of oxygen (PaO<sub>2</sub>)/fraction of inspired oxygen (FiO<sub>2</sub>) ratio in group A were higher than in group B (110.422 ± 28.305 vs 90.641 ± 31.169, P < 0.01 for PaO<sub>2</sub>; and 272.355 ±

**Table 4** Comparison of outcome variables reflecting post-operative recovery between groups A and B

	Group A (n = 17)	Group B (n = 85)	Z or t	P value
Extubation time (h)	12/7-32	15/5-504	-2.708	0.007
Time to initial passage of flatus (h)	59.71 ± 12.17	92.21 ± 40.96	-6.094	0.000
ICU length of stay (h)	40/13-219	60/8-600	-1.590	0.112

Values are given as the mean ± SD (median/range). ICU: Intensive care unit.

79.486 *vs* 219.649 ± 86.462, *P* < 0.01 for PaO<sub>2</sub>/FiO<sub>2</sub>).

The 17 patients who received a fluid balance ≤ -14 mL/kg on the first day and the second or the third day after operation served as group A and the other 85 patients who did not receive a fluid balance ≤ -14 mL/kg served as group B. Table 4 shows a comparison of outcome variables reflecting post-operative recovery between the two groups. As expected, both the extubation time and the time to initial passage of flatus in group A were shorter than in group B (*P* < 0.01). Table 5 shows the comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between the two groups. Both PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> in group A were higher than in group B (*P* < 0.01).

## DISCUSSION

The LT recipients in this study presented the following characteristics: (1) All had chronic liver cirrhosis with water-sodium retention to some degree; (2) Because of the operative injury for LT, the capillary leak syndrome (CLS) created a “third space” effect; (3) Intra-operative fluid overload was common in order to maintain stable hemodynamics; and (4) A large-dose methylprednisolone was used to avoid reject reaction during and after surgery. All these factors contributed to aggravated water-sodium retention. Therefore, a precise and individualized fluid therapy is strongly recommended.

Collective studies demonstrated the importance of intra-operative fluid management to maintain body fluid equilibrium by restricting the volume of fluid infusion. Excessive fluid administration was associated with a higher risk of post-operative complications<sup>[11-14]</sup>. Aduen *et al*<sup>[15]</sup> reported that pulmonary complications interfered with the peri-operative course of patients undergoing LT and portended a worse outcome. Meanwhile, the time for extubation, ICU stay and hospital stay were significantly prolonged.

The restrictive intra-operative fluid management may be advantageous because it reduces morbidity and mortality and shortens mechanical ventilation, the time to initial passage of flatus, intensive care, and hospital stay<sup>[16-21]</sup>.

In this study, binary logistic regression revealed that an intra-operative transfusion > 100 mL/kg (Table 2) was an independent risk factor for post-operative pulmonary complications. The incidence of post-operative pulmonary complications in group A was significantly lower than in group B (28.13% *vs* 54.29%, *P* = 0.014).

**Table 5** Comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between groups A and B

	Group A (n = 17)	Group B (n = 85)	t	P value
PH	7.436 ± 0.041	7.437 ± 0.055	-0.108	0.914
PaO <sub>2</sub>	117.471 ± 31.283	92.722 ± 30.105	3.075	0.003
PaCO <sub>2</sub>	44.094 ± 7.168	44.787 ± 12.038	-0.229	0.819
BE	4.459 ± 4.207	4.789 ± 4.062	-0.305	0.761
PaO <sub>2</sub> /FiO <sub>2</sub>	295.891 ± 92.741	224.243 ± 81.820	3.223	0.002

Values are given as the mean ± SD. PH: Power of hydrogen; PaO<sub>2</sub>: Arterial partial pressure of oxygen; PaCO<sub>2</sub>: Arterial pressure of carbon dioxide; BE: Base excess.

The extubation time and ICU length of stay were also significantly shorter than in group B (Table 3). Group A had higher PaO<sub>2</sub> and PaO<sub>2</sub>/FiO<sub>2</sub> than group B. These outcomes demonstrated that intra-operative fluid therapy was very important, which was associated with the incidence of pulmonary complications and the post-operative recovery.

Post-operative fluid overload is an independent risk factor for post-operative pulmonary complications after LT. Alsous *et al*<sup>[22]</sup> demonstrated that a fluid balance ≤ -500 mL on ≥ 1 d of the first 3 d of septic shock was associated with fewer pulmonary complications and better recovery. Our results demonstrated that it is a significant means to keep a fluid balance ≤ -14 mL/kg on the first day and the second or the third day after LT (Table 2). The incidence of post-operative early pulmonary complications in group A was lower than in group B (17.65% *vs* 51.76%, *P* = 0.01). This fluid therapy contributed to a better recovery (Tables 4 and 5) possibly because a fluid balance at ≤ -14 mL/kg within 3 d after LT could prevent edema, thus improving the blood supply and promoting recovery.

Of the four variables (fluid balance on the first post-operative day ≤ -14 mL/kg, fluid balance ≤ -14 mL/kg on the first day and the second or the third day after surgery, fluid balance ≤ -14 mL/kg on ≥ 1 d after surgery, and fluid balance ≤ -14 mL/kg on ≥ 2 d after surgery), only the fluid balance ≤ -14 mL/kg on the first day and the second or the third day after operation was included in the logistic regression analysis of outcome. It suggested that precise and individualized fluid balance in the peri-operative period should be accomplished as early as possible. In contrast, a negative fluid balance in the peri-operative period should be achieved to some extent.

The first 3 d after operation comprise the stress phase. Vascular recovery time varied from 36 h to 72 h after surgery. The transition points from positive to negative fluid balance also occurred. LT recipients need a large-dose methylprednisolone after operation, especially during the first few days. However, methylprednisolone would cause and aggravate water-sodium retention which may appear post-operatively, so the transition points would be postponed. Fluid balance during the first 3 d after surgery is crucial for recovery. If a positive fluid balance lasts too long, it is difficult for patients to recover. Thus, fluid bal-

ance should be properly managed as early as possible, and the quantity and time of negative fluid balance should be assessed individually.

Under the circumstances of stable hemodynamics, we should extrude the sequestered fluid from the peripheral circulation and the “third space” back to the central circulation. Whether or not a negative fluid balance is needed, the quantity and time of a negative fluid balance should be assessed by the following measurement: blood pressure, pulse, central venous pressure, HCT, the estimated volume of transfusion on the second day, and liquid intake and output volume of the preceding day. The fluid balance on the second post-operative day could be accomplished by adjusting the urine volume per hour. With administration of diuretics and colloid, urine volume could be controlled as needed. If hemodynamics were unstable, blood volume should be supplemented and inotropic drugs, such as dopamine, should be properly used.

We also found that Hb was an independent risk factor for post-operative pulmonary complications, which may be a significant factor for early recovery. When the patients with hepatic cirrhosis developed hypersplenism, which was always parallel with a reduction of hemocytes, their immune function became obviously lower and their hepatic function was poorer than other patients without a reduction of hemocytes. A low level of Hb may result in oxygen deficiency.

It is important to keep a fluid balance during the peri-operative period of LT. The precise and individualized fluid administration at the first 3 d after surgery significantly decreased the incidence of early pulmonary complications after LT. The strategies of an intra-operative transfusion  $\leq 100$  mL/kg and a fluid balance  $\leq -14$  mL/kg on the first day and the second or the third day after LT should be recommended. This study was limited by its small sample size. The shortcomings may limit the extrapolation of these results to all LT recipients. The hypothesis should be re-examined and verified in a much larger cohort before it is used for improving the prognosis and patient management. Nonetheless, if validated by future prospective studies, the fluid therapy used in this study would provide a simple and inexpensive method of augmenting the current prognostic indicators, and would be of obvious benefit for the anxious family members and care providers as well.

