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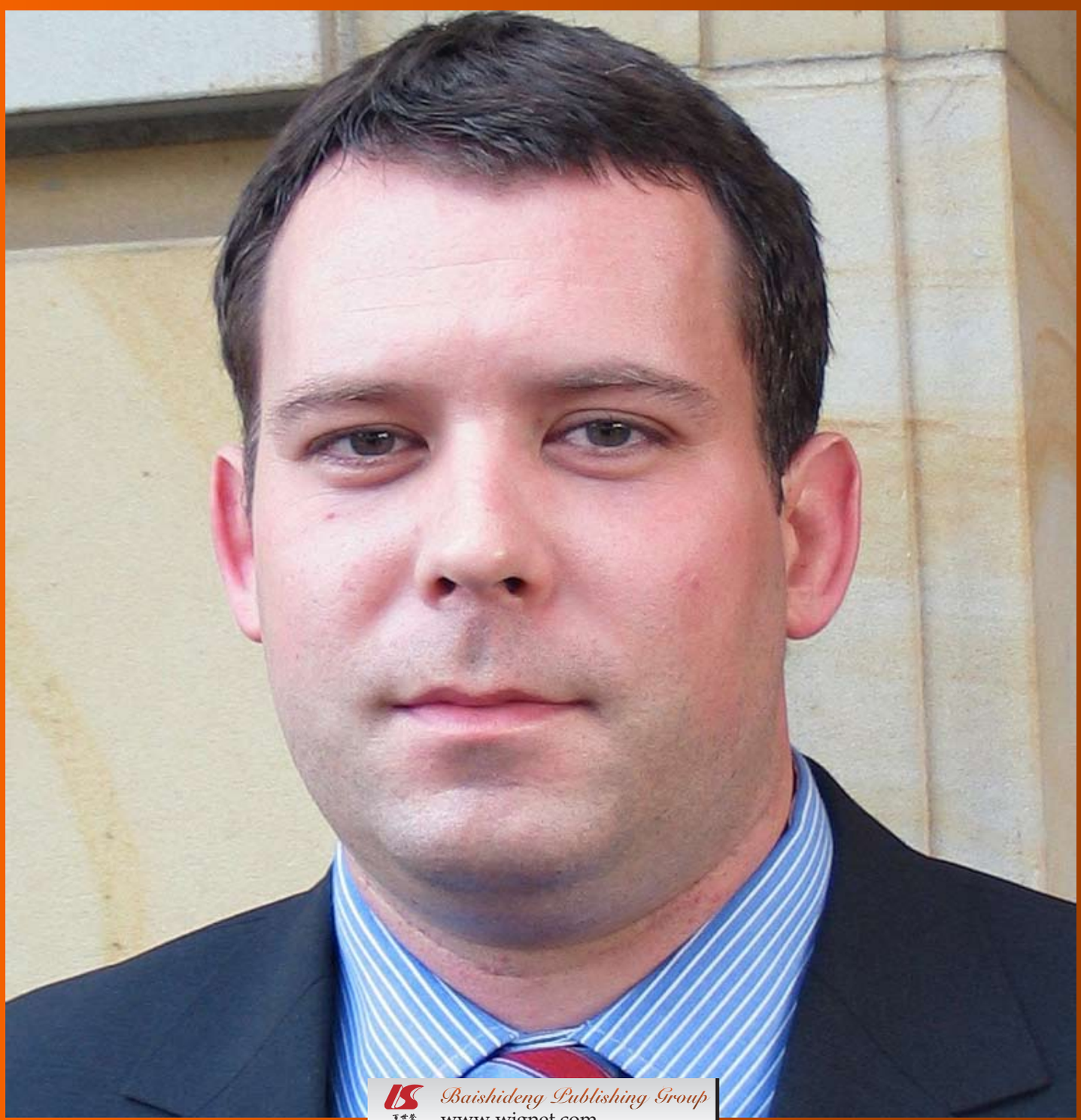

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EDITORIAL

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.

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[1].

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[2,3].

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[4-9].

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 β-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 overuse 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[10].

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

The World Society of Emergency Surgery (WSES) guidelines[11] 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[11].

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[10].

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[10].

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

According to the guidelines, Amynoglicosides should be reserved for patients allergic to b-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 β-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 Imipenem-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[12,13].

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

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[14].

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

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[14].

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*[14].

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[13].

The rapid spread of carbapenemases in *Klebsiella pneumoniae* (*K. pneumoniae*)[16] 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*[17] 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)[3] 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[12,13].

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[18].

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[11] 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[19,20].

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)
	+	
	Metronidazole	500 mg every 6 h (1-h infusion time)
With ESBL-associated risk factors	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 *via* continuous infusion or 4.5 g every 6 h (4-h infusion time)
With ESBL-associated risk factors	Meropenem	500 mg every 6 h (6-h infusion time)
	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 β-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)
	+	
	Metronidazole	500 mg every 6 h (1-h infusion time)
With ESBL-associated risk factors	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 *via* continuous infusion or 4.5 g every 6 h (4-h infusion time)
With ESBL-associated risk factors	Piperacillin	8 g LD then 16 g/d *via* 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)

ESBL: Extended-spectrum β-lactamase; LD: Loading dose.

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

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

Antimicrobial regimens recommended by WSES[11] 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 *via* 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 *via* 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	500 mg every 6 h (6-h infusion time)
	or	
	Imipenem	500 mg every 4 h (3-h infusion time)
	or	
	Doripenem	500 mg every 8 h (4-h infusion time)
	+	
	Teicoplanin	1.6 g *via* 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[24]. 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[11] 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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TOPIC HIGHLIGHT

Eduardo Garcia Vilela, Professor, PhD, *Series Editor*

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 imaging 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.

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 endoscopical 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[1]. 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[2-4]. In UC, ultimately, up to a third of patients with extensive disease will require a colectomy at some point during the disease's course[4].

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)[5]. 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[6]. Other disease activity indices include van Hees index, the Cape Town index, Oxford index and Talsted index, but none result in a better approach to identify relapses[7-10].

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 ℃	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

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[11].

In UC, the clinical activity index most commonly used to define the severity of disease was established by Truelove and Witts[12]. 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[13-16].

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 ℃, 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[17]. With the introduction of newer biological therapies, there might be potential for laboratory markers in selecting responders along with their role in monitoring therapy[17]. 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[18].

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-α and interleukin 1β[19]. It correlates well with clinical, endoscopic, radiological and cross-sectional activity markers in IBD, especially in CD, but not in UC[17,18,20,21]. 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[17]. 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[22,23]. In CD, ESR may correlate with colonic CD involvement[24]. 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[18].

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[17].

Other classical acute phase proteins that can be detected in the serum of IBD patients are α1-acid glycoprotein (orosomucoid), fibrinogen, serum amyloid A, β2-microglobulin, α2-globulin, and α1-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[25]. Most of these acute phase markers have been sparsely studied and do not show advantages over CRP in detecting and monitoring inflammation in IBD[17,26].

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[27-30]. 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[26,30-33].

Recently, it was observed that elevated serum levels of antibodies specific for certain carbohydrate structures might have a relationship with CD[34]. Malickova *et al*[35] evaluated anti-chitobiose carbohydrate antibody, anti-laminaribiose, carbohydrate antibodies, and anti-mannobiose carbohydrate antibodies in Central European patients with IBD and concluded that 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[35]. Another study, conducted by Rieder *et al*[36], 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[37-42]. IL-6 in active CD and IL-10 in recovery of CD have demonstrated a good correlation, which has been reproducible between studies[43].

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[17,18].

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[18].

Fecal occult blood (FOB) and α-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[44]. α-1 antitrypsin is considered a sensitive but non-specific parameter reflecting enteric inflammation in IBD and has been replaced by other fecal markers[45].

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[46,47]. 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[48].

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[49-51]. 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[52-54].

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[47]. Calprotectin is commercially available and an assay for S100A12 is under development[55]. 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[56-58]. However, calprotectin seems more predictive of relapse in UC than in CD[58,59]. 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[60].

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[19].

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[61-63]. 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[64]. 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[65].

Years later, Dapermo *et al*[66] 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[67-69]. 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[13,70-72]. 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*[73] 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[74]. 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*[75] 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[76,77].

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[78].

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[78].

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[79]. 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[80,81].

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[79,82]. Van Ostayen *et al*[83-86] 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[87-89].

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 assed by endoscopy or clinical and biochemical evaluation[90,91]. 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[92-96]. 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[97].

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[76]. The mesenteric blood flow in inferior mesenteric artery, although seemingly related to clinical end 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[98].

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 administrated iodinated contrast, in order to permit visualization of the small bowel wall, mucosa and lumen[99]. Then, apart from detecting extraluminal findings, CTE can delineate the extent and severity of bowel wall inflammation[100].

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[99,101].

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[102].

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[103].

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[103]. It has also been observed that the degree of bowel wall enhancement correlates with the severity of inflammation[104,105].

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[105].

CTE can also depict signs of chronic manifestations of CD, such as submucosal fat deposition, sacculations and fibrofatty proliferation[101]. 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)[106].

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[105,107]. Higgins *et al*[108] 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[109]. 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[110].

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[111].

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[112-114], although image quality is still better with CT. Motion artifacts from small bowel motility are more severe with MRE[112], but halting peristalsis by administering 1 mg of glucagon intramuscularly before contrast-enhanced imaging reduces blurring and artifacts related to bowel motility[115].

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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REVIEW

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.

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)[1], 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[2]. In fact, epigenetic modifications are as important as genetic modifications in regulating gene expression and controlling the onset of disease[3]. 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[4]. 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[5].

DNA METHYLATION

Definition of methylation

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

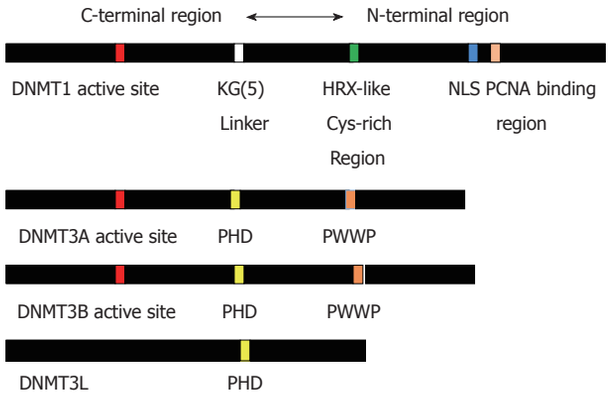

Figure 1 DNA methylation machinery. NLS: Nuclear localization signal; PCNA: Proliferating cell nuclear antigen.

ulation of gene expression without changing the genetic sequence. It is a flexible, heritable process that regulates some genes[7]. 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[8]. 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[9]. 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[10]. Methylation of other CpGs seems to have no biological functions[11].

There are four types of DNA methyltransferases (DNMTs), including NDMT1, DNMT3A, DNMT3B, and DNMT3L. DNMTs control the degree of methylation of the genome: NDMT1 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[12,13]. 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[14] (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[15].

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

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[18,19]. 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[20]. 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[21].

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	*hPer3* 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 *via* activation of CREB-binding protein

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

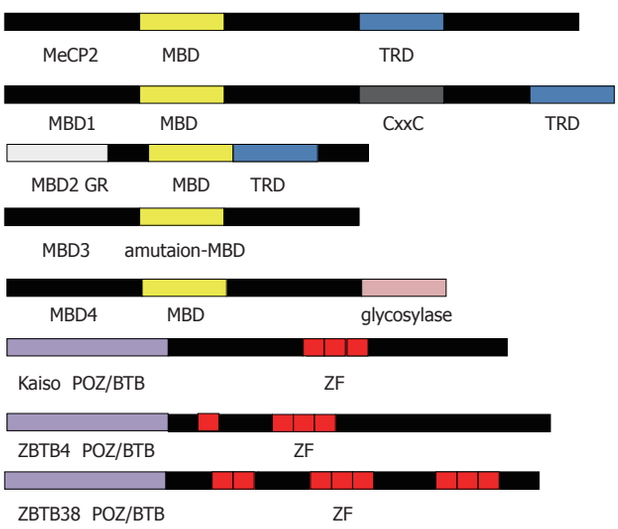

Figure 2 Proteins that bind the methylated DNA. MBD: Methylation-binding protein; TRD: Transcription repression domains.

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 cylindricalcast) 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[22] (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[23]. 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%[14,24]. 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[25]. 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[26].

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

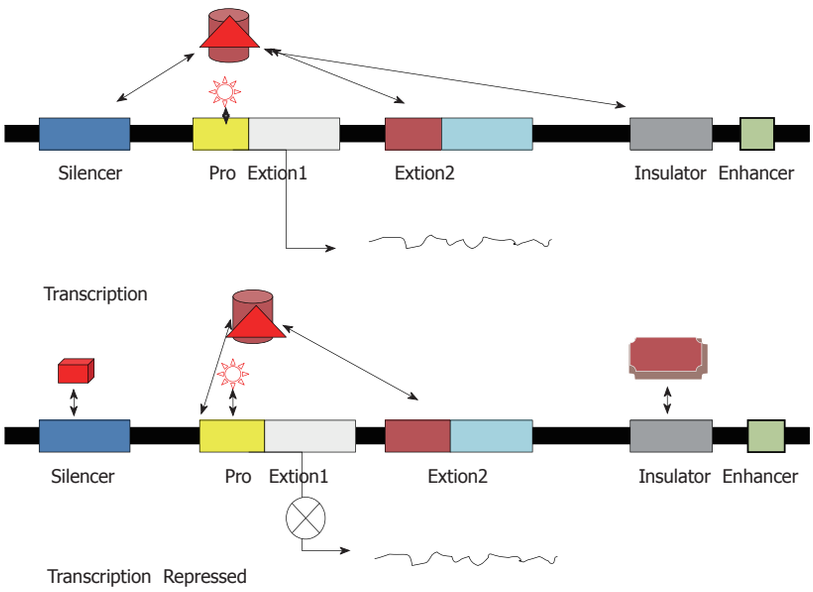

Figure 3 How methylation represses the gene expression..

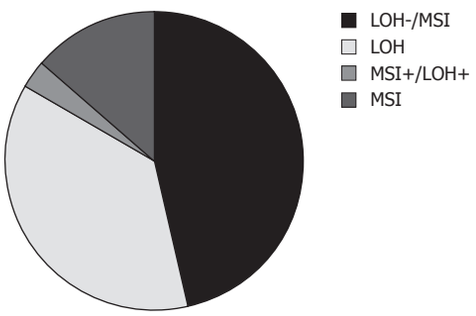

Figure 4 Factors for cancer development. LOH: Heterozygosity; MSI: Microsatellite instability.

expression. Abnormal patterns in DNA methylation often result in many diseases[27]. 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[28]. 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[1] (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 combination with the chromosomal instability and microsatellites instability[1].

In addition, it has been proposed that hypomethylation patterns of the genome exist and, more specifically, that hypomethylation and hypermethylation cooperate in cancer development[29]. 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[30,31].

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[32].

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 futher studied and will be a hotspot of research in the next few years[33-37].

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[38].

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[39-41]. The gene encoding BMP3 has been found to cause gastric carcinoma in Chinese population[42]. 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[43]. 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[11]. 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[44]. 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[45]. 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[46].

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[47]. Down-regulation of miR-212 may be related to gastric carcinogenesis through its target genes, such as *MECP2*[48]. Hypermethylation of hsa-miR-124a is present in gastric cancer[49]. 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[50]. 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[51]. 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[52].

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[30,53]. The two compounds do not, however, reverse the methylation entirely[54]. Both nucleoside analogs and non-nucleoside analogs can be used to demethylate the gene of interest, but with severe side effects[55,56]. 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[57].

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[58]. 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[59].

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 HpaⅡ-Msp Ⅰ and Sma Ⅰ-Xmal. 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[60].

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[61].

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[62].

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ORIGINAL ARTICLE

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.

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[1,2]. It provides both an accurate as-

sessment of the tumour (T) stage of a cancer and associated lymphadenopathy (N)[3-5].

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[6,7]. 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[8]. However, the sensitivity and specificity of these features in distinguishing malignant involvement only exceeds 80% when all four of these features are present[8].

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[9-13]. Initial studies assessing nodes with elastography used processing to produce a colour elastography image[14-16]. 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[17,18] as well as lymph nodes[15,18-20]. 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.

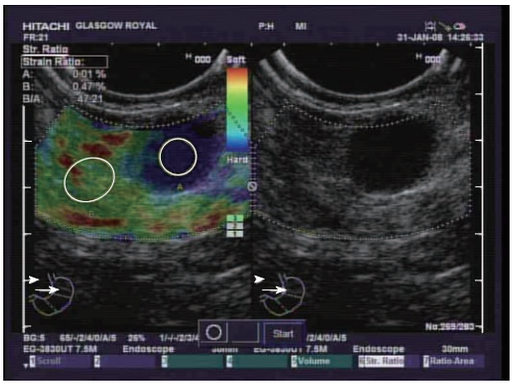

Figure 1 Endoscopic ultrasound image of a malignant appearing lymph node. The right-hand side of the image displays all 4 of the conventional endoscopic ultrasound criteria characteristics of malignant nodes with regard to size (> 1 cm), shape (round), density (hypodense) and distinction of border (clear 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.

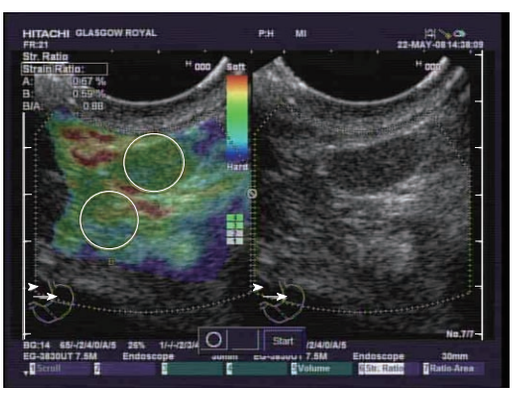

Figure 2 Endoscopic ultrasound elastography of a benign lymph node. The right-hand side of the image displays standard grey-scale endoscopic ultrasound images while on the left is a superimposed elastography image. In the elastography image window the strain ratio measurements of the two areas outlined 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.

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 recorded. 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

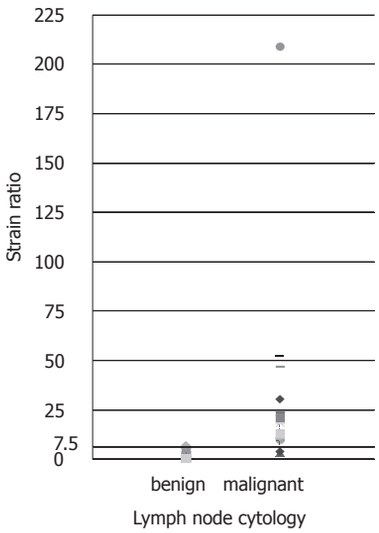

Figure 3 Plot of elastography strain ratio for cytologically proven benign or positive lymph nodes. The cut-off line of ⩾ 7.5 is the optimal strain ratio for discriminating between benign and malignant lymph nodes.

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 (χ^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[21]. 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 ⩾ 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 ⩾ 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

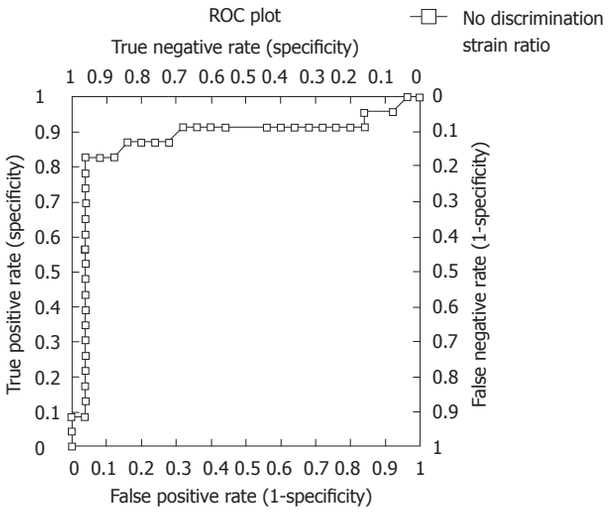

Figure 4 Receiver operating characteristic curve for elastography strain ratio. The receiver operating characteristic area under the curve was 0.87 ($P < 0.0001$).

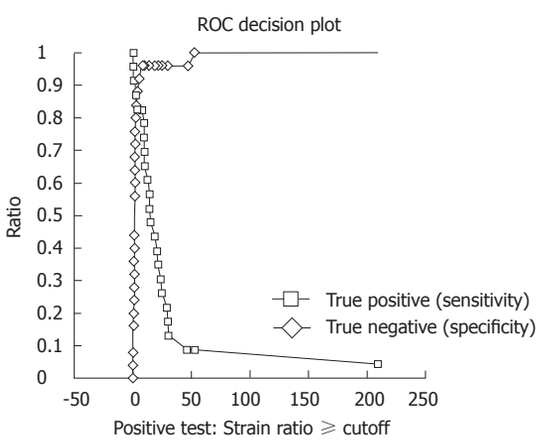

Figure 5 Receiver operating characteristic sensitivity, specificity based decision plot to determine the optimal elastography strain ratio cut-off point. The sensitivity and specificity lines cross at strain ratio 7.5.

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.

