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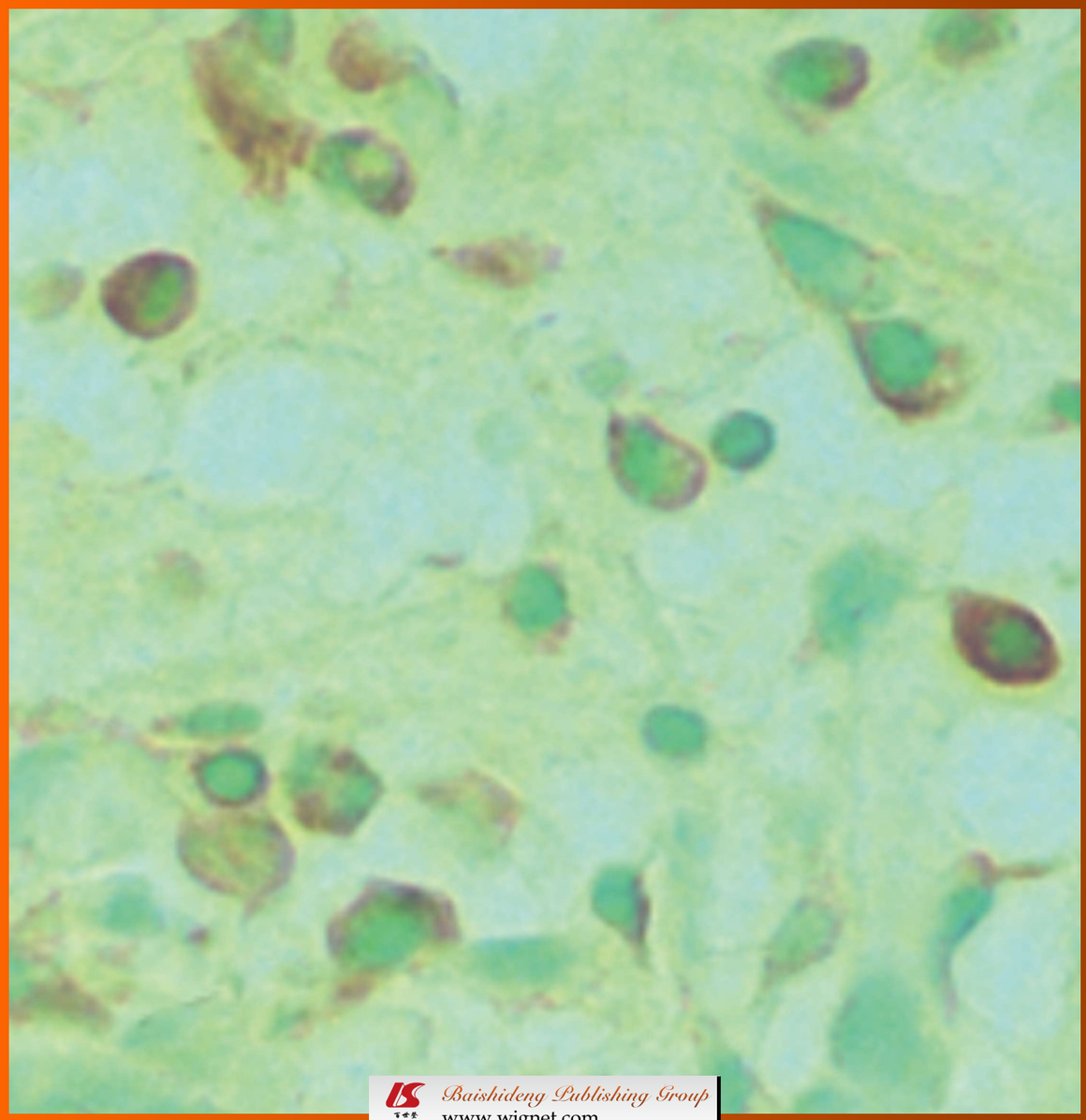

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Contents

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EDITORIAL

Betaine and nonalcoholic steatohepatitis: Back to the future?

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Abstract

Nonalcoholic steatohepatitis (NASH) is an important indication for liver transplantation in many Western countries. Obesity and insulin resistance are the two most common risk factors for NASH, which can lead to recurrent NASH after liver transplantation. There is currently no approved therapy for NASH, and treatment is directed at risk factor modification and lifestyle changes. Betaine has been used for NASH, with mixed results, and may show promise in conjunction with other agents in clinical trials.

Key words: Betaine; Nonalcoholic steatohepatitis; Cirrhosis; Obesity; Insulin resistance

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH), a subtype of nonalcoholic fatty liver disease, can lead to cirrhosis and is an increasingly important cause of liver transplantation in the United States[1]. It is thus subject to intense translational research. Despite a number of clinical trials on the treatment of NASH, there is still no approved therapy, and management is often directed at aggressive reduction of the two most common risk factors: obesity and insulin resistance. These limitations in management have led to renewed interest in the pathophysiology of this epidemic, as a prerequisite to embarking upon further clinical trials. However, most data in this area has been derived from animal models.

Betaine, a naturally occurring dietary compound, originally discovered in sugar beet juice, would appear to be an ideal agent for treating NASH. It is synthesized *in vivo* from the oxidation of choline, and has several effects that may impact the natural history of NASH. These include: (1) its role as a methyl donor for the conversion of homocysteine to methionine; (2) direct substitution for S-adenosylmethionine (SAM) for the direct methylation of phosphatidylethanolamine to phosphatidylcholine; (3) its downstream effects on oxidative stress and transsulfuration reactions; (4) activation of AMP-activated protein kinase; and (5) its properties as a lipotrope and osmolyte[2]. As a naturally acting agent, side effects with betaine would be expected to be minimal; however, in reality, this depends on whether it is administered as betaine anhydrous oral solution or as capsules.

Four clinical trials of betaine for the treatment of NASH have been reported. The first study by Miglio *et al*[3] was of limited value because histopathology was not used to diagnose NASH. Abdelmalek *et al*[4] first reported their experience with betaine in a pilot study of 10 patients treated for one year. Biochemical and histological improvement were noted, although three patients did not complete the study. Mukherjee *et al*[5] reported statistically significant improvement in liver function tests and histopathological

scores in their series of 35 patients treated with betaine for one year; however, this study was limited by the absence of a control arm. Abdelmalek *et al*[6] subsequently reported the results of their second study, which remains the largest and most robustly designed trial evaluating betaine for NASH. The primary aim of this study was to determine if 20 grams per day of anhydrous betaine improved liver function tests after one year of therapy. The secondary aim was to assess its impact on histology. Thirty-five patients completed this randomized placebo-controlled trial (17 betaine *vs* 18 placebo), which included pre- and post-measurement of serum anti-oxidant activity, adipokines, cytokines, homocysteine, S-adenosylhomocysteine (SAH), methionine, and liver biopsies scored according to the Brunt criteria[7]. These variables were analyzed according to the paired *t* test. At the conclusion of the study, betaine had no effect on aminotransferases, and of those patients who did show normalized aminotransferases, the proportion was similar with the placebo group. Betaine also had no effect on adiponectin, cytokine, and SAH levels. The impact of betaine on histology was also disappointing, with no change in fibrosis observed during the study. In addition, fewer patients treated with betaine versus placebo (29% *vs* 61%, $P < 0.01$) improved the steatosis grade by > 1 point. However, more betaine-treated patients compared to placebo (71% *vs* 22%, $P < 0.005$) had no change in steatosis over the study duration.

Such negative findings would appear to shut the door on betaine's therapeutic potential for NASH; however, several limitations in the study, rightfully acknowledged by the investigators, merit review. Probably the most important was the high number of patients who dropped out, which simply meant this randomized controlled study lacked power to detect a difference between the two groups. A large number of patients also had advanced fibrosis (stage 3-4), although descriptive statistics are lacking. As NASH is a chronic condition that normally takes several years to progress into cirrhosis, it is not surprising that no effect was noted after only 1 year of treatment[8]. Furthermore, it remains unclear what optimum dose and preparation of betaine are required for NASH, as the investigators extrapolated data used for homocystinuria. For example, study patients had a significantly higher incidence of gastrointestinal side effects (33% *vs* 9%, $P < 0.05$), which contributed to study withdrawal. It is plausible that a lower dose of anhydrous betaine or betaine capsules, which do not require addition with a solution before administration, might have led to improved compliance and fewer side effects. Serum betaine levels were also not measured and, although the ideal range remains to be determined, documentation would have confirmed compliance rather than accepting a subject's qualitative response in a possible attempt to appease an investigator[9].

A study by Kathirvel *et al*[10] aimed at understanding how betaine reverses hepatic insulin resistance in an animal model of nonalcoholic fatty liver disease may also provide support for re-considering betaine in future trials of NASH. It is more than likely that future trials of NASH will need to be of longer duration to fully assess the impact of treatment, and multiple medications may be required, given the multifactorial processes involved in its pathogenesis. However, betaine, by virtue of its multiple effects and low cost, strongly needs to be reconsidered in larger, prospective studies for NASH as monotherapy will enhance compliance during treatment which is likely to be prolonged in the majority of patients. Risk factor medication remains the mainstay of NASH management, but it is being increasingly recognized that NASH may develop in their absence, re-emphasizing the necessity of well- funded trials of appropriate duration (including cost-effectiveness analyses) for this silent epidemic[11].

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EDITORIAL

Ages of celiac disease: From changing environment to improved diagnostics

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Abstract

From the time of Gee's landmark writings, the recent history of celiac disease (CD) can be divided into many ages, each driven by a diagnostic advance and a deeper knowledge of disease pathogenesis. At the same time, these advances were paralleled by the identification of new clinical patterns associated with CD and by a continuous redefinition of the prevalence of the disease in population. In the beginning, CD was considered a chronic indigestion, even if the causative food was not known; later, the disease was proven to depend on an intolerance to wheat gliadin, leading to typical mucosal changes in the gut and to a malabsorption syndrome. This knowledge led to curing the disease with a gluten-free diet. After the identification of antibodies to gluten (AGA) in the serum of patients and the identification of gluten-specific lymphocytes in the mucosa, CD was described as an immune disorder, resembling a chronic "gluten infection". The use of serological testing for AGA allowed identification of the higher prevalence of this disorder, revealing atypical patterns of presentation. More recently, the characterization of autoantibodies to endomysium and to transglutaminase shifted the attention to a complex autoimmune pathogenesis and to the increased risk of developing autoimmune disorders in untreated CD. New diagnostic assays, based on molecular technologies, will introduce new changes, with the promise of better defining the spectrum of gluten reactivity and the real burden of gluten related-disorders in the population. Herein, we describe the different periods of CD experience, and further developments for the next celiac age will be proposed.

Key words: Antibodies; Autoimmunity; Celiac disease; Diagnostics; History; Intestinal mucosa

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INTRODUCTION

The first descriptions of celiac disease (CD) refer to a disorder of the gut (*koiliakos* in Greek), mainly characterized by fatty stools. While diarrhea was a symptom common to a number of diseases, fatty stools or steatorrhea was an uncommon symptom, characteristic of only a few dis-

eases, such as cystic fibrosis. The finding of steatorrhea in weaned children and in adults without cystic fibrosis was described as a single nosological entity by Samuel Gee, in a rapidly developing England at the end of the 19th century[1]. A similar disease was actually described by Aretaeus of Cappadocia, a physician active in Anatolia almost 2000 years earlier, during another period of rapid development, when agriculture had spread to the so-called region of the Fertile Crescent in the Middle-East.

We can refer to the Age from the first description by Aretaeus of Cappadocia to that of his English colleague 2000 years later as the "The Origins of the Celiac Age" (Figure 1 and Table 1). The cause of the disease was unknown, and the role of foods was conjecture. Gee described CD as a "chronic indigestion which is met with in persons of all ages, yet is especially apt to affect children between one and five years old. Signs of the disease are yielded by the feces; being loose, not formed, but not watery; more bulky than the food taken would seem to account for; pale in colour, as if devoid of bile; yeasty, frothy, an appearance probably due to fermentation; stinking, stench often very great, the food having undergone putrefaction rather than concoction". Gee described, for the first time, that the only cure for the disease would be dietary, even if he failed to identify the foods causing the disorder. With his description, we can start the second age of CD: a disease of the gut, diagnosed on the basis of clinical features and curable with diet.

THE AGE OF STEATORRHEA: FAT IN STOOLS AND DIETARY ADJUSTMENT AS A TREATMENT

The first decades after the initial description by Samuel Gee were characterized by a clear medical description of the gastroenterological symptoms and signs of CD, and by an increasing recognition and identification of new cases, both in children and in adults. Fatty stools, together with abdominal bloating and failure to gain weight were the leading symptoms of the disease, which suggested a malabsorption of food nutrients. Since that time, different attempts were made to cure CD by employing different types of diets. Although the frequent onset of the disorder occurring in babies immediately after weaning suggested a role for cereals as the offending food, the first hypothesis focused on amides, and not on the protein content of flours. In fact, in 1921 the disease was still considered an intolerance to carbohydrates. In 1949, the success of a banana-based diet eliminated carbohydrates as the cause (Sydney Haas). However, scientific methods to identify the specific offending food were applied only around the mid 20th century, thanks to advances in chemical sciences.

THE AGE OF GLUTEN: STILL A VERY RARE DISORDER

In 1950, Dicke observed for the first time, that many children with celiac syndrome might be successfully treated with a diet free from wheat and rye flours. Two years later Anderson demonstrated that the gluten in wheat and rye was the harmful factor[2]. The ability to measure the fatty acids in stools permitted the systematic evaluation of the efficacy of different diets, and eventually confirmed that wheat, barley and rye were harmful to those with CD[3].

This is not the end of this period. In fact, in the following years, the picture of CD continued to change every time improvements were made in diagnostics, which revealed new aspects of the disease. It will soon become evident that it is not just our knowledge changing over time, but CD itself, which may be due to the coincidence of several factors, including availability of large amounts of wheat with a high content of gluten, and changes in the epidemiology of gastro-enteric infections. In this Age, CD is still considered a rare disorder, affecting the gut directly as a consequence of a chronic indigestion of gluten. Late diagnosis, coincidence of malnutrition, and/or infections may account for the more severe form of the disease, the "celiac crisis", which can lead to shock and death. Currently, this form is extremely rare, while other types of presentation are increasingly described in the literature. This age can be also remembered as the age of the Crosby-Kugler capsule[4]. This instrument assisted in the diagnosis of CD allowing a mini-invasive withdrawal of fragments of the small bowel mucosa for histological analysis. The description of the typical picture of flat mucosa and *criptae* hypertrophy constituted at the same time a confirmation of diagnosis, and a tool for investigating the pathogenesis of the intestinal damage in CD. Repeat biopsies could confirm the healing of mucosa after a period of being on a gluten-free diet and the relapse after a new challenge with wheat, suggesting that sensitivity to gluten is a permanent condition in CD.

On the evolutionary scenario, the observation that mucosal damage greatly diminished the available surface for nutrient absorption could suggest a different expression of the disease depending on the available food supply. Indeed, some changes in the clinical expression of CD in different countries or in different periods may be related to the amount of food available, as well as to different epidemiology of infectious diseases, which can synergize with gluten to induce gut damage.

THE AGE OF GLIADIN ANTIBODIES: CELIAC DISEASE IS AN IMMUNE DISORDER RECALLING THE IDEA OF A "CHRONIC INFECTION BY GLUTEN"

The identification of gluten antibodies (AGA) in those affected by CD revolutionized the view of the disease in 1964[5]. Similar to what was found in the first years of the 20th century by von Pirquet in allergic diseases, CD appeared to be due to the immune response to gluten rather than to a direct action of the protein. However, it

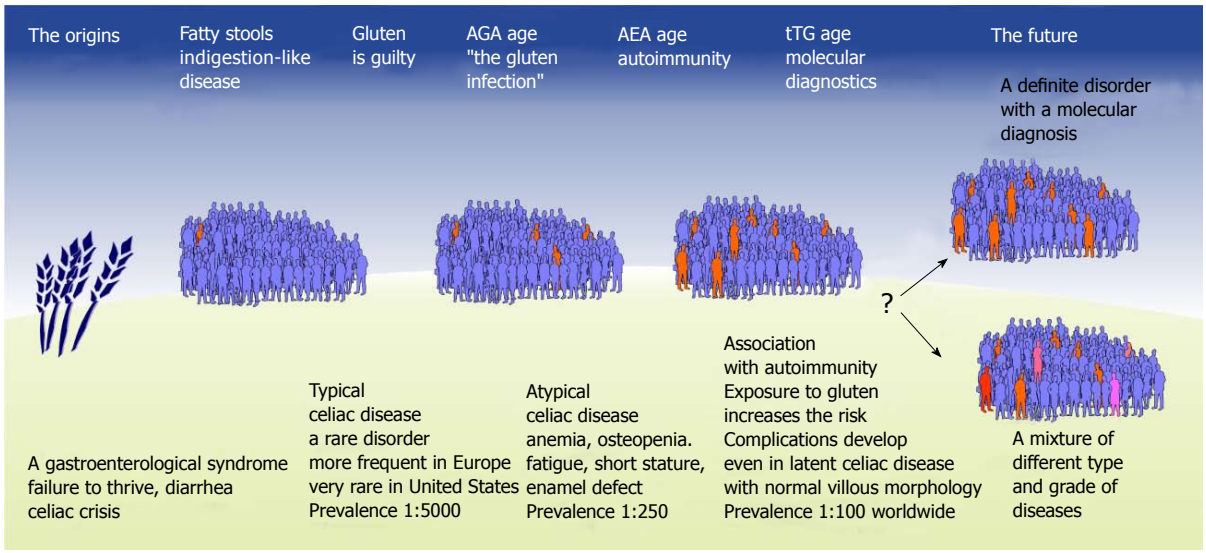

Figure 1 Changing epidemiology and clinics of celiac disease though the different ages.

Table 1 The ages of celiac disease

Age	Date	Clinics	Diagnosis	Pathogenesis
The origins	-1888	At the borders of the fertile crescent		
The age of steatorrhea Fat in stools and diet as a treatment	1888-1952	Intestinal syndrome	Fatty stools	Chronic indigestion Diet attempts
The age of gluten. An intolerance to a protein	1952-1965	Gut disease Celiac crisis	Crosby-kugler capsule assisted diagnosis Acid fat measure in feces allows diet follow-up A very rare disease	Wheat, barley and rye gluten is guilty Flat mucosa is responsible for a malabsorption syndrome The "three biopsies" approach suggested that gluten susceptibility in celiacs is a permanent condition
The age of AGA. CD as an immune disorder	1965-	AGA assay allowed characterization of the atypical form or even the diagnosis of asymptomatic subjects founded the celiac society	AGA Three biopsies	CD is an immune disorder, like a chronic infection by gluten Association to specific HLA variants
The Age of AEA. CD is associated with autoimmunity.	1973-	Definition of silent and latent CD The risk of autoimmunity in CD is, at least in part, related to the duration of exposure to gluten	AEA Three biopsies CD screening by means of AGA and AEA testing A disease more frequent than expected	Gluten is just the trigger, endomysium the target AEA and other autoantibodies in CD are gluten-dependen HLA DQ2 restricted anti-gluten T cells in biopsies The "celiac iceberg" model
The Age of transglutaminase: from target to diagnostic tool.	1997-	Widening spectrum of CD associated disorders	tTG antibodies One biopsy Screening on a few blood drops Anti-deaminated gluten peptides (DGP) antibodies ESPGHAN guidelines for diagnosis without biopsy	Tissue transglutaminase (tTG) is the autoantigen in endomysium tTG increase the affinity of gluten peptides for HLA DQ2 Interaction between tTG and gluten peptides could be responsible for autoimmune reactions and "antigen spreading"
The future. Will new tools identify new diseases?	2011-	A new definition for gluten intolerance with normal serum tTG antibodies	Mucosal tTG in potential CD	Mucosal assay for local tTG antibodies Phage display libraries to unravel CD pathology

CD: Celiac disease; AGA: Age of gliadin antibodies; AEA: Age of anti-endomysium anti-bodies; ESPGHAN: European Society for Paediatric Gastroenterology, Hepatology and Nutrition; HLA: Human leukocyte antigen.

was not an allergy, as it involved different mechanisms, which resemble more strictly the response to gut infections. Thus, the analogy of a chronic "gluten infection" substituted the definition of gluten "indigestion" previously used. The finding of gluten antibodies in CD was even more revolutionary, as it became evident that the measurement of these antibodies could allow an easier diagnosis of the disease, and a convenient follow-up by dietary change.

As a further confirmation of the role of the immune

system in the pathogenesis of CD, a close association between particular human leukocyte antigen (HLA) variants and the disease was observed. More importantly, the measurement of AGA, being a relatively non-invasive and low cost assay, allowed researchers to widen the search for CD in subjects with different clinical complaints, and to find that the disease could be associated with atypical, non-gastroenterological symptoms, such as anemia, short stature, or dermatitis herpetiformis[6-8]. Intolerance to gluten was in fact more frequent than previously expected, and could even be diagnosed in people without any evident symptoms (silent CD), but presenting the typical jejunal lesion of the disease.

THE AGE OF ANTI-ENDOMYSIUM ANTIBODIES: CELIAC DISEASE IS CONNECTED TO AUTOIMMUNITY

As a result of the AGA assay, CD was found to be more common in individuals with type 1 diabetes, and other autoimmune disorders, than in the general population. It was thus not surprising to find that serum from a person diagnosed with CD could contain autoantibodies. Anti-reticulin antibodies were identified in CD in 1971[9]. The finding that these antibodies behaved in a gluten-dependent manner, similar to AGA, was of particular interest as it represented an autoimmune reaction induced by foods[10]. Years before the relationship between gluten and reticulin was clarified, different assays for identifying CD-related autoantibodies entered clinical practice including reticulin antibodies in rat kidney; endomysium antibodies in monkey esophagus; and, later human umbilical cord sections (AEA)[11]. AEA were soon considered a specific sign of CD permitting the definition of a new kind of gluten intolerance, in the absence of overt mucosal lesion ("latent celiac disease")[12]. In these patients, the mucosal inflammation induced by gluten was only revealed by an infiltration of CD3-positive lymphocytes with an increase in the TCR-gamma/delta subset[13-15]. This is why we can also refer to the autoimmunity Age as the "AEA Age".

In the AEA Age, attention was focused on the particular relationship between CD and autoimmunity, which was initially thought to represent the clustering of different autoimmune disorders due to the sharing of the same HLA variants. More recently a multicenter study from the SIGEP suggested that in genetically predisposed subjects, the longer the exposure to gluten the higher the risk of developing autoimmune disorders[16]. In this picture, the risk of developing autoimmunity in CD could be higher in cases without the typical gastroenterological symptoms of the disease in patients who were more likely to be diagnosed later, and likely to remain exposed to gluten longer. It is noteworthy that postponing gluten intake in the first year of life could make the gastrointestinal symptoms less intense, thus delaying the diagnosis, and possibly increasing the risk of developing autoimmunity[17].

A milder gastroenterological presentation, because of a different environmental setting, could also be the cause of underestimating the prevalence of CD in the United States. However, the existence of AEA allowed for new screening and testing, which eventually demonstrated a similar prevalence of the disease in the United States, compared to many other countries, in the range of about 1%[18]. A good outcome of large screenings has been the increase in awareness of the disease in the population, making easier the clinical diagnosis and diet-based treatment.

The AEA Age ended with the idea that much still remained to be understood regarding CD, with the simile of the "celiac iceberg": while the tip is represented by cases with typical symptoms, the majority of individuals with gluten intolerance are under the water, and are difficult to identify because of atypical or even absent symptoms and/or due to apparently normal mucosa[19]. The iceberg idea was intriguing, as it suggested that a percentage of normal people exist, who can respond to gluten with different pathological reactions and that different diagnostic tools could unravel the disease. As a matter of fact, the AEA Age marked a major change in the knowledge of CD, from a rare gut disorder due to gluten and expressed with gastrointestinal complaints (just the tip of the iceberg), to a common autoimmune disorder triggered by gluten in the gut but expressed with a wide variety of clinical symptoms involving different systems. It is noteworthy that this submerged part of the iceberg is much bigger compared to the tip and, in the same way, clinical symptoms other than gastrointestinal are much more common than typical symptoms, where the disease itself is much more common than what was previously considered. Indeed, CD could be suspected in patients with a variety of autoimmune disorders such as diabetes, thyroiditis, dermatitis herpetiformis, autoimmune ataxia, alopecia, as well as symptoms directly due to malabsorption (Table 2).

THE AGE OF TRANSGLUTAMINASE: FROM TARGET TO DIAGNOSTIC TOOL

HLA variants DQ2 and DQ8 were the genetic factors most closely associated with CD. The isolation in duodenal biopsies of T cell clones recognizing gluten peptides in association with these HLA molecules further confirmed the pathogenic role of these genetic variants[20]. Furthermore, anti-gluten CD4 T cells produced large amounts of interferon gamma, which seemed to account for the typical mucosal damage seen in CD-affected mucosa. However, even this knowledge failed to explain why a HLA DQ2/DQ8 patient can present CD, yet another, not. In this scenario, the identification of the single antigen targeted in the Endomysial staining reaction was expected to permit a better knowledge of CD pathogenesis, and a better understanding of the origins of CD[21].

Thus, the search for the "endomysial antigen" represented an amazing adventure for most researchers involved in CD in the 1990s. In 1997, Dieterich and col-

Table 2 Old and new celiac disease before and after the identification of age of anti-endomysium anti-bodies

	Old CD, Pre-AEA age	New CD, Post-AEA age
Pathogenesis	Immune, intestinal	Autoimmune, systemic
Diagnosis	AGA + 3 biopsies	AEA + 1 biopsy
Prevalence	Rare 1:500-1:5000	Frequent 1:100 worldwide
Clinical picture	Malabsorption syndrome	Autoimmune disorders Malabsorption syndrome
Intestinal pathology	Severe villous atrophy and criptae hypertrophy	Severe villous atrophy and criptae hypertrophy or increased mucosal lymphocytes (latent celiac disease)

CD: Celiac disease; AGA: Age of gliadin antibodies; AEA: Age of anti-endomysium anti-bodies.

leagues found that the endomysial antigen involved in the autoimmune response in CD was the enzyme tissue transglutaminase or Type 2 transglutaminase (tTG or TG2)[22]. Indeed, tTG is present in the endomysial net, where it stabilizes the connective tissue by catalyzing the link between glutamine and lysine of different structural proteins. This activity is very important in tissue repair processes and an increased activity of the enzyme can be evidenced in damaged tissues, including the mucosa in CD. Furthermore, tTG plays another important role, in the packaging of debris after cell apoptosis, which allows for the correct removal of apoptotic bodies containing inflammatory response materials.

Ludwig Sollid was the first to publicly hypothesize a model linking gluten to tTG and to anti-tTG autoantibodies. Briefly, when large amounts of gluten enter the mucosa because of increased epithelial permeability (may be favored by other factors, such as infections), the anti-gluten response causes mucosal damage, causing the release and activation of tTG. Gluten itself, due to its high content in glutamine, can be a target of tTG and can be cross-linked with other proteins, including tTG. As a consequence, macromolecular complexes containing both gluten peptides and tTG can be recognized by AGA-producing B cells, as well as by AEA-producing B cells. According to the "Sollid hypothesis", B cells recognizing these macro-complexes, regardless of their antibody specificity will present gluten peptides to gluten-specific T cells. As a consequence, a single antigen can drive an immune response to many targets, overcoming the tolerance of the immune system, in a process also known as "antigen spreading".

Another finding connecting tTG and gluten relies on the capacity of the enzyme to deaminate gluten-derived peptides increasing their affinity to the DQ2 and DQ8 HLA, thus worsening the consequences of anti-gluten immunity[23,24]. Recently, measurement of the immune response to deaminated gliadin peptides (DGP)[25] has been utilized to increase the performances of the AGA assay[26]. This model could partly explain the role of environment, with gastrointestinal infections, in precipitating the pathogenic mechanisms of CD with a vicious circle of tissue damage, activation of tTG, entry and deamination of gluten, anti-gluten response and the spreading of autoantibodies. Hyper-production of IL-15 is associated with these mucosal changes, which could affect the production of the immunoregulatory cytokine TGF-beta[27,28]. Even if this model does not illuminate the specific relationship between CD and other autoimmune disorders, it describes a dysregulated mucosal immunity, which is likely to interfere with the normal mechanisms of immune tolerance.

Apart from contributing to pathogenic knowledge, the identification of the main CD autoantigen allowed for a further improvement of diagnostics for CD by using ELISA assays based on human recombinant tTG (htTG). Using htTG, population screening has been performed starting from finger puncture producing as little as a few drops of blood in children from primary schools[27], and more recently rapid tests have been produced for the consumer market. Due to the reliability of htTG assays, CD diagnosis can now be confirmed with just one jejunal biopsy without any need for repeating bioptic examinations after the start of the diet. In some cases, it is even thought that a confirmation by means of jejunal biopsy may not be necessary. In fact, considering that a strong correlation has been demonstrated between high levels of tTG antibodies and a higher grade of mucosal damage (Marsh score)[29,30], the ESPGHAN is currently evaluating the possibility of making the diagnosis without a confirmatory jejunal biopsy in patients who have symptoms that can be referred to CD, if IgA-tTG antibodies are > 10x the upper normal limit, AEA and HLA DQ2 and/or DQ8 are positive.

THE FUTURE AGE: WILL NEW TOOLS IDENTIFY NEW DISEASES?

Cut-off values generated for a quantitative tTG assay assume a semi-Gaussian distribution of the values in the healthy population, with a tail of high values representing true celiac patients. This means that positive results represent a statistical correlate of the disorder and are not to be confused with the disease itself. Even if these assays are very useful and reliable in assisting the diagnosis of CD, they just represent our best for today, not the confidence of identifying all individuals in whom a gluten-free diet could give measurable advantages. While it is almost certain that very high tTG antibody titers indicate the presence of the disease[29,30], it is less easy to give significance to low titers and border-line results. In fact, there are several lines of evidence that gluten-dependent pathology can develop even in some patients with negative tTG antibodies, albeit rarely. On the other hand, even some patients with positive tTG might not develop symptoms on a gluten-containing diet, the gluten-free diets should still be prescribed, as we are not able to predict the risk of developing pathology in individuals.

Indeed, CD is a multifactorial disorder. It just might be that, in considering the picture of the "celiac ice-

berg", there are different levels of intolerance to gluten, and exposure to gluten could have different consequences in each patient. In other words, we still know just a part of what made the iceberg. The forthcoming Age will clarify if we will be able to identify a single definite disorder by advances in molecular diagnostics, or if we will be faced with different forms of gluten intolerance (see Figure 1). Recently, it has been argued that intramucosal production of anti-tTG antibodies may precede their appearance in serum and could represent a specific indicator of gluten intolerance as well. An immunofluorescence technique on jejunal biopsies allows the detection of IgA deposits that co-localize with tTG in the villus connective tissue, which are considered *bona fide* tTG antibodies. These antibodies could be detected in patients with latent CD, regardless of their presence in serum, and have been shown to predict the development of villous atrophy and to disappear during a gluten-free diet[31]. Analysis of phage display antibody libraries confirmed that anti-tTG antibodies are indeed produced by mucosal lymphocytes and provided a further tool to identify latent CD, where tTG antibodies were produced in mucosa before that they can be found increased in serum[32]. These techniques have a role not only in research. In clinical practice, patients with a potential risk of developing gluten-related diseases, such as relatives of those diagnosed with CD, or with autoimmune disorders, may be positive for DQ2 HLA, but have normal levels of serum tTG antibodies. It was of particular interest to find that some of these patients did have mucosal tTG antibodies that behaved as gluten-dependent[33]. The characterization of such individuals, affected by intermediate or latent forms of CD is one of the goals of modern diagnostics and, a new key to better unravel knowledge on the disease. Further studies will address how gluten may interact with other environmental and genetic factors to condition the risk of developing different types of associated disorders.

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TOPIC HIGHLIGHT

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Risk factors for neoplastic progression in Barrett's esophagus

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Abstract

Barrett's esophagus (BE) confers a significant increased risk for development of esophageal adenocarcinoma (EAC), with the pathogenesis appearing to progress through a "metaplasia-dysplasia-carcinoma" (MDC) sequence. Many of the genetic insults driving this MDC sequence have recently been characterized, providing targets for candidate biomarkers with potential clinical utility to stratify risk in individual patients. Many clinical risk factors have been investigated, and associations with a variety of genetic, specific gastrointestinal and other modifiable factors have been proposed in the literature. This review summarizes the current understanding of the mechanisms involved in neoplastic progression of BE to EAC and critically appraises the relative roles and contributions of these putative risk factors from the published evidence currently available.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Metaplasia-dysplasia-carcinoma; Neoplastic progression; Risk factors

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INTRODUCTION

Barrett's esophagus (BE) describes a condition where native esophageal stratified squamous epithelium is replaced by metaplastic columnar epithelium, with cephalad displacement of the squamocolumnar junction. BE represents the only identified precursor lesion and most important risk factor for esophageal adenocarcinoma (EAC)[1]. Patients with BE have an estimated 30- to 125-fold greater risk of developing EAC than the general population[2]. A systematic review of 27 studies suggested annual progression rates of 0.5%[3], whereas a review of 8 UK studies by Jankowski *et al*[4] showed cancer risk of 1.0% per year.

BE PATHOGENESIS AND MECHANISMS OF NEOPLASTIC PROGRESSION

BE is an acquired condition where healing from esophageal mucosal injury [typically triggered by gastro-esophageal reflux disease (GERD)] is metaplastic, with replacement of damaged squamous cells by columnar epithelium. Ordinarily, esophageal healing involves regeneration of squamous cells; it remains unclear why the response is metaplastic in some individuals, since only a minority of patients with GERD develop BE. Progression of BE to EAC occurs by a metaplasia-dysplasia-carcinoma (MDC) sequence. Metaplastic columnar epithelial cells are predisposed to genetic damage with potential for developing

dysplasia[5]. Dysplasia represents a histological spectrum from low- to high-grade, defined by degree of cytological and architectural disruption present, with genetic instability resulting in progressive acquisition of genetic abnormalities towards a frankly neoplastic phenotype. These can be considered within the framework of Hanahan and Weinberg's[6] model of "cancer hallmarks" necessary for carcinogenesis, whereby cancer cells must acquire growth self-sufficiency, insensitivity to anti-growth signals, avoidance of apoptosis, limitless replicative potential, sustained angiogenesis, and invasive and metastatic potential[7].

Many genetic insults conferring these advantages in the BE MDC sequence have been characterized. Initiating events probably involve genes regulating cell cycle progression, notably *p16*. Mutations, loss of heterozygosity (LOH) or promoter hypermethylation (i.e. silencing) of *p16* have been identified in 80% of BE, whilst *p16* hypermethylation correlated with the degree of dysplasia in some studies[8]. Additional changes identified include upregulation of cyclins D1 and E, transforming growth factor-α and epidermal growth factor (EGF), each contributing towards growth autonomy[9,10]. These mutations should trigger apoptosis *via p53*-dependent pathways. However, subsequent accrual of *p53* lesions confers resistance to apoptosis, and has been identified in 52%-93% of EACs (compared with 1%-5% non-malignant BE cell lines)[11]. Inactivation of *p53* increases clonal genomic instability, predisposing to widespread DNA changes and evolution of ploidy lesions, late events in cancer progression. Many other genetic and molecular alterations have been described[8,9,12-64] (Table 1).

The concept of a linear, stepwise evolution of tumor suppressor gene mutations in which clonal expansion of a solitary mutated clone expands to fill the entire Barrett's segment has been termed the "selective sweep to fixation" model. However, an alternative model has been proposed by Leedham *et al*[65], who performed genetic analysis of individual crypts rather than a flow purified whole biopsy specimen. This technique permitted identification of certain mutations masked by whole biopsy segment analysis (attributed to dilution effect of the normal stroma on whole biopsy analysis), whilst also revealing a greater degree of genotypical and phenotypical heterogeneity within the same biopsy sample than previously appreciated. The demonstrated lack of a single founder mutation present in every crypt suggested that the clonal expansion arose from multiple independent clones rather than a single common founder mutation[65,66] (Figure 1).

This enhanced understanding prompted research into > 200 candidate novel biomarkers of disease progression in BE/EAC. Several, including 17p LOH, cyclin D1, tetraploidy and aneuploidy, have undergone phase 3/4 validation and in future might have clinical/prognostic utility as intermediate markers of progression[67]. However, Leedham's recent findings call into question the reliability of "surveillance" biomarker identification *via* genetic analysis of whole biopsy specimens, since minority clones within the sample (harboring neoplastic potential) might not be detected[65].

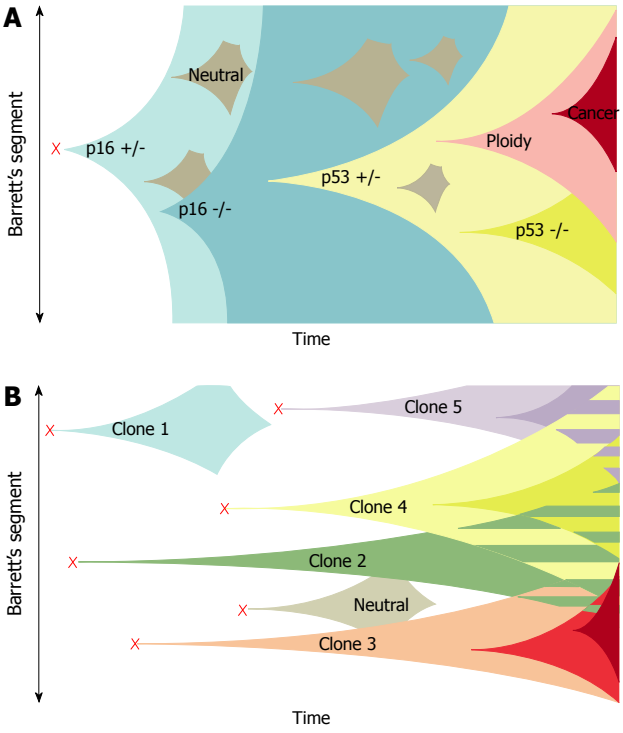

Figure 1 Clonal evolution models in Barrett's esophagus. A: The current model of clonal evolution adapted from Maley *et al*[66]. Founder mutation (red cross) occurs in a single progenitor and provides a growth advantage that predisposes to a selective sweep. Successive selective sweeps result in progression along the metaplasia dysplasia pathway. Clone bifurcation is responsible for the genetic heterogeneity in this model; B: The newly proposed model of evolution based on the mutation of multiple progenitor cells situated in esophageal gland squamous ducts located throughout the length of the esophagus (red crosses). Multiple independent clones then arise and evolve separately. The presence of multiple different clones gives rise to a mosaic interdigitating clonal pattern of the Barrett's segment represented as the striped areas[65].

Currently, dysplasia remains the only validated marker for identifying BE patients at risk, and forms the basis of EAC surveillance. However, this is imperfect. The tempo of progression towards EAC is highly variable and it remains unclear whether relentless progression through the MDC sequence is inevitable; some evidence suggests that high-grade dysplasia may remain stable for years or even regress[68]. Patients with BE may develop EAC during surveillance without detection of earlier MDC stages. This might relate to pace of progression, sampling error or lesions skipping directly from non-dysplastic disease to cancer. Other limitations of dysplasia as a prognostic marker include inter-observer variability in histological interpretation, and that inflammation may mimic dysplastic changes[69].

RISK FACTORS FOR NEOPLASTIC PROGRESSION

Until molecular biomarkers enter clinical practice it remains important to identify other clinical risk factors for malignant progression to effectively allocate resources and individualize surveillance programs, targeting those at highest risk. Identifying modifiable risk factors will also

Table 1 Published evidence from selected studies investigating genetic and epigenetic changes implicated in the metaplasia-dysplasia-carcinoma sequence of Barrett's esophagus

Factor	Summary of major findings/conclusions	Ref.
Growth self-sufficiency		
Cyclin D1	↑ nuclear cyclin D1 immunostaining in 46% BE specimens: -?cyclin D1 overexpression early event in MDC sequence	Arber *et al*[9]
	↑ nuclear cyclin D1 immunostaining in 64% EAC specimens	Arber *et al*[13]
	Cyclin D1 expression correlates with degree of dysplasia in BE	Coppola *et al*[14]
	Cyclin D1 expression 43% BE mucosa (*vs* 0% normal mucosa)	Umansky *et al*[15]
	Polyphenon E inhibits growth of BE and EAC cells *via* downregulation of cyclin D1 expression	Song *et al*[16]
Cyclin E	↑ cyclin E expression in neoplastic cells in BE	Coppola *et al*[14]
	Cyclin E expression 37% BE mucosa (*vs* 0% normal mucosa)	Umansky *et al*[15]
$p27^{Kip-1}$	83% EAC specimens displayed low p27 protein levels (despite high p27 mRNA): -p27 inactivated in most BE-associated EAC (post-transcriptional modification)→loss of cell cycle inhibition	Singh *et al*[17]
	Experimentally-induced BE and EAC development in mouse model significantly enhanced by p27 gene knockout	Ellis *et al*[18]
EGF (and EGF-R)	↑ EGF in cytoplasm of BE epithelial cells (*vs* gastric mucosa)	Jankowski *et al*[19]
	EGF-R expression area in inflamed mucosa (43.1%) significantly > normal mucosa (29.5%); all BE showed positive EGF-R staining	Jankowski *et al*[20]
	EGF/EGF-R expression significantly ↑ in BE and EAC mucosa (*vs* normal mucosa) by flow cytometry ($P < 0.01$)	Jankowski *et al*[21]
	EGF-R expression positive in 64% of BE-associated EAC; ↑ staining associated with poorer survival ($P = 0.004$)	Yacoub *et al*[22]
	EGF A61G G/G genotype associated with >double EAC risk in BE pts (*vs* A/A or A/G) (OR 2.2)	Lanuti *et al*[23]
TGF-α	↑ TGF-α expression in cells from BE and EAC mucosa (*vs* normal gastric mucosa) by flow cytometry ($P < 0.01$)	Jankowski *et al*[21]
	TGF-α expression positive in 100% of BE-associated EAC	Yacoub *et al*[22]
HGF (and HGF-R)	HGF expression significantly ↑ in BE specimens (*vs* normal esophageal mucosa)	Konturek *et al*[24]
	Intense HGF-R immunostaining in 100% EAC and dysplastic BE specimens (*vs* minimal staining in non-dysplastic BE or normal mucosa); HGF-R mRNA and protein levels ↑ in EAC cell lines	Herrera *et al*[25]
Erb family tyrosine kinases	Membranous c-erbB2 overexpressed in 26% EAC (*vs* 0% BE with dysplasia): -?later event in MDC sequence	Hardwick *et al*[26]
	c-erbB-2 gene amplification in 14% EAC *vs* 11% HG-dysplasia *vs* 0% metaplasia/LG-dysplasia specimens	Geddert *et al*[27]
FGF	Immunostaining intensity for FGF sequentially ↑ from metaplasia/LG-dysplasia (negligible)→HG-dysplasia (weak/moderate)→EAC (moderate/strong)	Soslow *et al*[28]
	FGF-1 mRNA and protein expression sequentially ↑ in HG-dysplasia/EAC (*vs* metaplasia/LG-dysplasia/controls)	Soslow *et al*[29]
Src family tyrosine kinases	Src-specific activity 3-4-fold ↑ in BE and 6-fold ↑ in EAC (*vs* controls): -?Src activation early event in MDC sequence	Kumble *et al*[30]
	Strong Src expression in 85% EAC *vs* 93% BE HG-dysplasia *vs* 72% BE LG-dysplasia *vs* 27% BE specimens	Iravani *et al*[31]
Insensitivity to anti-growth signals		
p16	9p21 (p16) LOH observed in 89% EAC specimens (*vs* 0% non-dysplastic BE); homozygous p16 deletion in only 25%	González *et al*[32]
	p16 promoter hypermethylation (inactivation) in 75% BE with HG-dysplasia *vs* 56% LG-dysplasia (*vs* 3% non-dysplastic BE)	Klump *et al*[8]
APC	5q (APC) LOH seen in 80% EAC specimens (and surrounding mucosa)	Barrett *et al*[33]
	APC gene LOH observed in 60% EAC specimens (*vs* 0% non-dysplastic BE)	González *et al*[32]
	APC promoter hypermethylation in 92% EAC *vs* 40% BE (*vs* 0% normal esophageal tissues)	Kawakami *et al*[34]
Avoidance of apoptosis		
p53	Positive p53 immunostaining in 87% EAC *vs* 55% BE with HG-dysplasia *vs* 9% LG-dysplasia *vs* 0% non-dysplastic BE	Younes *et al*[35]
	17p (p53) LOH found in 91% BE pts who developed aneuploid cell populations: -17p allelic losses precede aneuploidy	Blount *et al*[36]
	p53 overexpression in 64% EAC *vs* 31% dysplastic BE *vs* 0% non-dysplastic BE; trend of ↑ p53 expression with ↑tumour grade: -?p53 mutation early event in malignant progression	Symmans *et al*[37]
	p53 immunoreactivity only in EAC/BE with HG-dysplasia (not in BE with LG-/no dysplasia); mutated p53 in 69%: -?late event in MDC sequence (during transition to HG-dysplasia)	Rice *et al*[38]
	p53 protein expression in 85% EAC specimens *vs* 60% BE with HG-dysplasia *vs* 7% LG-dysplasia ($P < 0.001$)	Rioux-Leclercq *et al*[39]
	p53 mutations identified in 75% EAC specimens; p53 overexpression in 58% EAC *vs* 60% BE with HG-dysplasia *vs* 12% LG-dysplasia *vs* 0% non-dysplastic BE	Chung *et al*[40]
Fas (CD95)	↓ surface expression of Fas observed in EAC specimens; impaired translocation of Fas to membrane wild-type Fas protein retained in cytoplasm in EAC cell line: -?potential mechanism by which EAC cells evade Fas-mediated apoptosis	Hughes *et al*[41]
	↓ surface expression of Fas and resistance to Fas-mediated apoptosis observed in EAC cell lines	Mahidhara *et al*[42]
Bcl-xl/Bax/Bcl-2	Bcl-xl positive in all dysplasia and EAC cells, but negative in 47% non-dysplastic BE: -?switch to anti-apoptotic phenotype in transformation from metaplasia to EAC	van der Woude *et al*[43]

	Bcl-2 expression in 84% LG-dysplasia *vs* 0% HG-dysplasia or EAC	Rioux-Leclercq *et al*[39]
	Cytoplasmic Bcl-xl immunostaining in 59% EAC *vs* 71% BE/HG-dysplasia *vs* 60% LG-dysplasia *vs* 27% non-dysplastic	Iravani *et al*[31]
COX-2	↑ COX-2 mRNA levels in 80% BE and 100% EAC specimens (*vs* normal gastric controls) ($P < 0.001$); COX-2 immunostaining strongly positive in 100% BE samples (> gastric controls)	Wilson *et al*[44]
	COX-2 immunopositivity in 91% non-dysplastic BE *vs* 94% dysplastic *vs* 97% EAC	Lagorce *et al*[45]
	Natural/synthetic COX-2 inhibitors suppressed proliferation, induced apoptosis and blocked cell cycle in EAC cell lines	Cheong *et al*[46]
	Cox-2 mRNA strongly upregulated in experimentally-induced BE epithelium in rat model (*vs* absent in control animals); COX-2 overexpression observed in human BE patients with dysplasia	Majka *et al*[47]
Limitless replicative potential		
Telomerase	Telomerase RNA positive in 100% EAC/BE with HG-dysplasia *vs* 90% LG-dysplasia *vs* 70% non-dysplastic BE: marked ↑ telomerase RNA accompanies transition along MDC sequence	Morales *et al*[48]
	human telomerase reverse transcriptase (catalytic subunit of telomerase) expression ↑ at all stages of BE *vs* normal controls, and in EAC ($P = 0.003$) and dysplastic BE ($P = 0.056$) *vs* non-dysplastic BE	Lord *et al*[49]
	Telomerase activity (by telomeric repeat amplification protocol assay) ↑ in EAC samples *vs* adjacent mucosa ($P = 0.0002$) and in EAC *vs* BE ($P = 0.001$); no difference BE *vs* adjacent mucosa	Barclay *et al*[50]
	Telomerase inhibition (by small interference RNAs) induced senescence in 40% and apoptosis in 86% in BE cell lines	Shammas *et al*[51]
Sustained angiogenesis		
VEGF (and VEGF-R)	VEGF expression correlated with higher vascularisation in BE and EAC specimens	Couvelard *et al*[52]
	VEGF-A expressed in BE epithelium; VEGFR-2 strongly expressed in immature endothelial cells feeding BE epithelium; ↑ VEGF-C expression in BE (*vs* absent in normal epithelium); ↑ VEGFR-3 in EAC: ?aberrant neovasculature early in MDC sequence	Auvinen *et al*[53]
	VEGF expressed in 64% EAC specimens; significantly correlated with angiolymphatic invasion/survival	Saad *et al*[54]
	VEGF expression significantly ↑ in EAC (> dysplastic BE > BE > normal epithelium)	Griffiths *et al*[55]
Invasive/metastatic potential		
CAMs	↓ expression in EAC specimens of E-cadherin (in 74%), α-catenin (60%) and β-catenin (72%)	Krishnadath *et al*[56]
	Abnormal expression of β-catenin ($P = 0.022$), α-catenin ($P < 0.01$) and E-cadherin ($P = 0.049$) significantly associated with higher degrees of BE-related dysplasia	Washington *et al*[57]
	↓ expression of E-cadherin with progression along MDC sequence ($P < 0.01$); in contrast P-cadherin absent from BE (± dysplasia) but expressed in 67% EAC specimens	Bailey *et al*[58]
	Slug (E-cadherin repressor) immunostaining and mRNA levels overexpressed in EAC *vs* BE metaplasia specimens: -?Slug upregulation represents mechanism of E-cadherin silencing	Jethwa *et al*[59]
Cathepsins	Detected amplicon at chromosome 8p22-23 resulting in cathepsin B overexpression (observed in 73% EAC samples)	Hughes *et al*[60]
	↑ cathepsin C expression in EAC (*vs* BE *vs* normal) in rat model	Cheng *et al*[61]
CD44	Stepwise ↑ cathepsin D mRNA levels in GERD→BE→EAC tissue	Breton *et al*[62]
	CD44-H and -V6 variant frequently expressed in BE; differing expression patterns along spectrum normal→dysplastic BE→EAC: -?CD44H and V6 involved in carcinogenesis of BE mucosa	Lagorce-Pages *et al*[63]
	↓ CD44 expression in EAC/HG-dysplasia (*vs* BE/LG-dysplasia)	Darlavoix *et al*[64]

BE: Barrett's esophagus; MDC: Metaplasia-dysplasia-carcinoma; EAC: Esophageal adenocarcinoma; EGF: Epidermal growth factor; EGF-R: EGF receptor; pts: Patients; OR: Odds ratio; TGF: Transforming growth factor; HGF: Hepatocyte growth factor; HGF-R: HGF receptor; mRNA: Messenger RNA; FGF: Fibroblast growth factor; HG: High grade; LG: Low grade; LOH: Loss of heterozygosity; APC: Adenomatous polyposis coli; COX-2: Cyclooxygenase-2; VEGF: Vascular endothelial growth factor; VEGF-R: VEGF receptor; CAM: Cell adhesion molecule; GERD: Gastro-esophageal reflux disease.