## COMMENTS

### Background

Liver transplantation (LT) is the optimal therapy for end-stage liver disease. Early-phase complications after LT are common and known to contribute significantly to morbidity and mortality of the patients. Individualized fluid therapy during the peri-operative period may be a significant strategy to achieve a better early-phase recovery after LT.

### Research frontiers

Recently, more attention has been paid to peri-operative fluid therapy. Fluid therapy is an important factor related to pulmonary complications and patient recovery.

### Innovations and breakthroughs

This research focused on the LT recipients with cirrhosis-associated hepatocellular carcinoma, and fluid was administrated based on the body weight in kilograms so as to guarantee a precise and individualized fluid therapy peri-operatively.

### Applications

It is important to offer a precise and individualized fluid therapy during the peri-operative period to the patients undergoing LT for cirrhosis-associated hepatocellular carcinoma. With an intra-operative transfusion  $> 100$  mL/kg and a fluid balance  $\leq 14$  mL/kg on the first day and the second or the third day after LT can significantly improve the early-phase recovery after LT.

### Terminology

The capillary leak syndrome is a rare condition characterized by recurrent episodes of generalized edema and severe hypotension associated with hypo-proteinemia. A shift of fluid and protein from the intravascular to the interstitial space results in hypovolaemia. Attacks vary in frequency, severity, and duration, and can be fatal.

### Peer review

Authors have investigated the clinical significance of the correlation between peri-operative fluid therapy and early-phase recovery after LT. The topic is interesting and offers points of reflection. The manuscript is original and may be useful to clinicians.

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## Acute chylous peritonitis due to acute pancreatitis

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Following abdominal lavage and drainage, the patient was successfully treated with total parenteral nutrition and octreotide.

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**Key words:** Chylous ascites; Chyloperitoneum; Chyle; Peritonitis; Pancreatitis

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### Abstract

We report a case of acute chylous ascites formation presenting as peritonitis (acute chylous peritonitis) in a patient suffering from acute pancreatitis due to hypertriglyceridemia and alcohol abuse. The development of chylous ascites is usually a chronic process mostly involving malignancy, trauma or surgery, and symptoms arise as a result of progressive abdominal distention. However, when accumulation of "chyle" occurs rapidly, the patient may present with signs of peritonitis. Pre-operative diagnosis is difficult since the clinical picture usually suggests hollow organ perforation, appendicitis or visceral ischemia. Less than 100 cases of acute chylous peritonitis have been reported. Pancreatitis is a rare cause of chyloperitoneum and in almost all of the cases chylous ascites is discovered some days (or even weeks) after the onset of symptoms of pancreatitis. This is the second case in the literature where the patient presented with acute chylous peritonitis due to acute pancreatitis, and the presence of chyle within the abdominal cavity was discovered simultaneously with the establishment of the diagnosis of pancreatitis. The patient underwent an exploratory laparotomy for suspected perforated duodenal ulcer, since, due to hypertriglyceridemia, serum amylase values appeared within the normal range. Moreover, abdominal computed tomography imaging was not diagnostic for pancreatitis.

### INTRODUCTION

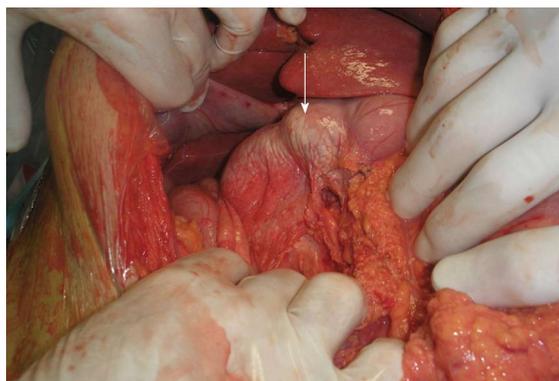
Accumulation of lymph within the peritoneal cavity is a rare pathological entity described as chylous ascites. Most cases occur progressively and the patient suffers from abdominal distention, nausea, vomiting, fatigue or low grade fever. A small number of cases have reported an acute development which may present as a surgical urgency mimicking appendicitis, hollow organ perforation and generalized peritonitis (acute chylous peritonitis). We hereby document a rare case of acute chylous peritonitis in a 46-year-old man with a history of alcoholism presenting with hypertriglyceridemia and acute pancreatitis.

### CASE REPORT

A 46-year-old man presented to the emergency department with an 8-h history of abdominal pain localized to the right abdomen. The pain was of acute onset and was primarily felt at the epigastrium. The patient also men-



**Figure 1** Preoperative abdominal computed tomography image showing inflammatory changes surrounding the head of the pancreas (arrow).



**Figure 2** Intraoperative photo showing the milky-like appearance of the wall of the distal portion of the stomach and the duodenum due to congestion of the intestinal lymphatic drainage (arrow).

tioned nausea and one episode of vomiting but no fever or diarrhea. Apart from a history of ankylosing spondylitis he also admitted systematical alcohol consumption over a period of several years (> 120 mg/d).

The patient's overall health condition was not gravely affected, showing a temperature of 37.2 °C, heart rate at 100 bpm and arterial pressure of 125/75 mmHg. On examination, no bowel sounds could be heard and right upper and lower abdominal quadrants appeared tender at palpation with rebound tenderness. Rectal digital examination did not reveal blood or tenderness.

Laboratory investigation showed no elevation of white blood cell count (7.740/mL with 57% polymorphonuclear leukocytes) while C-reactive protein values were only slightly affected (14 mg/L). The patient's biochemistry showed a mild elevation of hepatic enzymes (aspartate aminotransferase: 107 IU/L, alanine aminotransferase: 45 IU/L, alkaline phosphatase: 165 IU/L and total bilirubin: 1.6 mg/dL). Amylase values were normal both in blood (55 IU/L) and urine samples (404 IU/L). The laboratory notes mentioned that the blood sample of the patient was not totally appropriate for analysis because of a high concentration of lipids. Plain chest and abdominal X-rays (supine and elevated position) revealed no significant pathology such as free air or ileus. The computed tomography (CT) scan showed the presence of free fluid in the abdominal cavity, mainly at the subhepatic space and the right paracolic space, with mild inflammatory changes around the pancreatic head (Figure 1). No atrophy of the pancreas or calcification of the main pancreatic duct was demonstrated.

The patient was subsequently taken to the operating room for an exploratory laparotomy. In the peritoneal cavity a great amount of "milky" peritoneal fluid was discovered. Specimens were taken for biochemistry and microbiology. Careful examination of the abdomen revealed a bulky and rigid pancreatic head. Moreover, the surface of the distal stomach, duodenum and upper segment of posterior peritoneum had a white, milky-like appearance (Figure 2). A laceration in the peritoneum (at the root of the transverse mesocolon) was discovered, through which the milky fluid, apparently of retroperitoneal origin, entered the peritoneal cavity. Attempts to obtain a biopsy

specimen from the pancreas (with a biopsy needle) were unsuccessful because of hemorrhage after the first attempt due to the pancreatic edema. After a thorough peritoneal lavage and the insertion of 3 drain tubes the midline incision was closed.

