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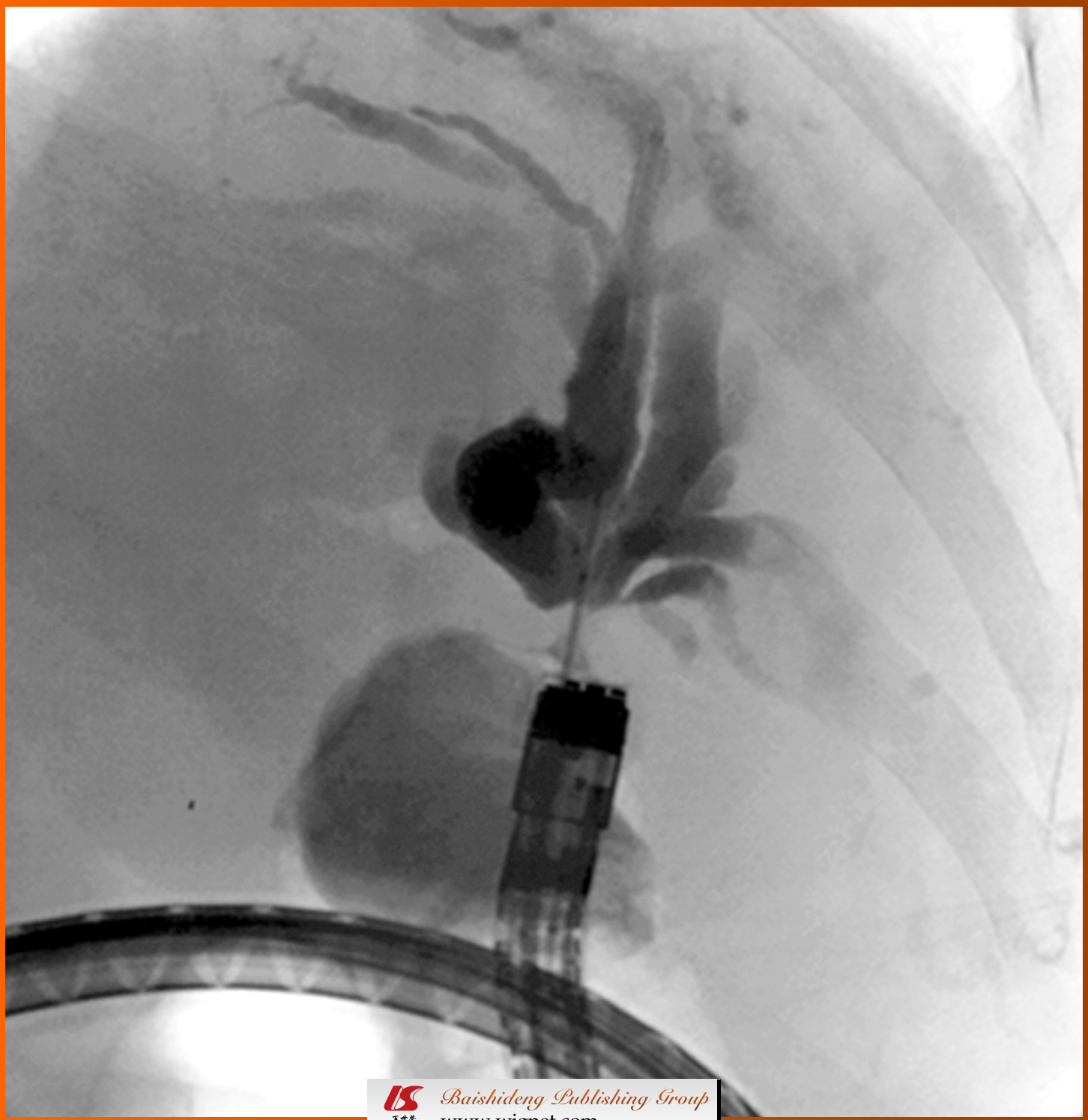

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EDITORIAL

Recent advances in celiac disease

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Abstract

Celiac disease now affects about one person in a hundred in Europe and North America. In this review, we consider a number of important and exciting recent developments, such as clinical associations, HLA-DQ2 and HLA-DQ8 predispositions, the concept of potential celiac disease, the use of new imaging/endoscopy techniques, and the development of refractory disease. This review will be of use to all internists, pediatricians and gastroenterologists.

Key words: Inflammation; Infection; Malabsorption; Pathophysiology; Physiology

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INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy characterized by intolerance to gluten. CD is usually characterized by various gastrointestinal (GI) symptoms (e.g. diarrhea, malabsorption, weight loss) associated with consumption of grains containing gluten (wheat, barley, rye). Although some CD patients may have primarily GI symptoms, CD may be detected due to associated extraintestinal disorders, even without GI symptoms, or due to screening for CD based on a positive family history. CD has a strong association with HLA-DQ2 and HLA-DQ8. Serological testing for antibodies to tissue transglutaminase (tTG) is usually positive (-95%) in the untreated patient. Endoscopic and histological damage seen in the proximal intestine is characteristic, but not diagnostic. As CD is defined to be a gluten-sensitive enteropathy, definitive diagnosis ultimately depends on a positive small bowel biopsy and demonstration of a response to a gluten-free diet (GFD)[1]. Common serological changes include the appearance of anti-tTG and other antibodies, e.g. endomysial antibodies (EMA). These antibodies have been reported in some, but not all studies, to decline or disappear in association with a clinical and/or histological response to a gluten-free diet.

The clinical spectrum of CD includes patients with classical gastrointestinal symptoms (e.g. diarrhea and weight loss), those who are detected on screening because of a family history of CD or having a CD-associated autoimmune condition, or those who have a predisposition for developing CD but at a particular time of testing, conceivably could have no symptoms, negative CD serology and a histologically normal small bowel biopsy. Now, a CD-specific quality of life instrument has also been developed and validated psychometrically[2], and may prove useful in everyday clinical practice.

EPIDEMIOLOGY

CD is highly prevalent in Caucasian populations and their descendants. The age of clinical onset (based on diagnosis) is often described by some experts as bimodal: the first peak is at 8 to 12 mo of age, and the second during the third to fourth decades of life[3]. Recent studies suggest, however, that CD should be considered as a disorder that has a risk of developing throughout life, even in the elderly. Overall, CD is twice as frequent among females as compared to males, possibly because the necessary HLA haplotypes, DQ2/DQ8, are more frequent in female than in male CD patients (i.e. 94% *vs* 85%)[4]. In addition, CD may be detected more frequently in females because females tend to seek medical care more often than males, usually at a younger age. With aging, however, this female predominant pattern disappears. In the elderly, the ratio of newly diagnosed males is equivalent to newly diagnosed females. In the few DQ2/DQ8 -negative CD patients, there is a male excess, and only inheritance of a paternal DQ2 haplotype leads to a daughter's predominance[4].

While it is estimated that CD affects up to 2% of Caucasians, the risk is higher in first-degree relatives of affected sibling pairs (17%), monozygotic twins (75%), and HLA-identical siblings (40%). Indeed, the single most important risk factor for celiac disease is having a first-degree relative with already-defined CD, particularly a sibling[5]. The estimated prevalence of CD in first-degree relatives living in Minneapolis, USA is 11% of all affected family members who carried at-risk genotypes (HLA-DQ2 in more than 90% of cases, and the remainder carrying HLA-DQ8). About half of these persons have clinically "silent disease", and yet, their small bowel biopsies may show severe architectural changes[6]. This apparent disconnection in some patients between severity of symptoms with CD and the severity of histological abnormalities (typically defined in the proximal small bowel) may reflect the variable extent of histological involvement further along the length of the small intestine.

The prevalence of CD appears to be increasing, particularly as the population ages[7]. CD in the elderly has been reviewed and also likely reflects increased recognition of undiagnosed CD in this age group[8-10]. The estimated prevalence of CD in individuals in the United Kingdom between 45 to 76 years of age is approximately 1.2%. About 20% of all newly diagnosed celiac cases are over 60 years of age. The clinical presentation in these older individuals is variable, ranging from "silent" disease, to vague abdominal complaints, to anemia. These limited symptoms could also lead to a delay in diagnosis.

Prolonged gluten exposure in undiagnosed CD is seen to increase the incidence of autoimmune diseases, such as diabetes and autoimmune thyroiditis. As far as gastrointestinal complications are concerned, it is important to rule out collagenous and lymphocytic colitis[11,12], which may be mistaken for non-compliance with a gluten-free diet (GFD). Eight percent of elderly persons in nursing homes or long-term care centers may have associated small bowel bacterial overgrowth, manifesting with malabsorption and diarrhea-like symptoms. Nutrient depletion in these individuals is common, and the incidence of osteopenic bone disease is increased. Finally, neurological disorders, such as dementia, are becoming increasingly recognized in elderly patients with CD[13].

Screening serological tests (IgA and IgG) appear to be age-independent in adults. However, the elderly seem to have an increased incidence of sero-negative CD. Interestingly, the fate of different celiac antibodies in genetically at-risk children on a normal diet has been assessed, and, remarkably, these appear to spontaneously disappear[14]. Specifically, antibodies to tTG and EMA were spontaneously lost in 49% and 45%, despite continuing gluten exposure. Although the procedural risk of endoscopy and biopsy may be marginally increased in the elderly, endoscopic biopsy remains crucial as the "gold standard" for diagnosis of CD in this age group as well as in all other age groups.

GENETICS

It is conceivable that CD could be a heterogeneous disorder, not only with differing clinical presentations, but also different degrees of pathological change in the small intestinal mucosa. Further morphometric and immunohistochemical studies from geographically and genetically diverse populations are needed to confirm observations on increased intraepithelial lymphocytes (IELs) in otherwise architecturally normal small intestine.

A small percentage of EMA-positive patients may have a small bowel biopsy in which the mucosa might be considered by some pathologists to be architecturally normal. From 409 children who were positive for celiac-related antibodies, 24 (5.9%) of the individuals were reported to have an architecturally normal small intestinal mucosa, 46% (11 of these 24 patients) had increased CD3+ intraepithelial lymphocytes, and 71% (17 of the 24) had an increased density of gamma-delta + cells[15]. In 17 of these 24 persons (70%), the number of lamina propria CD25+ cells was increased, and/or the expression of ICAM-1 and crypt HLA-DR was enhanced. Interestingly, in those persons with apparently normal jejunal histology, there appeared to be immunohistological evidence of immune activation in the epithelium, lamina propria and intestinal crypts. A GFD is usually not recommended in individuals with an architecturally-normal small bowel biopsy. Sophisticated immunohistochemical findings, such as these, raise the question as to whether definition of "normal" needs to be extended, and whether such subjects with abnormal antibodies in the intestinal biopsy may need further monitoring to determine if a GFD is indicated. In addition, duodenal mucosal tTG detection improves the sensitivity of diagnosis in CD for those with very mild histological changes, i.e. Marsh 1 lesions[16]. Thus, the definition of CD may conceivably extend to persons with an architecturally normal mucosal biopsy, but with abnormal CD-associated immunohistochemical changes. Additional studies are needed to confirm, evaluate and further elucidate these interesting observations.

Gluten has specific peptide sequences which show

HLA-DQ2 or HLA-DQ8 restrictive binding motifs across the various gluten proteins. About 40% of the heritability of CD includes the human leukocyte antigen HLA-DQ2 and HLA-DQ8 heterodimers. The HLA DQ2 and HLA-DQ8 molecules are necessary to develop CD, but are not, in themselves, sufficient for phenotypic expression of the disease. Indeed, HLA markers only explain an estimated 40% of the heritable risk for CD. Therefore, other non-HLA genes must also be involved. One of these may relate to genetic variants on chromosome 19, in the myosin IXB gene (i.e. MYO9B), and may potentially predict responsiveness to a GFD[17].

CD-associated HLA-DQ molecules bind and present gluten peptides to antigen-specific T-cells in the intestinal mucosa, and induce T-cell proliferation as well as cytokine secretion. Siblings who share HLA haplotypes have a greater likelihood of concordance with CD than the generally estimated risk for siblings. A small percentage of CD patients are DQ-2 negative, usually being DR4-positive for the class 2 antigen, DQ8. Carrying two copies of DQB1*02 is associated with an even greater risk for CD, but does not predict an earlier age of onset of disease or disease severity. This suggests that assessment of copy number of the DQB1*02 allele could permit stratification of risk[18].

In twin studies in which CD was diagnosed by small bowel biopsy and serology in one twin, monozygotic twin pairs had a high probability of being concordant with CD in the second twin: monozygotic and dizygotic co-twins had 70% and 9% cumulative probability of having symptomatic or "silent" forms of CD, respectively, within 5 years[19]. Under ACE (additive genetic, common and unshared environmental factors/models) with CD prevalences of 1/91 and 1/1000, heritability estimates were 87% and 57%, respectively[19].

The overall risk of siblings of children with CD developing CD was 10% in an Italian population, but the risk estimate ranged from 0.1% to 29% when HLA-DQ information of the proband, parents and sibling was considered[20]. The risk for the sibling developing CD was less than 1% for 40% of the sibs of the probands, 1% to 10% for 30% of the probands, and above 25% for the remaining 30% of the siblings. Thus, information about the risk for a second child to develop CD can be provided to parents with a child with CD. This antenatal estimate of the risk of CD in the child may be useful to provide early diagnosis and management, as well as providing focused and specific follow-up depending upon the risk stratification. Of importance, because CD is treated with a GFD and the resultant quality of life of the CD patient is high, the antenatal diagnosis of CD should not be used as a reason to consider termination of the pregnancy.

A double dose of DR3 (often with DQ2) is associated with an even higher risk of development of CD. HLA genotyping is thought to be useful to exclude CD in family members, or in persons in whom there is an increased risk of CD, such as those with Turner syndrome or Down's syndrome[21].

A strategy that combines gene expression profiling of intestinal biopsy specimens, linkage region information, and different bio-informatic tools for the selection of potential regulatory single-nucleotide polymorphisms (SNPs) has been used to search for novel candidate determinants of predisposition to CD in previously identified linkage regions[22]. Abnormalities in functional proteins have been observed in CD. By using genetic association analysis with a SNPs approach, the tight junction permeability barrier genes, KRD 3 (2SNPs) and MAGI 2 (2SNPs), were shown to be associated with CD in British and Dutch persons[23].

In addition to the involvement of HLA class I restricted CD8+ T-cells, the innate immune system may also be involved in CD. In the mucosa of untreated CD, there is an increase in activated CD8+ T-cells containing large granzyme-B (GrB)-positive granules, as well as cell surface expression of the Fas ligand (FasL). CD8+ T-cell cytotoxicity occurs in the mucosa of patients with active CD (through Fas and FasL-mediated killing of enterocytes). The gliadin interaction for this CD8+ T cell-mediated response (occurring through TCR/HLA class I) induces enterocyte apoptosis[24].

Variation among four closely linked genes on chromosome 4q27 represents a non-HLA genetic risk factor for CD, mapping to a region that contains IL2, IL21, TENR, and K1AA1109[25]. Also, multiple common variants for CD influencing immune gene expression have been defined with second generation genome wide association studies[26].

Genetic studies have also recently identified nine non-HLA loci that contribute to CD risk. Combining HLA and non-HLA risk genotypes increases the sensitivity of CD diagnosis by 6.2% compared with using only HLA for identification, with only a slight decrease in specificity[27]. There may be a quantitative relationship between the type and proportion of DQ heterodimers and the risk of CD[28].

PATHOGENESIS

Gliadins, derived from an alcohol soluble fraction of gluten, are storage proteins that are ingredients in wheat, barley and rye as well as other grains that contain gluten (albeit of less importance). Gliadins are characterized by a high content of glutamine and proline residues. Glutenins are insoluble in aqueous alcohol and are different in structure from the glutens. The early immune response in CD patients is directed towards several of these peptides, while the long-standing inflammatory response may be driven by gluten peptides deamidated or cross-linked by tTG and bound more tightly to HLA-DQ2 and HLA-DQ8. The tTG catalyzed modifications in gliadin are not restricted to single gliadin types or epitopes[29]. Prolamines in barley and rye are known as hordein and secalin, respectively. These barley and rye prolamines induce an mRNA interferon-gamma response in celiac mucosa[30]. The α-2 gliadin-33mer appears to cross the brush border membrane (BBM) of the jejunal enterocyte by a dose-dependent mechanism. Both the uncleaved as well as the degraded form of the 33mer translocate into the enterocyte[31]. Interferon-gamma enhances the trans-

location of the 33mer.

After passage across the BBM, gliadins trigger a Th-1 type-dependent inflammatory reaction. The effects of gliadin peptides and A-gliadin peptide P31-43 on cell lines and cultured small intestinal biopsies are mediated through epidermal growth factor receptor (EGFR) activation by interfering with EGFR endocytosis[32]. Gliadin has an immunogenic effect, but also directly affects cultured cells and intestinal preparations by way of separate peptides such as A-gliadin p31-43 (P31-43). The gliadin-induced delay of EGFR endocytosis in cultured intestinal biopsies suggests a role for EGFR activation in CD[32]. A 33 amino acid fragment of α-2 gliadin is an important trigger of the inflammatory process. In patients with active CD, there is transepithelial translocation of the attack 33mer, as well as incomplete degradation of the 33mer during intestinal transport.

In persons with active CD, there is a marked accumulation of polarized Th-1 cells that produce large amounts of interferon γ (IFNγ). T-bet, a member of the T-box family of transcription factors, is present in CD4+ and CD8+ mucosal T cells in patients with CD. Interleukin 21 (IL-21) is present in activated CD4+ T cells, as well as in natural killer T cells (NK T cells). IL-21 regulates the production of cytokines by T cell subsets. IL-21 increases the expression of Stat4 and T-bet, and stimulates the production of IFNγ in human T cells. In duodenal mucosal biopsies from patients with CD, there is enhanced IL-21 RNA and protein expression, and neutralization of IL-21 largely prevents peptic-tryptic and digest-enhanced IL-21 expression[33,34].

In persons with a genetic susceptibility to develop CD, gliadin interacts with the intestine to trigger disassembly of the inter-enterocyte tight junctions (TJs). The 2α-gliadin 20mer synthetic peptides of gliadin bind to the chemokine receptor, CXCR3. This binding induces MyD88-dependent zonulin release. In turn, zonulin release leads to increased intestinal permeability[35]. Increased intestinal permeability occurs prior to the onset of clinically apparent CD. Even on a GFD, the initially enhanced intestinal permeability does not necessarily return to normal.

Another important aspect related to the pathogenesis of CD may include the intestinal microflora that may be central to the clinical expression of the disease. In one recent report, enrichment in the mucosa-associated microbiota with rod-shaped bacteria in those that developed CD may have contributed to the so-called "epidemic" in Swedish children less than two years of age[36].

Finally, other recent studies related to CD pathogenesis have directly used intestinal biopsy specimens from CD and non-CD persons. For example, IL-15 receptor α mRNA expression is higher in duodenal biopsies from CD compared to non-CD persons, regardless of whether the CD subjects are or are not consuming gluten. IL-15 induces an intense immunological response in CD, with the production of nitrites and IFN gamma[37].

SEROLOGY

Serological markers of significance include EMA and tTG antibodies. The sensitivity of tTG is 98% and specificity 96%, whereas the EMA is 100% specific and sensitivity is greater than 90%[38]. Assays for tTG antibodies are largely based on the dominant antigen in the EMA test, however, tTG assays are more reliable and more reproducible, largely because the EMA is a qualitative assay and tTG assays are quantitative.

The antibodies to tTG and deamidated gliadin peptide (DGP) have been combined in a multiplex immunoassay of persons suspected as having CD, to potentially provide a complete antibody phenotype[39], and thereby to improve the performance characteristics of the serological testing. A meta-analysis has shown that the tTG antibody test out-performs the DGP antibody test, with a 5.2% greater sensitivity (93.0% *vs* 87.8%) and a 2.4% greater specificity (96.5% *vs* 94.1%), respectively[40].

DGP has been suggested to possibly be a better diagnostic test for CD before institution of a GFD than is the conventional gliadin antibody testing: the sensitivity, specificity, and accuracy of deamidated gliadin-IgA (74%, 95%, and 86%), deamidated gliadin-IgA (65%, 98%, and 84%), and deamidated gliadin-IgA + IgG (75%, 94%, and 86%), were superior to gliadin-IgA (63%, 90%, and 79%) ($P > 0.05$) and gliadin-IgG (42%, 90%, and 60%) ($P > 0.01$), and were similar to tTG-IgA (78%, 98%, and 90%)[39]. Further comparative evaluation with more modern serological assay methods would be useful, including tTG antibodies.

Because the small bowel biopsy in the person with CD does not have a pathognomic histological feature, serological testing may have an important supportive role in providing added information for the diagnosis of CD. Tissue transglutaminase (tTG) catalyzes the Ca^{2+}-dependant formation of cross links between protein-bound glutamine and glycine residues. The glutamine residue can be deamidated to glutamic acid by tTG, including specific glutamines in gluten-containing proteins. Deamidated gluten proteins have enhanced affinity for the HLA-DQ heterodimer of antigen-presenting cells. This activates T-lymphocytes and produces a T-helper type 1 response in the mucosa of celiac patients. The tTG-gluten complex is processed by B-cells, and presented to gluten-specific T cells, that give rise to tTG antibody T-helper type 2 response[41]. The tTG autoantibodies interact with extracellular membrane-bound transglutaminase, and thereby play an important role in proliferation of epithelial cells in persons with predisposition to CD.

The tTG is responsible for post-translational modification of proteins by introduction of lysine crosslinks, as well as deamidation. The IgA anti-tTG responses in CD and in dermatitis herpetiformis are focused on the region of tTG responsible for its transamidation and deamidation reactions, whereas the IgG response targets other regions of the enzyme[42].

The performance of serum anti-tTG may depend on clinical presentation of CD; e.g. classic symptomatic disease or silent asymptomatic disease. In patients estimated to have Marsh-Ⅲ A, B, or C degree of villous atrophy,

the sensitivity, specificity, positive and negative predictive values of the anti-tTG antibody test were 71%, 65%, 91%, and 30%, respectively[43]. The sensitivity was 90% for subjects with total villous atrophy, and only 42% for those with partial villous atrophy. In persons thought to have a high pretest probability of having CD based on symptoms such as weight loss, anemia, or diarrhea, 9.1% were anti-tTG negative[44], indicating that serological testing may miss a substantial number of cases of untreated CD that are antibody negative. Strongly positive tTG assay results without CD biopsy changes have also been recorded[45]. In the latter, it is not known if further biopsies at a later date will reveal the typical morphological changes of untreated CD.

The supply sources for EMA are limited to monkey esophagus or umbilical cord, and many assays are done "in-house" that may not be readily duplicated in other laboratories. EMA are considered to be highly sensitive and specific for serological changes seen in untreated CD. However, EMA assays are expensive, qualitative, and therefore subjective. EMA is increasingly being replaced by serological testing for antibodies to tTG, especially since the anti-tTG assay can be more precisely quantitated.

The EMA binding patterns and serum samples from CD patients are tTG-2 targeted, and the humoral response against tTG occurs at the level of the intestinal mucosa. tTG-2 targeted extracellular IgA deposits have been demonstrated by immunofluorescence in the small bowel mucosa in untreated celiac subjects, even when they are absent from the serum. In those subjects suspected of having CD but who are EMA and anti-tTG negative, finding the tTG-2 targeted antibody in the jejunal mucosa may help to make the diagnosis of CD[46].

Frozen sections of small bowel specimens were evaluated by immuno-fluorescence using rabbit antibody against human IgA. Although at best, semi-quantitative, these immunofluorescent deposits may be better initial markers for gluten sensitivity than small bowel mucosal IEL densities[47]. While architectural changes, such as villous atrophy, may lead to suspicion of untreated CD, tTG-2 specific IgA deposits may potentially be more useful. Further studies are needed.

Seronegative (EMA or tTG) CD occurs in less than 10% of celiacs, particularly in those with lesser degrees of villous atrophy. The presence of EMA in subjects with an architecturally-normal small bowel biopsy could indicate early developing CD. Serum and intestinal celiac anti-autobodies and intra-epithelial lymphocytes have been assessed as possible indicators of developing CD. Celiac autoantibody deposits have been recorded to provide a sensitivity and specificity of 93% and 93%, respectively, in detecting subsequent CD; this is compared to 59% and 57% for CD3+; 76% and 60% for gamma-delta+, and 88% and 71% for villous tip intra-epithelial lymphocytes[48].

Simple "in the office" anti-tTG tests have been developed commercially, and the blood drop-based assay for IgA anti-tTG was reported to have a sensitivity of 90% and a specificity of 95%[49]. The sensitivity and specificity of serum anti-tTG is laboratory-dependent, and assay results may differ for clinical compared to research laboratories. Because CD does not have a pathognomic histological feature, serology may have a supportive role in making the diagnosis. As CD is defined as a gluten-sensitive enteropathy, a clinical or serological response to a GFD is essential to establish a diagnosis of CD. Sometimes, re-biopsy after a GFD is necessary, or even further evaluation after a gluten challenge may be required.

A normal tTG level does not predict recovery of villous atrophy in celiac subjects on a GFD. For example, 16 of 48 (33%) subjects with CD on a GFD had persistent villous atrophy, but 7 of these 16 (44%) had a normal tTG[50]. In a multicenter, prospective study involving adult subjects attending one of several primary care practices, and in individuals not having symptoms or a condition known to be associated with CD, initial testing was done with anti-tTG. Those with elevated anti-tTG were tested for EMA (IgA), and then those in turn who were positive for EMA underwent an intestinal biopsy and HLA typing[51]. A positive anti-tTG was found in 3.1%, and the prevalence of CD in the serologically screened sample was 2.3%. When a similar study was performed in a university hospital, the prevalence of CD was 3.5%, and a negative HLA-DQ type excluded the diagnosis[52]. However, the "addition of HLA-DQ typing to TGA and EMA testing, and the addition of serological testing to HLA-DQ typing, provided the same measures of test performance as either testing strategy alone"[52].

Because the EMA and anti-tTG responses may remain elevated in CD on a GFD, it may be useful to measure soluble CD163, a scavenger receptor shed by tissue macrophages and correlated with the inflammatory lesion in CD. Those subjects with a more severe (Marsh grade 3) lesion had higher levels of CD163 than did those with a milder (Marsh grade 2, grade 1 or grade 0) lesion[53]. Further studies are needed.

There are three fatty acid binding proteins in the cytosol of the intestine: intestinal FABP (I-FABP), liver FABP (L-FABP), and ileal bile acid binding protein (I-BABP). These are present in increased amounts in the serum of persons with enterocyte damage from, for example, mesenteric thrombosis or necrotizing enteritis. Because I-FABP and L-FABP are found predominantly in the enterocytes in the upper portion of the jejunal villi, it is not surprising that their concentration is increased in the plasma of persons with CD. When measurements were made within one year of the introduction of a GFD, these initially increased I- and L-FABP levels fell to normal[54]. Together with following the patient's symptoms, quality of life, and celiac serology, assessing intestinal permeability may potentially prove to be a useful non-invasive test to follow the histological improvement of CD patients on a GFD.

The current standard for the assessment of adherence to a GFD in adult CD patients is largely based on a personal clinical evaluation. However, most serological assays appear to compare adequately in sensitivity and specificity to a thorough nutritional evaluation of the assessment of adherence to a GFD[55]. This is important, since only approximately 45%-80% of patients with CD adhere strictly to a GFD. This is thought to place them at increased risk

of developing metabolic bone disease, anemia, gastrointestinal symptoms, as well as impaired psychological well-being and quality of life.

MUCOSAL HISTOLOGY

Criteria for the diagnosis of CD include the initial demonstration of small bowel architectural changes including mucosal villous atrophy with crypt hyperplasia, along with increased intra-epithelial lymphocytosis. However, this may be a slowly developing process and the changes are not specific. In addition, some persons may suffer from CD symptoms before histological evidence can be documented. While some authors have suggested that an anti-tTG level can be defined which gives a positive predictive value of 100% for CD[56], it remains the standard of practice to always obtain a biopsy to determine if the histological changes of untreated CD are present before initiation of a GFD.

Some have noted that there may be significant differences between pathologists in mucosal biopsy interpretation[57]. The older Marsh classification, as modified by Oberhuber and colleagues, continues to be used by many pathologists. But for some, it may be considered complex because there are many different diagnostic categories. A simpler grading system has been proposed[57] based on three villous morphologies (A, non-atrophic; B1, atrophic, villous-ratio < 3:1; B2, atrophic, villi no longer detectable), and an intraepithelial lymphocyte count of > 25/100 enterocytes. Compared to the older classification system, this simpler classification schema was thought by the investigators to be superior.

However, the severity of villous atrophy based on histological analysis of biopsy specimens taken from the proximal intestine does not necessarily predict the severity of symptoms in CD for either children or adults. For example, when clinical symptoms of 18 CD patients with a good histological recovery were compared with 13 CD who had persistent small intestinal villous atrophy despite maintaining a GFD, symptoms could be absent despite the persistence of morphological abnormalities[58]. Other authors also noted the lack of association between the histological CD lesion and clinical manifestations[59]. Indeed, the lack of correlation between the degree of villous atrophy and symptoms was stressed in a further study of 499 CD patients, in which 44% had a classical presentation and 56% had atypical or silent CD[60]. These findings are not surprising, however, since the response to a GFD occurs initially in the most distal small bowel. Months to even years on a strict GFD may be needed before improvements in the proximal intestinal mucosa occur.

While duodenal biopsy represents the "gold standard" for the diagnosis of CD, capsule endoscopy (CE) has revealed that over a third of celiac patients have macroscopic mucosal changes extending beyond the duodenum, and in approximately 7%, the entire small bowel was involved[61]. As compared with duodenal biopsy for detecting changes in CD, the sensitivity of CE was reported to be 88%, specificity 91%, positive predictive value 97%, and negative predictive value 71%.