Table 2 Clinical and demographic risk factors for neoplastic progression of Barrett's esophagus

Innate factors	Gastrointestinal factors	Other modifiable factors
Age	Bile and acid reflux	Obesity
Gender	Anti-reflux surgery	Diet
Ethnicity	Proton pump inhibition	Alcohol
	Pharmacological lower esophageal sphincter relaxation	Smoking
	Salivary nitrates	Socioeconomic status
	Barrett's segment length	Pharmacological COX-2 inhibition

COX-2: Cyclooxygenase-2.

inform disease prevention strategies. Epidemiological studies of EAC have described a "birth cohort effect", with higher incidence rates observed in recent cohorts suggesting exposure to an exogenous risk factor in early life contributing increased risk in all ages of the cohort[70] (Figure 2). Multiple risk factors for neoplastic progression of BE have been investigated (Table 2).

INNATE HOST FACTORS

Age is a well-recognized risk for both BE and EAC. Corley *et al*[71] reported an incidence of BE of 2/100 000 for 21-30-year-old and 31/100 000 for 61-70-year-old, whilst El-Serag *et al*[70] calculated the risk of EAC to increase by 6.6% for each 5-year age increase. Evidence specifically linking age to risk of neoplastic progression within BE is lacking, but it seems intuitive to propose advancing age as an independent risk factor.

BE displays a male preponderance of approximately 2:1, rising to 4:1 for BE-associated EAC, suggesting an independent influence of gender on risk of neoplastic pro-

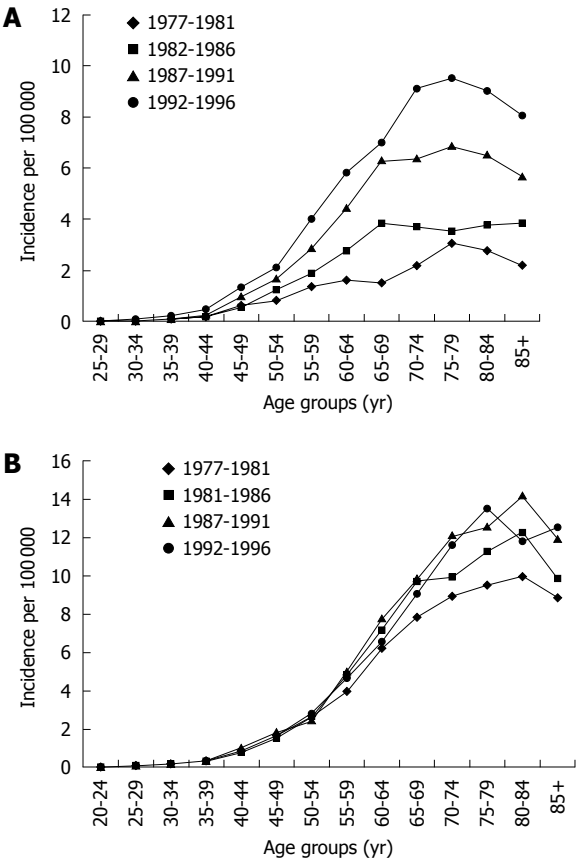

Figure 2 Age distribution of cases diagnosed with oesophageal adenocarcinoma (A) and gastric cardia adenocarcinoma (B) in the USA between 1977-1996, displaying the "birth cohort effect". Each individual curve represents the age-specific incidence rates in a five year period (from El-Serag *et al*[70]).

gression[71,72]. Why male gender should confer additional risk is unknown; some have speculated that male propensity toward visceral pattern of obesity might be relevant[73].

A higher prevalence of BE in Caucasians has long been recognized[74]; again, this association strengthens with development of BE-associated EAC[75]. Analysis of the US Surveillance, Epidemiology and End Results registry found that the annual incidence of EAC for Caucasian males was double that for Hispanic males and four times higher than Black, Asian, Pacific Island and Native American males[76]. Although selection bias and differing endoscopy uptakes between ethnic groups might partially explain this, other factors seem to be involved. Whilst environmental influences are probably important, hitherto-unidentified genetic variations influencing protection against reflux-induced mucosal damage seem likely. A US study found similar GERD prevalence in Caucasian and Black Americans from the same geographical population, yet the latter displayed significantly less esophagitis and almost no BE[77].

GASTROINTESTINAL FACTORS

Bile/acid reflux

The relationship between GERD and BE is well established, and whilst reflux of gastric acid is known to induce chronic mucosal esophageal injury the contribution of bile salts and acids (from duodenal refluxate) is increasingly recognized. Vaezi and Richter demonstrated patients with complicated BE (dysplasia/stricture/ulceration) reflux significantly greater amounts of both gastric and bile acids than those with uncomplicated BE, and postulated that complications might result from synergism between the two[78]. Bile salts induce esophageal injury over a wide pH range, and patients with BE display significantly more bile salts in aspiration studies than patients with mild reflux only[79]. Menges *et al*[80] observed a strong correlation between duration of esophageal exposure to acid and bile with severity of pathological change in BE. Furthermore, proton pump inhibitor (PPI) therapy predisposes to upper gastrointestinal bacterial colonization and consequent bile salt-deconjugation, which, in this high pH environment, has been linked to chronic inflammation[81].

Refluxate-mediated inflammation might promote carcinogenesis *via* both the arachidonic acid (AA) pathway and induction of oxidative stress. Low pH and bile salts promote expression of cyclooxygenase-2 (COX-2), catalyzing conversion of AA into various prostaglandins, including PGE_2. PGE_2 increases proliferation of BE epithelial cells and inhibits tumor surveillance through suppressing natural killer cell function. Consequently, abnormal cells displaying genomic instability may accumulate. COX-2 expression has been shown to increase with neoplastic progression of BE, supporting a role for the AA pathway in EAC carcinogenesis[44]. Chronic mucosal injury also induces production of reactive oxygen species (ROS), depletes antioxidants and increases expression of oxidative stress-related genes. High levels of oxygen radicals and lipid peroxidation products have been demonstrated in BE epithelial cells, with reduced levels of vitamin C and glutathione, indicating compromised oxidant defences[82]. ROS have well-established mutagenic capacity, whilst subsequent apoptosis of mutated cells is additionally suppressed by capacity of bile salts to induce proteasomal degradation of *p53*[83].

The Factors Influencing the Barrett's Adenocarcinoma Relationship (FINBAR) study suggested GERD symptom chronicity and frequency appeared better predictors for neoplastic progression than severity[84]. However, a significant proportion of EAC patients (40%-50%) do not recall ever having prior reflux[85]. Furthermore, reflux of gastroduodenal contents correlates poorly with heartburn symptoms, BE is frequently asymptomatic and development of less sensitive Barrett's epithelium may ameliorate symptoms. Thus, symptom-based risk scores for assessing progression risk have so far not proved useful in clinical practice.

PPIs

PPIs increase pH of gastric refluxate, attenuating acid-induced damage. Ouatu-Lascar *et al*[86] showed "normalization" of intraesophageal pH with acid suppression favors differentiation and reduces cellular proliferation in BE biopsy specimens. However, PPIs have not prevented

recent increases in EAC, and the observation of EAC with PPI administration in animal models raises concern they might actually favor progression of BE[87]. This might be mediated *via* interaction of gastrin with its cholecystokinin receptor, CCK_2R. PPIs elevate serum gastrin levels, which on binding to CCK_2R, stimulate expression of EGF and trefoil peptide, inducing COX-2 expression. Gastrin exposure increases proliferation in esophageal cell culture, and BE mucosa expresses more CCK_2R than normal squamous mucosa. CCK_2R stimulation also inactivates pro-apoptotic factors[88].

Despite this, the clinical relevance in humans remains unproven. Three large studies have examined PPI usage and EAC risk in BE patients, each reporting a strong inverse correlation. Two observed a decreased risk with longer duration of PPI, and one showed an increased risk with delayed PPI use[89]. Obszynska *et al*[90] investigated effects of hypergastrinemia induced by different PPI doses in cell models and BE patients. Despite increased cell proliferation *in vitro*, COX2 induction and enhanced epithelial restitution, they found no evidence of longer-term harm using surrogate biomarkers of proliferation or apoptosis *in vivo*. The Aspirin Esomeprazole Chemoprevention Trial (AspECT) is currently investigating effects of different PPI doses in combination with aspirin on EAC risk.

Anti-reflux surgery

Theoretically, anti-reflux surgery should prevent reflux of duodenal contents, against which PPIs have no effect, potentially mitigating against progression of BE. Unfortunately this is not supported by the available evidence. Two large cohort studies failed to show cancer protection in GERD patients[91,92], whilst a meta-analysis by Corey *et al*[93] concluded no reduction in progression risk for BE. However, different surgical procedures were employed and effectiveness of reflux control was not always assessed.

Lower esophageal sphincter-relaxing drugs

Pharmacological lower esophageal sphincter (LES) relaxation might promote development/progression of BE by increasing reflux, suggested by the observation that drugs with these effects (e.g. tricyclic antidepressants) have increased in use alongside the rise in EAC. A Swedish population-based study by Lagergren *et al*[94] reported a positive association between EAC and long-term use of LES-relaxing drugs, with the strongest association for anticholinergics; this association disappeared after adjustment for reflux symptoms.

Helicobacter pylori infection

An increase in BE-associated EAC alongside falling rates of *Helicobacter pylori* (*H. pylori*) infection has led some to propose a protective effect of *H. pylori*, mediated by its influence in reducing gastric acidity. The virulent cagA strain is particularly associated with high-grade gastric inflammation and atrophy[95]. A meta-analysis by Rokkas *et al*[96] reported statistically significant inverse relationships between *H. pylori* infection and both EAC and BE [odds ratio (OR), 0.52% and 0.64%, respectively]. Furthermore, a large prospective study of BE patients and GERD controls found less *H. pylori* infection with increasing "severity" of disease: 44% in GERD; 35% in uncomplicated BE; 14%-15% in BE with high-grade dysplasia/EAC[97].

However, another study, controlling for demographic and lifestyle factors, failed to demonstrate reduced EAC with cagA+ infection[98]. A confounding factor might be the degree of bile acid reflux, since excessive bile reflux may prevent *H. pylori* colonization and contribute to chronic mucosal injury[88]. The protective role for *H. pylori* is debatable and since *H. pylori* is a World Health Orgaisation class 1 mutagen for gastric adenocarcinoma it is difficult to argue against its eradication whenever it is detected.

Salivary nitrates

Dietary nitrate, concentrated in saliva and reduced to nitrites by oral flora, produces intraesophageal nitric oxide (NO) during reflux. Achlorhydria induced by PPI or atrophic gastritis may cause overgrowth of nitrate-reducing bacteria in the upper gut, providing another source of nitrite[88]. Clemons demonstrated the capacity of NO to induce double-strand DNA breaks in esophageal BE cells *in vitro*, which could promote neoplastic progression[99]. Increasing agricultural nitrate use in the latter 20th century caused significant increases in nitrate content of leafy vegetables and drinking water[100] and could have partially contributed to the increase in EAC incidence.

Barrett's segment length

Although EAC can develop in BE segments of any length, several observational studies support the intuitive notion that longer segments confer greater risk[101]. However, a meta-analysis by Thomas *et al*[102] showed only a non-significant trend towards reduced progression with shorter BE segments, and evidence remains insufficient to advocate surveillance strategies based on segment length alone.

OTHER MODIFIABLE RISK FACTORS

Obesity

Increasing obesity has also paralleled increased rates of BE and EAC. Strong links between obesity and both GERD and erosive esophagitis have been established[103]. It is logical that this might predispose to BE, but a meta-analysis specifically comparing body mass index (BMI) in BE cases with population controls showed only a modest risk increase[104]. However, elevated BMI is a strong risk factor for EAC (OR, 1.8 and 2.4 for BMI > 25 and BMI > 30, respectively)[105]. Increased risk may relate more to distribution of body fat than BMI alone, with visceral (abdominal) obesity conferring greater risk[106]. Other studies noted an association between obesity in early life and EAC risk, suggesting adiposity may act early in the disease process[84,107].

Although a small prospective study by Oberg and colleagues failed to identify any association between BMI

Table 3 Selected published evidence linking adipokines (and ghrelin) with Barrett's esophagus and progression to esophageal adenocarcinoma

Adipokine	Evidence in BE and EAC	
	Relevant study findings	Ref.
Adiponectin	↓ adiponectin receptors in Barrett's mucosa compared with normal mucosa from controls	Konturek *et al*[110]
(↓ in obesity)	↑ Bax (pro-apoptotic), ↓ Bcl-2 (anti-apoptotic) and ↑ apoptosis of EAC cell lines on incubation with adiponectin	Konturek *et al*[110]
	Plasma adiponectin levels inversely associated with BE risk in 50 matched cases (OR 4.7 for each 10 μg/mL ↓ in level) (independent of BMI)	Rubenstein *et al*[111]
	No difference in adiponectin levels between 51 BE patients and 67 controls	Kendall *et al*[112]
Leptin	Leptin receptors expressed in esophagus	Francois *et al*[113]
(↑ in obesity)	↑ proliferation and ↓ apoptosis (*via* various signalling pathways) in EAC cell lines	Ogunwobi *et al*[114]
	Leptin levels strongly associated with ↑ risk of BE in males (no association in females)	Kendall *et al*[112]
	Gastric (fundic) leptin levels positively associated with BE (no association with serum leptin)	Francois *et al*[113]
Ghrelin	↑ gastric emptying (so may ↓ gastric reflux)	Dornonville *et al*[115]
(↓ in obesity)	↓ TNF-α-induced COX-2 and interleukin-1-β expression in BE cell line	Konturek *et al*[110]
	Ghrelin expression negligible in archived EAC cell specimens (*vs* rich expression in normal mucosa)	Mottershead *et al*[116]
	↑ serum ghrelin associated with ↓ EAC risk (in overweight subjects)	de Martel *et al*[117]

BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; OR: Odds ratio; BMI: Body mass index; COX-2: Cyclooxygenase-2; TNF: Tumor necrosis factor.

and progression from BE to low- or high-grade dysplasia[108], it had limited power, and a larger study from the Seattle Barrett's Esophagus Program revealed strong correlations between waist-to-hip ratio and intermediate biomarkers of progression[109]; again, associations were less apparent for elevated BMI *per se*.

Obesity causes GERD through several mechanical and physiological mechanisms. However, part of the association between obesity and EAC is independent of GERD, suggesting a role for reflux-independent mechanisms, probably linked to important endocrine actions of adipose tissue. Many recent studies have linked several adipokines (metabolically active factors) to plausible actions in the MDC process[110-117] (Table 3).

Kristal *et al*[118] investigated whether weight loss (alongside other dietary measures) impacted upon measured biomarkers of cellular proliferation in BE. Despite weight loss (mean 3.6 kg) at 18 mo no differences in biomarkers were observed. This study was relatively small, and the lack of response might relate to the relatively modest weight loss, and/or choice of proliferation markers employed.

Diet

Several studies have shown an association between a diet high in fruit and vegetables and reduced EAC. A large population-based Swedish study found individuals in the highest exposure quartile of fruit and vegetable intake to have approximately 50% less EAC compared to the lowest quartile[119]. However, Kristal *et al*'s study observed no effect on biomarkers of BE cell proliferation despite a net increase in fruit and vegetable consumption[118], whilst the FINBAR study observed a reduction in EAC with increased fruit, but not vegetable, consumption[84]. A protective effect for the natural anti-oxidants in fruit was proposed. A well-controlled, prospective study by Dong *et al*[120] showed patients who took multivitamin pills had significantly decreased risk of tetraploidy [hazard ratio (HR), 0.19] and frank EAC (HR, 0.38). Significant inverse associations with EAC were also observed for supplemental vitamins C (HR, 0.25) and E (HR, 0.25), both well-recognized antioxidants.

Chen *et al*[121] observed a significant inverse association between zinc intake and EAC risk compared with controls (OR, 0.5); inverse associations were also noted for vitamin A, β-cryptoxanthin, riboflavin, folate, fiber, protein and carbohydrate, whilst saturated fat intake was positively associated with EAC. Rudolph *et al*[122] investigated selenium levels in 396 BE patients: those with levels in the upper three quartiles were less likely to display high-grade dysplasia (OR, 0.5), aneuploidy (OR, 0.4) or 17p LOH (OR, 0.5) than the lowest quartile. No association was observed with *p16* LOH (an early event in the MDC sequence), indicating selenium's protective effects might occur late in progression to EAC.

Alcohol

Data supporting links between alcohol and BE/EAC are sparse. The UK BE registry found no association between alcohol consumption in patients with BE compared with reflux esophagitis[123]. Although at least eleven studies have investigated the relationship between alcohol and EAC only six have shown a positive association, and in most it was weak[124-134]. One study even seemed to suggest wine to be protective[133].

Smoking

Studies of smoking and BE/EAC are contradictory. An Australian population-based case-control study found smoking was associated with 2- to 3-fold increased risk of BE and BE with dysplasia[135]. However, there was no dose-response effect. Other small studies found no clear association[131]. Whilst smoking is a strong risk factor for esophageal squamous cell carcinoma, studies of EAC have been inconsistent, yielding conclusions ranging from complete absence of association[132-134] to a significant OR of 3.4 for current smokers[128]. Problems with study methodology occur and certainly smoking has rarely been a primary endpoint for studies of BE/EAC.

Socioeconomic status

There are no clear associations between socioeconomic status and neoplastic progression of BE. Some studies suggest increased EAC risk in higher socioeconomic groups, others the reverse[72].

COX-2 inhibition

Given the role of the AA pathway in neoplastic progression, pharmacological inhibition of COX-2 might modify the natural history of BE. Various studies have investigated whether aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) might confer protection against EAC. A meta-analysis by Corley *et al*[136] including 1813 EAC patients suggested a protective association (OR, 0.67). Both intermittent and frequent use appeared advantageous, with evidence of a dose-effect, whilst aspirin conferred greater protection than NSAIDs.

However the Chemoprevention for Barrett's Oesophagus Trial randomized 100 BE patients with dysplasia to either celecoxib 200 mg twice daily or placebo, with negative results[137]. A retrospective analysis of the UK BE registry with a total follow-up of 3683 patient-years also failed to demonstrate a protective effect of aspirin[138]. AspECT should provide further useful information.

CONCLUSION

The etiology of progression of BE is probably multi-factorial, with contributions from environmental risk factors interacting with genetically-determined characteristics. Obesity and ongoing bile and acid reflux are emerging as potentially modifiable risk factors, though designing practical interventions has so far proved difficult. Developments in understanding the MDC process in BE may provide future testable therapeutic targets.

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REVIEW

Future of liver transplantation: Non-human primates for patient-specific organs from induced pluripotent stem cells

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Abstract

Strategies to fill the huge gap in supply *versus* demand of human organs include bioartificial organs, growing humanized organs in animals, cell therapy, and implantable bioengineered constructs. Reproducing the complex relations between different cell types, generation of adequate vasculature, and immunological complications are road blocks in generation of bioengineered organs, while immunological complications limit the use of humanized organs produced in animals. Recent developments in induced pluripotent stem cell (iPSC) biology offer a possibility of generating human, patient-specific organs in non-human primates (NHP) using patient-derived iPSC and NHP-derived iPSC lacking the critical developmental genes for the organ of interest complementing a NHP tetraploid embryo. The organ derived in this way will have the same human leukocyte antigen (HLA) profile as the patient. This approach can be curative in genetic disorders as this offers the possibility of gene manipulation and correction of the patient's genome at the iPSC stage before tetraploid complementation. The process of generation of patient-specific organs such as the liver in this way has the great advantage of making use of the natural signaling cascades in the natural milieu probably resulting in organs of great quality for transplantation. However, the inexorable scientific developments in this direction involve several social issues and hence we need to educate and prepare society in advance to accept the revolutionary consequences, good, bad and ugly.

Key words: Induced pluripotent stem cells; Hepatocytes; Tetraploid; Non-human primates; Anencephaly; Chimpanzee; Fumaryl acetoacetate hydrolase deficient; Hhex

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INTRODUCTION

There is a huge gap in supply *versus* demand of human organs for transplantation. Currently 108 614 patients in United States are waiting for an organ transplant according to United Network for Organ Sharing (UNOS)[1] against 7136 donors. There is a need to bridge this gap. Either we have to motivate more people to allow organ donation, or rely on alternative methods such as improved artificial organ support systems (dialysis machines, bioartificial liver, etc) or search for better ways to circumvent the problems, mainly immunological, with xenografts.

This includes improved methods for suppressing host immunity and growing "humanized organs" in animals. Recent developments with induced pluripotent stem cells (iPSC) have yielded a new option-growing organs from pluripotent stem cells derived from the patient's own tissues. Attempts have been made to grow

organs *in vitro* with mixtures of different stem cells and biocompatible scaffolds, but the development of an organ cannot be replicated *in vitro* due to its complexity. This is a major obstacle in the generation of organs, including those attempts to make organs derived from a patient's iPSCs, the ultimate goal in regenerative medicine. The straightforward method is to generate a fetus from iPSC in a surrogate mother for the sole purpose of organ harvesting, but obviously a host of ethical issues precludes this line of thought. Here, I will make an attempt to review the latest developments and discuss their prospects, taking the liver as a model organ.

Currently, more than 17 000 people in the United States are waiting for liver transplants. According to UNOS, about 5300 liver transplantations were performed in the United States in 2002.

BIOARTIFICIAL ORGANS-THE BIOARTIFICIAL LIVER: A WRONG ROAD?

The bio-artificial liver (BAL) is still in its infancy. BAL as a replacement for the normal liver is very unlikely. At most it is currently of use in bridging the gap between organ failure and transplantation or liver regeneration (as the liver has exceptional capacity to regenerate). BALs are largely unsuccessful because: (1) most of the liver cell lines are not functionally efficient and human iPSC-derived "hepatocytes" are not functional enough and difficult to obtain in sufficient quantities; (2) the special arrangement of hepatocytes into chords in sinusoidal spaces is important for their function; (3) the relationship and communication between hepatocytes themselves and between biliary epithelial cells, sinusoidal endothelial cells, etc, are quite important for functions of hepatocytes, such as active and passive transport of metabolites in the right direction and optimal gene regulation; (4) currently there is no source of functional hepatocytes in large quantities for bioreactors except from animals, which always pose a problem of infection, immune system activation, and functional incompatibility of essential proteins secreted by or possibly interacting with hepatocytes; (5) hepatocytes have a low life span under culture conditions, and it is difficult to maintain conditions close to that of the human microenvironment; and (6) difficulties in developing complex membranes which allow highly selective exchange of biologically important molecules.

The efficiency of bioartificial liver devices such as the "extracorporeal liver assist device" (ELAD) or non-biological devices such as the "molecular adsorbent recycling system" (MARS) and other models are not very different from dialysis alone. The current data show that only the MARS system reduces mortality in acute liver failure and in acute exacerbations of chronic liver failure, although this reduction is non-significant[2].

HUMANIZED LIVER IN ANIMALS

Pigs are the preferred animal for humanized organs, although primates like chimpanzees or gibbons would the ideal for the generation of "humanized" organs. We have made remarkable progress in the last 10 years in the field of xeno-immunology of pig-to-nonhuman primate transplantation, and we are expecting clinical trials in the near future. A common school of thought is for engineered animals lacking certain antigens so that their organs can be used for transplantation in human patients with a reduced chance of immune rejection[3]. Pigs can be genetically modified for xenotransplantation by alteration of immunologically important genes such as human decay-accelerating factor (hDAF), and CD46 (membrane cofactor protein), α-galactosyl transferase knockout (GT-KO), CD55 or CD46, CD59 transgenics, as well as human leukocyte antigen (HLA)-Ⅱ transgenics, including DP, DQ, and DR[3,4]. HLA-DR15+ transgenic pig skin pieces were grafted onto severe congenital immunodeficiency (SCID) mice reconstituted intraperitoneally with HLA-DR15+ human peripheral blood mononuclear cells. The dermal graft survived and was integrated[4]. Using GT-KO pigs and novel immunosuppressant agents, 2 to 6 months' survival of heterotopic heart xenotransplants has been achieved. The issue of hyper-acute rejection is more or less solved with hDAF and GT-KO pigs, but acute humoral xenograft rejection, injury to the endothelium leading to thrombotic microangiopathy and coagulation dysregulation, remains unsolved for a meaningful survival rate to be achieved. Baboons died following massive internal bleeding and profound thrombocytopenia post-transplantation of livers from GT-KO pigs transgenic for CD46[5,6].

STEM CELLS, IPSC AND SOPHISTICATED SCAFFOLDS MEET THE ANGIOGENESIS OBSTACLE FOR GENERATING ORGANS

The genesis of organs is a very complex process. Organs such as the brain, liver and kidney have extremely complicated architecture and contain several cell types. The relationship between cells, their specific orientation, and physical and chemical characteristics are of crucial functional importance. Thus, even if we generate genetically intact and fully functional hepatocytes, biliary epithelial cells, angiogenic precursor cells, sinusoidal endothelial cells, kupffer cells and so on, we are unlikely to regenerate (or generate *de novo*) a liver through co-culture of these cells, injecting these cells in a defined proportion into a damaged liver, or populating an appropriate scaffold or matrix. A highly sophisticated scaffold or matrix with spatial and temporal cues-chemical, mechanical, ionic, electric charge or surface properties-for homing of different cell types is unlikely to be successful in the near future, considering the complexity of the micro architecture of organs required for normal physiological function. One of the major barriers to successful generation of organs *in vitro* is our inability to generate the vascular architecture necessary for growth, development

and maintenance of any organ. Recently, attempts have been made to use natural scaffolds by decellularization of an entire organ, the liver in this example, and preserve its vascular network. Preliminary studies showed the possibility of being able to efficiently re-cellularize the bioscaffold using perfusion cell seeding with primary human fetal liver progenitor cells and endothelial cells in a bioreactor[7]. However, as noted above, numerous difficult technical issues remain to be addressed to efficiently deliver primary human liver progenitor cells to generate functional hepatic tissue. Availability of decellularized human liver scaffolds would be another problem.

Although iPSC technology offers wonderful possibilities for generating practically every cell type from adult somatic cells through a pluripotent stem cell intermediate, currently this has limited applications in, for example, regeneration of tissues of lesser complexity such as bone marrow and adipose tissue with a genetic modification [example: C-C chemokine receptor type 5 (CCR5) in the bone marrow stem cell gene therapy of acquired immunodeficiency syndrome (AIDS) or adipocyte gene therapy in inherited forms of diabetes or lipodystrophy][8] or without a genetic modification (as in the management of leukemia or degenerative disease, old age), or drug testing. For example, iPSC-derived hepatocyte-like cells, and proximal or distal renal tubular epithelium for hepatic or renal toxicity testing, respectively, are useful in new drug development or assessment of drug response to different human genotypes, a step towards personalized medicine.

Small organs or tissues can be engineered successfully using scaffolds, for example, blood vessels or urinary bladder. By culturing cells on a biodegradable scaffold such as polyglycolic acid, and later passing media in a pulsatile fashion under optimum pressure, was found useful in generating functional small-caliber arteries[9]. The pulsatile flow triggers collagen deposition and alignment of the fibers and this is critical for attaining mechanical maturity to withstand pressure met under natural conditions[10].

Growing larger organs is a major problem because oxygenation and metabolite exchange becomes difficult as the thickness increases. Self-assembly of cells, for example cardiomyocytes, can take place in thin sheets (< 80 μm), and increasing the thickness by sequential deposition of multiple cardiac sheets has to be slow enough to allow the host vasculature to sprout into and vascularize each layer before the next layer is deposited[11]. However this method is very impractical in humans because of the necessity of multiple surgeries. Furthermore, this approach is unlikely to be successful for more complicated organs like the liver, not only because the liver has different types of cells in a highly ordered manner, but it also has a complicated dual vasculature forming the sinusoids. Following a nature mimetic approach, a vascular tree should have a capillary network (10 μm-20 μm) which can be generated by induction of sprouting by cytokines and co-culture with related cells; the intermediate microvessels (50-500 μm) may be obtained by microfabrication-microfluidic techniques and finally the microvasculature (about 2 mm) is produced by a combination of tissue engineering methods[12]. Unfortunately, achieving vascularization in a tissue by assembling all these and finally generating a fully vascularized organ which is functional is a very complicated process making this approach undesirable.

MAKING GENETICALLY HUMAN ORGANS IN ANIMALS

It is an ingenious idea to generate genetically human organs in animals. With the recent advancement in iPSC technology, transgenic technology and embryo manipulation, it is possible to generate organs of one animal species in another one. The best achievement in this direction is reported by Kobayashi *et al*[13] in Cell 2010. Mouse wild-type iPSCs injected into Pdx1 -/- rat blastocysts (Pdx is a critical gene for genesis of the pancreas and hence Pdx1 -/- rats are pancreatogenesis-disabled) developmentally compensated for the vacancy of the pancreatic "developmental niche", generating almost an entirely iPSC-derived rat pancreas inside the mouse, and mouse iPSC-derived pancreas inside the Pdx-/- mouse. Similarly it could be possible to generate a human pancreas (and other organs) in animals, for example in monkey, pig or sheep, which are genetically modified to support implantation and development of an embryo containing cell clusters/organ of human origin.

Production of a chimeric embryo/fetus[14] was performed largely to study organogenesis, cell migration, cell lineages, cell destination, development and function of the immune system, rather than with the aim of generating live chimeric animals for organ/tissue harvesting. However, efforts to make live intergeneric chimeric animals (for example rat-mouse chimera) were unsuccessful because of incompatibility between the fetal parts of the placenta and the uterus[15,16]. The only exceptions we know are hybrids like geep (a sheep and a goat)[15-17]. Thus it is one of the major achievements of 2010 to produce a rat-mouse intergeneric chimera by injecting mouse pluripotent stem cells into rat blastocysts.

NEW WORLD MONKEYS COULD BE USED TO GENERATE GENETICALLY HUMAN ORGANS

Rats and mice belong to same family (Muridae) and subfamily (Murinae), but of a different genus, while human beings and chimpanzees belong to the same genus and there are seven species in the sub-family "Hominini" which contains man, chimpanzees, gorillas and orangutans. Man is closer to the chimpanzee than the rat is to the mouse (Table 1). Modern molecular studies have spectacularly confirmed this prediction and have refined the relationships, showing that the common chimpanzee

Table 1 Comparison between mouse and rat *versus* chimpanzee and human

	Mouse	Rat	Chimpanzee	Human
Size	20 g-40 g	250 g-520 g	35 kg-75 kg	45 kg-100 kg
Chromosomes	20 pairs	21 pairs	24 pairs	23 pairs
Genome similarity	96.50%		98%	
Gestation period	20 d	22 d	9 mon	9 mon
Birth weight	0.5 g-1.5 g	5 g-6 g	1.5 kg-2 kg	2 kg-4 kg
Liver	4-5 distinct lobes	4 distinct lobes	3 lobes which are not separate	3 lobes which are not separate
Gallbladder	Present	Absent	Present	Present

(*Pan troglodytes*) and bonobo (*Pan paniscus* or pygmy chimpanzee) are our closest living evolutionary relatives[18]. This opens an exciting possibility to generate and harvest human organs, genetically identical to the recipient, in new world monkeys. There is about 98% sequence similarity between human and chimpanzee genomes, and the global variation between humans at the single nucleotide level has been estimated at about 0.1%[19,20]. Chimpanzee body temperature, general blood biochemistry (glucose, sodium, potassium, calcium, phosphate, insulin, hemoglobin, urea, etc), red blood cell count, white cell count, platelet count, osmolarity, plasma protein composition, etc, falls within the range of human values[21]. It may be noted that rather than genetic differences, what makes humans unique are "aspects of human uniqueness which arose because of a primate evolutionary trend towards increasing and irreversible dependence on learned behaviors and culture"[20].

There are multiple possible approaches to generate a human organ in a chimpanzee or a higher primate. One approach is to make a true chimera by populating the chimpanzee donor blastocyst with patient specific human iPSC, which is modified to have genes for development of the liver but deficient in genes for brain development. This ensures that under no circumstances will a human brain develop inside an animal or grow with cells of animal origin. The chimpanzee donor blastocyst should be deficient in the genes which are critical and specific for the development of the organ in question (Pdx1 in pancreas and probably Hhex in liver). However, for the human liver we have yet to identify the most suitable liver-specific gene which can be knocked out without affecting general development of the fetus. Foxa1, 2 and 3, Gata-6, HNF-4a, HNF-1a, Hhex, Sox-9 are among key genes involved in foregut-liver development[22-26]. Alternatively, the blastocyst may be deficient in a protein which is metabolically important, and whose deficiency would result in selection pressure, such that only the iPSC-derived cells would survive. Taking the liver as a model organ, fumaryl acetoacetate hydrolase (FAH)-deficient chimpanzee blastocysts would be a good example which would be populated with human patient derived iPSC with the normal (wild type) FAH gene. During the development of the fetus, human liver cells expressing FAH would have a survival advantage over chimpanzee liver cells lacking FAH. Fumarylacetoacetate, a toxic metabolite, will accumulate in FAH deficient liver cells and kill them[27]. This selection process can be controlled at will using NTBC to facilitate a smooth and optimum rate of cell replacement without affecting the liver architecture. The introduction of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) inhibits p-hydroxyphenylpyruvate dioxygenase (the second enzyme in tyrosine degradation) and stops the formation of the toxic metabolites[27]. Thus, giving animals NTBC and slowly weaning them off might lead to a liver which is exclusively composed of human liver cells inside a chimpanzee fetus. One major worry in creating human organs in animals is the formation of germ cells from human donor cells in the gonads of the recipient animal, although the possibility is remote. Using animal blastocysts as well as the surrogate mother animal where the critical genes for spermatogenesis/oogenesis are knocked out, the theoretical possibility of germline transmission of human genes can be ruled out. A practical approach would be to use a mixture of human patient-derived iPSC with nonhuman primate embryonic cells/iPSC, in which some specific genes for fertility (with no effect on implantation or the development of the embryo, e.g., an acrosomal protein for male infertility) are knocked out for introduction into a tetraploid embryo (tetraploid complementation technique)[28]. It may be noted that there are reports of efficient generation of iPSC from non human primates[29]. There exist several methods to generate genetically intact 'virus free' iPSC from adult primate cells[30-34].

The proposal is very attractive but we can anticipate the following problems: (1) a chimpanzee-human mosaic fetus may not survive (though unlikely) because: (a) growth factors and transcription factors and/or signaling pathways may be incompatible; (b) cell adhesion molecules or response to directional molecule gradients may be different; and (c) the developing fetus may abort due to unforeseen reasons (e.g., failure of the tetraploid complementation technique or implantation and development); (2) possible immune rejection on transplantation even after perfusion washes and immune cell depletion due to small quantities of antigens, for example glycoproteins, adhering to the vessel walls, interstitial spaces, growth of some animal blood vessels into the "human" organ, etc; (3) a possibility that iPSC-derived organs are more prone to tumors; and (4) ethical issues involved in making human-chimpanzee mosaic embryos which might survive to near full term, even if it is ensured that human brain (or certain types of human neurons important in cerebral cortex for human identity) will not be present in the fetus by using human iPSC knockout for genes specific for brain development.

Despite these problems success is very likely because the genetic difference between mouse and rat is greater than that between human and chimpanzee and it was proved by Kobayashi *et al* that it is possible to generate a rat pancreas in a mouse. The immunological rejection

is less likely to be a major problem at least in the case of the liver because: (1) liver is a very immune tolerant organ compared with several other organs such as the kidneys[35,36]; (2) the patients own cells will be used to generate the new liver, ensuring 100% HLA matching; (3) better and less toxic immune-suppressants and immune-modulators are currently available; and (4) one can perform immunodepletion on the liver prior to harvest, first by treating with an immunocyte-specific mitogen and then treating with cyclophosphamide; this will push the immunocytes into mitosis which would then be preferentially killed by cyclophosphamide. There are more than a few ways to overcome the obstacle presented by the immune system in this setting, including the induction of immunological tolerance in the host[37]. Novel methods such as inhibition of leukocyte costimulatory molecules may offer a way to suppresses T cell activation resulting in immune suppression[38]. Several studies have found increased abnormal epigenetic changes, mutations in coding regions, and copy number variations in induced pluripotent cells compared with normal in a small proportion of cells[39-42].

It may be noted that iPSC is a relatively new technology and it might take another decade for the technology to mature. Similarly, newer screening methods which would facilitate selection of genetically intact cells, such as faster methods for whole genome scanning for mutations and epigenetic abnormalities are expected to resolve these issues.

Any research involving implantation of human embryos into the uterus after *in vitro* manipulation at any stage of development in humans or primates is illegal. However using a non-human primates (NHP) embryo to develop a human organ inside a NHP fetus inside the uterus of a NHP may not be illegal in many countries. The National Academy of Sciences (United States) Guidelines, recommends that human-nonhuman chimeras will not be allowed to breed, but this recommendation is only voluntary[43-45].

MAKING HUMAN ORGANS IN PARTLY *IN VITRO* SYSTEMS-ETHICAL ISSUES

Many ideas which are quite logical cannot be put into practice because of ethical concerns. One great example is therapeutic cloning. Commoditization of human oocytes and human sperm or human embryos and human organs is considered unethical in many countries. Any *in vitro* or *in utero* culture of intact human embryos, regardless of the method of its derivation, beyond 14 d or formation of the primitive streak, whichever is earlier, is illegal. One of the main concerns is the identity of the embryo as a human. However, human identity is technically the development of the brain and the nervous system which defines and determines all emotions, pain, memories, self respect, ethics and self identification. Growing an embryo which is anencephalic (without brain) for organ harvest would be a solution. However carrying an anencephalic fetus may be emotionally devastating for the surrogate mother in some cases. This can be avoided in turn by having a "*in vitro*-uterus" /semi-artificial uterus system (or uterus with some of the supporting organs) to facilitate the growth of the anencephalic system containing the organ of interest. However, these futuristic concepts are well beyond the consideration of current society for ethical reasons and the unpredictable social and medical consequences.

SCANNING AND PRINTING AN ORGAN

Development of a fetus from a zygote is an example of directed self-assembly processes, in which, through chemical or physical gradients, or predetermined cell-cell and cell-extracellular matrix interactions, the developing organism gradually acquires its final shape. Thus it is logical to assume that if we could provide the appropriate gradients, position and neighbors, then cells will migrate, self-assemble, and establish the correct connections to form the organ. This is quite true for tissues or organs of low complexity such as cartilages, bone, skin, urinary bladder or heart valves, but is unlikely for complex organs such as the liver or brain. Thus the human cornea, urinary bladder, etc. may be ideal tissues/organs for bio-artificial/"engineered" organs rather than complex organs such as the liver.

Most organs are composed of several types of cells in a very specific order in 3-dimensional space which is critical for their function. The concept of inkjet printing opens up a solution to this problem because it allows precise delivery of multiple cell types and matrix components into pre-determined sites with high precision. Multiple cell types in suspension are placed, instead of ink, into different "ink" chambers of a sterilized cartridge and the printer is directed to arrange or "print" these cells in a specific order. It is also possible to use conventional 2-dimensional printing to generate cells of different phenotypes with differential coatings of cell adhesion molecules printed in a specific pattern on extremely thin films with differential cell adhesion properties, which would result in a final pattern formation through minimization of configuration energy, the driving force in cell rearrangement. If we could use a suitable matrix, a chemical gradient also can be printed[46,47].

There is concern that bioprinting would result in non-functional tissues. However, in an elegant experiment by Jacob *et al*[48], synchronous macroscopic beating was demonstrated throughout a sheet obtained by the fusion of chick cardiac cell spheroids through bioprinting.