During the initial postoperative days, serum samples from the patient appeared to be of high lipidemic concentration, with normal serum amylase values. Only after serial dilutions with the assay buffer were we able to obtain a proper sample for analysis, showing serum amylase values of 870 IU/L. Total cholesterol reached as high as 618 IU/L (normal values up to 200) and triglycerides had a concentration greater than 1.000 mg/dL (normal values up to 175). No elevation of white blood cell count was documented at any time. Analysis of samples from free peritoneal fluid that was collected intraoperatively showed 337 mg/dL cholesterol and 2800 mg/dL triglyceride thus establishing the diagnosis of chylous ascites. Laboratory investigation of the fluid from the drain tubes showed values of amylase to be as high as 60 000 IU/L, decreasing to normal at postoperative day 7, at which time all of the tubes had been removed. Cancer markers  $\alpha$ -fetoprotein, carcinoembryonic antigen and carbonic anhydrase 19-9 were within normal values.

The patient received nothing po for 10 d and then was restored gradually to a full (fat-free) diet. Total fat-free parenteral nutrition was given intravenously from postoperative day 4 and for the subsequent 2 wk. Broad spectrum antibiotics were administered for 1 wk postoperatively and were not stopped because the patient suffered outbursts of fever during the end of the first week. Twenty five days after the first operation a second procedure was undertaken, this time due to persisting ileus. The laparotomy revealed adhesions in the peritoneal cavity. Octreotide (0.1 mg) was administered every 8 h from the day of the first surgery until the time of discharge, which came to be after 33 d of hospitalization.

## DISCUSSION

Under normal circumstances, lymph from the lower parts of the body as well as from the viscera is circulated

through lymphatic vessels that follow a retroperitoneal course before emptying in the cisterna chyli and finally the thoracic duct and the venous system. This fluid consists of converted long-chain triglycerides at high concentrations that originate from the gut during ingestion. In cases where a disruption to this normal flow occurs, the peritoneal cavity may be filled with a high-density, milky-like fluid that is called “chyle”. Although there are no unequivocal diagnostic criteria, it is generally agreed that a high concentration of lipids is indicative<sup>[1]</sup>. A sample of ascites fluid (either acquired by paracentesis or during laparotomy) showing values of triglycerides 2 to 8 times that of plasma is characterized as chyle and the situation “chylous ascites” or “chyloperitoneum”. Some authors have set absolute indicators such as a peritoneal lipid content greater than 200 mg/dL<sup>[2]</sup>. Other characteristics of chyle are a protein concentration ratio > 0.5 compared to that of plasma, a low cholesterol level (lower than plasma) and elevated amylase values in case of pancreatitis<sup>[3]</sup>.

The formation of chylous ascites is usually a chronic procedure, and the patient typically mentions symptoms of progressive and painless distention of the abdomen for some time before the diagnosis is established. Multiple causes for this relatively rare pathological entity have been described. Aalami *et al*<sup>[1]</sup> present a detailed classification of them in a comprehensive review published in 2000. Whereas congenital abnormalities in the formation of the lymphatic vessels (lymphatic hypoplasia or lymphangiectasia) are a frequent cause in infants, surgery and malignancy (especially lymphoma) are among the most common causes in adults<sup>[4]</sup>. In particular, surgery involving the thoracic cavity or the aorta and the retroperitoneal space has often been associated with the pathogenesis of chylous ascites by means of interrupting the normal lymphatic drainage<sup>[5]</sup>. Chyloperitoneum may also be the result of trauma to the intestines or mesentery. Idiopathic retroperitoneal fibrosis, sarcoidosis and abdominal or pelvic radiation therapy have also been mentioned in the literature. Infectious diseases are another possible cause, such as filariasis in tropical countries, and tuberculosis mostly in countries with low social and economical level<sup>[1]</sup>.

However, in rare cases the accumulation of chyle within the peritoneal cavity may occur rapidly and the patient may present with symptoms and signs of acute abdomen<sup>[6]</sup>. Vettoretto *et al*<sup>[6]</sup> found less than a 100 cases of acute chylous peritonitis in their review of 2008. The pain appears to be diffuse, possibly due to peritoneal distention and irritation of the root of the mesentery as the retroperitoneal space expands, since the fluid itself is not irritating to the peritoneum. During clinical examination, rebound tenderness and guarding may be documented, which is often localized at the right iliac fossa and this can possibly be explained by pooling of chyle at the right paracolic gutter. Thus the clinical picture may be misleading, with appendicitis, hollow organ perforation and visceral ischemia being the most commonly suspected diagnosis preoperatively<sup>[3,7-9]</sup>. Chyloperitoneum is usually discovered during exploratory laparotomy, and in some

cases this is the only intraoperative finding. Negative laparotomies have been reported<sup>[3,6,10,11]</sup>, in which the underlying cause was never discovered<sup>[11]</sup>.

Pancreatitis is a rare cause of chylous ascites formation<sup>[12]</sup>. It is believed that either lymph may actually leak through destroyed lymphatics due to pancreatic enzyme erosion or that chylous accumulation is the result of exudation of chyle, caused by the obstruction of lymphatic channel flow secondary to severe inflammatory changes that take place in the retroperitoneal space surrounding the pancreas<sup>[1]</sup>. Most cases involve chronic pancreatitis<sup>[1,13]</sup>, though acute pancreatitis has also been recognized as the causative reason, with the first such report dating back to 1984<sup>[12]</sup>. Since then, only a few cases of chylous ascites secondary to acute pancreatitis have been documented<sup>[8,13-15]</sup>. In almost all of them, the presence of chyle into the peritoneal cavity was discovered at some time after the episode of pancreatitis, usually days or weeks<sup>[13-15]</sup>. However, Khan *et al*<sup>[16]</sup> reported a case of acute hyperlipidemic pancreatitis-with normal serum amylase as in our case-that presented with acute chylous peritonitis and was treated conservatively. Smith *et al*<sup>[3]</sup> operated on a patient with relapsing pancreatitis and acute chylous ascites formation, due to a clinical resemblance with appendicitis.

Therapeutic choices may vary in accordance with the underlying pathology. Thorough lavage of the abdomen and adequate drainage has proven to be an excellent treatment modality for acute chylous peritonitis, since resolution of chylous ascites usually occurs within the next few days. However, successful conservative treatment has also been reported<sup>[13,16-18]</sup>. This requires proper preoperative diagnosis, which is often difficult due to the exceptional rarity of this pathological condition and its resemblance to other surgical urgencies that call for immediate laparotomy. Long-term fasting supported by total parenteral nutrition offers resolution in many cases. Alternatively, a high-protein and low fat diet has proven to be efficacious in reducing the amount of chyle produced. Administration of octreotide remains controversial<sup>[1,13]</sup>.

In our case, the localization of the pain at the epigastrium and its later course mimicking peritonitis, together with the mild inflammatory changes of the pancreatic head demonstrated on CT imaging, as well as normal amylase values, drew the attention towards a possible duodenal perforation, thus leading the patient to the operating room. Only in the subsequent day and after serial dilutions of the serum samples with the assay buffer were we able to detect an abnormally high level of serum amylase. The interference of excessive serum triglyceride concentrations in the measurement of serum amylase is a known reason for false-negative results and this was also the case with our patient<sup>[16]</sup>. Moreover, routine serum lipase measurement is not available in our hospital, which could otherwise serve as an adjunct in the diagnosis of acute pancreatitis. However, it should be noted that serum lipase levels too can appear normal in patients with triglyceride-rich serum<sup>[16]</sup>. In our patient, peritoneal lavage and adequate drainage offered sufficient treatment, while

the patient was gradually restored to a full fat-free diet in order to deal with the pancreatitis. Finally, the patient was encouraged to cease alcohol use.