"Latent CD" is defined as abnormal celiac serology and a normal small bowel biopsy (Marsh stage 0). These so-called "latent CD" patients have an increased hazard (HR) ratio for death comparable to those with Marsh 1-2 and Marsh 3: 1.35; 95% CI, 1.14-1.58, median follow-up, 6.7 years; HR, 1.72; 95% CI, 1.64-1.79; median follow-up, 7.2 years; HR, 1.39; 95% CI, 1.33-1.45; median follow-up, 8.8 years, respectively[62]. This corresponded with excess mortality of 1.7 per 1000 person-years in "latent CD", 10.8 in Marsh 1-2, and 2.9 in Marsh 3 stage CD. This raises the possibility that it may be important to diagnose very early CD. However, this label of "latent CD" may differ from the original definition of latent CD (without serological studies) where abnormal small intestinal architectural changes were induced with a high gluten-containing diet (initially reported in dermatitis herpetiformis) and then normalized on a GFD.

Given that duodenal biopsy is still the "gold standard" for the diagnosis of CD, it is of interest to know that when only two duodenal biopsies are obtained, diagnosis of untreated CD is confirmed in 90%, however, increasing the number of biopsies to 3 increased detection to 95%, and to 4 biopsies, 100% respectively[63].

While some present with symptoms and a small bowel biopsy is done to exclude CD, others will present with positive serology and a duodenal biopsy is then obtained. Occasionally lesions may be patchy or detected only in the duodenum, resulting in potential for sampling error and a false-negative result. Confocal endomicroscopy (CEM) is a novel method that permits magnification *in vivo* of the gastrointestinal mucosa by up to 1000-fold. In persons with known CD, accuracy of CEM in diagnosing CD was reported to be excellent, with receiver operator characteristics under the curve of 0.946, sensitivity of 94%, and specificity of 92%[64]. CEM was also sensitive in the detection of histological changes following treatment with a GFD.

"Lymphocytic enteritis" (Marsh 1) may be associated with symptoms, yet serological markers of CD appeared to be of limited value in identifying these individuals. In 130 of 221 first-degree relatives of HLA-DQ2-positive patients with CD, relatives were positive also for HLA-DQ2, and 49% were Marsh 0, 25% Marsh 1, < 1% Marsh 2 and 10% Marsh 3. Only 17 of 221 relatives had positive serological markers for CD[65]. These authors argued that the higher number of symptomatic patients with lymphocytic enteritis (Marsh 1) supports HLA-DQ2 genotyping strategy followed by duodenal biopsy in relatives of CD patients. Further studies to confirm these observations are needed.

Anti-tTG levels have continued to be used in assessing initiation and maintenance of a GFD. It is believed that tTG levels might be followed to reduce the risk of complications and monitor histological changes in the upper small bowel[66].

CLINICAL PHENOTYPES

The ESPGHAN (European Society of Pediatric Gastro-

enterology, Hepatology and Nutrition) criteria distinguish between three different forms of CD so that classification might be more precise: the latent or *potential* form defined by the presence of anti-celiac antibodies; the silent form (*asymptomatic*) defined by the presence of anti-celiac antibodies and villous atrophy of the small intestine; and the symptomatic form defined by the presence of anti-celiac antibodies, villous atrophy and clinical symptoms.

The adult height of children with classical CD (e.g. symptomatic with diarrhea) is influenced by their compliance to a GFD. Children diagnosed with CD after 4 years of age show a slower and less complete catch-up growth. A delayed diagnosis of CD may be associated with a shorter adult height in men, but not in women[67].

While abdominal symptoms may respond quickly to a GFD, it may take up to a year or more after the introduction of a GFD for persons with CD to achieve normalization of their initially abnormal small bowel biopsy. Elderly patients respond more slowly than younger patients to a GFD.

"Gluten sensitivity" may be defined as symptoms, such as diarrhea, apparently induced by gluten-containing foods. These have been reported in the absence of changes in small intestinal histology. In persons with diarrhea-predominant irritable bowel syndrome (D-IBS), stool frequency and the gastrointestinal symptoms score return to normal values in 60% of D-IBS subjects who were positive for HLA-DQ2 and CD-associated serum IgG after six months on a GFD, compared to only 12% who were negative[68].

Among the complications of undiagnosed and, therefore, untreated CD are growth failure in children, infertility, anemia, osteoporosis, small intestinal non-Hodgkin lymphoma[69], and a 3.9-fold increased all-cause mortality rate[70]. Potentially, this may underscore the importance of diagnosing and treating even latent CD.

Celiac patients were reported to have a 5.4-fold higher risk of non-Hodgkin's lymphoma, but no increased risk of Hodgkin's or chronic lymphatic leukemia. A shared susceptibility amongst siblings is observed[69]. It remains controversial whether there is an increased risk of developing lymphoma in CD if the disease is asymptomatic[58,71].

There is a 5-fold increase in risk of lymphoproliferative malignancy in CD in comparison to the general population[72].

CLINICAL ASSOCIATIONS

The prevalence of autoimmune diseases (e.g. autoimmune thyroid disease) is increased in persons with CD, as compared with the healthy control population. Conversely, CD is increased in persons with autoimmune diseases. The cumulative risk of autoimmune disease in patients with CD is 8% at age 15, and 16% at age 30 years. Factors associated with an increased risk of autoimmune diseases associated with CD include a family history of autoimmune disorders, and a diagnosis of CD before the age of 36[73]. Once the diagnosis of CD has been made, patients who are adherent to a GFD have a 6% risk of developing autoimmune disease at 10 years, *vs* 16% in those who are not compliant with a GFD. Expressed differently, the incidence of autoimmune disease is 5.4 per thousand patient years during adherence to a GFD, *vs* 11.3 per thousand patient years during non-adherence.

Asymptomatic CD is also seen in children and adults with autoimmune hepatitis and autoimmune bile duct disease. CD may be associated with asymptomatic increases in transaminase values. Persons with autoimmune liver disease should be examined for possible CD. In persons with CD who have acute hepatitis, an autoimmune cause should be suspected[74].

More and more clinical associations have been suggested for CD. For example, 42% of CD patients had oral soft tissue lesions, as compared to only 2% of non-CD patients[75]. Recurrent aphthous stomatitis disappeared in 89% of patients after one year of a GFD.

Biopsy-defined CD has a 4-fold higher prevalence in those with irritable bowel syndrome[76]. Mental disorders, non-compliance with the GFD, active medical co-morbidities, and dissatisfaction with doctor/patient communication were associated with reduced CD Questionnaire scores[77].

Up to 10% of patients with CD have neurological symptoms ranging from polyneuropathy, epilepsy, myoclonus, multifocal leukoencephalopathy, dementia, chorea, migraine, memory/attention impairment and peripheral axonal and demyelinating neuropathies as well as acetylcholine-antibody positive myasthenia gravis[78]. Autoimmunity may act as a mechanism triggering neurological dysfunction[79], and anti-neuronal, anti-gliadin and tTG antibodies may contribute to neurological impairment through Apaf-1 activation with Bax and cytochrome C translocation, leading to impairment of mitochondrial-dependent apoptosis. There is no statistically significant association between CD and subsequent development of Parkinson's disease, Alzheimer's disease, hereditary ataxia, symptoms of ataxia, Huntington's disease, or spinal muscular atrophy[80].

Adult but not pediatric patients with CD have an increased risk of sepsis, particularly pneumococcus infection[81]. In CD there is an increased prevalence of splenic hypofunction[82].

Using community-based cohorts and a record-linkage database, the adjusted relative risk of cardiovascular disease in CD was 2.5 for new EMA positive *vs* EMA negative individuals[83]. This suggests that CD may be associated with an increased risk of cardiovascular outcome. There may also be an association between CD and eosinophilic esophagitis[84].

Aldolase B deficiency causes hereditary fructose intolerance, and this may be associated with CD[85]. In CD patients as compared with individuals with dyspepsia or healthy controls, serum ghrelin concentrations are higher, not correlated to the severity of duodenal histological lesions, and revert to normal during the institution of a GFD, despite persistent duodenal lymphocytic infiltration[86]. It is not clear if these alterations in ghrelin concentrations have any biological importance in CD.

In 18 CD patients, there was increased intestinal 5-HT-enterochromaffin cell numbers, higher peak plasma 5-HT levels and postprandial area under the curve of 5-HT levels after a high-carbohydrate meal, as well as increased platelet 5-HT stores[87]. The authors suggested that serotonin excess may mediate dyspeptic symptoms in untreated CD. Further evaluation is required.

A meta-analysis of serological or histological diagnosis of CD in unselected adults with dyspepsia showed that the numerically increased prevalence was not statistically significant[88]. Another systematic review and meta-analysis by the same group, examining 14 studies with 2,278 persons diagnosed with IBS, had an approximately 4% prevalence of CD. The OR for biopsy-proven CD in IBS cases *vs* controls was 4.34[89]. In children with CD, 40% had elevated transaminase values; of those with elevated transaminase values, 95% were cryptogenic and normalized on a GFD, and 5% had autoimmune hepatitis that required immunosuppression plus a GFD to normalize clinical and biochemical parameters[90].

TREATMENT

Gluten free diet

What is the definition of a GFD? Even "gluten-free" products may not be completely free of gluten. In 1998, the World Health Organization/Food and Agriculture Organizations Commission proposed that foods which are said to be "gluten-free" could not contain more than 200 ppm of gluten. Each individual with CD may have a unique threshold or tolerance to the amount of gluten in the diet. Daily gluten intake of less than 10 mg is unlikely to cause significant histological abnormalities in the intestine of patients with CD[91]. Patients adhering to a GFD report an improved health related quality of life.

A systematic review has revisited the complications and need for long term follow-up in CD[92]. A 16-question disease-specific symptom index has been validated in adults with CD[93]. Such an index might be used to monitor the response of a CD patient to a GFD.

The treatment of CD is the life-long use of a GFD. A primary goal in the care of patients with CD is to improve the quality of their lives, through a collaboration of the stakeholders[94]. Lanzini *et al*[95] assessed in 465 consecutive CD patients, the histological outcome after a GFD consumed for a median of 16 mo. While CD serology became negative in 83% of CD patients with Marsh Ⅲ lesions on a GFD, mucosal biopsy histology normalized in only 8%, improved except for increased intraepithelial lymphocytes in 65%, was unchanged in 26% and worsened in 1%. The authors concluded that "complete normalization of duodenal lesions is exceptionally rare in adult celiac patients despite adherence to GFD, symptoms disappearance and negative CD related serology".

Early diagnosis and treatment are important in CD, as some of the associated complications may be irreversible, unless the CD is treated[96]. Growth retardation, osteoporosis and abnormal dentition will remain permanent if not treated early. The prevalence of associated depression is up to 37%, similar to that of persons with other chronic conditions[97].

In children with CD, long-term consumption of oats may be well tolerated[98], although concern has been expressed regarding possible contamination of oats with other gluten-containing grains. Other investigators have demonstrated that transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in CD[99]. It has always been assumed that patients with CD must remain on a GFD for life. However, up to 10% of CD patients diagnosed in childhood were reported to develop long-term latency of their CD when returning to a gluten-containing diet[100]. In patients who have been on a GFD and have no symptoms, even if there were small bowel CD-like histological abnormalities which remained, some had no evidence of clinical relapse even though they had been on a gluten-containing "normal" diet for more than 2 years.

Individuals with villous atrophy but no symptoms are said to have "silent" CD. Almost half of CD patients may clinically tolerate a gluten-containing diet, yet continue to have mucosal abnormalities. Indeed, approximately 10% of CD patients diagnosed in childhood may develop clinical tolerance to gluten. In the United Kingdom, about one-third of CD subjects are under no active follow-up[101].

Alternatives to a gluten free diet

There may be poor compliance with the GFD because it is difficult and gluten-free products are expensive. For these reasons, new approaches have been taken in the treatment of CD. Some of these include orally administered endopeptidase, antagonists to S100B protein, IL-15 blockers, elemental diets and transamidation of wheat flour. Lactobacilli added to sourdough for fermentation are able to break down the proline-/glutamine-rich gluten peptide. This may play a role in the future treatment of CD[102]. Supplementation of nutrients may become essential depending upon the severity of malnutrition. One double–blind placebo-controlled multicenter trial showed a significant improvement in general well being after 6 mo of supplementation with vitamin B[103].

Prolyl-endopeptidases are able to digest ingested gluten. Oral therapy with prolyl-endopeptidases, exogenous protease enzymes, represents a new approach to managing CD[104]. Bacterial prolyl-endopeptidase from *Flavobacterium meningosepticum* removes gluten toxicity by cleaving it into small fragments which lack T-cell stimulatory properties[105]. After prolonged exposure to high concentrations of bacterial prolyl-endopeptidase, the amount of immunostimulatory gliadin peptides reaching the local immune system in CD is decreased[106]. The prolyl-endoprotease from *Aspergillus niger* (AN-PEP) is a member of the serine peptidase family, and this degrades gluten peptides rapidly[107]. This AN-PEP is capable of accelerating the degradation of gluten in a gastrointestinal model that closely mimics *in-vivo* digestion[108]. The

pH optimum of the enzyme is compatible with that found in the stomach, and the enzyme is resistant to degradation by pepsin.

The gluten proteins may be purposely modified to abolish their capacity to stimulate the interferon gamma from CD4+ T-cells[109]. Another approach to the therapy of CD is the designing of non-toxic wheat, rye or barley based on their protein homology[110,111].

Other therapeutic approaches would include the binding of gluten to HLA-DQ2 or HLA-DQ8, or blocking the gluten-reactive T cells by immunotherapy (e.g. vaccination). For example, transamidation of wheat flour with a food-grade enzyme and an appropriate amine donor (microbial transglutaminase and lysine methyl ester) can be used to block the T-cell mediated gliadin activity[99]. Gluten contains many immunogenic peptides, but there may be weak varieties with a natural low number of T-cell-stimulatory epitopes[111].

Polymeric binders reduce the deleterious effects of gliadin on intestinal epithelium in cultured cells and transgenic mice[112]. These binders have a strong affinity for gliadin, inhibiting cytoskeleton disorganization and ultrastructural changes in intestinal epithelial cells. Their beneficial use in humans remains to be established.

Antigen-presenting cells include dendritic cells, macrophages and B-cells. A unique subset of dendritic cells appears to be responsible for local activation of gluten-reactive T-cells in the celiac lesion[113]. Enteric glial cells (EGC) release neurotropic factors and are activated by inflammatory insults. EGC-derived S100B protein released in astroglial cells is increased in the duodenum of patients with CD, with increased S100B messenger RNA and protein expression, increased iNOS protein expression, and increased nitrite production in treated CD[114]. This does not occur in those CD patients on a GFD, or in non-CD control subjects. Products derived to block EGC-derived S100B protein may have a therapeutic role.

IL-15 has proinflammatory and anti-apoptotic properties. IL-15 is over-expressed in the enterocytes and lamina propria mononuclear cells of untreated CD, where its level reportedly correlates with the degree of mucosal damage[115]. IL-15 also promotes IEL survival. Blocking IL-15 and suppressing uncontrolled IEL activation and survival has the potential to provide a new therapeutic approach to prevent tissue damage in CD. In the intestinal mucosa of CD patients, IL-15 impairs Smad3-dependant TGF-beta signaling in human T-lymphocytes downstream from Smad3 nuclear translocation[116]. There is upregulation of phosphor-c-jun. This provides further support to the suggestion of the potential therapeutic effect of blocking IL-15.

Intestinal permeability is increased in patients with CD, and is associated with alterations in tight junction proteins (e.g. zonulin). Addition of zonulin may prevent T-cell mediated stimulation in CD. In a double-blind randomized placebo-controlled study of milligram doses of AT-1001, an inhibitor of paracellular permeability derived from *Vibrio cholera*, prevented the expected increases in intestinal permeability in subjects with CD challenged with gluten[117]. AT-1001 use is also associated with a diminution in the anticipated rise of interferon-gamma levels.

REFRACTORY DISEASE

Recurrent symptoms sometimes develop in biopsy-proven CD patients on a GFD. The most common cause of non-responsive CD, which occurs in about 30% of CD patients, is non-adherence to a GFD. A Celiac Dietary Adherence Test (CDAT) consistency in a 7-item questionnaire was developed using a logistic regression, and validated against transglulaminase serology for the assessment of adherence to a GFD[118]. Usually, poor compliance to a GFD is thought to be responsible, although compliance may sometimes be difficult to establish. Intentional dietary indiscretion may be evident, but sometimes, there is limited awareness of gluten-containing substances. Gluten is ubiquitous, being documented in pill capsules and other materials, such as communion wafers.

Other causes, detailed elsewhere[119], may be responsible for recurrent symptoms even though the CD patient appears to be following a strict GFD. Some causes include associated primary or secondary pancreatic insufficiency, small intestinal bacterial overgrowth, collagenous sprue, or lymphocytic or collagenous colitis. Rarely, a complication may be responsible (e.g. lymphoma, carcinoma).

Sometimes, no initial response to a GFD ever occurs and symptoms persist. In these subjects, biopsies may be abnormal, but the gluten-dependent nature of the small bowel abnormalities was never documented. This has been labeled "unclassified sprue" or "sprue-like intestinal disease". Some of these persons may eventually prove to have a lymphoma. As many as approximately half of patients with CD on a GFD for more than two years may be able to tolerate a gluten challenge, even though they have mucosal abnormalities[100]. About 10% of CD patients diagnosed in childhood may develop temporary tolerance to gluten. However, because of the continuing mucosal abnormalities, they remain at risk of developing the complications of CD. Indeed, adolescents who do not adhere to a GFD have a lower quality of life[120].

Immunohistochemical labeling has helped to define an abnormal prognostic profile of intra-epithelial lymphocytes in the small bowel mucosa, so-called "refractory celiac disease, type 2 (RCD 2)" characterized by an aberrant clonal IEL population with loss of IEL antigens. About half of RCD 2 patients develop an enteropathy-associated T-cell lymphoma (EATL) within 5 years, and a particular HLA-DQ subtype, DQ2, if homozygous, predisposes to RCD 2[121].

Complete duodenal mucosal recovery in CD may be limited and may require prolonged periods of a GFD. In one study, remission was seen in 65% or no histological improvement was seen in 26% of patients[95]. This might be anticipated, however, if only duodenal biopsies are being taken since the proximal small intestine is most severely affected and varying periods of time are required for recovery to be significant. An apparent failure to histologically respond to a GFD may only reflect that duo-

denal, rather than more distal small intestinal biopsies were repeated.

Refractory CD has been defined as persistent or recurrent villous atrophy with crypt hyperplasia and increased intraepithelial lymphocytes (IELs) despite a strict GFD for greater than 12 mo (or if severe persisting symptoms necessitate intervention independent of the duration of the GFD)[122]. In assessing the GFD response, the site of re-biopsy and duration on a strict GFD are crucial to the definition of refractory disease. In some, as suggested by this definition, the opportunity for re-evaluation is very limited because of rapid progression of the intestinal disease.

RCD is usually manifested by recurrence of symptoms and intestinal abnormalities, despite adherence to a GFD. RCD can be further defined from a prognostic perspective by the histological appearance of monoclonal or polyclonal intraepithelial lymphocytes (RCD type 2) *vs* normal lymphocytes (type 1)[123]. These changes and the development of an EATL were shown to be adverse factors in the prognosis of CD, particularly in the first two years after CD has been deemed to be refractory[124].

Refractory celiac disease (RCD) type 2 but not type 1 shortens the sufferer's life expectancy[123].

Corticosteroids may improve clinical symptoms in some patients with RCD. Unfortunately, the histological response to steroids has not been consistent[123]. Patients with RCD 1 may benefit from immunosuppressive therapy, whereas those with RCD 2 may respond to Cladribine or to stem cell transplantation. Treatment with cladribine and anti-CD-52 has been shown to be associated with histological improvement. Azathioprine and anti-tumor necrosis factor-α have shown only limited success. The development of an overt lymphoma within 8 wk of treatment was seen in 3 out of 4 of these patients, thereby preventing further use. Alternative strategies that have been suggested include stem cell transplantation to replace the abnormal intra-epithelial lymphocyte population, and the blocking of IL-15[125-129].

CONCLUSION

Celiac disease is being increasingly diagnosed because of the recognition that the disease may be present without significant intestinal symptoms, may be associated with other autoimmune disorders and may be suspected from serological screening. Definition of the disease includes an intestinal biopsy before treatment with a GFD along with documentation of a definitive GFD response. In some patients, this may necessitate further intestinal biopsy after a period on a GFD. Serological testing may be useful in providing additional evidence that CD is present and may be useful in some patients to assess GFD compliance. Recent studies focused on the genetic basis and pathogenesis of CD have emerged to improve understanding of the complex molecular alterations that occur with CD.

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EDITORIAL

Management of liver cirrhosis between primary care and specialists

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Abstract

This article discusses a practical, evidence-based approach to the diagnosis and management of liver cirrhosis by focusing on etiology, severity, presence of complications, and potential home-managed treatments. Relevant literature from 1985 to 2010 (PubMed) was reviewed. The search criteria were peer-reviewed full papers published in English using the following MESH headings alone or in combination: "ascites", "liver fibrosis", "cirrhosis", "chronic hepatitis", "chronic liver disease", "decompensated cirrhosis", "hepatic encephalopathy", "hypertransaminasemia", "liver transplantation" and "portal hypertension". Forty-nine papers were selected based on the highest quality of evidence for each section and type (original, randomized controlled trial, guideline, and review article), with respect to specialist setting (Gastroenterology, Hepatology, and Internal Medicine) and primary care. Liver cirrhosis from any cause represents an emerging health issue due to the increasing prevalence of the disease and its complications worldwide. Primary care physicians play a key role in early identification of risk factors, in the management of patients for improving quality and length of life, and for preventing complications. Specialists, by contrast, should guide specific treatments, especially in the case of complications and for selecting patient candidates for liver transplantation. An integrated approach between specialists and primary care physicians is essential for providing better outcomes and appropriate home care for patients with liver cirrhosis.

Key words: Ascites; Family medicine; Hepatic encephalopathy; Hypertransaminasemia; Portal hypertension

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INTRODUCTION

Liver cirrhosis is defined in histology as a bridging fibrosis-a late stage of hepatic fibrosis-leading to deranged liver architecture and regenerative nodules. Liver cirrhosis is considered the end stage of a variety of chronic liver diseases, and is irreversible in its advanced stages[1]. Cirrhosis is characterized by poor life expectancy and is a leading cause of morbidity and mortality: in the United States liver cirrhosis is the 12th most common cause of death (9.5/100 000 individuals), while in Italy the incidence of liver cirrhosis is over 26 000 new cases each year, with a prevalence over 120 000 cases (7000 below 45 years), and 20 deaths/100 000 individuals[2,3]. Figures are likely to be even higher in Asia and Africa. Liver cirrhosis carries the

risk of life-threatening complications, partly due to a number of co-morbidities. Medical treatments that may halt the progression of compensated cirrhosis to decompensated cirrhosis are currently being developed[1]. Liver transplantation, however, is the only option in a selected subgroup of patients with end-stage disease. Because of the increasing prevalence of chronic viral hepatitis and (alcoholic-nonalcoholic) steatohepatitis and their high risk evolution toward liver cirrhosis and end-stage liver disease, preventive programs and early management of these conditions are considered an emerging health issue. It is essential that primary care physicians (PCPs) be optimally trained to identify patients with chronic liver disease as early as possible, and to properly manage those with liver cirrhosis[4]. A close interaction is therefore required between PCPs and specialists (i.e. gastroenterologists, hepatologists, and internists) who have a fundamental role as consultants and guides for specific treatments, i.e. in the case of complications and the management of patients approaching liver transplantation.

This article is based on a PubMed search to provide an updated view for comprehensive management of several aspects of liver cirrhosis in different settings.

DATA SOURCES

Full papers were searched on Medline (http://www.ncbi.nlm.nih.gov/PubMed) for guidelines, randomized controlled trials (RCTs), and authored review articles published in English-language journals in the past 25 years. The following MESH headings were used: "ascites", "liver fibrosis", "cirrhosis", "chronic hepatitis", "chronic liver disease", "decompensated cirrhosis", "hepatic encephalopathy", "hypertransaminasemia", "liver transplantation", and "portal hypertension". The reference list was updated as of November 2010. Authors independently assessed articles for relevance and study quality. For each section, evidence levels were scored as follows: (1) LEVEL I (at least one properly conducted RCT, systematic review, or meta-analysis); (2) LEVEL Ⅱ (other comparison trials, non-randomized, cohort, case-control, or epidemiologic studies, and preferably more than one study); and (3) LEVEL Ⅲ (expert opinion or consensus statements).

APPROACH TO PATIENTS WITH LIVER CIRRHOSIS

The clinical presentation of liver cirrhosis is often asymptomatic until complications appear. The presence of liver cirrhosis should be suspected in any patient with chronic liver disease and abnormal aminotransferases and/or alkaline phosphatase. Chronic liver disease stigmata should be searched for, and include vascular spiders, palmar erythema, and muscle wasting. Also, a palpable left lobe of the liver, hepatomegaly and splenomegaly are suggestive for liver cirrhosis. The diagnosis becomes much easier in the presence of signs of decompensation, namely jaundice, ascites, and asterixis. Additional laboratory tests include those exploring liver synthetic function, such as serum albumin and prothrombin time, while serum bilirubin investigates the ability of the liver to conjugate and excrete bilirubin. A low platelet count is suggestive of portal hypertension and hypersplenism. An AST/ALT ratio above 1 is indicative of liver cirrhosis, but its absence does not exclude cirrhosis (i.e. low specificity). The imaging studies include abdominal ultrasound, CT scan or magnetic resonance and might reveal a nodular liver and splenomegaly. The differential diagnosis of advanced chronic hepatitis relies on liver biopsy, which is still the gold standard for end-stage chronic liver disease. Percutaneous liver biopsy is not necessary in the presence of decompensated cirrhosis or when imaging studies have confirmed the presence of cirrhosis. Thus, liver biopsy is reserved for selected patients and can also be performed in out clinic settings[5,6]. Histology provides information on etiology, disease stage and grade of inflammation. Although the ultimate decision is not currently taken by PCPs, they should repeatedly check the patient with blood tests before referral for liver biopsy (at least two times and at least 2-3 mo apart). If abnormalities persist in spite of second step analyses and a liver ultrasonography has been inconclusive, the decision to perform a liver biopsy must be taken on an individual basis and rely on the patient's age and general health status, as well as the need for prognostic information (LEVEL Ⅲ)[7]. According to the American Association for the Study of Liver Disease (AASLD), liver biopsy has a major role in diagnosis, assessment of prognosis, assistance in therapeutic decisions, and reinforcement of the patient's compliance (LEVEL Ⅱ)[5]. Biopsy, however, is a costly procedure which is not free of potential side effects and risks, and is often refused by the patient. A French survey, which interviewed over one thousand PCPs, concluded that liver biopsy may be refused by up to 59% of patients with chronic hepatitis C and that 22% of PCPs share a similar concern[8].

Novel non-invasive methods might provide preliminary information with good diagnostic accuracy for further selection of patients at risk for progressive liver disease. For example, tests might help to evaluate the presence and extent of liver fibrosis, and to differentiate cirrhosis from chronic hepatitis (positive predictive values exceed 85%-90%)[9]. Such policy may be helpful in the primary care setting. Transient elastography (FibroScan®), for example, assesses liver stiffness, with some limitations in the case of morbid obesity, small intercostals spaces, and ascites[10]. Ongoing liver fibrosis is also predicted by using specific algorithms of surrogate serum markers or by the application of standardized procedures (e.g. APRI: the aspartate transaminase to platelets ratio index; FibroTest: haptoglobin, α2-macroglobulin, apolipoprotein A1, γGT, bilirubin: Hepascore: bilirubin, γGT, hyaluronic acid, α2-macroglobulin, age, gender; BARD: Body mass index (BMI), AST/ALT ratio, diabetes). A novel technique based upon ultrasound-based elastography (Fibroscan, Echosens, Paris, France) can assess mean hepatic tissue stiffness[11]. Results are expressed in kilopascals (kPa) and

Table 1 Diagnostic tests, suggested etiology, and current treatment for the most frequent forms of liver cirrhosis in adult patients

Abnormal test(s)	Etiology	Treatment
γGT (high), MCV (high)	Alcohol	Abstinence
HBsAg, HBV-DNA, HBc-IgM, HDV-RNA (positivity)	HBV + Delta virus infection	Interferon α-2b, nucleoside (Lamivudine, Telbivudine, Entecavir) and nucleotide (Adefovir, Tenofovir) analogues
HCV-RNA (positivity)	HCV infection	Interferon plus ribavirin
γGT (high), alkaline phosphatase (high), AMA (positivity)	Primary biliary cirrhosis	Ursodeoxycholate
ANA, ASMA, LKM (positivity)	Autoimmune hepatitis	Prednisone, azathioprine
Ferritin (high), transferring saturation index (> 45%), liver iron content (high), *HFE* gene mutation for hereditary hemochromatosis (C282Y, H63D)	Hemochromatosis	Phlebotomy, deferoxamine
Ceruloplasmin (low), serum (low) and 24 h urine copper excretion (high)	Wilson's disease	D-penicillamine, zinc
HDL-cholesterol (low), glucose (high), triglycerides (high)	NAFLD/NASH	Low caloric diet, exercise, drugs lowering insulin-resistance

AMA: Anti-mitochondrial antibody; ANA: Antinuclear antibody; ASMA: Anti-smooth-muscle antibody; γGT: γ- glutamyltransferase; HBV-DNA: Hepatitis B virus DNA; HCV-RNA: Hepatitis C virus RNA; HBsAg: Hepatitis B surface antigen; HDL: High density lipoprotein; HDV-RNA: Hepatitis delta virus RNA; LKM: Liver kidney microsomes; MCV: Mean corpuscular volume; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

the harder or stiffer the tissue, the faster a shear wave propagates, as a marker of hepatic fibrosis. Similar results have been reported with magnetic resonance elastography (MRE)[12]. Likely, the combination of elastography with one of these indices will also help specialists to better select patients suitable for liver biopsy[9,10].