Imagine a scanner that can scan in 3 dimensions in sub-nanometer resolution and store an enormous amount of data with spatial coordinates of each molecule in the scanned object! Similarly, imagine a 3-dimensional printer that can print at sub-nanometer resolution. If such a scanner and printer could exist, one could scan an entire organ no matter how complex it is, if not an entire human body and reconstruct (clone) it, perhaps so perfectly that it includes the memories! The printer

would be using all molecules which constitute the human body as its ink equivalent! This is science fiction today but tomorrow this may become a reality!

CONCLUSION

The development of iPSC technology has enabled us to generate cells which are very similar to pluripotent stem cells from adult cells. Improvements in this technology will have radical consequences in regenerative medicine, transplantation medicine, therapeutic cloning, and generation of patient-specific whole organs. Generation of iPSC-derived whole organs inside the uterus, making use of the natural developmental signals and environment may result in more natural and high quality organs for transplantation. In future, non-human primates or an "*in vitro*-uterus" may be useful for producing patient-specific organs such as the liver for transplantation. The society needs to be prepared in advance to accept the revolutionary consequences, good, bad and ugly, of these ongoing scientific developments.

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ORIGINAL ARTICLE

Induction of CD69 expression by *cag*PAI-positive *Helicobacter pylori* infection

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Abstract

AIM: To investigate and elucidate the molecular mechanism that regulates inducible expression of CD69 by *Helicobacter pylori* (*H. pylori*) infection.

METHODS: The expression levels of CD69 in a T-cell line, Jurkat, primary human peripheral blood mononuclear cells (PBMCs), and $CD4^+$ T cells, were assessed by immunohistochemistry, reverse transcription polymerase chain reaction, and flow cytometry. Activation of CD69 promoter was detected by reporter gene. Nuclear factor (NF)-κB activation in Jurkat cells infected with *H. pylori* was evaluated by electrophoretic mobility shift assay. The role of NF-κB signaling in *H. pylori*-induced CD69 expression was analyzed using inhibitors of NF-κB and dominant-negative mutants. The isogenic mutants with disrupted *cag* pathogenicity island (*cag*PAI) and *virD4* were used to elucidate the role of *cag*PAI-encoding type Ⅳ secretion system and CagA in CD69 expression.

RESULTS: CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. Although *cag*PAI-positive *H. pylori* and an isogenic mutant of *virD4* induced CD69 expression, an isogenic mutant of *cag*-PAI failed to induce this in Jurkat cells. *H. pylori* also induced CD69 expression in PBMCs and $CD4^+$ T cells. The activation of the CD69 promoter by *H. pylori* was mediated through NF-κB. Transfection of dominant-negative mutants of IκBs, IκB kinases, and NF-κB-inducing kinase inhibited *H. pylori*-induced CD69 activation. Inhibitors of NF-κB suppressed *H. pylori*-induced CD69 mRNA expression.

CONCLUSION: The results suggest that *H. pylori* induces CD69 expression through the activation of NF-κB. *cag*PAI might be relevant in the induction of CD69 expression in T cells. CD69 in T cells may play a role in *H. pylori*-induced gastritis.

Key words: CD69; T cells; *Helicobacter pylori*; *cag* pathogenicity island; Nuclear factor-κB

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INTRODUCTION

The leukocyte receptor CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after activation[1,2]. In healthy subjects, CD69

is not detected in peripheral blood lymphocytes, but is expressed on small subsets of T and B cells in peripheral lymphoid tissues[3]. In addition, CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses *in vivo*[4,5]. However, the biological significance of CD69-induced cell activation is poorly understood.

Helicobacter pylori (H. pylori) is a Gram-negative bacterium that colonizes the human stomach, as well as areas of gastric metaplasia in the duodenal bulb[6]. The precise role of *H. pylori* in gastric pathology, especially the mechanism responsible for the transition of chronic active gastritis to gastric carcinoma, has been studied by many researchers. The infection triggers a local cellular immune response resulting in chronic cellular infiltration with or without an active component of neutrophils, as well as the development of lymphoid follicles in the lamina propria[7]. Although the exact mechanisms of the induction of various diseases by *H. pylori* infection have not been elucidated, one factor strongly associated with *H. pylori* virulence and the development of peptic ulcers and gastric cancer is the *cag* pathogenicity island (PAI), which constitutes a gene cluster encoding a type Ⅳ secretion system (T4SS)[8].

Enarsson *et al*[9] examined the transendothelial migration of human lymphocytes in response to *H. pylori* with the use of the Transwell system, employing a monolayer of human umbilical vein endothelial cells. *H. pylori* induced a significant T-cell migration and the presence of the *H. pylori cag*PAI increased T-cell transendothelial migration. Overexpression of CD69 was noted on migrating T cells[9]. These results suggest that *H. pylori* infection induces the expression of CD69 on T cells.

The present study was designed to test the hypothesis that *H. pylori* can induce both the surface expression of CD69 antigen and the promoter activity of the CD69 gene in human T cells, and to investigate whether such induction involves the *cag*PAI-coding T4SS and the nuclear factor (NF)-κB pathway. The presence of NF-κB motifs within the proximal promoter region of the CD69 gene may account for the *H. pylori*-inducible promoter activity.

MATERIALS AND METHODS

Reagents and bacterial strains

N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and Bay 11-7082 were purchased from Sigma-Aldrich (St Louis, MO) and Calbiochem (La Jolla, CA), respectively. *H. pylori* ATCC 49503 (American Type Culture Collection, Rockville, MD) was used in most experiments described in this study. An isogenic *H. pylori* mutant lacking the *cag*PAI[9] or *virD4* also was employed, together with the parental wild-type strain (26695). *H. pylori* strains were plated on blood agar plates and incubated at 37 ℃ for 2 d under microaerophilic conditions. Using inoculating needles, bacteria harvested from the plates were suspended in 50 mL of brucella broth containing 5% fetal bovine serum (FBS) and then cultured in a liquid medium at 37 ℃ for 1 d in a controlled microaerophilic environment. Bacteria were harvested from the broth culture by centrifugation and then resuspended at the concentrations indicated below in antibiotic-free medium. All procedures were performed with the approval of the appropriate institutional biosafety review committee and in compliance with the guidelines for biohazards.

Cell culture

The human T-cell line, Jurkat, was maintained in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of a healthy donor using Ficoll-Hypaque gradients. PBMCs then were further purified using positive selection with immunomagnetic beads specific for CD4 (Miltenyi Biotec, Auburn, CA). On the day of the experiment, cells were refed with fresh antibiotic-free medium and cocultured with *H. pylori* for the time intervals indicated below.

Tissue samples

Stomach biopsy specimens from ten patients with *H. pylori* gastritis were examined histopathologically for CD69. The presence of *H. pylori* infection was confirmed by culture, serological analysis (with anti-*H. pylori* immunoglobulin G antibody), rapid urease test, and histological examination with Giemsa staining. Patients with *H. pylori* gastritis showed polymorphonuclear neutrophil infiltration in the gastric epithelium in conjunction with the presence of bacterial forms, which is consistent with *H. pylori* infection. All samples were collected after obtaining informed consent from each patient.

Reverse transcription-polymerase chain reaction

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand complementary DNA was synthesized from 1 μg total cellular RNA using a RNA-polymerase chain reaction (PCR) kit (Takara Bio, Otsu, Japan) with random primers. The specific primers used were as follows: for CD69, 5'-CATAGCTCTCATTGCCTTATCAGT-3'(forward primer) and 5'-CCTCTCTACCTGCGTATCGTTT-3'(reverse primer); for β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3'(forward primer) and 5'-CTCCTTAATGTCACGCACGATTTC-3'(reverse primer). Thereafter, cDNA was amplified using 30 and 28 cycles for CD69 and β-actin, respectively. The product sizes were 254 bp for CD69 and 548 bp for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Transfection and luciferase assay

The IκBαΔN- and IκBβΔN-dominant-negative mutants are IκBα and IκBβ deletion mutants lacking the N-terminal 36 and 23 amino acids, respectively[10,11]. The dominant-negative mutants of IκB kinase (IKK)α, IKKα (K44M), IKKβ, IKKβ (K44A), IKKγ, IKKγ(1-305), and

NF-κB-inducing kinase (NIK), NIK (KK429/430AA) have been described previously[12,13]. The CD69 promoter pXP2 luciferase reporter plasmid containing the wild-type sequence (position -255 to position +16), pAIM255-LUC, was described previously[14]. The internal deletion mutants of the NF-κB sites were constructed by deletion of the NF-κB sites of pAIM255-LUC. Jurkat cells were transfected with the appropriate reporter and effector plasmids by electroporation. After 24 h, *H. pylori* was added and incubated for 6 h. The cells were washed in phosphate buffered saline and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for reporter gene activity with the dual-luciferase assay system (Promega). Luciferase activities were normalized relative to the Renilla luciferase activity from phRL-TK.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by electrophoretic mobility shift assay (EMSA) as described previously[15]. The top strand sequence of the oligonucleotide probes or competitors are as follows: for the NF-κB element (κB1) of the CD69 gene, 5'-GATCCAGACAACAGGGAAAACCCATACTTC-3'; for the NF-κB element (κB2) of the CD69 gene, 5'-GATCCAGAGTCTGGGAAAATCCCACTTTCC-3'; for the NF-κB element of the interleukin-2 receptor α chain (IL-2Rα) gene, 5'-GATCCGGCAGGGGAATCTCCCTCTC-3'; and for the AP-1 element of the IL-8 gene, 5'-GATCGTGATGACTCAGGTT-3'. The oligonucleotide 5'-GATCTGTCGAATGCAAATCACTAGAA-3', containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. The above underlined sequences are the NF-κB, AP-1, and Oct-1 binding sites, respectively. To identify NF-κB proteins in the DNA-protein complex shown by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, p65, c-Rel, p52, and RelB (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical analysis

CD69 immunohistochemistry was performed using a mouse monoclonal antibody (clone FN50) to CD69 (BioLegend, San Diego, CA) after pretreatment of the deparaffinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA). The sections were counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene, and mounted. The stained cells were examined under a light microscope (Axioskop 2plus; Zeiss, Jena, Germany) with an Achroplan × 40/0.65 lens (Zeiss). Images were acquired with an AxioCam MRC camera and AxioVision 3.1 software (Zeiss). Gastric lymphocytes and macrophages were identified based on their morphological features.

Flow cytometry

Cells were washed with cell WASH (Becton Dickinson Immunocytometry Systems, San Jose, CA) and incubated for 30 min with phycoerythrin-labeled mouse monoclonal antibody against CD69 (clone TP1.55.3) or control mouse IgG2b, which were purchased from Beckman Coulter (Fullerton, CA). Cells were analyzed on an Epics XL flow cytometer.

RESULTS

Overexpression of CD69 in gastric lymphocytes and macrophages in H. pylori gastritis

We investigated the expression of CD69 by immunostaining in *H. pylori*-positive gastric tissues (n = 10). CD69 staining was detected in mucosal lymphocytes and macrophages (Figure 1A). In contrast, only a faint staining for CD69 was detected in the normal mucosa, and the expression level was much weaker than in *H. pylori*-positive gastric tissues (data not shown).

H. pylori increases CD69 mRNA levels in CD4$^+$ T cells

Using reverse transcription (RT)-PCR, we next examined the effect of coculture of Jurkat T cells (a transformed human T-cell line) with *H. pylori* ATCC 49503 on the induction of CD69 mRNA. Coculture with ATCC 49503 significantly enhanced the steady-state levels of CD69 mRNA in Jurkat cells. CD69 transcript levels clearly increased 1 h after the addition of ATCC 49503 to Jurkat cells (Figure 1B). In another series of experiments, in which Jurkat cells were infected with ATCC 49503 at different concentrations [i.e., the multiplicity of infection (MOI)] for 2 h (Figure 1B), *H. pylori* induced dose-dependent expression of CD69 mRNA. To characterize the effect of *H. pylori* infection on human T cells, we employed RT-PCR to examine CD69 mRNA expression in PBMCs and CD4$^+$ T cells in response to ATCC 49503. After 2-h infection, *H. pylori* induced CD69 mRNA expression in PBMCs and CD4$^+$ T cells, similar to the observation with Jurkat cells (Figure 1C).

To analyze whether the increase of mRNA synthesis results in elevated expression on the cell surface, direct immunofluorescent staining and flow cytometry were performed. Consistent with the RT-PCR analysis, the expression was upregulated in a dose-dependent manner (Figure 2B). The peak expression level of cell surface CD69 was noted at 8 h after infection (Figure 2A). *H. pylori* ATCC 49503 also enhanced cell surface CD69 expression on PBMCs (Figure 2C).

H. pylori-induced CD69 expression is cagPAI-dependent

The *cag*PAI, a cluster of about 28 genes, is one of the best known virulence factors; it encodes a T4SS that transports CagA protein, peptidoglycan, and possibly other molecules into host epithelial cells[16]. The *cag*PAI also encodes a homologue of the coupling protein *virD4*, which in *Agrobacterium tumefaciens* and conjugation systems is thought to deliver the T4SS substrates to the secretion machinery[17]. In *H. pylori*, *virD4* is necessary for CagA translocation but dispensable for the induction of

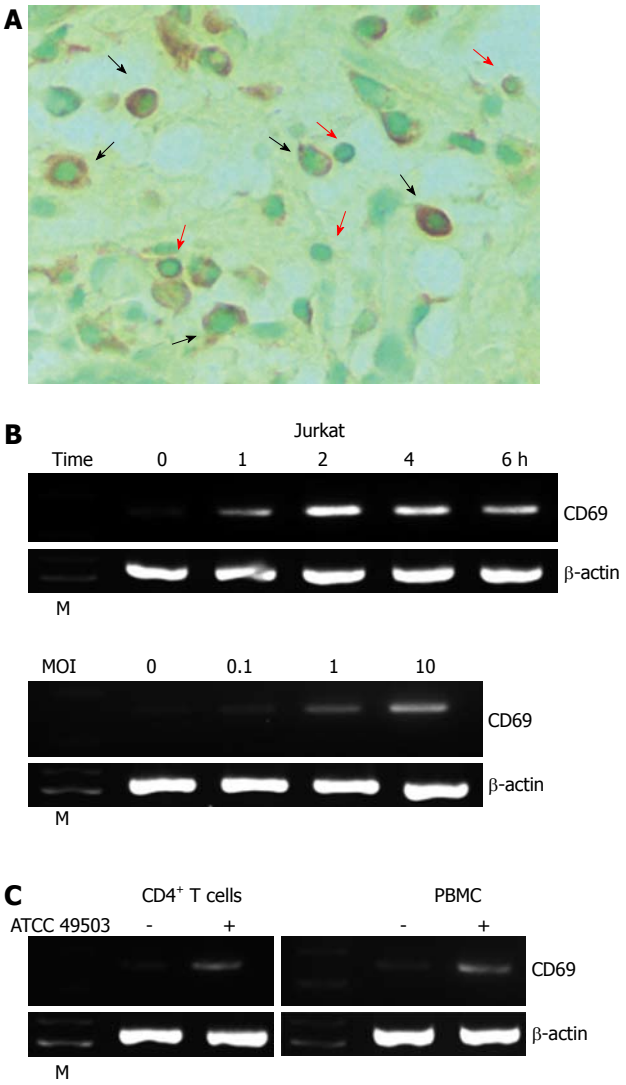

Figure 1 Expression of CD69 in *Helicobacter pylori*-infected T cells. A: Immunohistochemical detection of CD69 in tissues of patients with *Helicobacter pylori (H. pylori)*-positive gastritis. Serial sections of gastric biopsy specimens were stained with a mouse monoclonal antibody to CD69 and counterstained with methyl green. Shown is a representative example of mucosa from a patient with *H. pylori*-positive gastritis. Note the positive staining for CD69 in lymphocytes as well as macrophages. Original magnification, × 800. The red and black arrows indicate the surfaces of lymphocytes and macrophages, respectively; B: *H. pylori*-induced CD69 mRNA expression in Jurkat cells. Total RNA was extracted from Jurkat cells infected with *H. pylori* strain ATCC 49503 [the multiplicity of infection (MOI) of 100] for the indicated time intervals and used for reverse transcription-polymerase chain reaction (RT-PCR) (top). Jurkat cells were infected with the indicated concentrations of ATCC 49503 for 2 h. Total RNA was extracted and used for RT-PCR (bottom); C: *H. pylori*-induced CD69 mRNA expression in peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells. Total RNA was extracted from PBMCs and $CD4^+$ T cells infected with ATCC 49503 for 2 h and used for RT-PCR (MOI of 10). β-actin expression served as a control. Lane M: Markers.

IL-8[18,19]. Accordingly, we compared the abilities of the wild-type *H. pylori* strain 26695, an isogenic *cag*PAI mutant (Δ*cag*PAI), and a *virD4* mutant (Δ*virD4*), with regard to the induction of CD69 transcripts and expression of CD69 on the cell surface. Infection with wild-type strain 26695 induced CD69 mRNA expression in Jurkat cells, while the isogenic mutant that lacked *cag*PAI expression did not induce CD69 mRNA expression (Figure 2D). In contrast, the *virD4* mutant induced CD69 mRNA expression in Jurkat cells (Figure 2D). These results were confirmed by the cell surface expression of CD69 analyzed by flow cytometry (Figures 2B and E).

Role of NF-κB in H. pylori-induced activation of the CD69 promoter

In the next series of experiments, we investigated whether the *H. pylori*-mediated upregulation of CD69 gene expression directly enhances the activity of its promoter. Jurkat cells were transiently transfected with a reporter gene construct containing a segment from position -255 to position +16 of the CD69 upstream regulatory sequences. Coculture of *H. pylori* strain ATCC 49503 resulted in a dose-dependent increase in the activity of this CD69-driven reporter construct (Figure 3B). The NF-κB signaling pathway is activated in epithelial cells infected with *cag*PAI-positive *H. pylori* but not in cells infected with *cag*PAI-negative strains of *H. pylori*[20-22]. Two potential NF-κB binding sequences were identified at positions -160 (κB1) and -223 (κB2) (Figure 3A). κB1 and κB2 were identical to those found in the gene promoters of c-myc and IL-6, respectively[23]. To test the relative contribution of the NF-κB binding sites to the *H. pylori*-mediated activation of CD69, plasmids with internal deletion mutants of these sites of the CD69 promoter were transfected (Figure 3C). After *H. pylori* infection, single deletion of the κB2 site resulted in marked reduction of the inducible activity. Single deletion of the κB1 site and the combination of double deletions abolished *H. pylori*-mediated activation of this reporter construct. These data clearly indicate that the two NF-κB binding sites in the CD69 promoter regulate CD69-enhanced expression after infection with *H. pylori*.

H. pylori infection induces binding of NF-κB family proteins to the κB1 and κB2 motifs of the CD69 promoter in T cells

The data presented above indicate that *H. pylori*-induced CD69 expression is mediated by the κB1 and κB2 sites. To analyze whether these two putative NF-κB binding sites of the CD69 promoter could bind NF-κB family members, gel retardation assays were performed using as probes two double-stranded oligonucleotides (CD69 κB1 and CD69 κB2) containing these motifs. To characterize the NF-κB-related proteins that bind to the NF-κB sites of the CD69 promoter in CD69-expressing cells, the two oligonucleotide probes were incubated with nuclear extracts prepared from untreated Jurkat cells and from Jurkat cells infected with *H. pylori*. Jurkat cells were infected with *H. pylori* at different times after challenge, and nuclear protein extracts were prepared and analyzed to determine NF-κB DNA binding activity. As shown in Figure 4A, complexes were induced in these cells within 30 min after infection with *H. pylori* and were detected at 180 min after infection with both oligonucleotide probes. The amounts of these inducible DNA-protein complexes were *H. pylori* dose-dependent.

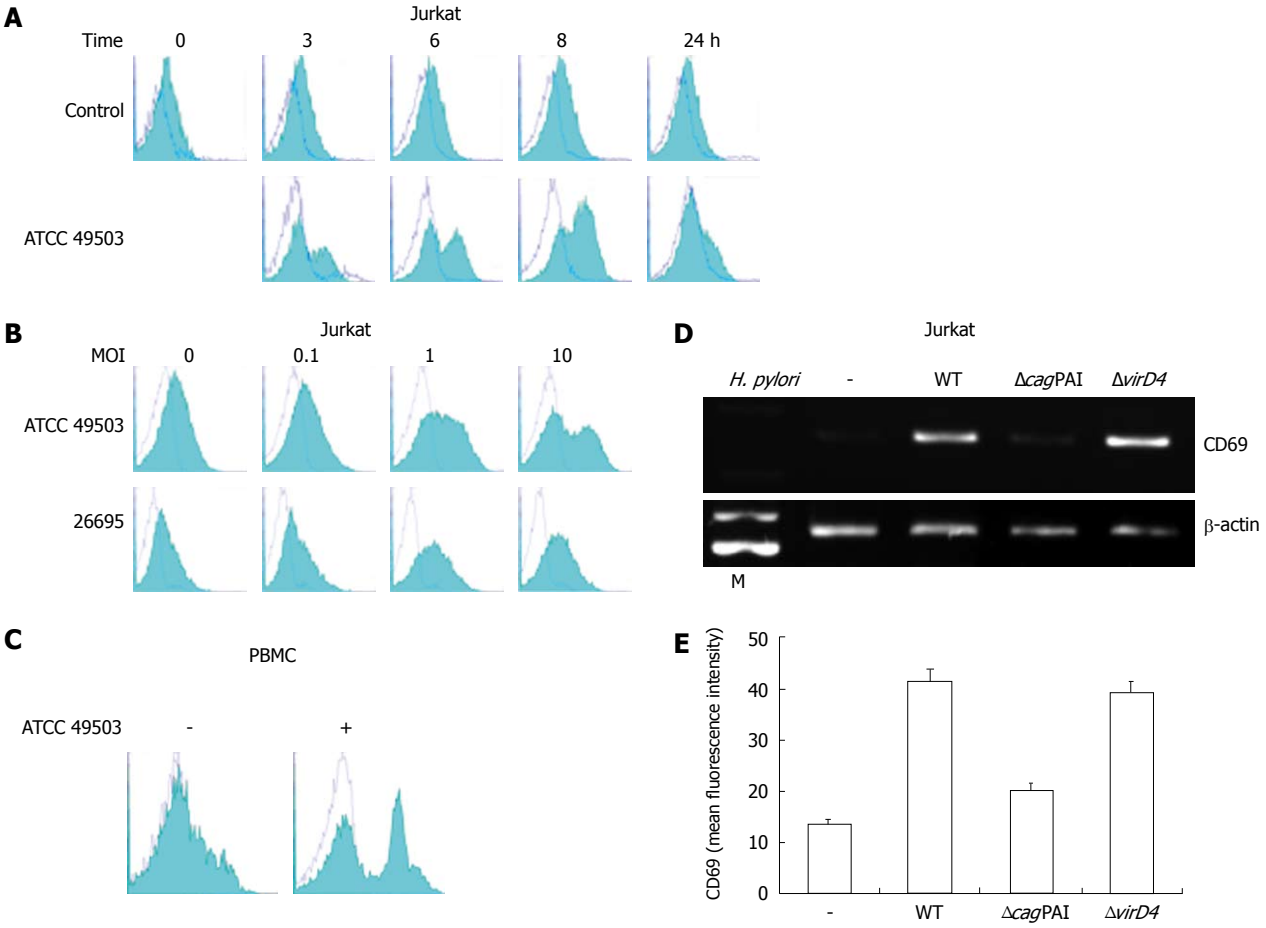

Figure 2 CD69 expression on Jurkat cells. A: Time course of cell surface expression of CD69 on Jurkat cells exposed to *Helicobacter pylori (H. pylori)*. Jurkat cells were cultured for the indicated times in culture medium (control) or in the presence of ATCC 49503 [the multiplicity of infection (MOI) of 10]. After cell harvest, CD69 expression on the cells was determined by flow cytometry; B: *H. pylori* infection increases cell surface expression of CD69 on Jurkat cells in a dose-dependent fashion. Jurkat cells were infected with different concentrations of *H. pylori* strains, ATCC 49503 and 26695, and CD69 levels were measured by flow cytometry on cells harvested after 8 h; C: *H. pylori* infection increases cell surface expression of CD69 on peripheral blood mononuclear cells (PBMCs). PBMCs were infected with ATCC 49503 (MOI of 10), and CD69 levels were measured on cells harvested after 8 h; D: *cag* pathogenicity island (*cag*PAI) products of *H. pylori* are required for the induction of CD69 mRNA expression. Total RNA was extracted from Jurkat cells that had been infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4* (MOI of 10) for 2 h and used for reverse transcription-polymerase chain reaction. Lane M: Markers; E: Flow cytometric analysis was carried out for the surface expression of CD69 in Jurkat cells infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4*. Jurkat cells were infected for 8 h with various *H. pylori* strains (MOI of 10). Cells were stained with phycoerythrin-labeled monoclonal antibody. Datas are mean ± SD of three experiments.

In both probes, the addition of an excess of unlabeled κB1 and κB2 oligonucleotides to the binding reaction completely abolished the formation of the inducible DNA-protein complexes (Figure 4B, lanes 3 and 4). Similarly, an equal amount of the oligonucleotide IL-2R κB, which contained the NF-κB motif of the IL-2R α chain gene, efficiently competed with the specific complexes (Figure 4B, lane 5). In contrast, the formation of these DNA-protein complexes was not blocked by the addition of an excess of the unrelated oligonucleotide AP-1 (Figure 4B, lane 6).

To identify the NF-κB family members that bind to the NF-κB motifs of the CD69 gene promoter, the binding reactions were preincubated with antibodies specific to p50, p65, c-Rel, p52, and RelB (Figure 4B). The anti-p50 antibody induced the supershifted bands or reduced the intensity of complexes κB1 and κB2 (Figure 4B, lane 7). The anti-p65 antibody induced supershifted bands or blocked the formation of complexes κB1 and κB2 (Figure 4B, lane 8). The anti-c-Rel antibody induced the supershifted band and reduced the intensity of only complex κB1 (Figure 4B, lane 9). In contrast, the anti-p52 or anti- RelB antibody did not interfere with the formation of any of these complexes (Figure 4B, lanes 10 and 11). These results indicate that the complexes κB1 and κB2 correspond to p50/p65/c-Rel and p50/p65, respectively. These results suggest that *H. pylori* infection seems to induce CD69 gene expression at least in part through the induced binding of NF-κB family members to the NF-κB sites in the CD69 promoter region.

NF-κB signal is essential for induction of CD69 expression by H. pylori in T cells

We also examined whether the *H. pylori*-mediated up-regulation of CD69 gene expression involves signal transduction components in NF-κB activation. Activation of NF-κB requires the phosphorylation of two conserved serine residues of IκBα (Ser-32 and Ser-36)

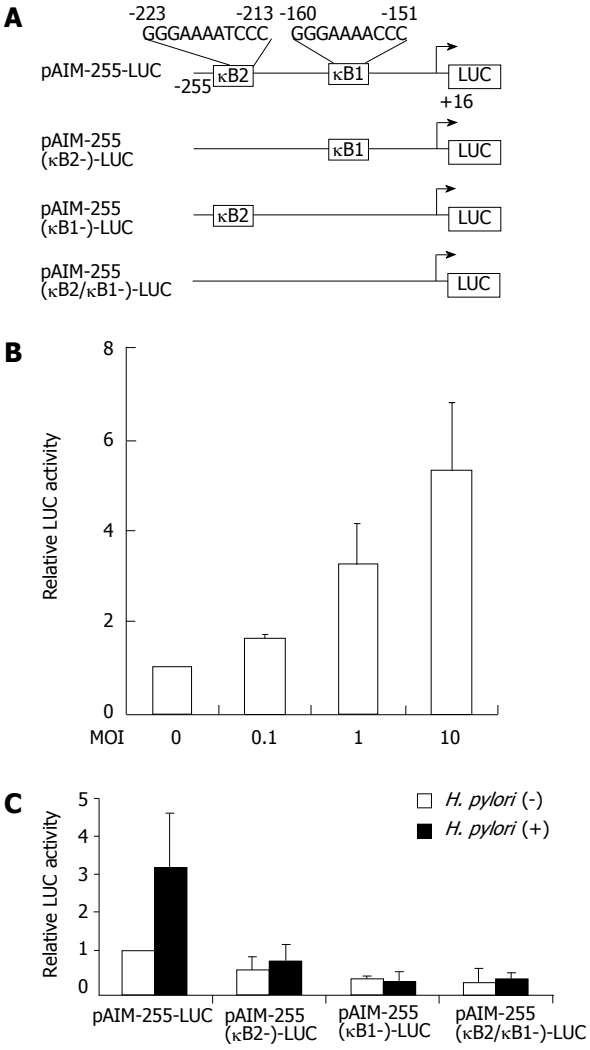

Figure 3 *Helicobacter pylori* activates the CD69 promoter through two nuclear factor-κB binding sites. A: Schematic diagram of the CD69 reporter constructs containing the wild-type (pAIM-255-LUC) and internal deletion mutants of κB1 and/or κB2 motifs. LUC: Luciferase; B: *Helicobacter pylori* (*H. pylori*) infection increases CD69 promoter activity in a dose-dependent fashion. pAIM-255-LUC was transfected into Jurkat cells, and the cells were subsequently infected with *H. pylori* ATCC 49503 for 6 h; C: The indicated CD69 reporter constructs were transfected into Jurkat cells, and subsequently the cells were infected with ATCC 49503 for 6 h (the multiplicity of infection of 10). The activity is expressed relative to that of cells transfected with pAIM-255-LUC without further *H. pylori* infection, which was defined as 1. Datas are mean ± SD of three experiments.

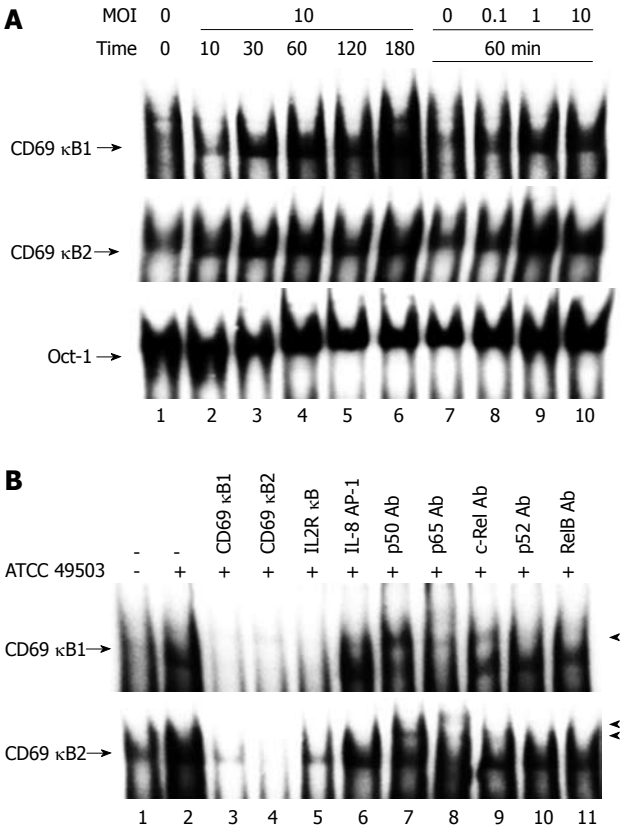

Figure 4 *Helicobacter pylori* infection induces nuclear factor-κB binding activity. A: Nuclear factor (NF)-κB activation in Jurkat cells infected with *Helicobacter pylori (H. pylori)*, as evaluated by electrophoretic mobility shift assay (Oct-1). Nuclear extracts from Jurkat cells infected with different densities [the multiplicity of infection (MOI)] of *H. pylori* ATCC 49503 (lanes 7 to 10) for the indicated times (lanes 1 to 6) were mixed with oligonucleotide probes CD69 κB1 (top) and CD69 κB2 (middle), which contained the putative NF-κB motifs located at positions -160 and -223, respectively; B: Competition assays were performed with nuclear extracts from Jurkat cells infected with ATCC 49503 (MOI of 10) for 180 min. Where indicated, the excess amounts of each specific competitor oligonucleotide were added to the reaction mixture with each oligonucleotide probe (lanes 3 to 6). A supershift assay of NF-κB DNA binding complexes in the same nuclear extracts was also performed. Where indicated, appropriate antibodies (Ab) were added to the reaction mixture before the addition of probe CD69 κB1 (top) or CD69 κB2 (bottom). Arrows indicate the specific complexes, while arrowheads indicate the DNA binding complexes supershifted by the antibodies.

and IκBβ (Ser-19 and Ser-23) within the N-terminal domain[24]. Phosphorylation leads to the ubiquitination and 26S proteasome-mediated degradation of IκBs, thereby releasing NF-κB from the complex and its translocation to the nucleus and activation of various genes[24]. The IKK complex, which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ), phosphorylates IκBs[24]. Previous studies indicated that members of the mitogen-activated protein kinase kinase kinase family mediate the physiologic activation of IKK[25]. These kinases include NIK[26]. IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and IKKα, IKKβ, and NIK kinase-deficient mutants were tested to determine their abilities to inhibit the *H. pylori*-mediated activation of the CD69-driven reporter gene. The expression of these various inhibitory mutants abolished *H. pylori*-induced CD69 expression (Figure 5A). These results emphasize the importance of signaling components involved in the activation of NF-κB in *H. pylori*-induced activation of the CD69 promoter.

Because activation of the CD69 promoter by *H. pylori* infection requires the activation of NF-κB, we blocked NF-κB activation with Bay 11-7082, an inhibitor of IκBα phosphorylation[27], or LLnL, a proteasome inhibitor[28]. The latter is known to inhibit the activation of NF-κB by blocking the degradation of the IκBα protein. Both Bay 11-7082 and LLnL markedly inhibited the *H. pylori*-induced expression of CD69 mRNA (Figure 5B).

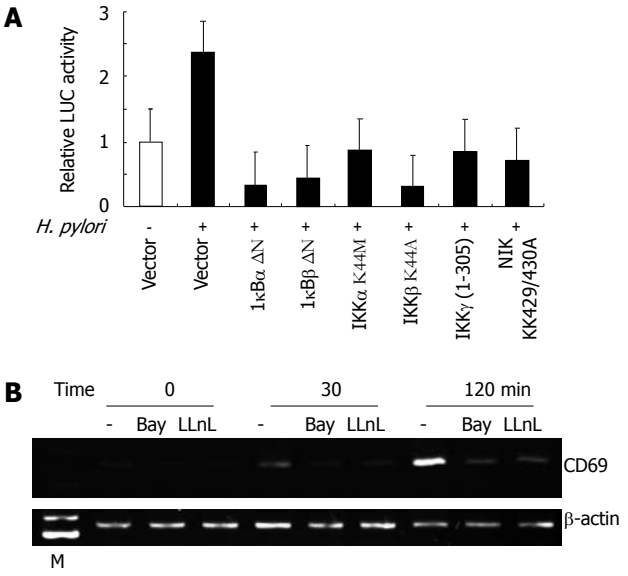

Figure 5 Nuclear factor-κB signal is essential for the activation of CD69 expression by *Helicobacter pylori* in T cells. A: Functional effects of IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants on *Helicobacter pylori (H. pylori)*-induced activation of the CD69 promoter. Jurkat cells were transfected with the CD69 reporter construct (pAIM-255-LUC) and the indicated mutant plasmids or empty vector (pCMV4) and then infected with *H. pylori* ATCC 49503 for 6 h. Open bar: Luciferase (LUC) activity of the CD69 reporter construct and pCMV4 without *H. pylori* infection. All values were calculated as the change (n-fold) in induction values relative to the basal level measured in uninfected cells. Data are mean ± SD of three independent experiments. B: Bay 11-7082 and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) inhibit CD69 mRNA expression induced by *H. pylori*. Jurkat cells were pretreated with Bay 11-7082 (20 μmol/L) or LLnL (20 μmol/L) for 2 h prior to *H. pylori* infection and subsequently infected with *H. pylori* ATCC 49503 for 30 or 120 min. CD69 mRNA expression on harvested cells was analyzed by reverse transcription-polymerase chain reaction. Lane M: Markers.

DISCUSSION

Early studies showed that CD69 regulates the immune response by modulating the expression of various cytokines; CD69-deficient mice show increased anti-tumor and autoimmune responses caused at least in part by increased production of proinflammatory cytokines and chemokines[29,30]. Although the functions of CD69 have been studied extensively, there is little or no information on its role in the immune response against microbial pathogens. Recently, CD69 was reported to be a critical negative regulator of immune activation during intracellular bacterial infection, protecting infected mice against lethal tissue damage[31]. The present study explores the way in which *H. pylori* infection controls the expression of CD69 gene in T cells.

The main findings of the study were: (1) *H. pylori* deregulated the expression of CD69 in T cells; (2) CD69 protein was upregulated in gastric lymphocytes of patients with *H. pylori* gastritis; (3) the importance of *H. pylori cag*PAI in the induction of CD69 expression in T cells; and (4) *H. pylori* stimulates the NF-κB signaling pathway to activate CD69 gene expression and also to activate the CD69 promoter.

This is the first report to demonstrate that CD69 gene expression is regulated by *H. pylori*. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated, and colonization generally is persistent. Factors that contribute to the failure of the immune response to clear the organism remain elusive[31]. Recent studies have suggested that CD69 may downregulate the immune response through the production of the pleiotropic cytokine, transforming growth factor-β[32]. Thus, CD69 expressed on T cells may regulate the immune responses against *H. pylori* infection.

It has been reported that the inducible expression of CD69 gene is tightly regulated by transcription factors of the NF-κB, AP-1, EGR, and ATF/CREB families, which are rapidly activated through different signaling pathways[14,33]. However, nothing is known about the regulation of CD69 expression in T cells infected with *H. pylori*. We demonstrate herewith that *cag*PAI-positive *H. pylori* can induce the expression of the CD69 antigen and that this induction is mediated by an increase in the CD69 promoter activity. Deletion of the sequences that contain the κB1 and/or κB2 motifs abolished the response to *H. pylori*. Pharmacologic inhibition of NF-κB, as well as IκBα-, IκBβ-, IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants, determined the role of NF-κB signaling molecules targeted by *H. pylori* to activate CD69 gene expression. Thus, our results suggest that NF-κB is essential for *H. pylori cag*PAI-mediated CD69 induction in T cells, and the two NF-κB sites (κB1 and κB2) appear to play an important role in this process.

Our results also demonstrated that the two NF-κB motifs of the CD69 promoter bind *H. pylori*-inducible NF-κB-related complexes. Antibodies directed against the different NF-κB proteins were used to identify the family members present in the DNA-protein complexes detected with the NF-κB motif-derived probes. These experiments demonstrated that the DNA-binding activities consisted of p50/p65/c-Rel and p50/p65 complexes binding to the κB1 and κB2 motifs, respectively. Although NF-κB clearly plays an important role in *H. pylori*-mediated induction of CD69, the role of CD69 in the control of immune responses against *H. pylori* infection needs to be further clarified. We are planning further studies using CD69-deficient mice to investigate the role of CD69 in the regulation of immune responses against *H. pylori* infection.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) is regarded as the major cause of various gastric diseases. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated. Factors that contribute to the failure of the immune response to clear the organism remain elusive. Recently, the leukocyte receptor CD69 was reported to be a critical negative regulator of immune activation during bacterial infection.

Research frontiers

CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses. However, whether and how *H. pylori* can induce the expression of CD69 has not been addressed in T cells. In this study, the authors demonstrate that *H. pylori* can induce CD69 expression through the activation of nuclear factor-κB and show that *cag* pathogenicity island (*cag*PAI) might be relevant in the induction of CD69 expression in T cells.

Innovations and breakthroughs

CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. *H. pylori* also induced CD69 expression in peripheral blood mononuclear cells and $CD4^+$ T cells *in vitro*. This is the first study to report the regulation of intracellular events leading to CD69 expression by *H. pylori* infection in T cells. The results also demonstrate that the two nuclear factor-κB motifs of the CD69 promoter are important in *H. pylori*-mediated induction of CD69.

Applications

By understanding how CD69 is induced and the role of CD69 in the control of immune responses against *H. pylori* infection, and by blocking its expression, this study may indicate a future strategy for elimination of *H. pylori*.

Terminology

CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after lymphocyte activation. The *cag*PAI is responsible for the secretion of the CagA effector through a type Ⅳ secretion system apparatus as well as transport of peptidoglycan.

Peer review

This article by Mori *et al* described induction of CD69 expression by *cag*PAI-positive *H. pylori* infection. According to my literature review, this might be the first study reporting to demonstrate that CD69 gene expression is regulated by *H. pylori*. This manuscript is scientific and well-written.

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ORIGINAL ARTICLE

RNAi knockdown of PIK3CA preferentially inhibits invasion of mutant PIK3CA cells

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Abstract

AIM: To explore the effects of siRNA silencing of PIK3CA on proliferation, migration and invasion of gastric cancer cells and to investigate the underlying mechanisms.

METHODS: The mutation of PIK3CA in exons 9 and 20 of gastric cancer cell lines HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45 was screened by polymerase chain reaction (PCR) followed by sequencing. BGC-823 cells harboring no mutations in either of the exons, and HGC-27 cells containing PIK3CA mutations were employed in the current study. siRNA targeting PIK3CA was chemically synthesized and was transfected into these two cell lines *in vitro*. mRNA and protein expression of PIK3CA were detected by real-time PCR and Western blotting, respectively. We also measured phosphorylation of a serine/threonine protein kinase (Akt) using Western blotting. The proliferation, migration and invasion of these cells were examined separately by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound healing and Transwell chambers assay.

RESULTS: The siRNA directed against PIK3CA effectively led to inhibition of both endogenous mRNA and protein expression of PIK3CA, and thus significantly down-regulated phosphorylation of Akt ($P < 0.05$). Furthermore, simultaneous silencing of PIK3CA resulted in an obvious reduction in tumor cell proliferation activity, migration and invasion potential ($P < 0.01$). Intriguing, mutant HGC-27 cells exhibited stronger invasion ability than that shown by wild-type BGC-823 cells. Knockdown of PIK3CA in mutant HGC-27 cells contributed to a reduction in cell invasion to a greater extent than in non-mutant BGC-823 cells.

CONCLUSION: siRNA mediated targeting of PIK3CA may specifically knockdown the expression of PIK3CA in gastric cancer cells, providing a potential implication for therapy of gastric cancer.

Key words: Gastric cancer; Metastasis; PIK3CA; PI3K/Akt pathway; RNAi

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INTRODUCTION

Gastric cancer is one of the most frequent cancers and is the second leading cause of cancer-related death worldwide[1-2]. Although diagnostic and surgical techniques as well as combined chemotherapy and radiotherapy for the treatment of gastric cancer have advanced in recent years, the overall 5-year survival rate is still less than 20%. Thus, it is necessary to further explore and investigate the tumorigenesis of gastric cancer and its novel therapy targets.

RNA interference (RNAi) refers to the inhibition of gene expression by small double-stranded RNA (dsRNA) molecules targeting specific mRNAs for degradation[3]. The discovery of RNAi has revolutionized our understanding of gene regulation, led to the development of new strategies for blocking gene function, and may yield RNA-based drugs to treat human disease[4]. To date, a great number of studies have demonstrated that RNAi-mediated gene silencing has promising therapeutic potential for cancer therapy[5].

PIK3CA encodes the key enzymatic subunit p110α of phosphatidylinositol 3-kinase (PI3K) and is located at 3q26.3[6]. Few studies have addressed PIK3CA expression in malignancies, although its mutation has been found in many human solid cancers, including breast, gastric and pituitary cancer[7-9] and it plays an essential role as an oncogene in tumor development and progression. Our previous studies have demonstrated that increased expression of PIK3CA in the cytoplasm of gastric cancer tissues was likely associated with lymph node metastasis[10]. However, the relationship between down-regulation of PIK3CA and proliferation as well as metastatic ability or invasion of gastric cancer cells and the mechanism underlying any such relationship remains largely unknown.

In this study, we investigated the effects of down-regulation of PIK3CA by small interfering RNA (siRNA) on proliferation, migration and invasion of two gastric cancer cell lines (BGC-823 and HGC-27) as well as p-Akt expression, with the aim of evaluating whether the expression of PIK3CA may be linked to tumor progression, and explored the underlying mechanisms.

MATERIALS AND METHODS

Detection of PIK3CA mutation in gastric cancer cell lines

Detection of PIK3CA mutation in exons 9 and 20 was performed in 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45), covering the majority of hot spots of PIK3CA gene mutation. The polymerase chain reaction (PCR) amplification primers were designed according to the study published by Lin *et al*[9]. PCR products were electrophoresed on 1.5% agarose gels to ensure their integrity before purification and DNA sequencing on an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, United States) by Biosune Ltd (Shanghai, China).

siRNA synthesis and transfection

The siRNAs were designed and synthesized by GenePharma (Shanghai, China). PIK3CA siRNA: sense 5'-GGC UAA AGA AAG CCU UUA UTT-3', antisense 5'-AUA AAG GCU UUC UUU AGC CTT-3'; Negative control siRNA: sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'. All sequences were submitted to National Institutes of Health Blast program to ensure gene specificity. Human gastric carcinoma BGC-823 cells were conventionally cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., Carlsbad, CA, United States) supplemented with 2 mmol/L of L-Glutamine and 10% FBS at 5% CO_2 and 37 ℃. The cells were divided into 3 groups: control group (containing only transfection reagent), negative control group (transfected with negative control siRNA) and experimental group (transfected with PIK3CA-siRNA). When cells reached 80-90% confluency, siRNA transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Total RNAs and proteins were prepared from samples collected before transfection and at 24 h and 48 h post transfection and used for real-time quantitative PCR or Western blotting analysis.