In conclusion, acute abdominal pain due to sudden accumulation of chyle in the peritoneal cavity is a rare situation that the clinician should be aware of in cases of acute abdomen.

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## Paroxysmal drastic abdominal pain with tardive cutaneous lesions presenting in Henoch-Schönlein purpura

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### Abstract

Henoch-Schönlein purpura (HSP) is a small-vessel vasculitis mediated by IgA-immune complex deposition. It is characterized by the clinical tetrad of non-thrombocytopenic palpable purpura, abdominal pain, arthritis and renal involvement. The diagnosis of HSP is difficult, especially when abdominal symptoms precede cutaneous lesions. We report a rare case of paroxysmal drastic abdominal pain with gastrointestinal bleeding presented in HSP. The diagnosis was verified by renal damage and the occurrence of purpura.

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**Key words:** Henoch-Schönlein purpura; Abdominal pain; Gastrointestinal bleeding

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Chen XL, Tian H, Li JZ, Tao J, Tang H, Li Y, Wu B. Paroxysmal drastic abdominal pain with tardive cutaneous lesions presenting in Henoch-Schönlein purpura. *World J Gastroenterol* 2012; 18(16): 1991-1995 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1991.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1991>

### INTRODUCTION

Henoch-Schönlein purpura (HSP) is the most common systemic vasculitis in children. The diagnostic criteria include palpable purpura with at least one other manifestation including abdominal pain, IgA deposition, arthritis or arthralgia, and renal involvement<sup>[1,2]</sup>. Immune complex deposits induce necrosis of the walls of small- and medium-sized arteries with infiltration of the tissue by neutrophils and the deposition of nuclear fragments, a process called leukocytoclastic vasculitis<sup>[3]</sup>. It is often associated with infection, certain medications, or tumors. It may coexist with or mimic Crohn's disease<sup>[4]</sup>. Periumbilical and epigastric pain worsen with meals due to bowel angina. Bleeding is usually occult or, less commonly, associated with melena. Intussusception is the most common surgical complication. Perforations, usually ileal, may occur spontaneously or be associated with intussusception. An ultrasound, recommended as the first diagnostic test, and computed tomography (CT) scans may reveal intussusception and asymmetric bowel wall thickening mainly involving the jejunum and ileum. There are a range of

possible endoscopic findings including gastritis, duodenitis, ulceration, and purpura, with the second portion of the duodenum characteristically being involved more than the bulb. Intestinal biopsies show IgA deposition and leukocytoclastic vasculitis in the submucosal vessels<sup>[3]</sup>. Superficial biopsies may show inflammation, ulceration, edema, hemorrhage, and vascular congestion, presumably due to vasculitis-induced mucosal ischemia<sup>[5]</sup>. The efficacy of corticosteroids in preventing severe complications or relapses is controversial. The majority of patients, however, improve spontaneously.

## CASE REPORT

A 15-year-old boy was referred from another hospital to our institution in November 2010. He was previously healthy without any remarkable past medical history and denied any recent non-steroidal anti-inflammatory drug use or illicit drug use. He complained of progressive epigastrium and periumbilical pain, nausea and mild diarrhea with melena that had lasted for 3 wk. No rash was noticed on the skin. On physical examination, his vital signs were normal, except for epigastric pressing pain and suspicious rebound pain. Importantly, no skin rash was observed.

Laboratory blood examinations showed the following indexes (normal range in parentheses): hemoglobin, 109 g/L (120-140 g/L); peripheral white cell count,  $20.09 \times 10^9/L$  ( $5-10 \times 10^9/L$ ); neutrophils, 83.7% (40%-60%); peripheral red cell count,  $5.43 \times 10^{12}/L$  ( $4.0-4.5 \times 10^{12}/L$ ); platelet count,  $267 \times 10^9/L$  ( $100-300 \times 10^9/L$ ); C-reactive protein, 68.0 mg/L (0-6.0 mg/L); erythrocyte sedimentation rate, 14 mm/h (0-20 mm/h); albumin, 24.8 mg/L (36-51 mg/L); and total immunoglobulin (Ig), 17.6 mg/L (25.0-35.0 mg/L); IgA, 2.292 g/L (0.7-3.3 g/L); total bilirubin, 6.3  $\mu\text{mol}/L$  (4-23.9  $\mu\text{mol}/L$ ); alkaline phosphatase, 38 U/L (35-125 U/L); c-glutamyl transpeptidase, 12 U/L (7-50 U/L); aspartate aminotransferase, 15 U/L (14-40 U/L); alanine aminotransferase, 21 U/L (5-35 U/L); prothrombin time, 13.8 s (11.0-14.5 s); creatinine, 453  $\mu\text{mol}/L$  (31.8-91.0  $\mu\text{mol}/L$ ), and blood urine nitrogen, 33 g/L (31.8-91.0 g/L). A routine urine test did not reveal any RBCs or proteins on the first day of hospitalization. Autoimmune-related indicators and tuberculosis (TB)-related antibodies were not found in the blood, and the TB-purified protein derivative (PPD) skin test was negative. Hepatitis B and C markers were also negative.

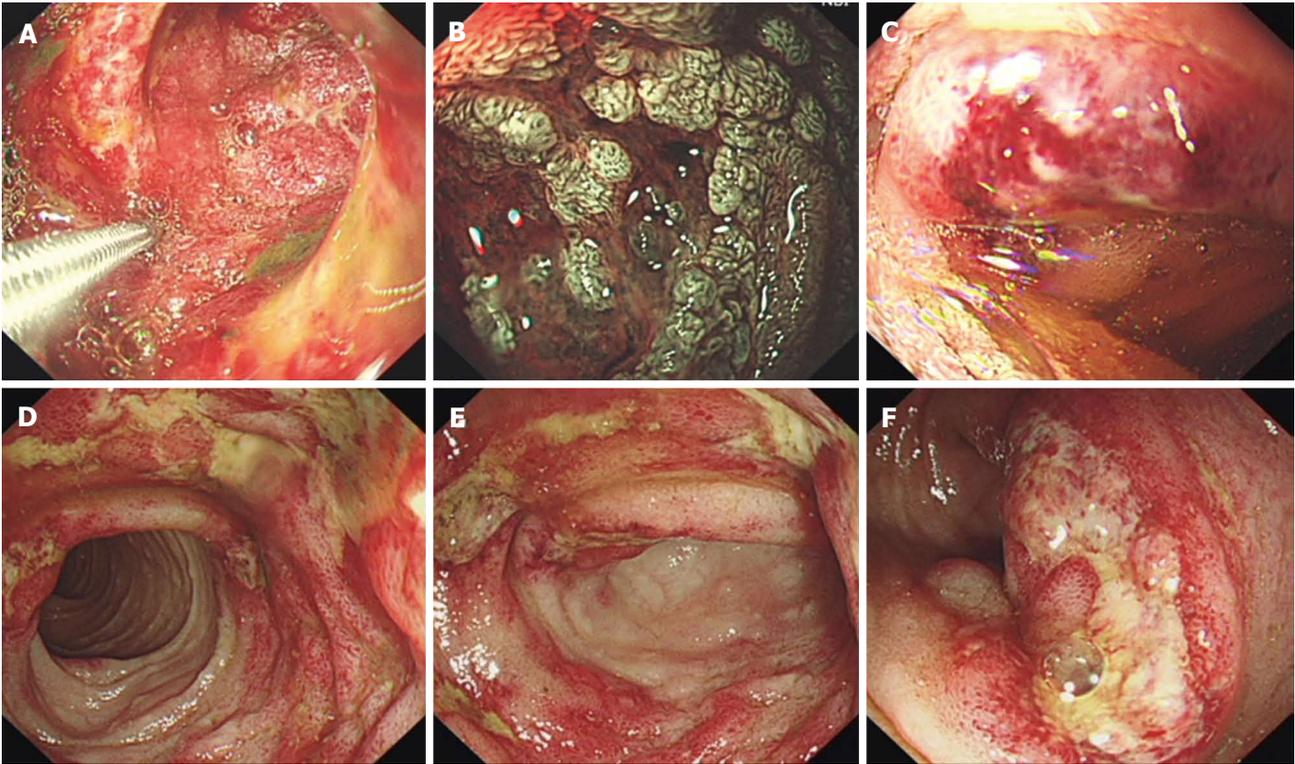
Plain abdominal radiography revealed incomplete small-intestine obstruction. Gastroscopy and colonoscopy revealed some mucosal swelling, erosion, and active ulcers with hemorrhage in the duodenum and terminal ileum (Figure 1), but not in the stomach or colon. The histopathology of duodenal mucosa and ileal mucosa showed a chronic mucosal inflammation with necrosis (data not shown). Ultrasound endoscopy (EUS) and abdominal CT examinations revealed the presence of thickened intestinal mucosa with submucosal hemorrhage and enlarged mesenteric and retroperitoneal lymph nodes (Figure 2). EUS-guided fine-needle aspiration (EUS-FNA)