Life expectancy and quality of life in patients with advanced cirrhosis remains poor, despite diagnostic advancement. Patients experience fatigue, pruritus, ascites, bleeding and encephalopathy. Dyspepsia and malnutrition are common. Whereas liver transplantation has changed life expectation for a number of patients, many transplantable patients still die due to long waiting lists. Targeted therapy is crucial in slowing or even halting disease progression and to provide standard medical care. PCPs should identify and address alcohol abusers early, while conditions like nonalcoholic steatohepatitis (NASH), B and C hepatitis, autoimmune disorders, and hemochromatosis should be appropriately counseled and treated. Attention should be given to active immunization, nutrition, and general healthcare.

MANAGEMENT OF PERSISTENT ASYMPTOMATIC ELEVATION OF SERUM TRANSAMINASES

Measurement of serum ALT is part of standard laboratory tests in asymptomatic outpatients, and is a sensitive screening tool for chronic liver disease[13]. Between one and four percent of asymptomatic subjects may have elevated ALT (LEVEL Ⅲ)[7,14,15]. In a recent survey in the Mediterranean area, the most likely cause of elevated serum ALT was an excessive alcohol intake (45.6%), nonalcoholic fatty liver disease (NAFLD) (24%), and HCV infection (18.6%)[14].

Over 20% of subjects with elevated ALT show signs suggestive of relevant chronic liver disease[2]. PCPs are required to carefully investigate most common causes of elevated ALT and for early identification of treatable chronic liver diseases[16,17]. Patient histories should focus on the use of medications, herbal extracts, and alcohol consumption. The presence of diabetes and thyroid disease (hypothyroidism) must be considered. The problem, however, may be underestimated as about 38% of patients with occasional ALT elevation show normal values at next measurement[16]. Despite the very high number of subjects showing such liver test abnormality in family practice, only a few will need referral, i.e. those patients with doubtful diagnosis after initial evaluation and patients with established diagnosis requiring therapy (LEVEL Ⅲ)[18].

THE IMPORTANCE OF IDENTIFYING ETIOLOGY

The identification of the cause underlying liver cirrhosis is essential in starting preventive measures and designing specific intervention (LEVEL Ⅰ). Table 1 shows the most appropriate tests for etiologic diagnosis of cirrhosis. Anti-mitochondrial antibodies are specific for primary biliary cirrhosis, HBV-DNA or HCV-RNA positivity for hepatitis B or C, low serum ceruloplasmin levels for Wilson's disease, and high serum ferritin and transferrin saturation index for hereditary hemochromatosis. Of note, liver cirrhosis may result from coexisting etiologic factors (i.e. alcohol and viral infection, obesity and virus, *etc*.).

HOW TO SCORE AND DEFINE PROGNOSIS

Once the diagnosis of liver cirrhosis has been formulated, a further important step is to score the disease. However, neither physical findings nor transaminases are helpful for defining prognosis or scoring the disease. Other laboratory tests (bilirubin, albumin, and prothrombin time), combined with the presence and severity of encephalopathy and ascites, are included in the Child-Pugh score (Table 2), the traditional scale used by many clinicians for assessing the liver disease severity (LEVEL Ⅰ). Another scoring system is the model for end-stage liver disease (MELD, http://www.mdcalc.com/meld) which provides robust information on mortality in cirrhosis, and is used for prioritizing candidates for transplantation[19] (LEVEL Ⅰ). Both scores

Table 2 Child-Pugh scoring system for liver cirrhosis and related indication priority for transplantation[20]

Score	1	2	3
Bilirubin (mg/dL)	< 2	2-3	> 3
Prothrombin time (INR)	< 4 sec. (< 1.7)	4-6 sec. (1.7-2.3)	> 6 sec. (> 2.3)
Albumin (g/dL)	> 3.5	3.5-2.8	< 2.8
Ascites	Absent	Mild	Severe
Encephalopathy	Absent	Mild	Severe

The Child-Pugh score is given by the sum of the score (1 to 3) of each of the five parameters. A score of 6 or lower defines the patient as class A, 7 to 9 as class B, and 10 or higher as class C.

can also be easily applied in primary care. Regardless of the cause, once decompensation has occurred, mortality without transplantation is 85% over 5 years[1]. In general, one-year survival rates for patients with Child-Pugh score A, B and C are 100%, 80% and 45%, respectively[20]. MELD score provides a more accurate prediction[21]. The hepatic clearance of exogenous administered substances, which provides an indication of residual liver functional mass[22,23], are easy to perform and may also meet future applications in family practice.

SELECTION FOR LIVER TRANSPLANTATION

Liver transplantation is considered as a viable treatment option for patients with acute liver failure and end-stage liver disease. In liver cirrhosis, transplantation is generally considered when a patient has suffered from either a complication of portal hypertension or a manifestation of compromised hepatic synthetic function[24]. However, given the high costs, mortality rate, and the paucity of donor organs, transplantation is currently justified only in the case of long-term prognosis, and psychological, intellectual, financial and family support. Accordingly, patients may be considered as current, future or inappropriate candidates. Selection consists of a search for contraindications and PCPs are actively involved in this process (i.e. alcohol and drug use)[25]. Currently, patients are generally put on a waiting list once Child-Pugh class B or a MELD score of over 13 is reached[21]. Onset of complications may anticipate referral, but severely decompensated or debilitated patients are generally discarded. Current indications and relative and absolute contraindications to liver transplantation are reported in Table 3.

Table 3 Current indications and contraindications to orthotopic liver transplantation in adult patients with liver cirrhosis

Indications	Contraindications
Advanced chronic liver failure	Relative
Child-Pugh score > 7	HIV seropositivity
Qualifying MELD score	Methadone dependence
	Stage 3 hepatocellular carcinoma
Acute liver failure	Absolute
Drug, toxins or virus induced fulminant hepatitis	Extrahepatic malignant disease
	AIDS
	Cholangiocarcinoma
	Severe, uncontrolled systemic infection
	Multiorgan failure
	Advanced cardiopulmonary disease
	Active substance abuse
General	
No alternative available treatment	
No absolute contraindications	
Willingness to comply with follow-up care and family assistance	

AIDS: Acquired immunodeficiency syndrome; HIV: Human immunodeficiency virus; MELD: Model for end stage liver disease.

TREATMENTS TO BE SHARED BETWEEN PCPs AND SPECIALISTS

Assistance is based on disease stage, complications and grade of self-sufficiency. Stable (compensated) patients are generally self-sufficient and a six month check (blood tests and liver ultrasonography) is indicated. Complicated and decompensated forms require an integrated approach with referral centers. Home care reduces costs[26] and should focus on a chronic care model of patient education and on empowering both the patient and the family to take responsibility for the care (Table 4)[17]. Several cirrhotic patients can benefit from treatments aimed to slow disease progression (Table 1)[27-31]. In particular, nucleoside (Lamivudine, Telbivudine, Entecavir) and nucleotide (Adefovir, Tenofovir) analogues have shown to be safe and effective in reducing the risk of decompensation and disease progression in patients with HBV infection, while interferon plus ribavirin is a therapeutic option for under-compensated liver cirrhotic patients with HCV infection.

SPECIFIC PROBLEMS

Monitoring alcohol and drug abuse

Alcohol abuse causes 25% of liver cirrhosis and contributes to another 25%-50% of cases. PCPs play a key role in the application of long-term detoxification programs, counseling, support, and monitoring. This step is crucial, since recovered abusers are considered for antiviral therapy or transplantation only after six months of continuous abstinence (LEVEL Ⅲ).

Ascites

Ascites is the most common complication and cause of hospitalization of cirrhotic patients, but it is also the complication which can be better treated at home. Portal hypertension, reduced albumin synthesis, decreased plasma oncotic pressure, and sodium retention are all determining factors. Paracentesis usually removes a transudative fluid (i.e. albumin < 1 g/dL; serum/ascites albumin gradient > 1.1). Patients exhibiting abdominal pain, tense ascites and fever may have a spontaneous bacterial peritonitis (SBP), a condition characterized by an ascitic granulocyte count exceeding

Table 4 Standard objectives for an efficient out clinic care of cirrhotic patients

1	Early diagnosis of chronic liver disease. Identification of etiology
2	Identification of patients with chronic liver disease at risk of cirrhosis
3	Evaluation of patient's general health status
4	Act on etiologic factors and on factors favoring disease progression. Identify treatment end-points and place the patient within his family and social setting
5	Promote family and cohabitants' participation to primary prevention for infective forms (health education), secondary prevention for inherited or metabolic disorders, support and surveillance for toxic forms (alcohol)
6	Suggest health-dietetic measures and therapeutic remedies
7	Check parameters of effectiveness and control side effects of specific treatments (antiviral, phlebotomy, immune-depressants, β-blockers, *etc.*)
8	Identify and treat associated conditions (diabetes, osteoporosis, malnutrition, *etc.*)
9	Avoid administration of hepatotoxic drugs, drugs promoting renal sodium retention and central nervous system depressants
10	Promote vaccination against flu and pneumonia, including transplanted patients, and against hepatitis A and B virus
11	Supervise for complications by promoting clinical, biochemical and instrumental follow-up
12	Assist specialists in identifying candidates for liver transplantation
13	Assist the patient requiring legal problems

250/mm^3. SBP can precipitate cirrhosis towards renal and liver failure. Therapy includes high doses of albumin to prevent renal failure and intravenous cefotaxime at doses of 2 g twice a day (LEVEL Ⅱ). Long term prophylaxis of SBP recurrence with norfloxacin is indicated in survived patients (LEVEL Ⅰ). Ascites is considered refractory if it persists despite the use of diuretic drugs at the maximum tolerable dose. Although some studies indicate the utility of bed rest as a remedy, no controlled trials have been performed in support to this practice. Therefore, initial treatment is dietary salt restriction[32,33] (LEVEL Ⅰ). Therapy starts with spironolactone at doses ranging from 100 to 400 mg/d. Furosemide may be added (40 to 160 mg/d) when spironolactone does not successfully improve fluid retention (LEVEL Ⅰ). Weight should be monitored daily and electrolytes should be frequently monitored. Albumin infusion is required to prevent post-paracentesis circulatory dysfunction[34] following large volume paracentesis[35]. Such treatments can be managed by PCPs or in an integrated care system with consultant specialists. Preventive measures include the avoidance of NSAIDs, since they promote sodium retention. In the case of recurrent or refractory ascites, before considering the patient for a transjugular intrahepatic portosystemic shunt (TIPS), large volume paracentesis is feasible at home. Paracentesis is safe and rarely precipitates hepatorenal syndrome (LEVEL Ⅱ). Patients with SBP or refractory ascites have a more advanced disease with a poorer prognosis, and so require hospitalization. Patients and their family have to be taught the importance of a daily body weight check, and to refer FD when it increases by 2-4 kg over a brief period of observation.

Hepatorenal syndrome (HRS) is a life-threatening complication in patients with refractory ascites. Diagnosis includes the following criteria: advanced chronic liver failure with portal hypertension; serum creatinine exceeding 1.5 mg/dL or a 24-h creatinine clearance of less than 40 mL/min; absence of shock, ongoing bacterial infection, or recent treatment with nephrotoxic drugs; no sustained improvement in renal function following diuretic withdrawal and the expansion of plasma volume with 1.5 L saline; less than 500 mg/dL proteinuria and no ultrasonographic evidence of obstructive uropathy or parenchymal kidney disease[36]. While awaiting transplantation, patients with HRS, eligible for transplantation, may improve with medications, namely albumin, terlipressin, and vasoactive drugs or TIPS[37].

Portal hypertension

Active variceal hemorrhage accounts for about one-third of all deaths related to cirrhosis. Steps related to the prevention and treatment of variceal hemorrhage includes: prediction of patients at risk, prophylaxis against a first bleed, treatment of an active bleed, and prevention of rebleeding. Diagnosing and treating portal hypertension is a way to prevent esophageal variceal bleeding, and PCPs may play an active role in this respect. Varices appearance should be checked by upper endoscopy every 2-3 years, with a follow-up after 2 years for low-risk bleeding or every year for high-risk bleeding. Non-selective β-blockers are effective in reducing the risk of bleeding by reducing the resting heart rate by 25% (LEVEL Ⅰ). Endoscopic band ligation is indicated for patients susceptible of high-risk bleeding and for those who have already bled[38] (LEVEL Ⅰ). TIPS is an alternative option for patients with previously failed treatments[39] (LEVEL Ⅱ). A recent study has shown that early use of TIPS is associated with significant reductions in treatment failure and mortality[40].

Hepatic encephalopathy

Hepatic encephalopathy is a chronically debilitating complication of hepatic cirrhosis and encompasses a wide spectrum of potentially reversible neuropsychiatric abnormalities seen in patients with liver dysfunction. This condition is deemed as the onset of brain dysfunction due to metabolic abnormalities, which occurs as a consequence of liver failure. Hepatic encephalopathy is mainly caused by a reduced clearance of gut-deriving neurotoxins, and is a potentially reversible condition ranging from subtle personality changes to coma, with flapping tremor as a frequent initial finding. PCPs should search for acid-base and electrolyte disturbances, constipation, infections, gastrointestinal bleeding, and inappropriate use of sedative medications. Treatment consists of identifying and correcting the precipitating factors, colon cleansing and acidification with lactulose (LEVEL Ⅱ). Dietary protein restriction is no longer advocated since it may facilitate malnutrition and the appearance of complications. Rifaximin, a minimally absorbed oral antibiotic, has an antimicrobial effect against enteric bacteria and has received approval from the United States Food and Drug Administration for reducing the risk of overt hepatic encephalopathy recurrence. In a randomized, double-blind, placebo-controlled trial, six-month rifaximin therapy at a dose of 550 mg twice daily was compared with a placebo

in patients with chronic liver disease who were in remission from recurrent hepatic encephalopathy. Rifaximin maintained remission more effectively than the placebo and also significantly reduced the risk of hospitalization for hepatic encephalopathy[41] (LEVEL Ⅰ). Venous infusion of branched-chain amino acids or flumazenil may be effective in the case of comas (LEVEL Ⅱ). Patients may be managed at home; admission to hospital is reserved for those who are non-responsive after 12 h treatment.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major complication of liver cirrhosis representing an increased cause of mortality; liver transplantation and cost management in most developed countries. As a consequence, screening for HCC is one of the most important tasks in patients with liver cirrhosis. American and European guidelines currently recommend at least one imaging screening/year for HCC (ultrasonography, triphasic CT). Serum alpha-fetoprotein has poor sensitivity and therefore is recommended only as an adjunctive screening marker[37,42]. Once HCC is detected, many treatment options are available, mainly depending on tumor size and number, and local expertise. Surgical resection can be effective; unfortunately most patients do not tolerate liver resection or have microscopic lesions, and so the best option for a cure remains liver transplantation. The Milan criteria are used as a guideline worldwide[43,44], and suggest that a four-year survival rate of 75 percent is achieved if liver transplantation is performed for either a single lesion of less than 5 cm in diameter, or up to 3 lesions with none larger than 3 cm. Outcomes are similar to the expected survival rates for patients undergoing transplantation for cirrhosis without HCC (LEVEL Ⅰ). Alternative treatments for patients who do not meet the criteria for resection or transplantation are ultrasound guided radiofrequency ablation, chemoembolization and alcohol ablation. These options are considered as a form of "bridging therapy" because it reduces tumor burden and delays tumor progression[45], and do not preclude future liver transplantation, if a donor organ becomes available.

Infections

Sepsis represents a high risk factor for mortality in cirrhotic patients which often do not present the typical signs and symptoms of infection (i.e. absence of leukocytosis due to severe leukopenia or even absence of fever). The active search for infections is important (cultures, X-ray, paracentesis, *etc.*). Most common infections concern the urinary tract (25%-55%), spontaneous bacterial peritonitis (10%-30%), and respiratory tract infection (20%). First line antibiotics include quinolones and cephalosporins[46] (LEVEL Ⅲ). Hospitalization is required for poor general health and/or the appearance of organ dysfunction.

SYSTEMIC PROBLEMS

Malnutrition

Malnutrition represents a negative prognostic factor for cirrhosis and consists of muscle wasting, hypoalbuminemia, decreased resistance to infections, and variceal bleeding. Causes include poor oral nutritional intake, malabsorption, ongoing alcohol use, chronic nausea, and early satiety due to abdominal compression from ascites. Nutritional status should be monitored in all cirrhotic patients; multivitamin supplementation is often indicated[47]. Nutritional support should be reserved only for severely malnourished patients scheduled for transplantation[48]. Oral supplementation with a branched chain amino acids has some utility by improving event-free survival in patients with decompensated liver cirrhosis[49]. Dental care is particularly important to allow adequate mastication.

Osteoporosis

In individuals with chronic liver disease, metabolic bone disease (hepatic osteodystrophy), is a potential complication of long-standing hepatic disease. It is therefore essential to prevent the development of fractures in individuals with advanced hepatic disease and those that have undergone liver transplantation[50]. In end-stage cirrhosis, vitamin D deficiency, hypoparathyroidism, and hypogonadism contribute to reduced bone formation. Osteopenia may occur early in patients with cholestasis or in those put on antiviral drugs[51]. This is also the case in patients after orthotopic liver transplantation[52]. Bisphosphonates, together with calcium and vitamin D_3, are effective in improving bone mineral density[53] (LEVEL Ⅱ).

Diabetes

Diabetes and cirrhosis are strictly interrelated, the first occurring with increased frequency in patients with NASH, hepatitis C or hemochromatosis. In a multivariate analysis, diabetes was an independent negative factor for liver disease evolution[54]. No controlled studies have tested the benefit of different regimens for cirrhotic patients with diabetes. Diet remains the first line remedy to control hyperglycemia. In the case of dietary failure, metformin is generally the first choice. Sulphonylureas can be used, but mindful of the risk of hypoglycemia. Glitazones are a new alternative, although no studies in liver cirrhosis have been performed. In any case, oral anti-diabetic drugs are not indicated in decompensated patients. Insulin represents the best approach, although this requires good self-monitoring (LEVEL Ⅲ).

PREVENTION

Primary prevention

The role of PCPs is important for this issue. The most attractive form of protection for liver cirrhosis is to prevent or slow the evolution of several risk factors triggering the hepatitis-fibrosis sequence. Mass infant vaccination has proven extremely effective in preventing hepatitis B infection. Screening blood donors effectively reduces hepatitis C transmission (LEVEL Ⅰ).

Secondary prevention

This step aims at preventing the appearance of cirrhosis

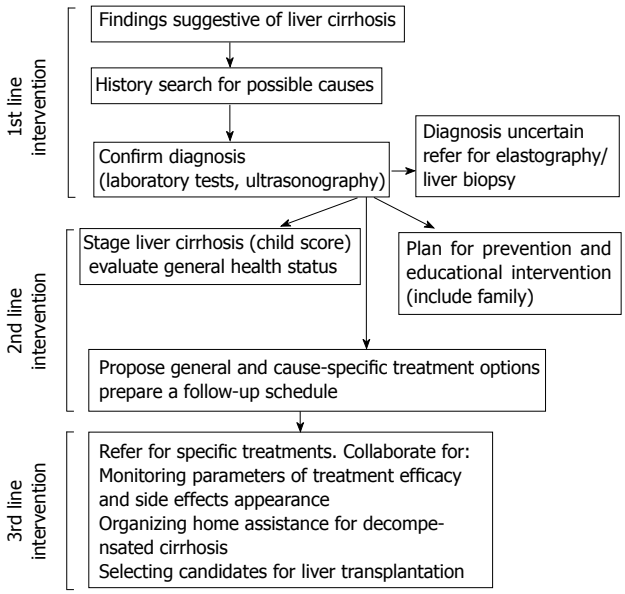

Figure 1 Algorithm for the management of patients with (or with suspected) liver cirrhosis in General Practice.

in patients with chronic liver disease and includes etiologic treatment for viral hepatitis, alcohol abstinence, phlebotomy in hemochromatosis, weight loss and improving insulin resistance in NASH patients[1]. Early detection of HCC by six-monthly ultrasonography and blood alpha-fetoprotein measurement may allow successful liver transplantation or mini-invasive treatments (LEVEL Ⅰ).

Prevention of infections

Vaccine immunization against hepatitis A and B, pneumococcus and influenza is important in preventing general status deterioration. SBP recurrence can be reduced by antibiotic prophylaxis (once-daily 400 mg norfloxacin or once-weekly 750 mg of ciprofloxacin)[55].

THE ROLE OF PCPs FOR OPTIMIZING CARE

The incidence of liver cirrhosis is expected to increase in the near future. Beside B and C viral infection and alcoholic cirrhosis, nonalcoholic liver steatosis (non-alcoholic fatty liver disease, NAFLD) is considered the hepatic manifestation of a new epidemic: the metabolic syndrome. This frequent condition is a cluster of risk factors for coronary heart disease and type 2 diabetes mellitus that includes visceral obesity, elevated blood pressure, insulin resistance, and dyslipidemia[56,57]. The onset of NAFLD represents a bridging condition between cardiovascular risk and potentially evolutive forms of liver diseases, namely steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma[58,59]. The PCPs are therefore asked to play a key role in programs involving prevention, treatment, surveillance, and home care of populations at risk (Figure 1)[4,60]. Referral of patients to specialists at least once is a good practice, since integrated management between PCPs and specialists is indeed associated with better outcomes[61]. Active cooperation is required for etiologic treatments, screening for complications and approaches to liver failure. However, appropriate timing for referral varies on an individual basis according to liver function and general health status, and this should include patient age, level of test abnormality, need for prognosis, and therapeutic decision. The need for a multidisciplinary approach should be considered, which includes feedback from dietitians, psychologists, and physical activity supervisors[62]. This integrated approach optimizes therapy adhesion, but necessitates the regular updating of health personnel[60].

Table 5 Features of home assistance in patients with liver cirrhosis

Advantages
Decreased number of hospitalization and re-admissions
Decreased costs of treatments
Assist the patient within his familiar comfort
Criteria of eligibility
Identification of a clinical status allowing home stay
Identification of priority criteria
Presence of a valid family support or of an active aid system
Selection criteria
Use the Karnofsky Performance Status[1] for patients with decompensated liver cirrhosis and limited self-sufficiency (set to < 50%)

[1]The Karnofsky Performance Scale Index allows patients to be classified as to their functional impairment. This scale is often used in the primary care setting to assess the prognosis in individual patients and to decide a treatment; the lower the score, the worse the survival.

PCPs can manage cirrhotic patients by checking therapy effectiveness and side effects. With the exclusion of major digestive bleeding, even severely decompensated patients or those in an irreversible coma or advanced HCC can be home managed (with the assistance of specialists and specialized nurses). Hepatic encephalopathy can be treated with lactulose (oral or rectal enema) and the minimally absorbed rifaximin, by controlling electrolytes, and treating infections. Ascites can be controlled with diuretics, albumin infusion or paracentesis. Albumin boosts the efficacy of diuretics, and reduces the number of hospital admissions[35]. Home care of cirrhotic patients should be encouraged since it allows a saving of up to two/third of the normal cost (Table 5) (LEVEL Ⅱ-Ⅲ).

CONCLUSION

Liver cirrhosis has an increasing prevalence worldwide, which matches the increasing diffusion of viral hepatitis infection, and metabolic steatohepatitis and fibrosis. Managing cirrhotic patients at home is challenging but cost-effective, although this policy requires active collaboration between PCPs and specialists, as well as nurses and paramedical staff. A set of conclusive key messages for practice are reported in Table 6.

Table 6 Key messages for best management of cirrhotic patients

	Statement	Evidence level
1	A compensated liver cirrhosis is suspected with abnormal liver function tests, low platelets count, and prolonged prothrombin time[63]	Ⅲ
2	Ultrasonography is a reliable, non-invasive, fast, and cost-effective test working as a first-line tool for diagnosing liver cirrhosis[64]	Ⅱ-Ⅲ
3	Child-Pugh and MELD scores assess the prognosis of liver cirrhosis[19,20]	Ⅰ
4	First-line treatment of patients with cirrhotic ascites includes diuretics and sodium restriction. Anti-aldosterone drugs are given with loop diuretics to increase diuretic response or when renal perfusion is impaired. Dietary salt intake should be restricted to approximately 88 mmol/day (2000 mg/d). Marked salt restriction can expose the risk of hyponatremia[32,37]	Ⅰ
5	Removal of less than 5 liters of fluid does not appear to have a hemodynamic consequence. For larger paracentesis, albumin (6 to 8 g/L of fluid removed) can be administered. Albumin is indicated in patients with PBS to prevent renal failure, and in patients with hepatorenal syndrome. Albumin can be also used to treat refractory ascites. Its infusion at home is safe and cost-effective[37,65]	Ⅱ
6	β-blockers (e.g. propranolol or nadolol) are recommended for prophylaxis of variceal bleeding at a dosage titrated to a 25 percent reduction in pulse rate[66]	Ⅰ
7	Liver transplantation is the only definitive care for patients with major complications (ascites, bleeding, HCC) and/or MELD above 13[1]	Ⅰ
8	Osteoporosis is an important systemic complication of end-stage liver cirrhosis. Management includes vitamin D and bisphosphonates[53]	Ⅱ
9	Malnutrition is a negative and independent predictor of survival in patients with liver cirrhosis[67]	Ⅱ
10	An integrated assistance of patients with liver cirrhosis has a better outcome than the management by generalists/specialists alone[61]	Ⅱ

LEVEL Ⅰ: At least one properly conducted RCT, systematic review, or meta-analysis; LEVEL Ⅱ: Other comparison trials, non-randomized, cohort, case-control, or epidemiologic studies, and preferably more than one study; LEVEL Ⅲ: Expert opinion or consensus statements (see text for details); MELD: Model for end-stage liver disease.

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TOPIC HIGHLIGHT

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Gastroenterology training in Latin America

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Abstract

Latin America is characterized by ethnic, geographical, cultural, and economic diversity; therefore, training in gastroenterology in the region must be considered in this context. The continent's medical education is characterized by a lack of standards and the volume of research continues to be relatively small. There is a multiplicity of events in general gastroenterology and in sub-disciplines, both at regional and local levels, which ensure that many colleagues have access to information. Medical education programs must be based on a clinical vision and be considered in close contact with the patients. The programs should be properly supervised, appropriately defined, and evaluated on a regular basis. The disparity between the patients' needs, the scarce resources available, and the pressures exerted by the health systems on doctors are frequent cited by those complaining of poor professionalism. Teaching development can play a critical role in ensuring the quality of teaching and learning in universities. Continuing professional development programs activities must be planned on the basis of the doctors' needs, with clearly defined objectives and using proper learning methodologies designed for adults. They must be evaluated and accredited by a competent body, so that they may become the basis of a professional regulatory system. The specialty has made progress in the last decades, offering doctors various possibilities for professional development. The world gastroenterology organization has contributed to the speciality through three distinctive, but closely inter-related, programs: Training Centers, Train-the-Trainers, and Global Guidelines, in which Latin America is deeply involved.

Key words: Training; Gastroenterology; Latin America

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INTRODUCTION

Latin America is home to over 50 countries and a population close to 600 million people. The two main languages are Spanish and Portuguese, but there is also long list of native languages. In the last 60 years, the economy of Latin America and the Caribbean grew by 4%, while the population increased by 2.1% annually. Although unemployment has dropped in recent years, it still averages 7.5%. Although the number of poor people in the overall population has dropped by more than 9% between 2002 and 2007, it is still

around 35%, which implies that 210 million people live in poverty in the region, i.e. prior to 1980, 39%of the population were poor. More positive results have been obtained for people living in extreme poverty; it is estimated that in 2007, 12.7% of the population lived in indigence, versus 18.6% in 1980; however, in absolute terms, the number of people affected increased from 62 million to 76 million in the same period. The mortality rate of children under five years of age is 27 per 1000 (27‰), that is one third of the average observed in developing countries (81‰). However, some nations like Bolivia (61‰) and Haiti (72‰) lag behind in this regard. With regard to income distribution, the region has experienced some modest progress. One of the elements that have been of concern to the regional economic authorities in 2007 and 2008 has been the rise of inflation in the region. According to ECLAC's (Economic Commission for Latin America and the Caribbean) estimates, since early 2006, and with greater impetus in 2007, consumer prices rose with increasing speed in most economies in the region, with annual increases of 7% to 30% in the various countries and an average close to 16%[1].

Most countries in Latin America and the Caribbean have succeeded in enforcing universal primary education, and they are experiencing an expansion of pre-school, secondary, and tertiary education.

In 2006, there were 16 million students enrolled in further education in Latin America and the Caribbean. The average rate of enrollment in universities went from 21% to 31% between 1999 and 2006, but varied from one country to another (3% in Belize to 88% in Cuba)[2].

To summarize, Latin America is characterized by ethnic, geographical, cultural, and economic diversity; therefore, training in gastroenterology in the region must be considered in the framework of this context.

CURRENT STATUS OF MEDICAL EDUCATION IN GASTROENTEROLOGY IN LATIN AMERICA

The continent's medical education is typically characterized by the following features: a large number of medical schools in some countries, most of which grant no local accreditations; teaching is done by various agencies in the country, with no common programs and without previously agreed requirements for the gastroenterologist's (GE) or the endoscopist's training. In sum, a lack of standards is its main feature.