Real time quantitative PCR

Transcript abundance of PIK3CA and β-actin (internal control) was relatively quantified by quantitative real time PCR (qRT-PCR) on total RNA isolated from the three cell groups. Briefly, 1 μg of total RNA was reverse transcribed in a 25 μL reaction volume using oligo dT (15) primers and M-MLV reverse transcriptase (Promega'Madison, WI, United States). The PCR amplifications and fluorescence detections were carried out using the ABI Prism 7500 Sequence Detection System following the manufacturer's instructions. For each sample, a relative quantity was calculated using the $2^{-\Delta\Delta C(T)}$ method[11]. Nucleotide sequences of specific primers for the selected genes were as follows: PIK3CA forward primer (5'-TGCTAAAGAGGAACACTGTCCA-3'), reverse primer: (5'-GGTACTGGCCAAAGATTCAAAG-3'); β-actin forward primer (5'-CTGAGCAGATCATGAAGAC-3'), reverse primer (5'-CTTGGTGGACGCATCCTGAG-3').

Western blotting

Cell lysates were prepared in a buffer containing 0.5 mmol/L Tris•HCl (pH 7.0), 0.1% beta-mercaptoethanol, 0.5 mmol/L ethylenediaminetetraacetic acid (pH 7.0), 0.5 mmol/L ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (pH 7.0), 2 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 2.5 mg/mL

Aprotinin, 1 mmol/L dithiothreitol and 0.5% Triton X-100. After protein quantitation using the Branford assay, 30 μg of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ, United States). The membranes were blocked using phosphate buffered saline (PBS) (pH 7.4) containing 5% nonfat milk for 1 h, probed with primary antibody (anti-PIK3CA) (Cell Signaling Technology, Beverly, MA, United States) overnight at 4 ℃. The membrane was then washed with PBST (PBS + 0.1% Tween-20) and incubated with a peroxidase-conjugated secondary antibody (goat anti-mouse IgG, Santa Cruz Biotechnology, Santa Cruz, CA, United States) for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent from BestBio (Shanghai, China). The membrane was stripped and reprobed with anti-phosphorylated Akt (Ser473), anti-Akt and anti-β-actin antibodies (Cell Signaling Technology).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The proliferation of BGC-823 and HGC-27 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measures the dehydrogenase enzyme activity in metabolically active tumor cells, as reflected by the conversion of MTT to formazan, whose absorbance can be quantified by measuring at the wavelength of 570 nm. The production of formazan is proportional to the number of living cells, with the intensity of the produced color serving as an indicator of cell viability. Briefly, approximately 5×10^3 cells/well from the three groups were respectively seeded in a 96-well microtiter plate, each group had six parallel wells. At 24, 36 and 48 h post-transfection, 20 μL of MTT (Sigma Chemical Co, MO, United States) (5 g/L) labeling reagent was added to the designated wells and cells were incubated at 37 ℃ for 4 h and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and 150 μL dimethyl sulfoxide (DMSO) (Sigma) was then added to each well. After shaking the plate for 15 min, the absorbance (A) at 570 nm was measured using Wellscan MK3 Automatic Microplate Reader. The blank control wells with medium only were set as zero absorbance. All experiments were performed at least three times.

Cell wound healing assay

To measure cell motility, 4×10^5 cells were seeded in 6-well plates. A central linear wound was created by scraping the cell monolayer with a 200 μL sterile pipette tip. The media were carefully changed to remove any floating cells and cultured at 5% CO_2 and 37 ℃. Migration of cells into the denuded areas in the scraped region was calculated at 24 h and 48 h, respectively. The wound at 0 h was considered 100% of the average gap.

Cell invasion assay

Cell invasion was assessed using Transwell chambers (Corning, NY, United States) with 50 μL sera-free DMEM containing 1 μg/μL Matrigel (BD, NJ, United States) in the upper chamber. Cells (4×10^4) were suspended with 200 μL DMEM without fetal bovine serum and placed onto the Matrigel. The lower chamber was filled with DMEM 500 μL containing 0.1 μg/μL fibronectin (Sigma). After 24 h incubation at 5% CO_2 and 37 ℃, the number of cells with Hematoxylin and Eosin (H and E) staining on the undersurface of the polycarbonate membranes (pore size 8 mm) was scored visually in 8 random fields using a light microscope.

Table 1 PIK3CA mutation in gastric cancer cell lines

Cell line	Nucleotide substitution	Amino acid change	Exon	Domain
HGC-27	G1633A	E545K	9	Helical
MKN-45	A3140G	H1047R	20	Kinase

Detection of PIK3CA mutation in exons 9 and 20 was screened by PCR followed by sequencing in 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45). Only two cell lines carried mutations of PIK3CA. HGC-27 cells contained the G1633A (E545K) mutation in exon 9 and MKN-45 contained the A3140G (H1047R) mutation in exon 20.

Statistical analysis

Data were analyzed by GraphPad Prism 4.0 and SigmaPlot 8.0 software. The results were expressed as mean ± SD. Two or multiple comparisons were performed with Student's *t*-test or a one-way analysis of variance (ANOVA), respectively. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Mutation analysis of PIK3CA

Among the 5 gastric cancer cell lines (HGC-27, SGC-7901, BGC-823, MGC-803 and MKN-45) analyzed, PIK3CA mutations in exon 9 or 20 were found in 2 of the 5 (40%) cell lines. HGC-27 cells harbored the G1633A (E545K) mutation in exon 9 and MKN-45 harbored the A3140G (H1047R) mutation in exon 20 (Table 1), which were consistent with a previous study in gastric cancer tissues[8].

To gain insight into the outcome through functional knockdown of PIK3CA, two gastric cancer cell lines (BGC-823 and HGC-27) harboring non-mutant and mutant PIK3CA, respectively, were selected to fulfill this task. The selection was based on the fact that in these three gastric cancer cell lines, the higher the expression of PIK3CA both at the mRNA and protein level, the more invasive the cells are[12].

RNAi decreases PIK3CA and p-Akt expression

We analyzed the expression of PIK3CA mRNA in BGC-823 cells transfected with siRNA by qRT-PCR. Before transfection, PIK3CA mRNA was abundantly expressed among the three groups of cells, with no statistical significance between them ($P < 0.05$) (data not shown). However, PIK3CA mRNA expression was

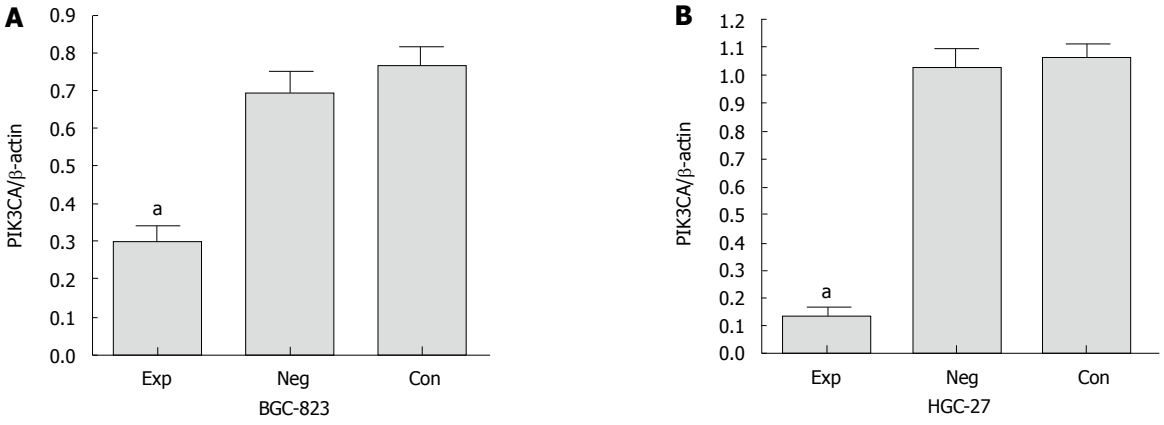

Figure 1 Expression analyses of PIK3CA mRNA determined by quantitative real time polymerase chain reaction at 24 h post-transfection. Values are shown as mean ± SD. Exp: Experimental group; Neg: Negative control group; Con: Control group. [a]$P < 0.05$ *vs* controls, $n = 3$.

markedly decreased by about 70% in the experimental group compared with the two control groups (Figure 1). Similarly, densitometric analysis showed that PIK3CA protein expression in the experimental group was about 2.5- and 2-fold lower than those in the control group and negative control group, respectively ($P < 0.05$), while no statistical difference in PIK3CA protein expression was found between these two control groups ($P > 0.05$) (Figure 2A and B). Interestingly, the level of p-Akt protein in the experimental group was also dramatically down-regulated compared with the two control groups as shown in Figure 2A and C. Additionally, the levels of PIK3CA mRNA and protein were dramatically reduced by about 85% and 80% in the experimental HGC-27 cells in comparison with the two control groups (Figure 1, Figure 2A and B). A similar difference was also observed when the HGC-27 cells were assessed for protein expression of p-Akt (Figure 2A and C). Interestingly, no significantly statistical difference in PIK3CA protein levels was found in experimental wild-type BGC-823 and mutant HGC-27 cells (Figure 2B). These data indicated that siRNA silencing of PIK3CA led to obvious inhibition of mRNA and protein expression of PIK3CA in these two gastric cell lines, and decreased activation of Akt was probably due to constitutive inactivation of PIK3CA rather than changes in its protein levels.

Effects of PIK3CA down-regulation on cell proliferation, migration and invasion

Because PIK3CA-siRNA was able to impair activation of the PI3K/Akt pathway, it was of significant interest and a priority to assess whether this siRNA had a functional effect on the biological properties of gastric cancer cells. To address this, we performed a MTT assay on gastric cancer cells transfected with siRNA against PIK3CA. As shown in Figure 3, transfection with PIK3CA-siRNA significantly decreased the proliferation of BGC-823 cells as compared with the controls ($P < 0.01$), while the proliferation of BGC-823 cells between the control group and the negative control group showed no statistical significance ($P > 0.05$), implying that knockdown of PIK3CA had an obvious inhibitory impact on the proliferation of BGC-823 cells. To examine the effect of PIK3CA-siRNA on cell motility, an *in vitro* wound-healing assay was performed. The results showed that the cells transfected with PIK3CA-siRNA had a reduced migration rate compared with the control groups at 24 h ($P < 0.05$) and 48 h ($P < 0.01$) (Figure 4). To further investigate the effect of PIK3CA-siRNA on cell invasion, we determined the invasion ability of the three groups of cells using the Transwell chambers assay. After incubation for 24 h, the number of control group and negative control group cells which had invaded the polycarbonate membrane of the Matrigel chamber was approximately 3.3- and 2.8-fold greater than that of the experimental group, respectively [(23.35 ± 1.37) and (20.24 ± 1.16) *vs* (6.98 ± 0.56)]($P < 0.01$) (Figure 5). The results of this experiment support the suggestion that PIK3CA-siRNA reduces invasion ability of gastric cancer BGC-823 cells. As expected, silencing of PIK3CA in mutant HGC-27 cells led to reduced cell proliferation and invasion to a greater extent than that in non-mutant BGC-823 cells (Figures 3-5), implying that PIK3CA knockdown may preferentially inhibit proliferation and invasion of mutant PIK3CA cells.

DISCUSSION

It is currently thought that gastric cancer develops through a complex process, such as the activation of oncogenes and/or the inactivation of tumor suppressor genes[13]. However, the critical underlying molecular mechanism of its progression is largely unclear. In recent years, many researchers have focused on signaling pathway deregulation in cancers. Among them, dysregulation of the PI3K/Akt pathway in a wide spectrum of human cancers has become a research hotspot.

The PI3Ks are heterodimers consisting of p110 catalytic and p85 regulatory subunits and have been linked to an extraordinarily diverse group of cellular functions, including differentiation, cell adhesion, apoptosis and tumor invasion[14]. Many of these functions relate to the

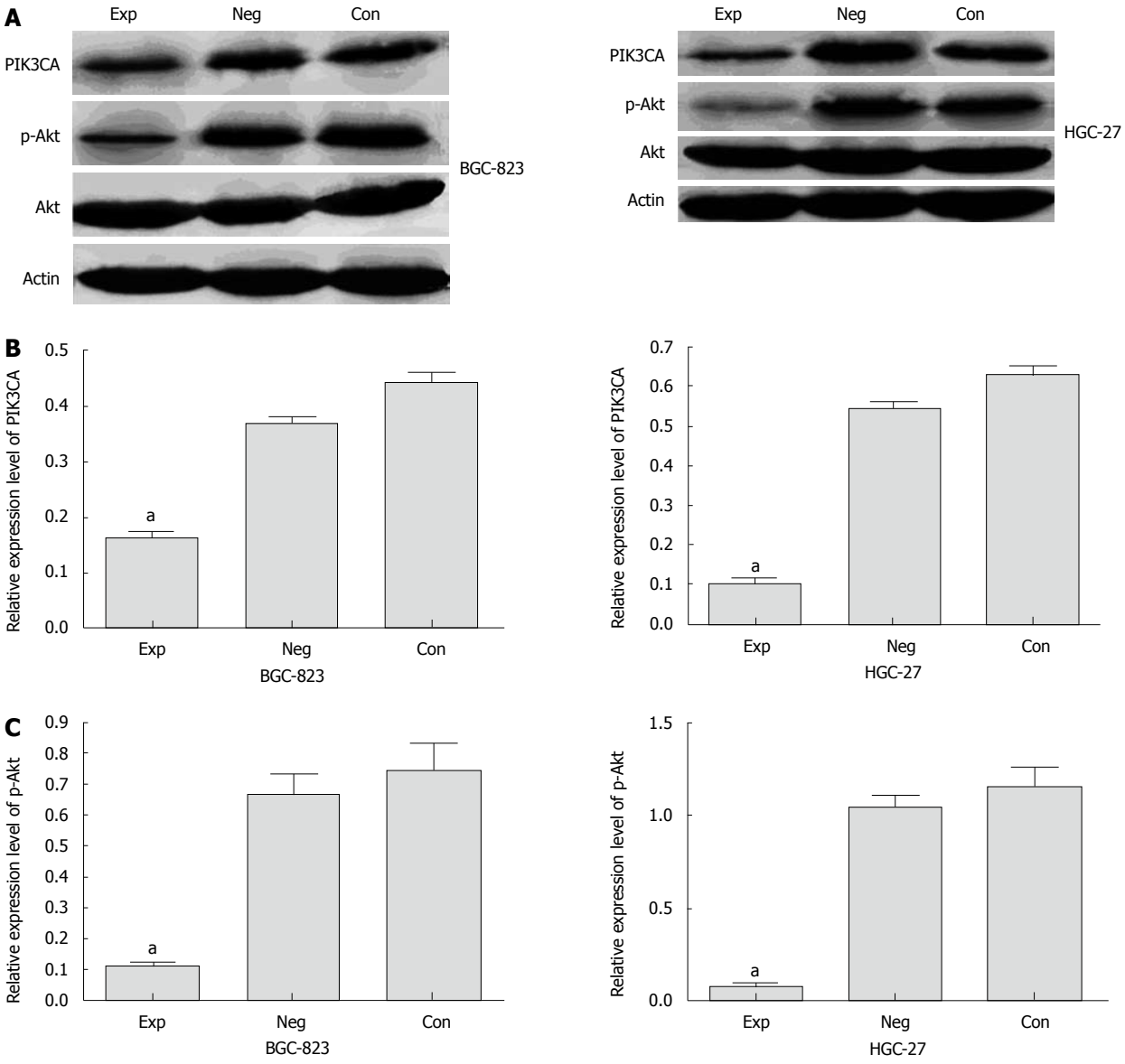

Figure 2 Protein expression of PIK3CA and p-Akt in gastric cancer cells. A: Expression analyses of PIK3CA and p-Akt protein in cells by Western blotting assay. The representative data are shown in triplicate experiments. β-actin: internal control protein; B and C: Statistical evaluation of relative expression of PIK3CA and p-Akt protein quantified by grey analyses with SigmaPlot 8.0 software. Exp: Experimental group; Neg: Negative control group; Con: Control group. [a]$P < 0.05$ *vs* controls, $n = 3$.

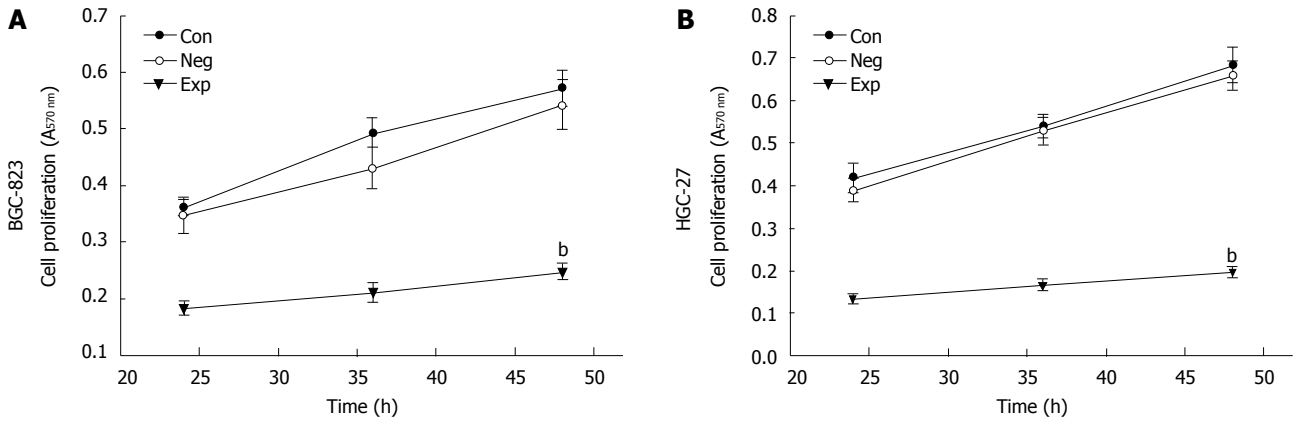

Figure 3 Cell proliferation assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values are mean ± SD. Con: Control group; Neg: Negative control group; Exp: Experimental group. [b]$P < 0.01$ *vs* controls, $n = 3$.

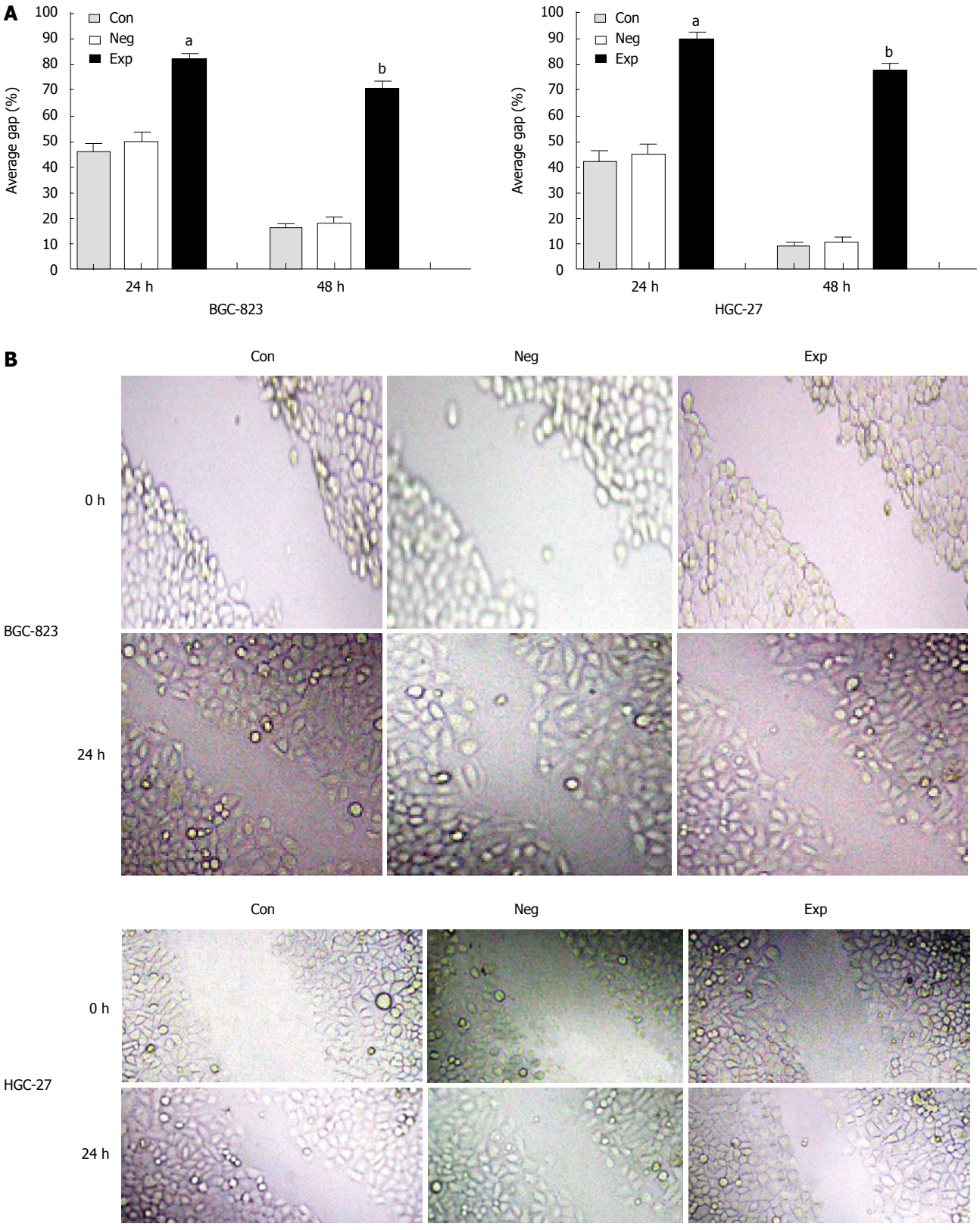

Figure 4 PIK3CA-siRNA reduced migration of BGC-823 and HGC-27 cells using an *in vitro* wound healing assay. A: The results were expressed as average gap and compared with the two control groups. The distance of the wound was measured at five reference points along the scratch wound. [a]$P < 0.05$ *vs* controls, [b]$P < 0.01$ *vs* controls, $n = 3$; B: A representative result of cell migration at 0 h and 24 h (original magnification, × 150). Con: Control group; Neg: Negative control group; Exp: Experimental group.

ability of PI3K to activate its key downstream effector Akt[15,16]. Many studies have shown that Akt activity is detectable in a variety of tumors[17-19], including gastric cancer shown by our group[10]. Elevated phosphorylated

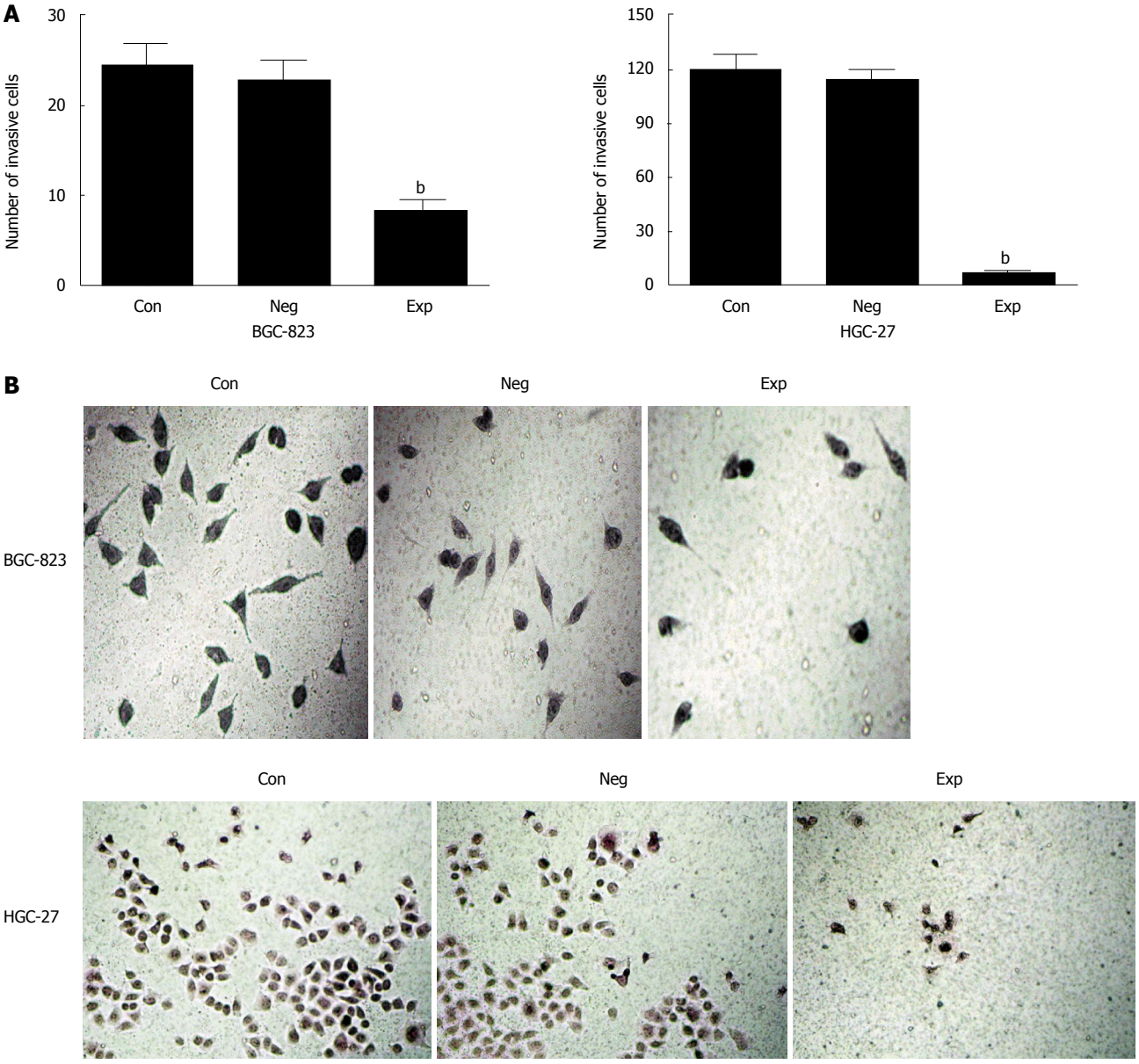

Figure 5 Effect of PIK3CA silencing on tumor cell invasion. A: PIK3CA knockdown reduces invasion ability of gastric cancer cells; B: A representative result of cell invasiveness among the three groups of cells (original magnification of BGC-823 and HGC-27 cells, × 200, × 150, respectively). Con: Control group; Neg: Negative control group; Exp: Experimental group. $^{b}P < 0.01$ *vs* controls, $n = 3$.

Akt (p-Akt), the activated form, has been demonstrated in multiple malignancies[20] and is often functionally linked to tumor progression, such as in thyroid cancer[21], and metastasis, such as in gastric cancer[10]. As reported by Grille *et al*[16], Akt activation in cancer cells increased the motility required for tissue invasion and metastases and was consequently associated with poor prognosis in many cancers. Our previous study[12] also demonstrated that different gastric cancer cell lines (HGC-27, BGC-823 and SGC-7901) varied in their invasiveness which was associated with their expression level of PIK3CA.

In the present study, our results revealed that both PIK3CA mRNA and protein were markedly inhibited in two cell lines transfected with PIK3CA-siRNA, which is consistent with many studies showing that the introduction of a 21 nt dsRNA into cancer cells strongly suppressed the expression of specific mRNAs[22,23]. Furthermore, RNAi-directed targeting of PIK3CA in these cells could reduce the capability of cell proliferation, migration and invasion. More importantly, a low level of p-Akt in the experimental group was detected compared with the two control groups. The above evidence indicates that a robust knockdown of PIK3CA by siRNA may result in decreased catalytic activity of PI3K, subsequent de-phosphorylation of the downstream effector Akt, and thus low activity or aberrant inactivation of the PI3K/Akt pathway in these cells. Our data are in agreement with previous observations that PI3-kinase activity is solely caused by gene-dependent expression of the catalytic subunit p110α (PIK3CA)[24,25].

Interestingly, the Transwell chambers assay showed that PIK3CA mutant HGC-27 cells had an approximate-

ly 5-fold increased ability to invade the Matrigel (Figure 5) compared to PIK3CA non-mutant BGC-823 cells, suggesting that PIK3CA mutation contributed to cell invasion, which is consistent with a previous study in which PIK3CA mutations occur late in glioma progression[26].

Taken together, siRNA targeting PIK3CA effectively inhibits the proliferation and invasion of gastric cancer cells *via* aberrant inactivation of the PI3K/Akt pathway, and would be expected to become a new strategy for the therapy of gastric cancer regardless of PIK3CA mutation. However, like all other newly developed therapeutic methods, applying RNAi *via* siRNAs to living animals, especially humans, poses many challenges such as their poor stability and different effectiveness in different cell types[27,28]. Further studies will be required to develop efficient approaches for the delivery of siRNA into target cells.

ACKNOWLEDGMENTS

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COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide, and no ideal approach is available to treat this disease. Thus, it is necessary to further explore and investigate the tumorigenesis of gastric cancer and its novel therapy targets. PIK3CA encoding the key enzymatic subunit p110α of phosphatidylinositol 3-kinase (PI3K), plays a vital role as an oncogene in tumor development and progression. Few studies have addressed PIK3CA expression in malignancies, although its mutation has been found in various human solid cancers. In addition, the relationship between PIK3CA expression and invasion of gastric cancer cells and the mechanism underlying any such relationship remains largely unknown.

Research frontiers

It is currently thought that gastric cancer develops through a complex process, such as the activation of oncogenes and/or the inactivation of tumor suppressor genes. However, the critical underlying molecular mechanism of its progression is largely unclear. In recent years, many researchers have focused on signaling pathway deregulation in cancers. Among them, dysregulation of the PI3K/Akt pathway in a wide spectrum of human cancers has become a research hotspot.

Innovations and breakthroughs

Previous studies have mainly focused on PIK3CA mutations in many human solid cancers. In the present study, the authors investigated the effects of the knockdown of PIK3CA by small interfering RNA on proliferation, migration and invasion of two gastric cancer cell lines with or without mutation of PIK3CA, with the aim of evaluating whether the expression of PIK3CA may be linked to tumor progression and may ultimately benefit from gastric cancer therapy regardless of the presence of PIK3CA mutations.

Applications

Functional knockdown of PIK3CA mediated by siRNA effectively inhibits the proliferation and invasion of gastric cancer cells with or without mutation of PIK3CA *via* aberrant inactivation of the PI3K/Akt pathway, and would be expected to become a new strategy for gastric cancer therapy. Further studies will be required to develop efficient approaches for the delivery of siRNA into target cells.

Terminology

PIK3CA gene, encoding the key catalytic subunit p110α of PI3K, is located on chromosome 3q26.3. AKT, a serine/threonine kinase, serving as the major downstream effector of PI3K, regulates many biological processes, such as proliferation, apoptosis and growth.

Peer review

The paper is well written and executed. The results are correctly described and commented.

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BRIEF ARTICLE

Advantage of autologous blood transfusion in surgery for hepatocellular carcinoma

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Abstract

AIM: To evaluate the significance of autologous blood transfusion (AT) in reducing homologous blood transfusion (HT) in surgery for hepatocellular carcinoma (HCC).

METHODS: The proportion of patients who received HT was compared between two groups determined by the time of AT introduction; period A (1991-1994, n = 93) and period B (1995-2000, n = 201). Multivariate logistic regression analysis was performed in order to identify independent significant predictors of the need for HT. We also investigated the impact of AT and HT on long-term postoperative outcome after curative surgery for HCC.

RESULTS: The proportion of patients with HT was significantly lower in period B than period A (18.9% *vs* 60.2%, $P < 0.0001$). Multivariate logistic regression analysis identified AT administration as a significant independent predictor of the need for HT ($P < 0.0001$). Disease-free survival in patients with AT was comparable to that without any transfusion. Multivariate analysis identified HT administration as an independent significant factor for poorer disease-free survival ($P = 0.0380$).

CONCLUSION: AT administration significantly decreased the need for HT. Considering the postoperative survival disadvantage of HT, AT administration could improve the long-term outcome of HCC patients.

Key words: Hepatocellular carcinoma; Surgery; Autologous blood transfusion; Homologous blood transfusion

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INTRODUCTION

Surgical resection is a safe and effective treatment for hepatocellular carcinoma (HCC). Because HCC usually develops in patients with liver cirrhosis, most of such patients present with bleeding tendencies related to

chronic liver dysfunction[1-3]. Therefore, surgery for HCC frequently requires intraoperative transfusion. Homologous blood transfusion (HT) is necessary for patients with excessive intraoperative bleeding, though this is still associated with risks of infections and/or immunological complications[4,5]. Moreover, evidence suggests that HT may be adversely associated with tumor recurrence and poor postoperative survival in various kinds of cancers[6-13]. Autologous transfusion (AT), which represents collection and reinfusion of the patient's own blood or blood components before surgery, and has been developed as a strategy to reduce the need for HT, is currently used for patients scheduled for surgery for various diseases including HCC[11,14-17]. It has been the policy in our hospital since 1995 to prepare for AT for patients scheduled for HCC surgery. To date, several investigators have examined the significance of AT in terms of reducing the need for HT and of postoperative outcome, but only a few were conducted with proper statistical analyses to identify the significance of AT[16,17].

In the present study, we reviewed the frequency of HT and AT administration in patients undergoing surgery for HCC, and statistically analyzed the significant factors that could predict the need for HT. We also compared the difference between the effects of AT and HT on long-term postoperative outcome after curative surgery for HCC.

MATERIALS AND METHODS

The present study included 294 patients with HCC who underwent hepatic resection at the Department of Surgery, Osaka University Hospital between January 1991 and December 2000. In 93 patients between 1991 and 1994 (period A), AT was not administered, and, when blood was needed, HT was administered. Between 1995 and 2000 (period B), AT was carried out preoperatively in the remaining 201 patients provided: (1) they agreed to the storage; (2) their hemoglobin (Hb) level was $\geqslant$ 11.0 g/dL before storage; and (3) they were free of severe cardiopulmonary and/or cerebrovascular diseases, or infection. Autologous blood was collected 1 to 3 times, with 200-400 mL of blood at a time. The blood was stored in a liquid state without freezing. Iron supplements were given daily to the patients who deposited the autologous blood in the post-storage period. In addition, if the total volume of the collected blood was $\geqslant$ 800 mL, recombinant human erythropoietin was administered. All through the study period, during hepatic resection, blood transfusion was carried out when the Hb level fell to < 8.0 g/dL in patients with normal cardiopulmonary function or < 9.0 g/dL in patients with severe cardiopulmonary or cerebrovascular diseases. In patients who had previously deposited autologous blood, autologous blood was first used prior to homologous blood. In this study, patients who required HT were defined as the HT group, irrespective of prior AT, and the remaining patients without HT were defined as the non-HT group. Furthermore, patients in whom only AT was performed were defined as the AT group, and patients without AT or HT were as defined as the non-transfusion group.

Hospital records were collected retrospectively to gather clinical information including clinical factors, tumor-related factors and surgery-related factors. In patients with autologous blood storage, preoperative Hb was indicated as Hb before the storage. The surgical procedure was selected based on the extent of the tumor and residual liver function. The indication for surgery and selection of surgical procedure were not different between period A and period B. The histological grade of differentiation of HCC was determined according to the Edmondson-Steiner classification, and was based on the areas of the tumor with the highest grade[18]. Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test or the Mann-Whitney *U* test. Survival rates were calculated according to the Kaplan-Meier method, and compared using the log-rank test. Multivariate logistic regression analysis was performed for the selection of significant variables. Statistical analysis was performed using StatView (version 5.0; SAS Institute Inc., Cary, NC). A P value < 0.05 was considered significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

RESULTS

Table 1 shows the clinicopathological characteristics of patients in period A (n = 93) and in period B (n = 201). The clinical features, tumor-related features, and surgery-related factors were not significantly different between patients of the 2 groups. HT was administered in 56 of the 93 patients (60.2%) in period A. In period B, HT was administered in 38 patients (18.9%) (HT group), AT in 134 patients (66.7%), and neither AT nor HT in 45 patients (22.4%) (non-transfusion group). In 134 AT patients, the amount of transfused autologous blood was 200 mL in 3 patients, 400 mL in 63 patients, 600 mL in 2 patients, 800 mL in 62 patients, 1000 mL in 1 patient, and 1200 mL in 3 patients. Among the 134 patients with AT, only AT was administered in 118 patients (58.7%) (AT group), and both AT and HT in the remaining 16 patients (8.0%). Figure 1 shows the distribution of patients according to blood transfusion. Thus, the proportion of patients who received HT was significantly lower in period B than in period A (P < 0.0001). With regard to disease-free survival examined only in patients with curative surgery for HCC, there were no significant differences between period A and period B; the 1-, 3-, 5-, and 10-year disease-free survival rates were 73.9%, 39.5%, 24.7%, and 7.2% for patients in period A, and 65.9%, 34.8%, 21.9%, and 7.2% for patients in period B (P = 0.5688), respectively. The 1-, 3-, 5- and 10-year overall survival rates were 85.7%, 75.6%, 63.1%, and 28.5% for patients in period A, and 92.9%, 70.6%,

Table 1 Clinicopathological characteristics of patients of periods A and B with hepatocellular carcinoma

	Period A (1991-1994) (*n* = 93)	Period B (1995-2000) (*n* = 201)	*P*-value
Clinical factors			
Gender (male/female)	81/12	161/40	0.144
Age (yr)[1]	61 ± 9	62 ± 9	0.102
HBs-Ag (±)	73/20	169/32	0.243
Anti-HCV Ab (±/unknown)	29/62/2	71/125/5	0.471
Child-Pugh classification (A/B)	79/14	160/41	0.275
Preoperative Hb (g/dL)[1]	13.6 ± 1.5	13.3 ± 1.6	0.213
Tumor-related factors			
Number of tumors (single/multiple)	70/23	146/55	0.635
Maximum tumor size (cm)[1]	3.8 ± 2.7	4.1 ± 3.1	0.450
Vascular invasion (±)	83/10	172/29	0.388
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	40/41/12	89/92/20	0.975
Surgery-related factors			
Procedure (nonanatomical/anatomical)	45/48	101/100	0.767
Operation time (min)[1]	291 ± 144	295 ± 151	0.853
Resected liver volume (g)[1]	218 ± 406	214 ± 289	0.925
Intraoperative blood loss (mL)[1]	2190 ± 5689	1621 ± 2209	0.219

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin.

Table 2 Clinicopathological characteristics of patients with hepatocellular carcinoma according to homologous blood transfusion

	Non-HT group (*n* = 200)	HT group (*n* = 94)	*P*-value
Clinical factors			
Gender (male/female)	162/38	80/14	0.390
Age (yr)[1]	62 ± 9	60 ± 9	0.084
HBs-Ag (±)	168/32	74/20	0.269
Anti-HCV Ab (±/unknown)	65/130/5	35/57/2	0.437
Child-Pugh classification (A/B)	167/33	72/22	0.157
Preoperative Hb (g/dL)[1]	13.5 ± 1.6	13.2 ± 1.7	0.171
AT administration (±)	82/118	78/16	< 0.0001
Tumor-related factors			
Number of tumors (single/multiple)	149/51	67/27	0.559
Maximum tumor size (cm)[1]	3.6 ± 2.4	4.9 ± 3.7	0.000
Vascular invasion (±)	177/23	78/16	0.193
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	91/88/21	38/45/11	0.446
Surgery-related factors			
Procedure (nonanatomical/anatomical)	111/89	35/59	0.004
Operation time (min)[1]	264 ± 130	356 ± 166	< 0.0001
Resected liver volume (g)[1]	159 ± 196	336 ± 490	< 0.0001
Intraoperative blood loss (mL)[1]	993 ± 707	3522 ± 6104	< 0.0001

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; AT: Autologous transfusion; HT: Homologous transfusion.

58.2%, and 40.3% for patients in period B ($P = 0.3202$).

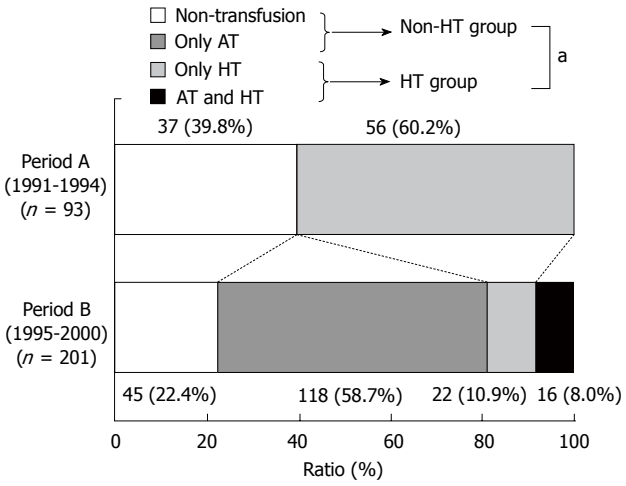

Figure 1 Distribution of patients according to transfusion status during periods A and B. The proportion of patients who received HT was significantly lower in period B than period A ([a]$P < 0.0001$). AT: Autologous transfusion; HT: Homologous transfusion.

In order to identify the factors that can predict the need for HT, various clinical parameters, tumor-related factors, and surgery-related factors were compared between the non-HT group and the HT group (Table 2). The preoperative Hb level was not significantly different between the 2 groups (13.5 ± 1.6 g/dL *vs* 13.2 ± 1.7 g/dL, $P = 0.1708$). The proportion of patients who received AT was significantly lower in the HT group than the non-HT group [59.0% (118/200) *vs* 17.0% (16/94), $P < 0.0001$]. The maximum tumor size was significantly larger in the HT group than in the non-HT group (4.9 ± 3.7 cm *vs* 3.6 ± 2.4 cm, $P = 0.0003$). As for surgery-related factors, there were significant differences in surgical procedure ($P = 0.0035$), operation time ($P < 0.0001$), resected liver volume ($P < 0.0001$), and intraoperative blood loss ($P < 0.0001$), suggesting that surgery in the HT group was major compared to that in the non-HT group.

To identify significant factors that could predict the need for HT, multivariate logistic regression analysis was performed (Table 3). The analysis was carried out using the 6 significant factors identified in the comparison of the non-HT group and the HT group. The analysis identified AT administration, intraoperative blood loss, and resected liver volume as significant independent predictors for the need of HT ($P < 0.0001$, $P < 0.0001$, $P = 0.0362$, respectively). Long-term postoperative outcome after surgery for HCC was examined. In this analysis, patients were limited to those with curative resection, which was defined as complete removal of all macroscopically evident tumors [non-HT group: 193 patients (non-transfusion group: 78 patients; AT group: 115 patients), HT group: 83 patients]. Among the 276 patients, 37 patients (13.4%) were followed-up for more than 10 years. The clinicopathological features of the groups are shown in Table 4. First, we compared the long-term postoperative outcome between the non-transfusion group and the AT group. The preoperative Hb level was significantly higher in the AT group than in the non-

Table 3 Results of multivariate logistic regression analysis for the need for homologous blood transfusion

		OR	95% CI	*P*-value
AT administration	±	28.571	9.615-83.333	< 0.0001
Maximum tumor size (cm)	< 5/≥ 5	1.126	0.500-2.538	0.774
Procedure	Nonanatomical/anatomical	1.016	0.449-2.202	0.967
Operation time (min)	< 300/≥ 300	0.986	0.435-2.242	0.974
Resected liver volume (g)	< 200/≥ 200	2.532	1.062-6.061	0.036
Intraoperative blood loss (mL)	< 2000/≥ 2000	30.303	9.346-100.000	< 0.0001

OR: Odds ratio; CI: Confidence interval; AT: Autologous transfusion.

Table 4 Clinicopathological characteristics of patients who underwent curative surgery for hepatocellular carcinoma

	Non-HT group (*n* = 193)	HT group (*n* = 83)	*P*-value (Non-HT *vs* HT)	Non-HT group		
				Non-transfusion group (*n* = 78)	AT group (*n* = 115)	*P*-value (Non-transfusion *vs* AT)
Clinical factors						
Gender (male/female)	156/37	70/13	0.488	63/15	93/22	0.986
Age (yr)[1]	62 ± 8	61 ± 9	0.115	62 ± 8	61 ± 9	0.878
HBs-Ag (±)	163/30	67/16	0.445	Nov-67	96/19	0.649
Anti-HCV Ab (±/unknown)	62/127/4	31/51/1	0.426	21/54/3	41/73/1	0.254
Child-Pugh classification (A/B)	161/32	65/18	0.262	66/12	95/20	0.833
Preoperative Hb (g/dL)[1]	13.5 ± 1.5	13.4 ± 1.6	0.425	12.8 ± 1.8	14.1 ± 1.1	< 0.0001
Tumor-related factors						
Number of tumors (single/multiple)	147/46	64/19	0.866	62/16	85/30	0.372
Maximum tumor size (cm)[1]	3.5 ± 2.4	4.8 ± 3.7	0.000	3.3 ± 2.2	3.6 ± 2.4	0.287
Vascular invasion (±)	172/21	70/13	0.268	73/5	99/16	0.101
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ/unknown)	89/83/21	33/41/9	0.304	36/34/8	53/49/13	0.412

[1]Data are expressed as number of patients and mean ± standard deviation. HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; AT: Autologous transfusion; HT: Homologous transfusion.

transfusion group (14.1 ± 1.1 g/dL *vs* 12.8 ± 1.8 g/dL, $P < 0.0001$). Tumor-related factors were similar in the 2 groups. There were no significant differences in the disease-free survival rates between the AT group (1-, 3-, 5-, and 10-year: 70.6%, 37.1%, 22.3%, and 11.2%, respectively) and the non-transfusion group (73.1%, 41.3%, 30.7%, and 9.6%, respectively) ($P = 0.3874$) (Figure 2A). Next, we compared the long-term survival rates of the non-HT group and the HT group. Although the cumulative disease-free survival rate of the non-HT group was significantly better than that of the HT group ($P = 0.0305$) (Figure 2B), since the maximum tumor size was significantly different in the comparison (3.5 ± 2.4 cm *vs* 4.8 ± 3.7 cm, $P = 0.0004$), additional comparison was also performed based on the tumor size. The disease-free survival rates for the non-HT group (1-, 3-, 5-, and 10-year: 75.6%, 42.6%, 29.4%, and 10.8%, respectively) was significantly better than those of the HT group (69.0%, 31.6%, 16.7%, and 4.5%, respectively) of the subgroup with tumor size 5.0 cm or smaller than 5.0 cm ($P = 0.0452$) (Figure 2C), but not in patients with tumor size larger than 5.0 cm (1-, 3-, 5-, and 10-year: 56.4%, 24.1%, 10.8%, and 5.4% in the non-HT group, and 39.4%, 26.3%, 13.1%, and 0.0% in the HT group, respectively, $P = 0.7391$) (Figure 2D). Furthermore, multivariate analyses using significant factors identified in the univariate analyses demonstrated that transfusion status (non-HT/HT) was one of the independent significant factors for disease-free survival ($P = 0.0380$) (Table 5), suggesting disadvantages of HT on postoperative prognosis.