biopsy of the enlarged lymph nodes revealed an inflammatory reaction without lymphoma (data not shown). Positron emission tomography and computed tomography also indicated that the enlarged lymph nodes were a sign of inflammation, not of a malignant tumor (data not shown).

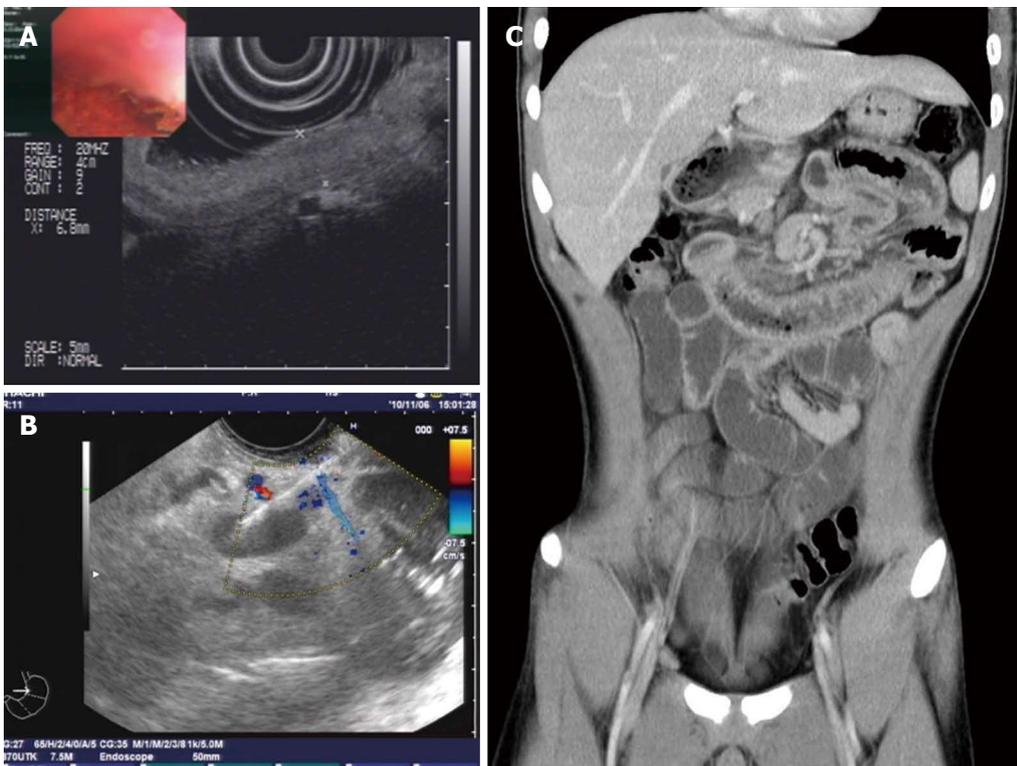
During the first two weeks of hospitalization, the patient accepted treatment with antibiotics and a proton pump inhibitor (pantoprazole 40 mg, twice daily), but his abdominal pain was not alleviated, and the paroxysmal abdominal pain worsened progressively. After two weeks of hospitalization, the patient's urine test revealed significant albuminuria. Levels of urine albumin increased to 1170 mg/L (0-20 mg/L), and 24-h total urine protein increased to 4.08 g (0-0.12 g). Further, the urine protein analysis showed a remarkable increase in kappa and lambda light chain levels to 62.5 mg/L (0-7.1 mg/L) and 28.8 mg/L (0-3.9 mg/L), respectively. The level of urine IgA increased to 102 mg/L (0-17.5 mg/L). Therefore, the diagnoses were considered to be an autoimmune-related disorder such as Crohn's disease and multiple vasculitis or lymphoma. The patient was treated with corticosteroid therapy in the form of intravenous methylprednisolone (40 mg/d). By the next day, the drastic abdominal pain was rapidly alleviated, and the melena also gradually disappeared. After one week of methylprednisolone therapy, the treatment was changed to the oral administration of prednisolone therapy (30 mg/d). During the fourth week of corticosteroid therapy, palpable purpura appeared over the patient's lower extremities. A diagnosis of HSP was ultimately established. After four weeks of corticosteroid treatment, endoscopy and EUS showed that the patient's mucosal damage had improved significantly, and the mucosal edema was observably mitigated (Figure 3). A percutaneous renal biopsy revealed focal segmental endocapillary crescent-like proliferation (Figure 4). Positive immunofluorescence staining for mesangial IgA was also observed (data not shown). The histopathology of renal biopsies supported the diagnosis of anaphylactoid purpura nephritis (APN), which was classified as ISKDC IV according to the classification criteria of the International Study of Kidney Disease in Children<sup>[6]</sup>.