Current status of scientific research in gastroenterology in Latin America

Despite the efforts of several groups, especially those working in Mexico, Brazil, Peru, Chile, and Argentina, the volume of research continues to be relatively small, highlighting some contributions in the field of *Helicobacter pylori* and Celiac Disease, among others. There is still much to learn and report about the continent, especially with regard to epidemiology.

Table 1 Number of citable papers in all gastroenterology fields indexed at the SCOPUS database originating from three South American countries

Year	Argentina	Brazil	Colombia
2000	20	100	3
2008	28	220	46

Data were extracted from SCImago-Journal and Country Rank (retrieved on July 13, 2010, from http://www.scimagojr.com).

Nevertheless, continued investment in scientific research by government agencies in some countries, such as CONICET (Argentina), CNPq (Brazil) and COLCIENCIAS (Colombia) has been associated in recent years with a remarkable increase in the output of indexed papers in all fields, including gastroenterology, coming from these countries (Table 1).

Scientific events in gastroenterology in Latin America

There is such a multiplicity of events, that they might even be considered excessive. There is usually a succession of general gastroenterology events as well as others that deal with sub-disciplines, both at regional and local levels. Although this results in an undesirable fragmentation of the field, the positive consequence is that these events ensure that many colleagues have access to information, with the caveat that such information may not always be necessarily reliable or of good quality. It is important to address the role of the pharmaceutical industry and medical equipment in the continuing professional development programs (CPDPs).

Needs: Medical education programs must be based on a clinical vision and be considered in close contact with the patients. They should require previous training in Internal Medicine (via a two-year internship or a full postgraduate fellowship).

Proper supervision of the programs must be ensured. It is essential for each institution that the courses be accredited and to have links with the School of Medicine, to ensure that the premises are adequate, and that there is the necessary equipment and clinical support.

It is important to guarantee that the gastroenterologist's (GE's) training course is of an adequate duration (two to three years) with a minimum dedication of six hours a day. The recommendation is to prioritize the training of trainers that have no teaching background.

Moreover, it is essential for the programs to be appropriately defined and evaluated on a regular basis. The programs should guarantee the GE's training concerning the development of their skills and attitudes. The importance of electronic methods (e-teaching, e-learning, the use of the internet) must be emphasized. The syllabus should also contemplate the teaching of basic administration skills.

The disparity between the patients' needs, the scarce resources available, and the pressures exerted by the health systems on doctors are frequently cited by those that complain about their poor professionalism.

Teaching development can play a critical rol in ensuring the quality of teaching and learning in universities. "*A good university teacher is not the one who prepares their students to pass an exam, it is the one who obtains a students valuation of learning and a critical thought. He is also the one who encourages them to solve problems with creativity and curiosity and with ethical commitment as well as with a desire of improving their knowledge in a specific subject.*"[3].

Briefly put, the CPDP activities must be planned on the basis of the doctors' needs, with clearly defined objectives, using proper learning methodologies designed for adults. Andragogy, defined as "the art and science of helping adults learn", is based on five assumptions about how adults learn and their atitude towards, and motivation for, learning: (1) adults are independent and self directing; (2) they have accumulated a great deal of experience, which is a rich resource for learning; (3) they value learning that integrates with the demands of their everday life; (4) they are more interested in immediate, problem centred approaches then in subject centred ones; and (5) they are more motivated to learn by internal drives than by external ones.

The CPDP activities must also be evaluated and accredited by a competent body, so they may become the basis of a professional regulatory system (recertification or others). One of the greatest challenges is to balance the needs of professional employers and health care systems with those of the patients.

WHAT SHOULD THE FUTURE GASTROENTEROLOGIST LEARN?

Future specialists naturally aim at following their vocation, working with dignity and ethics, applying their knowledge, maintaining an ongoing training, and at times, they may seek academic development (teaching or research). The specialty has made progress in the last decades, offering the doctors various possibilities for professional development; it is no longer the outlook faced by the clinical gastroenterologist 50 years ago, or by more recent endoscopists. Today's GE can choose to develop an in-depth knowledge on certain sub-specialties, such as nutrition, hepatology, transplantations, interventional endoscopy, capsule endoscopy, NOTES (Natural Orifice Translumenal Endoscopic Surgery), motility, etc. In sum, the Latin American GE's training must be comprehensive, taking into account both society's interests, as well as the doctors' legitimate objectives.

SOME EXAMPLES ON THE TRAINING OF GASTROENTEROLOGISTS IN LATIN AMERICA

In Argentina, there are no unified criteria to be met to graduate as a gastroenterologist. The courses are dictated by the Argentine Society of Gastroenterology in partnership with the University of Buenos Aires, or by private or state-run schools of medicine in the interior of the country. Overall, there are eight courses that train GEs lasting for two to three years. Entry to the courses requires the completion of two years of internal medicine. There is no single accreditation body, nor are there any unified requirements for the training of endoscopists or liver experts[4].

In Uruguay, the specialists' training depends on the state School of Medicine's School of Graduates. It does not request a post-graduate degree in internal medicine. The gastroenterology course takes three years on a part-time basis, and it includes theoretical and hands-on activities that also include liver diseases. A degree in endoscopy has been recently created.

In Chile, gastroenterology is viewed as part of internal medicine. A GE is an internist that must go through three additional years in the specialty. There are several university programs in gastroenterology. The universities are recognized by the National Medical Certification Board.

Both Argentina and Chile have implemented mechanisms to regularly renew accreditation to specialists.

In Brazil, specialist training in gastroenterology has been regulated since 1977 by federal legislation, which established a two-year program in accredited institutions for candidates who have already completed a previous two-year basic training in Internal Medicine. Thus, the typical training takes four years, but most programs affiliated to university hospitals offer an additional elective year in sub-specialties, such as endoscopy or hepatology. After completing the specialist training, gastroenterologists are eligible to apply for certification, conferring the specialist title, which is provided by the Brazilian national gastroenterology society, known as Federation of Gastroenterology (FBG), to candidates passing the relevant examinations. Accreditation of institutions is provided by a federal agency, the National Commission for Medical Residency, on the basis of the characteristics of both the institution (infrastructure, number of hospital beds, average of outpatients visits, certified personnel, *etc*) and the program quality (balance between inpatients and outpatients activities, supervision, hours of endoscopy training, *etc*).

CME (Continuous Medical Education) in Brazil has been a compulsory requirement for the renewal of the specialist title since 2005, following the creation of the professional update certificate CAP (acronym for Certificado de *Atualização* Professional). Starting in January 2011, the titles will be renewed only to those who obtained their CAP or acquired a minimum of 100 CME credits in the previous five years. Those who fail have to sit an additional exam to keep their title. The national societies of each specialty, such as the Brazilian FBG, participate in the National Accreditation Committee (CNA) with other organizations, such as the Brazilian Medical Association or the Medical Federal Board. The CNA evaluates organizers' applications for activities that intended to grant CME credits and validates the credits already obtained by doctors. The national societies are requested to organize activities to provide a minimum of 40 credits a year. This is followed strictly by the FBG, which has the organization of CPDP meetings and distance learning activities as one of its main aims. Regarding the Medical Schools and the University hospitals, de Almeida Troncon *et al*[5] thinks that they do not devote enough time

and effort to comply with that requirement. His concerns about the support of the CME activities by the biomedical industry are based on the lack of independence of the programs. Lack of evaluation of the activities is another weakness of the program in Brazil, where the cognitive aspects are emphasized over the acquisition of skills and attitudes. Evidence that the CME activities in Brazil are positive for the quality of medical work is still scarce. Troncon suggests that new ways of implementing CME have to be found: funding must come from the Medical Schools and the working institutions, which should adopt the principles of adult learning, especially in identifying the needs of learners, developing the objectives of the activities, and finally designing the curricula. Finally, he stresses that the evaluation is a key issue in that process.

Venezuela: There are approximately seventy students within the sixteen post-graduate programs in gastroenterology in Venezuela. All of them differ in curriculum, academic and research structure, and even in their own graduation profile. The group led by Lizarzábal *et al*[6] tested their individual and global quality by conducting a users-satisfaction survey, questioning students and program directors. The sample included 46 students who answered anonymously. The students' results showed that 13% of the programs were considered to be of excellent quality (A) and 8.7% of the programs were graded as B (good). An important group (71.7%) was graded as C (bad) and 6.5% as D (very bad). Users' perception differed from the perception of directors, who evaluated the quality of more than half (57%) as A-B, while only 21.7% were graded A-B by the users.

In view of the above results, the Society of Gastroenterology of Venezuela made some recommendations to improve the quality of the postgraduate teaching programs, which are summarized as follows: (1) Implementing structured and explicit curricular designs in 100% of the Venezuelan Gastroenterology Postgraduate Programs; the programs that already have such designs (60%) will be asked to apply consistently unified criteria; (2) Request that 100% of the programs be accredited by the National Universities Council (CNU) before pursuing certification and re-certification; (3) Strengthen a culture of research; (4) Develop evaluation strategies to allow monitoring of the service provided; (5) Improve proficiency of the human resources available. Most of the staff lack the academic training needed to implement an adequate curricular design, evaluation, research, and learning strategies; and (6) Plan and reach consensus on education in Gastroenterology with a broad participation of the stakeholders. The profile and training of the Venezuelan gastroenterologist is still to be defined.

CONTRIBUTIONS OF THE WGO (WORLD GASTROENTEROLOGY ORGANIZATION) TO THE SPECIALITY IN LATIN AMERICA

The current objectives of WGO are enshrined in its mission statement: "to promote, to the general public and health care professionals alike, an awareness of the worldwide prevalence and optimal care of digestive disorders through the provision of high quality, accessible and independent education and training", which signals the commitment of WGO to address two challenges: firstly, providing the gastroenterologist of the future with an optimal training and, secondly, bringing the benefits of digestive health care to those who currently struggle or, indeed, fail to achieve access to it. The primary emphasis of WGO, therefore, is on education and training. These objectives are achieved through three distinctive, though closely interrelated, programs: Training Centers, Train-the-Trainers, and Global Guidelines.

Training Centers most directly address the issue of training specialists in gastroenterology or individuals with additional expertise in gastroenterology to serve previously underserved areas. Each centre represents a direct collaboration between local experts, international faculties, and national and regional societies from Europe and North America to deliver regionally relevant training to those who have limited, or in some cases, no access to such opportunities. The centers in Latin America: La Paz, Bolivia; La Plata, Argentina; Santiago, Chile; Mexico City, Mexico; San José, Costa Rica; and Bogota, Colombia, provide training of variable duration to several hundred young and aspiring gastroenterologists and digestive surgeons from underserved nations in the region. The newest center was inaugurated two years ago in Ribeirao Preto, State of Sao Paulo, Brazil, and is dedicated solely to training in gastrointestinal motility techniques. The centers offer an in-depth view of the various aspects of the field (clinical, endoscopic, and motility). On the other hand, the Inter American Association of Gastroenterology (Asociación Interamericana de Gastroenterología: AIGE) has created scholarships to facilitate the access of six gastroenterologists to the WGO's teaching centers annually.

Finally, it is an important aim for WGO to create an electronic network among all its teaching centers that will include the seven in Latin America. This network should be accessible to all who seek to train in our specialty; thereby, ensuring the highest standards of care for those who suffer from digestive disorders through the world.

Likewise, the WGO has already organized four courses called "Train the Trainers" (TTT) in South America (Uruguay, Brazil, Chile, and Peru), in an attempt to remedy a global problem, i.e. the local faculties' lack of training on educational methodology to teach at university level. Train-the-Trainers courses are uniquely devoted to bringing the very latest in educational techniques to those who will train the gastroenterologists of the future, including those who teach and train at the Training Centers. The TTTs are developed in several modules aimed at teaching educational skills to faculties, in a user-friendly manner, in an informal and friendly atmosphere.

Even if gastroenterologists should nowadays be fluent in English, language can be an issue in such TTT courses in Latin America. The WGO has decided to begin TTTs in Spanish in 2011.

Another activity pushed forward by the WGO, and in which Latin America has been deeply involved, is the devel-

opment of the Clinical Guidelines (CG), with one peculiarity: they are the only ones to consider the availability of resources globally, through the so-called cascade mechanism that enables a professional to adapt the situation to a specific patient, in the patient's own context. To make guidelines more applicable to different resource environments, the concept of "cascades" has been developed. A cascade is a collection of related diagnostic and treatment options arranged hierarchically in terms of conditions and available resources. Whilst guidelines should continue to summarize best known practice, they could also include alternatives for clinicians with limited funding. These alternatives are usually on the basis of cost, but could also take account of local availability, technology, and infrastructure[7].

The complete texts of all the CGs are fully available at the site of the WGO (www.worldgastroenterology.org) in six languages, including Spanish and Portuguese. Outstanding Latin American gastroenterologists were involved in the development of several CGs, such as the one on Celiac Disease or *Helicobacter pylori* in the developing world.

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REVIEW

Silybin and the liver: From basic research to clinical practice

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Abstract

Herbal products are increasingly used, mainly in chronic liver disease. Extracts of milk thistle, Silymarin and silybin, are the most prescribed natural compounds, with different indications, but with no definitive results in terms of clinical efficacy. This review analyzes the available studies on the effects of the purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease. We searched PUBMED for articles pertaining to the *in vitro* and *in vivo* effects of silybin, its antifibrotic, anti-inflammatory, and antioxidant properties, as well as its metabolic effects, combined with the authors' own knowledge of the literature. Results indicate that the bioavailability of silybin phytosome is higher than that of silymarin and is less influenced by liver damage; silybin does not show significant interactions with other drugs and at doses < 10 g/d has no significant side effects. Experimental studies have clearly demonstrated the antifibrotic, antioxidant and metabolic effects of silybin; previous human studies were insufficient for confirming the clinical efficacy in chronic liver disease, while ongoing clinical trials are promising. On the basis of literature data, silybin seems a promising drug for chronic liver disease.

Key words: Milk thistle; Silymarin; Liver disease; Hepatic fibrosis; Hepatic inflammation; Radical species

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INTRODUCTION

The terms milk thistle, flavonoids, silymarin, and silybin are generally used interchangeably; however, each of these compounds has specific characteristics and actions, with an intrinsic beneficial or toxic effect. In the last 10 years, about 12 000 papers have been published on these substances, used as antioxidants or chemopreventives and anticancer agents, and especially as hepatoprotectants. This publication volume indicates that scientific interest in these molecules, or classes of molecules, is high worldwide. In the US and Europe, about 65% of patients with liver disease take herbal preparations; in Europe, the cost of the use of silymarin reaches $180 million in Germany alone. Despite the wealth of literature, no firm clinical evidence exists to recommend the use of these substances in clinical practice[1-10]. This discrepancy is attributable to various factors, such as quality of clinical trials, heterogeneity of diagnoses, lack of standardized preparations, and frequently inconsistent dosing and outcome parameters. At a time when the use of herbal products is increasing, whether driven by individual choice or industry promotion, in our opinion it is necessary to focus more intently on these compounds that may have beneficial, placebo, or toxic effects.

This review analyzes studies of the effects of the

purified product silybin, both as a free and a conjugated molecule, on liver cells or on experimentally induced liver damage, and in patients with liver disease.

DEFINITION AND CHARACTERISTICS OF SILYBIN

As reported, silybin and silymarin are not synonymous[1,3,6]. Silymarin is a complex of at least seven flavonolignans that are the most common class of compounds present in milk thistle extract, and one flavonoid, taxifolin. The relative abundance of each compound may vary depending on the source of botanical material, supplier, and extraction processes. Silybin represents about 50% to 70% of the silymarin extract. Silybin can be resolved into two 1:1 diastereoisomers, silybin A and silybin B. In addition, silybin may be present as isosilybin, a 1:1 mixture of two diastereoisomeric compounds, isosilybin A and isosilybin B[11-17]. The concentrations of silybin in the main pharmaceutical products containing silymarin present in the US and other countries range from 20% to 40%[16].

PHARMACOKINETICS AND PHARMACODYNAMIC ASPECTS

Flavonolignans are known for their poor and erratic bioavailability; for example, silymarin absorption rate levels vary between 20% and 50%. Silybin has been separated commercially as a pure substance[11-16], and the study of silybin pharmacokinetics properties using an HPLC method has shown that the concentration-response relationship is linear over a concentration range of 0.5-100 μg/mL[16]. After administration to rats, the disposition of silybin in the plasma and bile fluid is due to rapid distribution and equilibrium between the blood and hepatobiliary system, and the bile levels of unconjugated and total silybin are greater than those in plasma[18-22].

Similarly to other flavolignans, limiting factors for the use of silybin are its low solubility in water, low bioavailability, and poor intestinal absorption. To counteract this aspect, different more soluble derivatives of silybin have been synthesized, such as silybin bis-hemisuccinate, β-cyclodextrin complex, silybin-N-methyl-glucamine, silybin 11-O-phosphate, and silybin-phosphatidylcholine. Another strategy for improving silybin solubility is represented by the enzymatic synthesis of its β-glycosides, such as silybin β-galactoside, silybin β-glucoside, silybin β-maltoside, and silybin β-lactoside. A soluble silybin prodrug has been finally synthesized with a high aqueous soluble polymeric carrier (polyethylene glycol)[23-30].

Some conjugations may in part affect the activity of silybin. For example, the radical-scavenging activity of the silybin 20-O-β-D-glucuronide is considerably lower than that of free silybin. In contrast, a considerable increase in radical-scavenging activity is observed in the other silybin, 7-O-β-D-glucuronide, in which position C-20 is free. The increase in the radical-scavenging activity of this latter conjugate cannot be simply ascribed to the addition of the glucuronyl moiety. The change in antiradical activity is, therefore, the result of the overall structural change. It is also quite interesting that one diastereoisomer of silybin (B) undergoes conjugation faster than the other (A); this difference indicates that the two silybin diastereoisomers are metabolized at different rates. These findings will aid the development of improved silybin formulations designed to inhibit the C-20 conjugation or, alternatively, to contribute to a modified dosage scheme that maintains free plasma silybin at sufficient levels[31-35]. Both free and conjugated silybin have a rapid plasma and tissue distribution that reaches maximum levels within one hour after 50 mg/kg silybin administration in mice[36]. The protein binding of silybin in rat plasma is 70.3% ± 4.6%[21].

In humans, the pharmacokinetics of silybin was evaluated after the administration of variable doses of silymarin, pure silybin, and silybin conjugated with phytosome in healthy volunteers. Hoh *et al*[37] for the first time identified the silybin plasma metabolites and measured the silybin tissue levels in humans who had ingested silybin. They demonstrated that silybin undergoes multiple conjugation reactions in humans and clearly identified the conjugated species: silybin monoglucuronide, silybin diglucuronide, silybin monosulfate and silybin glucuronide sulfate. The administration in humans of 240 mg of pure silybin induces a peak concentration of 240 ± 54 ng/mL in about 2 h that persists for 4 h. After administration of a single oral dose of 560 or 600 mg silymarin (about equivalent in total to 240 mg of silybin), the fractions of the free, sulfated, and glucuronidated silybin in human plasma are about 17%, 28%, and 55% of the total dose, with a higher plasma percentage of glucuronidated silybin B (71%) than of glucuronidated silybin A. The total percentage of dose recovered in urine, as free and conjugated, is very low, ranging from 1% to 7% of the dose (mean: 2.8 ± 0.6 ng/mL)[29,38-42]. The complex silybin phytosome (silybin + phosphatidylcholine) was recently formulated with the addition of vitamin E (Indena, IBI-Lorenzini spa Italy: Realsil®). This conjugation induces a greater solubility of silybin, as reported in Table 1. In 12 healthy volunteers aged 20-53 years, silybin was administered as Realsil® in two pharmaceutical forms (capsules and granules, both corresponding to 47 mg of silybin). Data were compared to those obtained by administering, in a cross-over manner after 1 wk of wash-out, a silymarin capsule containing 58 mg of silybin and silymarin granules containing 80 mg of silybin[43]. As summarized in Tables 1 and 2, the global results of the pharmacokinetic analysis indicated that the bioavailability of silybin phytosome is much higher than that of silymarin.

EFFECT OF LIVER DAMAGE ON PHARMACOKINETICS OF SILYBIN

In patients with well-compensated liver cirrhosis, silybin was administered as silybin phytosome at a dose of 120 mg

Table 1 Pharmacokinetics of silybin phytosome and silymarin in healthy participants[38-43]

	Silybin phytosome	Silymarin
Peak concentration (ng/mL)	298 ± 96	102 ± 22[a]
Time of peak (h)	1.6 ± 0.3	1.4 ± 0.3
Mean residence time (h)	3.6 ± 0.4	3.5 ± 0.4
AUC (ng/mL/h)	881 ± 207	257 ± 66[b]

[a]$P < 0.05$, [b]$P < 0.01$. NB: The analyses were performed using HPLC.

Table 2 Pharmacokinetics of silybin after administration to 12 healthy volunteers as silymarin or silybin-phosphatidylcholine-vitamine E complex (RA)[43]

Product	Cmax (ng/mL)	Tmax (h)	AUC (ng/mL per hour)
RA granules (47 mg silybin)	213 ± 166	0.5	246 ± 114
RA capsules (47 mg silybin)	117 ± 93	1.0	161 ± 85
Silymarin gran (58 mg silybin)	18 ± 17	0.5	25 ± 18
Silymarin caps (80 mg silybin)	5 ± 7	1.0	11 ± 5

× 3 /d, or as silymarin containing 84 mg of silybin 4 fold/d. The results showed that liver cirrhosis does not modify the kinetics of silybin and that in liver patients, the bioavailability of silybin is higher when it is administered as a phytosome adduct[44]. Similar data were obtained in rats with experimentally induced cirrhosis[45]. Table 3 summarizes the data obtained in humans.

More recently, the pharmacokinetics of silybin has been evaluated in patients with chronic hepatitis [hepatitis C virus (HCV) or non-alcoholic fatty liver disease (NAFLD)] and compensated liver cirrhosis[46,47]. A single, 600 mg p.o. dose of milk thistle extract was administered to healthy volunteers and to three patient cohorts, and blood samples were obtained over 24 h. Silybin A and B accounted for 43% of the exposure to the sum of total silymarin flavonolignans in healthy volunteers and only 31% to 38% in liver disease cohorts as a result of accumulation of silychristin (20%-36%). Area under the curve (AUC, 0-24 h) for the sum of total silymarin flavonolignans was 2.4, 3.3, and 4.7-fold higher for the HCV or NAFLD ($P \leqslant 0.03$) and HCV cirrhosis cohorts ($P \leqslant 0.03$), respectively, compared with healthy volunteers. Silymarin kinetics was correlated with plasma levels of caspases as an index of liver inflammation; caspase 3/7 activity correlated with the AUC (0-24 h) for the sum of all silymarin conjugates among all participants ($R^2 = 0.52$) and was 5-fold higher in the HCV cirrhosis cohort ($P \leqslant 0.005$ versus healthy participants). These findings suggest that the presence of liver damage, particularly as chronic inflammation, may affect the bioavailability of the different components of silymarin, possibly explaining the low beneficial effects of flavonoids in patients with liver damage.

Interactions and toxicity

There are substantial differences between silymarin and silybin in their interactions with metabolizing enzymes, and the reasons for these differences remain unknown. Silybin alone or as silybin β-galactoside, β-glucoside, β-lactoside, or β-maltoside at a final concentration of silybin 100 μmol/L has been tested *in vitro*. Under these conditions, silybin showed slow inhibitory effects (IC_{50} > 200 μmol/L) on marker substrates of CYP2E1, CYP2D6, CYP2C19, and CYP2A6. Silybin and silybin β-glycosides did not induce expression of CYP1A2 and CYP3A4 and did not affect the inducible expression of either of these enzymes[48-53].

Table 3 Comparison between silybin phytosome and silymarin pharmacokinetics in patients with liver cirrhosis[44]

	Silybin phytosome (360 mg)	Silymarin (336 mg)
C_{max} (ng/mL)	860 ± 166[b]	83 ± 15
Tmax (h)	2.7 ± 0.7[b]	2.6 ± 2.1
/2 (h)	3.3 ± 0.7[b]	2.6 ± 0.4
AUC	515 ± 665[b]	262 ± 39
Total bioavailability	252 ± 39[b]	19 ± 23

[b]$P < 0.001$.

In *in vivo* work, Sridar *et al*[54] investigated metabolic interactions involving silybin at doses ranging from 25 to 250 μmol/L and substrates metabolized by CYP3A4 or CYP2C9, showing that silybin may be a modulator/inactivator of P450s 3A4 and 2C9. What remains to be elucidated is whether this effect is brought about by competition at the site or inhibition for substrate binding and for metabolism at a particular binding pocket in the active site; another possibility is that it results from a conformational change in the active site. Despite these results, silybin has shown no effect on the metabolism of indinavir[55,56], which is mediated by CYP3A4, and others[57-60] have documented that silybin, at a concentration of 100 μmol/L, has no effect on either basal or inducible expression of CYP3A4 mRNA.

In any case, the silybin-drug interaction of these enzyme substrates is not clinically relevant, and the inhibitory effects of silybin occur only at concentrations that massively exceed the physiologically used doses[60-62]. *In vitro*, silybin inhibits the UGT glucuronyl transferases UGT1A6 and UGT1A9, but it is 14- to 20-fold more selective for UGT1A1[48,49,61-64] (see Table 4 as summary). The clinical relevance of this phenomenon is presently unknown because there are no published reports indicating the existence of a clinical interaction with bilirubin. UGT1A1 is the only enzyme responsible for bilirubin glucuronidation, and it contributes to the glucuronidation of several drugs. In animals, silybin affects the hepatobiliary elimination of various drugs[52,53,57,58,64]; oral feeding of pure silybin at doses of 100 and 200 mg/kg/bw/d showed a moderate to highly significant increase in both glutathione-S-transferase and quinine reductase activities in the liver, lung, stomach, skin, and small bowel in a dose- and time-dependent manner[58]. Recently, Flaig *et al*[27] provided the best evidence that silybin can be administered to humans at doses producing anticancer-relevant concentrations, with minimal or no side effects.

Table 4 Interference of silybin with cytochromes[48-64]

Interference	Possible interference	No interference
UGT1	CYP3A4	CYP2E1
	CYP2	CYP2D6
		CYP2C19
		CYP1A2
		CYP2A6

That study employed the largest doses ever used, ranging from 2.5 to 20 g of silybin-phosphatidylcholine (Indena's Siliphos® "silybin phytosome"), given daily in three divided doses for 4 wk to 13 men with a history of prostate carcinoma. At a dose escalation from 15 to 20 g/d, silybin was discontinued because of asymptomatic hyperbilirubinemia, most likely because of inhibition of the glucuronyl transferase UGT1A1. However, in all patients, this mild hyperbilirubinemia improved with treatment cessation.

The limitations of available clinical trials with regard to establishing safety are the same as those for establishing efficacy. Clinical trials testing safety are poor predictors of the fate of extracts in real-world settings, where patients ingest multiple drugs and herbs, take different formulations of the same product, and add alcohol and other compounds, often for extremely long periods. In the randomized trials that reported adverse effects, their frequency was approximately equal in the silybin and control groups. The majority of the observed adverse events were unrelated to the product or difficult to separate from the concomitant disease, and in available reports, causality is rarely addressed. There are no safety data in children or older adults, as there are no reported studies in children and very few studies that included patients older than 65 years. Adverse effects associated with oral ingestion of silybin include mainly gastrointestinal problems, but these are rare. Headache/dizziness and pruritus were reported in one clinical trial. Asymptomatic liver toxicity has been observed in recent clinical trials performed in cancer patients, in whom hyperbilirubinemia and increases in alanine aminotransferase (ALT) levels were observed; however, these effects were present only when very high dosages of silybin phytosome (between 10 and 20 g/d) were used. At high doses, a laxative effect of silybin phytosome is possible because of increased bile secretion and bile flow. Mild allergic reactions have also been noted, but they were not serious. Therefore, the available data indicate that milk thistle has few side effects when doses lower than 5 g/d are used, with possible adverse effects at doses greater than 10 g/d[6,7,62].

The documented effects of silybin in basic research

In liver cells, as well as in other types of cells, the common effects of silybin may be summarized as follows: (1) Antioxidant; (2) Direct and/or indirect (through the antioxidant capability) modulator of inflammation and fibrogenesis; and (3) indirect and/or direct modulator of some intrahepatic metabolic pathways.

Antioxidant action

The antioxidant effects of silybin have been demonstrated in all cells studied. Silybin acts as an antioxidant because it inhibits radical formation, binds some radical species (scavenger), interferes with lipid peroxidation of membranes (and therefore modulates membrane permeability), and increases the intracellular content of scavengers[65]. In fact, in the presence of oxidative and nitrosative stress, silybin inhibits the formation of superoxide anion radicals and of nitric oxide (NO), increases ATP content through the phosphorylation of ADP, decreases the content of malondialdeyde (MDA) and totally abolishes the decrease of glutathione, of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase[66-73]. These results, which are dose-dependent, have been documented in isolated rat Kupffer cells, hepatocytes, HEPG2 cells, isolated mitochondria from rat hepatocytes, and in models of ischemia-reperfusion of rat liver. In experimentally induced liver damage, one hour after intragastric administration to rats of conjugated silybin (0.6 g/kg bw), the content of silybin in the microsomes measured using a specific HPLC assay was approximately 2.5 μg/mg of protein, corresponding to a final concentration of 10 μmol/L of silybin. Under these conditions, lipid peroxidation, mitochondrial permeability and respiration, and membrane potential as well as cell death were reversed by silybin, particularly if it was conjugated as silybin + phosphatidylcholine. This last compound, at a dose of 20 μmol/L, directly scavenges hydroxyl, hydroxyethyl, lipodienyl, methyl, and trichloromethyl radicals[74,75].