DISCUSSION

The results of the present study demonstrated a reduction in HT administration in surgery for HCC after the introduction of AT. Our results are in agreement with those of previous reports which emphasized the significance of AT in reducing the need for HT in surgery for HCC[16,17]. However, in these previous reports, only 20-30 patients were included in the AT group. Furthermore, although the Hb level immediately before surgery was reported in the AT group, the Hb level before storage was not indicated, suggesting a different clinical background of patients who received HT and those of other groups. On the other hand, in the present study, despite its retrospective design, the clinicopathological background, including the Hb level, was similar in the 2 groups as shown in Table 1. In this regard, the present study is significant as it identified the benefits of AT in the reduction of HT administration.

In the present study, we analyzed the data for significant predictors of HT use. The results showed that AT administration was an independent significant predictor of the need for HT, and support the significance of AT in reducing the need for HT. In the analysis, preoperative Hb, which is reported to be significantly associated with the need for HT[19,20], was not an independent significant factor. While the reason for this difference in the results

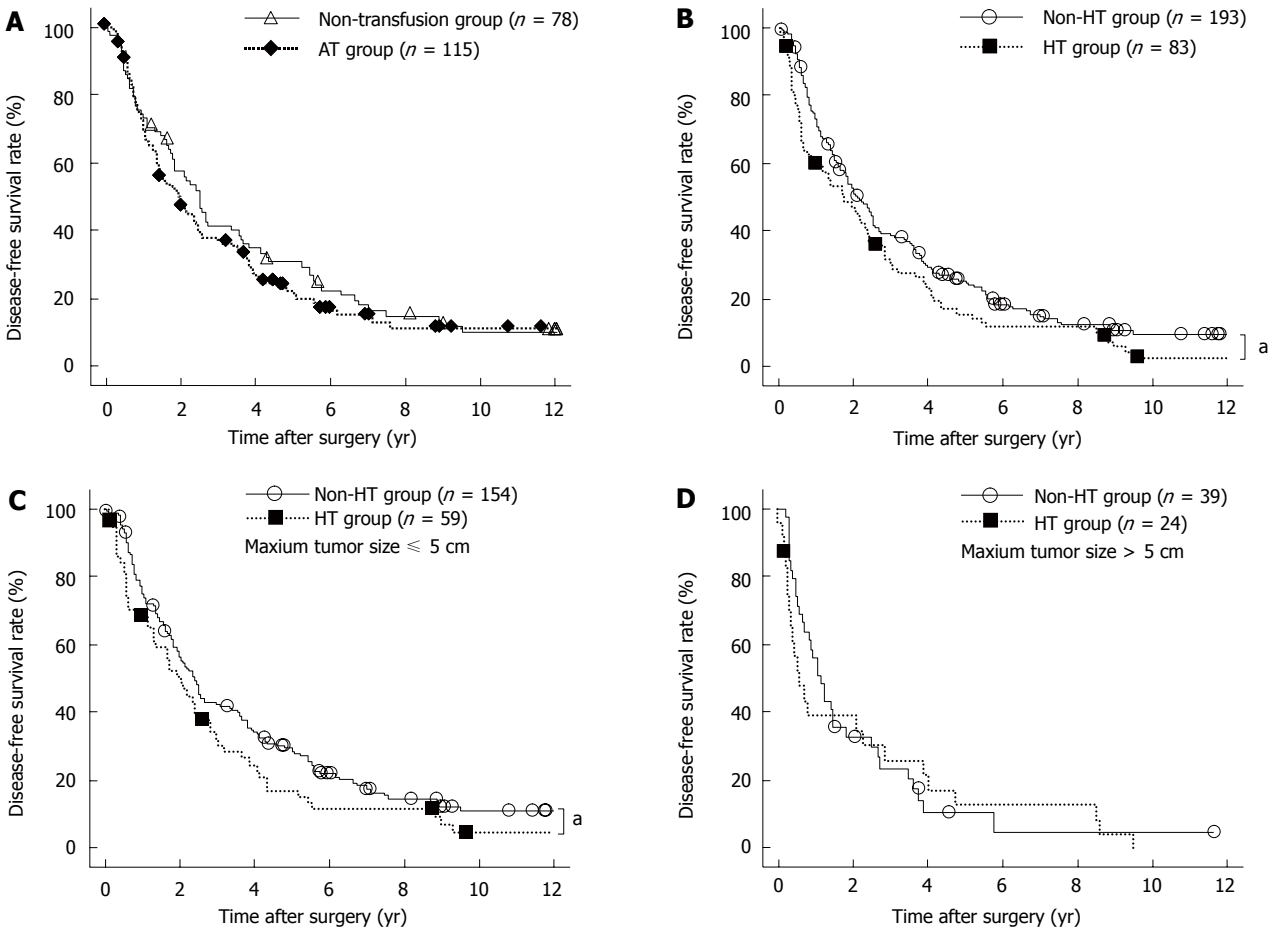

Figure 2 Disease-free survival after curative surgery for hepatocellular carcinoma. A: There were no significant differences between the non-transfusion group (solid line) and the Autologous transfusion (AT) group (dotted line) (P = 0.3874); B: The cumulative disease-free survival in the non-Homologous transfusion (HT) group (solid line) was significantly better than in the HT group (dotted line) (^aP = 0.0305); C: The disease-free survival in the non-HT group (solid line) was significantly better in than the HT group (dotted line) in patients with maximum tumor size of ≤ 5.0 cm (^aP = 0.0452); D: No significant differences were noted between the non-HT group (solid line) and the HT group (dotted line) in patients with the maximum tumor size > 5.0 cm (P = 0.7391).

Table 5 Statistical analysis of disease-free survival of patients with curative resection for hepatocellular carcinoma

	Univariate		Multivariate	
	P-value	OR	95% CI	*P*-value
Clinical factors				
Gender (male/female)	0.840			
Age (yr) (≤ 63/> 63)	0.402			
HBs-Ag (±)	0.279			
Anti-HCV Ab (±)	0.045	1.401	1.032-1.901	0.031
Child-Pugh classification (A/B)	0.079			
Preoperative Hb (g/dL) (≤ 12/> 12)	0.824			
Transfusion (non-HT group/HT group)	0.031	1.372	1.018-1.849	0.038
Tumor-related factors				
Number of tumors (single/multiple)	0.000	1.819	1.290-2.564	0.001
Maximum tumor size (cm) (≤ 5/> 5)	0.001	1.07	0.750-1.525	0.709
Vascular invasion (±)	< 0.0001	2.473	1.606-3.806	< 0.0001
Histological grade (Ⅰ, Ⅱ/Ⅲ, Ⅳ)	0.017	1.188	0.898-1.570	0.227

OR: Odds ratio; CI: Confidence interval; HBs-Ag: Hepatitis B surface antigen; Anti-HCV Ab: Anti-hepatic C virus antibody; Hb: Hemoglobin; HT: Homologous transfusion.

remains unclear, it could be due to the effect of recombinant human erythropoietin administered after the storage of autologous blood. Alternatively, it is possible that, since the subjects of the above previous studies did not receive AT, the significance of preoperative Hb is overestimated. Thus, the present study is significant in terms of identifying the effect of AT in reducing HT using appropriate statistical analysis.

We also investigated the effects of AT and HT on postoperative outcome after curative surgery for HCC. The study revealed that the disease-free survival rates were comparable between the non-transfusion group and the AT group when the clinical background was similar. Furthermore, the disease-free survival rates of the HT group were significantly worse than those of the non-HT group, based on the results of univariate analysis. Since there was a significant difference in the maximum tumor size between the 2 groups, which suggests the possibility of different tumor biology and recurrences between the HT group and the non-HT group, the survival rate was compared in subgroups based on tumor size, and showed significant differences in the pa-

tients with tumor size ≤ 5.0 cm. In addition, the difference was confirmed to be independently significant by multivariate analyses.

Since the report of a survival advantage of HT in patients undergoing colectomy for colon cancer[21], some investigators have indicated that HT triggers recurrence in various kinds of cancers[6-8]. This HT-induced disadvantage is speculated to be derived from transfusion-associated immunomodulation. Actually, several investigators suggested that HT induces downregulation of natural killer cell activity and cytotoxic T-cell function, resulting in a subclinical state of anergy or tolerance[22-24].

The correlation has been reported also in patients with HCC[9-14,25]. Although the results of the present study were comparable to these previous reports, we think that the present study reports a new finding based on the inclusion of patients who were followed-up for more than 10 years. With regard to the long-term survival advantages, to our knowledge, there are only a few reports describing the survival advantage of HT on long-term prognosis (> 10 years). Hirano *et al*[14] investigated the long-term (> 10 years) survival disadvantage of HT over AT, but their reports did not include the clinical background of patients and described the results of only univariate analysis, suggesting inadequate analysis. Also in this regard, the present study provides significant data.

Thus, the present study revealed that AT is significant in reducing the need for HT, which is associated with a long-term postoperative survival disadvantage after HCC surgery. In this study, however, in order to investigate the long-term postoperative outcome for more than 10 years, we limited inclusion in the study to patients who underwent surgery between 1991 and 2000. Based on this limitation, it is possible that the selected time period does not reflect recent advances in both surgical and anesthetic techniques, which could explain the recent decrease in intraoperative blood loss. Considering such recent advances affecting intraoperative blood loss, one can speculate that there are increasingly more patients with HCC who do not need AT. It was also reported recently that the practice of using autologous blood requires more administrative work and laborious collection procedures, and is not without disadvantages[26-29]. Taken together, AT actually has advantage an over HT, but currently, it may be necessary to deliberate on the need for AT itself during surgery for HCC.

In summary, the present study showed that AT administration significantly decreased the need for HT in surgery for HCC, and that AT was one of the significant independent predictor of the need for HT. Considering that HT was disadvantageous with regard to long-term postoperative survival, one can assume that AT administration can lead to improvement in the long-term postoperative outcome of patients with HCC.

COMMENTS

Background

Some evidences suggest that homologous blood transfusion (HT) may be adversely associated with tumor recurrence and poor survival in various kinds of cancers, and autologous blood transfusion (AT) is currently used for patients scheduled for surgery. To date, several investigators have examined the significance of AT in terms of reducing the need for HT and postoperative outcome, but few were conducted with proper statistical analyses to identify the significance of AT in surgery for hepatocellular carcinoma (HCC).

Research frontiers

The authors compared the proportion of patients who received HT between 2 groups determined by the time of AT introduction; period A (1991-1994, *n* = 93) and period B (1995-2000, *n* = 201), and performed multivariate logistic regression analysis for identification of independent significant predictors of the need for HT. Furthermore, they investigated the impact of AT and HT on long-term postoperative outcome after curative surgery for HCC.

Innovations and breakthroughs

The present study showed that the proportion of patients having HT was decreased after AT introduction, that AT administration was a significant independent predictor of the need for HT, and identified HT administration as an independent significant factor for poorer disease-free survival.

Applications

Considering the results of the present study, it could be suggested that AT administration could improve the long-term outcome of patients with HCC.

Peer review

This is a large series of patients treated in several ways with respect to the need for blood transfusion during their surgery for HCC. Unfortunately the authors have a mix of numbers that they have used in different ways to make the conclusion they wanted to make.

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BRIEF ARTICLE

Potential risk factors for nonalcoholic steatohepatitis related to pancreatic secretions following pancreaticoduodenectomy

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Author contributions: Choi SH designed the research study; Song SC, Choi DW, Heo JS, Kim WS, and Kim MJ conducted the research; Song SC provided analytical tools and analyzed the data; and Song SC prepared the manuscript.

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Abstract

AIM: To identify risk factors for nonalcoholic steatohepatitis following pancreaticoduodenectomy, with a focus on factors related to pancreatic secretions.

METHODS: The medical records of 228 patients who had a pancreaticoduodenectomy over a 16-mo period were reviewed retrospectively. The 193 patients who did not have fatty liver disease preoperatively were included in the final analysis. Hepatic steatosis was diagnosed using the differences between splenic and hepatic attenuation and liver-to-spleen attenuation as measured by non-enhanced computed tomography.

RESULTS: Fifteen patients (7.8%) who showed postoperative hepatic fatty changes were assigned to Group A, and the remaining patients were assigned to Group B. Patient demographics, preoperative laboratory findings (including levels of C-peptide, glucagon, insulin and glucose tolerance test results), operation types, and final pathological findings did not differ significantly between the two groups; however, the frequency of pancreatic fistula (P = 0.020) and the method of pancreatic duct stenting (P = 0.005) showed significant differences between the groups. A multivariate analysis identified pancreatic fistula (HR = 3.332, P = 0.037) and external pancreatic duct stenting (HR = 4.530, P = 0.017) as independent risk factors for the development of postoperative steatohepatitis.

CONCLUSION: Pancreatic fistula and external pancreatic duct stenting were identified as independent risk factors for the development of steatohepatitis following pancreaticoduodenectomy.

Key words: Nonalcoholic fatty liver diseases; Nonalcoholic steatohepatitis; Pancreatic duct stenting; Pancreatic fistula; Pancreatic surgery

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Song SC, Choi SH, Choi DW, Heo JS, Kim WS, Kim MJ. Potential risk factors for nonalcoholic steatohepatitis related to pancreatic secretions following pancreaticoduodenectomy. *World J Gastroenterol* 2011; 17(32): 3716-3723 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i32/3716.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i32.3716

INTRODUCTION

Steatohepatitis refers to a spectrum of nonalcoholic fatty liver diseases (NAFLD) ranging from simple triglyceride

deposition and accumulation with or without fibrosis to the development of cirrhosis, end-stage liver failure, and even hepatocellular carcinoma[1-6]. Nonalcoholic steatohepatitis (NASH), first described by Ludwig *et al*[7] at the Mayo Clinic in 1980, refers to hepatic lobular or portal inflammation and focal necrosis with fatty changes in patients without a history of alcohol abuse[4,8-12]. Steatohepatitis is associated with lipodystrophy, metabolic syndrome (dyslipidemia, insulin resistance, and diabetes mellitus), genetic susceptibility, environmental factors, and hepatocyte apoptosis associated with mitochondrial dysfunction and the production of reactive oxygen species, which can lead to hepatic fibrogenesis and inflammation[10,13-15].

Fatty liver disease refers to either the accumulation of fat in hepatocytes in excess of 5% of the total liver weight or the fatty degeneration of more than one-third of the total number of cells in the liver[16]. In the general population, the prevalence of NAFLD is 6%-40% among asymptomatic patients, and the incidence of fatty liver disease is 60%-75% in obese patients and 84%-96% in morbidly obese patients who undergo bariatric surgery, with 25%-55% of these patients also having NASH[1,3-5,9,17].

Glucose intolerance in response to insulin resistance induces an elevation in blood levels of glucose and insulin and results in increased synthesis of hepatic free fatty acids[5]. The oxidation of free fatty acids can lead to the production of reactive oxygen free radicals that can be cytotoxic to DNA, mitochondria, and other cellular structures and can lead to the production of pro-inflammatory cytokines. Steatohepatitis reportedly develops after a "first hit" involving triglyceride accumulation and a "second hit" involving oxidative stress, lipid peroxidation, pro-inflammatory cytokines, and mitochondrial dysfunction[4,18].

Previous studies in humans or murine models have identified independent risk factors for hepatic fibrosis, including advanced age, obesity, hypertension, Type Ⅱ diabetes, insulin resistance, dyslipidemia, an aspartate transaminase (AST)/alanine transaminase (ALT) ratio greater than 1, hyperinsulinemia, altered lipid homeostasis, and pancreatic steatosis[4,7-9,17-20]. Additional risk factors that might contribute to disease progression include increased transferrin saturation, long-term total parenteral nutrition leading to choline deficiency, jejunoileal bypass surgery for morbid obesity, environmental toxins, and drugs such as chemotherapeutic agents or glucocorticoids[3,5,18].

Ductal adenocarcinoma of the pancreatic head is the most predominant tumor in the pancreas, and pancreaticoduodenectomy is the treatment of choice[11]. Pancreaticoduodenectomy is also used to treat various other malignancies of the periampullary region, the bile duct, and the duodenum or the borderline diseases of the pancreas[21]. Side-effects associated with pancreaticoduodenectomy include weight loss, abdominal pain, fatigue, and exocrine and endocrine insufficiencies. Pancreaticoduodenectomy also has a high rate of morbidity and mortality, including the postoperative development of steatohepatitis. Only a few reports have explored the relationship between pancreaticoduodenectomy and the development of steatohepatitis[22]. Therefore, we aimed to identify the risk factors for steatohepatitis after pancreaticoduodenectomy, with a particular focus on factors related to pancreatic secretions.

MATERIALS AND METHODS

Patient demographics and clinical variables

All study procedures were approved by the Institutional Review Board (No. 2010-09-082, Samsung Medical Center). The study included 228 patients who had pylorus-preserving pancreaticoduodenectomy (PPPD), Whipple' s procedure, or hepato-pancreato-duodenectomy (HPD) between January 2009 and April 2010. Electronic medical records and data were retrospectively reviewed. Exclusion criteria were: (1) patients without non-enhanced computed tomography (CT) findings (n = 12); (2) a preoperative diagnosis of fatty liver disease by non-enhanced CT (n = 19); and (3) mortalities resulting from postoperative pseudoaneurysm (n = 4). Thirty-five patients who consumed more than 150 g of alcohol per week were not excluded from the study because they did not have a diagnosis of preoperative fatty liver disease by non-enhanced CT. The final study group therefore consisted of 193 patients that were divided into two groups: Group A consisted of 15 patients who developed postoperative steatohepatitis, and Group B consisted of 178 patients who did not develop postoperative steatohepatitis.

Data were collected on patient demographics, operative procedures, pathologies, and perioperative clinical variables, including levels of insulin, C-peptide, and glucagon, and results from an oral glucose tolerance test conducted preoperatively. Data were also collected on postoperative liver function and the postoperative attenuation ratios for the liver and spleen.

Data on pancreatic enzyme levels in serum on postoperative day 7, pancreatic duct size, pancreatic fistula, pancreatic duct stenting, and type of stenting were collected and considered as potential parameters associated with pancreatic secretions. Pancreatic fistula was diagnosed according to the International study group pancreatic fistula (ISGPF) definition[23]. External pancreatic duct stenting was usually placed during the first postoperative month. Post-discharge pancreatic enzyme supplementation was administered routinely to all patients who had a pancreaticoduodenectomy.

Evaluation of steatohepatitis

Fatty liver disease was defined according to the difference between the splenic and hepatic attenuation ratios (CT_{S-L}) and the liver-to-spleen attenuation ratio ($CT_{L/S}$). To minimize sampling error, we used two CT images from the liver, one from the right lobe and one from the left lobe, and we excluded images from the periphery of the liver. Perioperative steatohepatitis was presumed when CT_{S-L}

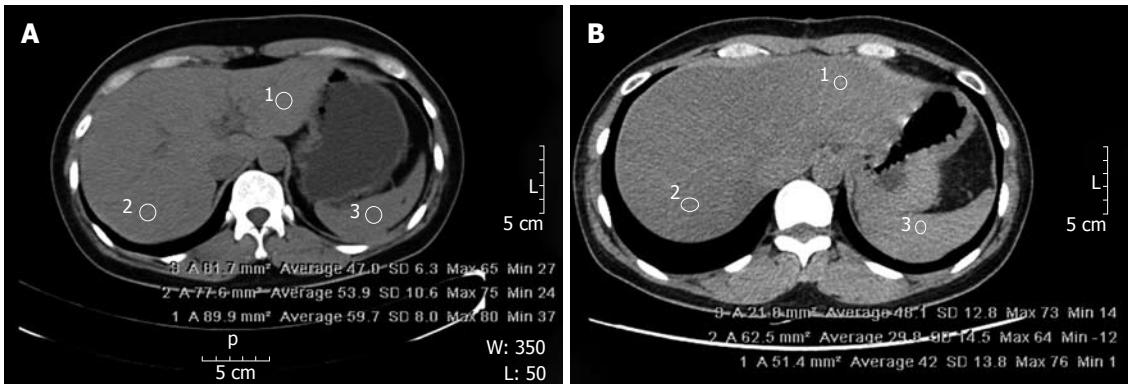

Figure 1 Preoperative and postoperative non-enhanced computed tomography images of a representative patient demonstrating different attenuation values for the spleen and liver. The inclusion criteria were: (1) $CT_{S-L} \geqslant 10$ Housefield unit (HU); or (2) $CT_{L/S} \leqslant 0.9$ HU [L: Mean attenuation value for two random points (1, 2) of the liver; S: Attenuation value for one random point (3) of the spleen]. A: Preoperative; B: Postoperative.

Table 1 Preoperative characteristics and laboratory findings for the two groups

	Group A(n = 15)	Group B (n = 178)	P
Gender (M:F), n	7:8	112:66	0.270
Age in years, mean (range)	58.7 (40-74)	61.8 (15-81)	0.295
BMI (kg/m^2) ± SD	20.6 ± 2.9	22.0 ± 3.3	0.082
Hepatitis B viral infection (+), n (%)	0 (0)	6 (3.4)	1.000
Type Ⅱ diabetes, n (%)	2 (13.3)	34 (19.1)	0.582
Alcohol consumption > 150 g/wk, n (%)	3 (20.0)	32 (18.0)	0.738
Biliary drainage, n (%)	4 (26.7)	74 (41.6)	0.245
Albumin/globulin ratio (range)	1.79 (1.2-4.1)	1.53 (0.8-2.4)	0.234
Total cholesterol (mg/dL), mean (range)	209.3 (136-309)	200.6 (73-470)	0.641
Serum amylase (U/L), mean (range)	91.3 (22-263)	129.8 (15-1361)	0.388
Serum lipase (U/L), mean (range)	170.4 (24-707)	336.7 (7-13562)	0.593
AST (U/L), mean (range)	134.3 (11-547)	111.7 (12-1230)	0.621
ALT (U/L), mean (range)	156.1 (11-551)	144.0 (9-1371)	0.826
ALP (U/L), mean (range)	315.9 (64-913)	267.3 (34-2236)	0.518
INR, mean (range)	0.99 (0.85-1.11)	1.07 (0.81-8.78)	0.585
Total bilirubin (mg/dL), mean (range)	5.7 (0.3-18.8)	6.2 (0.2-44.3)	0.799
Fasting glucose (mg/dL), mean (range)	140 (93-150)	135 (47-458)	0.735

BMI: Body mass index; SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio.

was equal to or greater than 10 Housefield Units (HU) or when $CT_{L/S}$ was equal to or less than 0.9 HU (Figure 1).

CT images were obtained with a 64-channel, 4-multidetector, CT scanner (General Electric®, NY, United States). The parameters for non-enhanced CT were: 100-300 mAs; rotation speed of 0.6 s; table speed of 3 mm; noise index of 11.57; detector coverage of 40 mm; pitch-to-speed ratio (mm/rot) of 0.984:1; and helical thickness of 5 mm. CT images were reviewed on a Picture Archiving Communication System workstation (General Electric®).

Statistical analysis

For continuous variables, the paired-sample Student's t test was used to compare the two patient groups. For categorical variables, Chi Square analysis or Fisher's exact test was used to compare the two groups. Two-way Analysis of Variance was used to analyze group differences in repeated measures of the levels of glucose, insulin, C-peptide, and glucagon. Pearson's correlation coefficient test was used to determine the correlation between the postoperative difference of CT_{S-L} and the postoperative liver function test results. Multivariate analysis of risk factors was conducted using multivariate Cox proportional hazards modeling. Statistical analyses were performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, United States), and P values < 0.05 were considered statistically significant.

RESULTS

Perioperative clinical characteristics

The mean period between the operation and the patient's postoperative follow-up appointment was 3.2 ± 2.0 mo (range: 1-11). For each group, the average period was 2.4 mo in Group A and 3.3 mo in Group B (P = 0.106). Fifteen patients (7.8%) who showed postoperative hepatic fatty changes were included in Group A,

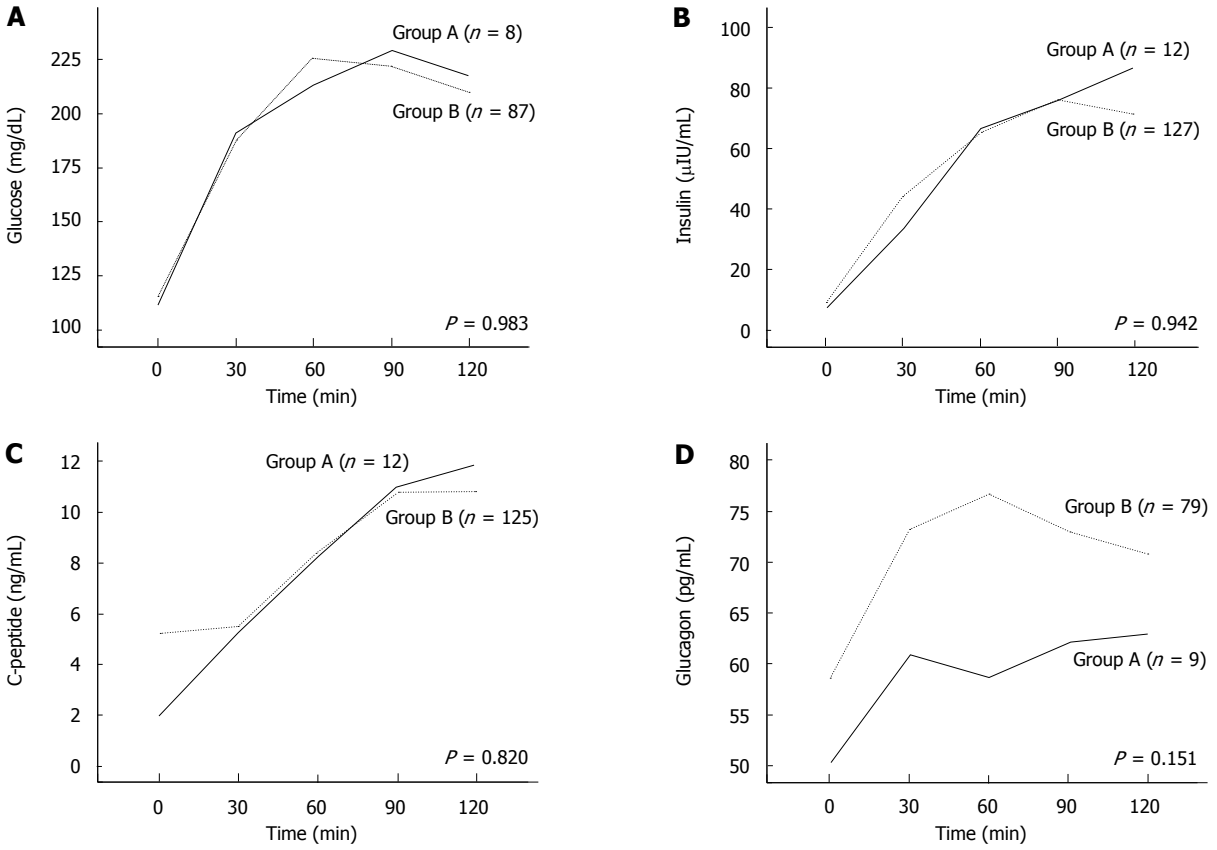

Figure 2 Preoperative oral glucose tolerance test results, continuous stimulation test results for insulin, C-peptide, and glucagon levels. A: Glucose levels; B: Insulin levels; C: C-peptide levels; D: Glucagon levels.

Table 2 Operative treatments and final pathological findings for the two groups

	Group A (n = 15)	Group B (n = 178)	P
PPPD	12	126	
Whipple's procedure	3	47	0.482
HPD	0	5	
Pancreatic cancer	7	50	
Common bile duct cancer	3	45	
Ampulla of vater cancer	5	27	
Duodenal cancer	0	11	
IPMN of pancreas	0	23	
NET of pancreas or duodenum	0	3	0.665
MCN or SPT of pancreas	0	5	
Duodenal GIST	0	2	
Hilar cholangiocarcinoma	0	4	
Colon cancer with duodenal invasion	0	4	
Gallbladder cancer with duodenal invasion	0	1	
Pancreatitis	0	3	

PPPD: Pylorus-preserving pancreaticoduodenectomy; HPD: Hepato-pancreato-duodenectomy; IPMN: Intraductal papillary mucinous neoplasm; NET: Neuroendocrine tumor; MCN: Mucinous cystic neoplasm; SPT: Solid pseudopapillary tumor; GIST: Gastrointestinal stromal tumor.

and the remaining 178 patients (92.2%) were included in Group B (Table 1). Seventy-eight patients (40.4%) had preoperative biliary drainage, including percutaneous transhepatic biliary drainage, endoscopic retrograde biliary drainage, endoscopic nasobiliary drainage, and biliary stenting. None of the patients had undergone bariatric surgery (data not shown).

Preoperative patient characteristics and laboratory findings, including liver function test results and levels of pancreatic enzymes, were similar in the two groups (Table 1). PPPD or Whipple's procedure was performed on 15 patients (100%) in Group A and 173 patients (97.2%) in Group B (P = 0.842) (Table 2). HPD was performed on 5 patients (2.8%) in Group B. Based on final pathologic reports, all patients (100%) in Group A were diagnosed with malignant disease, whereas patients in Group B were diagnosed with a variety of diseases (Table 2). Preoperative oral glucose tolerance test results, continuous stimulation test results for insulin, C-peptide and glucagon levels were not significantly different between the two groups (Figure 2). For patients with malignant disease, the two groups were similar in terms of cancer stage (P = 0.190), perineural invasion (P = 0.259), and vessel invasion (P = 1.000). The liver function test for all patients showed that postoperative $CT_{L/S}$ values correlated with postoperative ALT levels (γ = -0.149, P = 0.039) but not with postoperative AST or ALP levels (Table 3).

Factors associated with pancreatic secretions

Serum levels of pancreatic enzymes on postoperative day 7, pancreatic duct size, and the proportion of patients that received a pancreatic duct stent were similar in the two groups (Table 4); however, the proportion

Table 3 Pearson's correlation coefficients for the correlation between postoperative liver function and the difference in the postoperative attenuation values between the spleen and liver (CT_{S-L}) in all patients

Postoperative liver function test result	γ	*P*
AST	-0.138	0.056
ALT	-0.149	0.039
ALP	-0.023	0.755

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

of patients that developed a pancreatic fistula postoperatively ($P = 0.020$) or had an external pancreatic duct stent placed ($P = 0.005$) was higher in Group A than in Group B (Table 4). A multivariate analysis of risk factors identified the postoperative development of a pancreatic fistula (HR = 3.332, $P = 0.037$) and the postoperative placement of an external pancreatic duct stent (HR = 4.530, $P = 0.017$) as independent risk factors for postoperative steatohepatitis (Table 5). Among the 41 patients who showed postoperative pancreatic fistula in the two groups, 32 and 9 patients showed grade A and B fistulas guided by ISGPF definition, respectively. Grade A fistula was observed in 6 patients (85.7%) in group A, and in 26 patients (76.5%) in group B ($P = 1.000$).

DISCUSSION

Steatohepatitis associated with pancreatic secretions

Duct-to-mucosal anastomosis and pancreatic duct stenting are used to prevent pancreatic leakage. In our institution, pancreatic stenting is usually performed when the diameter of the pancreatic duct is 2-5 mm, although there is some variability according to the attending surgeon's preferences. The methods chosen for pancreatic duct stenting in association with steatohepatitis involve several considerations. The remnant pancreas secretes several enzymes that are associated with digestion, including enterokinase, trypsinogen, chymotrypsin, amylase, lipase, cellulose, phospholipase, and esterase[24-26]. External pancreatic duct stenting induces an earlier impairment in the secretion of pancreatic enzymes into the bowel lumen. Phospholipases A1 and A2 cleave fatty acids from phospholipids, and esterase hydrolyzes cholesterol esters. We hypothesize that hepatic fibrosis can be prevented by inhibiting the entry of free fatty acids into hepatocytes. Furthermore, lipid absorption by bile acid denaturation, which can lead to fat accumulation in hepatocytes, might be prevented by impairing bicarbonate secretion within the acidic gastric environment.

Adipokines such as leptin, resistin, adiponectin, and tumor necrosis factor (TNF)-α are known to regulate hepatic and peripheral glucose levels and lipid metabolism[27]. Decreased serum levels of adiponectin and increased serum levels of leptin or TNF-α are associated with NASH. Also, the hydrolysis of starches and glycogen into disaccharides or trisaccharides by amylolytic pancreatic enzymes can be impaired by external pancreatic duct drainage. These endocrine abnormalities are accelerated by the loss of insulin and glucagon, and they can induce insulin insensitivity and abnormal glucose metabolism.

Reduced motilities of the stomach and duodenum after pancreaticoduodenectomy can lead to the development of diabetes (20%-40%) with hyperglycemia and delayed gastric emptying (15%-40%), and a reduction in the release of pancreas-stimulating hormones from the duodenum (100%)[28]. These effects can aggravate insulin resistance. Moreover, motility dysfunction can be induced by intestinal bacterial overgrowth as well as anatomic alterations that may result from anastomotic procedures. Wu *et al*[29] found that decreased small intestinal motility was associated with delayed intestinal transit followed by bacterial overgrowth (*Escherichia coli*) in a rat model of NASH. Furthermore, patients who have undergone a pancreaticoduodenectomy are at increased risk of developing an ascending infection through hepaticojejunostomy and jejunojejunostomy. The effectiveness of antibiotics for decreasing elevated liver enzymes in NASH needs to be further investigated, however.

A previous study[23] identified the duration of untreated jaundice, malignancy, small pancreatic duct size, and soft pancreatic texture as risk factors for pancreatic fistula. The relationship between pancreatic fistula and steatohepatitis is still questionable, but the use of long-term total parenteral nutrition or the development of a secondary infection or sepsis after a pancreatic fistula might influence hepatic function.

Other factors related to steatohepatitis

High levels of low density lipoprotein-cholesterol (LDL-C) and low levels of high density lipoprotein-cholesterol (HDL-C) are established risk factors for atherogenesis in patients with diabetic dyslipidemia[30]. Insulin resistance is a key factor in the development of metabolic syndrome involving dyslipidemia and Type Ⅱ diabetes. Dyslipidemia is associated primarily with low levels of HDL-C, high levels of LDL-C, and hepatic overproduction of triglyceride-rich very low density lipoprotein-cholesterol (VLDL-C)[31]. In the present study, LDL-C levels tended to be higher in Group A than in Group B, but this apparent difference was not significant (data not shown); therefore, the relationship between LDL-C and steatohepatitis remains to be determined.

Bariatric surgery is often considered for patients who are morbidly obese. Roux-en-Y gastric bypass, gastroplasty, or adjustable gastric banding are commonly performed, and jejunoileal or ileoileal bypass surgeries are no longer preferred[4,5]. Biliopancreatic diversion that involves a small bowel bypass procedure to form a short common channel from the ileocecal valve can induce metabolic derangement and is associated with a high incidence of postoperative hepatic steatosis[3,32]. This is caused by a combination of malnutrition and malabsorption of vitamins, iron, ferritin, and calcium. Pancreaticoduodenectomy involving a Roux-en-Y jejunojejunal bypass appears

Table 4 Perioperative clinical variables related to pancreatic secretions

	Group A (n = 15)	Group B (n = 178)	P
Serum amylase[1], mean (range)	44.5 (9.0-119.0)	51.4 (6.0-266.0)	0.558
Serum lipase[1], mean (range)	31.9 (6.0-266.0)	30.0 (1.0-215.0)	0.793
Pancreatic duct size, mean (range) (mm)	4.5 (2-18)	3.5 (1-11)	0.401
Pancreatic fistula[2], n	7	34	0.020
Placement of pancreatic duct stent, n	13	131	0.363
Internal, n	4	95	0.005
External, n	9	36	

[1]Postoperative day 7; [2]Diagnosed according to International study group pancreatic fistula criteria[23].

Table 5 Multivariate analysis of risk factors for the development of steatohepatitis after pancreaticoduodenectomy[1]

Variables	HR	95% confidence interval	P
Serum amylase[2]	0.990	0.973-1.007	0.262
Serum lipase[2]	1.014	0.986-1.041	0.332
Pancreatic duct size[3]	0.882	0.635-1.224	0.452
Pancreatic fistula[4] (-)	1.000	-	-
Pancreatic fistula[4] (+)	3.332	1.075-10.321	0.037
Internal pancreatic duct stenting	1.000	-	-
External pancreatic duct stenting	4.530	1.312-15.643	0.017

[1]Analyzed by logistic regression; [2]Postoperative day 7; [3]Dichotomized for categorical variables using a median split; [4]Diagnosed according to International study group pancreatic fistula criteria[23]. HR: Hazard ratio.

to be associated with the same side-effects as small bowel bypass surgery. We also found that all patients with postoperative steatohepatitis had malignant pathological findings, but the pathogenesis remains uncertain.

Diagnostic methods to identify fatty liver disease

Histological evaluation through liver biopsy remains the gold-standard method for distinguishing NASH from simple fatty liver disease and for estimating intrahepatic fat content, the extent of necroinflammatory lesions and fibrosis. However, liver biopsy is associated with sampling errors and the risk of bleeding, infection, and biliary leakage[1,6,8,20,33]. Kleiner *et al*[15] proposed a semi-quantitative scoring system (the NAFLD activity score) to assess the histological features of NAFLD and to discriminate between NASH and non-NASH fatty liver disease. Five features-steatosis, hepatocellular ballooning, lobular inflammation, fibrosis, and the absence of lipogranulomas-were independently associated with the accurate diagnosis of NASH using adult liver biopsies.

Ultrasonography, non-enhanced CT, magnetic resonance imaging, and proton magnetic resonance spectroscopy (^{1}H MRS) are radiological, non-invasive methods to diagnose hepatic steatosis[1,4,16], but these methods cannot accurately distinguish NASH from simple fatty liver disease or objectively quantify fat content[1,6]. Recently, fatty infiltration of the liver was detected using chemical-shift imaging and a selective fat-suppression technique, acquired by the percentage of relative signal intensity loss on magnetic resonance T1- or T2-weighted images, the ratio of peak lipid to water by ^{1}H MRS, dual-energy multi-slice spiral CT, and non-enhanced CT measuring tissue density as a radiographic attenuation that can be objectively measured in Housefield units[16,34]. In a study by Nomura *et al*[22], non-enhanced CT was found to be useful for diagnosing steatohepatitis with established accuracy and for evaluating $CT_{L/S}$ and CT_{S-L}. Other studies identified a correlation between $CT_{L/S}$ and CT_{S-L} and histological findings of steatohepatitis, and some reports defined steatohepatitis as $CT_{L/S} < 0.9$ HU or $CT_{S-L} \geqslant 10$ HU[16,35]. Unlike the study by Nomura *et al*[22], which identified a relationship between postoperative AST and CT_{S-L}, our study found that CT_{S-L} correlated significantly with postoperative ALT levels, not AST levels. Kato *et al*[36] proposed a NAFLD scoring system that was based on the development of pancreatic adenocarcinoma, the pancreatic resection line, and postoperative diarrhea. This group diagnosed NASH by percutaneous liver biopsy after pancreaticoduodenectomy and revealed a significant correlation between their scoring system and CT findings.

Limitations

The present study has several limitations that should be considered when interpreting the results. First, the study was retrospective, and the period between the operation and the postoperative follow-up CT was not uniform and averaged 3.2 mo, which is short. Nevertheless, if we consider the prevalent period for the development of steatohepatitis postoperatively, the results could present useful information.

Second, there were only 15 patients (7.8%) who developed steatohepatitis after surgery in our study, whereas in the study by Nomura *et al*[22] 33% of asymptomatic patients without severe obesity had decreased hepatic attenuation meeting the criteria for steatohepatitis after pancreaticoduodenectomy. The reason for the difference in the incidence between our study and that of Nomura *et al* is not clear, but it might reflect differences in the rates of obesity and the timing between the operation and the follow-up CT scan. Our study had a very small number of obese patients (24 patients with a BMI $\geqslant$ 25 kg/m^2 and 2 patients with a BMI $\geqslant$ 30 kg/m^2).

Third, we used a radiological method to diagnose steatohepatitis or NAFLD without histopathological evidence. Non-enhanced CT was reported to have a sensitivity of 73%-100% and a specificity of 95%-100% for the detection of moderate or severe steatohepatitis, al-

though hepatic iron overload might alter these rates[1,37].

Finally, the location and the extent of the pancreatic resection and postoperative patient-related factors such as steroid use, weight loss, and exercise were not included in our statistical analyses. In addition, the effect of adjuvant chemoradiotherapy, which was administered to 7 patients (46.7%) in Group A and 80 patients (44.9%) in Group B, was not included in our analysis.

Future prospective controlled studies with a larger sample size based on histopathological findings are needed to verify the relationships identified in the present study. Postoperative steatohepatitis might not be a significant problem, especially in late-stage malignant patients. Nevertheless, this preliminary report provides evidence for operation-related causes of steatohepatitis following pancreaticoduodenectomy, ruling out other factors causing hepatic fatty change or injury.

COMMENTS

Background

Only a limited number of reports have examined operation-related causes of postoperative steatohepatitis following pancreaticoduodenectomy.

Research Frontiers

To identify the risk factors for steatohepatitis after pancreaticoduodenectomy, with a particular focus on factors related to pancreatic secretions.

Innovations and breakthroughs

This preliminary report helps to identify operation-related causes of steatohepatitis following pancreaticoduodenectomy, and it is the first study to identify potential risk factors related to pancreatic secretions.

Applications

In this study, pancreatic fistula and external pancreatic duct stenting significantly influenced the development of steatohepatitis following pancreaticoduodenectomy. These findings have clinical implications and could be used to design future clinical trials.

Peer review

This is very interesting clinical research about the mechanism of post-operative steatohepatitis development following pancreaticoduodenectomy.

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BRIEF ARTICLE

Characteristics and outcomes of acute upper gastrointestinal bleeding after therapeutic endoscopy in the elderly

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Abstract

AIM: To characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after endoscopic therapy among patients aged ⩾ 65 years admitted for acute upper gastrointestinal bleeding (UGIB) compared with those aged < 65 years.

METHODS: Medical records and an endoscopy database of 526 consecutive patients with overt UGIB admitted during 2007-2009 were reviewed. The initial presentations and clinical course within 30 d after endoscopy were obtained.

RESULTS: A total of 235 patients aged ⩾ 65 years constituted the elderly population (mean age of 74.2 ± 6.7 years, 63% male). Compared to young patients, the elderly patients were more likely to present with melena (53% *vs* 30%, respectively; $P < 0.001$), have comorbidities (69% *vs* 54%, respectively; $P < 0.001$), and receive antiplatelet agents (39% *vs* 10%, respectively; $P < 0.001$). Interestingly, hemodynamic instability was observed less in this group (49% *vs* 68%, respectively; $P < 0.001$). Peptic ulcer was the leading cause of UGIB in the elderly patients, followed by varices and gastropathy. The elderly and young patients had a similar clinical course with regard to the utilization of endoscopic therapy, requirement for transfusion, duration of hospital stay, need for surgery [relative risk (RR), 0.31; 95% confidence interval (CI), 0.03-2.75; $P = 0.26$], rebleeding (RR, 1.44; 95% CI, 0.92-2.25; $P = 0.11$), and mortality (RR, 1.10; 95% CI, 0.57-2.11; $P = 0.77$). In Cox's regression analysis, hemodynamic instability at presentation, background of liver cirrhosis or disseminated malignancy, transfusion requirement, and development of rebleeding were significantly associated with 30-d mortality.

CONCLUSION: Despite multiple comorbidities and the concomitant use of antiplatelets in the elderly patients, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy.

Key words: Adverse outcomes; Elderly; Therapeutic end-oscopy; Upper gastrointestinal bleeding

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INTRODUCTION

The 2003 World Health Report highlighted the accelerated aging of the global population, as the number of elderly people will double in the next few decades[1]. Upper gastrointestinal bleeding (UGIB) affects a substantial number of elderly people and is a potentially life-threatening clinical event. Age has been considered as a significant prognostic factor for adverse outcomes, including rebleeding and mortality, from acute UGIB in numerous clinical risk models[2-6]. However, it is unclear if the role of age in UGIB is due to a more severe disease or differences in the treatment received. Generally, the elderly have often been treated less aggressively than younger patients because of an assumption of increased risk of any therapeutic procedures, secondary to comorbid conditions. However, recent studies of gastrointestinal endoscopy conducted in elderly patients reported an overall procedural success and morbidity similar to that reported for the general population, even in patients undergoing upper endoscopy for the evaluation of acute gastrointestinal bleeding[7,8].