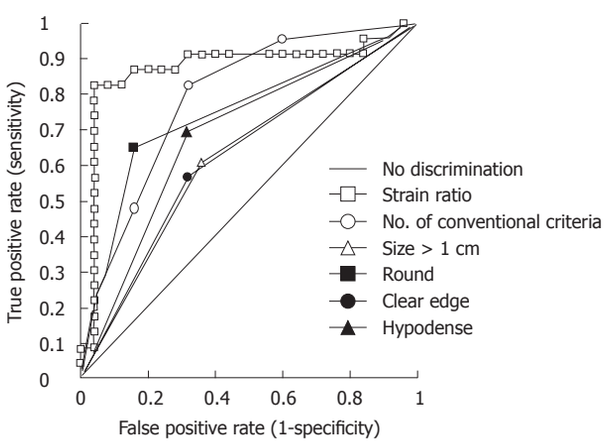

Figure 6 Receiver operating characteristic curve comparison of elastography strain ratio against conventional endoscopic ultrasound criteria both in combination and individually (size > 1 cm, round, hypodense, clear edge).

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[1]
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

[1]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[17,18]. There has also been encouraging, though limited, data on lymph node assessment[13,18-20].

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[8,19,22]. However, using EUS elastography with a strain ratio cut off of $\geqslant$ 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 $\geqslant$ 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[19,23]. 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%[24,25]. 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[26]. 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[18,23]. 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*[23] 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[19].

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[27-30]. 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

We would like to acknowledge Professor Donald McMillan of the University of Glasgow for his comments and guidance on writing the statistical analysis section of this paper.

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 esophago-gastric lymph nodes but other malignancies as well.

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ORIGINAL ARTICLE

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* $HLR^-/HLA\text{-}DR^+$ 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.

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[1-4]. 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[5-7], muconodular cancer[8], very-well-differentiated tubular cancer with an intestinal[9] or gastric[10] phenotype, and a low-grade subtype of diffuse desmoplastic cancer[11]. Similarly, various kinds of cancer with poor outcome have been identified, from poorly differentiated neuroendocrine carcinoma, small to large cell[12,13], to anaplastic diffuse cancer[11] or hepatoid[14], chorioncarcinomatous[15] and adenosquamous carcinoma[16]. In addition, comparative genomic hybridization analysis has shown a clear relationship between the number and severity of genomic alterations and tumor histotype and prognosis[17,18].

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[19]. 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[19] 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 cancers, due to discrepancies concerning: (1) the impact of histology or stage on mucinous cancer prognosis[20-23]; 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[5-7,24-27]. 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[2]. 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[28]. No antiblastic 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[7,19,29].

Molecular analysis

In situ hybridization for the *EBER1* gene of EBV was performed as described previously[3,7]. 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[7,19,30].

Morphological analysis

Tumor histotypes were identified as described previously[8-11,19]. 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 $CD8^+$ 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[7,31], and an evaluation of dendritic cells was added[27]. 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 $CD8^+$ T cells and $CD11c^+$ 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)[11,12]. 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[19]. 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 κ value of 0.84 concerning interobserver agreement for five main histotypes (cohesive, diffuse, mucinous, anaplastic and HLR), a κ of 0.81 agreement for nine subtypes (HLR, VWDT, ordinary cohesive, low-grade diffuse desmoplastic, ordinary diffuse, muconodular, ordinary mucinous, invasive mucinous and anaplastic), and a κ of 0.79 agreement for the three histotype-based grades (low, intermediate and high).

From the previously investigated Pavia series of 294 cases[19], 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[1].

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

				Cox survival analysis		
Stage	*n* (%)	Death rate	95% CI	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[a]
Ⅲ	68 (34)	26.04	19.74-34.46	9.49	4.45-20.24	< 0.001[b]
Ⅳ	31 (15.5)	52.66	36.11-76.79	18.90	8.41-42.46	< 0.001[c]

[a]*P* = 0.001 *vs* Ⅲ + Ⅳ; [b]*P* = 0.015 *vs* Ⅱ ; [c]*P* = 0.004 *vs* Ⅲ. CI: Confidence intervals; HR: Hazard ratio.

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_2 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

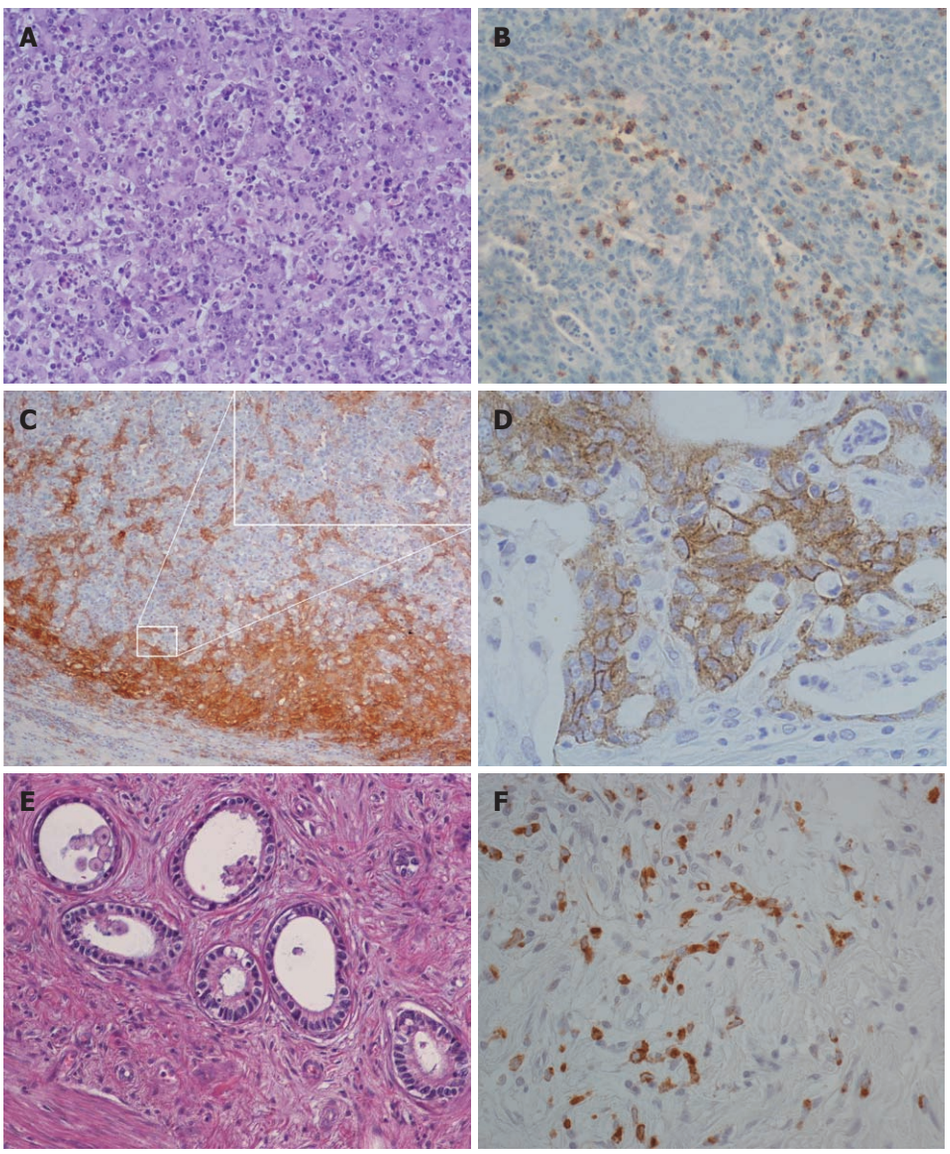

Figure 1 Histological and histochemical aspects of high lymphoid response cancers. A: Lymphoepithelioid pattern of an HLR EBV$^+$ tumor (hematoxylin-eosin, × 200); B: High intratumor T8 cells infiltration in an HLR EBV$^-$/MSI$^-$ case (CD8 immunoperoxidase-hematoxylin, × 200); C: Peritumor dendritic cell rich demarcating band in an HLR MSI-H tumor (CD11c immunoperoxidase-hematoxylin, × 100); in the inset, focal enlargement of image C to show interaction of CD11c-reactive dendritic cells with unreactive neoplastic cells (× 400); D: HLA-DR reactivity of an HLR MSI-H case (immunoperoxidase-hematoxylin, × 400); E: Low-grade very-well-differentiated tubular carcinoma (hematoxylin-eosin, × 200); F: Low-grade desmoplastic tumor with spindle neoplastic cells interspersed among fibroblast rich stroma (CAR5, immunoperoxidase-hematoxylin, × 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[1] shows a significantly worse prognosis for the diffuse compared to intestinal and unclassified types [model: χ^2 (2γ) = 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: χ^2 (2γ) = 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 concordance (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,

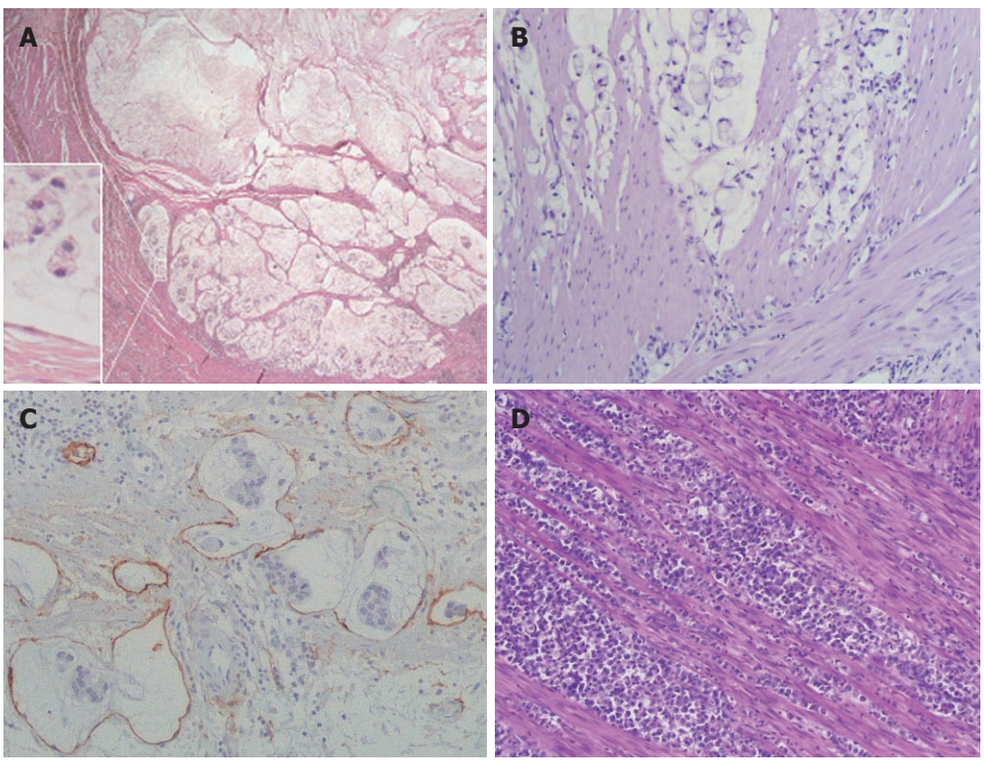

Figure 2 Muconodular, well-differentiated tubular, diffuse desmoplastic and high lymphoid response. A: Low-grade muconodular cancer with expansile growth (hematoxylin-eosin, × 20); in the inset, note tumor cells floating inside mucin, free of contact with stroma (× 400); B: intermediate-grade, locally 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) .

Table 2 Survival analysis of 200 gastric cancers according to histotype-based grade and according to Lauren classification

				Cox survival analysis			
						P value	
	n (%)	Death rate	95% CI	HR	95% CI	Univariate	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[a]	< 0.001[b]
Lauren type[1]							
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

[a]*P* = 0.001 *vs* grade 2; [b]*P* = 0.019 *vs* grade 2. Harrell's *c* = 0.63. [1]F: Univariate model: χ^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.

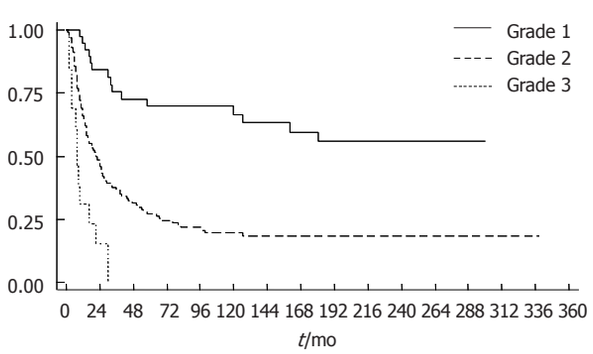

Figure 3 Kaplan-Meier survival estimate of 200 gastric cancers according to histotype-based grade.

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 (%)				Cox survival analysis			
							P value	
	Varese series	Joint series	Death rate	95% CI	HR[1]	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[a]	0.987[b]
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[c]	< 0.001[c]
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

[1]For grades, based on grade 1; for histotypes, based on high lymphoid response (HLR) type. [a]$P = 0.005$ *vs* muconodular and $P < 0.001$ *vs* mucinous. [b]$P = 0.037$ *vs* muconodular and $P < 0.001$ *vs* mucinous. [c]$P < 0.001$ *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)

				Cox survival analysis			
						P value	
	n (%)	Death rate	95% CI	HR	95% CI	Univariate	With stage
HLR^+							
EBV^+	24 (29.6)[1]	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^-							
MSI-H	38 (48.7)[2]	12.29	8.24-18.33	3.64	1.78-7.47	< 0.001	0.015

[1]One EBV^+/MSI^- case omitted; [2]% of all MSI-H cases, HLR^+ or $^-$. 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^-/MSI^- subset, while a trend for worse behavior of the EBV^+ compared to the other subsets was noted by univariate analysis, which disappeared with stage-inclusive bivariate analysis (Table 4). EBV^+ 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 $HLA\text{-}DR^-$ cases, a behavior probably accounted for by the $HLR^+/HLA\text{-}DR^+$ 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$^+$ 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[19], 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[19]. This is especially true for rare histotypes like muconodular[8], VWDT[9,10] or low-grade diffuse desmoplastic[11] 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[1-4]. 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[19] 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$^+$ (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[6,7,32], compared to both the MSI-H and the MSI$^-$/EBV$^-$ 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[26].

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[19], 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$^+$[5,7,19,26] and HLA-DR$^+$ cancers[24], it might interfere with therapies potentially affecting antitumor immune response[26].

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[22], 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[31,33]. Moreover, the few MSI$^+$ 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[25,34].

Several studies have shown that mucinous gastric cancers have a worse prognosis[20-22]. However, it is uncertain whether a higher stage at diagnosis[23], 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)[4] or a variety of promising molecular tools[17,18,35], 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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ORIGINAL ARTICLE

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)-α 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-α 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-α suppression.

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[1,2]. Various techniques and devices for liver resection have been employed to improve the perioperative outcome[3-7], although the clamp-crush technique used by a skilled surgeon still has the most favorable outcome according to recent systematic reviews[8,9]. Liver surgeons focus on reducing bleeding during liver resection, which leads to shorter operative time. Heat-assist devices such as the harmonic scalpel[10], Ligasure[11], saline-linked monopolar cautery[12], microwave coagulator[13], and radiofrequency (RF) devices[14], 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[15]. Some randomized trials have indicated the merits and demerits of using heat-assist devices[4,7].

The greatest merit is the sealing effect to reduce perioperative morbidity[7,16]. A second merit is enhancement of the surgical margins of tumors located near the cutting surface of the liver[17]. On the other hand, a necrotic zone remains in the cutting surface of the residual liver[14]. Although cryoablation causes lethal systemic responses with high levels of cytokines[18,19], 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)-α and IL-6 prime and facilitate hepatocyte replication[20-22]. Although the humoral factors increase and influence each step of LR in a very short time[21], each cytokine activates subsequent molecular signals to complete LR[22]. RF ablation, in particular, excessively increases plasma cytokines such as TNF-α and IL-6 compared to simple liver resection[23,24]. Due to superphysiological stimulation of these cytokines, the heat effect of RF ablation may impair liver regeneration[25]. On the other hand, fast recovery of liver function in LR after RF ablation has been reported in major clinical hepatectomy[6]. Therefore, the exact effect of RF ablation on LR remains unclear.

Steroid administration has been proved to attenuate surgical stress following liver resection[26,27]. In addition, steroid pretreatment has been proved to decrease plasma cytokine levels and the therapeutic dose of the steroid does not inhibit hepatocyte proliferation[28]. Although previous investigations showed that steroid treatment could ameliorate excessive surgical stress of extended hepatectomy[26-28], 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 institutional 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 ℃) 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[29,30]. 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 μg/kg body weight)[31]. 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 ℃ 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-α, 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.)[31]. 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[31]. 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 ± 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 ℃ 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 μg/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 WesternBreeze® 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[30].

STAT3 DNA-binding activation assay

STAT3 activation was quantified using a TransAM™STAT3 Kit (Active Motif, Funakoshi Co., Tokyo, Japan)[32]. 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 ± 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-α 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 ± 3.43 *vs* 29.02 ± 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 ± 4.18 *vs* 7.05 ± 1.54, $P < 0.001$, and 12.55 ± 3.14 *vs* 6.03 ± 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 (× 10^3 μL)	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
PH	4.95 ± 0.74	4.70 ± 0.81	6.55 ± 1.26	6.15 ± 0.81	7.43 ± 0.99	5.48 ± 1.14
PHA	5.80 ± 1.09	5.70 ± 0.84	7.68 ± 1.32	6.60 ± 1.49	7.65 ± 1.41	6.73 ± 0.81
P values	NS	NS	NS	NS	NS	NS
TP (g/dL)						
PH	5.55 ± 0.24	4.71 ± 0.29	4.58 ± 0.09	4.87 ± 0.29	5.18 ± 0.21	5.43 ± 0.34
PHA	5.62 ± 0.17	4.43 ± 0.25	4.28 ± 0.17	4.05 ± 0.13	4.68 ± 0.24	5.31 ± 0.36
P values	NS	NS	0.022	0.002	0.019	NS
ALB (g/dL)						
PH	4.10 ± 0.14	3.73 ± 0.21	3.45 ± 0.13	3.58 ± 0.21	3.80 ± 0.22	4.08 ± 0.15
PHA	4.20 ± 0.18	3.55 ± 0.21	3.18 ± 0.17	2.85 ± 0.21	3.10 ± 0.22	3.78 ± 0.28
P values	NS	NS	0.042	0.003	0.004	NS
AST (U/L)						
PH	77.3 ± 3.3	591.8 ± 111.8	211.0 ± 23.6	126.8 ± 23.7	117.3 ± 14.5	105.5 ± 13.4
PHA	79.3 ± 4.6	2441.8 ± 501.8	366.5 ± 136.9	224.0 ± 110.2	163.5 ± 59.2	100.8 ± 11.9
P values	NS	0.001	NS	NS	NS	NS
ALT (U/L)						
PH	48.5 ± 13.4	734.3 ± 187.4	207.8 ± 108.7	71.0 ± 21.1	59.3 ± 9.4	47.5 ± 13.7
PHA	49.3 ± 9.6	1603.3 ± 313.7	503.8 ± 207.8	102.5 ± 23.1	66.0 ± 8.9	51.3 ± 11.1
P 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.

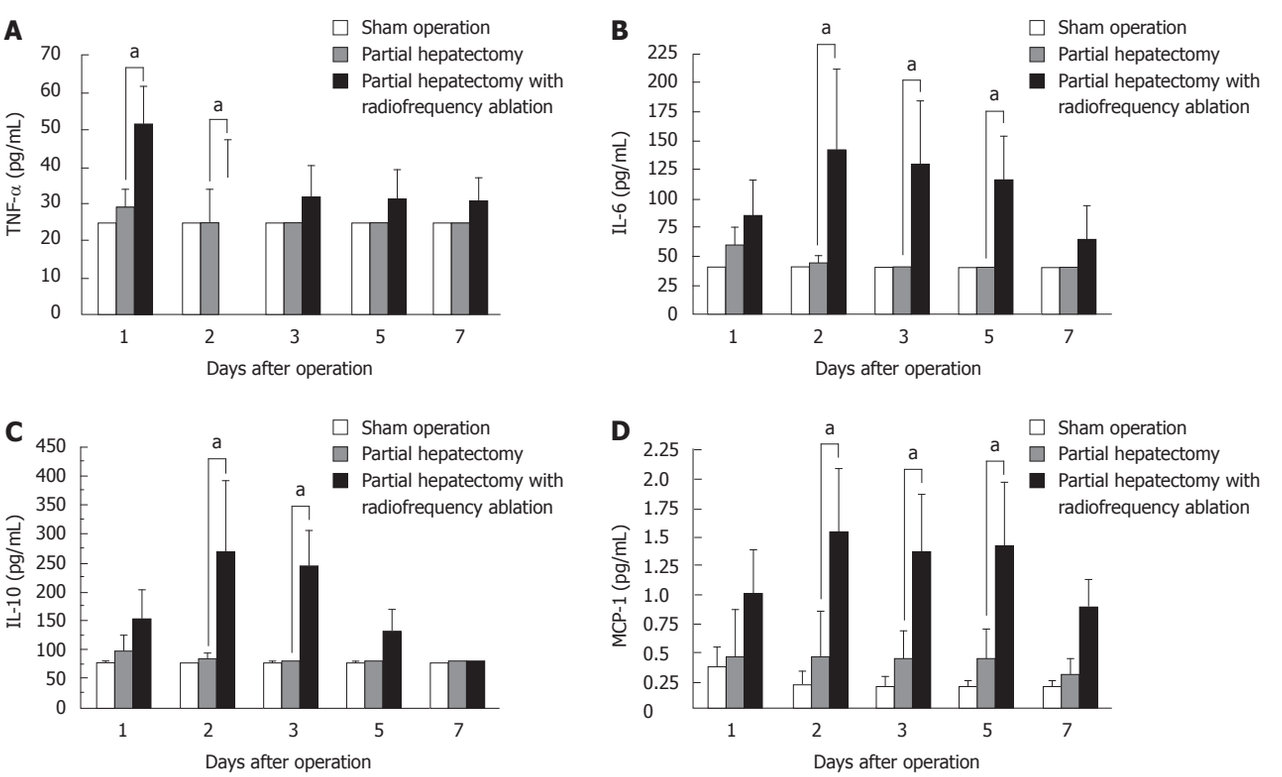

Figure 1 Changes in serum tumor necrosis factor-α (A), IL-6 (B), IL-10 (C), and monocyte chemoattractant protein-1 (D) levels after the operations. $^aP < 0.05$ between groups. TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemoattractant protein-1.