## DISCUSSION

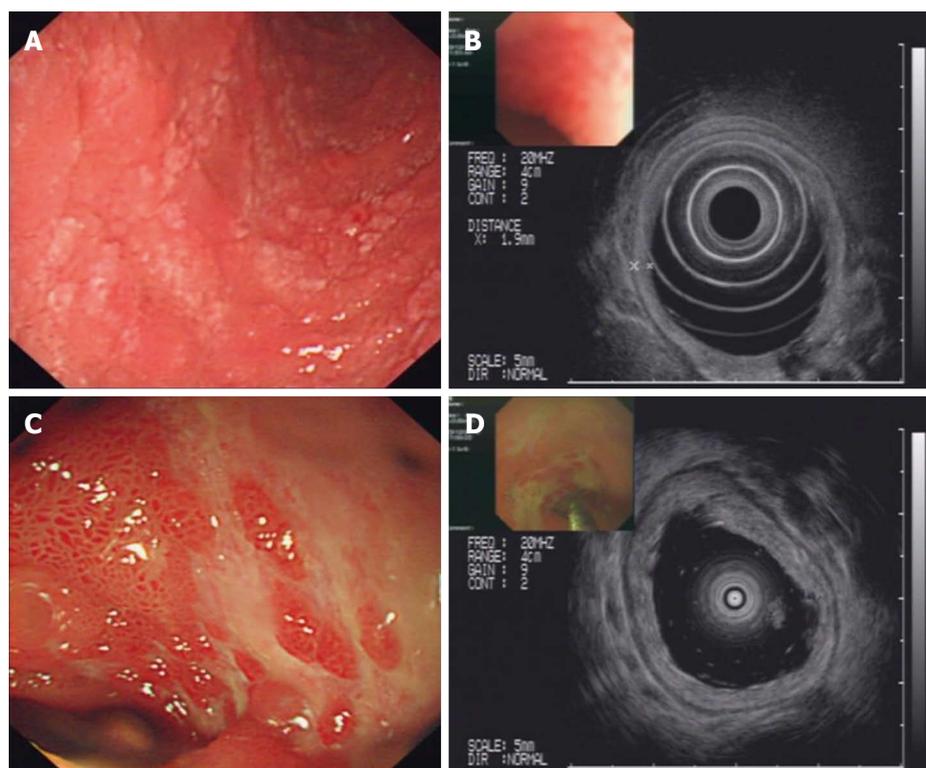
HSP is a form of blood vessel inflammation or vasculitis, also referred to as anaphylactoid purpura. Vasculitis is involved in many conditions<sup>[1]</sup>. Each of the forms of vasculitis tends to involve certain characteristic blood vessels. HSP is a multisystem disorder predominantly affecting the skin, joints, gastrointestinal tract and kidneys, but other organs can be affected as well. Neurological, pulmonary, cardiac and genitourinary complications rarely occur. HSP results in a skin rash (most prominent over the buttocks and behind the lower extremities) associated with joint inflammation (arthritis) and sometimes cramping pain in the abdomen. The condition primarily affects children (over 90% of cases); occurrences in adults have



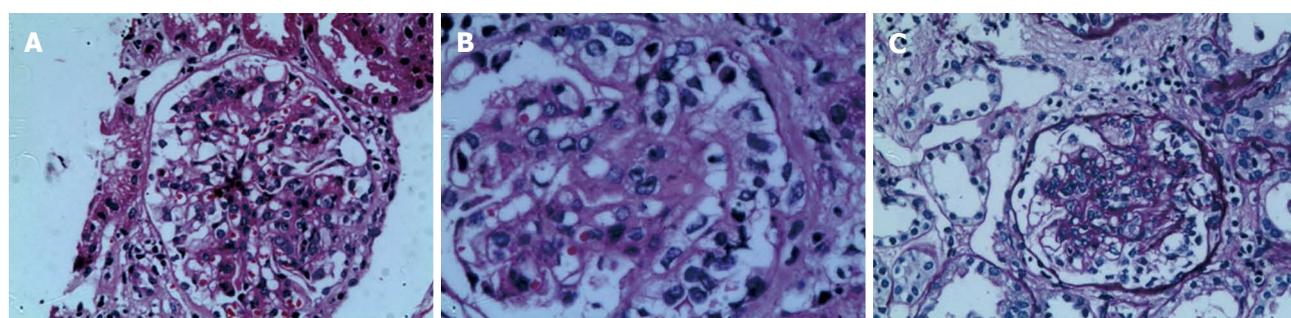
**Figure 1** The endoscopy examination revealed intestinal damage. A-C are gastroscopic images, which show significant damage to the duodenum. A: Diffuse redness, swelling, hemorrhage and petechiae in the mucosa; B: Distortion and proliferation of the duplicature (narrow-band image); C: Ulcer; D-F: Colonoscopic images and demonstrate significant damage to the terminal ileum; D: Mucosal hemorrhage and petechiae; E, F: Ulcers.



**Figure 2** The ultrasound endoscopy and the computed tomography scan indicated the presence of thickened intestinal mucosa and enlarged abdominal lymph nodes. A, B: Ultrasound endoscopy scan images; C: Computed tomography scan images.



**Figure 3** The extent of damage to the intestinal mucosa improved after treatment. A: Gastroscopic image of the duodenum; B: Ultrasound endoscopy (EUS) image of the duodenum; C: Colonoscopic image of the terminal ileum; D: EUS image of the terminal ileum.



**Figure 4** A percutaneous renal biopsy revealed focal segmental endocapillary crescent-like proliferation. A: HE staining, 200 ×; B: HE staining, 400 ×; C: Periodic acid-Schiff staining, 200 ×. HE: Hematoxylin and eosin.

rarely been reported (3.4 to 14.3 cases per million)<sup>[3]</sup>. HSP occurs most often in the spring and frequently follows an infection of the throat or breathing passages. HSP seems to represent an unusual reaction of the body's immune system in response to this infection (either bacterial or viral). In addition to infection, drugs can trigger the condition. HSP occurs most commonly in children, but people of all age groups can be affected.

HSP is usually diagnosed based on skin, joint, and kidney findings. Throat culture, urinalysis, and blood tests for inflammation and kidney function are used to determine the diagnosis. A biopsy of the skin, and less commonly, the kidneys, can be used to demonstrate the presence of vasculitis. Special staining techniques (direct immunofluorescence) of the biopsy specimen can be used to document antibody deposits of IgA in the blood

vessels of involved tissue. Renal involvement is rarely severe and is observed in approximately 50% of patients<sup>[1,2]</sup>.

According to the diagnostic criteria of the European League against Rheumatism and the Paediatric Rheumatology European Society published in 2006, palpable purpura often presents with one of the following: diffuse abdominal pain, a biopsy showing predominant IgA expression, acute arthritis/arthralgia, or renal involvement defined as any hematuria or proteinuria. However, when gastrointestinal manifestations occur alone or precede dermatological or renal disease, the diagnosis is difficult<sup>[7,8]</sup>. Typically, palpable purpura will not precede renal involvement<sup>[9,10]</sup>. However, in this case, purpura occurred during the seventh week of onset, and renal involvement occurred during the fourth week of onset, which delayed the diagnosis of HSP. HPS may coexist with Crohn's

disease or mimic its symptoms (e.g., ileitis or ulcerative colitis)<sup>[4,11,12]</sup>. The clinical manifestations of HSP in the gastrointestinal tract are similar to gastrointestinal tuberculosis, lymphoma, inflammatory bowel disease, and other autoimmune disorders, which renders the diagnosis of HSP difficult. In this case, paroxysmal drastic abdominal pain with gastrointestinal bleeding was presented as the princeps clinical situation. The characteristic endoscopic findings included diffuse mucosal redness, small ring-like petechiae, hemorrhagic erosions and ulcers. These manifestations created confusion until the diagnosis of HSP was verified by the presence of renal damage and purpura. Mucosal lesions develop anywhere within the gastrointestinal tract. The small intestine is considered to be the most frequently affected site, and duodenal involvement was more prominent in the second part of the duodenum than in the bulb and stomach or colon.

Gastrointestinal involvement is frequent in HSP. The diagnosis of HSP may be difficult, especially when abdominal symptoms precede the characteristic palpable purpura. Typical endoscopic findings may alert gastroenterologists to the need to consider this diagnosis early in treatment and thus avoid unnecessary laparotomy.