Over the last decades, clinical considerations related to the diagnosis and treatment of UGIB has changed dramatically. Paramount to these changes have been the increased involvement of acute care specialists during resuscitation, advances in diagnostic and therapeutic endoscopy, the use of powerful acid suppressive and vasoactive agents, and more selective and less invasive surgical approaches that may offer a promising outcome for patients. Hence, outcome studies on the appropriate approach to UGIB in the elderly are needed. We therefore conducted the current study to characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after pharmacologic and endoscopic therapy with regard to the transfusion requirement, duration of hospital stay, need for surgical intervention, rate of rebleeding, and 30-d mortality among a large cohort of patients aged $\geq$ 65 years who were hospitalized for acute UGIB compared with those aged < 65 years.

MATERIALS AND METHODS

Patient population

This retrospective study was approved by the Institutional Review Board of the Hospital and was conducted at Siriraj Hospital, a tertiary academic medical center for the Bangkok metropolitan area and surrounding communities. All consecutive adult patients who were hospitalized for acute UGIB (e.g., hematochezia, melena, coffee-ground vomiting or hematemesis with or without hypotension) and who underwent endoscopy between January 2007 and December 2009 were included in the study. For the purposes of this study, we defined "elderly" as those older than 65 years of age.

Management of Acute UGIB

At our institution, patients who presented with acute UGIB were given appropriate initial resuscitation followed by diagnostic and therapeutic measures. Empiric therapy using either an intravenous proton pump inhibitor (PPI) or somatostatin analogue infusion was given before the endoscopy for suspicion of peptic ulcer and varices, respectively. Urgent endoscopy within the first 12 h after admission was performed in patients with signs of ongoing bleeding as determined by gastroenterologists. Endoscopic treatment was given in the form of injection therapy with epinephrine, coaptive thermocoagulation, hemostatic clip or combination therapy in patients with active bleeding, nonbleeding visible vessels or adherent clots. High-dose PPI was administered by infusion for 72 h after endoscopy in patients who required endoscopic intervention. Bleeding esophageal and gastric varices were treated with band ligation and cyanoacrylate injection, respectively, in addition to the use of vasoactive drugs and intravenous antibiotics. After the procedure, the patients were subsequently transferred to a medical ward for monitoring. Endoscopy was repeated in the event of rebleeding. Patients underwent surgery if bleeding persisted or if rebleeding occurred after two therapeutic endoscopies.

Clinical, Endoscopic, and Laboratory Data

Medical records and an endoscopy database of all patients were reviewed. Patient demographics, clinical presentations, initial vital signs, the presence of comorbid conditions, drugs taken at the time of admission and initial laboratory tests were obtained. We abstracted data describing the endoscopic management, including endoscopic diagnosis and the presence of stigmata of recent bleeding, endoscopic hemostasis, and medication use following endoscopy. Outcome data describing the overall course within 30 d after the initial endoscopic treatment with specific attention to rebleeding, the need for surgery, a requirement for blood transfusion, the length of hospital stay, and mortality were gathered.

The presence of hemodynamic instability was defined as systolic blood pressure < 100 mmHg, a heart rate > 100 beats/min and/or orthostatic changes in systolic blood pressure (a decrease of > 10%) or heart rate (an increase of > 10%) between a supine and seated position. Rebleeding was defined by the presence of hematemesis or melena with signs of hemodynamic instability or a decrease in hemoglobin level > 2 g/dL in a previously stable patient. Endoscopic grading of ulcer lesions was categorized according to the Forrest's classification[9]. The stigmata of recent bleeding included arterial spurting or pulsatile bleeding from the ulcer base, a non-bleeding visible vessel, and an adherent clot covering the base of an ulcer. Grading of varices was carried out using the classification of the Italian Liver Cirrhosis Project[10].

Statistical analysis

Data were summarized using descriptive statistics. Continuous variables were compared using the *t* test or the

Table 1 Characteristics of patients for the entire group and for each cohort

	Total (n = 526)	Patients aged ≥ 65 yr (n = 235)	Patients aged < 65 yr (n = 291)	P value
Age (yr)	60 ± 15.9	74.2 ± 6.7	48.4 ± 11.1	< 0.001
Male: n (%)	370 (70)	148 (63)	222 (76)	< 0.001
Presenting symptoms: [1]n (%)				
Hematemesis	207 (39)	65 (28)	142 (49)	< 0.001
Melena	214 (41)	126 (53)	88 (30)	< 0.001
"Coffee ground" vomiting	86 (16)	36 (15)	50 (17)	0.57
Hematochezia	19 (4)	8 (4)	11 (4)	0.82
Clinical findings: n (%)				
Red blood on nasogastric lavage	97 (18)	41 (17)	56 (19)	0.60
Systolic blood pressure < 100 mmHg	163 (31)	62 (26)	101 (35)	0.04
Heart rate > 100 beats/min	192 (37)	63 (27)	129 (44)	< 0.001
Presence of hemodynamic instability	313 (60)	114 (49)	199 (68)	< 0.001
Comorbid illness: [1]n (%)				
Cardiovascular disease	109 (21)	81 (34)	28 (10)	< 0.001
Cerebrovascular disease	49 (9)	34 (14)	15 (5)	< 0.001
Chronic renal failure	37 (7)	17 (7)	20 (7)	0.87
Liver cirrhosis	140 (27)	45 (19)	95 (33)	< 0.001
Cancer	64 (12)	33 (14)	31 (11)	0.24
Diabetes mellitus	126 (24)	87 (37)	39 (1)	< 0.001
Hypertension	186 (35)	125 (53)	61 (21)	< 0.001
Alcohol drinking: n (%)	246 (47)	66 (28)	180 (62)	< 0.001
Previous use of medications: [1]n (%)				
Low-dose aspirin	111 (21)	82 (35)	29 (10)	< 0.001
Clopidogrel	33 (6)	30 (13)	3 (1)	< 0.001
Warfarin	38 (7)	22 (9)	16 (6)	0.09
NSAID other than aspirin	123 (23)	57 (24)	66 (23)	0.67
Laboratory features at presentation:				
Hemoglobin (g/dL)	8.7 ± 4.7	8.4 ± 2.3	9.0 ± 6.0	0.19
White blood count (10^3/μL)	11.8 ± 8.1	11.2 ± 5.3	12.3 ± 9.7	0.15
Platelets (10^3/μL)	232 ± 125	242 ± 116	223 ± 131	0.09
Prothrombin time (s)	13.4 (11.4-248)	13.2 (11.4-160)	13.9 (11.4-248)	0.84
Creatinine (mg/dL)	1.1 (0.2-11.8)	1.3 (0.4-10.8)	0.9 (0.2-11.8)	0.44

Categorical variables are presented as number and percentage, and continuous variables are presented as the mean ± SD or median and range when appropriate. [1]Some patients presented with more than 1 symptom or comorbid illness and used more than 1 drug. NSAID: Non-steroidal anti-inflammatory drug.

Mann-Whitney test. Categorical variables were compared using the χ^2 or Fisher exact test. The Kaplan-Meier method with the log-rank test was used to compare differences in the rates of rebleeding and death within 30 d after primary endoscopic treatment. Cox's regression analysis was used to detect possible prognostic variables on recurrent bleeding and survival. All statistical testing was performed at the conventional 2-tailed α level of 0.05.

RESULTS

Patient population

During a three-year period, a total of 526 patients (370 men, 156 women) with acute UGIB were identified. The age distribution at presentation is shown in Figure 1. Acute UGIB occurred in patients aged 18-40 years (13%), 41-50 years (15%), 51-60 years (20%), 61-70 years (24%), 71-80 years (19%) and > 80 years (9%). Two hundred thirty-five patients were at least 65 years of age and constituted the elderly population, with a mean age of 74.2 ± 6.7 years. Two hundred ninety-one patients were < 65 years old and constituted the young population, with a mean age of 48.4 ± 11.1 years. The patient demographics and clinical characteristics of the entire group and each of the cohorts are shown in Table 1.

Clinical characteristics

Patients ≥ 65 years of age were more likely to present with melena, receive antiplatelet agents, and have comorbid conditions including cardiovascular disease, cerebrovascular disease, cirrhosis, diabetes, and hypertension compared with the young population (Table 1). The rates of antiplatelet use were increased with older age (P < 0.001). In contrast, patients < 65 years old presented with hematemesis (49% *vs* 28%, respectively; P < 0.001) and hemodynamic instability (68% *vs* 49%, respectively; P < 0.001) more commonly than the elderly. There were no differences in terms of 'coffee ground' vomiting, hematochezia, red blood on the initial nasogastric lavage, the use of anticoagulants and non-steroidal anti-inflammatory drugs (NSAIDs), and laboratory indices at presentation between the two groups (Table 1).

Endoscopic findings

The endoscopic findings for the entire patient group and each of the cohorts are shown in Table 2. Distributions of peptic ulcer and varices as the source of bleeding among each age range at presentation are summarized in Figure 1. Bleeding peptic ulcers were identified more

Table 2 Endoscopic findings for the entire group and for each cohort

	Total (n = 526)	Patients aged ⩾ 65 yr (n = 235)	Patients aged < 65 yr (n = 291)	P value
Peptic ulcer as source of bleeding: [1]n (%)				
Active bleeding	19 (4)	11 (5)	8 (3)	0.20
Non-bleeding visible vessel	59 (11)	25 (11)	34 (12)	0.71
Clot with underlying vessel	16 (3)	8 (3)	8 (3)	0.66
Flat, pigmented spot	41 (8)	23 (10)	18 (6)	0.13
Clean base	224 (46)	112 (48)	112 (38)	0.03
Portal hypertensive related-lesions as source of bleeding: [1]n (%)				
Esophageal varices	137 (26)	41 (17)	96 (33)	< 0.001
Gastric and duodenal varices	17 (3)	4 (2)	14 (5)	0.051
Portal hypertensive gastropathy	58 (11)	17 (7)	41 (14)	0.01
Other endoscopic findings: [1]n (%)				
Esophageal ulcer	19 (4)	9 (4)	10 (3)	0.81
Esophagitis	36 (7)	17 (7)	19 (7)	0.75
Gastropathy, duodenitis, or erosions	129 (25)	61 (26)	68 (23)	0.49
Mallory-Weiss tear	26 (5)	5 (2)	21 (7)	0.007
Gastric cancer	15 (3)	6 (3)	9 (3)	0.71
Dieulafoy's lesion	11 (2)	6 (3)	5 (2)	0.51
Angiodysplasia	2 (0.004)	1 (0.4)	1 (0.3)	0.88
No clinically significant finding	11 (2)	3 (1)	8 (3)	0.24

[1]Some patients presented with more than 1 endoscopic finding.

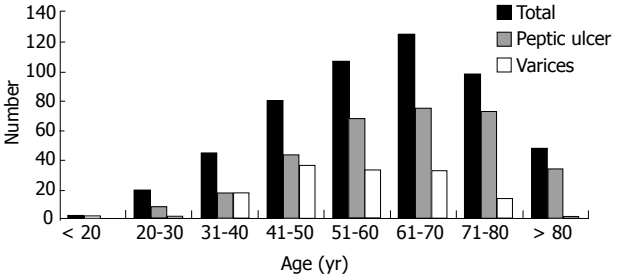

Figure 1 Age distribution of patients with acute upper gastrointestinal bleeding.

frequently in patients aged 60-80 years, suggesting that peptic ulcers are a more common source of bleeding in the elderly than in patients aged < 65 years (68% *vs* 56%, respectively; P = 0.006). Among those with peptic ulcer bleeding, clean base ulcers were seen more frequently in the elderly compared with young patients (48% *vs* 38%, respectively; P = 0.03). The numbers of active bleeding, non-bleeding visible vessels, clots, and flat pigmented spots did not differ significantly between the two groups (Table 2). Of these, 45 (28%) elderly patients required therapeutic endoscopy compared with 55 (34%) young patients (relative risk for the elderly, 0.84; 95% confidence interval (CI), 0.60 to 1.16; P = 0.29) (Table 3).

Variceal bleeding tended to decrease after the fourth decade of life. As a result of higher alcohol consumption, esophageal varices (33% *vs* 17%, respectively; P < 0.001) and Mallory-Weiss tears (7% *vs* 2%, respectively; P = 0.007) were noted more frequently in patients < 65 than in those > 65 years of age. Furthermore, young patients had a trend toward higher numbers of bleeding from gastric and duodenal varices compared to the elderly population (Table 2). Of those with variceal bleeding, 27 (66%) elderly patients received therapeutic endoscopy compared with 75 (75%) young patients (relative risk for the elderly, 0.89; 95% CI, 0.69 to 1.14; P = 0.34) (Table 3).

Treatment outcomes

Urgent endoscopy was performed in 47 patients aged ⩾ 65 years and in 64 patients aged < 65 years. Hemodynamic instability at presentation was less frequent in the elderly patients compared to the young patients (49% *vs* 68%, respectively; P < 0.001). One of the elderly patients and four of the young patients underwent emergency surgery because of failure to achieve hemostasis during endoscopy. The mean number of units of packed erythrocytes transfused prior to endoscopy and during hospitalization was similar in both groups. In addition, the length of hospital stay was not significantly different between the two groups.

Rebleeding occurred within 30 d after the initial endoscopic therapy in 36 patients (15%) aged ⩾ 65 years and in 31 patients (11%) aged < 65 years (P = 0.1) (Figure 2A). When we analyzed the rebleeding rate according to the source of bleeding, the probability of rebleeding within 30 d after endoscopic therapy among patients with peptic ulcer bleeding was similar in both groups (P = 0.35) (Figure 2B), but the rebleeding rate among patients who had ulcers with stigmata of recent bleeding was higher in the elderly patients (P = 0.02) (Figure 2C). The rate of recurrent variceal bleeding was also higher in the elderly patients than in the young patients, although the difference was not statistically significant (P = 0.08) (Figure 2D).

Sixteen patients (7%) aged ⩾ 65 years and 18 patients (6%) aged < 65 years died within 30 d after the initial endoscopic treatment. The observed survival was virtually identical for both groups (P = 0.8) (Figure 3A). However, all deaths tended to occur in a greater proportion of elderly patients who had ulcers with stigmata of recent bleeding (P = 0.3) (Figure 3C) and varices (P = 0.1)

Table 3 Clinical outcomes after pharmacologic and endoscopic therapy

	Patients aged ⩾ 65 yr (*n* = 235)	Patients aged < 65 yr (*n* = 291)	*P* value	Relative risk (95% CI)
Urgent Endoscopy: *n* (%)	47 (20)	64 (22)	0.58	0.91 (0.65-1.27)
For peptic ulcers bleeding	22 (14)	29 (18)	0.33	0.78 (0.47-1.29)
For variceal bleeding	16 (46)	30 (37)	0.36	1.25 (0.79-1.98)
Endoscopic therapy for bleeding peptic ulcers: [1]*n* (%)	45 (28)	55 (34)	0.29	0.84 (0.60-1.16)
Epinephrine injection	36 (80)	50 (91)	0.12	
Coaptive thermocoagulation	28 (62)	39 (71)	0.36	
Hemostatic clip	11 (24)	14 (25)	0.91	
Combined therapy	29 (64)	42 (76)	0.19	
Endoscopic therapy for variceal bleeding: [1]*n* (%)	27 (66)	72 (75)	0.34	0.89 (0.69-1.14)
Esophageal band ligation	25 (93)	64 (89)	0.59	
Cyanoacrylate injection	3 (11)	10 (14)	0.72	
The 72-h infusion of PPI after endoscopic therapy for bleeding peptic ulcers: *n* (%)	47 (29)	49 (30)	0.84	0.97 (0.69-1.35)
The 3-5 d infusion of vasoactive agent after endoscopic therapy for variceal bleeding: *n* (%)	27 (66)	73 (76)	0.22	0.87 (0.68-1.11)
Units of blood transfused:				
Before endoscopy	1.8 ± 1.4	1.8 ± 1.6	0.88	
During hospitalization	2.7 ± 0.2	2.9 ± 0.2	0.33	
Hospital stay (d)	5 (1-14)	4 (1-13)	0.84	
Hospital stay < 3 d: *n* (%)	85 (36)	106 (36)	0.95	0.99 (0.79-1.25)
Recurrent bleeding: *n* (%)				
Within 3 d	11 (5)	11 (4)	0.61	1.24 (0.55-2.81)
Within 7 d	22 (9)	17 (6)	0.13	1.60 (0.87-2.95)
Within 30 d	36 (15)	31 (11)	0.11	1.44 (0.92-2.25)
Emergency surgery: *n* (%)	1 (0.4)	4 (1.4)	0.26	0.31 (0.03-2.75)
Death within 30 d: *n* (%)	16 (7)	18 (6)	0.77	1.10 (0.57-2.11)

Categorical variables are presented as number and percentage, and continuous variables are presented as the mean ± SD or median and range when appropriate. CI: Confidence interval. [1]Some patients were treated with more than 1 endoscopic modality.

(Figure 3D). The cause of death in the elderly patients was profound shock at presentation caused by a spurting hemorrhage from the ulcer that failed endoscopic therapy (two) and refractory variceal bleeding after endoscopic band ligation (one). The deaths in the remaining 13 patients were related to their comorbid illnesses: nosocomial pneumonia (five), septicemia (two), spontaneous bacterial peritonitis (one), myocardial infarction (one), congestive heart failure (one), liver failure (two), and primary liver cancer (one). The causes of death in the young patients were profound shock at presentation caused by active variceal bleeding (five) and ulcer bleeding (one), which failed endoscopic intervention. Twelve patients died of their comorbid illnesses: nosocomial pneumonia (four), septicemia (three), renal failure as a consequence of massive gastrointestinal bleeding (four), and metastatic biliary cancer (one).

Prognostic Factors for Rebleeding and Death

By univariate analysis, an increased risk of rebleeding after endoscopic hemostasis was associated with the presence of endoscopic stigmata of recent bleeding ($P < 0.001$) and high blood transfusion requirement before the endoscopy ($P = 0.003$). The risk of rebleeding was not associated with age ⩾ 65 years ($P = 0.11$) or even with age ⩾ 85 years ($P = 0.53$), male gender ($P = 0.75$), the presence of comorbid illness ($P = 0.23$), the use of antiplatelet agents ($P = 0.78$), the presence of hemodynamic instability at presentation ($P = 0.19$), hematemesis ($P = 0.27$), hematochezia ($P = 0.75$), peptic ulcer bleeding ($P = 0.33$), variceal bleeding ($P = 0.14$), and medium to large variceal size ($P = 0.57$). In the multivariable Cox regression model, the number of blood transfusions before endoscopy and stigmata of recent bleeding remained significantly associated with rebleeding (Table 4).

In the univariate analysis, the following variables had a significant influence on patient survival within 30 d: the presence of hemodynamic instability at presentation ($P < 0.001$), liver cirrhosis ($P < 0.001$), disseminated malignancy ($P < 0.001$), variceal bleeding ($P < 0.001$), the total number of blood transfusions ($P = 0.02$), and the occurrence of rebleeding ($P < 0.001$). The risk of death was not significantly associated with age ⩾ 65 years ($P = 0.80$) or even with age ⩾ 85 years ($P = 0.13$), male gender ($P = 0.74$), hematemesis ($P = 0.09$), hematochezia ($P = 0.51$), and the endoscopic stigmata of recent bleeding ($P = 0.89$). In the multivariate analysis, the presence of hemodynamic instability at presentation, a background of liver cirrhosis or disseminated malignancy, a transfusion requirement during admission, and the development of rebleeding remained significantly associated with 30-d mortality (Table 4).

DISCUSSION

The current study shows that age is associated with a steep rise in the incidence of acute UGIB. The elderly patients had different sources and clinical presentations

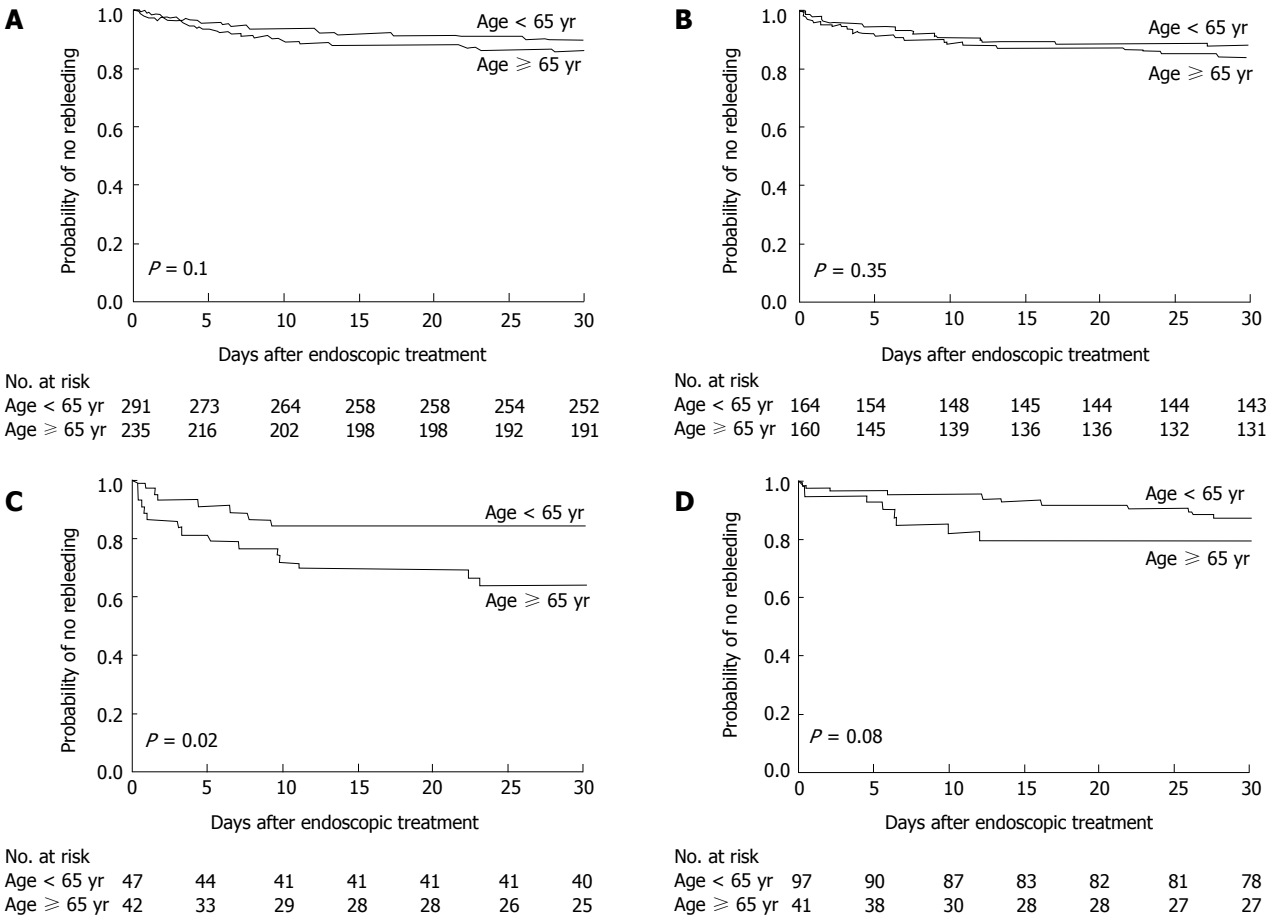

Figure 2 Kaplan-meier estimates of the likelihood that bleeding would not recur within 30 d after endoscopic treatment. A: Among all causes for bleeding; B: Among peptic ulcers as the source of bleeding; C: Among peptic ulcers with stigmata of recent bleeding; D: Among varices as the source of bleeding.

Table 4 Prognostic factors for rebleeding and mortality in acute upper gastrointestinal bleeding

Variable	Parameter estimate	Standard error	Hazard ratio (95% CI)	*P* value
Prediction for Recurrent bleeding				
Number of blood transfusions before endoscopy	0.22	0.07	1.25 (1.08-1.42)	0.003
Stigmata of recent bleeding	0.99	0.26	2.68 (1.58-4.41)	< 0.001
Prediction for Mortality				
Hemodynamic instability	0.95	0.50	2.57 (1.05-7.76)	0.04
Liver cirrhosis	1.33	0.62	3.77 (1.10-12.2)	0.03
Disseminated malignancy	1.8	0.65	6.06 (1.37-18.8)	0.02
Number of blood transfusions	0.11	0.03	1.12 (1.03-1.19)	0.004
Recurrent bleeding	1.62	0.39	5.07 (2.30-10.9)	< 0.001
Variceal bleeding	0.51	0.61	1.66 (0.55-5.79)	0.39

CI: Confidence interval.

of acute UGIB compared with the young patients. The risk for rebleeding correlated with endoscopic stigmata of recent bleeding and the severity of bleeding, as reflected by blood transfusion requirement, but not with advanced age. Furthermore, the elderly patients did not show a significant difference in clinical course from the young patients with regard to the utilization of endoscopic therapy, transfusion requirement, the duration of hospital stay, the need for surgery, the rate of rebleeding, and mortality.

Aging may result in various physiologic changes in the gastrointestinal tract[11], which may increase the risk for the development of acid-related disorders. Our study confirms that the incidence of acid-related bleeding increases with increasing age[12-14]. Consistent with previous studies, we found that approximately 80% of UGIB occurring in patients aged ⩾ 65 years is derived from acid-related disorders[12-15]. One of the major factors that

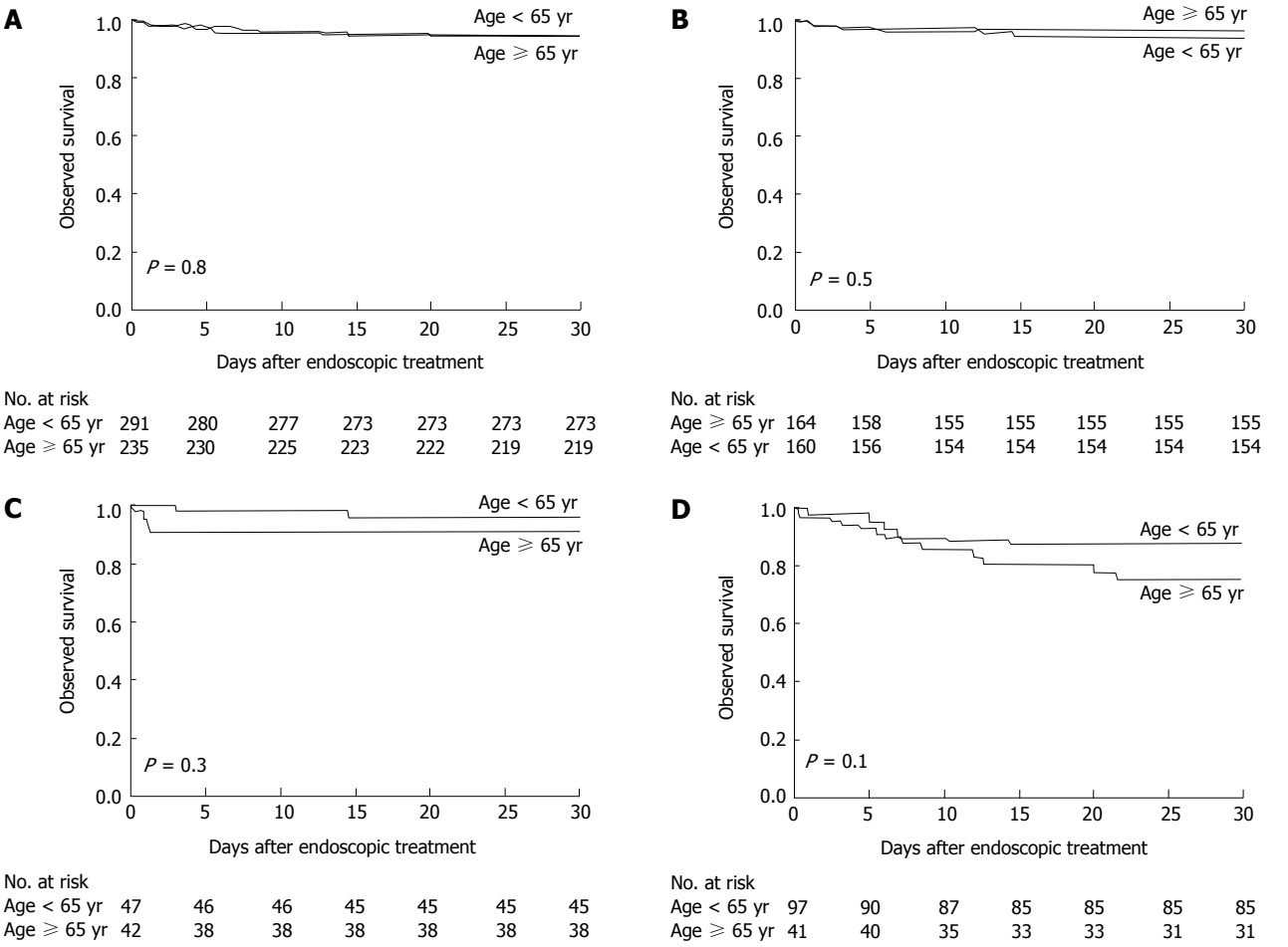

Figure 3 Kaplan-meier estimates of observed survival within 30 d after endoscopic treatment. A: Among all causes for bleeding; B: Among peptic ulcers as the source of bleeding; C: Among peptic ulcers with stigmata of recent bleeding; D: Among varices as the source of bleeding.

might explain this feature in the elderly population is the increased prescribing of gastroduodenal-damaging drugs, including aspirin, clopidogrel, and NSAIDs. As expected, 60% of our elderly population used either antiplatelet agents or a prescription NSAID, which was 2 times greater than that seen in the young population. Furthermore, some investigators have hypothesized that a mechanism underlying various manifestations of gastrointestinal bleeding in the elderly may involve ischemic damage to gastrointestinal mucosa[16,17]. In the current study, while direct measurements of visceral atherosclerosis were not available, we examined whether clinically recognized cardiovascular or cerebrovascular diseases were associated with the risk of ulcer-related bleeding. We found no association between clinical atherosclerotic disease and the risk of ulcer-related bleeding (data not shown).

Patients with acute UGIB typically present with vomiting of fresh blood or coffee ground-like material or the rectal passage of blood. Compared with young individuals, our elderly patients commonly presented with mild symptoms or subtle bleeding. One possible explanation could be that these patients were more likely to be hospitalized for non-life-threatening bleeding for close medical attention given their vulnerability and multiple comorbid diseases. Therefore, it is possible that patient comorbidity may result in the early recognition or management of UGIB rather than reflecting the etiology of the bleeding. Our experiences are in agreement with prior evidence that suggests an association between hospitalized gastrointestinal bleeding and poor health[16,18,19]. Moreover, we cannot exclude the possibility that the incidence of hemodynamic instability from UGIB was underestimated in this study because the use of beta blockers, which is common in the elderly who have multiple comorbid conditions, can mask tachycardia in patients with UGIB.

Patient risk stratification can be performed based on predictive factors for rebleeding, and resources can be allocated accordingly. There are numerous studies that have reported the predictive factors for rebleeding[2-6]. The current study confirms that endoscopic stigmata of recent bleeding is the most important predictor of rebleeding and influences other important end points such as transfusion requirement[20-23]. In contrast to prior studies, our data do not show a significant association between older age and the risk of rebleeding. It is possible that the current study included all patients with UGIB regardless of etiology in the analysis, and thus, the effect of stigmata of recent bleeding in elderly patients with peptic ulcers could have been diluted by the inclusion of a large number of patients with clean-based ulcers that

are at low risk of rebleeding. When we restricted our analysis according to the endoscopic findings, we found that age ⩾ 65 years was associated with an increase in the risk of ulcer rebleeding among those with high-risk ulcer stigmata. Although the reason for this observation was unclear, we hypothesize that it may be related to impaired hemostasis caused by platelet dysfunction because elderly patients are more likely to have received antiplatelet therapy or NSAIDs before admission. The late rebleeding seen in our elderly patients also suggests that there may be an unknown pathogenic process that adversely affects the healing of peptic ulcers (Figure 2C). In addition, there was a trend toward a higher rate of variceal rebleeding in elderly patients. A larger study size may be required to clarify the possible prognostic factor of older age for variceal rebleeding.

Despite advances in the management of UGIB during the past decade, the reported mortality for patients over 60 years of age with UGIB is 12%-25% and nearly 35% in those over 80 years of age[24,25]. The lack of change is probably explained by the associated comorbidities with increasing age. These patients are also more vulnerable to a physiological challenge from an acute bleeding episode. However, the mortality of our elderly cohort for acute UGIB was 7%, which was lower than those of previous reports. The leading cause of death in the elderly is sepsis followed by multiorgan failure. This study also reports a low rate of surgical intervention, which was seen in approximately 1% of all patients. The decrease in surgical requirement and mortality in our patients could reflect the increasing use of endoscopic hemostasis and likely underlines the systematic use of potent antisecretory agents for acid-related bleeding and vasoactive agents for variceal bleeding after therapeutic endoscopy. These therapeutic measures have been reported to improve the outcome of patients with UGIB[26,27].

Several bleeding scoring systems have been developed to predict the outcomes for patients with UGIB[2-6] and have shown that the risk for adverse outcomes increases when the patients are older. However, our study showed that age ⩾ 65 years did not influence the transfusion requirement, duration of hospital stay, need for surgery, and mortality. Furthermore, multivariate analysis showed that comorbid illnesses with liver cirrhosis or disseminated malignancy, severe bleeding represented by significant hemodynamic change requiring multiple blood transfusions and the development of rebleeding were significant predictive factors for mortality. These findings are consistent with the reported predictive models on mortality for UGIB in the literature[2-6].

Some factors may limit the generalizability of our findings. First, our patients awaiting endoscopy, who were suspected to have a high risk of ulcer or variceal rebleeding, received the preemptive use of high-dose intravenous PPI or vasoactive agents, respectively. It is possible that this management could influence endoscopic findings and the course of UGIB. Second, we cannot exclude referral bias, which may select patients with severe diseases. However, we would not expect the age-related differences in clinical presentation and the source of UGIB to be a large artifact of this bias. The similar outcome in young and elderly patients after endoscopic therapy indicates that a selection bias for severe disease does not have an adverse impact on treatment outcome.

In conclusion, the etiology of UGIB in the elderly has changed little in recent years. Despite multiple comorbidities and the concomitant use of antiplatelet therapy in elderly patients, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy. Morbidity and mortality from UGIB in the elderly are determined by the nature of the bleeding lesions and the presence of comorbid conditions. With the growth of older populations, a coordinated approach to diagnosis and management of acute UGIB should optimize favorable outcomes in this vulnerable patient population similar to those in younger people.

COMMENTS

Background

Upper gastrointestinal bleeding (UGIB) affects a substantial number of elderly individuals and is a potentially life-threatening clinical event. Advanced age has been considered a significant prognostic factor for adverse outcomes from acute UGIB; however, it remains unclear if this is due to the severity of disease or differences in the treatment received. A better understanding of the prognostic significance of age should enhance the accuracy during triage and could lead to the more efficient use of critical care resources for the management of acute UGIB.

Research frontiers

The aim of the research was to characterize the effects of age on clinical presentations and endoscopic diagnoses and to determine outcomes after pharmacologic and endoscopic therapy with regard to transfusion requirement, the duration of hospital stay, the need for surgical intervention, the rate of rebleeding, and 30-d mortality among a large cohort of patients aged ⩾ 65 years who were hospitalized for acute UGIB compared with those aged < 65 years.

Innovations and breakthroughs

In the current study, the authors demonstrated that the elderly patients had different sources and clinical presentations of acute UGIB compared with the young patients. The risk for rebleeding correlated significantly with endoscopic stigmata of recent bleeding and the severity of bleeding but not with advanced age. Furthermore, the clinical course did not significantly differ between the elderly and young patients with regard to the utilization of endoscopic therapy, transfusion requirement, the duration of hospital stay, the need for surgery, the rate of rebleeding, and mortality.

Applications

In conclusion, advanced age does not appear to influence adverse outcomes of acute UGIB after therapeutic endoscopy. The promising outcomes of acute UGIB in the elderly may be due in part to the increased involvement of acute care specialists during resuscitation, advances in diagnostic and therapeutic endoscopy, the use of powerful acid suppressive and vasoactive agents, and more selective and less invasive surgical approaches. Therefore, the authors recommend a coordinated approach to manage acute UGIB, which should serve to optimize favorable outcomes in this vulnerable patient population similar to those in young people.

Peer review

The authors did not discuss beta blockers usages in the patients. Beta blocker usages are common in the elderly who have multiple comorbid conditions. They select heart rate as one of a predictor of hemodynamic instability. Use of beta blockers can mask tachycardia in the patients with UGIB. This would underestimate the incidence of hemodynamic instability from UGIB in the study.

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BRIEF ARTICLE

Moxibustion activates mast cell degranulation at the ST25 in rats with colitis

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Abstract

AIM: To investigate the effects of moxibustion on the morphology and function of mast cells (MC) at Tianshu (ST25) in rats with trinitro-benzene-sulfonic acid (TNBS)-induced colitis.

METHODS: A total of 53 male Sprague-Dawley rats were randomly divided into a normal group and experimental group. In the experimental group, a rat model of TNBS-induced colitis was established, and the rats were then randomly divided into a model group, moxibustion group, moxibustion plus disodium cromoglycate (M + DC) group and moxibustion plus normal saline (M + NS) group. Rats in the moxibustion group received suspended moxibustion at bilateral ST25 for 10 min, once a day for 7 d. Rats in the M + DC and M + NS groups were pretreated with disodium cromoglycate and normal saline at bilateral ST25, respectively, and were then concurrently subjected to the same treatment as rats in the moxibustion group. The hematoxylin-eosin staining method was used to observe histology of the colon and the toluidine blue-improved method was used to observe mast cells at ST25 acupoint areas.

RESULTS: An improvement in colonic injury in the moxibustion group was observed and the degranulation ratio of MC at ST25 acupoint was markedly higher in the moxibustion group than in the model group (45.91 ± 11.41 *vs* 32.58 ± 8.28, $P < 0.05$). After inhibition of degranulation of MC at ST25 by disodium cromoglycate, no improvement in colon tissue injury was observed.

CONCLUSION: Moxibustion exerted its effect on healing impaired colonic mucosa in rats with TNBS-induced colitis by increasing the degranulation ratio of local MC, but had little effect on the morphology of MC at ST25 acupoint.

Key words: Disodium cromoglycate; Colitis; Mast cell; Moxibustion; ST25 acupoint

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INTRODUCTION

Acupuncture, one of the alternative or complementary therapies, is receiving increasing acceptance in Western medicine for the treatment of certain medical conditions[1-6]. Moxibustion, involving warm stimulation by moxa combustion at acupoint areas, is one of the acupuncture-moxibustion therapies for treating certain disorders in the clinic[7-10]. It was shown that the effective mechanism of acupuncture stimulation is closely related to the activation of mast cells (MC) at acupoint areas[11]. Acupoints have complex structures and are composed of nerve endings, plexuses, blood vessels, lymphatic vessels and connective tissues[11-14]. However, the correlation between the effect of moxibustion and the response of MC at acupoint areas is still unclear and merits further study.

MC are common effector cells and are widespread in connective tissues, especially in subcutaneous and submucosal loose connective tissues. MC act in several ways including changes in degranulation and the release of various bioactive mediators [5-hydroxytryptamine (5-HT), P substance (SP), heparin, and leukotriene][15-17]. It was reported that acupuncture stimulation markedly increased the density of local MC and activated MC degranulation at needle acupoints, which led to downstream effects in activating certain cellular pathways[11-14,18]. Pretreating the acupuncture point with disodium cromoglycate not only counteracted degranulation, it also reduced the effect of acupuncture[18].

Previous studies by our research team have indicated that moxibustion has a beneficial effect on inflammatory bowel disease (IBD)[8-10]. Moreover, we found that moxibustion at ST25 can heal impaired colonic mucosa in a rat model of colitis created by an immunological method associated with local stimulation. ST25 is the primary large intestinal meridian point of hand Yangming, which regulates the function of the large intestine, spleen, and stomach. ST25 is an efficacious point in the clinical treatment of patients with IBD.

In this study, we established a colitis rat model induced by trinitro-benzene-sulfonic acid (TNBS). The hematoxylin-eosin staining method was adopted for histological assessment of colonic mucosal injuries after moxibustion intervention and the toluidine blue-improved method was used to observe morphology and degranulation of MC at ST25 acupoint areas.

MATERIALS AND METHODS

Animals

Fifty-three male Sprague-Dawley rats (SPF class), weighing 100-140 g, were supplied by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (TCM), and randomly divided into a normal group (n = 11) and an experimental group (n = 42). All rats were housed at a constant temperature and humidity with free access to food and water. All studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the International Association and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

Establishment of the colitis rat model

An experimental colitis rat model was established according to the TNBS-induced method reported by Morris[19]. After weighing and administering anesthesia (1% sodium pentobarbital; i.p., 45 mg/kg), the 42 experimental rats were injected with TNBS/ethanol (100 mg/kg TNBS + 50% ethanol 0.25 mL) into the anus *via* a rubber tube; the solution was retained in the gut cavity at a depth of 6 cm-8 cm. Rats in the normal control group were given an enema with 0.9% NaCl of the same volume as given to the experimental rats. The rats were subsequently held upside down before removing the enema apparatus, and were kept in this position for 1 min to prevent the solution from flowing out.

After colitis was induced, one rat from the normal group and two rats from the model group were dissected to remove colon tissue. The tissue was stained with hematoxylin-eosin to confirm the establishment of the experimental colitis model. The remaining rats in the experimental group were then randomly divided into four groups: a model group, moxibustion group, moxibustion plus pretreated disodium cromoglycate (M + DC) group and moxibustion plus pretreated normal saline (M + NS) group.

Treatment

Location of ST25 acupoints in the rats were based on an anatomic method referenced in the "Map of Animal Acupoints" from Shi Yan Zhen Jiu Xue written by Lin WZ. In the moxibustion group, moxibustion was administered at bilateral ST25 acupoints using a fine moxa stick with the smoldering end 2 cm away from the acupoints for 10 min once daily for 7 d in total. In the M + DC group, bilateral ST25 acupoints were injected with disodium cromoglycate (55 mg/kg 0.2 mL; 0.1 mL for each acupoint) before moxibustion. In the M + NS group, bilateral ST25 acupoints were injected with normal saline (0.2 mL; 0.1 mL for each acupoint) before moxibustion.

Observation of colonic mucosa by hematoxylin eosine method

Following sacrifice of the animal and laparotomy, the inflamed segment of colon approximately 8 cm from the anus was removed, washed with iced saline, fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, dehydrated in 95%, 90% and 70% ethanol, cleared in xylene, mounted in Permount or Histoclad, and observed under a microscope.

Observation of MC at ST25 acupoints by the toluidine blue-improved method

Sequential paraffin slices 4-μm thick were prepared after 48 h of fixation at 4 ℃ in fixing solution (10% formalin).

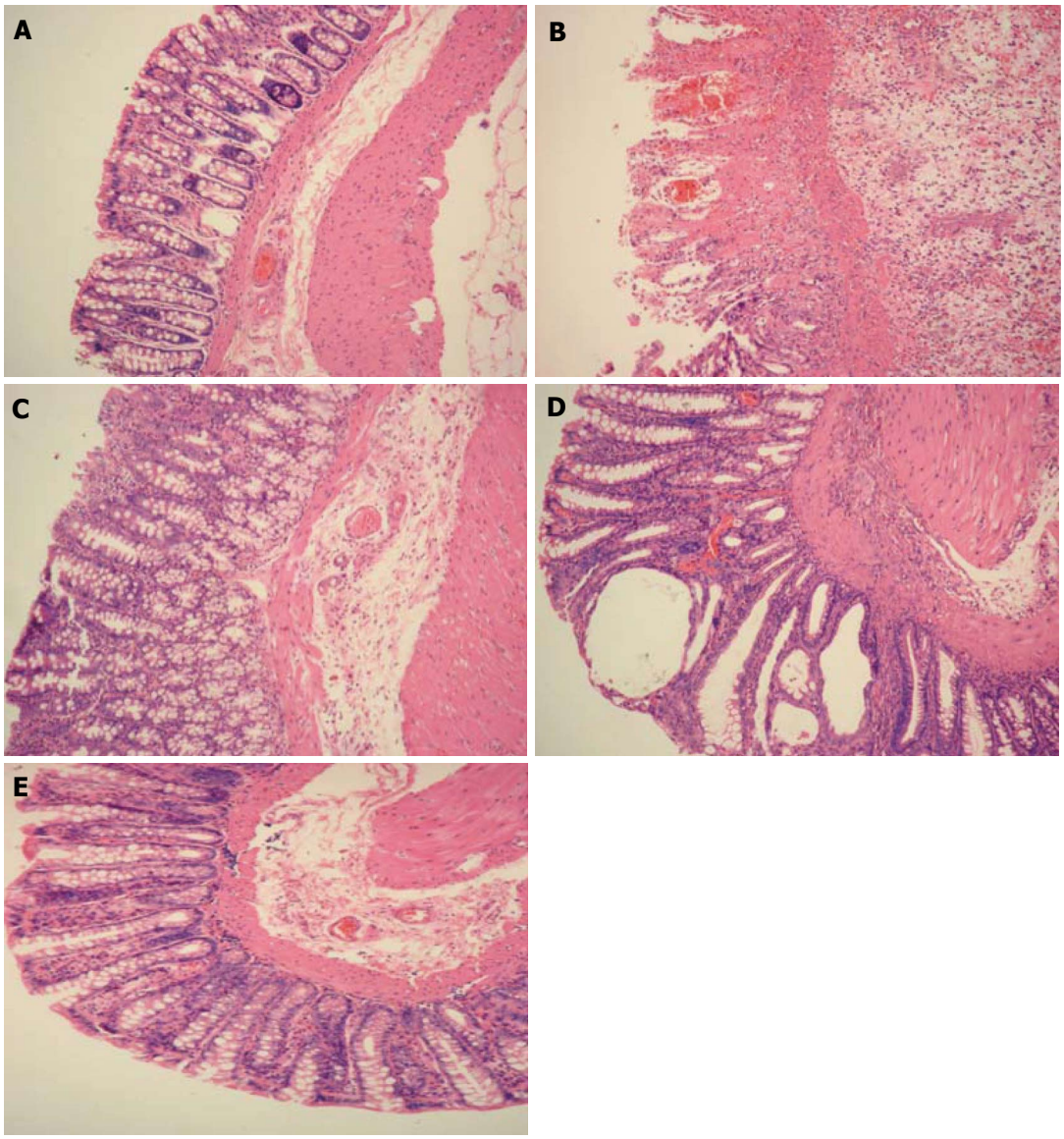

Figure 1 Results of hematoxylin eosine staining of rat colonic tissue (× 100). A: Normal group: the colonic mucosa was complete and the colonic gland was regularly arranged, with no apparent inflammatory cell infiltration; B: Model group: damage to colonic mucosa was found and there were monocytes and a large number of inflammatory cells infiltrating the mucosa or submucosa; C: Moxibustion group: the colonic gland was regularly arranged compared with the model group and ulceration was covered by regenerated epithelium; D: Moxibustion plus disodium cromoglycate group: slight congestion of colonic mucosa and fibroplasia of submucosa were found and a large number of infiltrating inflammatory cells, however, this was not as serious as in the model group; E: Moxibustion plus normal saline group: the colonic gland was regularly arranged and inflammatory cell infiltration of the submucosa was noted.