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).

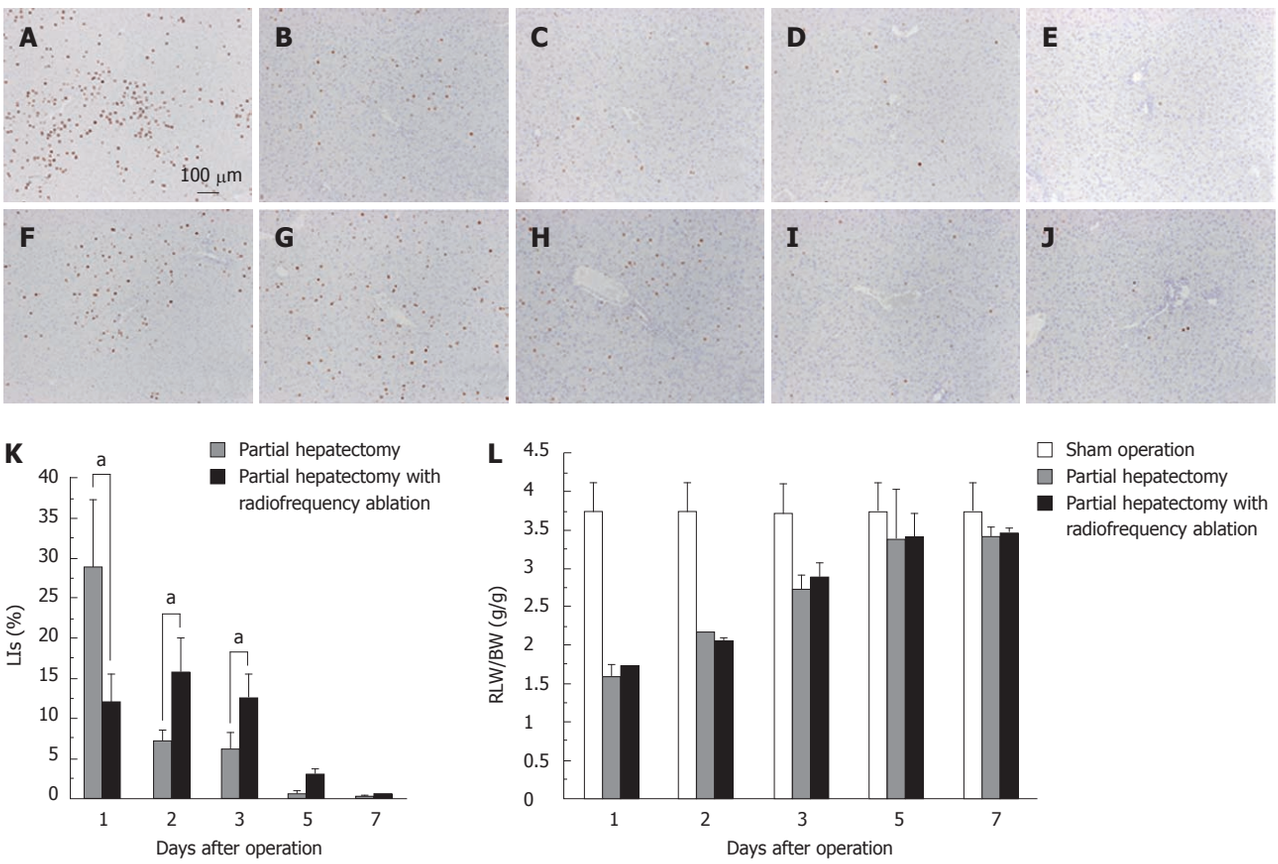

Figure 2 Immunohistochemistry for 5-bromo-2-deoxyuridine in the partial hepatectomy group (A-E) and in the partial hepatectomy with radiofrequency ablation group (F-J) at 1 d (A and F), 2 d (B and G), 3 d (C and H), 5 d (D and I) and 7 d (E and J) after the operations. K: Comparison of labeling indices 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. [a]*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 ± 8.28 *vs* 14.87 ± 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-α 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 ± 4.18 *vs* 46.5 ± 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 ± 0.042 *vs* 0.298 ± 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

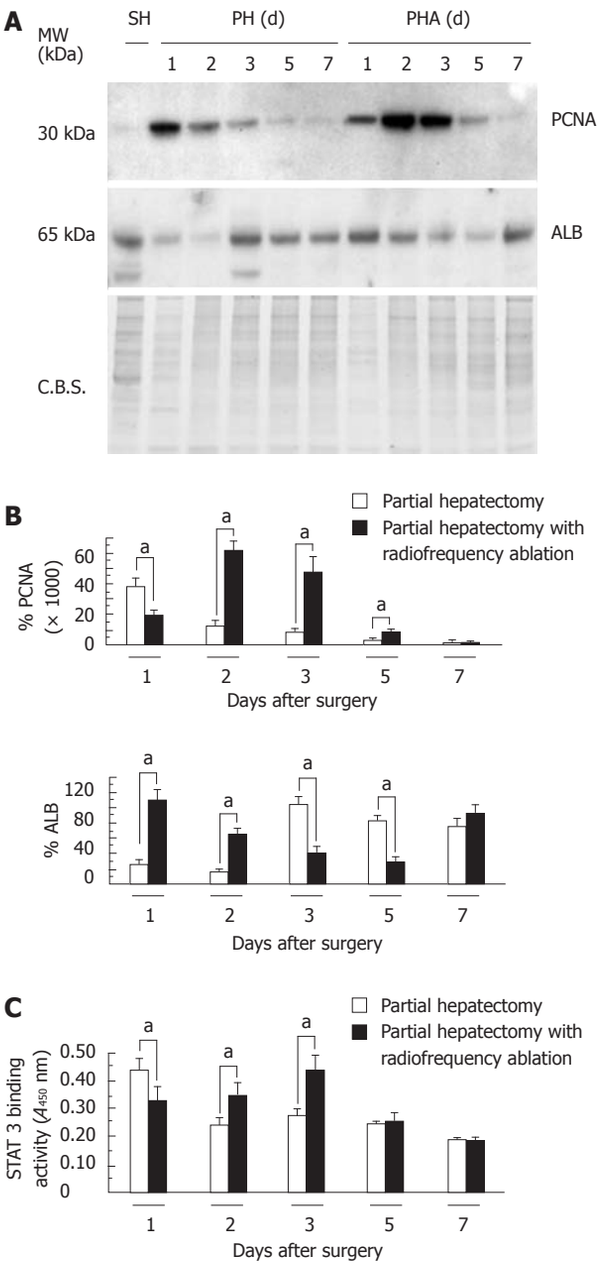

Figure 3 Proliferation cell nuclear antigen expression in the nuclear protein and albumin expression in the cytosol after the operations. A: Western blotting analysis for proliferation cell nuclear antigen expression (PCNA); B: Densitometric analysis of each protein signal; C: STAT3 DNA-binding activity in the nuclear protein after the operations. $^{a}P < 0.05$ 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[33] and can be used for hemostasis during liver resection[34]. 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[25]. The use of murine models to investigate RF ablation has been limited[25,35]. 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[24]. Furthermore, high serum cytokine levels after ablation have been reported in human studies[24,36]. 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[37,38]. Necrotic tissue after RF ablation could also modulate the systemic immune response to specific cancer cells[39]. On the other hand, Meredith *et al*[40] 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[25]. 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[24,35,36], 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[41]. Delayed LR after RF ablation affected the serum albumin level. Albumin production was suppressed when hepatocytes began to proliferate[42]. 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-α and

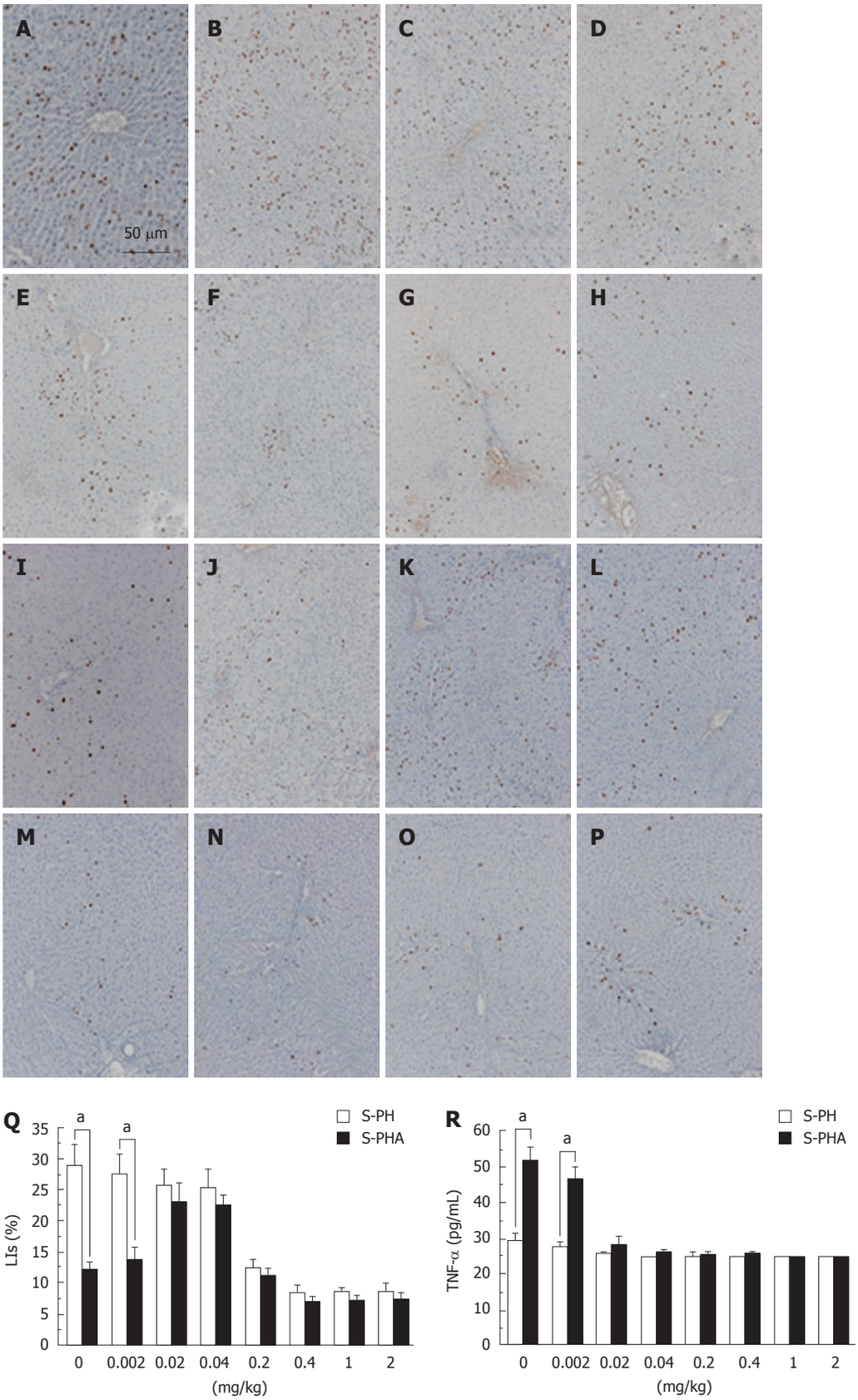

Figure 4 Immunohistochemistry for 5-bromo-2-deoxyuridine staining in the partial hepatectomy with dexamethasone pretreatment group (A-H) and partial hepatectomy after radiofrequency ablation with dexamethasone pretreatment group (I-P) at 1 d after the operations. Animals were pretreated with 0 mg/kg (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-α levels at 1 d after the operations. The horizontal axis presents each concentration of dexamethasone pretreatment. [a]*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.

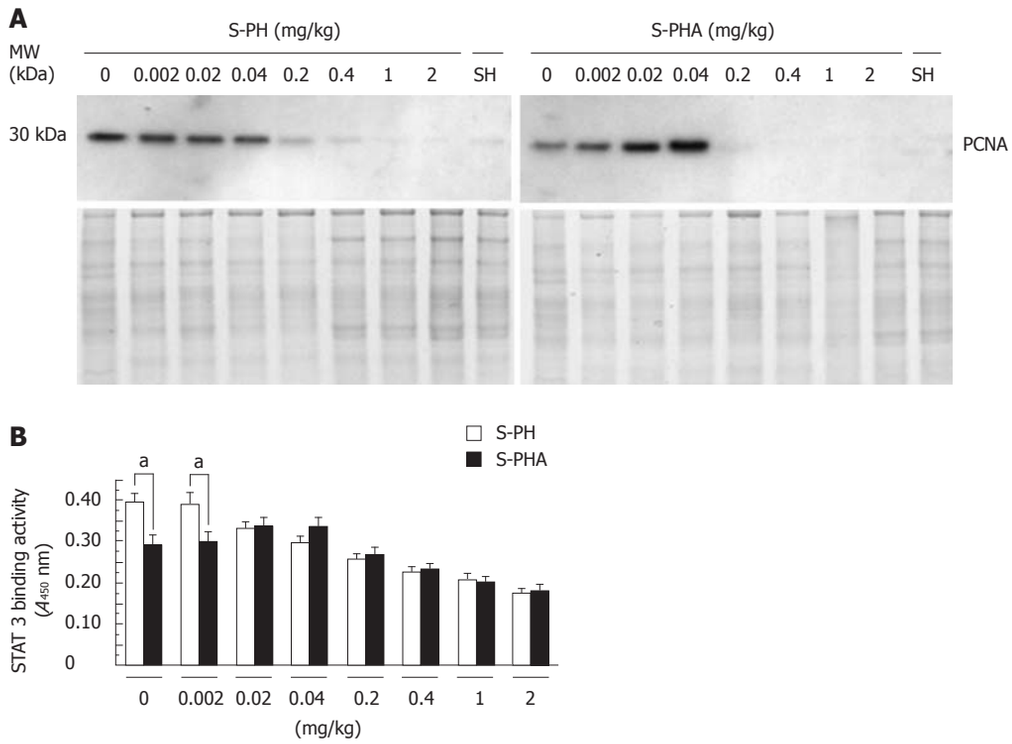

Figure 5 Proliferation cell nuclear antigen expression in the nuclear protein at 1 d after the operations. A: Western blotting analysis for proliferation cell nuclear antigen (PCNA) expression. Animals were pretreated with various concentrations of dexamethasone 30 min prior to the hepatectomy; B: STAT3 DNA-binding activity in the nucleus at 1 d after the operations. [a]*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[43], although knockout murine models targeting TNF-α receptor and *IL-6* genes have demonstrated that these cytokine signals are necessary to accomplish LR[44,45]. 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-α level, which gradually decreased. In addition, steroid pretreatment in the PHA group showed that only the TNF-α 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-α than by other cytokines/chemokines. Thus, TNF-α activation should be observed within a short time after simple hepatectomy[21]. 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-α activation after RF ablation, our results strongly suggested that the TNF-α could play a major role in LR after RF ablation. Further study is needed to determine whether TNF-α 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-α and IL-6 production[46-48], although moderate stimulation by TNF-α and IL-6 is necessary to complete LR[21]. 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[42]. 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-α suppression. Further clinical study is needed to confirm that low-dose steroid administration has a clinical benefit for LR after RF ablation.

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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-α 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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ORIGINAL ARTICLE

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 esophagus 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.

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

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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[1]. 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[2-4]. 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[5-8]. In ESCC, loss of E-cadherin expression is associated with tumor invasiveness, metastasis and prognosis[2,9,10]. 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[11-13]. 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[14]. Interestingly, elevated levels of S100A4 are closely associated with the process of metastasis in several human solid cancers including gastric cancer[15,16], colorectal adenocarcinoma[17,18], and breast cancer[19]. 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[20]. 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)[21]. All the cells were maintained in 50 mL/L CO_2 atmosphere at 37 ℃ 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 1×10^6 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 (-ΔΔCt) method[22].

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'-TCAGAACTAAAGGAGCTGCTGACC-3', reverse 5'-TTTCTTCCTGGGCTGCTTATCTGG-3'), E-cadherin (forward 5'-GGAAGTCAGTTCAGACTCCAGCC-3', reverse 5'-AGGCCTTTTGACTGTAATCACACC-3'), GAPDH (forward 5'-AATCCCATCACCATCTTCCAGGAG-3', reverse 5'-GCATTGCTGATGATCTTGAGGCTG-3'). The cDNA was amplified with an initial denaturation at 94 ℃ for 3 min followed by the sequential cycles of denaturation at 94 ℃ for 50 s, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 1 min for 30 cycles, with final extension at 72 ℃ 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 ℃ 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 (Amasham, 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 × 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[23]. 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-μm pore size Transwell Biocoat Control inserts (Becton Dickinson). In brief, 1×10^4 cells were seeded on a transwell containing numbers of 8-μm pores for invasion assay. The chamers were put into the incubator at 37 ℃, 50 mL/L CO_2. 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 chected 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/kytjzcwj/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 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.

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	

Statistical analysis

All statistical analyses were performed using SPSS 11.0 software. The results were presented as mean ± 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 representive (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 μg of messenger RNA and standardized with β-actin; Figure 1C and D). We found that transcript copy numbers for S100A4 were 46.3 ± 9.4 for M tumor and 10.5 ± 3.6 for N tumor ($P = 0.036$); for E-cadherin, they were 13.62 ± 2.3 for M tumor and 40.17 ± 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

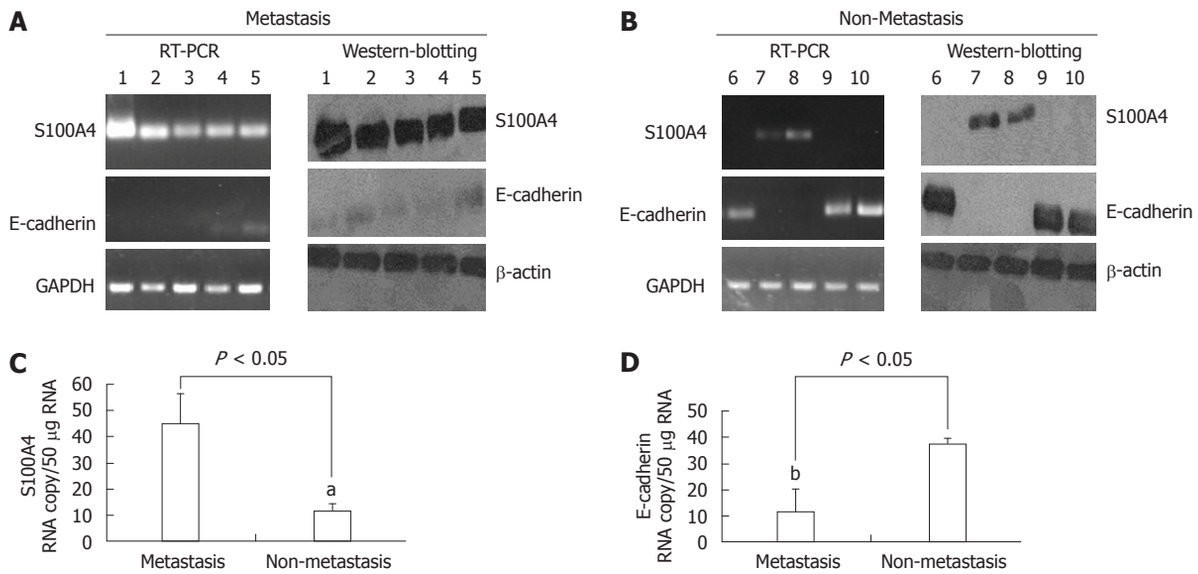

Figure 1 S100A4 and E-cadherin expression in metastasis and non-metastasis tissue. A: The representive reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting results for S100A4 and E-cadherin in metastasis tissue; B: The representive RT-PCR and Western blotting results for S100A4 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 copynumber 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, $^{a}P < 0.05$ *vs* metastasis group and $^{b}P < 0.05$ *vs* non-metastasis group.