Finally, silybin acts as an antioxidant because it also serves as an iron chelator[76-78]. More recently, it has been suggested that dehydrosilybin (DHS), an oxidized form of silybin, has greater antioxidant activity than silybin (about three times better), probably because of the presence of unsaturated bonds that contribute to hydrogen-donating capacity. The better scavenger activity of DHS may also be the result of its greater ability to react with cell membranes because it has higher lipophilicity than silybin[32,65].

Table 5 summarizes the main antioxidant effects of silybin.

Anti-inflammatory action

In general terms, silymarin and silybin interfere with the NF-κB-controlled transduction cascade. NF-κB is an inducible and ubiquitously expressed DNA-binding protein, acting as a transcription factor for genes involved in inflammation, cell survival, differentiation, and growth[79,80]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by interaction with inhibitory protein 1 κ B α (IκBα). Upon activation from oxidative stress, NF-κB dissociates from IκBα, and IκBα is degraded. NF-κB translocates to the nucleus and, through kinase phosphorylation, drives the activation of genes supporting inflammation. Consistent with its antioxidant activity, silybin has been demonstrated to inhibit NF-κB activation and translocation through suppression of IκBα phosphorylation and degradation[81-83]. In an acute model of liver damage

Table 5 Antioxidant effects of silybin[65-75]

Doses	Cells	Targets
From 10 to 100 μmol/L	Hepatocytes	Decreased formation of reactive oxygen species from mitochondria; iron chelator
	HepG2	Decreased formation of superoxide anion
Mean dose with documented effects: 20 μmol/L	Kupffer cells	Decrease of NO production
	Monocytes	Scavenger of lipodienyl, methyl, trichloromethyl radicals
	Endothelial cells	Decrease of hydrogen peroxide concentration
	Cancer cells	Block of membrane lipid peroxidation

Table 6 Anti-inflammatory/antifibrotic effects of silybin[79-99]

Doses	Cells	Targets
From 5 to 50 μmol/L	Hepatocytes	Inhibition of NF-κB-mediated signaling
Mean: 15 μmol/L	Endothelial cells	Suppression of IκBα phosphorylation
	Platelets	Inhibition of protein kinase kinase
	Cancer cells	Inhibition of c-jun N-terminal kinase
	Phagocytes	Inhibition of leukotriene formation
	Stellate cells	Inhibition of release of cytochrome c
	HepG2	Inhibition of ERK, MEK, and Raf phosphorylation; inhibition of release of caspase 9 and 3, IL-8, and of PDGF- and TGF-β-mediated signaling; decrease of MMP2; increase of TIMP2; inhibition of HCV replication

in which mice were treated with concanavalin A, silybin reduced plasma levels of transaminases and the plasma and liver content of pro-inflammatory cytokines, inhibited hepatic NF-κB activation, and increased plasma and tissue levels of IL-10[84]. In rats with dimethyl-nitrosamine-induced chronic liver damage, silybin conjugated with phosphatidylcholine and vitamin E (Realsil®, Ibi-Lorenzini, Italy) administered by gastric gavage could prevent loss of body and liver weight, as well as reducing the degree of liver injury, as determined by ALT values and necroinflammatory scores. This outcome was associated with reduced hepatic stellate cell activation and proliferation both after 1 and 5 wk of treatment[85]. The anti-inflammatory action of silybin is also related to its interference with multiple cytokine-induced signaling pathways to down regulate inducible nitric-oxide synthase (iNOS) expression[86-88]and to the inhibition of cyclooxygenase (COX)-2 expression and activity and leukotriene formation in human platelets, white blood cells, and endothelial cells. Finally, silybin inhibits activation of the protein kinases and of a c-jun N-terminal kinase[89-92].

In addition to its antioxidant and anti-inflammatory actions, silybin also shows an antiviral effect. In fact, at a concentration of 20 μmol/L, it inhibits the protein expression and the replication of HCV virus in infected polymorphonucleated cells derived from patients with chronic HCV infection[93-95].

Antifibrotic action

In an *in vitro* model of human hepatic fibrogenesis, silybin demonstrated both direct and indirect antifibrotic properties. In fact, in stellate cells from human liver, silybin reduced platelet-derived growth factor (PDGF)-induced DNA synthesis and cell proliferation at a dose of 25 μmol/L. Silybin also reduced PDGF-induced cell migration in a dose-dependent fashion. Finally, pre-treatment with 25-50 μmol/L of silybin significantly reduced the TGF-β-induced *de novo* synthesis of procollagen type I in cell supernatants[96].

To investigate the role of silybin in modulating the pro-inflammatory properties of hematopoietic stem cells, cells were stimulated with IL-1β (20 ng/ml), a potent pro-inflammatory cytokine; silybin inhibited, in a dose-dependent manner, IL-1-induced synthesis of human MCP-1 (monocyte chemoattractant protein 1) and human IL-8 as detected in cell supernatants. This effect was related to the effect of silybin on the inhibition of IκBα phosphorylation and to its capability to inhibit ERK, MEK, and Raf phosphorylation at any concentration used[96]. Antifibrotic effects were also documented in experimental animals and in humans[85,97-99].

Table 6 summarizes the main anti-inflammatory and antifibrotic effects of silybin.

Metabolic effects

Silybin interferes with some mechanisms of action of insulin. In fact, it modulates the uptake of glucose in adipocytes by blocking the insulin-dependent glucose transporter 4. In rat hepatocytes, silybin, in concentrations ranging from 25 to 100 μmol/L, lowers glucose formation from different gluconeogenic substrates through an inhibitory effect on pyruvate kinase activity[100]. As previously reported in cultured hepatocytes, low doses of silybin reduce reactive oxygen species formation from mitochondria; this reduction leads to a decrease in oxidation of carbons arising from glycolysis[101]. Moreover, silybin inhibits, in a dose-dependent manner, gluconeogenesis and glycogenolysis, both in basal conditions and after a glucagon-dependent stimulation, by blocking glucose-6-phosphate hydrolysis. This effect was demonstrated using different substrates, such as dihydroxyacetone, lactate/pyruvate, glycerol, and fructose[102] (Table 7).

Table 7 Effects of silybin on hepatic metabolism of glucose at doses of 25-100 μmol/L in hepatocytes[100-102]

Doses	Cell	Targets
25-100 μmol/L	Hepatocytes	Inhibition of pyruvate kinase Inhibition of glycolytic flux Inhibition of glucose-6-phosphate hydrolysis Inhibition of glucose-6-phosphatase

Table 8 Other effects of silybin on cellular signaling[106-122]

Induction of apoptosis through an inhibition of IGF-IR
Down regulation of survivin and an increase in p53 expression
Block of cycle-regulator cyclins and promoter activities
Decrease of MMP-1 production
Decrease of angiogenesis
Inhibition of VEGF expression
Inhibition of HIF-1α
Increase of IGFBP-3

In an animal model of type 1 diabetes mellitus[103], in a 6-mo, double-blind, randomized trial in patients with poorly controlled non-insulin-dependent diabetes mellitus and alcoholic liver disease[104], and in a randomized, double-blind, placebo-controlled trial in patients with type Ⅱ diabetes[105], silybin significantly affected plasma levels of glucose and triglycerides, with a trend toward lower hemoglobin A1c levels.

Other effects on cellular signaling

In cancer cells, silybin alters cell cycle regulators and induces apoptosis, both through antioxidant and anti-inflammatory properties and through the inhibition of growth factor receptor-mediated mitogenic and cell survival signaling, particularly related to the activation of tyrosine kinases[106-111]. PDGF receptor (PDGFR), epidermal growth factor receptor (EGFR), Bcr-Abl, and KIT are examples of tyrosine kinases overexpressed in most human cancers. Silymarin or silybin are particularly effective in inhibiting EGFR signaling with suppression of cyclin-dependent kinase expression (i.e. CDK4) and up regulation of CDK inhibitors (CDKIs). These effects lead to G1 and G2-M arrest in cancer cells[112-119], as recently confirmed in a model of human colon cancer[120,121]. In fact, at a dose ranging from 40-75 μmol/L, silybin significantly inhibited cell proliferation through cell cycle arrest *via* inhibition of cyclin promoter activity.

Because it inhibits constitutive NF-κB activation, silybin can induce apoptosis, consistent with a significant decrease in its nuclear level of p65 subunit. In addition, it activates caspase 3 and caspase 9 and decreases survivin levels[122].

Angiogenesis refers to the growth of capillary vessels from existing blood vessels and is considered obligatory for the growth and progression of solid tumors. Angiogenesis critically depends on several conditions; in fact, endothelial cells must: (1) proliferate to provide the necessary number of cells for the growing vessels; (2) secrete matrix metalloproteinases (MMPs), which are required to break down surrounding tissue matrix; and (3) be capable of movement and migration. In addition, the angiogenic stimuli like hypoxia and the production of angiogenic cytokines, such as vascular endothelial growth factor (VEGF), must be sustained. Silybin treatment decreases secreted VEGF levels and shows a strong, concentration-dependent inhibition of capillary tube formation on matrigel, retraction, and disintegration of preformed capillary network, inhibition of matrigel invasion and migration, and a decrease in MMP-2 secretion[92,106-122].

Table 8 summarizes the main cellular signaling affected by silybin.

The use of silybin in the clinical setting: efficacy and suggestions

The use of silymarin in the clinical setting has a long history; in its native Mediterranean region, it has been employed for liver damage since the Greco-Roman era. As mentioned in the introduction, "herbal therapy" use is consistently increasing worldwide, and some of the most common herbal supplement preparations are derived from the milk thistle plant (*Silybum marianum*). Despite a long history of its use and the large number of people who consume this substance, no conclusive data on its clinical efficacy can be identified. In fact, only a few well designed clinical trials have been performed. Most studies have been conducted using silymarin and with inclusion of patients with alcoholic or viral cirrhosis. Stickel *et al*[8] identified the 10 main controlled trials conducted in liver patients, with numbers of patients ranging from 20 to 200 and doses of silymarin ranging from 210 to 450 mg/d for 7 to 730 d. These clinical studies generally have suffered from the same shortcomings found in many other trials on herbal medicines, such as a small sample size, lack of appropriate randomization and of allocation concealment or blinding, very different periods of treatment, lack of information about type and dose of extract used as well as product characterization, ill-defined patient population, and a lack of etiology, severity of disease, and discussion of potential confounders.

We could argue that the history of studies on silybin reflects, in part, the epidemiological history of liver diseases. When liver cirrhosis was the only clearly identified manifestation of liver damage and alcohol was the only known pathogenetic factor, silymarin and other plant extracts represented the most frequently used drugs. Knowledge about the pathogenetic relationships between hepatitis viruses and liver damage induced researchers and industry to focus attention on antiviral drugs. In response to the discovery of metabolic liver diseases, as well as increased knowledge about the cellular and subcellular mechanisms of both induction and progression of liver damage, researchers in academia and industry have begun to reappraise natural products and to evaluate their therapeutic efficacy using appropriate methods.

The clinical experience with silybin is mainly related to its properties as a detoxifying agent and as a hepatoprotective compound in different acute and chronic liver diseases.

Silybin as a detoxifying and hepatoprotective substance

Several chemotherapeutic agents are metabolized by the liver and can exert hepatotoxicity, with the net result of drug reductions or withdrawal. Chemotherapeutic agents that are likely to produce hepatotoxicity include dactinomycin, daunorubicin, docetaxel, gemcitabine, imatinib, 6-mercaptopurine, methotrexate, and oxaliplatin. Cancer patients taking these therapies often self-medicate with milk thistle because of its reputation as a liver protectant. Clinicians also prescribe it to cancer patients for the same purpose. The rationale of milk thistle use is to provide support to the liver while it performs multiple functions, including responding to the increased metabolic demands caused by tumor growth, assisting in metabolizing products generated when a tumor is killed or reduced by chemotherapy and radiation, and aiding in the processing of drugs prescribed to cancer patients.

Silybin is also considered a potent inhibitor of human intestinal β-glucuronidase, blocking the release and reabsorption of free xenobiotics and their metabolites from their glucuronide conjugates. Because the liver is the primary organ that cleanses and detoxifies the blood, many detoxification programs include a component of liver support or what is often called liver cleansing. For these reasons, silybin is commonly included in detoxification regimens[6,7,62,108-111]. However, only one randomized, double-blind study has reported the effects of milk thistle in patients receiving cancer therapy; 50 children with acute lymphoblastic leukemia and grade 2 or higher hepatic toxicity were randomized to receive a milk thistle supplement (Siliphos, Thorne Research, Dover, Idaho) (5.1 mg/kg/day) or placebo for 28 days. The authors reported significant reductions in AST levels ($P < 0.05$) and a trend toward a significant reduction in ALT levels ($P < 0.07$). A significantly larger number of children in the milk thistle group developed a > 50% reduction in total bilirubin at day 28 compared to placebo ($P < 0.0069$)[123].

Silybin in acute liver damage

The administration of silybin within approximately 48 h after poisoning produced by the mushroom *Amanita phalloides* (death cap) seems to be an effective measure to prevent severe liver damage[124]. A retrospective analysis of 205 cases of clinical poisoning from 1971 to 1980 documented the efficacy of milk thistle silybin extract in increasing survival rates in adults and children exposed to this potentially lethal mushroom[125]. In January 2007, six family members in California suffering from aflatoxin poisoning caused by *A. phalloides* mushrooms were treated with intravenous milk thistle, provided by Madaus Pharma (Brussels, Belgium; division of Madaus AG, Cologne, Germany). The US Food and Drug Administration granted permission for the use of milk thistle after considering that all patients were at high risk of dying of liver failure. Ultimately, one of the six patients died, while the others had a full recovery after treatment. A case report mentions that silybin may offer protection from liver toxicity caused by the pharmaceutical drug phenytoin[126].

Silybin in chronic liver disease

The various flavonoid compounds that are included in the general term "milk thistle" have been recognized as a "safe and well-tolerated herb" with a limited adverse event profile[1-10]. As previously reported, the majority of studies have been conducted using silymarin and including patients with alcoholic or viral cirrhosis. The Cochrane Collaboration group[4,127] evaluated the studies related to the use of silymarin/silybin in patients with different types of acute and chronic liver diseases. Various items were analyzed to define the quality of trials, such as the number of participants, the criteria of randomization, the blinding, the modalities of follow-up, and the statistical methods. From 1,831 references, 67 publications addressed patients with alcoholic and/or hepatitis B or C liver diseases treated with milk thistle; of these, only 26 were selected according to the agreed criteria, and these were associated with 13 trials. The active treatment consisted of silymarin per os in 10 randomized clinical trials, silybin phytosome per os in two randomized clinical trials, and silybin + phosphatidylcholine in one randomized clinical trial. The Cochrane Collaboration studies concluded that milk thistle does not seem to significantly influence the course of the disease in treated patients. However, all causes of mortality were reduced by 50% in patients with alcoholic liver disease without HCV antibodies who took milk thistle extracts compared to placebo ($P < 0.05$); in trials that studied liver-related mortality, there was a significant effect of silybin (relative risk, 0.50; 95% CI 0.29 to 0.88; $P = 0.02$)[4,8,9,127, 128].

Seeff *et al*[5,129] examined the spontaneous use of herbal products in the US in patients affected by HCV chronic infection, including non-responders to a previous treatment with interferon and ribavirin. In 1145 patients, about 50% used herbal products; of these, silybin was used by 70%. Even if no changes in liver tests and/or in HCV-RNA serum levels were observed, the univariate analysis documented a lower incidence of symptoms and a better quality of life in patients who consumed silybin relative to those who did not. On multivariate analysis, adjusted for age, gender, educational status, alcohol use (n. drinks/d), physical activity, body mass index, and smoking, silybin positively affected more than one aspect of quality of life. An examination of the total number of patients treated with silymarin/silybin in both well-conducted trials and in pilot studies with a high quality identified about 2000 patients with liver cirrhosis or with chronic hepatitis of different etiology, with a mean duration of treatment of six months and a dose of silybin ranging from 160 to 360 mg/d (except in two cases, see below).

The main results regarding the efficacy of silybin in

Table 9 Main studies performed by using purified silybin as drug

Authors	Type of study, number of patients	Drug used, dose and duration of treatments	Outcomes	Results	Clinical relevance
Vailati *et al*[146]	A phase II randomised, open trial on 60 patients with chronic alcoholic or viral hepatitis	Three doses (160, 240, 360 mg) of silybin and phosphatidylcholine (IdB 1016, Indena, Italy) for two weeks. No placebo or no intervention group was used	Liver tests	Improvement of liver enzymes with all used doses	Scarce
Buzzelli *et al*[147]	Double blind with identical placebo. Twenty patients with HBV and/or HCV chronic active hepatitis	IdB1016 (complex with phosphatidylcholine and silybin) two capsules, twice a day (equivalent to 120 mg of silybin in each capsule) (480 mg/d). Duration of treatment and of follow-up: two months in total	Mortality. Liver biochemistry	Improvement of liver enzymes and bilirubin	Scarce
Buzzelli *et al*[148]	Unclear, described as double blind but the method to achieve this was not described. Trial characteristics: cross over design. Patients were assigned to the Silipide group for two months treatment, and one month washout. Ten patients with chronic hepatitis C. (non-responders) to a previous treatment with recombinant interferon α	Silipide (IdB1016) capsules 360 mg/d. Control group: placebo capsules. Duration of treatment and follow-up: two months of treatment and one month of washout	Mortality. Liver biochemistry	Results were not reported separately, only overall results. Improvement of liver tests	Data published only in abstract form
Lirussi *et al*[104]	Blinding: adequate, double blind with placebo of identical appearance. Sixty out-patients with chronic alcoholic liver disease and non-insulin dependent type 2 diabetes	Silybin-β-cyclodextrin (135 mg silybin) sachets t.i.d Duration of treatment: 6 mo	Mortality. Liver biochemistry	Decrease of fasting glucose and lipid peroxidation markers	Good
Bares *et al*[138]	Randomized study to 1 of 3 oral doses. Thirthy seven patients with chronic hepatitis C non responders to a previous IFN treatment	IdB1016 at 314, 628, 942 mg t.i.d.(120,240 and 360 mg t.i.d. silybin equivalents, respectively) for 12 wk	Effects on serum markers of iron status	There was a significant decrease in serum ferritin, that was independently associated with the stage Ⅲ-Ⅳ of liver fibrosi	Good
Falasca *et al*[134]	Observational study on forthy naïve HCV positive patients (30 treated and 10 observed without treatments)	Silybin-Vitamine E-Phospholipid Complex (Realsil®- Ibi-Lorenzini-Italy) in a dose of 4 pills per day (each pill: 47 mg of silybin) for 3 mo	Hepatoprotection and anti-inflammatory effect by determining cytokine pattern and markers of liver disease	Improvement of liver enzymes and of Il2 plasma levels. Improvement of insulin resistance markers in patients with contemporaneous liver steatosis	Medium
Federico *et al*[141]	Observational study on 85 outpatients: 59 with NAFLD and 26 with HCV related chronic hepatitis in combination with NAFLD, non-responders to previous antiviral treatment. 53 (39 NAFLD and 14 HCV) were treated, while the other 32 patients (20 NAFLD and 12 HCV) served as a control group (no treatment)	The complex silybin-vitamin E-phospholipids (Realsil®), 4 pieces/d for six months followed by another six months of follow up	Effects on insulin resistance and liver damage	US steatosis, liver enzymes, hyperinsulinaemia, and indices of liver fibrosis were improved in both treated groups	Suggestive
Ferenci *et al*[139]	Observational study on 36 patients with HCV chronic hepatitis non responders to IFN + ribavirin. Duration of the study: 7 d	Silybin i.v. (Madaus, Germany) at 5, 10, 15 and 20 mg/kg per day for 14 d	Effect on viral load. Safety	Good compliance, no side effects and potent antiviral effect against HCV	High

liver disease patients are summarized as follows.

In the past, most studies were focused on liver cirrhosis, particularly alcoholic cirrhosis, and the efficacy of silybin was evaluated in terms of improvement in liver test abnormalities and/or in mortality rate (see above for results)[126-128]. Silybin β-cyclodextrin has been studied as an antidiabetic drug in patients with alcoholic liver disease and concomitant non-insulin-dependent diabetes mellitus; in these patients, the drug (at a dose of 135 mg/d) did not influence liver function test results or insulin secretion but significantly reduced fasting glucose ($P < 0.03$) and serum triglyceride levels ($P < 0.01$) compared to placebo. The effects seem to be related to a reduction in insulin resistance[104].

More recently, studies have focused on chronic hepatitis, particularly that induced by HCV. Silybin phytosome, at a dose ranging from 240 mg/d to 942 mg t.i.d. (the highest dose used in patients with liver damage) was well tolerated without adverse effects and significantly improved liver damage as expressed by transaminase levels, oxidative stress (serum malondialdehyde levels) and both ferritin serum levels and iron global body storage[130-138]. Today, the attention of researchers is focused on a possible antiviral effect of silybin against HCV infection.

Polyak *et al*[95] tested the anti-inflammatory and antiviral action of different extracts from silymarin on polymorphonuclear cells from patients with chronic HCV infection, documenting an anti-inflammatory and antiviral effect of milk thistle extract, mainly for silybin, which presented the strongest anti NF-κB and anti-HCV replication effect. Ferenci *et al*[139] and Biermet *et al*[140] have demonstrated that silybin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. These studies also showed that very high doses of silybin i.v. (from 5 to 20 mg/kg/d for 14 d) were free of toxic effects.

Finally, non-alcoholic fatty liver disease (NAFLD) may occur as an expression of a metabolic syndrome or in association with HCV chronic infection. The simultaneous presence of NAFLD in this latter group of patients may negatively affect the progression of fibrosis and the response to antiviral treatment. Patients affected by primitive NAFLD and/or chronic HCV-related hepatitis in combination with NAFLD, all non-responders to previous antiviral treatment, were treated with four pieces/d of the complex silybin-vitamin E-phospholipids corresponding to about 200 mg of pure silybin/d for three or six months. The treatment induced a significant reduction of plasma markers of chronic inflammation (C-reactive protein and cytokines), metabolic parameters (triglycerides, cholesterol, insulin resistance), liver tests (transaminases and gamma-glutamyl transpeptidase), degree of ultrasonographic liver steatosis and, finally, of main indices of liver fibrosis TGF-β, hyaluronic acid and metalloproteinase 2)[99,141,142].

In a rodent model of non-alcoholic steatohepatitis, the same silybin phospholipid complex prevented mitochondrial dysfunction[143], and preliminary results in a large, multicenter Italian trial *vs* placebo indicate that complex silybin-vitamin E-phospholipids significantly improved liver damage in patients with NAFLD and markers of liver fibrosis in patients with HCV chronic hepatitis[144].

In Table 9 the main studies performed with silybin are reported and discussed.

CONCLUSION

The data reported in this review clearly indicate the increasing interest in silybin and its compounds as well as the continuous improvement in knowledge about the molecular actions of this substance. However, in the clinical setting, there is currently a lack of definitive data about its efficacy in patients with chronic liver disease. The only well-defined finding is the absence of adverse events at high doses. Generally, all clinical studies on herbal products suffer from similar limitations, in part related to the fact that well-designed trials require resources and natural products industries do not sponsor them with significant budgets. In addition, in the majority of cases, herbal products differ from pharmaceutical compounds because multiple ingredients could act through multiple pathways to therapeutically affect the host. In the case of silybin, clinical studies on the pure extract and/or its derivatives are few and with a limited number of patients. Silybin is the most active flavonolignan in silymarin, and most capsules are standardized to this compound, but many variations of complex mixtures and single extracts are available and may critically affect clinical outcome. A full phytochemical and biological profile is preferable before commencing any clinical study. Today, analytical techniques that examine a suite of compounds, including their respective ratios, provide a more rational approach to authentication and quality assessment of the products. There must be assurance that the administered dose increases plasma and/or target tissue concentrations consistent with those required to produce effects *in vitro*. One primary fault of many clinical studies of botanicals is that adequate pharmacokinetic analyses are not completed before initiating efficacy trials. Some botanicals may fail in efficacy trials, not because the botanical is itself without activity but because the dosing was not sufficient to achieve pharmacologically meaningful concentrations.

Several silybin clinical trials are ongoing at this time (see also online at www.nccam.nih.gov). In addition, manufacturers of milk thistle extracts are conducting clinical trials with their own products that will elucidate the effects of specific preparations.

There is a need to enhance the funding opportunities to evaluate these long-used products with evidence-based knowledge, but there is also a need to reiterate Kroll *et al*[60], "As biological studies progress, it remains important to make the distinction between silymarin and silybin and their respective and distinct compositions". This latter point should also be considered when clinical investigators turn to pooling existing studies for meta-analyses. The epidemiology of chronic liver disease is changing

worldwide: viral infections are declining, and patients with HCV/HBV chronic hepatitis are older; NAFLD and alcoholic liver diseases are increasing, and generally patients with these pathologies are younger. Finally, alcohol-related problems, metabolic disruptions, and viral infections frequently coexist in the same patient[145]. Therefore, in clinical practice, there is the need for drugs that can be used in the long term without serious adverse events. Researchers should definitively demonstrate if silybin has potential in this regard. Lastly, the absence of significant adverse effects of silybin even at high doses, the good compliance by the patient, the availability of a purified form of the compound, represent characteristics that allow to obtain commercially available products containing almost 600 mg of pure silybin to ensure a good concentration in tissues.

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ORIGINAL ARTICLE

Double-balloon-enteroscopy-based endoscopic retrograde cholangiopancreatography in post-surgical patients

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Abstract

AIM: To evaluate double balloon enteroscopy (DBE) in post-surgical patients to perform endoscopic retrograde cholangiopancreatography (ERCP) and interventions.

METHODS: In 37 post-surgical patients, a stepwise approach was performed to reach normal papilla or enteral anastomoses of the biliary tract/pancreas. When conventional endoscopy failed, DBE-based ERCP was performed and standard parameters for DBE, ERCP and interventions were recorded.

RESULTS: Push-enteroscopy (overall, 16 procedures) reached enteral anastomoses only in six out of 37 post-surgical patients (16.2%). DBE achieved a high rate of luminal access to the biliary tract in 23 of the remaining 31 patients (74.1%) and to the pancreatic duct (three patients). Among all DBE-based ERCPs (86 procedures), 21/23 patients (91.3%) were successfully treated. Interventions included ostium incision or papillotomy in 6/23 (26%) and 7/23 patients (30.4%), respectively. Biliary endoprosthesis insertion and regular exchange was achieved in 17/23 (73.9%) and 7/23 patients (30.4%), respectively. Furthermore, bile duct stone extraction as well as ostium and papillary dilation were performed in 5/23 (21.7%) and 3/23 patients (13.0%), respectively. Complications during DBE-based procedures were bleeding (1.1%), perforation (2.3%) and pancreatitis (2.3%), and minor complications occurred in up to 19.1%.

CONCLUSION: The appropriate use of DBE yields a high rate of luminal access to papilla or enteral anastomoses in more than two-thirds of post-surgical patients, allowing important successful endoscopic therapeutic interventions.

Key words: Double balloon enteroscopy; Endoscopic retrograde cholangiopancreatography; Choledochojejunostomy; Hepaticojejunostomy; Pancreaticojejunostomy; Percutaneous cholangiodrainage

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INTRODUCTION

With the technique of push-and-pull enteroscopy by a double balloon endoscope, it is possible to advance much deeper into the small intestine than using a conventional push-enteroscope[1-3]. Double balloon enteroscopy (DBE) has been successfully applied for diagnosis and treatment of various small intestinal diseases, such as mid-gastrointestinal bleeding, polyposis syndromes, Crohn's disease, lymphoma, foreign body impaction, or other inflammatory or neoplastic diseases in the jejunum or ileum[1-3]. Although the introduction of DBE by Yamamoto has brought a significant benefit for the management of various small intestinal diseases, its value in the diagnosis and treatment of biliary or pancreatic diseases in patients after complex abdominal or bilio-pancreatic surgery has recently been reported in some case studies of selected patients[4-10]. The emerging role of DBE in postoperative endoscopic procedures arises from the fact that conventional endoscopy using side viewing endoscopes, forward viewing push-enteroscopes, or (pediatric) colonoscopes has often been reported to be unsatisfactory in patients after partial or total gastrectomy (Billroth Ⅱ gastrojejunostomy, Roux-en-Y reconstruction), Whipple resection or bilio-pancreatic reconstructions (pancreaticojejunostomy, choledocho-choledochostomy, hepaticojejunostomy)[4,5,10-12]. For example, in the pre-DBE era, conventional endoscopic access to the afferent loop and/or choledocho-, hepatico- or pancreaticojejunostomy was extremely difficult because of various lengths of bowel to be traversed, unfortunate locations of low jejunal anastomoses, jejunal loops of differing lengths, fixed jejunal loops, angulation or postoperative strictures and changes[4,5,10-12].

Failure of endoscopic access and therapy in post-surgical patients with normal papilla, choledocho-, hepatico- or pancreaticojejunostomy often results in more invasive and cost-intensive procedures such as percutaneous transhepatic cholangiodrainage (PTCD), computed tomography (CT)-guided pancreatic drainage, or repeated surgery. A training model for balloon-assisted enteroscopy and hepatobiliary interventions has been established by our group to learn, facilitate and adequately perform modern enteroscopic interventions[13-17]. Therefore, this study describes our clinical results from the prospective use of DBE in performing cholangio- and pancreatography, including therapeutic interventions of the biliary and pancreatic tract in a group of 37 consecutive post-surgical patients.

MATERIALS AND METHODS

Patient population

Between August 2005 and December 2008, 45 consecutive patients after complex abdominal surgery were admitted to the Department of Medicine 1 of the University Erlangen-Nürnberg because of abdominal pain, cholestasis, inflammatory symptoms, cholangitis, choledocholithiasis, or for an enlarging pancreatic pseudocyst. During this study period, eight patients with partial gastrectomy (Billroth Ⅱ) and both afferent and efferent loops at the gastrojejunostomy were excluded from the study, because six could initially be successfully treated using the treatment gastroscope and two using the side-viewing duodenoscope.