The subcutaneous tissue samples were stained with 0.5% toluidine blue. The numbers of MC per microscopic view (0.16 mm^2 at × 200 magnification) were counted at 4 areas per slice and then averaged. Mast cells with more than 3 granules outside the cell shape or with empty cavities in the cytoplasm were considered to be degranulated. The ratios of degranulated to total MC were calculated. Representative photomicrographs were obtained at a magnification of × 400 for morphological evaluation.

Statistical analysis

Experimental data were expressed as mean ± SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., United States). Differences in mean were compared by one way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Improvement of colonic ulceration in rats treated with moxibustion

In the stained colon tissue slices, colonic glands and caliciform cells were observed by light microscopy (Figure 1). In the normal group, the colonic mucosa epithelium was complete and the colonic gland was regularly arranged, with no apparent inflammatory cell infiltration (Figure 1A). In the model group, damage to colonic mucosa was observed, mucosal villi were damaged or missing, there was congestion and edema in the submucosa, the gland was damaged or missing, caliciform cells were reduced, monocytes or mast cells were present, a large number of infiltrating inflammatory cells was present in the mucosa

Table 1 Average diameter and size of mast cells at ST25 acupoint areas (mean ± SD)

Group	*n*	MC average diameter	MC average size
Normal	10	14.0266 ± 2.1240	163.9111 ± 51.3831
Model	10	14.6944 ± 2.8082	182.1338 ± 79.6975
Moxibustion	10	12.8357 ± 3.5726	148.2694 ± 88.3540
M + DC	10	15.2929 ± 1.5578	194.111 ± 35.6967
M + NS	10	14.2835 ± 1.5379	162.4508 ± 28.2383

MC: Mast cells; M + DC: Moxibustion plus disodium cromoglycate; M + NS: Moxibustion plus normal saline.

Table 2 Degranulation ratio of mast cells at ST25 acupoint areas (mean ± SD)

Group	*n*	MC total number	MC degranulation number	Degranulation ratio (%)
Normal	10	7.16 ± 1.27	1.22 ± 0.29	17.01 ± 4.11
Model	10	10.38 ± 2.52	3.44 ± 1.27[a]	32.58 ± 8.28[a]
Moxibustion	10	13.22 ± 4.40[a]	5.78 ± 1.97[a,b]	45.91 ± 11.41[a,b]
M + DC	10	10.14 ± 4.26	3.36 ± 1.64[a,c]	33.41 ± 9.56[a,c]
M + NS	10	12.24 ± 4.34[a]	5.12 ± 2.26[a,b]	42.41 ± 7.71[a,b]

MC: Mast cells; M + DC: Moxibustion plus disodium cromoglycate; M + NS: Moxibustion plus normal saline.. [a]$P < 0.05$ *vs* normal group; [b]$P < 0.05$ *vs* model group; [c]$P < 0.05$ *vs* moxibution group.

or submucosa, and ulceration was noted (Figure 1B). In the moxibustion group, the colonic gland was regularly arranged, ulceration was covered by regenerated epithelium, submucosal edema was found, and a small number of infiltrating inflammatory cells was observed (Figure 1C). In the M + DC group, the colonic gland was distended and irregularly arranged, slight congestion of colonic mucosa and fibroplasia of the submucosa were found, and a large number of infiltrating inflammatory cells was noted, however, this was not as serious as in the model group (Figure 1D). In the M + NS group, the colonic gland was regularly arranged, there was slight congestion of the colonic mucosa, and edema and inflammatory cells had infiltrated the submucosa (Figure 1E).

Effects of moxibustion on the morphology and function of MC

There were no significant differences among the groups in the average diameter and size of MC at ST25 acupoint areas (Table 1). The number of degranulated MC and the ratio of MC in the normal group were lower than those in the other groups ($P < 0.05$); the degranulation ratio of MC in the moxibustion group was greater than those in the model group and the M + DC group ($P < 0.05$); no significant difference was found in the degranulation ratio of MC between the moxibustion group and the M + NS group (Table 2, Figure 2).

DISCUSSION

It has been suggested that MC could play a primary role in the effective mechanism of moxibustion at acupoint areas. An improvement in colonic injury in the moxibustion group was demonstrated and the degranulation ratio of MC at ST25 acupoint areas was remarkably higher in the moxibustion group than in the model group, which indicated a correlation between MC and moxibustion effects. Following inhibition of the degranulation of MC at ST25 acupoints by injection of the MC stabilizer, disodium cromoglycate, we found that not only did this treatment counteract degranulation of MC, but there was no improvement in colon tissue injury after moxibustion. In addition, it was found that moxibustion had no obvious influence on the size of MC at ST25 acupoints, which further suggested that the degranulation of MC at ST25 acupoints could participate in the mechanisms of moxibustion therapy.

The degranulation of MC at acupoint areas has been confirmed to participate in the analgesic activity of acupuncture[20-22]. These studies revealed that there are many more MC degranulated at acupoints following acupuncture intervention. The shapes of the degranulated MC were irregular with a vague boundary and the granules in the cytoplasm were scattered and small in size, and some granules released by MC spread over the entire tissue space. It has been shown that the analgesic effect of acupuncture could be significantly attenuated by repression of the degranulation of MC at acupoint areas using sodium cromoglycate[23]. Kimura found that the region of moxibustion treatment instantly received a large number of immunocyte infiltrations, which consisted of lymphocytes, monocytes, some granulocytes and MC[24]. Menjo showed that there was immediate degeneration of the epidermal cell layer and increased amounts of MC were observed after moxibustion treatment[25]. In this study, we also demonstrated a correlation between the activation of MC at moxibustion acupoint areas and the effects of moxibustion in TNBS-induced colitis rats.

MC, as resident cells in human loose connective tissue, are usually found gathered around small vessels and collaterals, and are particularly rich at nerve endings and nerve plexuses, forming a complex system of intercellular communications[26-30]. The cytoplasts of MC are filled with metachromatic basophilic granules in which various bioactive mediators are resident. Under acupuncture and moxibustion stimulation, large quantities of these bioactive mediators are released by activated MC to interact with surrounding tissues，producing the original so-called Qi sensation[20,22,30-32]. These bioactive substances (histamine, SP and 5-HT) in the granules penetrate into the tissue spaces, and on the one hand, transmit to other MC through the tissue fluid in a direction flowing along the meridian line, which induces further degranulation of MC[33]; on the other hand, these bioactive substances directly stimulate peripheral nerve receptors or nerve endings causing neuraxial reflection, releasing substance P, which can induce MC degranulation, stimulating the adjacent nerve endings further. Moxibustion signals are integrated and modification occurs in different stages from acupoint areas to the center and target organs, by which the moxibustion effect is achieved and target organs adjust.

It has also been reported that there was a significant-

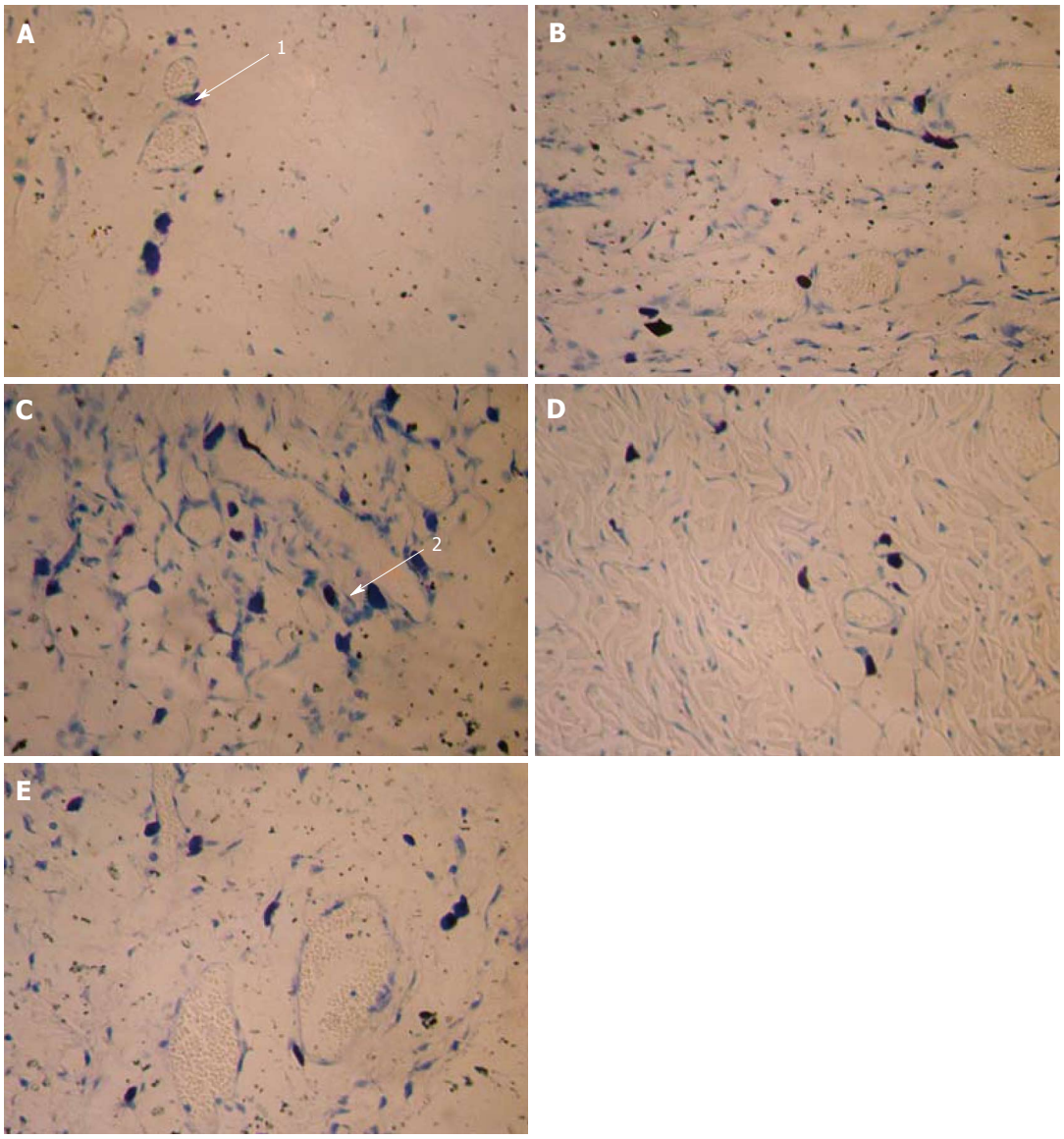

Figure 2 Results of the mast cells toluidine blue-improved method at ST25 (× 400). Normal group (A), model group (B), moxibustion group (C), moxibustion plus disodium cromoglycate group (D) and moxibustion plus normal saline group (E). Arrow 1: Intact mast cells (MC); Arrow 2: Degranulated MC. MC plasma stains purple, and nucleus is shown as dark blue, scattering in subcutaneous loose connective tissues, or gathering in a group or lining up; cell shape appears round, oval, shuttle-like, erose; small cells had little plasma and a clear shape, large cells had more plasma and an unclear shape.

ly different effect between acupuncture and moxibustion on the quantities of degranulated cells and the distribution of MC at acupoint areas. Furthermore, the effect of moxibustion is stronger than that of acupuncture, and this may be attributed to heat radiation and some chemical substances released from the burning of moxa, which possibly stimulates the MC at acupoint areas by the penetrating effect of moxibustion heat.

In conclusion, moxibustion stimulation may exert its effect on TNBS-induced colitis rats by triggering the degranulation of local MC at ST25 acupoints.

COMMENTS

Background

Previous studies on the effective mechanism of acupuncture stimulation show that it is closely related to the degranulation of mast cells (MC) at acupoint areas. The research has indicated that moxibustion stimulation has a beneficial effect on inflammatory bowel disease, and ST25 is an efficacious point in the clinical treatment of patients with inflammatory bowel disease. However, the correlation between the effect of moxibustion and the response of MC at acupoint areas is still unclear.

Research frontiers

With further study on the mechanism of moxibustion, more and more data show that MC at acupoints play an important role in bridging acupoint areas and target organs, which had become a the hot topic of study.

Innovations and breakthroughs

The results of the authors' study have proved that moxibustion is effective in TNBS-induced colitis rats. Moxibustion therapy exerts its effect on healing impaired colonic mucosa by triggering degranulation of local MC at ST25 acupoints.

Applications

The experimental data has important clinical significance and can be used in the further study of moxibustion therapy in the treatment of inflammatory bowel disease.

Peer review

This study investigated the relationship between the mast cell degranulation at the Tianshu (ST25) acupoint of moxibustion and the development of trinitrobenzene-sulfonic acid-induced colitis in rats. This is a study supported by The National Basic Research Program of China (973 program), etc, attempting to explore the mechanism of moxibustion. This kind of research would have important clinical significance and should certainly be encouraged. In general, this paper was well written.

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BRIEF ARTICLE

Sleeve gastrectomy prevents lipoprotein receptor-1 expression in aortas of obese rats

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Abstract

AIM: To investigate the effects of sleeve gastrectomy on adipose tissue infiltration and lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) expression in rat aortas.

METHODS: Twenty-four rats were randomized into three groups: normal chow (control), high fat diet (HD) and high fat diet with sleeve gastrectomy (SG). After surgery, the HD and SG groups were fed a high fat diet. Animals were sacrificed and plasma high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were determined. LOX-1 protein and LOX-1 mRNA expression was also measured. Aortas were stained with Nile red to visualize adipose tissue.

RESULT: Body weights were higher in the HD group compared to the other groups. HDL levels in control, HD, and SG groups were 32.9 ± 6.2 mg/dL, 43.4 ± 4.0 mg/dL and 37.5 ± 4.3 mg/dL, respectively. LDL levels in control, HD, and SG groups were 31.8 ± 4.5 mg/dL, 53.3 ± 5.1 mg/dL and 40.5 ± 3.7 mg/dL, respectively. LOX-1 protein and LOX-1 mRNA expression was greater in the HD group *versus* the other groups. Staining for adipose tissue in aortas was greater in the HD group in comparison to the other groups. Thus, a high fat diet elevates LOX-1 protein and mRNA expression in aorta.

CONCLUSION: Sleeve gastrectomy decreases plasma LDL levels, and downregulates LOX-1 protein and mRNA expression.

Key words: Sleeve gastrectomy; Morbid obesity; High fat diet; Aorta; Lipoprotein receptor-1 expression

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Bai J, Wang Y, Liu Y, Geng DH, Liu JG. Sleeve gastrectomy prevents lipoprotein receptor-1 expression in aortas of obese rats. *World J Gastroenterol* 2011; 17(32): 3739-3744 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i32/3739.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i32.3739

INTRODUCTION

Morbid obesity is a serious health problem worldwide. The incidence of diet-induced obesity in the United States has risen to 32%[1]. Approximately 127 million individuals are overweight, of which 60 million are obese and 8-10 million have morbid obesity with serious medical comorbidities, such as increased disability, morbidity

and early mortality[1-3].

Atherosclerosis is an important comorbidity of obesity that accounts for over 500 000 deaths annually in the United States. Diseases associated with atherosclerosis, such as myocardial infarction and stroke, account for the majority of deaths in industrialized countries. Atherosclerosis is a complex, multifactorial disease with both genetic and environmental determinants.

In clinical trials on atherosclerosis and hypertension, researchers found a direct association between the amount of weight loss and blood pressure reduction following a 36 mo weight loss intervention[4,5]. Prospective cohort studies have also found that the prevalence of atherosclerosis and hypertension decreases with weight loss[6-8]. Additionally, several researchers have found a positive relationship between weight gain and atherosclerosis[6,9-12].

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) is a type of oxidized low density lipoprotein (OX-LDL) receptor[13]. After binding with OX-LDL, LOX-1 can induce vascular smooth muscle cell migration to the tunica intima via extracellular signal-regulated kinase (ERK) activation. It can also promote vascular smooth muscle cell proliferation and increase lipid intake, and thereby pathological vascular changes that significantly affect the formation and progress of atherosclerotic disease[14].

Thus, we hypothesized that sleeve gastrectomy would result in weight loss, and thereby prevent LOX-1 protein and LOX-1 mRNA expression, as well as adipose tissue infiltration in the aorta.

MATERIALS AND METHODS

Twenty-four male, 8-week-old, Wistar rats weighing 180 g-200 g (Beijing Laboratory Animal Research Center, China) were acclimatized for 7 d, and then randomized into three groups: normal chow (control), high fat diet (HD) and high fat diet with sleeve gastrectomy (SG). The normal diet consisted of 10% kcal of fat (D12450B diet, Research Diets Inc, New Brunswick, NJ), whereas the high fat diet consisted of 60% kcal of fat (D12492 diet, Research Diets Inc, New Brunswick, NJ). Throughout the study, rats were kept in individual metabolic cages with a natural light/dark cycle, at a temperature of 18 ℃ ± 2 ℃ and humidity of 50% ± 2%.

Rats were anesthetized with an intraperitoneal injection of 300 mg/kg chloral hydrate and placed in the supine position on a surgical board with their extremities immobilized. An epigastric incision of approximately 1.5 cm-2 cm in length was made. The incision was kept open with a blade retractor, and the gastric omentum dissociated to reveal the gastric cardium. The gastric cavity was then closed with vascular clamps and cut off with a cauterizer, which also induced hemostasis. A gastric tube was made from the distal antrum (1.5 mm-2 mm from the pylorus) to the Hiss angle using an 8-0 unabsorbable suture. The fundus was completely removed (i.e., 70%-80% of total stomach). After the gastric tube was constructed, the peritoneal cavity was cleaned with saline and closed with a 6-0 silk suture. In the control group, a sham operation was performed as described above with the exception of the stomach incisions. All animals were given 5 mL of sterile, warmed saline subcutaneously to avoid dehydration, and allowed to recover from anesthesia and surgery. Rats were then returned to their home cages, and provided with food and water *ad libitum* 24 h after the surgery.

Following the surgery, rats in the HD and SG groups received a high fat diet for 30 d, whereas rats in the control group received normal chow. Body mass was checked in all rats prior to the operation and sacrifice. Thirty days after surgery, all rats were sacrificed and blood samples were collected to measure high-density lipoprotein (HDL) and low-density lipoprotein (LDL) using fast-phase liquid chromatography (FPLC) and their respective colorimetric assay kits.

Aortas were homogenized and centrifuged at 15 000 rpm at 4 ℃ for 15 min. Protein concentrations were determined with a protein assay (Thermo Fisher Scientific Inc., IL, United States). Forty micrograms of protein were separated by electrophoresis *via* a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Gels were then blotted onto nitrocellulose membranes, which were blocked with 5% skimmed milk for 1 h and then blotted overnight at 4 ℃ with Rbt polyclonal primary antibody (ab60178, Abcam, Unit 225A and 225B, 2/F Core Building 2, No. 1 Science Park West Avenue, Hong Kong Science Park, Shatin, N.T., Hong Kong). After blotting with goat anti-Rbt secondary antibody, immunecomplexes were visualized using an electrochemiluminescence Western blotting analysis system (FUJI film, United States).

Real-time quantitative polymerase chain reaction (PCR) analysis was carried out using an iQ5 Real-Time PCR Detection System (Bio-rad, CA, United States). The total amount of RNA used in reverse transcription was 1 μg. The following steps were performed to synthesize cDNA: samples were placed at 25 ℃ for 10 min, then 42 ℃ for 50 min, then 85 ℃ for 5 min, then chilled on ice, then 1 μL of *Escherichia coli* RNase H was added, and lastly the samples were incubated at 37 ℃ for 20 min. LOX-1 primers were as follows: sense: 5'-GACTGGATCTGGCATAAAGA-3'; antisense: 5'-CCTTCTTCTGACATATGCTG-3'.

GAPDH sequences were as follows: sense 5'-CACCCTGTGCTGCTCACCGAGGCC-3'; antisense 5'-CCACACAGATGACTTGCGCTCAGG-3'.

Real-time PCR parameters were as follows: 2 min at 50 ℃, 2 min at 95 ℃, followed by 40 cycles of 15 s at 95 ℃, 30 s at 55 ℃ and 30 s at 60 ℃. All measurements were performed in triplicate and each series of experiments was repeated twice. All quantifications were standardized to the amount of GAPDH amplification.

Aortas were taken out and frozen (Leica CM1850, Germany). A frozen slide (5 μm) was made, and stained immediately with 0.5 mL of Nile red (1 mg/mL) (Sigma

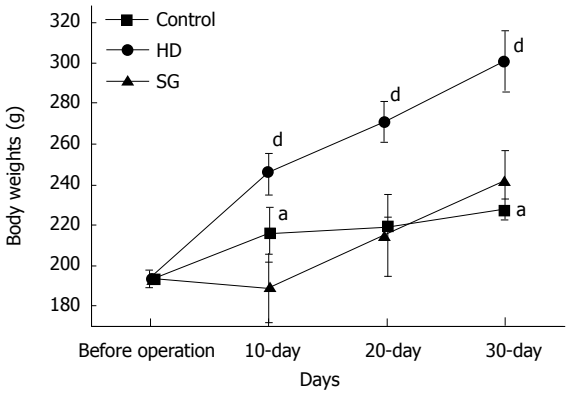

Figure 1 Body weights (g) of control, high fat diet, and high fat diet plus sleeve gastrectomy groups. $^{a}P < 0.05$ *vs* SG group; $^{d}P < 0.01$ *vs* other groups. SG: Sleeve gastrectomy; HD: High fat diet.

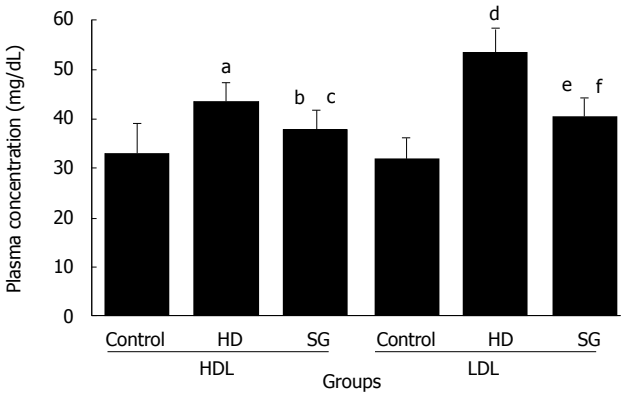

Figure 2 Plasma high-density lipoprotein and low-density lipoprotein concentration (mg/dL). $^{a}P < 0.01$ *vs* control; $^{b}P < 0.05$ *vs* control; $^{c}P > 0.05$ *vs* HD; $^{d}P < 0.01$ *vs* control; $^{e}P < 0.05$ *vs* control; $^{f}P < 0.01$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; HDL: High density lipoprotein; LDL: low density lipoprotein.

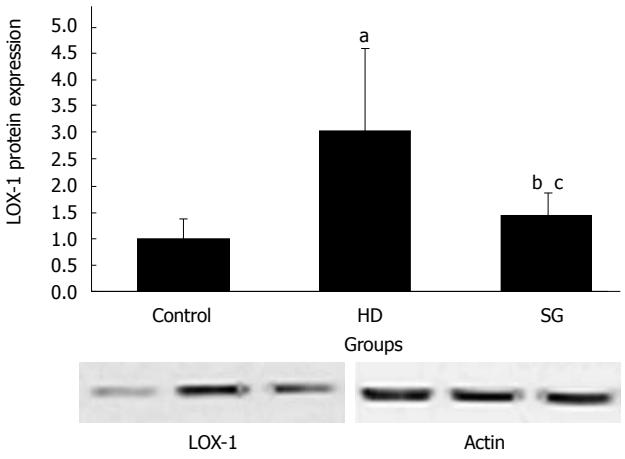

Figure 3 Lipoprotein receptor-1 protein expression compared to control levels. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; LOX-1: Lipoprotein receptor-1.

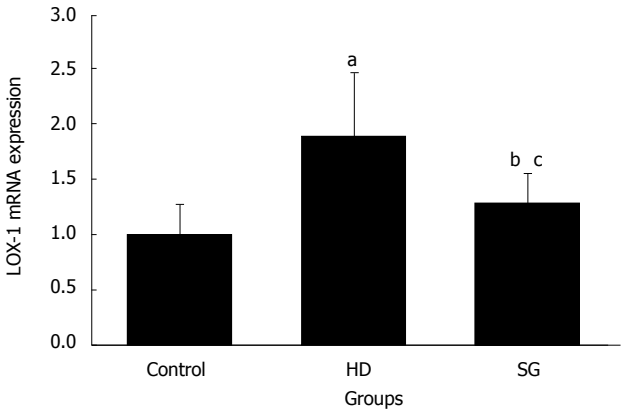

Figure 4 Lipoprotein receptor-1 mRNA expression compared to control levels. $^{a}P < 0.01$ *vs* control; $^{b}P > 0.05$ *vs* control; $^{c}P < 0.05$ *vs* HD. HD: High fat diet; SG: Sleeve gastrectomy; LOX-1: Lipoprotein receptor-1.

Co, St Louis, MO, United States) for 5 min in the dark[15]. Fluorescence microscopy (Olympus IX51 10X10, Japan) was used to visualize adipose tissue in the slides.

RESULTS

All of the rats survived and recovered from the gastrectomy. Body weights were significantly higher in the HD group compared to the control and SG groups ($P < 0.05$) (Figure 1). HDL levels in control, HD and SG groups were 32.9 ± 6.2 mg/dL, 43.4 ± 4.0 mg/dL and 37.5 ± 4.3 mg/dL, respectively. However, there were no statistical differences between the HD and SG groups ($P > 0.05$). LDL levels in the control, HD and SG groups were 31.8 ± 4.5 mg/dL, 53.3 ± 5.1 mg/dL and 40.5 ± 3.7 mg/dL, respectively (Figure 2). There was a statistically significant difference in LDL between the HD and SG groups ($P < 0.01$). LOX-1 protein expression in the HD and SG groups was 3.0 ± 1.6-fold and 1.5 ± 0.4-fold higher compared to control (Figure 3). There was a statistically significant difference between the HD and SG groups ($P < 0.05$). Furthermore, LOX-1 mRNA expression was 1.9 ± 0.6-fold and 1.3 ± 0.3-fold greater in the HD and SG groups versus control (Figure 4). There was a statistically significant difference between the SG and HD groups ($P < 0.05$). Nile red staining of control, HD and SG aortas is illustrated in Figures 5.

DISCUSSION

Obesity is associated with low-grade inflammation[16,17], which has been shown to be an initiating factor in endothelial dysfunction and atherosclerosis, and thus may cause arterial stiffness[17,18].

A direct association between the amount of weight loss and blood pressure reduction has been reported in some clinical trials[4,5]. Prospective cohort studies have also found that the prevalence of atherosclerosis and hypertension decreases with weight loss[6-8]. Additionally, several researchers have found a positive association between weight gain and obesity with atherosclerosis and hypertension[6,9-12].

Pro-inflammatory cytokines may play a role in the development of insulin resistance, which can be reversed by anti-inflammatory agents. These findings suggest that inflammation may be directly involved in the pathogenic

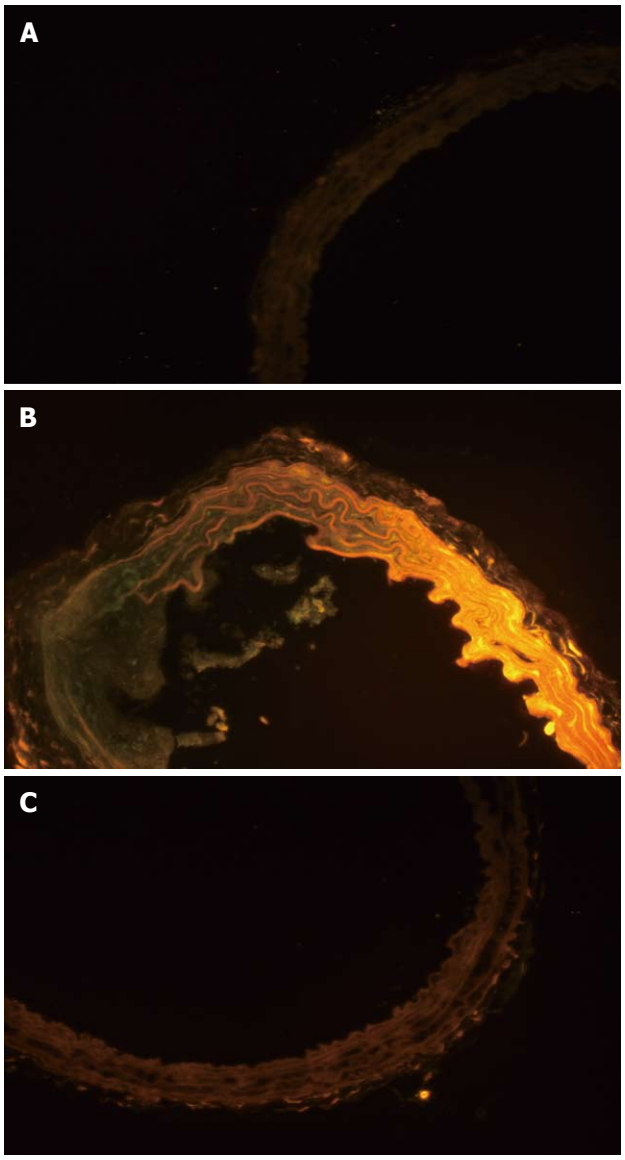

Figure 5 Nile red staining of an aorta from the three groups (× 100). A: Nile red staining of an aorta from the control group; B: Nile red staining of an aorta from the high fat diet group; C: Nile red staining of an aorta from the sleeve gastrectomy group.

properties of cytokines. Evidence suggests that both macronutrient intake and obesity may activate inflammatory signaling pathways in cells[19].

In the present study, LOX-1 protein expression in the aorta was upregulated in the HD group, and was prevented by gastrectomy in the SG group. Furthermore, LOX-1 mRNA expression was downregulated in the SG group *versus* the HD group. LOX-1 is a major receptor of ox-LDL and LDL in the vascular endothelium. The role of LOX-1 in atherogenesis is supported by several lines of evidence. LOX-1 demonstrates a strong affinity for binding, internalizing and degrading OX-LDL[20]. The oxidized form of LDL (OX-LDL) is thought to be more important in atherogenesis than the native LDL form[21]. OX-LDL injures the endothelium and is an important mediator in atherogenesis[22]. OX-LDL activates LOX-1 and induces endothelial dysfunction and apoptosis[23,24]. There are other mediators of atherosclerosis, such as angiotensin Ⅱ, cytokines, sheer stress and advanced glycation end-products, that upregulate LOX-1. Furthermore, LOX-1 is dynamically upregulated by pro-atherogenic conditions, such as diabetes, hypertension and dyslipidemia. LOX-1 is present in atheroma-derived cells, and in human and animal atherosclerotic lesions[25-28].

To date, surgery has been proven to be the only effective method for treating morbid obesity[29,30]. Observational studies suggest that weight-loss surgery is associated with a 60% to 80% diabetes remission rate in severely obese individuals, and that earlier interventions are more likely to provide remission[31]. Additionally, there are concerns regarding the lack of evidence, as well as the safety, invasiveness, and cost-effectiveness of such surgical weight-loss procedures. Providing appropriate evidence has been problematic due to the invasive nature of the surgery, which makes recruitment difficult. However, with the advent of safer, less invasive surgical weight-loss procedures, randomized clinical trials are now feasible.

Sleeve gastrectomy, a type of bariatric surgery, was performed in this experiment. In the SG group, body weights were significantly lower than those of the HD group. As a result, LOX-1 protein and mRNA expression levels, as well as LDL levels, were significantly lower in the SG group *versus* the HD group. SG is a type of purely restrictive surgery, where a moderate restriction is created, while the integrity of the duodenum, pylorus, antrum, lesser curvature and vagal nerve, and a relatively normal eating behavior, are maintained. Recent findings also suggest that SG might be a safe, beneficial, and effective stand-alone approach[32-34]. Moon *et al* reported that SG resolves all comorbidities of obesity in over 90% of subjects over a 12-mo period, with the exception of dyslipidemia, which is resolved in 65% of subjects[33]. Moreover, there was a dramatic loss of appetite in more than half of the patients postoperatively[33]. Karamanakos *et al*[35] found that SG preserved the integrity of the pylorus and did not induce an intestinal bypass. Furthermore, LDL levels, as well as liver enzymes, were decreased significantly in SG patients.

In summary, a high fat diet elevates LOX-1 protein and mRNA expression in the aorta. Sleeve gastrectomy can prevent increases in plasma LDL levels, as well as an upregulation in LOX-1 protein and mRNA expression associated with a high fat diet.

ACKNOWLEDGMENTS

The authors wish to thank Professor Yun Yu for revising this manuscript.

COMMENTS

Background

Morbid obesity is a serious health problem worldwide. Atherosclerosis is an important comorbidity of obesity. Diseases associated with atherosclerosis, such as myocardial infarction and stroke, account for the majority of deaths in industrialized countries. Studies have found a direct association between the amount of weight loss and the prevalence of atherosclerosis. Lectin-like

oxidized low density lipoprotein receptor-1 (LOX-1) is a type of oxidized low density lipoprotein (OX-LDL) receptor. After binding with OX-LDL, LOX-1 can affect the formation and progress of atherosclerotic disease. Thus, the authors hypothesized that sleeve gastrectomy would result in weight loss, and thereby prevent LOX-1 protein and LOX-1 mRNA expression, as well as adipose tissue infiltration in the aorta.

Research frontiers

The hotspot about this paper is the treatment of atherosclerosis in obese animals after bariatric surgery.

Innovations and breakthroughs

It was difficult to control morbid obesity and its comorbidities, such as atherosclerosis and non-alcoholic steatohepatitis, before bariatric surgery was used in the clinic. This kind of surgery can decrease body weight and reverse many comorbidities caused by obesity.

Applications

These results could expand the indication of bariatric surgery in the clinic and many obese patients with atherosclerosis could undergo surgery in order to decrease body weight and cure atherosclerosis.

Terminology

Bariatric surgery: Bariatric surgery, or weight loss surgery, includes a variety of procedures performed on people who are obese. Weight loss is achieved by reducing the size of the stomach with an implanted medical device (gastric banding) or through removal of a portion of the stomach (sleeve gastrectomy or biliopancreatic diversion with duodenal switch) or by resecting and re-routing the small intestines to a small stomach pouch (gastric bypass surgery); Atherosclerosis: Atherosclerosis (also known as arteriosclerotic vascular disease) is a condition in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels; a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells and promoted by low-density lipoproteins (plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins. It is commonly referred to as a hardening or furring of the arteries.

Peer review

With great interest I have read the article entitled: "Sleeve gastrectomy prevents LOX-1 expression of aortas in obese rats". This is a well-performed study with some interesting findings concerning the influence of obesity surgery on atherosclerosis.

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BRIEF ARTICLE

Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of Toll-like receptor (TLR) 3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma (ESCC).

METHODS: Reverse transcription-polymerase chain reaction and immunohistochemistry were used to analyze the expression of TLR3, TLR4, TLR7 and TLR9 mRNA and protein in samples from 87 esophageal cancer patients consisting of both tumor and normal tissue.

RESULTS: A significant increase in TLR3, TLR4, TLR7 and TLR9 mRNA levels was detected in ESCC samples. Tumors exhibited high TLR protein expression, (70.1%, 72.4%, 66.7% and 78.2% for TLR3, TLR4, TLR7 and TLR9, respectively, $P < 0.05$). Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells (48.3%) and TLR9 expression in fibroblast-like cells (60.9%). Tumors with high TLR3 expression in tumor cells or high TLR4 expression in mononuclear inflammatory cells were significantly associated with a higher probability of lymph node metastasis and increased depth of invasion. However, tumors with high TLR9 expression in fibroblast-like cells were associated with low probabilities of invasion and metastasis. There was no significant variation between the expression of TLR3, TLR4, TLR7 and TLR9 among different ethnic groups.

CONCLUSION: TLR3, TLR4, TLR7 and TLR9 expression appears important to the biological pathogenesis of ESCC. TLRs may represent therapeutic targets for ESCC.

Key words: Esophageal squamous cell carcinoma; Invasion; Metastasis; Prognosis; Toll-like receptor

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INTRODUCTION

Esophageal carcinoma (EC) remains a major threat to health worldwide, with a 5-year survival rate below 10%, and in China, EC is characterized by its distinct geographic distribution and differences in ethnic preva-

lence[1]. Xinjiang, in Western China, has one of the highest prevalences of esophageal squamous cell carcinoma (ESCC) in the world, and the ratio in ESCC incidence between different ethnic groups is as large as 13.4:1. ESCC has become the main cause of tumor-related deaths in the Kazak ethnic group in Xinjiang[2]. Despite advances in clinical treatment, ESCC prognosis remains poor due to its relapse and metastasis characteristics. For these reasons, prognostic factors are essential to improve the classic risk classification in ESCC.

Chronic infection and inflammation can induce cancer formation *via* cytokines and chemokines, which play vital roles in promoting angiogenesis and metastasis, the most important factors contributing to cancer development and growth. Toll-like receptors (TLRs) comprise an important family of pattern recognition receptors that allow immune cells to recognize pathogens and trigger inflammatory responses, as they are expressed not only in a variety of immune cells but also in non-immune cells such as fibroblasts and epithelial cells. These responses include the secretion of cytokines that increase the resistance of infected cells as well as the release of chemokines that recruit immune cells to necrotic cells. Chronic inflammation can promote carcinogenesis by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation. Research has demonstrated that basement membrane changes induced by chronic inflammation are correlated with the aberrant proliferation of esophageal epithelia[3]. The TLR signaling pathway activates several different signaling elements, including nuclear factor kappa B (NF-κB), extracellular signal regulated kinase, and Jun-NH-kinase/p38, which regulate many immunologically related proteins[4]. Several researchers also found that MyD88 (the TLR-mediated signaling adapter protein) plays an important role not only in the pathway of TLR-mediated inflammation but also in Ras-MAPK signaling, cell-cycle control, and cell transformation, which promote carcinogenesis[5,6]. Evidence indicates that TLR expression in tumor cells can promote inflammation and cell survival in the tumor microenvironment[7-9]. These results suggested that TLR stimulation could lead to tumor progression. These findings may be useful in elucidating potential prognostic markers.

The purpose of the present study was to investigate the expression of TLR3, TLR4, TLR7 and TLR9 in ESCC as well as their association with the clinicopathologic characteristics of ESCC. To this aim, we analyzed the protein levels of TLR3, TLR4, TLR7 and TLR9 by immunohistochemical techniques and their mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Clinical samples

A total of 87 formalin-fixed and paraffin-embedded tissue blocks were obtained from esophageal carcinoma patients who had not received pre-operative radiotherapy or chemotherapy; all patients were treated at the Department of Thoracic Surgery of the First Affiliated Hospital in Medical University of Xinjiang from June 2007 to March 2009, and the borderline tumor tissues were used as controls. Patient ethnicity was as follows: Han, 30 patients; Uyghur, 25 patients; and Kazak, 32 patients. The mean age of the patients was 50.5 years; the youngest patient was 39 years old, and the oldest patient was 73 years old at the time of surgery. Each specimen was histologically examined, and the tumor was graded by at least two experienced pathologists. The main characteristics of ESCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the TNM (American Joint Committee on Cancer, 4th edition) as follows: (1) 20 cases; (2) 34 cases; and (3) 33 cases. Among the 87 tumors were 30 well-differentiated tumors, 29 moderately differentiated tumors, and 28 poorly differentiated tumors. Fifty-three patients had lymph node metastases. In addition, 40 frozen biopsies that included 20 normal esophageal epithelia and 20 ESCC samples were subjected to RT-PCR for the detection of TLR3, TLR4, TLR7 and TLR9 mRNA expression. All patients were enrolled by written informed consent, and the study was approved by the Ethical Committee of the Medical University of Xinjiang.

Immunohistochemical studies

Sections of 3-μm-thick paraffin-embedded tissue were deparaffinized in xylene and then rehydrated in a graded ethyl alcohol series (100%, 95%, 80% and 70%). For increased specificity and sensitivity, tissues were microwave-treated at 95 ℃ for 15 min to retrieve the antigen. After cooling and rinsing in distilled water, endogenous peroxide activity was blocked with 3% H_2O_2 for 15 min, after which samples were rinsed in 0.01 mol/L phosphate-buffered saline [phosphate buffered saline (PBS), pH 7.4] for 10 min and then preincubated with a protein blocking solution for 10 min. Primary antibodies (mouse monoclonal anti-human TLR3, TLR4, TLR7 and TLR9 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, United States). Antibodies were diluted at 1:200 in PBS and applied at 4 ℃ overnight in a humid chamber. Slides were washed three times in PBS and then incubated with secondary biotinylated antibody for 15 min at room temperature. Antigen-antibody complexes were detected using the streptavidin-peroxidase method (15-min exposure) with diaminobenzidine [diaminobenzidine (DAB)] as the chromogen substrate (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, United States). The peroxidase signal was visualized by treatment with a DAB substrate-chromogen system for 8 min. Finally, the sections were stained lightly with hematoxylin, and PBS was used in place of the primary antibody as a negative control. All immunostained sections were coded and independently examined by two investigators using light microscopy. The results were scored on a scale from 0 to 4 for the percentage of positive cells and from 0 to 3 for the intensity of positive cells. The percentage of positive cells was scored as follows:

Table 1 Polymerase chain reaction primers used for the detection of Toll-like receptors

TLR	Forward	Reverse
TLR3	AGTGCCGTCTATTTGCCACACA	AACAGTGCACTTGGTGGTGGAG
TLR4	TCTTCAACCAGACCTCTACATTCCA	GGAACATCCAGAGTGACATCACAG
TLR7	CCGTGACAATTACCTGGCCTTC	CAGGGCCTTCAGCTGGTTTC
TLR9	AGGATGATGCCAGGATGATGTC	TCAGGTCCAGGTTCTTGGTTGAG
β-actin	GGCACCCAGCACAATGAAG	CCGATCCACACGGAGTACTTG

TLR: Toll-like receptor.

≤ 10%, 11%-25%, 26%-50%, 51%-75%, and ≥ 76%. The intensity of staining was scored as follows: absent, weak staining, moderate staining, and intense staining. The overall score (percentage of stained cells ×intensity of staining) was then used to identify the mean score by using an Excel spreadsheet [≥ mean score for positive (+); < mean score for negative (-)], in line with a previous study[10].

RNA extraction and RT-PCR

Total RNA was extracted from fresh frozen tissue using TRIzol (Invitrogen, Carlsbad, CA, United States) as described by the manufacturer. mRNA was reverse-transcribed with RevertAid (MBI Fermentas, Burlington, Ontario, Canada) at 42 ℃ for 60 min, and the synthesized cDNA (20 ng) was subjected to polymerase chain reaction (PCR) (95 ℃ for 1 min, 25 or 30 cycles of 95 ℃ for 3 s, 60 ℃ for 30 s, and 68 ℃ for 1 min, and a single extension at 68 ℃ for 10 min). PCR products were separated on a 4% agarose gel and visualized with ethidium bromide. Each analysis was repeated at least twice to ensure reproducibility. mRNA for β-actin was used as a normalization control in RT-PCR and as a loading control in conventional PCR. Forward and reverse primer pairs are listed in Table 1, and their products were 181 bp, 198 bp, 172 bp and 97 bp.