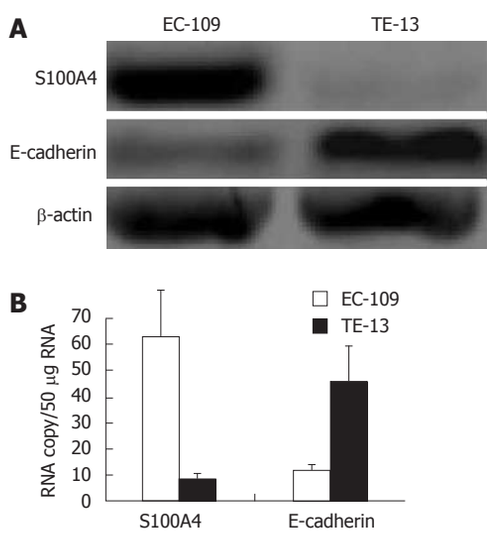

Figure 2 Analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines. A: Western blotting analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines; B: Reverse transcription-polymerase chain reaction analysis for S100A4 and E-cadherin in EC109 and TE13 cell lines. The levels of S100A4 were 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 biological function is hardly known. Many studys found that S100A4-induced invasiveness in malignant tumor cells is caused or partially caused by down-regulation of E-cadherin[24-29]. Kwak *et al*[30] 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 stablely 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

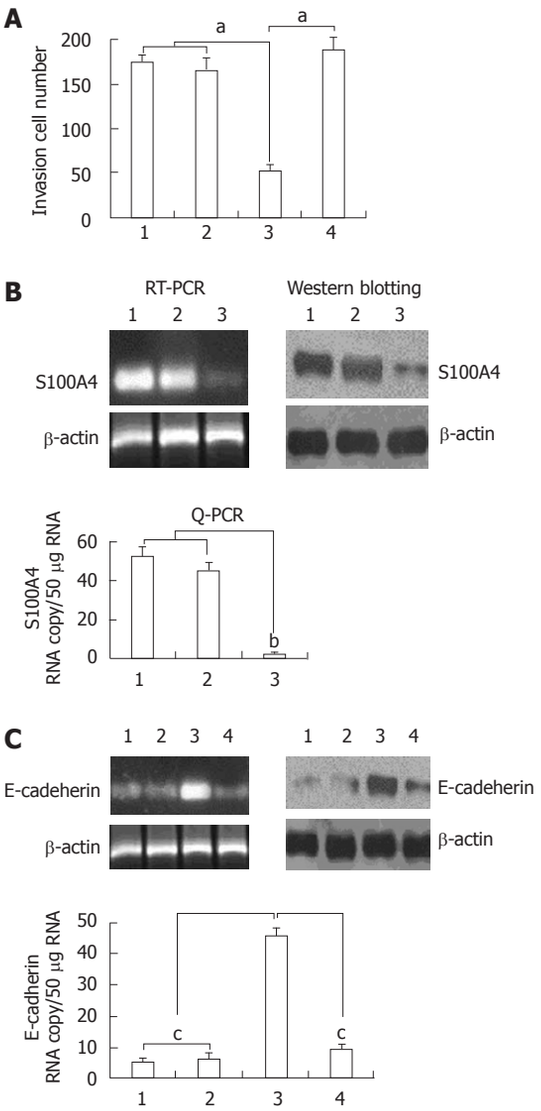

Figure 3 Knockdown of S100A4 inhibits invasive capability of EC109 cells. A: Transmigration cells of control, mock siRNA, S100A4 siRNA and S100A4 siRNA + S100A4 cDNA cells was calculated from three independent experiments. The indicated cells (1×10^4) were seeded on 8-mm porous transwell chambers. After 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; $^aP < 0.05$ *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, $^bP < 0.05$ *vs* control or mock siRNA group; C: Western blotting, RT-PCR and Q-PCR analysis for E-cadherin and mRNA and protein expression. $^cP < 0.05$ *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

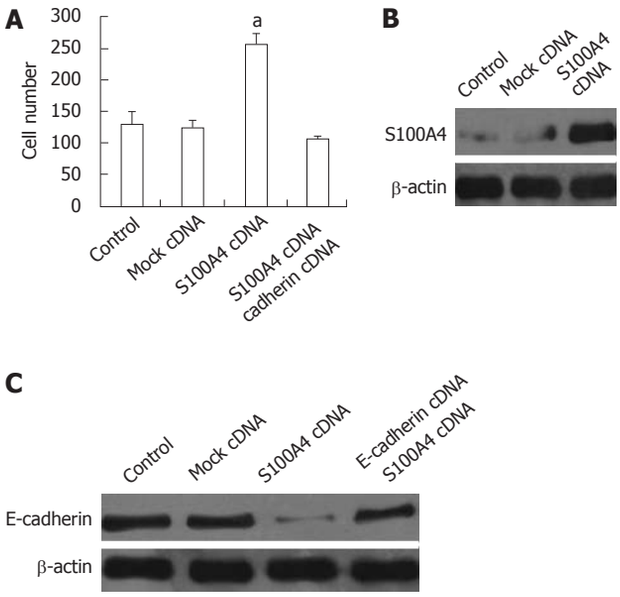

Figure 4 S100A4 overexpression promotes invasive capability of TE13 cells. A: Transmigration cells of control, mock cDNA, S100A4 cDNA and S100A4 cDNA + E-cadherin cDNA cells was calculated from three independent experiments. The indicated cells (1×10^4) were seeded on 8-mm porous transwell chambers. 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; $^aP < 0.05$ *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 latter, the mice were killed , autopsy was carried out to remove organs. The number of the seeded tumor naked in the speen, 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

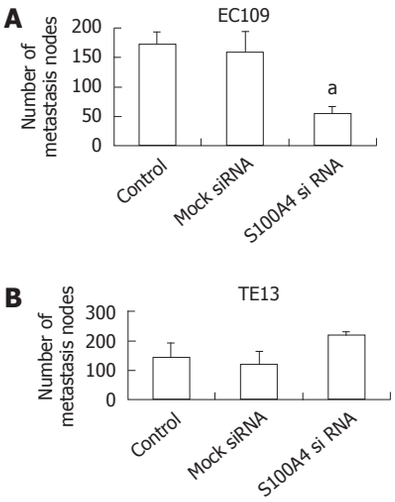

Figure 5 Knockdown of S100A4 inhibits metastasis of xenograft tumors (*n* = 6 per group). A: The total metastasis nodes in EC109 tumors ($^aP < 0.05$ *vs* control or mock siRNA); B: The metastasis nodes in TE13 tumors.

malignancy is manifested by the ability of tumor cells to traverse tissue barriers and invade surrounding tissues[31]. 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[32,33]. Recent studies have shown S100A4 is up-regulated in many types of epithelial cancers, including esophageal squamous cell[17-20]. 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[34,35].

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[36]. Currently, EMT is thought to be a key step for cancer metastasis[37]. 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[2,38-40]. Several studies have recently described a direct interaction and/or reciprocal influence between S100A4 and E-cadherin[24-30].

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 suggestd 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[41-44]. 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[45].

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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BRIEF ARTICLE

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% ± 0.1% in high-fat *vs* 2.8% ± 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.

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[1]. 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[2,3]. Impaired gut barrier function is related to several disease states such as steatohepatitis, fatty liver dis-

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

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[13]. 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[14,15]. In mice, an orally fed secondary bile acid deoxycholic acid (DCA) induces intestinal inflammation[16], and *in vitro* bile acids provoke permeability of intestinal cell monolayers[17,18]. In contrast, the most hydrophilic bile acid, ursodeoxycholic acid (UDCA), may counteract increased intestinal permeability[19].

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[20]. Mice deficient in FXR show compromised barrier function and localization of neutrophils in their intestinal villi[21].

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_2 (70%) and O_2 (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^2 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 $NaHCO_3$, 1.8 mmol/L Na_2HPO_4, 0.2 mmol/L NaH_2PO_4, 1.25 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 10 mmol/L glucose) on each side. The system was water-jacketed to 37 ℃ and carbonated with a carbogen (95% O_2, 5% CO_2; 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^2 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 ℃. 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*[22] 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 ℃. 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 ΔΔC(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

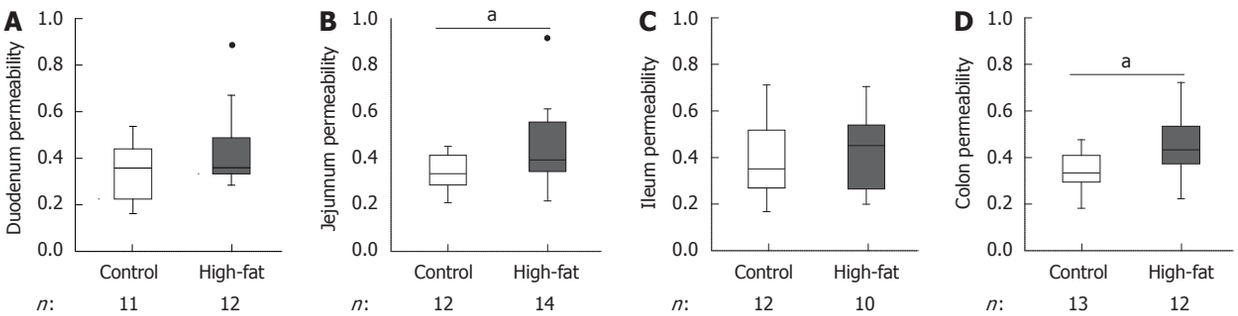

Figure 1 Effect of high-fat-feeding on intestinal permeability. Intestinal permeability in duodenum (A), jejunum (B), ileum (C), and colon (D) of high-fat-fed *vs* control mice. Permeability was measured in an Ussing chamber. Results are shown as per mille translocated dextran. Box plots show median,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

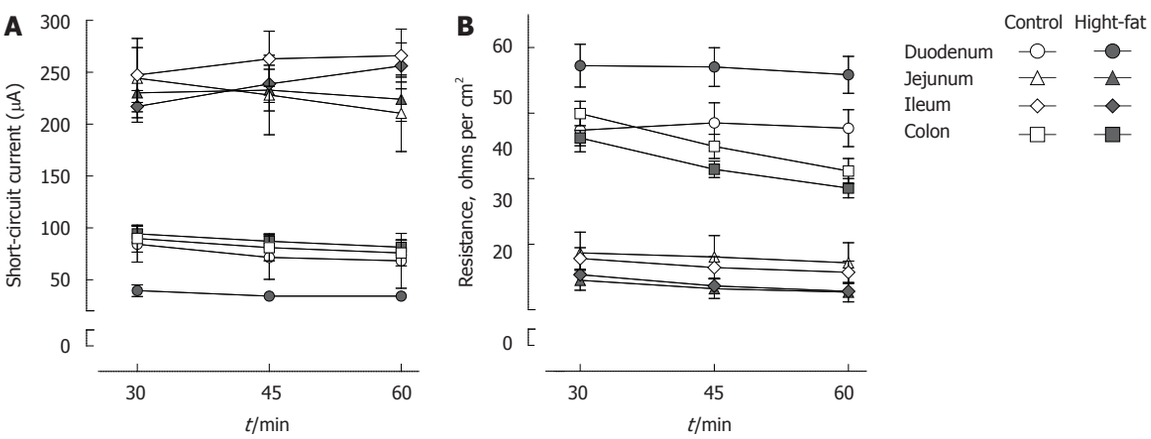

Figure 2 Stability of Ussing chamber experiments. Short-circuit current (A) and resistance (B) in Ussing chamber experiments. Bars indicate mean and SEM. n = 9-15/group.

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-

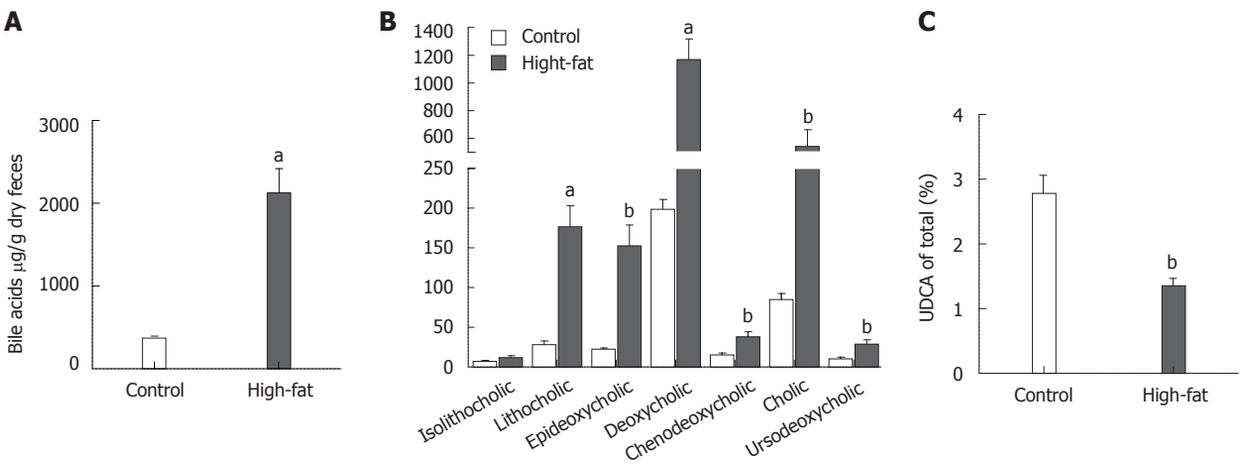

Figure 3 Fecal bile acids. A: Concentration of total measured bile acids; B: Concentration of measured bile acids in feces; C: Proportion of ursodeoxycholic acid (UDCA) in total measured bile acids. Bars indicate mean and SEM. n = 6/group, $^{a}P < 0.001$, $^{b}P < 0.01$ between high-fat and control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

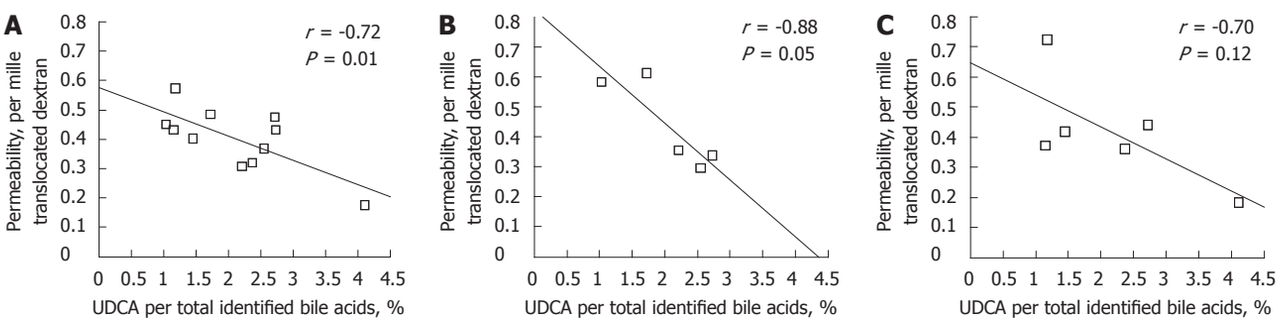

Figure 4 Correlation of proportion of ursodeoxycholic acid with intestinal permeability. Pearson's correlation of fecal proportion of ursodeoxycholic acid (UDCA) percentage to overall permeability of intestine (A), jejunal permeability (B), and colonic permeability (C). Each dot represents an individual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

Table 1 Gene expression of farnesoid X receptor and tumor necrosis factor (mean ± SEM)

	Control		High-fat		*P* value
	n	expression	*n*	expression	
FXR					
Jejunum	14	0.57 ± 0.051	12	0.74 ± 0.049	0.03
Colon	10	1.36 ± 0.234	14	1.49 ± 0.159	0.63
TNF					
Jejunum	14	0.21 ± 0.041	12	0.27 ± 0.040	0.30
Colon	10	0.42 ± 0.068	14	0.82 ± 0.148	0.02

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

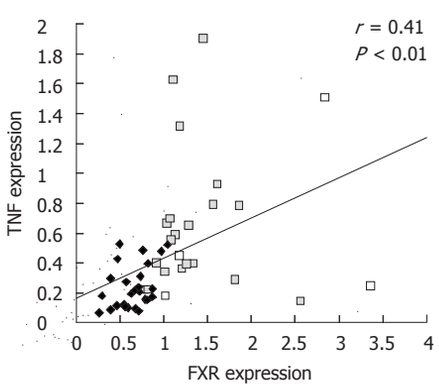

Figure 5 Correlation of tumor necrosis factor and farnesoid X receptor expression. Pearson's correlation of tumor necrosis factor (TNF) and farnesoid X receptor (FXR) expression. Jejunal values are shown as black diamonds and colonic values as grey squares. Each dot represents an individual animal.

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[23], 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 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 ± 0.049 for high-fat *vs* 0.57 ± 0.051 for control, P = 0.03) and colonic TNF expression was doubled (0.82 ± 0.148 for high-fat *vs* 0.42 ± 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[12]. Also a carbohydrate-free diet containing an extremely high amount of fat (72E%) increased intestinal permeability in mice[9]. 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[7,12].

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*[12,17,18], as well as recovery from tissue damage *ex vivo*[24], and are elevated in serum of high-fat-fed rats[12]. Our data indicate that high-fat-feeding modifies gut permeability by a mechanism related to bile acids, as previously hypothesized[12].

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*[21] 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-κB route[25], 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[16], 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*[26-29], and anti-TNF antibodies restore barrier function *in vivo*[30,31]. 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[18]. On the contrary, it is capable of counteracting intestinal barrier dysfunction[19], protecting against chemically induced colitis[19,32,33] and colitis-associated adenocarcinoma[34] in rodents. Its role may be especially relevant in comparison to DCA, because UDCA protects mitochondria against DCA-induced reactive oxygen species production[35]. 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[10]. It has also been proposed that translocation of lipopolysaccharides occurs transcellularly *via* chylomicrons[36]. 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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BRIEF ARTICLE

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-β-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 ± 1.1 mL *vs* 20.1 ± 1.7 mL) but not in the tap water group (19.0 ± 1.4 mL *vs* 19.4 ± 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 ± 1.24 μmol *vs* 4.25 ± 1.00 μmol) but not in the tap water group (3.41 ± 0.46 μmol *vs* 2.91 ± 0.56 μmol). 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.

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

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INTRODUCTION

Atherosclerosis, colelithiasis and obesity are very frequent and interrelated diseases among postmenopausal women[1-4].

High serum triglycerides and total/low-density lipoprotein (LDL) cholesterol are important risk factors for atherosclerosis, heart attack and stroke[5]. 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-β-hydroxycholesterol and 7-ketocholesterol, are specific, sensitive and reliable markers of systemic oxidative stress *in vivo*[6-8]. In addition, oxysterol coupled to vitamin E measurement in plasma can be used for estimating systemic oxidant stress/antioxidant balance[8].

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[3]. Cholesterol gallstone pathogenesis is complex and multifactorial, involving genetic defects and environmental factors[9-11]. 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[12-15].

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

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[22-26]. 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 Committee 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[27,28].

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[29].

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 ℃, 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 ℃ 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[30] 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[31].

Plasma and serum were stored at -80 ℃. Plasma levels of α-tocopherol were analyzed by high performance liquid chromatography (HPLC), and 7-β-hydroxycholesterol and 7-ketocholesterol were measured from the same sample by mass spectrometry using an isotope dilution method[8]. 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 ℃ (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	*P* 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^2)	24.9 ± 0.9	24.0 ± 0.6	NS
Diagnosis n (%)			
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[32].

BA standards were obtained from Sigma Aldrich (St. Louis, United States). Total serum BA concentration was determined enzymatically by the 3α-hydroxysteroid-dehydrogenase assay (Stereognost 3a, Pho, Nycomed, AS, Torsov, Norway). The qualitative and quantitative BA composition was assessed by an HPLC-electrospray-mass spectrometry method, as previously reported with a slight modification[33]. 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 ℃ 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 instrument. 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 χ^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-β-hydroxycholesterol, 7-ketocholesterol, α-tocopherol, γ-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.

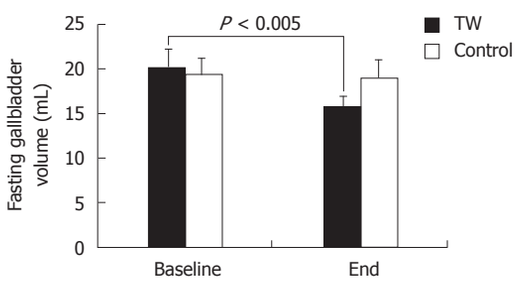

Figure 1 Fasting gallbladder volume at baseline and at the end of the study in the thermal water and in the control group. TW: Thermal water.

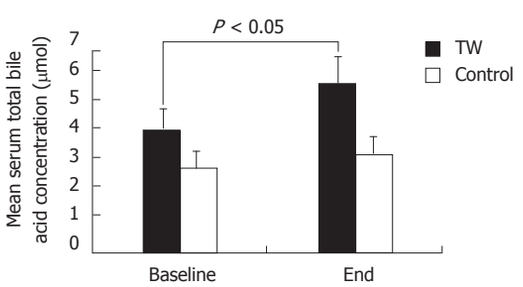

Figure 2 Mean serum total bile acid concentration at baseline and at the end of the study in the thermal water and in the control group. TW: Thermal water.

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 ± 1.1 mL *vs* 20.1 ± 1.7 mL) but not in the CTRL group (19.0 ± 1.4 mL *vs* 19.4 ± 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 ± 1.24 μmol *vs* 4.25 ± 1.00 μmol) but not in the CTRL group (3.41 ± 0.46 μmol *vs* 2.91 ± 0.56 μmol).