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## Melena-associated regional portal hypertension caused by splenic arteriovenous fistula

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### Abstract

Regional portal hypertension is a rare cause of upper gastrointestinal bleeding. We reported an extremely rare case in which regional portal hypertension was associated with both the splenic arteriovenous fistula and chronic pancreatitis. In June 2010, our patient, a 41-year-old man, was admitted to a local hospital due to a sudden melena and dizziness without haematemesis and jaundice. The splenic arteriovenous fistula in this patient was successfully occluded through transcatheter arterial embolization. At the 12-mo follow-up, our patient was in good condition.

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**Key words:** Regional portal hypertension; Splenic arteriovenous fistulas; Pancreatitis; Transcatheter arterial embolization; Upper gastrointestinal bleeding

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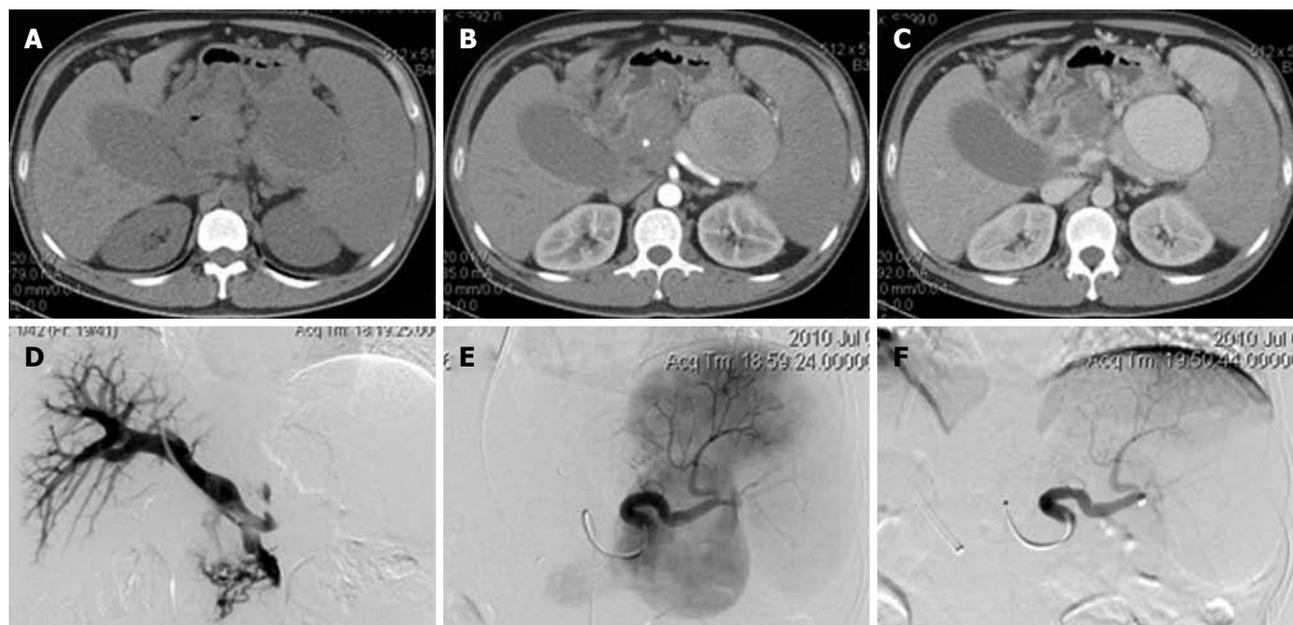
Chen B, Tang CW, Zhang CL, Cao JW, Wei B, Li X. Melena-associated regional portal hypertension caused by splenic arteriovenous fistula. *World J Gastroenterol* 2012; 18(16): 1996-1998  
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i16/1996.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i16.1996>

### INTRODUCTION

Regional portal hypertension is a rare cause of upper gastrointestinal bleeding, with pancreatitis disease being the most frequently reported cause in the literature<sup>[1,2]</sup>. Splenic arteriovenous fistulas are also rare. Until now, there are approximately 126 reported cases of splenic arteriovenous fistula in the database of PubMed. Herein, we reported an extremely rare case in which regional portal hypertension was associated with both the splenic arteriovenous fistula and chronic pancreatitis.

### CASE REPORT

A 41-year-old man was admitted to a local hospital in June 2010 due to a sudden melena and dizziness without haematemesis and jaundice. He was managed conservatively, with fluid support and blood transfusion. No melena occurred again, and the symptom of dizziness was reduced. With the melena of unknown causes, he was referred to our institution for further diagnosis and treatment. On admission, his temperature was 36.7 °C, pulse was 85 times/min, respiration was 19 times/min, and blood pressure was 124/76 mmHg. Splenomegaly was found in physical examination. The liver was not palpable and no signs of jaundice were observed. The patient had a history of alcohol abuse, but no history of liver disease, trauma and surgery. Five years ago, the patient had an acute pancreatitis which recurred for five times. The details of laboratory tests were as follows: red blood



**Figure 1** The main findings of enhanced computed tomography and angiography. A: An oval cystic low-density lesion located in the area of splenic hilum; B: The lesion was enhanced significantly on the arterial phase; C: Significantly enhanced on the portal phase of contrast-enhanced scan; D: A normal portal pressure and an increased pressure of splenic vein of 23.5 mmHg; E: Splenic arteriovenous fistula manifested by angiography; F: Splenic arteriovenous fistula being totally occluded after embolization.

cell  $4.13 \times 10^{12}/L$ , hemoglobin 119 g/L, platelet  $246 \times 10^9/L$ , white blood cell  $6.90 \times 10^9/L$ , total bilirubin  $13.3 \mu\text{mol}/L$ , albumin 42.9 g/L, creatinine (CREA)  $572.9 \mu\text{mol}/L$ , glucose (GLU)  $0.953 \text{ mmol}/L$ , GLU120  $21.75 \text{ mmol}/L$ , prothrombin time 12.0 s, and alpha-fetoprotein 1.36 ng/mL. Tests for HBsAg, HBeAg, anti-HBe, anti-HBc, anti-HCV and anti-HBs were all negative, except for anti-HBs. Gastroscopy revealed severe gastric varices and non-atrophic gastritis with bile reflux. Unenhanced computed tomography (CT) scanning of upper abdomen showed an oval cystic low-density lesion located in the area of splenic hilum, which was easily misdiagnosed as pancreatic pseudocyst on unenhanced CT (Figure 1A) and as pseudoaneurysm on contrast-enhanced scan. The lesion was significantly enhanced on the arterial phase of contrast-enhanced scan (Figure 1B). And on the portal phase of contrast-enhanced CT scan, this lesion was further enhanced and collateral vessels could be shown at the fundus of the stomach (Figure 1C). Additionally, thrombosis in the splenic vein was also found on the portal phase of contrast-enhanced CT scan. Biopsy of the liver was not performed. The current diagnosis was regional portal hypertension considered to be related to chronic pancreatitis. Under local anesthesia, a percutaneous transjugular approach was used to estimate the portal venous pressure; the portal pressure was normal and an increased pressure of splenic vein was 23.5 mmHg (Figure 1D). Through a percutaneous femoral approach, splenic artery angiography showed, on the arterial phase, a smaller splenic artery, splenic vein aneurysmal expansion, and esophageal and gastric varices, suggesting the formation of splenic arteriovenous fistula (Figure 1E).