Statistical analysis

All statistical analyses were performed with the SPSS statistical software package (version 15.0). The chi-square test was used to compare the differences in cumulative TLR3, TLR4, TLR7 and TLR9 expression between normal and ESCC groups, and to determine whether the clinicopathologic variables were associated with the levels of TLR3, TLR4, TLR7 and TLR9 as well as compare the mRNA expression in fresh frozen ESCC tissues with that of normal samples as determined by RT-PCR. P values < 0.05 were considered statistically significant.

RESULTS

TLR protein expression in ESCC and their association with ESCC clinicopathologic characteristics

Immunohistochemistry (IHC) staining of 87 primary ESCC lesions and normal esophageal tissues was performed using anti-TLRs antibodies (Table 2). Representative staining patterns for TLRs are shown in Figure 1. IHC staining demonstrated that TLRs were localized in the cytoplasm, but TLR3 was also expressed in the cell membrane. Positive staining for TLR4 and TLR9 was generally observed within normal esophageal surface epithelium, but weak or no TLR4 and TLR9 staining was detected in stromal cells. However, in ESCC, TLRs were strongly expressed not only in cancer cells, but also in some stromal cells, such as fibroblast-like cells and mononuclear inflammatory cells. The positive rates of TLR3, TLR4, TLR7 and TLR9 expression in the normal esophageal surface epithelium were 8.0%, 5.7%, 9.2% and 4.6%, respectively. These values sharply increased to 70.1%, 66.7%, 72.4% and 78.2%, respectively, in ESCC lesions (P < 0.05 compared with the positive rate in healthy tissue). Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells (48.3%) and TLR9 expression in fibroblast-like cells (60.9%). Table 3 summarizes the percentages of TLR staining in each cellular type. TLR3 and TLR7 were mainly expressed in esophageal tumor cells, and there was a statistically significant difference compared with the expression in the control group.

We also evaluated the possible relationship between the expression of TLRs in tumor cells and the clinicopathologic characteristics of ESCC including tumor stage, histological grade, lymph node metastasis, and depth of invasion. TLR3 expression in tumor cells was significantly associated with depth of invasion and lymph node metastasis. TLR4 expression in tumor cells was significantly associated with lymph node metastasis. TLR7 expression in tumor cells was significantly associated with tumor grade. TLR9 expression was found to gradually increase with worsening histopathological grade (P < 0.005, Table 2). However, the TLR9 IHC staining scores did not correlate with the depth of invasion and lymph node metastasis.

We analyzed the association between the expression of TLR4 and TLR9 in tumor stromal cells and poor prognostic indicators because a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells. We found that carcinoma patients with higher TLR4 expression in the stromal compartment had a significantly higher risk of disease progression. TLR4 expression in mononuclear inflammatory cells (48.3%) was significantly associated with the depth of invasion and lymph node metastasis. Conversely, TLR9 expression in fibroblast-like cells (60.9%) was significantly associated with reduced depth of invasion and lymph node metas-

Table 2 Statistical analysis of Toll-like receptor expression and clinicopathologic factors in esophageal carcinoma

Characteristics	TLR3 Positive (%)	*P*	TLR4 Positive (%)	*P*	TLR7 Positive (%)	*P*	TLR9 Positive (%)	*P*
Normal control (*n* = 87)	7 (8)		5 (5.7)		8 (9.2)		4 (4.6)	
Tumor differentiation	61 (70.1)		63 (72.4)		58 (66.7)		68 (78.2)	
W (*n* = 30)	22 (75.3)	0.889	22 (75.3)	0.539	14 (46.7)	0.003	18 (60.0)	0.004
M (*n* = 29)	20 (69.1)		19 (65.6)		19 (65.6)		23 (79.3)	
P (*n* = 28)	19 (67.9)		22 (78.6)		25 (89.3)		27 (96.4)	
Depth of invasion								
≤ muscularis (*n* = 37)	18 (48.6)	< 0.001	27 (73.1)	0.92	23 (62.2)	0.002	27 (73.1)	0.314
≥ adventitia (*n* = 50)	43 (86.0)		36 (72.0)		35 (66.0)		41 (82.0)	
LN metastasis								
Negative (*n* = 34)	18 (52.9)	0.005	20 (58.8)	0.023	26 (76.5)	0.12	29 (85.3)	0.197
Positive (*n* = 53)	43 (81.1)		43 (81.1)		32 (60.3)		39 (73.6)	
Ethnic groups								
Han (*n* = 30)	23 (76.7)	> 0.05	22 (73.3)	> 0.05	17 (56.7)	> 0.05	23 (76.7)	> 0.05
Uyghur (*n* = 25)	18 (72.0)		20 (80.0)		16 (64.0)		20 (80.0)	
Kazak (*n* = 32)	20 (62.5)		21 (65.6)		25 (78.1)		25 (78.1)	

All values are presented as the number of cases, with percentages in parentheses. W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated; LN: Lymph node; TLR: Toll-like receptor.

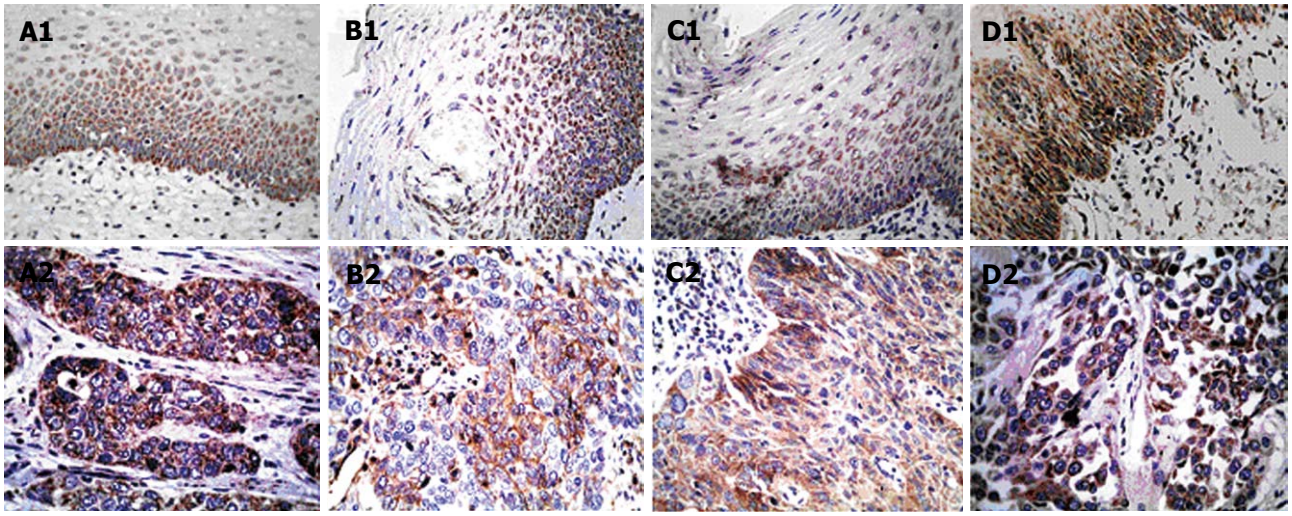

Figure 1 Immunohistochemistry staining of esophageal lesions with Toll-like receptor-specific mAbs. A1 to D1 show the expression of Toll-like receptor (TLR) 3, TLR4, TLR7 and TLR9 in normal esophageal epithelium, respectively. A2 and C2 show positive staining for TLR3 and TLR7 in esophageal squamous cell carcinoma cells. B2 shows positive TLR4 staining in tumor cells and mononuclear inflammatory cells, and D2 shows positive TLR9 staining in tumor cells and fibroblast-like cells (Original magnification, × 400).

Table 3 The percentage expression of Toll-like receptors in each cellular type within esophageal squamous cell carcinoma tissues

Factors	Tumor cells Positive cases (%)	Fibroblast Positive cases (%)	MICs Positive cases (%)
TLR3	61 (70.1)	3 (3.4)	4 (4.6)
TLR4	63 (72.4)	8 (9.2)	47 (48.3)
TLR7	58 (66.7)	5 (5.7)	2 (2.3)
TLR9	68 (78.2)	53 (60.9)	12 (13.8)

All values are presented as the number of cases, with percentages in parentheses. MICs: Mononuclear inflammatory cells; TLR: Toll-like receptor.

tasis (Table 4). We also observed variations between the expression of TLR3, TLR4, TLR7 and TLR9 in tumor cells among different ethnic groups in Xinjiang, although the differences were not statistically significant.

TLR mRNA expression in ESCC and normal controls

To confirm the IHC results, TLR3, TLR4, TLR7 and TLR9 mRNA expression in esophageal biopsies was detected by RT-PCR (Figure 2). Similar to the IHC results, TLR3, TLR4, TLR7 and TLR9 mRNA expression was increased in ESCC tissues. TLR3 and TLR7 gene expression was quantified in 15 ESCC and 3 normal esophageal tissues. TLR4 mRNA expression was higher in ESCC samples than in normal controls after normalization to β-actin expression. The percentages of TLR9 mRNA positivity in ESCC and normal tissues were 55% and 15%, respectively. Although the sample size was limited,

Table 4 Analysis of the relationship between the expression of Toll-like receptors in each cellular type with the clinicopathologic characteristics of esophageal squamous cell carcinoma *n* (%)

Factors	Tumor Grade			Depth of invasion		LN metastasis	
	W	M	P	⩽ muscularis	⩾ adventitia	Negative	Positive
Number of cases	30	29	28	37	50	34	53
TLR4 MICs (+)	19 (63.3)	16 (55.2)	12(42.9)	12(32.4)	35(70)	14(41.2)	33(62.3)
P		0.245		0.001		0.018	
TLR9 fibroblast (+)	20 (66.7)	18 (62.1)	17(60.7)	30(81.1)	23(43.4)	29(85.3)	24(45.3)
P		0.885		0.001		0.006	

All values are presented as the number of cases, with percentages in parentheses. TLR: Toll-like receptor; LN: Lymph node; MICs: Mononuclear inflammatory cells; W: Well differentiated; M: Moderately differentiated; P: Poorly differentiated.

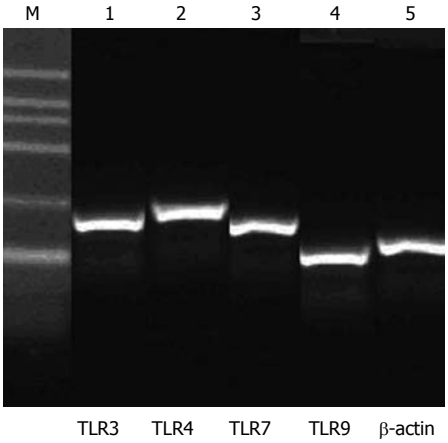

Figure 2 mRNA expression patterns of Toll-like receptor 3, Toll-like receptor 4, Toll-like receptor 7 and Toll-like receptor 9. M: 100-600 bp marker ladder. Lanes 1 to 4 show the expression of Toll-like receptors, and lane 5 shows the expression of β-actin.

the differences in TLR3, TLR4, TLR7 and TLR9 mRNA expression levels between normal esophageal epithelia and ESCC were statistically significant ($P < 0.05$).

DISCUSSION

This study demonstrated that samples of recurrent EC exhibited significantly higher mRNA levels of TLR3, TLR4, TLR7 and TLR9 than normal tissue. ESCC tumors exhibited high TLR protein expression in cancer cells. Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells. Tumors with high TLR3 expression in tumor cells or high TLR4 expression in mononuclear inflammatory cells were significantly associated with poor prognosis. However, tumors with high TLR9 expression in fibroblast-like cells were associated with a low probability of metastasis.

In this study, high TLR3 expression in esophageal cancer cells was associated with a high probability of lymph node metastasis. Similar observations were made in different cancer types. Studies on breast and prostate carcinomas demonstrated that high TLR3 expression was significantly associated with higher probabilities of metastasis and biochemical recurrence[10,11], which is in agreement with previous studies indicating that TLR3 expression is related to tumor aggressiveness[12,13]. Although the precise effect of increased TLR3 expression requires further investigation, our work suggests that TLR3 plays a vital role in esophageal carcinogenesis. Therefore, TLR3 may represent a good therapeutic target in esophageal cancer.

This study also demonstrated that the expression level of TLR4 in tumor cells was significantly associated with depth of invasion. TLR7 and TLR9 expression was positively associated with tumor grade in ESCC. Moreover, studies on TLR4 and TLR7 expression in gastric and lung cancer cells have suggested that high TLR expression results in increased tumor progression[14,15] and stimulation with TLR7 agonists lead to NF-κB activation, upregulated expression of the antiapoptotic protein Bcl-2, increased tumor cell survival, and chemoresistance[16,17]. It has also been reported that TLR9 expression gradually increased during the progression from normal cervical squamous epithelial tissues to cervical intraepithelial neoplasia and invasive cervical cancer[18]. These findings indicated that increased TLR protein expression may interfere with normal TLR signaling pathways and function and may represent useful markers of the malignant transformation of cancer cells. In addition, cancer cells activated by TLR signals may release cytokines and chemokines that in turn recruit and stimulate immune cells to release additional cytokines and chemokines. This process results in immune tolerance, cancer progression, and propagation of the tumor microenvironment.

In this study, we also observed the expression of TLR3, TLR4, TLR7 and TLR9 in tumor stromal cells as well as their association with the clinicopathologic characteristics of ESCC. Tumor stromal cells such as fibroblast-like cells, mononuclear inflammatory cells, and numerous intracellular mediators comprise the tumor microenvironment. These factors actively participate in tumor progression and infiltration, where the tumor microenvironment not only responds to and supports carcinogenesis but also contributes to tumor initiation, progression, and metastasis. The interaction between transformed cells and the microenvironment determines the fate of the tumor. Another interesting finding was TLR4 expression

in mononuclear inflammatory cells and TLR9 expression in fibroblast-like cells, which are associated with ESCC prognostic factors. TLR4 expression in mononuclear inflammatory cells was associated with an increased incidence of lymph node metastasis and depth of invasion, and TLR9 expression in fibroblast-like cells was associated with a low rate of lymph node metastasis. These findings are supported by research that demonstrated the importance of tumor stromal cells in tumor behavior through the release of various growth factors, proteases, and extracellular matrix proteins, which induce gene mutations, inhibit apoptosis, and stimulate angiogenesis and cell proliferation[19-21].

Metastatic relapse attributable to the presence of tumor cells within lymph nodes is the most frequent cause of cancer-related death in patients with esophageal tumors[22]. In the current study, the high expression of TLR4 by mononuclear inflammatory cells was associated with an increased incidence of lymph node metastasis and depth of invasion, suggesting that the regulation of the immune response within the tumor microenvironment might be another consequence of TLR activation. TLR9 expression by fibroblast-like cells was associated with good patient prognosis, suggesting that the surrounding connective tissue of the tumor is important for preventing tumor spread. Therefore, our results also suggest the existence of different phenotypes of stromal cells that influence prognosis depending upon the expression pattern of TLRs. In this study, TLR3, TLR4, TLR7 and TLR9 expression appeared important to the biological pathogenesis of esophageal cancer. However, different phenotypes of TLR expression in stromal cells can lead to different results and as a series of candidate prognostic factors, the function of these markers in ESCC should be further investigated.

COMMENTS

Background

The prognosis of esophageal carcinoma remains poor due to its relapse and metastasis characteristics. Toll-like receptors (TLRs) comprise an important family of pattern recognition receptors, and the TLR signaling pathway activates several different signaling elements, including nuclear factor kappa B, extracellular signal regulated kinase, and Jun-NH-kinase/p38, which regulate many immunologically related proteins, alter the microenvironment of tumors, and promote tumor progression and metastasis. Esophageal squamous cell carcinoma (ESCC) progression is associated with TLR stimulation, a crucial event in immune escape and metastasis.

Research frontiers

TLR expression is a common event in several cancers; this can promote carcinogenesis by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation. However, TLR expression has not been reported in esophageal carcinoma. In this study, the authors demonstrated that the overexpression of TLR3, TLR4, TLR7 and TLR9 in ESCC appears to be important in the biological pathogenesis of ESCC.

Innovations and breakthroughs

This is the first study to report that TLR3, TLR4, TLR7 and TLR9 overexpression appears important in the biological pathogenesis of ESCC. Furthermore, high TLR4 expression in mononuclear inflammatory cells was significantly associated with a higher probability of lymph node metastasis and depth of invasion, and high TLR9 expression in fibroblast-like cells was associated with low probabilities of invasion and metastasis.

Applications

TLR3, TLR4, TLR7 and TLR9 expression may represent potential prognostic markers and therapeutic targets for ESCC.

Terminology

TLRs, a family of pattern recognition receptors expressed in immune and non-immune cells, play a crucial role in the innate immune response and the subsequent induction of adaptive immune responses against microbial infection or tissue injury. Furthermore, TLR expression in cancer cells is associated with tumor proliferation and invasion.

Peer review

This manuscript by Dr. Sheyhidin *et al* investigated the expression of TLR3, TLR4, TLR7 and TLR9 in ESCC tissues from a good number of patients. The resulted data confirmed upregulation of TLRs in ESCC and its association with clinic pathological characteristics. This study is clinical significance because it included a good number of patients including patients from various ethnic groups. Nevertheless, the data generated from this study are confirmative in nature.

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BRIEF ARTICLE

Repeated anastomotic recurrence of colorectal tumors: Genetic analysis of two cases

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Abstract

AIM: To investigate genetics of two cases of colorectal tumor local recurrence and throw some light on the etiopathogenesis of anastomotic recurrence.

METHODS: Two cases are presented: a 65-year-old female receiving two colonic resections for primary anastomotic recurrences within 21 mo, and a 57-year-old female undergoing two local excisions of recurrent anastomotic adenomas within 26 mo. A loss of heterozygosity (LOH) study of 25 microsatellite markers and a mutational analysis of genes *BRAF*, *K-RAS* and *APC* were performed in samples of neoplastic and normal colonic mucosa collected over the years.

RESULTS: A diffuse genetic instability was present in all samples, including neoplastic and normal colonic mucosa. Two different patterns of genetic alterations (LOH at 5q21 and 18p11.23 in the first case, and LOH at 1p34 and 3p14 in the second) were found to be associated with carcinogenesis over the years. A role for the genes *MYC-L* (mapping at 1p34) and *FIHT* (mapping at 3p14.2) is suggested, whereas a role for *APC* (mapping at 5q21) is not shown.

CONCLUSION: The study challenges the most credited intraluminal implantation and metachronous carcinogenesis theories, and suggests a persistent, patient-specific alteration as the trigger of colorectal cancer anastomotic recurrence.

Key words: Anastomotic recurrence; Colorectal cancer; Allelic loss; Genetic alterations

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INTRODUCTION

Local recurrences (LRs) from colorectal cancer are often inoperable and have poor prognoses, with an estimated 5-year survival of 10 percent and a median survival of 16 mo[1]. LRs are defined as being perianastomotic (when

rising in the extramural tissue) or primitively anastomotic[2]. These latter may be due to implantation of exfoliated cancerous cells in the suture line[3,4] or to metachronous carcinogenesis[5].

We report two singular cases of patients repeatedly developing recurrent tumors (adenocarcinoma, adenoma) at the suture line and/or in the contiguous colonic mucosa within 21 and 26 mo of left hemicolectomy and anterior rectal resection for colorectal adenocarcinoma, respectively. To clarify the molecular mechanism(s) implicated in such a singular feature and, more in general, the development of anastomotic recurrence, we performed an extended genetic analysis of patients' tumor tissues and colonic mucosa obtained from surgical specimens and follow up endoscopy. The investigation was focused on the chromosomal alterations most frequently associated with colorectal cancer development, including mutational analysis of *BRAF*, *K-RAS* and *APC* genes and loss of heterozygosity (LOH) analysis of 25 chromosomal sites known to be involved in colonic carcinogenesis. This is the first genetic study performed on anastomotic recurrence of colorectal cancer.

MATERIALS AND METHODS

Case 1

In November 1998, a 65-year-old woman underwent a left hemicolectomy with a stapled colorectal anastomosis for a 4.5 cm × 3 cm fungating tumor of the sigmoid colon, 32 cm from the anal verge. Preoperative workup did not show any local infiltration or liver/pulmonary metastases. Histological examination showed a moderately differentiated adenocarcinoma infiltrating the whole colonic wall up to the pericolic fat tissue with uninvolved mucosa 23 cm proximal and 20 cm distal to the tumor edges, and 20 tumor-free lymph nodes (pT2N0M0).

In accordance with our follow-up policy[6], the patient underwent clinical and ultrasound evaluation and circulating carcino-embryonic antigen (CEA) determination every three months, as well as computed tomography (CT) scan and colonoscopy one year postoperatively. The latter procedure identified a recurrence involving half the circumference of the colorectal anastomosis. No local or distant metastases were disclosed by a CT scan, and CEA level was normal. A colorectal resection with mesorectal excision and stapled colorectal anastomosis by the double-stapling technique[7] was performed 6 cm from the anal verge. The resected specimen showed a moderately differentiated adenocarcinoma infiltrating the muscle layer with 12 tumor free lymph nodes (pT2N0M0) and normal mucosa, 9 cm proximal and 6 cm distal to the tumor.

The patient presented nine months later with rectal bleeding. At colonoscopy the anastomosis showed a circumferential tumor recurrence and five polyps. The CEA level was normal and a CT scan of the abdomen and thorax did not disclose distant metastases. A colorectal resection with double-stapled coloanal anastomosis 2 cm from the anal verge was performed with a defunctioning ileostomy which was later closed. The patient received adjuvant radiotherapy (45 G) to the pelvis three weeks later. The histopathologic examination showed a moderately differentiated adenocarcinoma infiltrating the muscle layer (pT2N0M0) and five adenomas (3 located proximal and 2 distal) within 3 cm of the suture line, < 1 cm in size, with severe dysplasia.

The regular yearly follow up revealed no further sign of local recurrence or distant metastases and the patient is in good health 11 years after the initial resection. In January 2010, the patient underwent endoscopic exploration with biopsy.

Case 2

In May 2006, a 57-year-old woman underwent anterior rectal resection with coloanal anastomosis and ileostomy for a 4 cm polypoid lesion of the lower rectum (5 cm from the anal verge); on histological examination of endoscopic biopsies, this proved to be an adenocarcinoma arising in a villous adenoma. Neither regional nor distant spread was present at preoperative CT scan. In the resected specimen the histological diagnosis of adenocarcinoma developing from a high grade villous adenoma was confirmed, with initial invasion of the submucosa (early colorectal cancer), and free lymph nodes (n = 27) and surgical margins (pT1N0M0). After ileostomy closure, an anastomotic substenosis was easily resolved by 2 mechanical dilatations.

The patient was submitted to regular follow-up[6]. Twenty-two months after surgery, a colonoscopy revealed an asymptomatic anastomotic 3 cm polyp, which was completely removed by transanal resection. Histological examination showed a tubulo-villous adenoma with high grade dysplasia.

At the subsequent colonoscopy, 4 mo later, a second anastomotic 2 cm polyp was removed by transanal resection, again revealing an adenoma with high grade dysplasia. Neither local recurrences nor distant metastasis were detected at further follow-up. In September 2009, the patient underwent endoscopic exploration with biopsy.

Tissue processing and genetic analysis (Table 1)

In case 1 the LOH study (see Table 1) and the mutational analysis for *BRAF*, *KRAS* and *APC* (see below) were performed on the following samples: (1) primary adenocarcinoma and the corresponding peritumoral, distal and proximal mucosa; (2) first recurrence and peritumoral and distant mucosa (12 mo postoperatively); (3) second recurrence and adenoma (21 mo after initial surgery); and (4) anastomotic and distant colorectal mucosa (134 mo after initial surgery).

In case 2 the LOH study (see Table 1) and the mutational analysis for *BRAF* and *KRAS* were performed on: (1) primary tumor and peritumoral mucosa; (2) villous adenoma (22 mo postoperatively); (3) recurrent anastomotic adenomas (26 mo after initial surgery); and (4) anastomotic mucosa (40 mo after initial surgery).

Table 1 Microsatellite markers used in the loss of heterozygosity study, with relevant cytogenetic locations, putative genes involved and their function, and references to papers describing a role for colonic carcinogenesis

Microsatellite Markers	Cytogenetic band	Gene	Function	Ref.
BAT40	1p13.1			[8]
MYC-L	1p34			[8]
BAT 26	2p16.3	*hMSH2*	Mismatch repair enzyme	[8]
D2S123	2p16			[8]
D3S1481	3p14	*FHIT*	Histidine triad gene family (purine metabolism)	[9]
D4S2397	4p15.2			[10]
D5S346	5q21	*APC*	Antagonist of the Wnt signaling pathway	[8]
D10S1671	10q25			
D10S169	10q26.3	*MGMT*	DNA defense *vs* O6-methylguanine	[11]
D10S1765	10q23.3	*PTEN*	Protein tyrosine phosphatase	[12]
D16S421	16q22	*CDH1*	Ca++ dependent cell-cell adhesion glycoprotein	
D16S402	16q23-q24			
D16S507	16q23.2			
D17S250	17q21			[8]
TP53ALU	17p13.1	*TP53*	Tumor protein "guardian of the genome"	
TP53	17p13	*TP53*	Tumor protein "guardian of the genome"	[13]
D18S452	18p11.23			[14]
D18S53	18p11.22-p11			[13]
D18S64	18q21	*DCC*	Receptor for netrin 1	[15]
D18S857	18q22.1	*DCC*	Receptor for netrin 1	
DXYS233	Xp22.32-Yp11.3			[16]
SHOX	Xp22.3			[16]
DXYS154	Xqter-Yqter			[16]
DXS8009	Xq25-q26			
DXS8098	Xq24-q25			

Using 5 μm haematoxylin stained sections of tissue specimens routinely formalin fixed and paraffin embedded, DNA was isolated by manual microdissection and extracted using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany). Only tumor samples containing more than 70% tumor cells were included in the study. All microdissection were conducted in close collaboration with the pathologist to ensure consistency with histological diagnoses and accurate dissection for tumor cell enrichment. For each patient DNA extracted from normal lymphocytes was used as reference DNA. DNA quality was assessed by polymerase chain reaction (PCR) amplification of the human beta-globin gene.

Polymerase chain reaction

The molecular analysis was performed with a panel of 25 polymorphic microsatellite markers located on chromosomal regions potentially involved in colorectal cancer development and progression and listed in Table 1. Primer sequences and amplification conditions were in accordance with the Genome Database information (http://www.ncbi.nlm.nih.gov/genemap99). Forward primers were synthesized with a fluorescent tag (WellRed dyes from Research Genetics, Huntsville, AL, United States).

The target sequences were amplified by PCR in a 25 μL reaction mixture containing 2 μL DNA sample, 10x buffer (10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP (Promega, Madison, WI), 0.4 μmol/L of each primer and 1.25 U Taq Polymerase (Promega, Madison, WI). Microsatellites were submitted to 35-40 cycles of amplification at different annealing temperatures (range 57 ℃-61 ℃). The presence and correct size of amplimers were evaluated by 2% agarose gel electrophoresis. The fluorescently labelled PCR products were subjected to electrophoresis on an automated DNA sequencer CEQ 8000XL (Beckman Coulter Inc., Fullerton, CA), and the fluorescent signals from the different sized alleles were recorded and analyzed using CEQ 10000XL analysis software (Beckman Coulter).

Definition of LOH and allelic imbalance

The LOH was defined as the ratio of relative allelic peak height in the tumor DNA to relative allelic peak height in the corresponding normal DNA. The formula employed for the calculation was T2: T1/N2: N1, where T1 and N1 are the height values for the smaller allele and T2 and N2 are the height values for the larger allele of the tumor (T) and normal (N) samples respectively. For informative markers LOH was scored when the signal reduction for one allele was of 40%. This degree of allelic imbalance (AI) indicates that a substantial proportion of the cells within a sample contains the same DNA abnormality and likely represents the presence of a clonal population. Abnormal results were demonstrated at least twice with equivalent results. At certain loci AI probably reflects increased copy number rather than loss of an allele. Distinguishing between these possibilities is important conceptually, but would not change data analysis. Therefore, all AIs were labelled as LOH.

The same areas of chromosomal regions showing LOH were repeated in an independently microdissected sample from different paraffin blocks when sufficient tissue was available.

Microsatellite instability

The novel appearance in the tumor DNA of one or more alleles, i.e. new peaks in the electropherogram, not present in its paired normal DNA, was considered as an indicator of microsatellite instability (MSI). Samples were classified as microsatellite stable or unstable according the revised Bethesda Criteria[8].

BRAF, KRAS and APC mutation analysis

Direct sequencing was performed to identify *BRAF* V600E mutations, *KRAS* codon 12/13 mutations and *APC* exon 15. Primer sequences for *BRAF* and *KRAS* were: *BRAF-F* (5'TGCTTGCTCTGATAGGAAAATGA-3'), *BRAF-R* (5'-TGGATCCAGACAACTGTTCAAA-3'), *KRAS-F* (5'-GCCTGCTGAAAATGACTGAA-3') and *KRAS-R* (5'-AGAATGGTCCTGCACCAGTAA-3'), which generated fragment lengths of 165 and 167 bp respectively. *APC* mutation analysis was performed using three sets of primers, amplifying two overlapping portions of exon 15 in accordance with Su *et al*[17]: *APC-1F* (5'-CATCAGCTGAAGATGAAATAGGA-3') and *APC-1R* (5'-GCAATCGAACGACTCTCAAA-3'), codons 1281–1402, 364 bp; *APC-2F* (5'-ATGTTCAGGAGACCCCACTC-3') and *APC-2R* (5'-CACTCAGGCTGGATGAACAA-3'), codons 1376–1508, 396 bp; and *APC–3F* (5'-GGGTCCAGGTTCTTCCAGAT-3') and *APC-3R* (5'-TTGCCACAGGTGGAGGTAAT-3'), codons 1478-1607, 387 bp.

DNA sequencing was performed using EurofinsMWGOperon/M-Medical (Milano, Italy). Sequencing results were verified in our laboratory in both directions using DNA STAR PC software (Lasergene, Madison, WI, United States). The presence of mutations was determined through alignment with normal sequences as reported in NCBI/Blast Human Genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

LOH analysis

The results of LOH analysis for all 25 chromosomal markers investigated are reported in Table 2.

Case 1

A generalised genetic instability at various sites in tumoral and non-tumoral, histologically normal mucosal samples was seen. Chromosomes 5q, 16q and 18 presented with the highest frequency of LOH. In particular, LOH at 5q21 and 18p11.23 loci was consistently found in all tumor samples, including primary and recurrent adenocarcinomas and the late occurring adenoma, but was not found in any sample of non-tumoral mucosa. In contrast, LOH at 16q23-24 was consistently present in all extratumoral mucosa samples (except one showing MSI), being absent in all tumor specimens. LOH at 10q26.3 and 18q21 loci was also found to be restricted to non-tumoral mucosal samples, but only in those collected at the time of the initial surgery. The primary tumor, but not the neoplasms observed at the time of recurrences, showed LOH at 1p13.1 and 1p34.

When investigated for MSI, all samples showed a stable phenotype (in accordance with the Bethesda revised criteria[8]) except for one individual (proximal) sample of normal mucosa at the initial surgery, which was characterized by MSI in 5 of the chromosomal markers analyzed (low-MSI). The mutational analysis of exon 15 of *APC* gene demonstrated the presence of a single nucleotide polymorphism in the codon 1493 ACG > ACA (T1493T) in all tumoral and non-tumoral samples.

Case 2

LOH at 3p14 was found to be a consistent, specific tumor change occurring in all neoplastic specimens but not in samples of non-neoplastic mucosa. Furthermore, allelic loss was seen at the locus 1p34 of tumor specimens (except for the third adenoma) but was also observed in the peritumoral non-neoplastic mucosa, whereas LOH at 10q23.3 was restricted to the primary tumor and corresponding peritumoral mucosa. Scattered LOH changes were also found in homologous pseudoautosomal regions (DXYS233, DXYS154, SHOX) of the sex chromosomes X-Y in the normal mucosa and the third adenoma. No evidence for MSI was yielded by any of the samples analyzed in this case.

Mutation analysis

The sequence analyses for *K-RAS* and *BRAF* mutation performed in tumor and normal tissues of both cases showed a wild type phenotype in all samples. Mutation analyses for exon 15 of *APC*, performed in Case 1, in which LOH was present at the gene locus in 5q21, yielded negative results.

DISCUSSION

Sixteen percent of patients undergoing colorectal resection for colon cancer present with a local recurrence[18], and, since in 12% of cases[19] the recurrences occur primarily at the site of the anastomosis, it may be estimated that roughly 2% of patients undergoing a colorectal resection for cancer will eventually develop an anastomotic recurrence. The mechanism(s) involved in the development of anastomotic recurrence are poorly understood. The present study has focused on genetic alterations occurring in primary and recurrent tumors as well as in the extra-tumoral colonic mucosa of two patients with repeated and rather early recurrence of anastomotic/perianastomotic neoplasms. To this end, a search was carried out for allelic losses at 25 chromosomal sites known to be involved in colonic carcinogenesis (Table 1) and for mutational events in three genes (*K-RAS*, *BRAF* and *APC*) commonly altered in colorectal cancer. This extensive genetic analysis included the normal mucosa at the time of the resection of the primary tumor ("genetic predisposition") and the potential changes in the genetic pattern of recurrent tumors and/or colonic mucosa pos-

Table 2 Results of loss of heterozygosity analysis with 25 microsatellite markers amplified in the present cases

	Case 1 (adenocarcinoma of the sigmoid colon – pT2N0M0)												Case 2 (adenocarcinoma of the rectum – pT1N0M0)					
	Initial surgery (left hemicolectomy)				Second procedure (colorectal resection) 12 mo after initial surgery (a.i.s.)			Third procedure (colorectal resection) 21 mo a.i.s.		Follow up (colonoscopy) 134 mo a.i.s.			Initial surgery (proctocolectomy)			Second procedure (endosc. resection) 22 mo a.i.s.	Third procedure (endosc. resection) 26 mo a.i.s.	Follow up (colonoscopy) 40 mo a.i.s.
	Prim. tumor	Peritu. mucosa	Distal mucosa	Prox. mucosa	First recurr.	Peritu. mucosa	Distant mucosa	Second recurr.	Aden	Anast. mucosa	Rectal mucosa	Colon mucosa	Prim. tumor	Peritu. mucosa	Aden.	Adenoma	Adenoma	Anast. mucosa
1p13.1	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
1p34	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	LOH	LOH	LOH	NO LOH	NO LOH
2p16.3	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
2p16	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
3p14	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	LOH	LOH	LOH	NO LOH
4p15.2	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NI	NI	NI	NI	NI	NI
5q21	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q23.3	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q25	NI	NI	NI	MSI	NI	NI	NI	NI	NI	NI	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
10q26.3	NO LOH	NO LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
16q22	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
16q23.2	NO LOH	NO LOH	NO LOH	MSI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
16q23 -q24	NO LOH	LOH	LOH	MSI	NO LOH	LOH	LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	
17p13	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
17p13.1	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
17q21	NO LOH	NO LOH	NO LOH	MSI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
18p11.23	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
18p11.22 -p11	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
18q21	NO LOH	LOH	MSI	LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	LOH	NO LOH	NI	NI	NI	NI	NI	NI
18q22.1	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xp22.32-Yp11.3	NI	NI	NI	MSI	NI	NI	NI	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xp22.32-Yp11.3	NO LOH	NO LOH	LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	LOH	LOH
Xqter-Yqter	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NO LOH	LOH	NO LOH	NO LOH	NO LOH	NO LOH
Xq24-q25	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Xq25-q26	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NI	NI	NI	NI
FAL (%)	23.5	12.5	29.4	13.3	11.1	11.1	5.9	11.1	16.7	16	10.5	5.2	20	26.7	13.3	13.3	13.3	6.25

LOH: Loss of heterozygosity; NO LOH: Retention of heterozygosity; NI: Not informative; MSI: Microsatellite instability; FAL%: Fractional allelic loss (No. of markers with LOH/total No. of informative markers).

sibly involved in tumor progression and recurrences.

The LOH analysis (Table 1) showed in both cases a diffuse genetic instability at various sites both in tumor tissue and in extra-tumoral mucosa, although the affected loci largely differed between neoplastic and non-neoplastic samples as well as between non-neoplastic samples taken from different colonic regions. The fractional allelic loss did not significantly vary in the tumors

as compared to the normal mucosa or in the peritumoral as compared to distant mucosa (Table 2). In both cases a noticeable persistence of genetic changes both in the primary and recurrent tumors was found even though the affected chromosomal loci differed from one case to the other, being 5q21 and 18p11.23 in Case 1 and 1p34 and 3p14 in Case 2. Since these changes (with the exception of 3p14 in the first samples of Case 2) were consistently absent in the extra-tumoral mucosa, they may reasonably be considered as reflecting chromosomal alterations responsible for tumor development. Their consistent appearance in primary and recurrent tumors (with the exception of the third adenoma in Case 2) supports the supposition of an identical genetic mechanism for anastomotic recurrences. The potential involvement of the *APC* gene, which maps at 5q21, in colonic carcinogenesis of Case 1 is not supported by the lack of detectable mutations in the gene exon 15, which is more commonly altered in colo-rectal cancers. In Case 2, the LOH at 1p34 and 3p14.2 sites, the loci of mapping of the *MYC-L* and *fragile histidine triad* (*FHIT*) genes respectively suggest a key role of these two genes in this patient' s tumor development. In this regard it is worth noting that 1p34 LOH occurs in both tumoral and non-tumoral samples, whereas 3p14.2 LOH is absent in normal mucosa. This may suggest that the *MYC-L* alteration may reflect a "mark" of a proliferative instability leading to carcinogenesis, whereas the alteration of the *FHIT* gene may suggest its role in the events occurring at the early phase of carcinogenesis (i.e., transformation from normal mucosa to adenoma).

The two most credited theories to explain the occurrence of anastomotic recurrence are the intraluminal implantation of exfoliated cancerous cells[3,4] and the metachronous carcinogenesis[5], possibly triggered by modifications of the microenvironment around the suture depending on the surgical technique[20,21] or the materials used[22-24]. Both theories fail to satisfactorily explain our findings. Indeed, in both cases the intraluminal implantation theory, though supported by the presence of consistent genetic alterations in primary and recurrent tumors, is contradicted by other genetic alterations (such as LOH at 1p13.1 and 1p34 loci in case 1, and at 10q23.3 in case 2) that are present in the primary but not in the recurrent neoplasms. Moreover, the recurrence of perianastomotic benign tumors (adenomas) with overlapping genetic changes is also in contrast with the implantation theory. On the other hand, the metachronous carcinogenesis theory by itself cannot explain the short time needed to develop new tumors and their location at the suture line or within a short distance from it, since metachronous carcinogenesis *per se* implies the chance onset of a second adenoma/adenocarcinoma in any segment of the colonic remnant at an interval of years. Also the hypothesis that the anastomosis' surgical techniques[20,21] and/or the materials used[22-24] may be implicated in carcinogenesis by altering DNA at specific sites seems to be confounded by our findings, since the same genetic alterations found in recurrent neoplasms were present in the primary tumors, whose development obviously cannot be associated to previous surgical procedures. Moreover, such a hypothesis does not explain why recurrences occur in a very small minority of patients, in spite of the standardized surgical procedures performed (including the materials used) in all patients affected by colorectal cancer.

A genomic instability of DNA in tumor and adjacent tissues has already been described in breast cancer, where independent mutational events were observed[25-27]. Considering colon cancer, Ahlquist *et al*[28] found various epigenetic changes in mucosa surrounding colorectal neoplastic lesions, and hypothesised that the tumor itself may have caused a "field cancerization" of the contiguous mucosa. This phenomenon, in our opinion, is unlikely to have occurred in the present cases, owing to the large discrepancy in genetic changes between the tumors and the normal extratumoral mucosa in spite of a diffuse, tumor-independent genetic instability in the colonic mucosa of our patients. Independently of its aetiology, Umeto *et al*[29] suggested that colonic genetic instability associated with microenvironmental changes may "predispose" to metachronous carcinogenesis by altering several genes implicated in colon cancer development. In our cases, the consistency of genetic alterations among primary and recurrent tumors, even if separated by an interval of years, suggests a persistent, patient-specific alteration rather than a generic, diffuse DNA instability, as the trigger of recurrent carcinogenesis after radical resection of colorectal cancer.

In conclusion, although the mechanism of elective recurrent carcinogenesis in the anastomotic and/or perianastomotic areas still remains unsolved, since genetic alteration patterns differ in the two cases, our study supports a role for the genes found to be altered. Further studies in larger series of patients are warranted for assessing the potential role of these gene changes in the detection of patients at risk of developing an early anastomotic recurrence, and for confirming the patient-specificity of genetic alterations responsible of cancerogenesis, regardless of other genetic alterations occurring in colonic mucosa through years.

COMMENTS

Backgrounds

Local recurrences (LRs) from colorectal cancer are often inoperable and have poor prognoses, with an estimated 5-year survival of 10 percent and a median survival of 16 mo. LRs are defined as being perianastomoticor primitively anastomotic. These latter may be due to implantation of exfoliated cancerous cells in the suture line or to metachronous carcinogenesis.

Innovations and breakthroughs

This is the first genetic study performed on anastomotic recurrence of colorectal cancer.

Applications

Although the mechanism of elective recurrent carcinogenesis in the anastomotic and/or perianastomotic areas still remains unsolved, since genetic alteration patterns differ in the two cases in the study,it supports a role for the genes found to be altered.

Peer review

The conclusion needs to be modified given that the findings are based on a sample of two patients.

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Role of diaphragm in pancreaticopleural fistula

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Abstract

A pancreatic pleural effusion may result from a pancreatopleural fistula. We herein discuss two interesting issues in a similar case report of a pleural effusion caused after splenectomy, which was recently published in the *World Journal of Gastroenterology*. Pancreatic exudate passes directly through a natural hiatus in the diaphragm or by direct penetration through the dome of the diaphragm from a neighboring subdiaphragmatic collection. The diaphragmatic lymphatic "stomata" does not contribute to the formation of such a pleural effusion, as it is inaccurately mentioned in that report. A strictly conservative approach is recommended in that article as the management of choice. Although this may be an option in selected frail patients, there has been enough accumulative evidence that a pancreaticopleural fistula may be best managed by early endoscopy in order to avoid complications causing prolonged hospitalization.

Key words: Fistula; Pleural effusion; Pancreatic surgery

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TO THE EDITOR

We have read with great interest the article by Shu-Guang Jin *et al*[1] that presented a case of pancreatic pleural effusion caused after splenectomy. In their work, the authors support the notion that the leaking fluid from a pancreatic duct disruption may reach the thoracic cavity by the lymphatic system and stomata. They also conclude that an active conservative treatment should be carried out in the early period of this complication to reduce the need for endoscopy or surgery. We feel that both of these statements need further discussions.

Pancreaticopleural fistula is a rare occurrence. This internal pancreatic fistula is usually caused by a chronic pancreatitis or, more rarely, it is a traumatic consequence. A pancreatic pleural effusion develops due to a direct passage of pancreatic exudate through a natural hiatus in the diaphragm[2] or by direct penetration through the dome of the diaphragm[3] from a neighboring subdiaphragmatic collection.

The most common cause of pancreaticopleural fistula is a pseudocyst formed in the lesser sac from an anterior disruption of the pancreatic duct that erodes the overlying diaphragm.

Although studies of the pathways of peritoneal fluid absorption indicate that the peritoneal surface of the diaphragm is the main site of drainage, this does not nec-

essarily suggest that this mechanism is implicated in pancreatic fluid transportation into the pleural cavity. Pleural liquid is a filtrate from capillaries in the parietal pleura lining the chest wall. Drainage from pleural space occurs *via* the lymphatics in the parietal pleura[4]. Peritoneal fluid enters the lymphatic lacunae (a rich plexus of flattened terminal lymphatics) *via* special mesothelial openings, the so called "stomata"[5]. This fluid is further transported *via* the parasternal route to the mediastinal nodes and then to the terminal thoracic duct or the right lymphatic duct[6] and not to the pleural cavity. The "stomata" system provides a direct route between the peritoneal cavity and lymphatics[7].

In the presented case of pancreatic pleural effusion after splenectomy, a left subphrenic encapsulated fluid collection was clearly revealed by an abdominal computed tomography. A pancreatic pseudocyst such as the aforementioned is almost invariably implicated in these rare cases of pancreatopleural fistula[8]. This communication happens through normal orifices or diaphragmatic erosion.

The protein-rich fluid with an elevated amylase content drained by the thoracocentesis was a great indicator of the pancreatopleural fistula which, as speculated, was the result of a posterior pancreatic duct rupture due to an intraoperative injury. The authors proposed that a purely medical treatment was appropriate for their patient, in order to reduce the need for endoscopy or surgery. This policy was recommended in their conclusions. Although the medical treatment proved effective in their case, one has to bear in mind the adverse consequences that may be caused by such an approach. Such a notion has been extensively emphasized by many researchers in the field. This therapeutic option usually requires prolonged hospitalization which contributes substantially to morbidity and cost. On the contrary, an early instituted endoscopic retrograde cholangiopancreatography (ERCP)[9] combined with either a papillotomy, a stent or a nasopancreatic tube may be an optional initial treatment. The role of early therapeutic endoscopy is constantly expanding[10] as it has proved beneficial instead of long-term conservative treatment. Up to 90% of the patients with pancreatic fistulas can be successfully treated by this modality, with minimal morbidity and no mortality[11].

Although formal treatment recommendations have not been adopted, the first line of treatment supported by most of the authors in the field includes drainage of the effusion, inhibition of pancreatic secretion with octreotide and ERCP plus stenting of the pancreatic duct.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

World Journal of Gastroenterology

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

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Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

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

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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