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 ± 0.35 μmol *vs* 0.59 ± 0.07 μmol, respectively), taurocholic acid (TCA) (0.15 ± 0.04 μmol *vs* 0.05 ± 0.02 μmol, respectively) and glycocholic acid (GCA) (0.39 ± 0.10 μmol *vs* 0.14 ± 0.02 μmol, 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 ± 0.85 μmol *vs* 1.15 ± 0.35 μmol). 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 ± 0.07 μmol *vs* 0.75 ± 0.21 μmol). 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 ± 0.14 μmol *vs* 0.27 ± 0.09 μmol) but not in the TW group (0.50 ± 0.12 μmol *vs* 0.42 ± 0.18 μmol). 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 ± 0.16 μmol *vs* 0.45 ± 0.13 μmol) but not in the TW group (1.03 ± 0.44 μmol *vs* 0.67 ± 0.16 μmol). 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 ± 0.70 μmol *vs* 1.95 ± 0.58 μmol) but not in the CTRL group (1.46 ± 0.20 μmol *vs* 1.40 ± 0.35 μmol). 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 ± 0.14 μmol *vs* 0.52 ± 0.11 μmol) but not in the TW group (1.04 ± 0.20 μmol *vs* 0.81 ± 0.27 μmol).

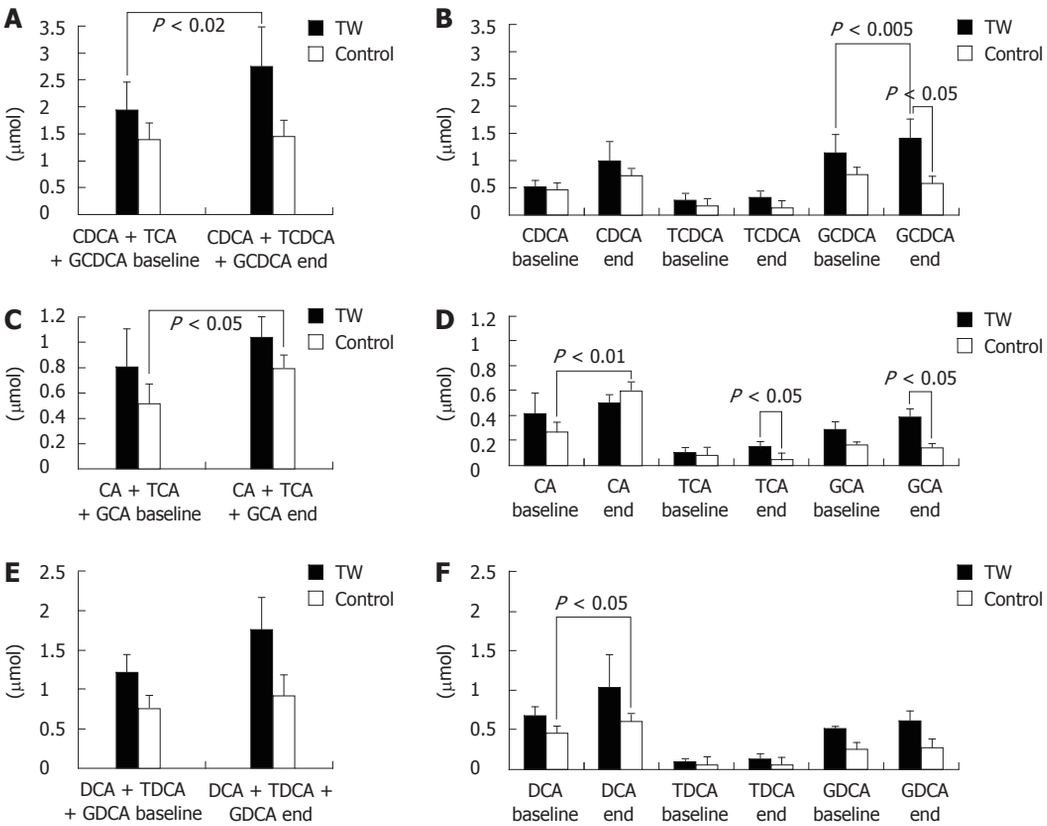

Figure 3 Mean serum bile acid molecular species concentration at baseline and at the end of the study in the thermal water and in the control group. A: Total chenodeoxycholic acid (CDCA); B: Free CDCA, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA); C: Total cholic acid (CA); 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 ± 0.057 *vs* 0.893 ± 0.055, respectively). Body weight did not differ between the TW and the CTRL group both at baseline (64.4 ± 2.4 kg *vs* 61.1 ± 1.5 kg, respectively) and at the end of the study (64.3 ± 2.4 kg *vs* 61.3 ± 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[14,34-36].

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[37,38]. 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[39]. In addition, pharmacological CDCA administration has been used as a litholytic/preventive treatment against gallstones and ameliorates cholesterol solubility in BA[40].

The beneficial effect of sulphate-bicarbonate-calcium TW administration on gallbladder motility has been already demonstrated, but the underlying mechanisms are not clear[41]. 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[42] and should be secondary to an osmotic mechanism, although the warm temperature of the TW could also play a role[41]. 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)[43]; (2) a positive correlation between serum CDCA and intestinal transit (the higher the concentration the faster intestinal transit)[14]; and (3) that CDCA administration accelerates colonic transit in healthy volunteers and in female patients with constipation-predominant irritable bowel syndrome[43,44]. 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[42] 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[21].

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 α-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-β-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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BRIEF ARTICLE

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 $\geqslant$ 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.

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[1-3].

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[4-7].

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 *linitis 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) $\geqslant$ 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[8]. 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 $\geqslant$ 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).

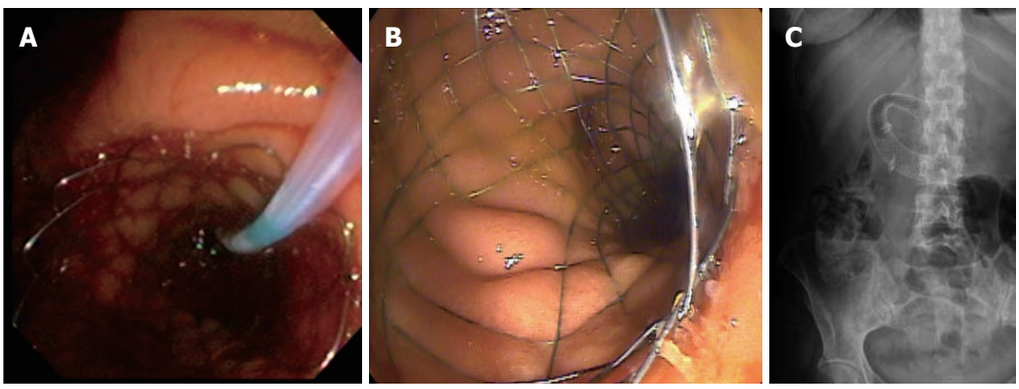

Figure 1 Use of self-expandable metal stents in gastroduodenal malignant disease. A and B: Endoscopic view of deployed self-expandable metal stents (SEMS); C: Radiologic view of duodenal SEMS.

A

	Pre	Discharge	15 dias	30	60	120	180
	2	1	1	3			
	0		1				
	0	1	1	1			
	0	2	1				
	1	1	2	1			
	0		1				
	0		1	3	2	2	1
	0	1	1	3	2		
	0	1	1	3	2		
	0	1	1				
	0	2	2	2	1	0	0
	1	1	1	3	3	3	2
	2	2	1	1			
	0	2	2	2	2	1	
	0		2	0			
No oral intake	11			1		1	1
Liquids only	2	7	11	3	1	1	1
Soft solids	2	4	4	2	4	1	1
Low residue or full diet	0			5	1	1	

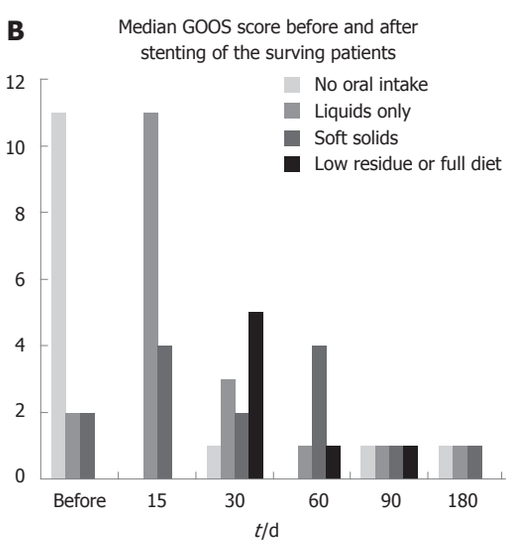

Figure 2 Bar graph showing median gastric outlet obstruction scores before the procedure and after 30 d, 60 d, 90 d and 180 d of stenting of the surviving patients.

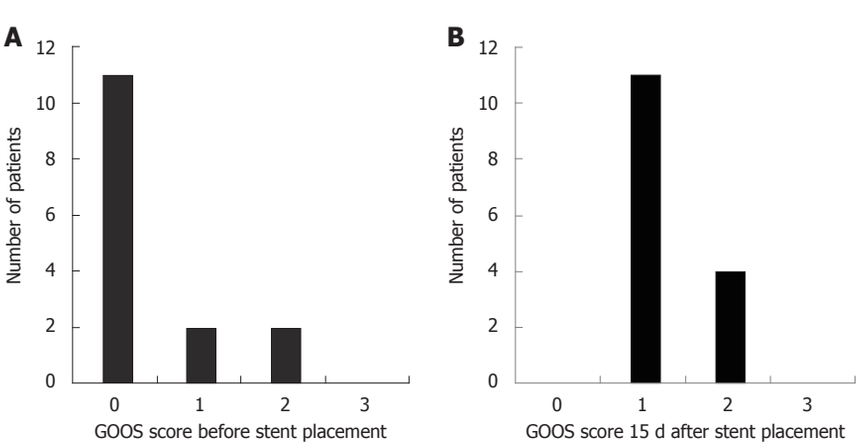

Figure 3 Bar graphs showing the gastric outlet obstruction scores. A: Before stent placement; B: 15 d after stent placement.

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[9,10]. 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[1,8,11]. 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[7,12,13].

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[14]. 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[15].

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[1,15].

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[16-18].

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[17].

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[19-21]. 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[18,21-25].

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[25].

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[22,25]. 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[22,25-27].

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[18-20]. 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[28,29], 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 ≥ 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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BRIEF ARTICLE

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

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

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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 cholecystolithiasis, 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[1]. 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)[2]. 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)[2]. 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)[3]. 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)[3].

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

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[1]
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^3)
Palpable tender mass in the right upper quadrant
Duration of complains > 72 h[2]
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 ≥ 5 μg/kg per minute, or any dose of dobutamine)
Neurological dysfunction (decreased level of consciousness)
Respiratory dysfunction (PaO2/FiO2 ratio < 300)
Renal dysfunction (oliguria, creatinine > 2.0 mg/dL)
Hepatic dysfunction (PT-INR > 1.5)

[1]Acute hepatitis, other acute abdominal disease, and chronic cholecystitis should be excluded; [2]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.

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[1]
Abnormal liver function tests[2]
C: Imaging findings
Biliary dilation, or evidence of etiology (stricture, stone, stent, *etc.*)
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[3]
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 ≥ 5 μg/kg per minute, or any dose of dobutamine
Nervous system: disturbance of consciousness
Respiratory system: PaO_2/FiO_2 ratio < 300
Kidney: serum creatinine > 2.0 mg/dL
Liver: PT-INR > 1.5
Hematological system: platelet count < 100 000/μL

Compromised patients, for example, elderly (> 75 years old) and patients with comorbidity, should be monitored closely. [1]Abnormal white blood cell count, increased serum C-reactive protein level, and other changes including inflammation; [2]increased serum alkaline phosphatase, γ-glutamyltransferase, aspartate aminotransferase and alanine aminotransferase levels; [3]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^3 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[4].

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[5]; 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. Pneumoperitoneum 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 ± SD, whereas nonparametric summary statistics are presented as medians with interquartile ranges. Categorical data were analyzed using the χ^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.

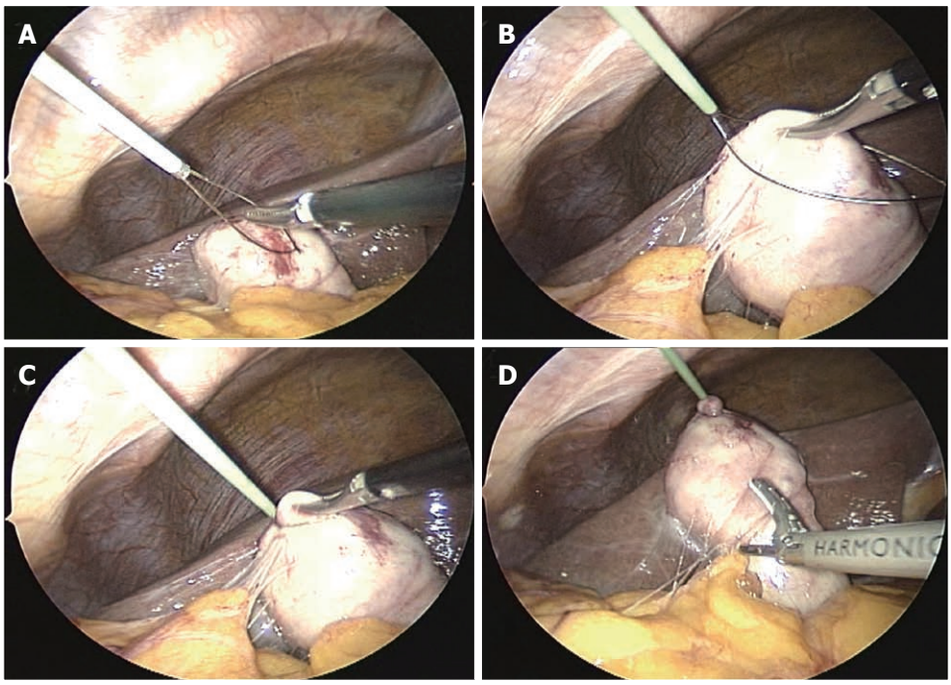

Figure 1 The way to grasp by wire loop retractor. A: Insert the grasper into the loop of wire; B: Grasp the tissue needed for retraction; C: Wring the wire; D: Retract the same as for the grasper in wire loop retractor.

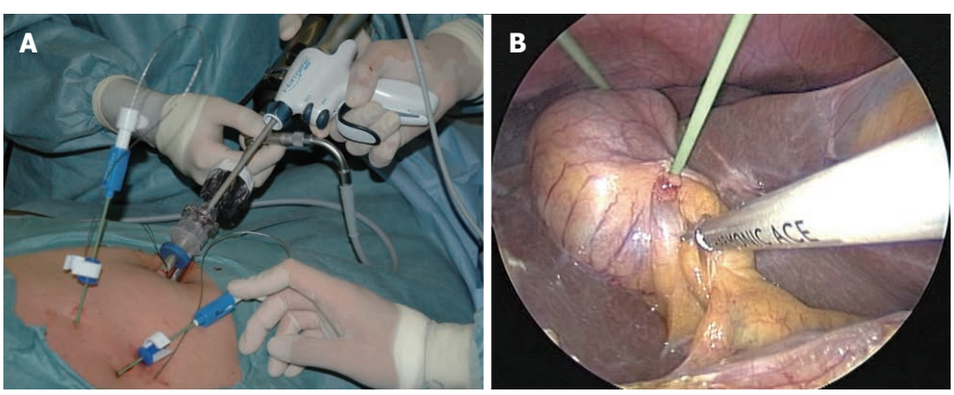

Figure 2 External and internal view of our original single-incision laparoscopic cholecystectomy. A: External view of the placement of trocars and wire loop retractors; B: Internal view of the original technique.

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
n	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 Choledochlithiasis 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 none to mild/moderate/severe	8/15/3	52/27/15	0.02
Gallbladder wall thickening none to mild/moderate/severe	16/2/8	66/14/4	< 0.01
IOC completion[1]	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	

[1]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^3, 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^3 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[6]. However, the conversion and complication rates of LC for AIG were greater than those of elective LC for other indications[7,8]. 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[6-11]. 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			
n	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			0.78
WBC > 14 000	5	13	
WBC < 14 000	21	41	
Max CRP in acute phase			0.44
CRP > 10	6	18	
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)			0.12
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[1]	23/24	42/49	0.26
Conversion to open cholecystectomy	2	5	1
Bile spillage	9	14	0.44
Complication	1	7	0.26

[1]Excluded open converted cases. ASA: American Society of Anesthesiologists; BMI: Body mass index; WBC: White blood cell; CRP: C-reactive protein; IOC: Intraoperative 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[6-11].

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[6-11].

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 convinced 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[12]. 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[13].

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[14,15]. 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 cholecystolithiasis, 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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BRIEF ARTICLE

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.

Key words: Bile reflux; Choledochal cyst; Encoscopic retrograde cholangio-pancreatography; Gadolinium-eth-oxybenzyl-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[1-3].

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[4,5].

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 pancreatico-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 pancreatico-biliary reflux. In this study, we present six patients with anomalous union of the pancreatico-biliary duct (AUPBD) who exhibited biliopancreatic bile reflux and pancreatico-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 pancreatico-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[6-8].

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 path- ology 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 pancreatico-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

					T2-MRC	Gd-EOB-DTPA-enhanced MRC			
Sex	Age	Type of choledochal cyst[1]	Type of AUPBD[2]	Surgery	CBD stone	BP	PB	Travel extent of contrast on pre-prandial images[3]	Travel extent of contrast on post-prandial images[4]
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[5]	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[6]	CBD

[1]Todani classification of choledochal cyst; [2]Kimura classification of anomalous union of pancreatico-biliary duct (AUPBD); [3]extent of contrast material on gadoxetic acid-enhanced magnetic resonance cholangiography (MRC) 60 min after administration; [4]Extent of contrast material on gadoxetic acid-enhanced MRC 30 min after a fatty meal; [5]filling defect of contrast material in common bile duct (CBD); [6]filling defect of contrast material in CBD. Biliary amylase level: 133 000 (IU/L). Gd-EOB-DTPA: Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acidBP: 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)[9-11].

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 Todani 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

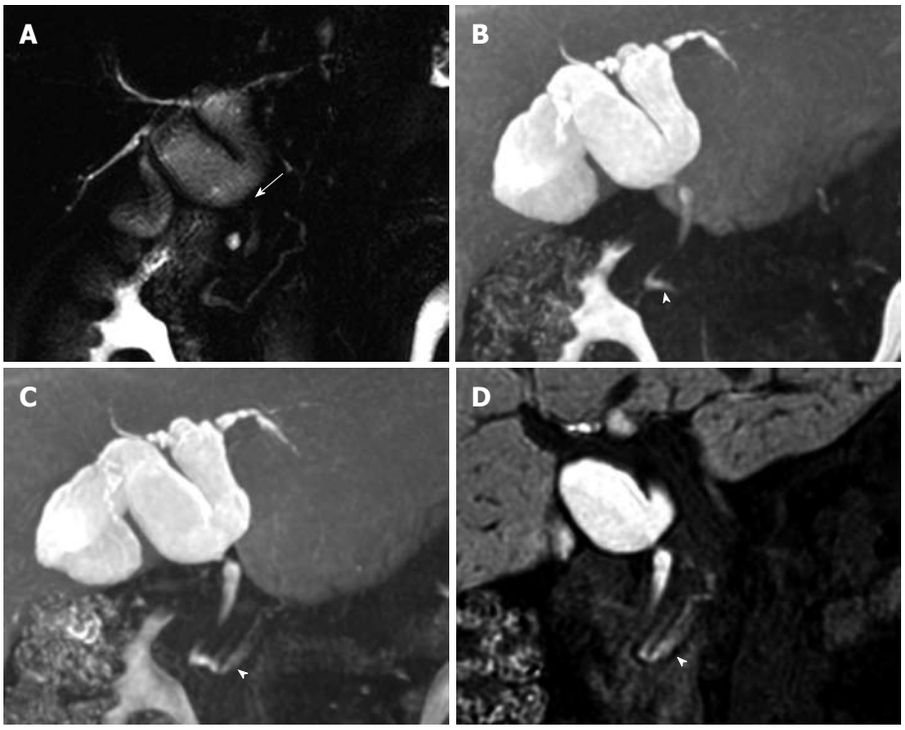

Figure 1 A 52-year-old woman with anomalous union of the pancreatico-biliary duct and a type Ⅰ a choledochal cyst. A: Fusiform dilation of the common hepatic and cystic ducts with a focal stricture in the common bile duct (arrow) on T2-magnetic resonance cholangiography (MRC); B: Maximum intensity projection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

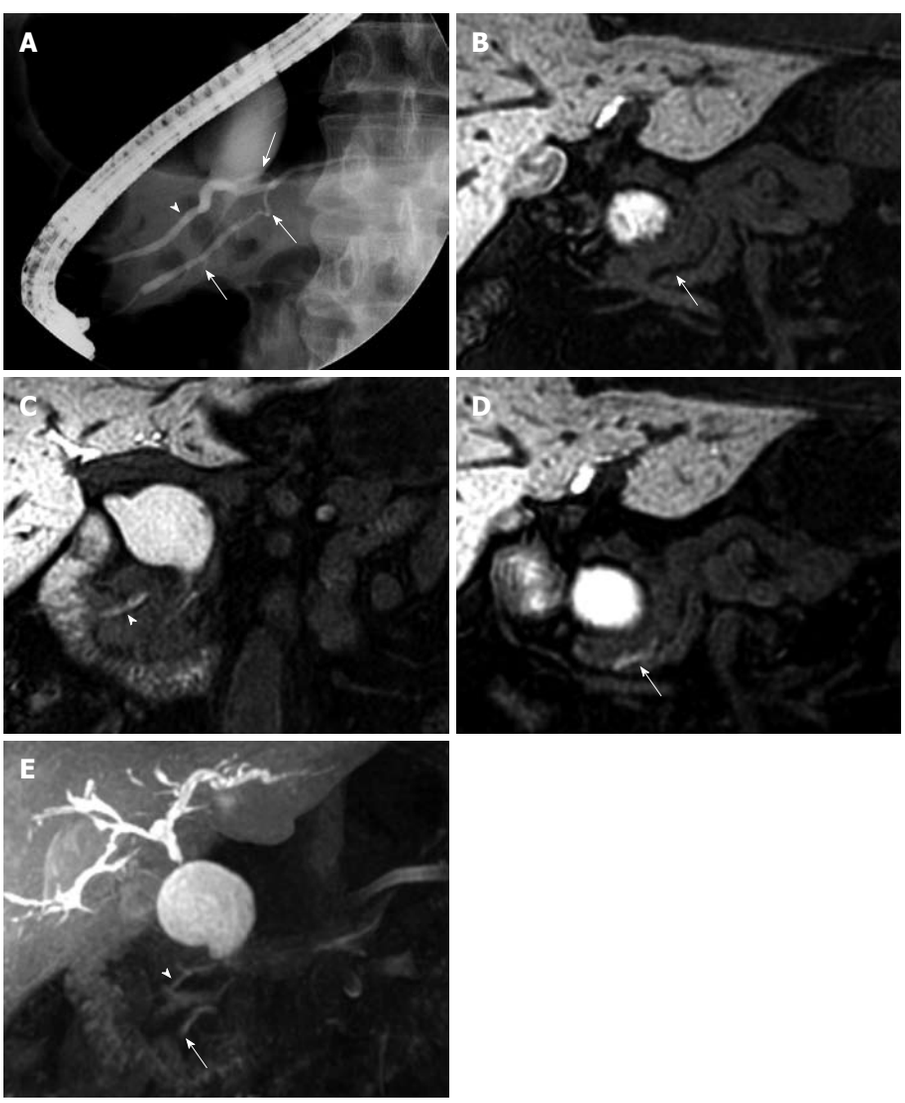

Figure 2 A 64-year-old man with anomalous union of the pancreatico-biliary duct and a type Ⅰ a choledochal cyst. A: Endoscopic retrograde cholangiopancreatography shows cystic dilation of the extrahepatic bile duct with a focal stricture in the distal common bile duct. The end of the long ventral pancreatic 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 gadoxetic acid-enhanced magnetic resonance cholangiography (MRC) coronal image does not visualize the main pancreatic duct (arrow); C and D: Gadoxetic 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: Gadoxetic acid-enhanced MRC maximum intensity projection reconstruction images taken 30 min after a fatty meal show major and minor pancreatic ducts.