Thirty-seven consecutive post-surgical patients were included in this study after having obtained informed consent and agreement to participate and for scientific documentation of the examination results. This clinical trial was carried out in accordance with the Helsinki declaration. The different indications for ERCP, previous surgery, localization of foot-point anastomosis, and depth of papilla or ostium localization are listed in Tables 1 and 2. In this prospective protocol, all patients underwent first usual, conventional endoscopy at least once using esophago-gastroduodenoscopy (GIF-Q160, GIF-1T140; Olympus, Hamburg, Germany), side-viewing duodenoscopy (TJF160; Olympus) and push-enteroscopy (PE; SIF Q140; Olympus) to exclude other diseases and to document postoperative anatomy, type of surgery, depth of anastomoses and, if possible, of papilla or biliary or pancreatic enteroanastomoses. Thirteen percent of all patients had two PEs in order to clarify the post-surgical situation and to reach the entero-anastomosis.

If this approach by conventional endoscopy failed to gain access to the papilla, the ostium of the bilio-digestive or pancreatico-digestive anastomosis, push-and-pull enteroscopy (DBE, EN-450T5; Fujinon Europe, Willich, Germany) was tried before admitting the patient for re-operation, CT-guided drainage or PTCD. Among these DBE examinations, the p-type enteroscope (EN-450P5/20; Fujinon Europe) was used in 13.7% and the t-type enteroscope (EN-450T5) in 86.2% of the patients.

All enteroscopic procedures were performed during conscious sedation (midazolam/pethidine or propofol/pethidine) by two experienced examiners (> 1500 ERCP) and two endoscopy assistants. Butylscopolamine was only used after reaching the end of the afferent loop for ERCP or at withdrawal of the enteroscope, respectively, in cases of vigorous peristalsis, to identify postoperative anatomy, hidden ostium or to facilitate cannulation of the ostium of the biliodigestive anastomosis.

PE

PE was started in the left lateral position using the Olympus SIF-Q140 forward-viewing enteroscope (working length 2.50 m, no elevator lever) without overtube[18]. If PE failed to come forward, the patient was turned to the prone position and X-rays were used to localize loops, to straighten the enteroscope, to direct manual compression to guide the enteroscope forward, or to minimize pain by adequate withdrawal of the enteroscope[18-21]. Post-surgical

anatomy, location of the foot-point anastomosis and the route to the afferent loop were each exactly documented, as well as time requirements for each diagnostic and therapeutic step. Foot-point anastomosis and the afferent loop were marked by India ink. Forward-viewing PE-based ERCP was performed using the typical ERCP technique as described previously[18-21].

DBE

DBE was performed using a standard technique, starting in the left lateral position, and thereafter changing to the prone position as described by Yamamoto and other authors[1-4]. At times, manual compression to guide the enteroscope in the abdomen and radiography were necessary. Provided that the anatomical situation and access to papilla or ostium of the enteroanastomoses were clarified, the afferent loop in proximity to the foot-point anastomosis was marked with clips and Indian ink on retraction of the enteroscope, so that this location would be found quicker in a future examination. Using a standardized protocol, the advance was exactly documented during DBE, and the respective anatomical depth of foot-point anastomosis, and papilla and ostium region were determined with the retracted and (as much as possible) straightened enteroscope. The time taken for this procedure and the whole procedure were also recorded. If during enteroscopy, advance failed, the enteroscope slid back, or if pain was experienced by the patient, radiography was applied to avoid kinking, to straighten loops and to retract the enteroscope carefully.

DBE-based ERCP

When papilla or pancreatico-, choledocho-, or hepaticojejunostomy were needed, ERCP was applied using the push-and-pull enteroscope, a forward-viewing endoscope of 2 m working length, without elevator lever[19-21]. This was assisted by X-rays for radiographic imaging of bile ducts and/or pancreatic ducts or a pancreatic cyst. Appropriate stabilization of the enteroscope with the overtube and/or enteroscope balloon was often required before performance of ERCP.

After administration of contrast medium and diagnosis, papillotomy or, an initial bougienage and/or incision of a stenotic ostium of the hepaticojejunostomy was performed. This was achieved by the use of a 5 and 6 Fr Huibregtse catheter and/or a 6 Fr papillotome (Olympus, intended for SIF Q140 enteroscope), or a snare. Further interventions aided by a 5-m guide wire (Metro guide wire; Cook, Limerick, Ireland) were implantation of endoprostheses (5-8 Fr) or of biliary 7 Fr nasobiliary probes, stone removal, or ostium and papilla dilation using either a CRE-dilation balloon (CRE 8-10mm balloon; Cook) or a basket.

With regard to prosthesis change, the old prosthesis was at first mobilized with a foreign-body forceps or a loop, and extracted and placed in the afferent loop. After DBE-ERCP implantation of the new prostheses was completed, the old prostheses were fixed again with the loop and extracted from the patient during the final retraction of the double balloon enteroscope.

RESULTS

Patient population

During the period between August 2005 and December 2008, 45 post-surgical patients were admitted to hospital for endoscopy. Eight of these patients with partial gastrectomy (Billroth Ⅱ, without Roux-en-Y reconstruction) could initially be successfully treated with gastroduodenoscopy or side-viewing duodenoscopy alone, and were therefore excluded from the prospective study. In the remaining 37 patients with complex abdominal surgery, neither a gastroscope nor duodenoscope gained initial access to the papilla or ostium, such that PE, and if it failed, then DBE were necessary.

Previous types of abdominal surgery

Previous abdominal surgery of the remaining 37 patients (Table 1) was partial gastrectomy in eight patients (Bilroth Ⅱ-resection, 21.6%, four patients had further resections after B-Ⅱ-resection, five patients with Roux-en-Y reconstruction); total gastrectomy with Roux-en-Y loop in seven patients (18.9%), and classical or modified Whipple operation with Roux-en-Y loop in seven patients (18.9%). Fifteen patients had normal stomach anatomy after biliary surgery with reconstruction of a choledocho- or hepaticojejunostomy *via* Roux-en-Y loop (40.5%).

Thus, 34 patients had previously undergone Roux-en-Y construction (91.8%), whereas only three had an end-to-side gastrojejunostomy that contained an afferent and efferent loop (8.1%).

Among all post-surgical patients, 24/37 patients (64.8%) had a final diagnosis of choledocho- or hepaticojejunostomy (23 Roux-en-Y, one dorsal gastrojejunostomy), while 13 patients (35.1%) still had a normal papilla. The pancreaticojejunostomy had to be searched additionally in only three of these patients (8.1%) (Table 2).

Indications for ERCP and interventional procedures

With regard to the indication, it was necessary to radiograph the bile ducts of 34 patients (91.8%), because these patients were admitted for cholestasis (59.3%), cholangitis (28.1%), or choledocholithiasis (13.3%), with a view to PTCD or re-operation. Radiography of the pancreatic duct was required in only three patients (8.1%), because of the presence of a pancreatic pseudocyst and suspected or advanced chronic pancreatitis, respectively (Table 1).

Due to the complex anatomical situation in seven patients (18.9%) with recurrent disease, 37 PTCDs had already been performed in these individuals before the introduction of DBE-ERCP (Table 2).

Access to papilla and entero-anastomoses by PE and DBE

The individual endoscopic accessibility and anatomical

Table 1 Characteristics of post-surgical patients receiving push-enteroscopy or double balloon enteroscopy-endoscopic retrograde cholangiopancreatography

Pts.	Age/sex	Indication	Previous surgery	Access by G/T/P
1	72 f	Recurrent cholangitis	LTX, Roux Y, hepaticojejunostomy	No
2[3]	76 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ)	No
3	60 m	Liver abscesses	Whipple resection, Roux Y, hepaticojejunostomy	P
4	66 m	Benign cholestasis	CHE, Roux Y, hepaticojejunostomy	P
5[3]	52 f	Benign cholestasis	Complicated CHE, Roux Y, hepaticojejunostom	No
6	79 f	Postsurgical bile duct leakage	Complicated CHE partial gastrectomy (B Ⅱ)	P
7	38 m	Recurrent cholangitis	Congenital bile duct atresia Roux Y, hepaticojejunostomy	No
8	66 m	Pancreatitis with pseudocyst	Pylorus preserving pancreatic head resection, Roux Y, hepatico-& pancreaticojejunostomy	No
9	58 f	Benign cholestasis abdominal pain	Total gastrectomy, Roux Y, hepaticojejunostomy	No
10	64 f	Benign cholestasis with cholangitis	CHE, right hemihepatectomy, Roux Y, hepaticojejunostomy	No
11	50 f	Benign cholestasis, bile ducht stones	Dorsal gastroenterostomy with hepaticojejunostomy	G[1]
12	51 f	Benign cholestasis	CHE, partial gastrectomy (B Ⅱ) with Roux Y	No
13	81 f	Malignant cholestasis	CHE, partial gastrectomy (B Ⅱ) with Roux Y	No
14[3]	52 f	Benign cholestasis	Compliated CHE, Roux Y, hepaticojejunostomy	No
15[3]	71 m	Malignant cholestasis	Complicated CHE, partial gastrectomy (B Ⅱ), Roux Y	No
16	69 f	Recurrent cholangitis	CHE, Roux Y, hepaticojejunostomy	No
17	47 f	Cholangitis, malignant cholestasis	Total gastrectomy, Roux Y, hepaticojejunostom	T[2]
18	67 m	Benign cholestasis	LTX, bile duct revision, Roux Y, hepaticojejunostomy	No
19	51 f	Benign cholestasis, bile ducht stones	LTX, bile duct revision, Roux Y, hepaticojejunostomy	No
20	68 f	Benign cholestasis, chronic pancreatitis	Total gastrectomy, Roux Y	No
21	71 m	Recurrent cholangitis	Modified Whipple resection, Roux Y, hepaticojejunostomy	No
22	68 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ) with Roux Y	No
23[3]	64 f	Malignant cholestasis	CHE, small bowel & colon resection, Roux Y, hepatico-jejunostomy	No
24	61 m	Suspected malignant cholestasis	Modified Whipple resection, Roux Y, hepaticojejunostomy	No
25	62 m	Malignant cholestasis	Total gastrectomy, Roux Y	P
26	73 m	Benign cholestasis	Pylorus preserving pancreatic head resection, Roux Y, hepatico& pancreaticojejunostomy	No
27	76 m	Benign cholestasis	Total gastrectomy, Roux Y	No
28[3]	76 f	Malignant cholestasis	Total gastrectomy, Roux Y	No
29	84 m	Malignant cholestasis	Partial gastrectomy (B Ⅱ) with Roux Y	No
30	54 m	Choledocholithiasis, cholangitis	Complicated CHE, Roux Y, choledochojejunostomy	No
31	74 m	Choledocholithiasis	Total gastrectomy, Roux Y	No
32	61 m	Recurrent cholangitis	LTX, bile duct revision, Roux Y, choledochojejunostomy	P
33[3]	55 m	Suspected malignant cholestasis, chronic pancreatitis	Whipple resection, Roux Y, hepatico- & pancreatico-jejunostomy	No
34	34 f	Biliary colics, benign cholestasis hepatitis C	LTX, Roux Y, hepaticojejunostomy	P
35[3]	64 m	Suspected malignant cholestasis, chronic pancreatitis	Whipple resection, Roux Y, hepatico- & pancreatico-jejnostomy	No
36	51 f	Suspected choledocholithiasis,right abdominal pain	LTX, Roux Y, choledochojejunostomy	No
37	61 m	Recurrent cholangitis	Complicated CHE, Roux Y, hepaticojejunostomy	No

[1]Only after previous double balloon enteroscopy; [2]Only after previous double balloon enteroscopy and by use of a short-specialised, large caliber overtube (16.8 mm); [3]Patients indicate initial failure of DBE-based ERCP. G: Gastroscope; T: Side-viewing duodenoscope; P: Push-enteroscope; CHE: Cholecystectomy: B Ⅱ: Billroth Ⅱ resection; LTX: Liver transplantation; DBE: Double balloon enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography.

depth of the anastomoses, as well as of the papilla and the ostium of the choledocho- or hepaticojejunostomy and of the pancreaticojejunostomy using PE and DBE are described in Tables 1 and 2. The average depth of all anastomoses (three Billroth Ⅱ gastrojejunostomy, 34 footpoint anastomoses jejunojejunostomy) was 71 ± 21 cm, and the length of the afferent loop to the papilla or enteroanastomosis measured a further 53 ± 26 cm.

In total, a median of four (2-19, 25th-75th percentile) balloon-assisted enteroscopic cycles had to be performed after the passage of the anastomosis in the afferent loop, until the papilla or ostium were reached by DBE. Manual compression to guide the enteroscope was necessary in most patients.

The push-enteroscope could reach the papilla or the enteroanastomoses in only 6/37 cases (16.2%), while DBE had to be applied in 31 post-surgical patients (83.7%).

With DBE, access to papilla, choledocho-, hepatico- or pancreaticojejunostomy could be successfully and repeatedly achieved in 23 out of 31 patients (74.1%).

A total of 86 DBE-ERCPs were undertaken in those 31 patients, who failed to be successfully examined by PE. Seventy-five of the 86 DBE examinations (87.2%) were successfully carried out as a diagnostic or therapeutic

Table 2 Results of push-enteroscopy and double balloon enteroscopy-endoscopic retrograde cholangiopancreatography: postoperative anatomy and final diagnosis

Pts.	Foot-point anastomosis (cm)	Papilla/ ostium (cm)	ERCP diagnosis	PTCD before /after DBE
1	84	162	Stenotic hepaticojejunostomy (mucosal and intramural stricture 3 mm), putrid cholangitis	(2) Yes
2[1]	67	Not found	Swelling of anastomosis, afferent loop not found	No
3	65	90	Stenotic hepaticojejunostomy (mucosal, 11 mm stricture), cholangitis	No
4 P	75	110	Sludge, stenotic hepaticojejunostomy (mucosal, 3 mm stricture)	No
5[1]		Not found	PTCD stenotic hepaticojejunostomy (12 mm stricture)	(8) Yes(6)
6 P	52 (B Ⅱ)	78	Distal bile duct leakage and adhesion to abd. drainage	No
7	80	165	Stenotic hepaticojejunostomy (mucosal, 2 mm stricture), cholangitis	No
8	85 85	107 118	Normal choledochojejunostomy pancreaticojejunostomy with 10 mm diameter, 10 mm pancreatic Duct stricture, pancreatic pseudocyst	No
9	85	130	Normal hepaticojejunostomy, bile duct kinking	No
10	77	142	Stenotic hepaticojejunostomy (intramural, 4 mm) and stricture, common hepatic duct 4mm, bilioma	No
11	46	62	Obstructed hepaticojejunostomy by sludge/stones (hepaticolithiasis)	No
12	70	105	Papilla stenosis, bile duct kinking and stricture 3 mm	No
13	60	84	Bile duct stricture 18 mm due to papilla tumor	Yes (2)
14[1]	95	Not found	PTCD stenotic hepaticojejunostomy (12 mm stricture)	(12) Yes (6)
15[1]	57	110	PTCD edematous, tumorous papilla	Yes (2)
16	65	120	Stenotic hepaticojejunostomy (mucosal, 4 mm stricture)	(10) Yes
17	65	92	Malignant proximal bile duct stricture 22 mm	No
18	100	175	Hepaticolithiasis, normal hepaticojejunostomy	No
19	70	120	Stenotic hepaticojejunostomy (intramural, 12 mm stricture), cholestasis due to bile duct bleeding	(1) Yes
20	60	78	Papilla & bile duct stenosis due to chronic, pancreatitis, pancreatic duct stenosis	No
21	55	85	Stenotic hepaticojejunostomy,(mucosal, 2 mm stricture) & intrahepatic stricture	No
22	75	110	Distal bile duct stricture 45 mm due to ampullary tumor	No
23[1]		Not found	PTCD complete malignant stricture of hepaticojejunostomy due to progredient metastasis	Yes (1)
24	60	120	Hilar and hepatic duct strictures 9 and 26 mm, normal hepatico jejunostomy	No
25 P	65	110	Malignant obstruction biliary metal stent, sludge, cholangitis	(4) Yes
26	110	158	Stenotic hepaticojejunostomy, (intramural, 10 mm stricture)	No
27	76	112	22 mm bile duct stricture due to chronic pancreatitis	(2) Yes
28[1]	88	145	Polypoid papilla tumor	Yes (4)
29	100	140	Distal bile duct stricture 35mm due to suspected pancreatic tumor	No
30	105	151	Bile duct with sludge, normal choledochojejunostomy	No
31	51	165	Choledocholithiasis	(2) Yes (2)
32 P	78	147	Stenotic choledochojejunostomy, (intramural, 6 mm stricture) and bilioma segment Ⅳ	No
33[1]	66 66	Not found 126	PTCD: malignant stenotic hepatico-jejunostomy (filia), but normal pancreaticojejunostomy and chronic pancreatitis -	Yes (3)
34 P	80	132	Stenotic hepaticojejunostomy & hilar stenosis in ischemic cholangiopathy	No
35[1]	68 68	114 131	PTCD: recurrence of pancreatic tumor with malignant stenosis at hepaticojejunostomy, bile ducts and small intestine normal pancreaticojejnostomy and chronic pancreatitis	Yes (5)
36	70	131	Normal choledochojejunostomy	No
37	78	139	Stenotic hepaticojejunostomy (mucosal 2mm stricture)	No

[1]Patients indicate initial failure of double balloon enteroscopy (DBE)-based endoscopic retrograde cholangiopancreatography (ERCP). PTCD: Percutaneous transhepatic cholangiodrainage; PE: Push-enteroscopy.

DBE-ERCP (Tables 1-3), while 11 examinations (12.7%) in eight patients were unsuccessful.

After the initial, successful DBE-ERCP in two patients, the papilla and ostium of the hepaticojejunostomy, respectively, could be reached afterwards with the side-viewing endoscope or gastroscope. However, both treatments only worked after previous DBE, during which a large caliber overtube (17 mm, length 110 cm; Fujinon Europe) was inserted as a guide bar and the hepaticojejunostomy, located in an intestinal loop, was made visible through an inserted prosthesis.

Failure of PE and DBE to reach papilla or enteroanastomoses

In 8/31 patients (25.8%), despite DBE application, access to the bile ducts could not be achieved for a number of reasons (Tables 1 and 2): the anastomosis region was considerably swollen (one patient) or not visible because of metastasis (one patient); the afferent loop was technically not intubatable (one patient); the papillary or ostial region was infiltrated or covered by a tumor (four patients); or the ostium of the hepaticojejunostomy could not be found (one patient). Seven of these 8 patients (87.5%)

Table 3 Results of push-enteroscopy and double balloon enteroscopy-endoscopic retrograde cholangiopancreatography: therapeutic measures and (means ± SD) of sedation, X-rays and procedure time

Pts.	Push ERCP-/DBE-ERCP		Sedation		X-ray		Procedure Time (min)
	Procedures	Therapy	Dose (mg)	Drug	Time (min)	Dose (10^3 cGy/cm^2)	
1	7	Ostium incision (snare, papillotome) dilation, 2 stents inserted, regular change of 2 stents/1 yr	12.8 ± 3 132 ± 31	Midazolam Pethidine	19 ± 11	3.4 ± 2	122 ± 158
2	1	Not successful, re-operation	10.0 100 120	Midazolam Pethidine Butylscopolamine	3.3	1.0	82
3 P	3	Ostium incision (papillotome), dilation, stent insertion, regular change of stent/1 yr	15.0 ± 1 125 ± 35 40	Midazolam Pethidine Butylscopolamine	7.5 ± 7	1.8 ± 1.9	115 ± 79
4 P	4	Stent insertion, regular change of stent/1 yr	12 ± 2 137 ± 25 5	Midazolam Pethidine Diazepam	20 ± 29	3.1 ± 1.6	110 ± 171
5	2	Not successful, PTCD	12 ± 1 150 5 40	Midazolam Pethidine Diazepam Butylscopolamine	2.8 ± 1	4.0 ± 0.2	77 ± 11
6 P	2	Stent insertion, closure of bile duct leakage	7.8 ± 0.4 100	Midazolam Pethidine	4.0 ± 1	0.4 ± 0.1	135 ± 71
7	9	Ostium incision (papillotome), 2 stents inserted, regular change of stents/1 yr	1691 ± 867 135 ± 74 40	Propofol Pethidine Butylscopolamine	7.1 ± 6	1.8 ± 2.4	168 ± 131
8	4	Bougienage pancreaticojejunostomy, stent insertion into pancreatic duct and pseudocyst; normal hepatico-jejunostomy	13.3 ± 2 158 ± 38 40 ± 28	Midazolam Pethidine Butylscopolamine	11.8 ± 9	2.0 ± 2	161 ± 92
9	1	Normal hepaticojejunostomy	14 150	Midazolam Pethidine	10.1	0.5	91
10	4	3 stents inserted, one change of 2 stents	11.2 ± 5 133 ± 28	Midazolam Pethidine	12.6 ± 9	0.6 ± 0.4	61 ± 12
11	4	Insertion nasobiliary probe, dilation, stone extraction, insertion of stent	9.5 ± 1 125 ± 35 20	Midazolam Pethidine Butylscopolamine	8.1 ± 2	0.7 ± 0.4	61 ± 22
12	8	Bougienage, papillotomy, papilla dilation 8-10mm, stent insertion, regular change of stents/18 months	1082 ± 476 156 ± 77 47 ± 11	Propofol Pethidine Butylscopolamine	14 ± 8	3.1 ± 1.8	113 ± 97
13	3	Stent insertion, regular change of stent unsuccessful due to progredient papilla tumor, PTCD	10.8 ± 3 91 ± 52 40 ± 28	Midazolam Pethidine Butylscopolamine	13 ± 4	5.9 ± 2.9	177 ± 61
14	2	Not successful, PTCD	25 ± 7 175 ± 35	Midazolam Pethidine	5.4 ± 1	0.8 ± 0.1	155 ± 21
15	2	Not successful, PTCD	7.8 ± 3 100 ± 25 40	Midazolam Pethidine Butylscopolamine	5.9 ± 2	1.5 ± 0.3	122 ± 46
16	1	Ostium incision (papillotome), 2 stents inserted (perforation)	14 150 5	Midazolam Pethidine Diazepam	15.7	1.7	155
17	5	Papillotomy*, bougienage, nasobiliary probe; insertion of 2 stents, regular change of 2 stents/9 mo	16.8 ± 4 210 ± 74 16.7 ± 10 30 ± 11	Midazolam Pethidine Diazepam Butylscopolamine	11.6 ± 11	2.5 ± 2.6	198 ± 98
18	1	Stone extraction	19 200	Midazolam Pethidine	24.4	5.3	178
19	4	Extraction sludge & blood coagel, insertion nasobiliary probe, extraction of percutaneous drainage & insertion of 2 stents (rendezvous), regular change of 2 stents/ 9 mo	13 ± 1 116 ± 28 5 ± 5	Midazolam Pethidine Diazepam	9.7 ± 9	2.0 ± 1.8	82 ± 31
20	4	Papillotomy, stent insertion pancreatic duct, regular change of stent/6 mo, hemostasis with injection therapy	695 ± 275 75 ± 50 70 ± 14	Propofol PSSSethidine Butylscopolamine	8.7 ± 1	0.7 ± 0.4	61 ± 13
21	3	Insertion of 2 stents, regular change of 2 stents/6 mo	12 ± 1.8 158 ± 62	Midazolam Pethidine	15 ± 7	4.5 ± 1.9	185 ± 32
22	1	Papillotomy, insertion of 2 stents	19 200 40	Midazolam Pethidine Butylscopolamine	17.2	4.5	113

23	1	Not successful, PTCD	16 50 20	Midazolam Pethidine Butylscopolamine	0.6	0.2	63
24	2	Stent insertion	17.5 ± 2 100 ± 70	Midazolam Pethidine	18.9 ± 15	5.6 ± 2.8	150 ± 61
25 P	3	Stone/sludge extraction, dilation, biliary metal stent and malignant bile duct stricture, stent insertion, regular change of stent/9 mo	9 ± 4 200 ± 65	Midazolam Pethidine	12.9 ± 2	3.3 ± 1.1	54 ± 12
26	2	Ostium incision (papillotome), bougienage, stent insertion	7 ± 4 75 ± 35 40 ± 20	Midazolam Pethidine Butylscopolamine	4.5 ± 2	1.2 ± 0.6	61 ± 23
27	3	Papillotomy, extraction of percutaneous drainage and insertion of 2 stents (rendezvous)	5.7 ± 1 83 ± 28 40	Midazolam Pethidine Butylscopolamine	5.0 ± 1	1.0 ± 0.1	71 ± 12
28	1	Not successful, PTCD	5 50	Midazolam Pethidine	2.1	0.6	109
29	2	Papillotomy, bougienage, stent insertion	9 ± 2 150 ± 25	Midazolam Pethidine	9.2 ± 2	4.4 ± 0.3	113 ± 21
30	1	Sludge extraction, insertion nasobiliary	2.5 50	Midazolam Pethidine	16.4	7.8	123
31	2	Papillotomy, stone extraction, extraction of percutaneous drainage and insertion of stent (rendezvous)	7 ± 2 100 ± 25 80	Midazolam Pethidine Butylscopolamine	2.2 ± 0.5	1.9 ± 0.4	96 ± 31
32 P	1	Stent insertion	10 200 20	Midazolam Pethidine Butylscopolamine	27.1	7.9	161
33	2	Not successful, PTCD diagnostic pancreatography, extraction of percutaneous drainage with both ostium incision and insertion of 2 stents (rendezvous)	12 ± 5 150 ± 70 40 ± 28	Midazolam Pethidine Butylscopolamine	10.6 ± 9	3.3 ± 2.5	97 ± 80
34 P	3	Insertion of 2 stents, regular change of stents/12 mo	10 ± 7 183 ± 124 10 ± 5 20 ± 20	Midazolam Pethidine Diazepam Butylscopolamine	19.9 ± 10	3.6 ± 2.3	98 ± 33
35	1	Not successful, PTCD diagnostic pancreatography	11 150 40	Midazolam Pethidine Butylscopolamine	0.3	0.1	86
36	1	Normal choledochojejunostomy	7 50 20	Midazolam Pethidine Butylscopolamine	2.1	1.8	51
37	1	Ostium incision (papillotome), insertion of 2 stents	8.5 150	Midazolam Pethidine	4.4	2.0	72

Pts overall	Total number PE/DBE	Mean sedation dose per examination		Total x-ray time	Total x-ray dose	Total examination time
37	16 PE 86 DBE	11.7 ± 2.8 124 ± 45 20 ± 20 1156 ± 593	Midazolam Pethidine Butylscopolamine Propofol	9.0 ± 5.5	2.5 ± 1.3	111 ± 54

P: Push-enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography; DBE: Double balloon enteroscopy; PTCD: Percutaneous transhepatic cholangiodrainage; PE: Push-enteroscopy.

underwent subsequent PTCD or surgery (one patient, 12.5%).

Diagnosis, results and interventions at normal and malignant choledocho- and hepaticojejunostomy

In choledocho- or hepaticojejunostomies, 14 out of 24 (58.3%) were cicatricially changed, three were infiltrated by malignant tissue (12.5%), and seven (29.1%) appeared normal in width and were intact (Table 2).

DBE was able to achieve access to 15 of the 24 choledocho- or hepaticojejunostomies (62.5%), while PE reached only four out of 24 (16.6%), and the remaining five patients with failure of the enteroscopic approach (20.8%) had to undergo PTCD.

Among the seven normal appearing ostium of the choledocho- or hepaticojejunostomies (29.1%), sludge and

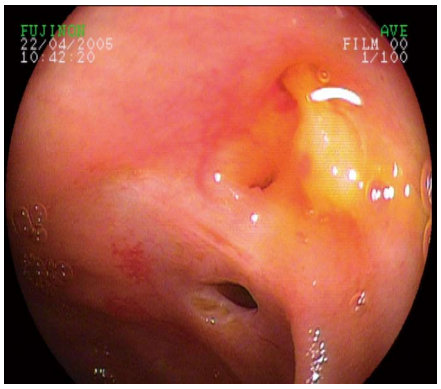

Figure 1 Endoscopic finding of stenotic hepaticojejunostomy in recurrent cholangitis with putrid secretion after careful ostium incision during double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique.

concrements had to be removed from one normal choledocho- and three normal hepaticojejunostomies in one patient suffering from cholangitis and choledocholithiasis, and three patients with hepaticolithiasis, respectively. In addition, endoprosthesis and/or nasobiliary probe insertion *via* the normal choledocho- or hepaticojejunostomy were necessary in two of these patients and in one with hilar and hepatic duct strictures, respectively.

Out of three tumor-induced malignant ostium stenoses (12.5%), the precise location of the enteroanastomosis could be identified twice, but in neither case could the stenosis be passed by a flexible hydrophilic guidewire and successfully treated. All three patients with tumorous hepaticojejunostomies required PTCD.

Diagnosis and results in post-surgical stenotic choledocho- and hepaticojejunostomy

Eight patients out of 14 (57.1%) with cicatricial ostial stenosis at the choledocho- or hepaticojejunostomy were treated successfully *via* DBE-ERCP, and a further four *via* PE (28.5%), while the remaining two patients (14.2%) required PTCD (Tables 2 and 3).