With splenic artery being extremely tortuous, it was difficult for conventional catheter to track the orifice of the fistula through target vessels, therefore embolization of the fistula was performed using a micro-catheter. Post-operative angiography revealed that splenic arteriovenous fistula had been totally occluded (Figure 1F).

## DISCUSSION

Regional portal hypertension, also known as sinistral or left-sided portal hypertension, is a rare cause of upper gastrointestinal bleeding, with pancreatitis disease being the most frequently reported cause in the literature<sup>[1,2]</sup>. Splenic arteriovenous fistula is a rare but potentially curable cause of portal hypertension. The fistula may be congenital or acquired. It is thought that they arise from rupture of a splenic artery aneurysm or after abdominal trauma, surgery or pancreatitis<sup>[3]</sup>. To our best knowledge, splenic arteriovenous fistulas related to pancreatitis have been rarely reported and its pathogenesis remains unclear<sup>[4]</sup>. In this case, the fistula arose from pancreatitis, and regional portal hypertension was caused by splenic arteriovenous fistula and splenic vein thrombosis, with the former playing a major role. As for regional portal hypertension, the key point is to make the correct diagnosis as soon as possible, because it is curable. It should be considered in the presence of gastrointestinal bleeding with normal liver function tests and splenomegaly. Surgical excision of splenic arteriovenous fistula is technically difficult, and is sometimes unsuccessful because of the remote location of the lesion, presence of numerous portal collaterals, and adhesion. Interventional radiologic technique, such as transcatheter arterial embolization, has

been demonstrated to be a safe and effective alternative to surgery<sup>[5]</sup>. The splenic arteriovenous fistula in our patient was successfully occluded through transcatheter arterial embolization. At the 12-mo follow-up, this patient was found in good condition.

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## Events Calendar 2012

January 13-15, 2012  
 Asian Pacific *Helicobacter pylori*  
 Meeting 2012  
 Kuala Lumpur, Malaysia

January 19-21, 2012  
 American Society of Clinical  
 Oncology 2012 Gastrointestinal  
 Cancers Symposium  
 San Francisco, CA 3000,  
 United States

January 19-21, 2012  
 2012 Gastrointestinal Cancers  
 Symposium  
 San Francisco, CA 94103,  
 United States

January 20-21, 2012  
 American Gastroenterological  
 Association Clinical Congress of  
 Gastroenterology and Hepatology  
 Miami Beach, FL 33141,  
 United States

February 3, 2012  
 The Future of Obesity Treatment  
 London, United Kingdom

February 16-17, 2012  
 4th United Kingdom Swallowing  
 Research Group Conference  
 London, United Kingdom

February 23, 2012  
 Management of Barretts  
 Oesophagus: Everything you need  
 to know  
 Cambridge, United Kingdom

February 24-27, 2012  
 Canadian Digestive Diseases Week  
 2012  
 Montreal, Canada

March 1-3, 2012  
 International Conference on  
 Nutrition and Growth 2012  
 Paris, France

March 7-10, 2012  
 Society of American Gastrointestinal  
 and Endoscopic Surgeons Annual  
 Meeting  
 San Diego, CA 92121, United States

March 12-14, 2012  
 World Congress on  
 Gastroenterology and Urology  
 Omaha, NE 68197, United States

March 17-20, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 Orlando, FL 32808, United States

March 26-27, 2012  
 26th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

March 30-April 2, 2012  
 Mayo Clinic Gastroenterology and  
 Hepatology  
 San Antonio, TX 78249,  
 United States

March 31-April 1, 2012  
 27th Annual New Treatments in  
 Chronic Liver Disease  
 San Diego, CA 92121, United States

April 8-10, 2012  
 9th International Symposium on  
 Functional GI Disorders  
 Milwaukee, WI 53202, United States

April 13-15, 2012  
 Asian Oncology Summit 2012  
 Singapore, Singapore

April 15-17, 2012  
 European Multidisciplinary  
 Colorectal Cancer Congress 2012  
 Prague, Czech

April 18-20, 2012  
 The International Liver Congress  
 2012  
 Barcelona, Spain

April 19-21, 2012  
 Internal Medicine 2012  
 New Orleans, LA 70166,  
 United States

April 20-22, 2012  
 Diffuse Small Bowel and Liver  
 Diseases  
 Melbourne, Australia

April 22-24, 2012  
 EUROSON 2012 EFSUMB Annual

Meeting  
 Madrid, Spain

April 28, 2012  
 Issues in Pediatric Oncology  
 Kiev, Ukraine

May 3-5, 2012  
 9th Congress of The Jordanian  
 Society of Gastroenterology  
 Amman, Jordan

May 7-10, 2012  
 Digestive Diseases Week  
 Chicago, IL 60601, United States

May 17-21, 2012  
 2012 ASCRS Annual Meeting-  
 American Society of Colon and  
 Rectal Surgeons  
 Hollywood, FL 1300, United States

May 18-19, 2012  
 Pancreas Club Meeting  
 San Diego, CA 92101, United States

May 18-23, 2012  
 SGNA: Society of Gastroenterology  
 Nurses and Associates Annual  
 Course  
 Phoenix, AZ 85001, United States

May 19-22, 2012  
 2012-Digestive Disease Week  
 San Diego, CA 92121, United States

June 2-6, 2012  
 American Society of Colon and  
 Rectal Surgeons Annual Meeting  
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 United States

June 18-21, 2012  
 Pancreatic Cancer: Progress and  
 Challenges  
 Lake Tahoe, NV 89101, United States

July 25-26, 2012  
 PancreasFest 2012  
 Pittsburgh, PA 15260, United States

September 1-4, 2012  
 OESO 11th World Conference  
 Como, Italy

September 6-8, 2012  
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Neurogastroenterology and Motility  
 Meeting  
 Bologna, Italy

September 7-9, 2012  
 The Viral Hepatitis Congress  
 Frankfurt, Germany

September 8-9, 2012  
 New Advances in Inflammatory  
 Bowel Disease  
 La Jolla, CA 92093, United States

September 8-9, 2012  
 Florida Gastroenterologic Society  
 2012 Annual Meeting  
 Boca Raton, FL 33498, United States

September 15-16, 2012  
 Current Problems of  
 Gastroenterology and Abdominal  
 Surgery  
 Kiev, Ukraine

September 20-22, 2012  
 1st World Congress on Controversies  
 in the Management of Viral Hepatitis  
 Prague, Czech

October 19-24, 2012  
 American College of  
 Gastroenterology 77th Annual  
 Scientific Meeting and Postgraduate  
 Course  
 Las Vegas, NV 89085, United States

November 3-4, 2012  
 Modern Technologies in  
 Diagnosis and Treatment of  
 Gastroenterological Patients  
 Dnepropetrovsk, Ukraine

November 4-8, 2012  
 The Liver Meeting  
 San Francisco, CA 94101,  
 United States

November 9-13, 2012  
 American Association for the Study  
 of Liver Diseases  
 Boston, MA 02298, United States

December 1-4, 2012  
 Advances in Inflammatory Bowel  
 Diseases  
 Hollywood, FL 33028, United States

**GENERAL INFORMATION**

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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. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar 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]

*No volume or issue*

- 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 dis-

eases. *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/index.htm>

**Patent (list all authors)**

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

**Statistical data**

Write as mean  $\pm$  SD or mean  $\pm$  SE.

**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).

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

**Italics**

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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