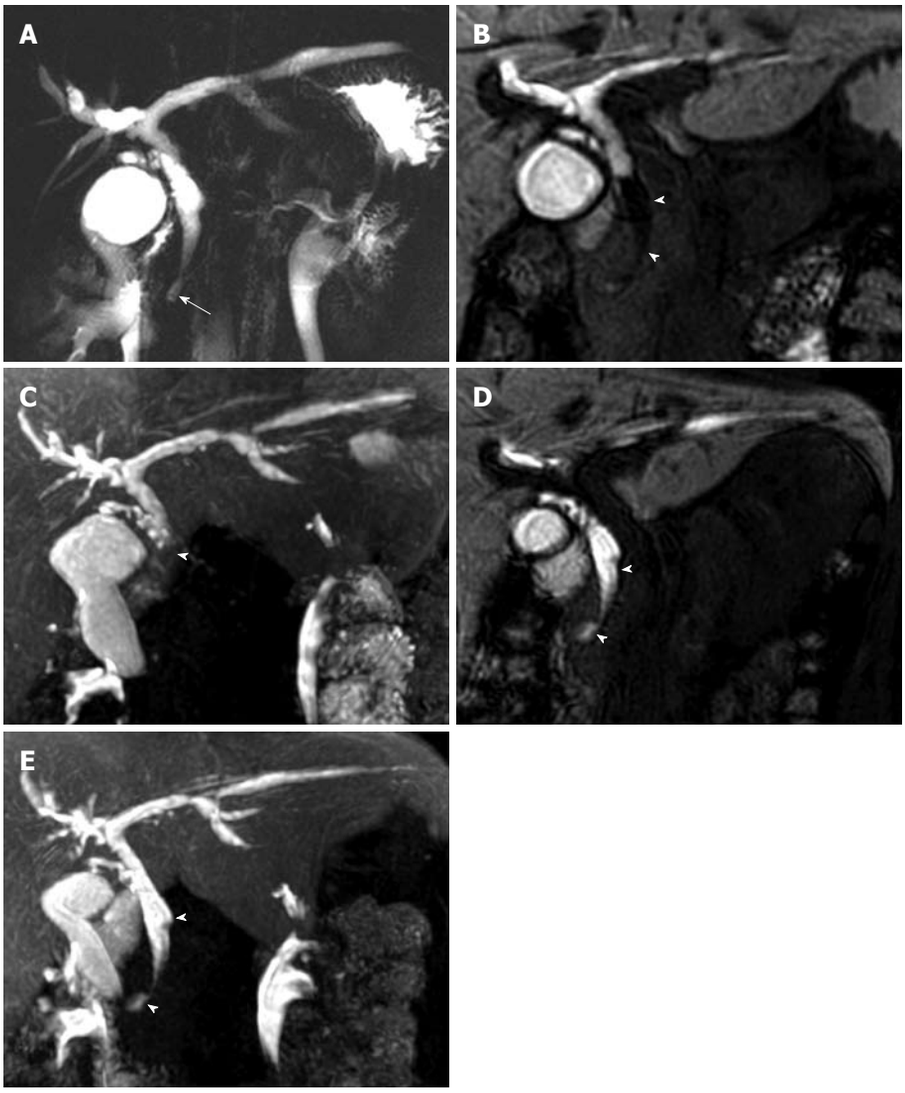

Figure 3 A 40-year-old woman with anomalous union of the pancreatico-biliary duct and a type Ⅰc choledochal cyst. A: T2-magnetic resonance cholangiography (MRC) shows a long common channel (arrow), with diffuse bile duct dilation; B: Sixty-minute delayed gadoxetic acid-enhanced MRC coronal image; C: 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.

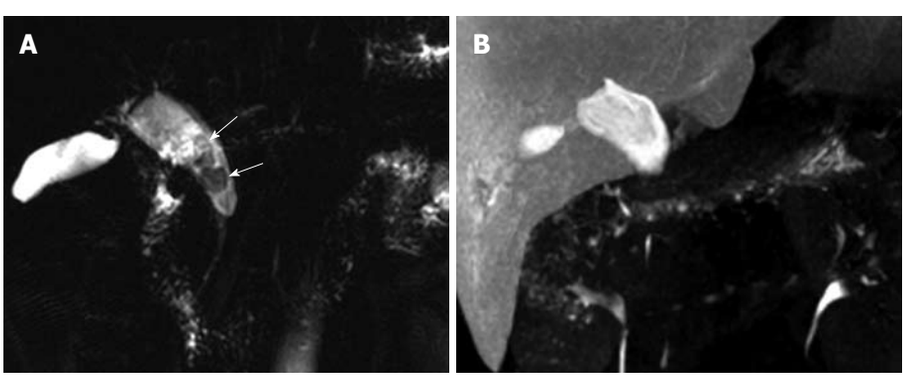

Figure 4 A 35-year-old woman with a type Ⅰa choledochal cyst and multiple common bile duct stones. A: Fusiform dilatation of the extrahepatic bile duct is seen on T2-magnetic resonance cholangiography (MRC) and multiple nodular filling defects (arrows) are seen in the common bile duct (CBD) which represent 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)[6]. 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[12,13]. 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[14].

Various methods have been reported to confirm biliopancreatic reflux, including operative or postoperative T-tube cholangiography, computered 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[12,15,16]. 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[15,17]. However, these methods are limited by their levels of invasiveness, the time required, patient discomfort, and adverse effects of contrast materials[18-20].

Gadoxetic acid is widely used because of its diagnostic efficacy in focal lesions of the liver and its safety[21-24]. 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[4-5]. 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[4,5,25,26]. 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 assessment 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[27]. Sai *et al*[28] 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*[27] 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[29,30]. 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[31].

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[32]. 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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BRIEF ARTICLE

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 ± SD (range) age of 55.8 ± 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.

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[1]. In 1854,

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

Bezoars can be classified as phytobezoars (undigested vegetables), trichobezoars (hairs), lactobezoars (milk) and pharmacobezoars (medications) according to their composition[3]. 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 ± 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 ± SD (range) age of 55.8 ± 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 ± 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%[4-8]. 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[9,10]. 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 mon to 30 years[4-7]. In our study, the mean interval between surgery and bezoar detection was 7.4 ± 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[11]. In 1986, Krausz *et al*[4] reported that 91.2% of 113 patients with phytobezoars had a history of persimmon intake. Erzurumlu *et al*[12] 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[13]. 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 *at al*[12] 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[14].

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[11,15]. However, findings from recent studies suggest that sonography or computerized tomography (CT) can assist radiologists in diagnosing bezoars before surgery[6,16]. 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[17]. 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[17]. 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[2]. 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[18], all operations were conducted as open surgery in our study.

Intestinal bezoars are often found in association with gastric bezoars[6]. Coexisting gastric bezoars was reported in 17%-21% of patients[19-21]. 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*[22] 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 commons types of articles considering this problem.

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BRIEF ARTICLE

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 coinfected 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.

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

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INTRODUCTION

Hepatitis E virus (HEV) is a single-strand, positive-sense RNA, non-enveloped virus which is classified into the *Hepeviridae* 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[1]. Genotype 1 and 2 of mammalian strains are predominant in humans and associated with large waterborne epidemics in endemic regions[2]. 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[3], and do not cause severe liver diseases[4]. In mainland China, HEV genotype 4 has become the dominant genotype instead of genotype 1 since 2004[5].

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[6-10]. 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[11].

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%[12]. HEV could result in severe disease and a poor outcome in patients with pre-existing liver diseases[13,14]. 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 coinfected 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[14-16]. 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 ℃ 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[10]. 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 resemblings 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-1Hyo04 (AB189070), swArkell (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 (μmol/L)	DBIL (μmol/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.

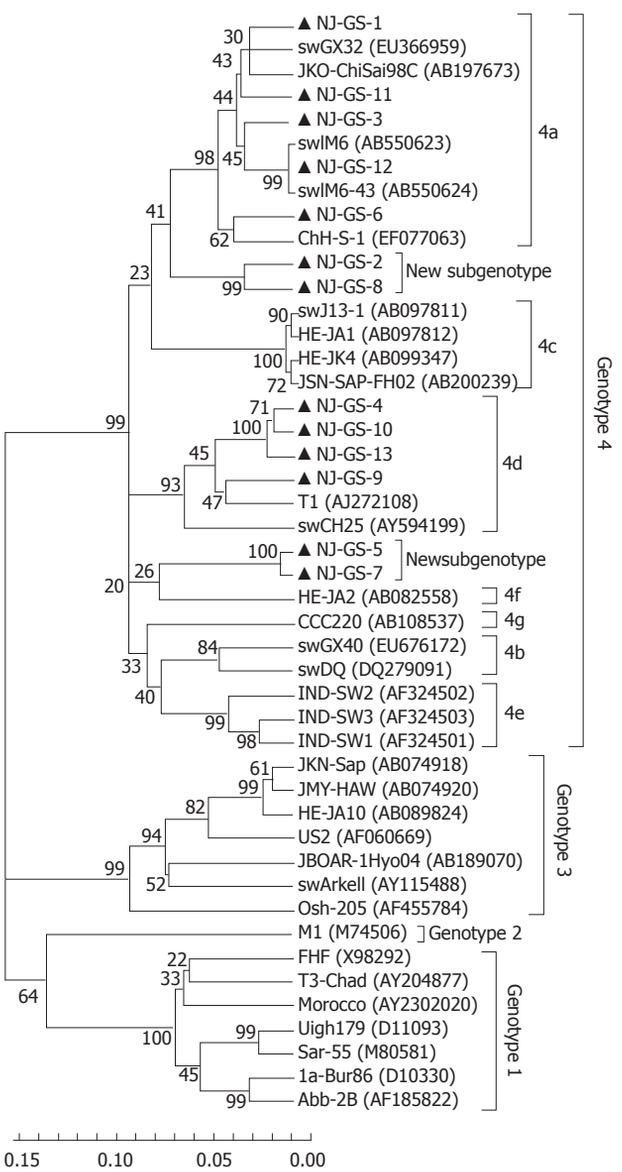

Figure 1 Phylogenetic tree based on 286 bp of open reading frame 2 of hepatitis E virus genotypes 1-4, 13 human sequences. Human hepatitis E virus isolated in this study were signed with solid triangle.

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-

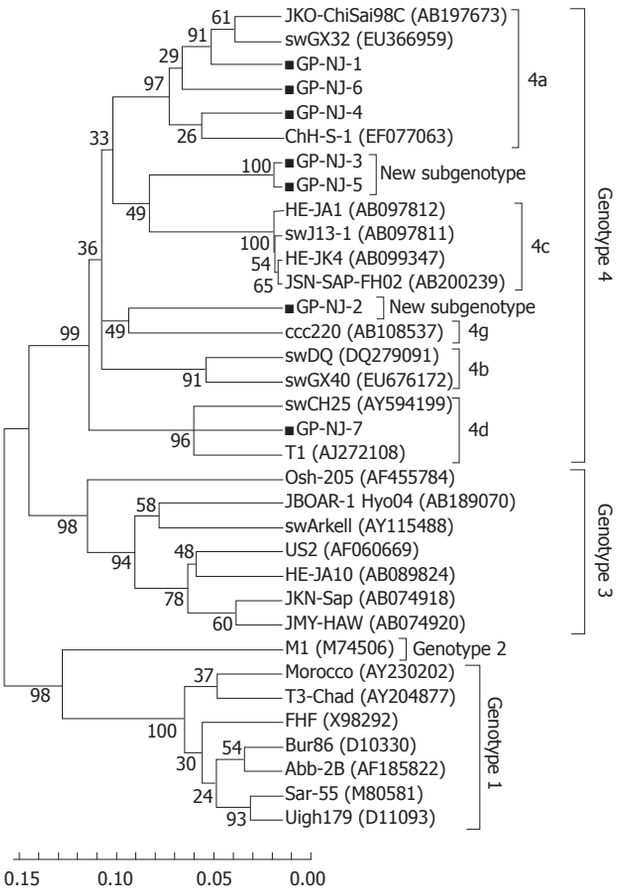

Figure 2 Phylogenetic tree based on 243 bp of open reading frame 1 of hepatitis E virus genotypes 1-4, 7 human sequences. Human hepatitis E virus isolated in this study were signed with solid square.

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[17]. 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 coinfected with HEV had more severe liver diseases with higher rates of compensated cirrhosis, hepatic failure, and complications[13,14]. 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[18,19]. Transmissions through blood transfusions, mother-to-fetus, and person-to-person were also reported[20,21], 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[6,10,17,22], 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[16]. 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[4,23]. Recently, the Wantai kit used in this study was proved to be more sensitive than GeneLab kit (98% *vs* 56%)[24], which has a high specificity for diagnosis of acute infection of HEV[25,26]. Detection of rising IgG or RNA was considered diagnostic with a specificity of 100%; the specificity of IgM was found to be 99.4%[15]. 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 or were treated at other hospitals at early period of illness, and the period of viraemia is short[3], 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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BRIEF ARTICLE

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 pared 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 $Foxp3^+$ (forkhead box P3) staining could be detected in CRC tissues and patients' blood. Interestingly, in the 33 samples (15 cases of B7-$H1^{high}$ CRC tissues and 18 cases of B7-$H1^{low}$ CRC tissues) of freshly isolated mononuclear cells from CRC tissues, the percentages of $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ regulatory T cells were found remarkably higher in B7-$H1^{high}$ CRC tissues than in B7-$H1^{low}$ 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 ($CD4^+Foxp3^-$/$CD8^+Foxp3^-$), which was thought to contribute to the anti-tumor immune response, highly expressed PD-1; while regulatory T cells ($CD4^+Foxp3^+$/$CD8^+Foxp3^-$) 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 ($CD4^+Foxp3^-$ T cell, $P < 0.0001$; $CD8^+Foxp3^-$ 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.

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[1]. 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[2,3]. Recent evidence showed that experimental depletion of Tregs improves immune-mediated tumor clearance and enhances the response to immune-based therapy[4,5]. Tregs have been shown to suppress tumor-specific T cell immunity and therefore may contribute to the progression of human tumors[6,7]. Furthermore, tumor Tregs are associated with a reduced survival of the patients with ovarian carcinoma and brain tumors[8,9]. In contrast, it has been found in Hodgkin lymphoma that decreased number of infiltrating $Foxp3^+$ cells in conjunction with increased infiltration of cytotoxic T lymphocytes predicts an unfavorable clinical outcome[10]. Although previous studies have suggested that tumors could induce $CD25^+Foxp3^+$Tregs from naïve CD4 T cells in the absence of thymus, the cellular and molecular mechanisms for that, however, are still not well understood[11,12]. Optimal activation of antigen-specific lymphocytes requires specific antigen recognition by lymphocytes and costimulatory signals[13]. 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[14-19]. 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[15]. B7-H1 was thought to inhibit T cell growth and cytokine production by ligation of the PD-1 receptor[20], which is expressed on activated T and B cells[15,21]. Zou *et al*[22] 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[23-25]. To make the situation more complicated, B7-H1 can also function as a receptor to transmit signals to T cells and tumor cells[26,27]. 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[28]. 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[29]. 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[30-32]. 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 ElivisionTM 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-μm-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 ℃ 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[21], respectively, at 4 ℃ 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 negative 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 (× 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 (× 40), then the infiltrating T lymphocytes were counted and recorded at high power field (HPF, × 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 ℃. 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 (eBioscince, United States) at room temperature, then incubated with PE-cy5 conjugated anti-Foxp3 anti-

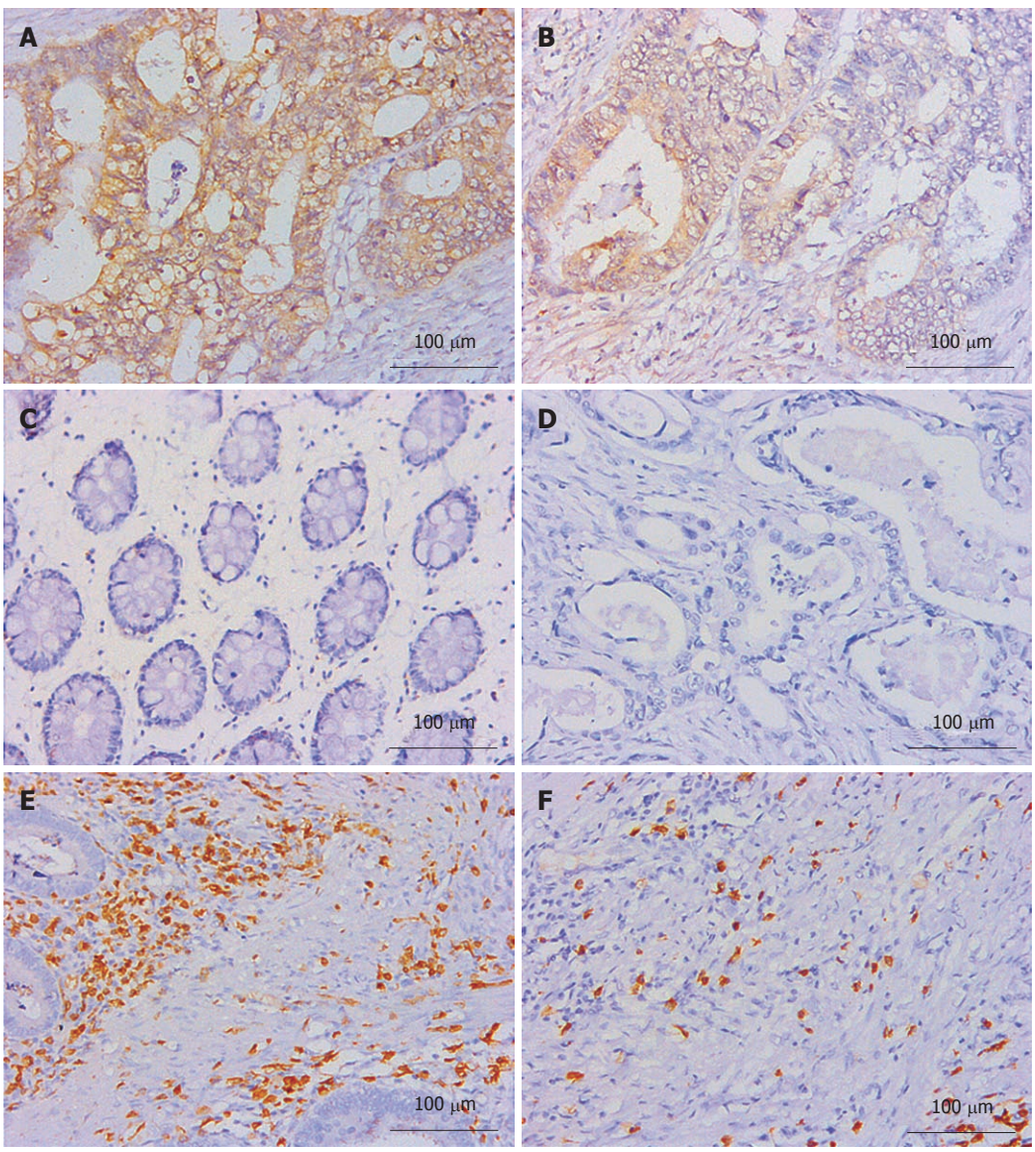

Figure 1 B7-H1 and CD3 immunostaining of colorectal carcinoma tissues. A and B: B7-H1 immunostaining in colorectal carcinoma tissues (A: Magnification 400 ×; B: Magnification 200 ×); C: B7-H1 immunostaining in normal colorectal tissues; D: Negative control in colorectal carcinoma tissues; E and F: CD3 stained infiltrating T lymphocytesb (E: Tumor stroma; F: Tumor nest).