In one case with stenotic hepaticojejunostomy and previous PTCD (suspected hepaticolithiasis) at an outlying hospital, DBE-ERCP revealed blood in the afferent loop, bile duct bleeding from PTCD, and obstruction of the stenotic ostium including bile ducts due to blood clots. Thus, extraction of sludge and blood clots was performed, and insertion of a temporary nasobiliary drainage for irrigation of the bile duct. Then, after 3 d, a first DBE-based rendezvous technique was applied *via* the PTCD with successful extraction of the percutaneous drainage and endoscopic insertion of two internal stents.

Of note, a successful rendezvous technique was further achieved in three patients with non-malignant disease who were admitted to our hospital after construction of a PTCD, and in one patient with initial failure of DBE (Table 3). Thus, these four patients had most significant benefit from DBE-ERCP because they had endoscopically inserted endoprotheses and lost their percutaneous drainage within 1 wk.

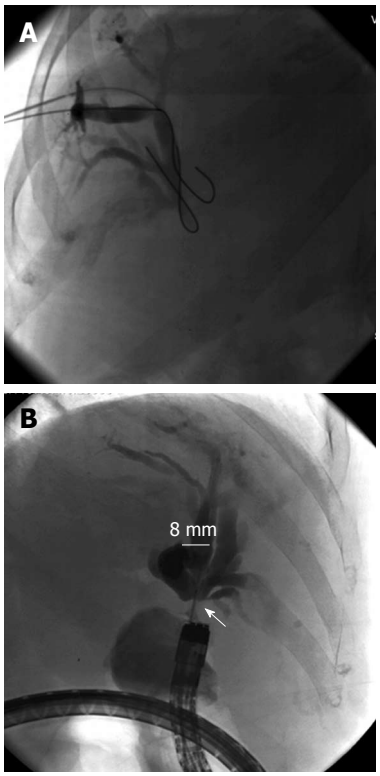

Figure 2 Radiological findings of stenotic hepaticojejunostomy in recurrent cholangitis with unsuccessful percutaneous drainage (A), but selective access to dilated bile ducts (width 8 mm) through a high-grade stricture (3 mm long, arrow) by double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique (B).

Ostium incision and dilation and endoprosthesis insertion at post-surgically strictured choledocho- and hepaticojejunostomy

Initial endoscopic interventions at the non-malignant post-surgical biliary anstomosis (choledocho- or hepaticojejunostomy), which could not be cannulated by a flexible guidewire, included a careful, 1-3-mm ostium incision (by snare and/or 6 Fr papillotome) of each narrowed ostium in 6 out of 12 cases (50.0%) during DBE-ERCP. Five ostial incisions were made during DBE-ERCP, and one during PE-based ERCP. All incisions resulted in significant widening of the ostium with subsequent successful cannulation and intervention in the biliary system. Perforation occurred in one of the 5 patients treated with ostial incision by DBE-ERCP (20.0%), which had to be treated surgically. None (0%) of the ostial incisions caused relevant bleeding, but in two cases (40.0%), pus was discharged from the opened ostium (Figures 1 and 2).

The other six patients (50.0%) with post-surgically strictured choledocho- or hepaticojejunostomy were initially cannulated using a guidewire and were treated either with a bougienage *via* a papillotome or nasobiliary probe, to widen the ostium ready to implant subsequently a prosthesis, or by dilation using a colonic CRE balloon.

Overall, in patients with cicatricial changed choledocho- or hepaticojejunostomies, on average 1.5 ± 0.7 endoprostheses were implanted per DBE-ERCP examination

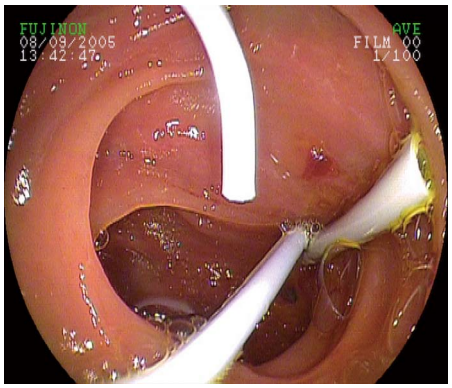

Figure 3 Endoscopic finding of stenotic hepaticojejunostomy in recurrent cholangitis after ostial incision and insertion of two endoprotheses during double balloon enteroscopy-endoscopic retrograde cholangiopancreatography in prograde technique.

(one double pigtail 5 Fr, 18 double pigtail 7 Fr and three double pigtail 8 Fr, as well as four straight 7 Fr endoprostheses and two 7 Fr nasobiliary probes; Figure 3).

At present, four patients with cicatricially changed ostium of the choledocho- and hepaticojejunostomy were treated several times by DBE-ERCP over a period of 1 year, with a regular exchange of prostheses every 3 mo (Table 3). After prosthesis implantation, all four patients had no further problems with cholangitis and cholestasis. In three out of four patients (75%), a sufficient widening of the ostium was achieved after the 1-year prosthesis therapy. Consequently, prosthesis therapy was no longer required and the cholestasis parameters stayed within the normal range over a prolonged period of time. However, the prosthesis exchange proved to be more difficult than the initial prosthesis implantation, because this procedure carries varying degrees of difficulty. In addition, an average treatment time of 12 ± 41 min had to be calculated for prostheses extraction and their temporary placing in the intestines.

DBE-ERCP with interventions at the pancreatic anastomosis

Among the 31 post-surgical patients, pancreaticojejunostomy was also found *via* DBE in three patients (9.6%) because of recurrent abdominal pain, inflammatory symptoms and an expanding cystic lesion in the pancreatic region. This could only be achieved successfully by DBE (Tables 1 and 2). The pancreaticojejunostomies (mean insertion depth: 128 ± 7 cm) were located mostly at 3-8 cm aborally of the biliodigestive anastomosis, and hence, required 1 ± 1.7 balloon-assisted cycles more to identify the pancreaticojejunostomy and to stabilize the DBE in front of it.

During the DBE-based pancreatography, two duct systems in patients with recurrent pancreatic tumor presented a similar appearance to those with chronic pancreatitis (clotted side branches, duct irregularities, but no acute strictures). In addition, one significantly dilated residual pancreatic duct was detected merging into a cystic lesion (pseudocyst). In the latter case, for the first time a 7 Fr double pigtail prosthesis had to be inserted for drainage of the pseudocyst *via* DBE-ERCP, because the patient suffered evidently from pain, weight loss, and inflammatory symptoms. After 2 d, the patient was free of symptoms. However, a mild lipase increase occurred post-interventionally, but there was no manifestation of post-ERCP pancreatitis. Within a week, the pseudocyst regressed noticeably, which was sonographically controlled and later documented with endoscopic ultrasound and CT. The prosthesis was removed 2 mo after insertion.

DBE-ERCP with interventions *via* the afferent loop at the papilla

Thirteen (41.9%) of the 31 patients still had a normal papilla. In 11 out of 13 patients (84.6%), the papilla was accessible *via* a Roux-en-Y loop, and only in two patients (15.3%) was it directly accessible from the Billroth Ⅱ stomach anastomosis *via* the afferent loop (Table 1).

The papilla could be reached with conventional PE in two of these 13 (15.3%) cases, and ERCP could be successfully performed with this forward-viewing enteroscope.

In the remaining 11 patients (84.6%) with normal papilla and prior abdominal surgery, the papilla had to be searched by push-and-pull-enteroscopy. DBE-ERCP could only be performed after appropriate stabilization of the enteroscope in front of the papilla, partly by use of the balloons. The DBE-ERCP and treatment was successful in eight of the 11 cases (72.7%; Tables 2 and 3), while in three cases (27.2%), DBE-based endoscopic retrograde cholangiography (ERC) failed because of tangential position to the papilla, or because of a papillary tumor (re-operation in one patient, and PTCD in two).

In the eight successful DBE-ERCs, seven patients (87.5%) had papillotomies of 3-7 mm in length using a 6 Fr papillotome, whereby moderate pancreatitis and bleeding (14.2% for each) occurred as side effects. In total, 1.2 ± 0.4 endoprostheses were successfully placed *via* the forward-viewing enteroscope (four double pigtail 7 Fr prostheses, one double pigtail 8 Fr prosthesis, seven straight 7 Fr endoprosthesis, and one 7 Fr nasobiliary probe).

In addition, apart from bougienage with the 6 Fr papillotome, dilatations using a CRE dilation balloon (8-10 mm, Cook) and removal of 5 ± 11 concrements and sludge using baskets were carried out in cases of papillary or distal bile duct stenoses. For treatment of purulent cholangitis with concrements, a nasobiliary drainage for irrigation was also placed *via* the enteroscope and left for 3 d to perform endoscopic shockwave lithotripsy and clean the bile system.

Laboratory results before and after DBE-ERCP with interventions

Before intervention, laboratory testing determined that the patients presented with distinct cholestasis and bilirubin elevation (2.8 ± 3.1 mg/dL) and/or inflammatory symptoms (leukocytes 12800 ± 10200/μL, C-reactive protein 51 ± 37 mg/L). By performing DBE-ERCP with ostial incisions, papillotomies and/or implantation of biliary endoprostheses, a clear reduction of cholestasis and chol-

angitis parameters was obtained. Values for bilirubin (1.6 ± 2.0 mg/dL), leukocytes (6800 ± 4000/μL) and C-reactive protein (18 ± 21 mg/L) decreased significantly ($P < 0.05$).

Complications of DBE-ERCP with interventions

Among 86 DBE-ERCPs, post-interventional cholangitis was not observed in any of the 31 patients treated by DBE-ERCP. However, after six of 86 examinations (6.9%) in 31 patients (19.3%), a lipase increase of more than twice the norm was seen on the day after DBE, whereas clinically significant post-ERCP pancreatitis (one mild and one moderate) was only seen after two examinations (2.3%) in two patients.

Post-interventional bleeding occurred in one of 86 examinations (1.1%) in 31 patients (3.2%) after papillotomy, which required emergency endoscopy, intensive care treatment, and blood transfusion.

Post-interventional stomach pain was experienced after six of 86 examinations (6.9%) in 31 patients (19.3%), whereas perforation occurred in two DBE-ERCPs (2.3%). One perforation developed immediately after ostial incision, while the second became evident 8 h later, with ileal perforation. Both perforations could be treated surgically, and no patient died due to complications of DBE-ERCP. No other fatalities following DBE-ERCP were recorded.

After two of 86 examinations (2.3%), two patients complained of abdominal pain that lasted > 24 h, and raised temperature developed on the day after the examination. Of note, one patient developed tonsillitis after DBE-ERCP (1.1%). No other serious side effects occurred.

Examination and radiography times and premedication during DBE-ERCP

The average duration of all DBE-ERCPs was 111 ± 54 min, and radiography took 9.0 ± 5.5 min with a dose of 2465 ± 1295 cGy/m^2. The individually required examinations for each patient are listed in Table 3, which included the exact therapeutic procedures, time measurements, and premedication.

With regard to premedication, an average of 11.7 ± 2.8 mg midazolam and 124.9 ± 45 mg pethidine or 1156 ± 593 mg propofol was needed per patient undergoing DBE-ERCP. In addition, butylscopolamine was administered at an average dose of 44.8 ± 20 mg. During conscious sedation for DBE-ERCP, one patient each developed hypoxia induced by midazolam/pethidine or propofol, which led in each case to abortion of the examination.

DISCUSSION

The difficulties involved with endoscopic access to the bile ducts and the pancreas in patients with prior abdominal surgery before the introduction of DBE have been described previously[4-6,10-12,19-21]. The success rate of ERCP with a side-viewing endoscope, push-enteroscope or pediatric colonoscope in patients with previous surgery depends on a number of factors, e.g. type of previous surgery, length of afferent loop, post-surgical changes, or experience of the endoscopist. Usually, results tend to be very variable (e.g. success rate of Billroth Ⅱ gastrojejunostomy up to 92%, Roux-en-Y reconstruction, 33%, and pancreaticojejunostomy, 8%) accompanied by high complication rates[4,6,19-21].

Access through conventional endoscopy was particularly difficult in our patients after several rounds of complex abdominal surgery (91.8% Roux-en-Y reconstruction, 8.1% gastrojejunostomy), and initially, access or treatment by gastroscope or duodenoscope was not possible. As recently outlined by several other investigators in small patients series[5-10,22-24], our stepwise approach with PE and DBE in 37 non-selected, consecutive post-surgical patients found that DBE-ERCP was clearly more efficient than PE. By the appropriate use of DBE in over two-thirds of cases, enteroanastomoses or papilla could be repeatedly reached, identified and satisfactorily visualized. The enteroscope could be stabilized also for bilio-pancreatic intervention. DBE-ERCP could be successfully conducted in 74.1% of the cases *via* the enteroscope, while PE reached biliary anastomoses or papilla in only 16.2% of the patients, which resulted in successful ERCP in only a minority of patients. Both results are in good agreement with recently published data for the approach by double- or single-balloon enteroscopy[5-10,22-26], as well as for earlier published data on postoperative or PE-based ERCP[4,11,19-21].

However, until a successful DBE-ERCP was achieved, several balloon-assisted enteroscopic cycles over an average length of 124 ± 47 cm of the small intestine, application of X-rays, and manual guidance of the enteroscope were necessary. In addition, a substantial effort in time, staffing and sedation had to be afforded. Compared with PE, the push-and-pull method by DBE proved to be markedly more effective, because pushing and stretching of small intestinal loops is reduced by regular retractions of the DBE cycle. The threading of the small intestine onto the DBE and the option to block the balloons at the enteroscope provides the enteroscope tip with a greater possibility of movement for identifying the biliary or pancreatic anastomoses or the papilla. In addition, sliding back of the enteroscope may be prevented by inflated balloons, which, compared with PE, explains the significantly higher effectiveness of interventions during DBE-ERCP.

Out of the 37 post-surgical patients with significant cholestasis and cholangitis, PE achieved a successful bile duct drainage in six (16.2%), whereas, before DBE was introduced, a far more invasive procedure, either PTCD or surgery, would have been carried out in the remaining 31 patients. PTCD carries a significantly higher morbidity and mortality risk compared to the endoscopic procedure[12,14-17,27,28], therefore, all consecutive patients with previous abdominal surgery were included in this prospective treatment protocol after DBE had been introduced in August 2005 at the University of Erlangen–Nuremberg. Of note, DBE facilitated successful ERCP with biliary

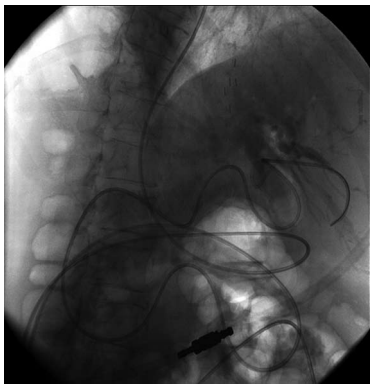

Figure 4 Radiological finding of insertion of a nasobiliary probe for irrigation in recurrent cholangitis with sludge after liver transplantation and hepaticojejunostomy by double balloon enteroscopy-endoscopic retrograde cholangiopancreatography through 120 cm of small bowel.

interventional procedures leading to significant reduction of cholestasis or cholangitis in 23 of 31 patients (74.1%). Thus, PTCD could be avoided in those 23 post-surgical patients, because endoscopic biliary drainage was achieved.

In comparison to reported PTCD-induced complication and infection rates of up to 55%, and even mortality[12,14-17,27,28] only one case of post-papillotomy bleeding (3.2%), two of post-ERCP pancreatitis (6.4%) and two perforations (6.4%) occurred following DBE-ERCP, but no cholangitis or mortality has been recorded to date. Thus, this first prospective investigation from a university tertiary referral center confirms that DBE-ERCP has considerable potential to treat successfully benign (postoperative) or malignant biliary and papillary stenoses, bile duct concrements, and cholangitis, even in non-selected post-surgical patients[4-10], and it helps to reduce the number of percutaneous approaches. Only in eight of 31 patients (25.8%), in whom the biliary or pancreatic anastomoses or papilla could not be found *via* DBE, was PTCD finally necessary. Even when the biliodigestive anastomoses could not be found and/or DBE-ERCP failed because of tumor-changed papilla or choledocho- and hepaticojejunostomy, a change in treatment procedure could be attempted after construction of PTCD by using DBE. After introduction of the percutaneous tube into the small intestine, percutaneous drainage was successfully changed in four patients to internal drainage inserted *via* DBE (Table 3). This was achieved by application of a DBE-PTCD rendezvous procedure, which was performed for the very first time in Erlangen in 2006. Before the DBE era, a longer-lasting bougienage and Yamakawa prosthesis therapy or biliary metal stent implantation were often indicated after the initial PTCD puncture[12,14-17]. By the use of DBE-ERCP, however, the external drainage could be extracted from all four patients after 1 wk. Practically, methylene blue injected externally through the PTCD helps to identify the afferent loop and/or biliary anastomoses or papilla, so that these are more easily and quickly detected by the subsequent DBE.

The key benefits of DBE-ERCP in the care of post-surgical patients with cholestasis/cholangitis and patients with installed percutaneous drainage are somewhat limited by the small caliber of bile duct prostheses that are applied *via* the enteroscope. According to the present state of technology, only an implantation of 5-8 Fr prostheses through an operating channel of 2.8 mm is possible. Consequently, several prostheses (1.5 ± 0.7) were implanted in our patients. In the case of strongly soiled bile ducts and concurrent cholangitis or sump syndrome, it is recommended first to apply a nasobiliary probe for irrigation of the bile ducts (Figure 4) to prevent rapid clogging of the small caliber bile duct prostheses.

The sequential coupling of two examinations (DBE and ERCP) explains the lengthy examination times, high doses of sedation, and applied fluoroscopy dosage. Considering the enormous benefit of DBE-ERCP with an approximately 74% successful biliary drainage and a significantly smaller complication rate than PTCD[11,12,14-17,27-29], the effort involved in such an examination seems justified.

In comparison to the more frequent cholestatic patients, only three of 37 patients also required radiography and interventions of the pancreatic duct after pancreatic resection. Overall, only a limited view could be gained as to which role DBE-ERCP might play in this area. In all three patients, the position of the pancreaticojejunostomy was only reached by DBE and was located deeper in the small intestine or considerably closer to the blind end of the afferent loop than was the choledocho- or hepaticojejunostomy. The technical conduction of the endoscopic retrograde pancreatography *via* DBE was undertaken in the same manner as described for ERCP. The ostium, however, was smaller, but in none of the cases stenotic. The main pathological changes of chronic pancreatitis were limited to the remaining pancreatic duct in the corpus area. During DBE-based pancreatography, a cystic lesion (pseudocyst) could be successfully drained *via* insertion of a 7 Fr double pigtail prosthesis for the first time, which led to a noticeable improvement of the patient, and regression of the pseudocyst within a week. Therefore, DBE offers also a novel option for pseudocyst drainage in postsurgical patients.

In conclusion, this prospective study from a single university tertiary referral center confirms the results from other investigators and shows that DBE-ERCP achieves a high rate of successful cholangiography and drainage in post-surgical patients[5-10,22-26,29], allows further treatment of pancreatic cystic lesions *via* pancreaticojejunostomy, and offers new possibilities in patients with PTCD as DBE-based rendezvous techniques are applicable.

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COMMENTS

Background

Abdominal surgery involving the stomach, small bowel, pancreas, liver or biliary tract may change significantly the anatomy of these organs, with construction of small bowel anastomoses and small bowel limbs of differing length, angles or fixation. Thus, postoperative endoscopy with conventional endoscopes to reach the biliary tract or pancreas through small bowel limbs has often been described as unsatisfactory in postoperative disease.

Research frontiers

Balloon-assisted endoscopy has been developed since 2004, with the introduction of a double balloon enteroscopy (DBE) system, followed later by single balloon endoscopy or balloon-guided enteroscopy techniques. All balloon-assisted endoscopy techniques have the potential to access more deeply into the small bowel than conventional endoscopes, and they allow one to examine the whole small bowel (4-7 m long). Thus, this study investigated the value of the DBE for examination of postoperative patients with diseases of the biliary tract or pancreas.

Innovations and breakthroughs

Before the era of balloon-assisted endoscopy, only 20%-30% of patients with diseases of the biliary tract or pancreas (e.g. tumor, stones, inflammation, stenosis) could be effectively managed by conventional endoscopy, whereas the other 70%-80% had to be treated by more invasive percutaneous puncture techniques, external tube insertion, drainage procedures, and more cost-intensive computed tomography (CT)-based therapies, or even re-operation. This paper describes, in a large number of consecutive patients, successful use of DBE to perform effective endoscopic treatment in a majority (74%) of post-surgical patients with bilio-pancreatic diseases.

Applications

DBE-based examination of the biliary tract or pancreas represents a further important endoscopic treatment modality for postoperative patients after complex abdominal resections. It allows successful application and interventions in post-surgical patients with bile duct stenosis, obstruction, stones or pancreatic diseases (chronic inflammation, tumor) in terms of performing incision of the bile duct ostium, or papillotomy, endoprosthesis insertion, or stone extraction.

Terminology

DBE-based examination of the biliary tract and pancreas is achieved by forward-viewing optics in post-surgical patients, and requires examination of the small bowel by DBE, and includes endoscopic–radiological examination of the bile duct and/or pancreatic duct, with the aim of performing interventions in the case of bile duct, liver or pancreatic disease. This whole procedure is called DBE-based retrograde cholangiopancreaticiography and is indicated only when conventional endoscopy fails to reach the biliary tract or pancreas.

Peer review

This study describes the utility of modern enteroscopy, especially DBE, in symptomatic patients with cholestasis and cholangitis after complex abdominal surgery. A high rate of enteroscopic access and successful biliary interventional procedures, with a new intervention, ostial incision at biliary anastomoses is presented, which resulted in a substantial reduction in more invasive procedures such as transhepatic percutaneous biliary interventions or CT-guided punctures.

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ORIGINAL ARTICLE

(-)-Epigallocatechin-3-gallate inhibits VEGF expression induced by IL-6 *via* Stat3 in gastric cancer

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Abstract

AIM: To demonstrate that (-)-Epigallocatechin-3-gallate (EGCG) inhibits vascular endothelial growth factor (VEGF) expression and angiogenesis induced by interleukin-6 (IL-6) *via* suppressing signal transducer and activator of transcription 3 (Stat3) activity in gastric cancer.

METHODS: Human gastric cancer (AGS) cells were treated with IL-6 (50 ng/mL) and EGCG at different concentrations. VEGF, total Stat3 and activated Stat3 protein levels in the cell lyses were examined by Western blotting, VEGF protein level in the conditioned medium was measured by enzyme-linked immunosorbent assay, and the level of VEGF mRNA was evaluated by reverse transcription polymerase chain reaction (RT-PCR). Stat3 nuclear translocation was determined by Western blotting with nuclear extract, and Stat3-DNA binding activity was examined with Chromatin immunoprecipitation (ChIP) assay. IL-6 induced endothelial cell proliferation was measured with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide assay, *in vitro* angiogenesis was determined with endothelial cell tube formation assay in Matrigel, and IL-6-induced angiogenesis *in vitro* was measured with Matrigel plug assay.

RESULTS: There was a basal expression and secretion of VEGF in AGS cells. After stimulation with IL-6, VEGF expression was apparently up-regulated and a 2.4-fold increase was observed. VEGF secretion in the conditioned medium was also increased by 2.8 folds. When treated with EGCG, VEGF expression and secretion were dose-dependently decreased. IL-6 also increased VEGF mRNA expression by 3.1 folds. EGCG treatment suppressed VEGF mRNA expression in a dose-dependent manner. EGCG dose-dependently inhibited Stat3 activation induced by IL-6, but did not change the total Stat3 expression. When treated with EGCG or AG490, VEGF expressions were reduced to the level or an even lower level in the tumor cells not stimulated with IL-6. However, PD98059 and LY294002 did not change VEGF expression induced by IL-6. EGCG inhibited Stat3 nucleus translocation, and Stat3-DNA binding activity was also markedly decreased by EGCG. Furthermore, EGCG inhibited IL-6 induced vascular endothelial cell proliferation and tube formation *in vitro* and angiogenesis *in vitro*.

CONCLUSION: EGCG inhibits IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer, which has provided a novel mechanistic insight into the anti-angiogenic activity of EGCG.

Key words: Epigallocatechin-3-gallate; Vascular endothelial growth factor; Signal transducer and activator of transcription 3; Angiogenesis; Gastric cancer

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INTRODUCTION

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and active component of green tea, has shown to have chemopreventive and chemotherapeutic properties for a variety of cancers[1,2]. Previous studies demonstrated that EGCG inhibited tumor growth by anti-angiogenesis, as well as by inhibiting proliferation and inducing apoptosis[3,4]. Angiogenesis is necessary for the growth and metastasis of solid tumors, and vascular endothelial growth factor (VEGF) is the most potent angiogenic factor. EGCG inhibited angiogenesis mainly by targeting VEGF signaling pathway[5-7]. Recent studies have shown that EGCG directly inhibits VEGF expression in multiple tumors[8-10]. We also demonstrated that EGCG reduced VEGF production in gastric cancer, and the inhibitory effect was at transcriptional level, suggesting that EGCG inhibited VEGF expression by reducing VEGF gene transcription[11]. However, the detailed molecular mechanism underlying the inhibitory effect of EGCG on VEGF expression is not fully understood.

VEGF expression associates with a variety of cytokines, growth factors, transcription factors, and oncoproteins, such as interleukin-6 (IL-6)[12,13]. Significantly, many of these molecules transmit signals through signal transducer and activator of transcription 3 (Stat3), a member of Janus-activated kinase (JAK)/STAT signaling pathway[12-15]. Activation by phosphorylation of tyrosine residue is required for the activity of Stat3, which is normally a transient and tightly regulated process. Once activated, Stat3 translocates into nucleus, binds to specific DNA promoter sequence and induces downstream gene expression[15]. Aberrant activation of Stat3 is found in a variety of tumors and contributes to oncogenesis by enhancing proliferation and preventing apoptosis[16,17]. Recent studies showed that abnormal Stat3 activation directly promoted VEGF expression and angiogenesis, and blockage of Stat3 activation inhibited these effects[18-20]. Abnormal Stat3 activation is also found in various gastric cancer cell lines and specimens, and associated with tumor status[21-23]. Phosphorylated Stat3 expression is significantly correlated with VEGF expression and microvessel density in gastric cancer, and is an independently prognostic factor of poor survival[22,23]. Blockade of Stat3 activation induced cell apoptosis and growth inhibition in gastric cancer[21,22]. Furthermore, gastric cancer cells transfected with dominant-negative Stat3 exhibits a decreased VEGF expression and less angiogenic phenotype[23], suggesting that blockade of Stat3 activation could inhibit VEGF expression and angiogenesis in gastric cancer. Our recent study showed that EGCG inhibited Stat3 activation and VEGF expression in gastric cancer[11]. Previous studies also demonstrated that EGCG inhibited activation of Stat3 in multiple tumor cells[9,24,25]. However, to our knowledge, whether EGCG inhibits VEGF expression and angiogenesis *via* Stat3 remains to elucidate.

An etiologic relation between high risk of gastric cancer and chronic gastritis with *Helicobacter pylori* has been firmly established[26]. Consequently, various cytokines have been implicated in the pathogenesis of gastric cancer. As a multifunctional cytokine, IL-6 has received particular attention. IL-6 promotes tumor growth and metastasis by up-regulating VEGF expression and VEGF-mediated angiogenesis, and is closely associated with disease status and outcome of gastric cancer[27,28]. Recent studies demonstrated that IL-6 induced VEGF expression and angiogenesis *via* Stat3 in multiple tumors[29-31] and gastric cancer[32]. Blocking Stat3 signaling pathway down-regulated VEGF promoter activity, and effectively abolished IL-6-induced VEGF expression and angiogenesis[33,34]. Therefore, this study was designed to demonstrate that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer in an attempt to further understand the molecular mechanism underlying the anti-angiogenic activity of EGCG.

MATERIALS AND METHODS

Cell culture

Human gastric cancer (AGS) cells (Cell Bank of Sun Yet-San University, Guangzhou, China) were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), (Gibco BRL, Gaithersburg, MD) and incubated at 37℃ in a humidified incubator at 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were prepared from fresh human umbilical cord obtained from the Department of Obstetrics and Gynecology, First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China as described previously[11], and grown in human endothelial-serum free medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100U penicillin, streptomycin and fungizone, and incubated at 37℃ in a humidified incubator at 5% CO_2. To maintain a uniform condition, all experiments were carried out between cell passages 4-6.

Western blotting

After serum starvation for 24 h, AGS cells (5×10^5 cells/well)

seeded in 90 mm plates were stimulated with IL-6 (50 ng/mL, R&D systems, Minneapolis, Minn., USA) in the presence of EGCG (Sigma-Aldrich Chemical Co., St Louis, MO, USA) at concentrations indicated for another 24 h to determine the VEGF protein level or for 1 h to determine the Stat3 protein level. Total protein was extracted from the cell lysates with mammalian cell lysis kit (Bio Basic Inc., Ontario, Canada). Protein level was quantified with Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Total protein (100 μg) was separated in 12% sodium dodecyl sulfate (SDS)-PAGE, and transferred onto PVDF membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk and incubated at 4℃ overnight with a rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit polyclonal anti-Stat3 antibody (Santa Cruz), or a goat polyclonal anti-p-Stat3 antibody [Phospho-Stat3 (tyr-705); Santa Cruz]. After being washed with 0.1% Tween 20 in Tris-saline three times, the membrane was incubated with biotin-labeled anti-rabbit or anti-goat IgG for 1 h at room temperature with agitation. The probe proteins were detected using enhanced chemiluminescence system (Amersham International, Piscataway, NJ, USA). The same membrane was stripped and re-blotted with an antibody specific to β-actin (Santa Cruz). Protein expression levels were normalized by β-actin.