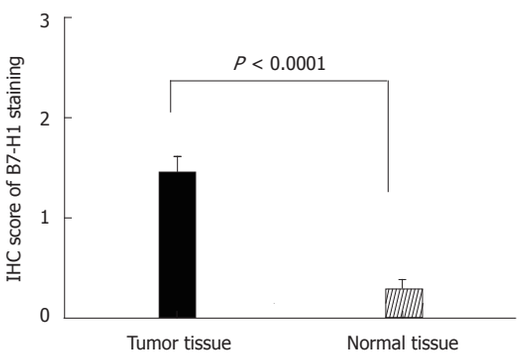

Figure 2 B7-H1 expression level in colorectal carcinoma tissues and adjacent normal tissues from 33 patients evaluated by immunohistochemisty. IHC: Immunohistochemisty.

body (eBioscince, 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 Multicomp 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 χ^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-

Table 1 Correlation between infiltrating T lymphocytes and B7-H1 expression in colorectal carcinoma tissues

Infiltrating T lymphocytes in colorectal carcinoma tissues	Cases	B7-H1 expression		χ^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[21], 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[30]. 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^{high} colorectal carcinoma tissues were infiltrated with elevated numbers of CD4$^+$/CD8$^+$ regulatory T cells

To evaluate the effect of tumor B7-H1 on intratumoral regulatory T cells, we first characterized the percentage of $CD4^+Foxp3^+$ T cells and $CD8^+Foxp3^+$ 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 $CD4^+$ or $CD8^+$ T cells, a population with a clear $Foxp3^+$ could be detected (Figure 3).

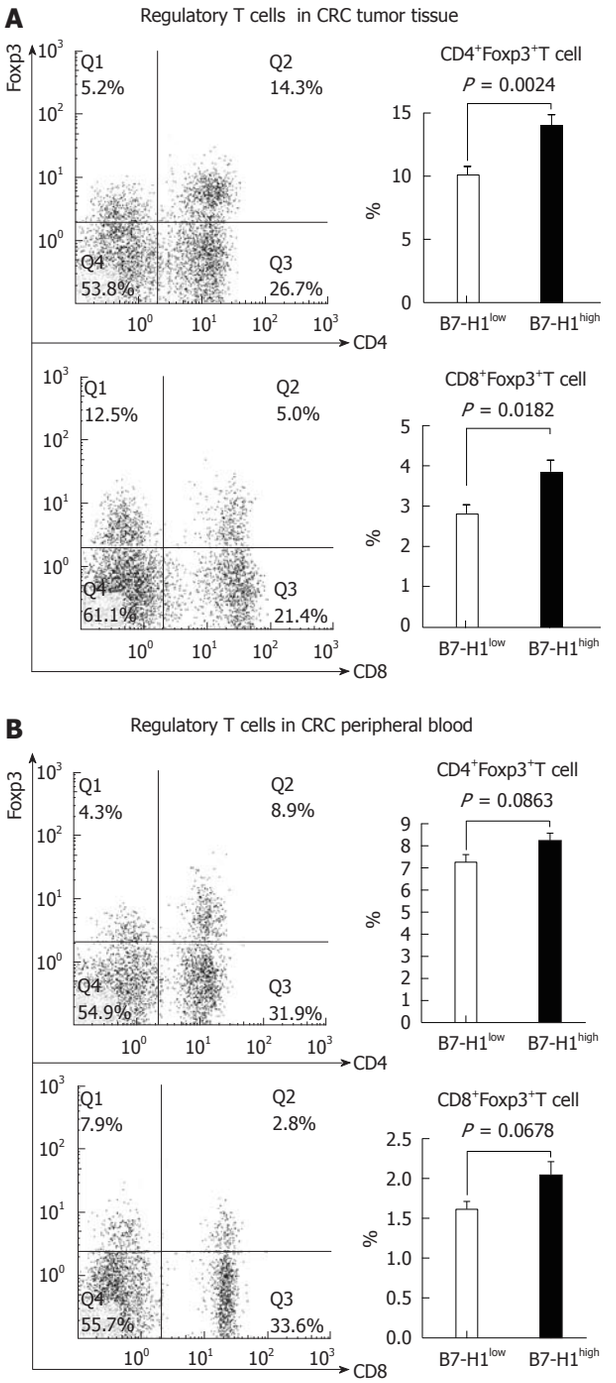

Figure 3 An elevated $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$T cell amount observed in B7-H1^{high} colorectal carcinoma tissues. Mononuclear cells were harvested from fresh tumor tissues (A) and the peripheral blood (B) of the same colorectal carcinoma patient. The percentage of the $Foxp3^+$ T cells was determined by fluorescence-activated cell sorting analysis. A: The population of $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$T cells was increased remarkably in B7-H1^{high} colorectal carcinoma (CRC) tissues compared with B7-H1^{low} CRC tissues; B: In peripheral blood, there was no significant diversity of regulatory T cells between the B7-H1low and B7-H1high 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^{high} group ($n = 15$) and B7-H1^{low} group ($n = 18$)

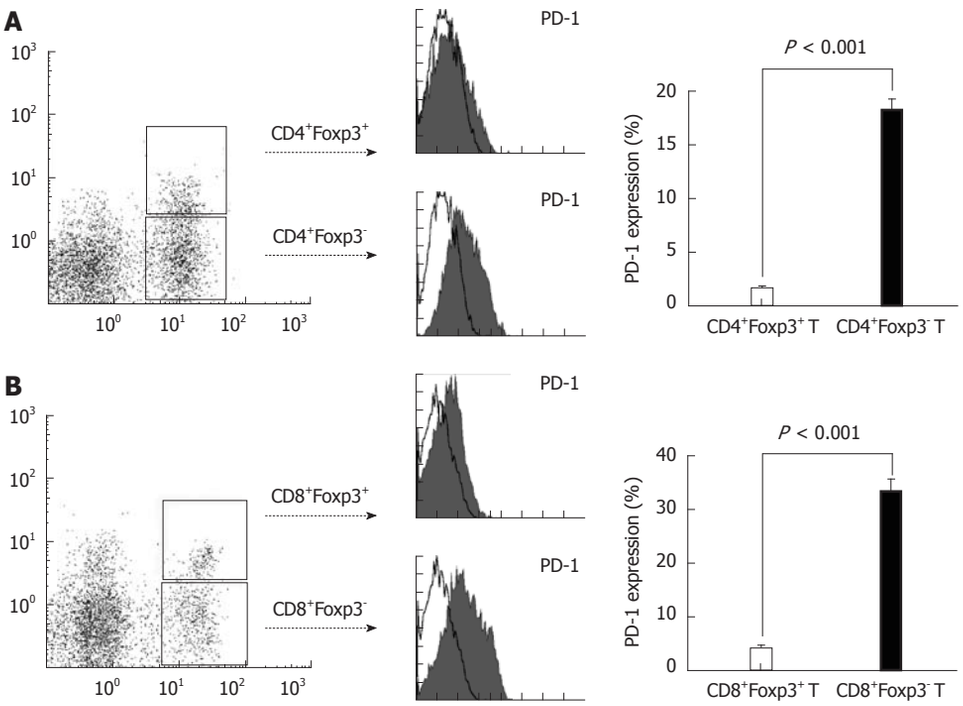

Figure 4 PD-1 expression on $CD4^+Foxp3^+$ or $CD8^+Foxp3^+$ regulatory T cells and conventional T cells in colorectal carcinoma tissues. Mononuclear cells were harvested from fresh tumor tissues of the same colorectal carcinoma patient. The PD-1 expression was determined by fluorescence-activated cell sorting analysis, gated on $CD4^+Foxp3^{+/-}$ or $CD8^+Foxp3^{+/-}$ T cells. A: $CD4^+Foxp3^+$ regulatory T cells could hardly express PD-1 on the cell surface, while PD-1 expression level was significantly higher on the conventional $CD4^+$T cells; B: $CD8^+Foxp3^+$ regulatory T cells almost failed to express PD-1, while PD-1 expression was significantly higher on the conventional $CD8^+$T cells as well.

according the B7-H1 expression in tumor tissues. The results showed that in the CRC tumor tissues, the percentages of $CD4^+Foxp3^+$ T cells and $CD8^+Foxp3^+$ T cells in B7-$H1^{high}$ group were remarkably higher than in the B7-$H1^{low}$ 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-$H1^{high}$ group and B7-$H1^{low}$ 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[33]. 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 $CD4^+Foxp3^+$/$CD8^+Foxp3^+$ regulatory T cells and $CD4^+Foxp3^-$/$CD8^+Foxp3^-$ conventional T cells in CRC tissues. We found a high percentage of $CD4^+Foxp3^-$/$CD8^+Foxp3^-$ conventional T cells expressing PD-1 on the cell surface (Figure 4, $P < 0.0001$). However, the $CD4^+Foxp3^+$/$CD8^+Foxp3^+$ regulatory T cells could hardly express PD-1 (Figure 4, $P < 0.0001$), which could allow the survival of regulatory T cells in B7-$H1^{high}$ 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[33]. 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 $CD4^+CD25^+$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[6-10]. 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[34]. In the present study, we detected the $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ regulatory T cell population in the CRC tumor tissues and peripheral blood. The percentage of regulatory T cells was significantly elevated in B7-$H1^{high}$ 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[30,35]. 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 ($CD4^+Foxp3^-$ /$CD8^+Foxp3^-$) expressed PD-1 on the cell surface at a high level, while regulatory T cells ($CD4^+Foxp3^+$ / $CD8^+Foxp3^+$) 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-$H1^{high}$ 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 $CD25^+Foxp3^+$ regulatory T cells (Treg) from naïve 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 theraputic 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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BRIEF ARTICLE

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-κB 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.

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[1,2]. 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[3]. It has been suggested that tumor cells produce IL-8 as an autocrine growth factor, which promotes tumor growth, tissue invasion and metastatic spread[4,5]. Moreover, IL-8 expression correlates with vascularity in gastric carcinoma[6]. In human moderately differentiated gastric adenocarcinoma cancer cell line SCG-7901[7] and poorly differentiated adenocarcinoma cancer cell line TMK-1[8], 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 ℃ in a humidified chamber containing 5% CO_2.

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'-GAUGGUUCCUUCCGGUGGUdTdT-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 asfollows: sense: 5'-UUCUCCGAACGUGUACGUdTdT-3'; antisense: 5'-ACGUGACACGUUCGGAGAAdTdT-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 (1×10^6 cells per well) were plated in a 6-well plate and incubated at 37 ℃ 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 1×10^5 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 ℃ 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[9]. Cells were trypsinized and seeded at 1×10^4 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 (1×10^5 cells/well) 48 h before adhesion assay. Cells (1×10^5 cells/well) were seeded onto HUVEC monolayers and incubated at 37 ℃. 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^2 overnight at 4 ℃. 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[10] using Transwells (8 μmol/L pore size polycarbonate membrane) obtained from Corning. Cells (1×10^5) 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 ℃ with 5% CO_2. 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 (5×10^5 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-μm pore BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions. Cells (2.5×10^5) were added to chambers and incubated for 24 h at 37 ℃. 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 Technol-ogies). First-strand cDNA was synthesized using 2.5 μg 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'-TCTCAGCCCTCTTCAAAAACTTCT-3'. As a control, each cDNA sample was simultaneously subjected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primers: GAPDH-L: 5'-CCACCCATGGCAAATTCC ATGGCA-3'; GAPDH-R: 5'-TCTAGACGGCAGGTCA GGTCCACC-3'. The threshold cycle (Ct) of each sample was determined, and the relative level of a transcript ($2^{\Delta Ct}$) was calculated by obtaining ΔCt (test Ct - GAPDH Ct) and then expressed as arbitrary units ($1/2^{\Delta Ct}$ 1×100) = 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 μg 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 ℃ 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-κB-p65, NF-κB-p65, phospho-Akt, Akt, ICAM-1, VCAM-1, CD44, and β-actin (Cell Signaling Technology, Inc., Danvers, MA). The bound antibodies were visualized using an electrochemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

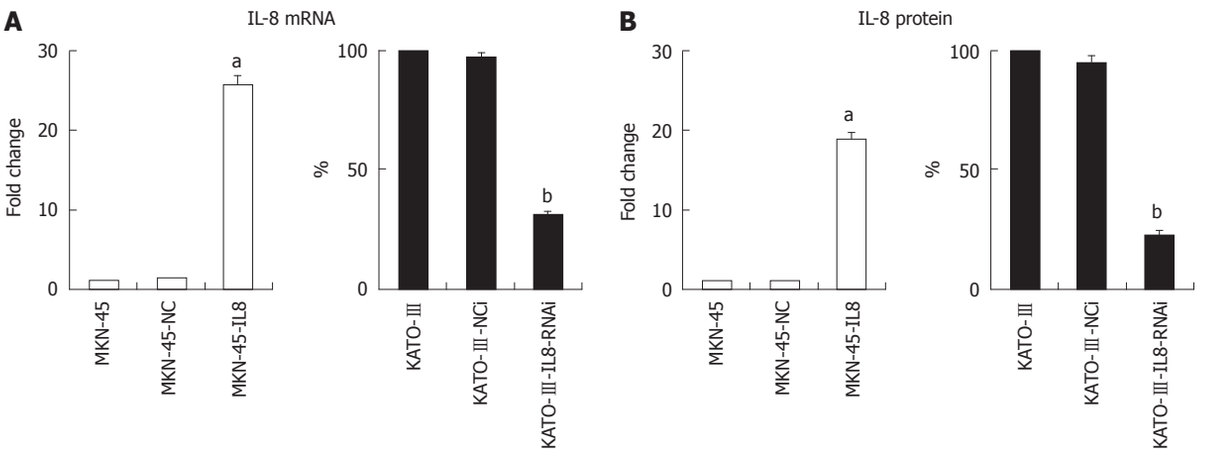
Statistical analysis

Data were presented as means ± 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

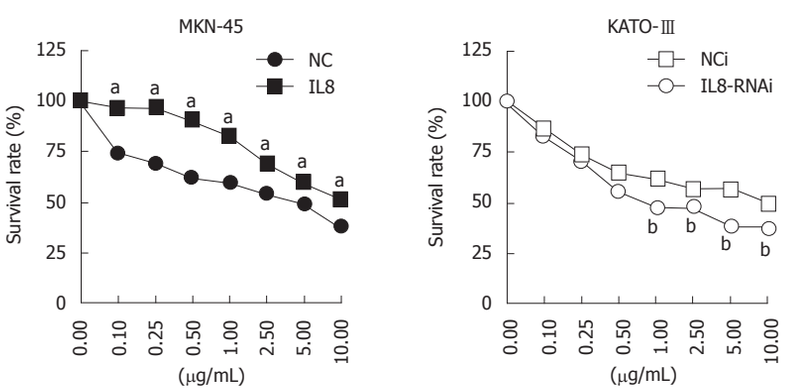
To investigate the effect of IL-8 in GC, the mRNA expression of IL-8 was quantified in three GC cells (Figure 1A). MKN-45 cell lines had lower expression of IL-8 in comparison with KATO-Ⅲ cells, which is consistent with a previous study by Kitadai *et al*[11]. To increase the IL-8 expression, MKN-45 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).



	IL-8 mRNA	SD		IL-8 mRNA	SD
MKN-45	0.98	0	KATO-Ⅲ	100.00	0
MKN-45-NC	1.34	0.04	KATO-Ⅲ-NCi	97.49	1.63
MKN-45-IL8	25.64	1.259	KATO-Ⅲ-IL8-RNAi	31.18	1.259

	IL-8 protein	SD		IL-8 protein	SD
MKN-45	0.99	0	KATO-Ⅲ	1.00E + 02	0
MKN-45-NC	1.03	0.04	KATO-Ⅲ-NCi	9.50E + 01	2.4
MKN-45-IL-8	18.75	0.9	KATO-Ⅲ-IL8-RNAi	22.43	1.8

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 ΔΔCt 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 ± SD ($^aP < 0.05$ *vs* MKN-45, $^bP < 0.05$ *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 ± SD ($^aP < 0.05$ *vs* MKN-45, $^bP < 0.05$ *vs* KATO-Ⅲ).



	NC	IL8
0.00	100	100
0.10	73.9434	96.8807
0.25	69.395	96.6221
0.50	61.9587	90.9916
1.00	59.9102	82.852
2.50	54.2774	68.6101
5.00	48.9984	59.7491
10.00	37.996	50.8949

	NCi	IL8-RNAi
0.00	100.00	100.00
0.10	86.80	82.90
0.25	73.60	70.70
0.50	65.10	56.05
1.00	62.10	47.60
2.50	57.30	47.60
5.00	57.30	38.29
10.00	50.20	37.44

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 ± SD. All experiments were repeated three times with similar results ($^aP < 0.05$ *vs* MKN-45-NC, $^bP < 0.05$ *vs* KATO-Ⅲ-NCi).

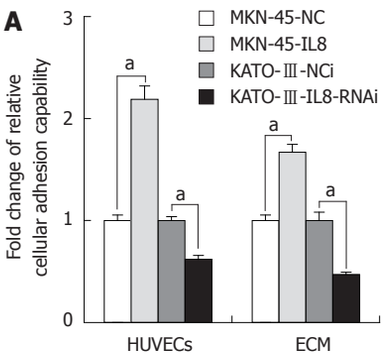
Interleukin-8 insignificantly affects proliferation of gastric cancer cells

Since IL-8 has been reported to be an autocrine growth factor[12], 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).

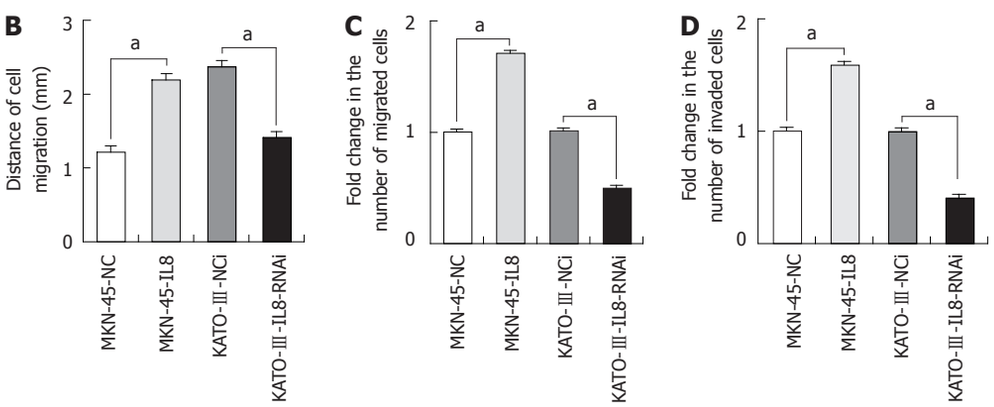
Interleukin-8 increases resistance to oxaliplatin in gastric cancer cells

Previous reports indicated that increased IL-8 was associated with chemoresistance, such as oxaliplatin[13-15]. 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 μg/mL to 10 μg/mL ($P <$



	MKN-45-NC	MKN-45-IL8	KATO-Ⅲ-NCi	KATO-Ⅲ-IL8-RNAi
HUVECs	1	2.19	1	0.62
ECM	1	1.67	1	0.47
SD				
HUVECs	0.05	0.13	0.04	0.04
ECM	0.05	0.08	0.08	0.02



	Distance	SD
MKN-45-NC	1.22	0.07
MKN-45-IL8	2.19	0.11
KATO-Ⅲ-NCi	2.37	0.11
KATO-Ⅲ-IL8-RNAi	1.41	0.08

	Fold chage	SD
MKN-45-NC	1.00	0.05
MKN-45-IL8	1.71	0.09
KATO-Ⅲ-NCi	1.01	0.05
KATO-Ⅲ-IL8-RNAi	0.50	0.02

	Fold chage	SD
MKN-45-NC	1.00	0.05
MKN-45-IL8	1.59	0.05
KATO-Ⅲ-NCi	1.00	0.06
KATO-Ⅲ-IL8-RNAi	0.41	0.03

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 ± SD. All experiments were repeated three times with similar results ($^aP < 0.05$). 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 μg/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-κB 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-κB transcriptional activity, and activated the phosphoinositide-3-kinase and cascade. As shown in Figure 4, the levels of phospho-NF-κB-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-κB and Akt activation.

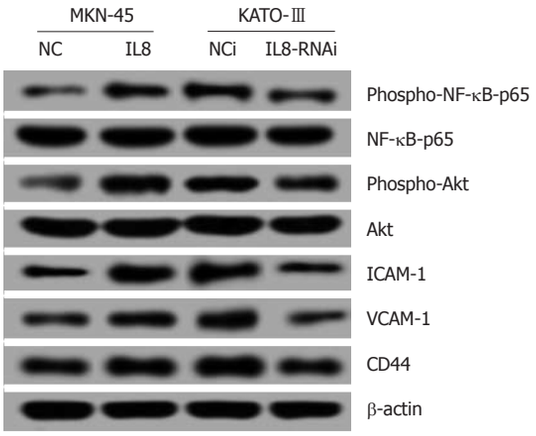


Figure 4 Effect of Interleukin-8 on NF-κB and Akt activities and adhesion molecules ICAM-1 and VCAM-1, and CD44 expression in gastric cancer cells. The protein expression levels of phospho-NF-κB-p65, NF-κB-p65, phospho-Akt, Akt, ICAM-1, VCAM-1, and CD44 were determined by Western blotting. All experiments 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[16-18], prostate cancer[14,19], breast cancer[20], and colon cancer[13]. A study confirmed that IL-8 increases angiogenesis of human gastric carcinoma[6]. 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*[7], 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[6,7].

The NF-κB and Akt signaling pathway activations are involved in cellular transformation, survival, proliferation, 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[13] and prostate cancer cells[14]. 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 caner is dependent on the capacity of cancer cell adhesion and migration[21]. 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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BRIEF ARTICLE

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.

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[1-5]. 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[6-8].

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)[9,10].

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-liked immunosorbent assay (ELISA) for IgG and IgM with anti-HEV activity described previously[11,12]. 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 Qiagen viral RNA kit (Qiagen) according to the manufacturer's instructions. The viral RNA was finally dissolved in 20 μL 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[13]. The external primers were P1 (5'-CCGACAGAATTGATTTC GTCGGC-3') and P4 (5'-CCGTAAGTCGACTGGTCG-TACTC-3'). The internal primers were P2 (5'-GTTGTCTC-GGCCAATGGCGAGCC-3') and P3 (5'-TCGGCGGCG-GTGAGAGAGCCA-3'). The first-round and the second-round amplifications were carried out according to the following cycling program denaturation at 9 ℃ for 45 s, annealing at 52 ℃ for 60 s, extension at 72 ℃ 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/BioInfo/Clustal X /Top.html), and the phylogenetic analysis was performed using the MEGA program, version 3.1 (Pennsylvania State University).

Statistical analysis

The Pearson χ^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 ± 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 *n* (%)	OR (95% CI)	*P* 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 showed 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 showed in Table 2. HEV infection prevalence was significantly correlated with the increasing age in total participants ($\chi = 2.91$, $P = 0.004$) and female participants ($\chi = 1.97$, $P = 0.048$).

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 (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).

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		*P* 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 (*n* = 124)	HEV negative IgG (*n* = 422)	*P* 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.

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[14].

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[15,16].

Although no statistically significant association was observed between HEV seropositivity and blood-borne hep-

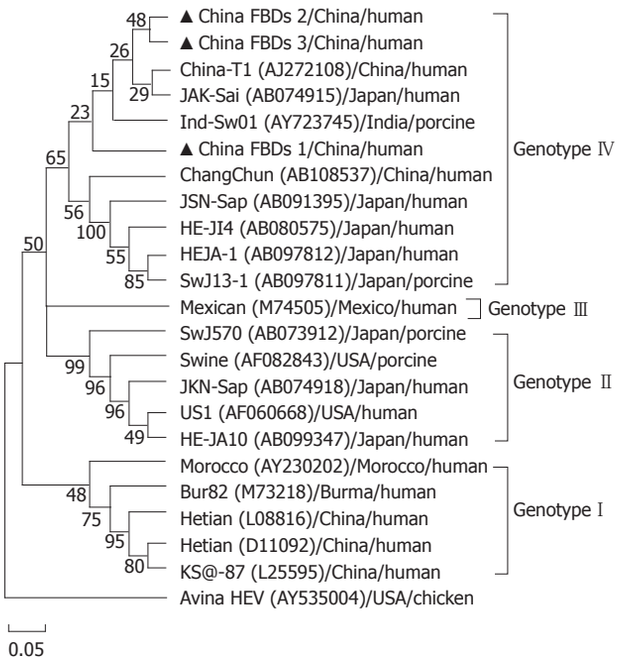

Figure 1 A phylogenetic tree is constructed using the neighbor-joining method based on the 236-nt ORF2 sequences of four genotypes hepatitis E virus isolates. The isolates were named by the accession number in parentheses, the name of the country of origin and species from which it was isolated. Bootstrap 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[17].

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)[18]. 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[19-21]. Schlauder *et al*[22] 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[23], 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 wildly 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 calici2-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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BRIEF ARTICLE

Prophylaxis of chronic kidney disease after liver transplantation - experience from west China

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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^2, 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 $\leqslant$ 6 ng/mL while 65.8% (79 of 120 cases) of which were treated with CSA had a concentration $\leqslant$ 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.

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[1]. 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[2-7]. Cohen *et al*[8] 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*[9] 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[2,4-7]. Its nephrotoxicity is seen by kidney biopsy, which includes severe tubular atrophy, interstitial fibrosis and focal hyalinosis of small renal arteries and arterioles[10,11]. Lee *et al*[12] 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[4,6,7,13,14]. Shenoy *et al*[13] 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. Cantarvich *et al*[15], 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 × creatinine $(mg/dL)^{-1.154}$ × $(age)^{-0.203}$ × (0.742 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[16]. CKD was defined as GFR < 60 mL/min per 1.73 m^2 for at least 3 consecutive months. Hepatorenal syndrome was defined as Salerno *et al*[17] 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^2.

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 ⩾ 6.22 mmol/L, hypertriglyceridemia as triglyceride ⩾ 2.26 mmol/L[18]. Diabetes mellitus (DM) was diagnosed if

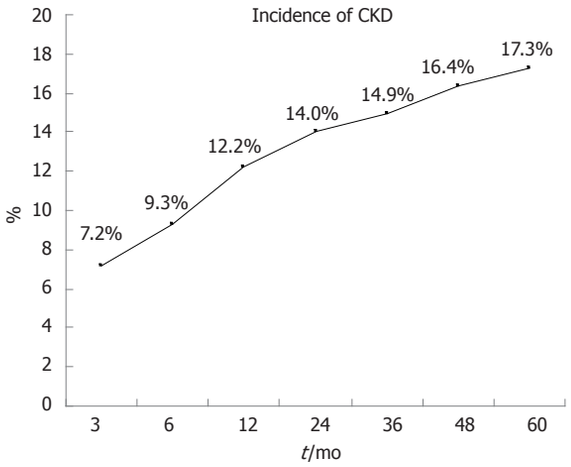


Figure 1 Incidence of chronic kidney disease 5 years after liver transplantation. The estimated glomerular filtration rate (eGFR) was calculated by the abbreviated Modification of Diet in Renal Disease formula after each visit of a patient. And once met the criterion of chronic kidney disease (CKD) (eGFR < 60 mL/min per 1.73 m^2), 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 ⩾ 11.1 mmol/L or fasting plasma glucose ⩾ 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 ± 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 χ^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 diseas recipients *n* (%)

Clinical features	CKD group (*n* = 99)	Non-CKD group (*n* = 473)	*P* 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 ⩽ 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 (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 administrated 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).

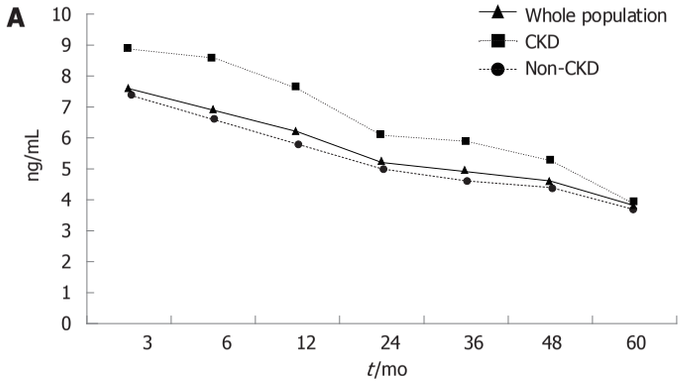
DISCUSSION

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[4,6,7,13,14]. Both were used. However, because of the CNI withdrawal time and dose reduction level differed among different centers, no agreement was reached.

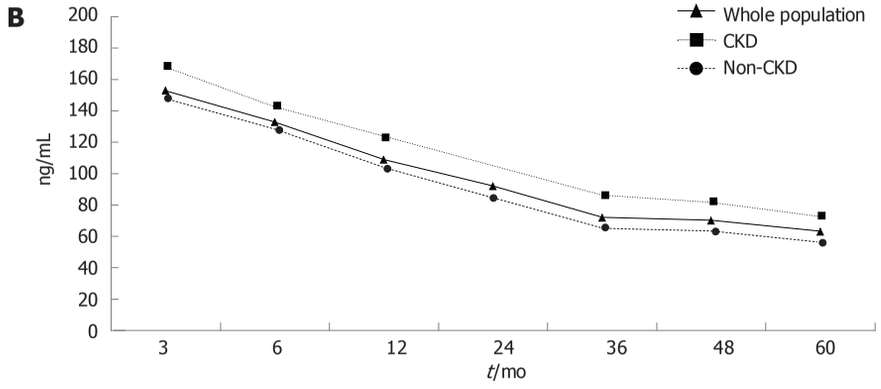
Unlike the strategies mentioned above, in our center, an initial low CNI dose was administered. And by intensely monitoring of the graft function and gradually CNI dose reduction, a low blood concentration of CNI was maintained thereafter. The results displayed that over half the patients managed to 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[2-7]. We defined CKD as GFR < 60 mL/min per 1.73 m^2 in this research, a level at which the prevalence of complications of CKD begins to increase[19,20]. 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



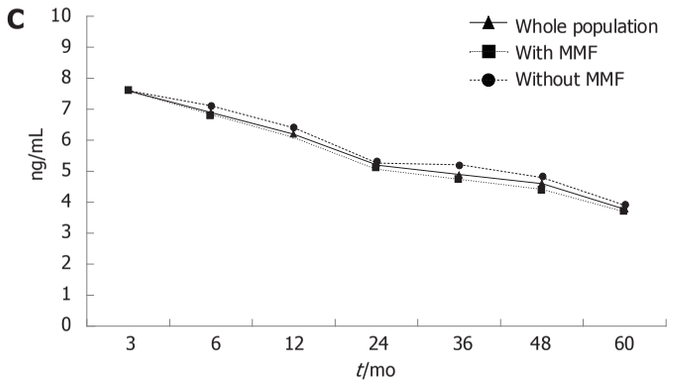
Cases:	429	355	330	216	125	76	54
CKD:	8.9 ± 3.7	8.6 ± 3.1	7.6 ± 3.1	6.1 ± 2.0	5.9 ± 1.7	5.3 ± 1.3	3.9 ± 1.9
Non-CKD:	7.4 ± 2.6	6.6 ± 2.5	5.9 ± 2.1	5.0 ± 1.5	4.6 ± 1.3	4.4 ± 1.3	3.7 ± 0.8
P value:	0.002	0.001	0.001	0.003	0.001	0.011	NS



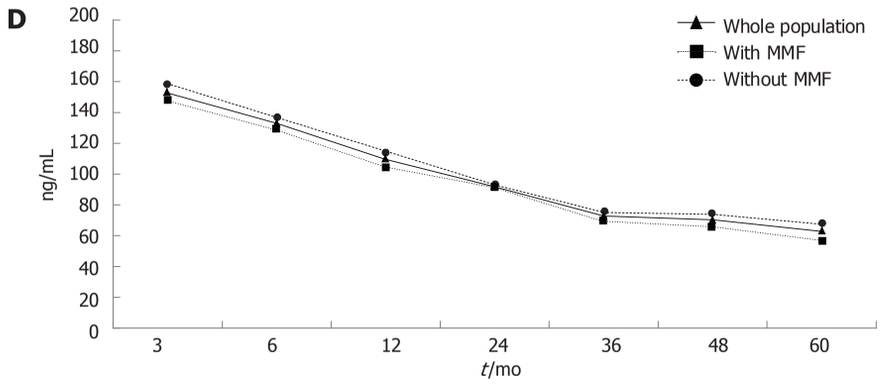
Cases:	143	120	120	93	66	48	35
CKD:	167.8 ± 56.3	142.7 ± 44.7	122.0 ± 36.7	104.4 ± 36.3	86.2 ± 37.5	81.5 ± 9.6	73.3 ± 30.0
Non-CKD:	147.6 ± 53.7	129.2 ± 40.7	104.3 ± 28.9	86.5 ± 22.4	65.1 ± 14.2	63.0 ± 12.7	56.3 ± 20.4
P value:	NS	NS	0.007	0.021	0.020	0.001	NS

A	Whole population	CKD	Non-CKD
3	7.6	8.9	7.4
6	6.9	8.6	6.6
12	6.2	7.6	5.9
24	5.2	6.1	5
36	4.9	5.9	4.6
48	4.6	5.3	4.4
60	3.8	3.9	3.7

B	Whole population	CKD	Non-CKD
3	153	168	148
6	133	143	129
12	109	122	104
24	92	104	87
36	72	86	65
48	70	82	63
60	63	73	56



Cases:	429	355	330	216	125	76	54
MMF:	7.6 ± 3.0	6.8 ± 2.7	6.1 ± 2.4	5.1 ± 1.5	4.7 ± 1.5	4.4 ± 1.0	3.9 ± 1.4
No MMF:	7.6 ± 2.5	7.1 ± 2.6	6.4 ± 2.4	5.3 ± 1.9	5.2 ± 1.5	4.8 ± 1.6	3.7 ± 1.1
P value:	NS	NS	NS	NS	NS	NS	NS



Cases:	143	120	120	93	66	48	35
MMF:	148.4 ± 33.3	129.4 ± 33.9	104.4 ± 25.6	91.5 ± 24.8	69.5 ± 24.3	64.8 ± 13.2	56.5 ± 19.1
No MMF:	155.8 ± 67.2	135.3 ± 47.7	112.9 ± 36.2	92.3 ± 31.7	74.6 ± 27.7	74.3 ± 14.7	68.4 ± 29.2
P value:	NS	NS	0.007	0.021	0.020	0.001	NS

C	Whole population	With MMF	Without MMF
3	7.6	7.6	7.6
6	6.9	6.8	7.1
12	6.2	6.1	6.4
24	5.2	5.1	5.3
36	4.9	4.7	5.2
48	4.6	4.4	4.8
60	3.8	3.7	3.9

D	Whole population	With MMF	Without MMF
3	153	148	158
6	133	129	135
12	109	104	113
24	92	91.5	92.3
36	72	69	75
48	70	65	74
60	63	56	68

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*[21] 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[3,6,7,22]. In this study, both renal dysfunction pre-operation and AKI post-operation proved to be important risk factors for CKD. Velidedeoglu *et al*[23] 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[24-26].

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-β and other profibrotic mediators[27].

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[4,6,7,13,14,28]. 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[5,29].

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[12]. 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[9,30]. 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 administrated with only a few patients[31]. 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 posttransplantation DM could reach 14.9% in the living donor liver transplantation[32]. 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[33,34], 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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CASE REPORT

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 understanding 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.

Key words: Bowel angioedema; Bowel thickening; Computed tomography; Contrast media; Small bowel anaphylaxis

Peer reviewer: Arthur J Segal, Professor, Rochester General Hospital, Department of Diagnostic Imaging, 1425 Portland Ave, Rochester, NY 14621, United States

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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[1,2]. 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[3-5]. 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 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.

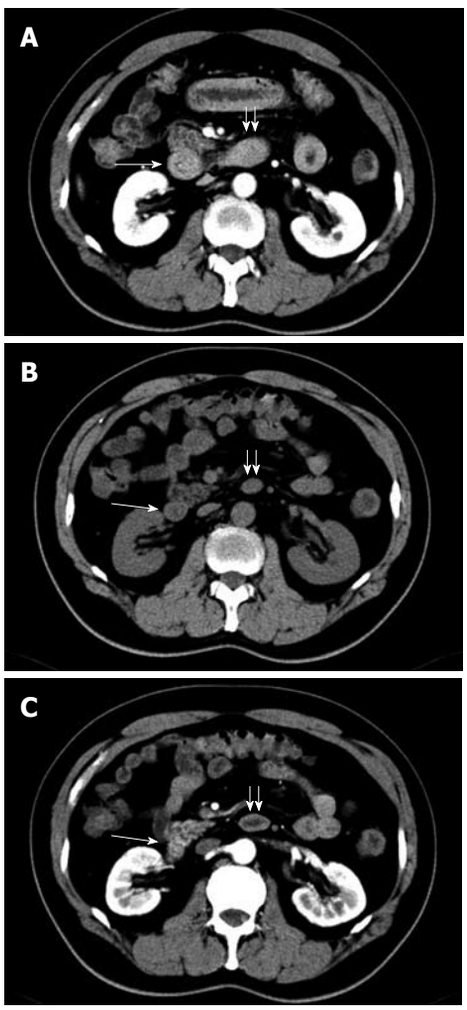

Figure 1 A 55-year-old man. A: Circumferential thickening of the proximal small bowel in a long segment including the descending duodenum (long arrow) and horizontal duodenum (double short arrows) in venous phase image; B and C: This bowel segment appears normal in unenhanced image (B) and arterial phase image (C).

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

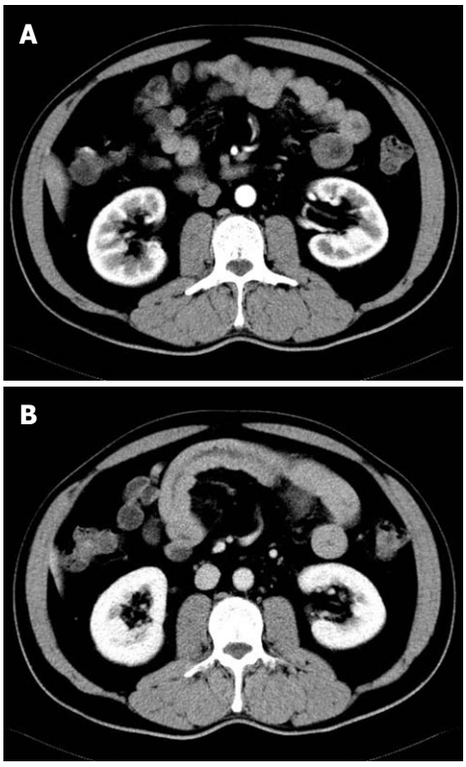

Figure 2 A 46-year-old man. A: The proximal bowel segment is slightly thickened in arterial phase image; B: The proximal bowel segment shows marked circumferential thickening in venous phase.

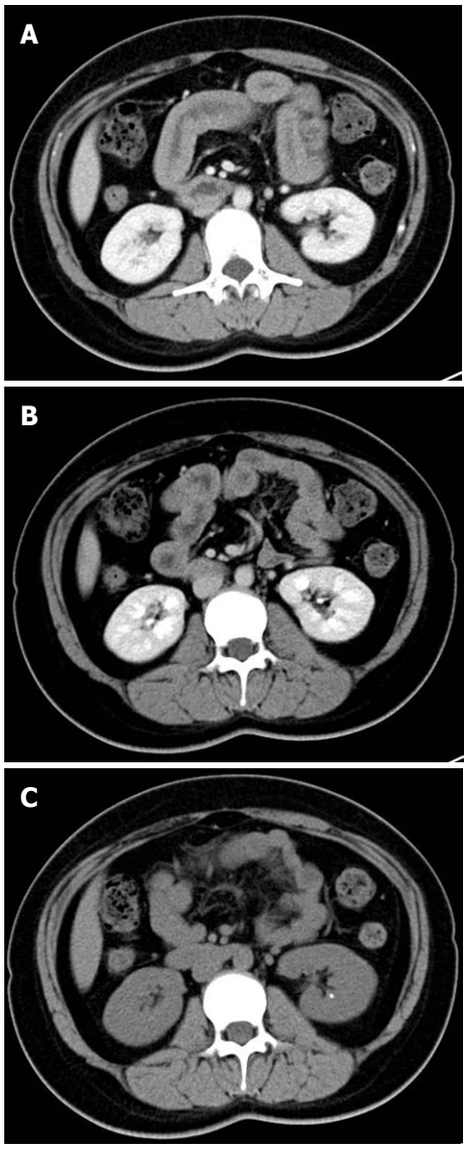

Figure 3 A 32-year-old woman. A: Venous phase image reveals an edematous proximal small bowel segment, including the descending and horizontal duodenum; B: The edematous wall resolved after the 40-s delayed image; C: This same bowel segment presented as normal on the unenhanced image.

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[3,4]. 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[6]. 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[4].

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 compared with pruritus and mild urticaria in affected patients[1]. 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[7]. 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[4,5]. 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[8,9]. 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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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

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International Conference on Nutrition and Growth 2012
Paris, France

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Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
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Barcelona, Spain

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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

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/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

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