Enzyme-linked immunosorbent assay

AGS cells were seeded in 90 mm plates at 5×10^5 cells per well and stimulated with IL-6 (50 ng/mL) for another 24 h in the presence of EGCG at concentrations indicated after serum starvation for 24 h. The conditioned media were harvested and centrifuged. VEGF concentrations in the supernatant were measured using VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D systems).

Reverse transcription polymerase chain reaction

AGS cells (5×10^5 cells/well) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 12 h in the presence of EGCG at concentrations indicated. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The levels of human VEGF mRNA were evaluated by reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription was performed with 1 μg total RNA and 100 pmol random hexamers in a total volume of 20 μL to produce first-strand cDNA. PCR experiments were performed with 1 μL of the first-strand cDNA in a 50 μL reaction mixture. Human VEGF cDNA was amplified with specific primers (sense primer, 5'-TGCATTCACATTTGTTGTGC-3'; antisense primer, 5'-AGACCCTGGTGGACATCTTC-3'; a 200 bp product) and β-actin specific primers (sense primer, 5'-TCATCACCATTGGCAATGAG-3'; antisense primer, 5'-CACTGTGTTGGCGTACAGGT-3'; a 150 bp product). Amplification protocol was as follows: denaturation at 94℃ for 1 min, annealing at 60℃ (for β-actin, 55℃) for 1 min, and extension at 72℃ for 1 min. All PCRs were linear up to 30 cycles. The PCR products were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and quantified by densitometry using the Image Master VDS system and associated software (Pfizer, NY, USA).

Signaling inhibitors blocking the VEGF induction by IL-6

AGS cells were seeded into a 90 mm plate at 5×10^5 cells per well. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for another 24 h in the presence or absence of 50 μmol EGCG or signaling inhibitors: 20 μmol AG490 (Calbiochem, La Jolla, Calif., USA), 25 μmol PD98059 (Sigma) or 25 μmol LY294002 (Sigma). AG490 is a JAK2 inhibitor, PD98059 is a MAPK/ERK kinase (MEK) inhibitor, and LY294002 is a phosphatidylinositol-3-kinase (PI3K) inhibitor. Total proteins were extracted from the cell lysates and subjected to Western blotting analysis for VEGF expression.

Stat3 nuclear translocation

AGS cells (5×10^5 cells/well) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 1 h in the presence or absence of 50 μmol EGCG. The cells were washed with cold phosphate buffered saline and collected with a policeman cell scraper. The cells were suspended in a hypotonic buffer (10 mmol HEPES, 2 mmol $MgCl_2$, 10 mmol KCl, 0.1 mmol EDTA, 1 mmol DTT, 0.5 mmol phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40) and incubated on ice for 10 min. The cell lysates were then centrifuged at 15000 × *g* for 5 min and the pellets were resuspended in a high salt buffer (50 mmol HEPES, 300 mmol NaCl, 50 mmol KCl, 0.1 mmol EDTA, 1 mmol DTT, 0.5 mmol phenylmethylsulfonyl fluoride and 10% glycerol), and then incubated with rotation for 30 min at 4℃. Lysates were centrifuged at 15000 × *g* at 4℃ for 30 min. The supernatant was collected as a nuclear fraction and used for Stat3 nuclear translocation assay with an anti-p-Stat3 antibody, Phospho-Stat3 (tyr-705; Santa Cruz) by Western blotting.

Chromatin immunoprecipitation assay

AGS cells (1×10^7 cells) were seeded in 90 mm plates. After serum starvation for 24 h, the cells were stimulated with IL-6 (50 ng/mL) for 6 h in the presence or absence of 50 μmol EGCG. Chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described[35]. Briefly, cells were cross-linked using 1% formaldehyde at room temperature for 10 min. After sonication, the soluble chromatin was diluted 10-fold with ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mmol EDTA; 16.7 mmol Tris-HCl, pH 8.1; 167 mmol NaCl), and precleared with Protein A beads blocked with 1% salmon sperm DNA and 1% BSA. The precleared chromatin solution was immunoprecipitated by anti-p-Stat3 antibody (Santa Cruz) overnight at 4℃ with rotation. The immunoprecipitates were then pelleted, washed, and the antibody/protein/DNA complex was eluted off the beads by resuspending the pellets in 50 mmol $NaHCO_3$ and 1% SDS for 30 min. Cross-linking was reversed, and protein and RNA were removed by

adding 10 μg Proteinase K and 10 μg RNase A, followed by incubation at 42℃ for 3 h. Purified DNA was subjected to PCR with primers for VEGF promoter as follows: forward: 5'-AGACTCCACAGTGCATACGTG-3' and reverse: 5'-AGTGTGTCCCTCTGACAATG-3', which amplify 235 bp fragments flanking the Stat3 binding element. The final products were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and quantified by densitometry.

HUVECs proliferation and tube formation assay for in vitro angiogenesis

AGS cells seeded in 90 mm plates at 5×10^5 cells per well were stimulated with 50 ng/mL IL-6 for another 24 h in the presence or absence of 50 μmol EGCG or 20 μmol AG490 after serum starvation for 24 h. The conditioned media were generated from the supernatants centrifuged using Amicron® Ultra-15 Centrifugal Filter Devices (Millipore Filter, Bedford, Mass., USA) at 4000 × *g* for 15 min.

For proliferation assay, HUVECs were seeded in 96-well plates pre-coated with 1% gelatin at 5×10^3 cells per well and cultured with 100 μL conditioned media supplemented with 2% FBS in the presence or absence of 10 ng/mL VEGF neutralizing antibody. After cultured for 48 h, the viable cells were quantified by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide assay.

For tube formation assay, Matrigel was thawed at 4℃ in an ice-water bath. Matrigel (300 μL per well) was carefully added to a pre-chilled 24-well plate using a cold pipette and allowed to polymerize for 1h at 37℃. After polymerization, HUVECs (5×10^4 cells/well) in the conditioned media supplemented with 2% serum in the presence or absence of 10 μg/mL VEGF neutralizing antibody were layered on the top of the polymerized gel. Cells were incubated for 48 h at 37℃ in a humidified incubator at 5% CO_2, and the formed tubes were fixed with 10% buffered neutral formalin, stained with Diff-Quick Solution Ⅱ (Baster, McGraw Park, IL), and photographed (100 ×). For quantification of tube formation, the total length of tubes formed in a unit area was measured using national institute of health image program.

Matrigel plug assay for in vivo angiogenesis

Matrigel plug assay was performed as described previously with some modifications[36]. Briefly, 6-8-wk female BALB/c nude mice, weighing 18-22 g (Experimental Animal Center of Sun Yet-san University, Guangzhou, China) were subcutaneously injected with 0.5 mL of a liquid mixture composed of Matrigel (350 μL, 10mg/mL) and the conditioned media (150 μL) prepared as described above with or without 10 μg/mL of VEGF neutralizing antibody near the abdominal midline. The Matrigel was quickly polymerized *in vivo* to form a single and solid gel plug, which allowed the angiogenic factors to release and stimulate angiogenesis. One week later, the Matrigel plugs were harvested, weighed, and then minced and digested in 5 mL Drabkin reagent (Drabkin reagent kit 525; Sigma) for hemoglobin content measurement. Final hemoglobin concentration was calculated from a standard calibration curve.

Statistical analysis

All data were presented as means ± SE. Statistical significance was calculated using unpaired Student's *t* test. A *P* value less than 0.05 was considered statistically significant. All analyses were performed using SPSS version 13.0 (SPSS Inc, USA).

RESULTS

EGCG inhibits VEGF induction by IL-6 in AGS cells

To assess the effect of EGCG on VEGF expression induced by IL-6 in human gastric cancer, we first examined VEGF expression induced by IL-6 in AGS cells treated with EGCG. AGS cells were treated with EGCG at different concentrations and stimulated with IL-6 (50 ng/mL) for 24 h. VEGF protein levels in tumor cell lysates were analyzed by Western blotting. As shown in Figure 1A, there was a basal expression of VEGF in AGS cells. After stimulation with IL-6, VEGF expression was apparently up-regulated and a 2.4-fold increase was observed. When treated with EGCG, VEGF expressions were dose-dependently decreased. This inhibitory effect was not due to the toxic effect of IL-6, because IL-6 at the concentration less than 100 ng/mL did not cause growth inhibition of AGS cells within 24 h (data not shown).

VEGF secretion is a crucial step for tumor-induced angiogenesis, so we further evaluated the effect of EGCG on VEGF secretion induced by IL-6. VEGF protein levels in the conditioned medium were measured by ELISA. IL-6 also induced VEGF secretion in AGS cells and a 2.8-fold increase was observed. Consistent with the result of Western blotting analysis, the secreted proteins of VEGF induced by IL-6 in the conditioned media were reduced by EGCG in a dose-dependent manner (Figure 1B). These data provided direct evidence that EGCG inhibited VEGF production induced by IL-6 in gastric cancer cells.

EGCG inhibits IL-6-induced VEGF expression at transcription level

To determine whether the inhibitory effect of EGCG on IL-6-induced VEGF expression was at transcriptional level in gastric cancer, we examined VEGF mRNA expression in AGS cells by RT-PCR. We found that IL-6 induced VEGF mRNA expression in AGS cells. When stimulated with IL-6 (50 ng/mL) for 12 h, a 3.1-fold increase in VEGF mRNA expression was observed. Treated with EGCG, VEGF mRNA expressions were dose-dependently reduced (Figure 1C). These findings suggested that EGCG inhibited VEGF expression induced by IL-6 in gastric cancer cells at transcriptional level.

EGCG inhibits IL-6-induced VEGF expression *via* Stat3 pathway

IL-6 is known to signal through Stat3, MAPK and PI3K in gastric cancer[32]. To elucidate the signaling pathway that

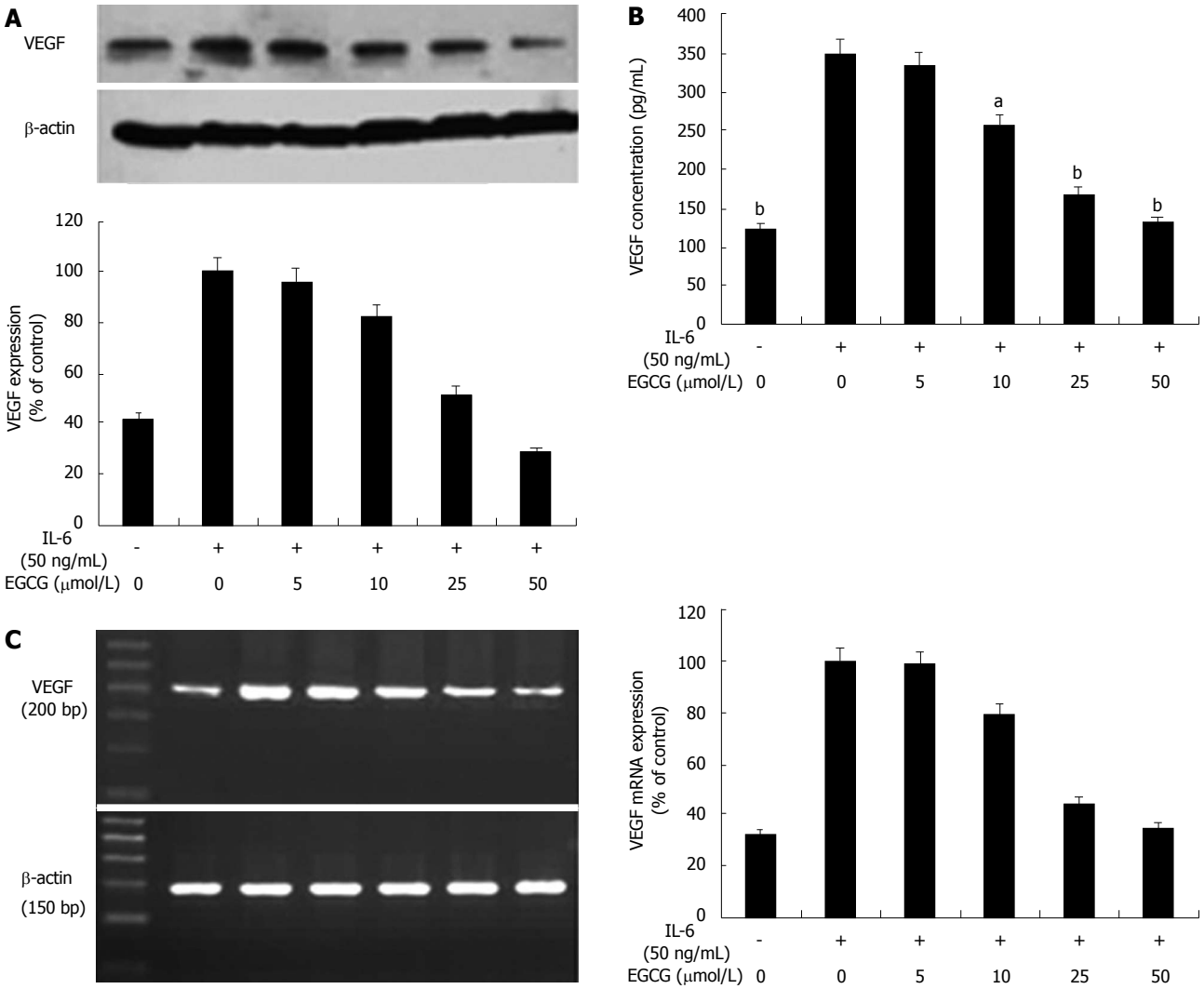

Figure 1 (-)-Epigallocatechin-3-gallate inhibits vascular endothelial growth factor production induced by interleukin-6 in human gastric cancer cells. A: When treated with (-)-Epigallocatechin-3-gallate (EGCG), vascular endothelial growth factor (VEGF) expression was dose-dependently decreased; B: Enzyme-linked immunosorbent assay showed that interleukin-6 (IL-6) induced VEGF secretion in human gastric cancer (AGS) cells and a 2.8-fold increase was observed. EGCG treatment dose-dependently reduced VEGF protein level in the conditioned medium. C: IL-6 also induced VEGF mRNA expression and a 3.1-fold increase in VEGF mRNA expression was observed. When treated with EGCG, VEGF mRNA expression was dose-dependently decreased. Values are expressed as percent of control (means ± SE, $n = 3$, $^{a}P < 0.05$, $^{b}P < 0.01$).

EGCG inhibited VEGF expression in gastric cancer, we tested the effect of several signaling pathway inhibitors, including JAK/STAT, MAPK and PI3K signaling pathway. IL-6 markedly increased VEGF expression in AGS cells. When treated with 50 μmol EGCG or 20 μmol AG490, VEGF expressions were reduced to near the basal level or even lower. However, the other two groups treated with PD98059 or LY294002 still exhibited enhanced VEGF expression, approximating the level of that stimulated with IL-6 (Figure 2). Because PI3K, MEK/ERK, and Stat3 activities could all be suppressed by EGCG, we further analyzed the combined effect of EGCG and the specific inhibitors on IL-6-induced VEGF expression. We found that treatment with 50 μmol EGCG or 20 μmol AG490 alone apparently inhibited IL-6-induced VEGF expression. However, EGCG treatment combined with AG490 did not suppress VEGF expression, and EGCG or AG490 treatment combined with PD98059 or LY294002 did not suppress VEGF expression either (data not shown). These data suggested that EGCG reduced VEGF expression induced by IL-6 *via* Stat3 signaling pathway in gastric cancer.

EGCG reduces IL-6-induced Stat3 activation in AGS cells

We previously demonstrated that EGCG inhibited Stat3 activation without changing Stat3 expression in gastric cancer[11]. In this study, we further assessed the effect of EGCG on Stat3 expression and activation induced by IL-6 in gastric cancer. AGS cells were treated with or without EGCG at different concentrations and stimulated with 50 ng/mL IL-6 for 1 h. Total Stat3 and phospho-Stat3 protein levels were examined using Western blotting with anti-Stat3 antibody to detect total Stat3 protein expression, and with anti-p-Stat3 antibody (specific for tyr-705) to detect phospho-Stat3, respectively. As shown in Figure 3, Stat3 was constitutively activated in AGS cells. When stimulated

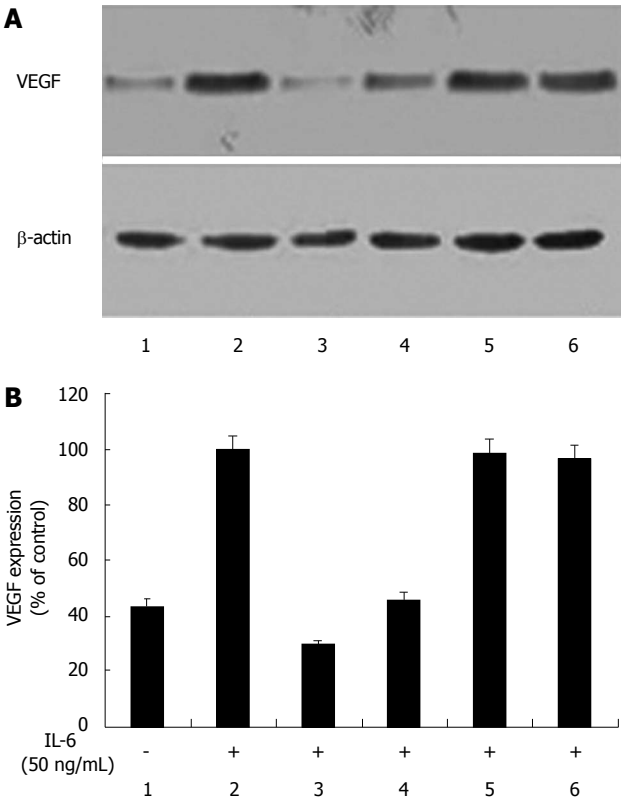

Figure 2 (-)-Epigallocatechin-3-gallate inhibits interleukin-6-induced vascular endothelial growth factor expression in human gastric cancer cells *via* JAK/STAT pathway. Human gastric cancer (AGS) cells were stimulated with interleukin-6 (IL-6) (50 ng/mL) for 24 h in the presence of 50 μmol (-)-epigallocatechin-3-gallate (EGCG) or signaling inhibitors. Vascular endothelial growth factor (VEGF) protein levels in tumor cell lysates were analyzed by Western blotting. IL-6 markedly increased VEGF expression in AGS cells. When treated with EGCG or AG490, VEGF expression was significantly reduced. PD98059 and LY294002 did not change IL-6-induced VEGF expression. 1: Without IL-6 stimulation; 2: Stimulated with IL-6; 3-6: Treated with 50 μmol EGCG or signaling inhibitors of 20 μmol AG490, 25 μmol PD98059 or 25 μmol LY294002.

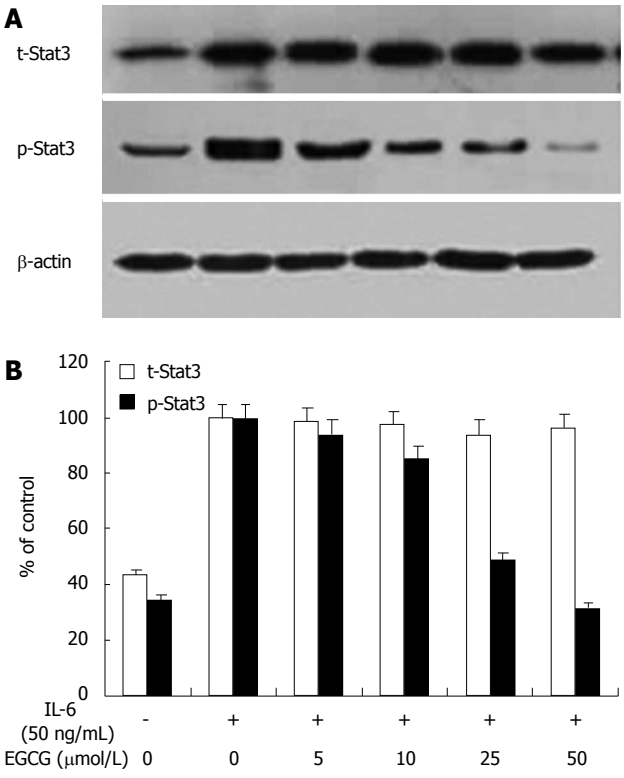

Figure 3 (-)-Epigallocatechin-3-gallate inhibits signal transducer and activator of transcription 3 activation induced by interleukin-6 in human gastric cancer cells. Human gastric cancer (AGS) cells were stimulated with interleukin-6 (IL-6) (50 ng/mL) in the presence of (-)-epigallocatechin-3-gallate (EGCG) at concentrations indicated for 1 h. Total signal transducer and activator of transcription 3 (Stat3) and activated Stat3 were examined by Western blotting. Stat3 was constitutively activated in AGS cells. After stimulated with IL-6, total Stat3 and activated Stat3 expression were both markedly increased by 2.3 folds and 2.9 folds, respectively. When treated with EGCG, activated Stat3 expression decreased in a dose-dependant manner, but total Stat3 expression remained unchanged.

with IL-6 (50 ng/mL) for 1 h, phosphorylation of Stat3 at tyrosine 705 increased by 2.9 folds and the total Stat3 expression increased by 2.3 folds. EGCG treatment inhibited IL-6-induced activation of Stat3 in a dose-dependant manner, without affecting total Stat3 expression. These findings suggested that EGCG reduced IL-6-induced VEGF expression in gastric cancer by inhibiting Stat3 activation instead of Stat3 expression.

EGCG inhibits Stat3 nuclear translocation and DNA binding activity

Our results showed that EGCG inhibited Stat3 activation and reduced VEGF expression at transcriptional level in gastric cancer. Activated Stat3 acts as a transcription activator, and is capable of translocating into nucleus, binding to the Stat3 consensus sequence in VEGF promoter region, thereby up-regulating VEGF expression[15]. To clarify whether EGCG affected Stat3 nuclear translocation and DNA binding activity, we first performed Western blotting with extraction of nuclear protein to visualize the nuclear translocation of phospho-Stat3 after IL-6 stimulation. As shown in Figure 4A, before IL-6 stimulation, less phospho-Stat3 was localized in the nucleus. Once stimulated with IL-6 for 1 h, phospho-Stat3 was apparently increased and translocated into the nucleus. After EGCG treatment, phospho-Stat3 in the nucleus was markedly decreased.

To further evaluate the Stat3-DNA binding activity, ChIP assay was performed. Immunoprecipitation was conducted with an anti-p-Stat3 antibody followed by PCR using oligonucleotide primers that amplified a 235 bp region spanning Stat3 binding site in VEGF promoter. IL-6 apparently increased this band in AGS cells, suggesting that IL-6 up-regulated VEGF expression by promoting Stat3 binding to VEGF promoter and activating VEGF transcription. When treated with EGCG, Stat3-DNA binding activity was markedly decreased (Figure 4B). Taken together, these data provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 through inhibiting Stat3 translocating into nucleus and binding to VEGF promoter in gastric cancer.

EGCG inhibits IL-6-induced angiogenesis in vitro

We further evaluated the effect of EGCG on IL-6-induced angiogenesis *in vitro* by assessing proliferation and

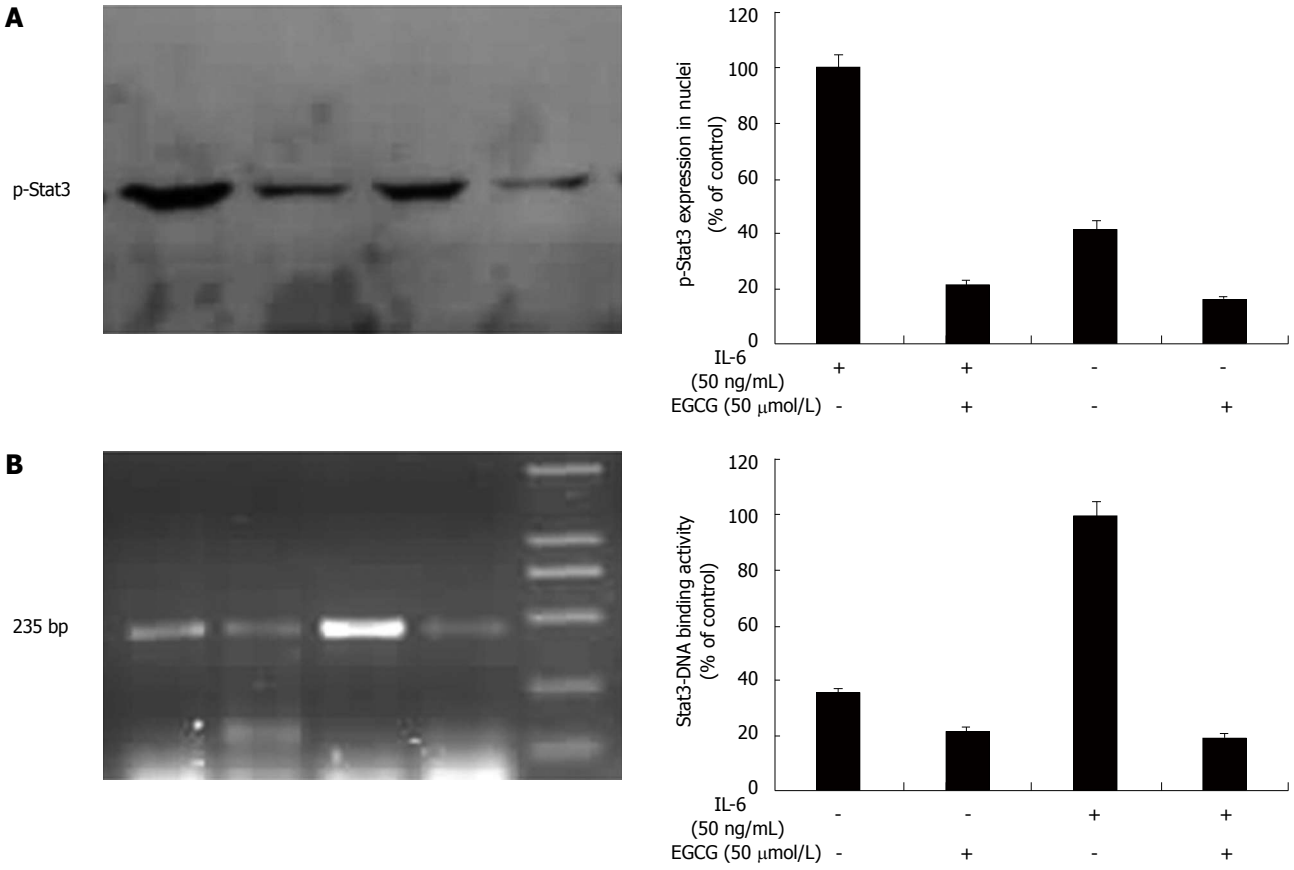

Figure 4 (-)-Epigallocatechin-3-gallate inhibits signal transducer and activator of transcription 3 nuclear translocation and DNA binding activity in human gastric cancer cells. Signal transducer and activator of transcription 3 (Stat3) nuclear translocation was determined by Western blotting with extraction of nuclear proteins. A: After treated with 50 μmol (-)-epigallocatechin-3-gallate (EGCG) and interleukin-6 (IL-6) (50 ng/mL) for 1 h, phospho-Stat3 in the nucleus was visualized with an anti-p-Stat3 antibody; B: IL-6 apparently increased phospho-Stat3 expression in the nucleus, but EGCG treatment markedly decreased this effect. Stat3-DNA binding activity was determined by chromatin immunoprecipitation (ChIP) assay. Immunoprecipitation was conducted with Stat3 antibody followed by polymerase chain reaction (PCR) using oligonucleotide primers that yielded a 235 bp band spanning Stat3 binding site in vascular endothelial growth factor promoter. IL-6 apparently increased Stat3-DNA binding activity. When treated with EGCG, Stat3-DNA binding activity was also markedly decreased.

tube formation of HUVECs cultured in the conditioned media. As shown in Figure 5A and B, the conditional media stimulated with IL-6 promoted HUVECs proliferation and tube formation when compared with the non-stimulated cell culture media. VEGF neutralizing antibody effectively blocked the enhancement of proliferation and tube formation of HUVECs cultured in the IL-6-stimulated conditional media, suggesting that IL-6 increased the angiogenic ability of AGS cells through up-regulating VEGF induction. Treatment with EGCG or AG490 also abrogated the effect of IL-6 on HUVECs cell proliferation and tube formation. These data further confirmed that EGCG inhibited angiogenesis induced by IL-6 through targeting Stat3/VEGF signaling pathway.

EGCG inhibits IL-6-induced angiogenesis in vivo

The effect of EGCG on angiogenesis induced by IL-6 *in vivo* was assessed using Matrigel plug assay. Matrigel plugs were harvested on the 7th d and examined by measuring the density of hemoglobin, as an indicator of vascularization. Hemoglobin concentrations were determined and normalized to represent the vascular densities in the plug. Grossly, the Matrigel plugs embedded with the conditioned media from IL-6-stimulated AGS cells developed substantial vasculature, as compared with the non-stimulated media. However, plugs that contained the conditioned media treated with VEGF neutralizing antibody, EGCG and AG490 exhibited considerably less vascularization (Figure 5C). Collectively, Matrigel plug assay demonstrated that IL-6 markedly potentiated AGS cells to enhance angiogenesis *in vivo* by inducing VEGF production, and EGCG abolished the pro-angiogenesis ability of AGS cells induced by IL-6 through inhibiting VEGF induction *via* Stat3.

DISCUSSION

Both VEGF over-expression and Stat3 over-activation occur at high frequency in human tumors[12-23], and IL-6 has shown to induce VEGF expression and angiogenesis by promoting Stat3 activity[29-31]. In gastric cancer, IL-6 induces VEGF expression *via* Stat3 signaling pathway[32], and is associated with tumor angiogenesis and disease status[27,28]. Previous studies have indicated that EGCG inhibits VEGF expression and Stat3 activation in multiple cancers[9,11,24,25]. However, whether EGCG inhibits

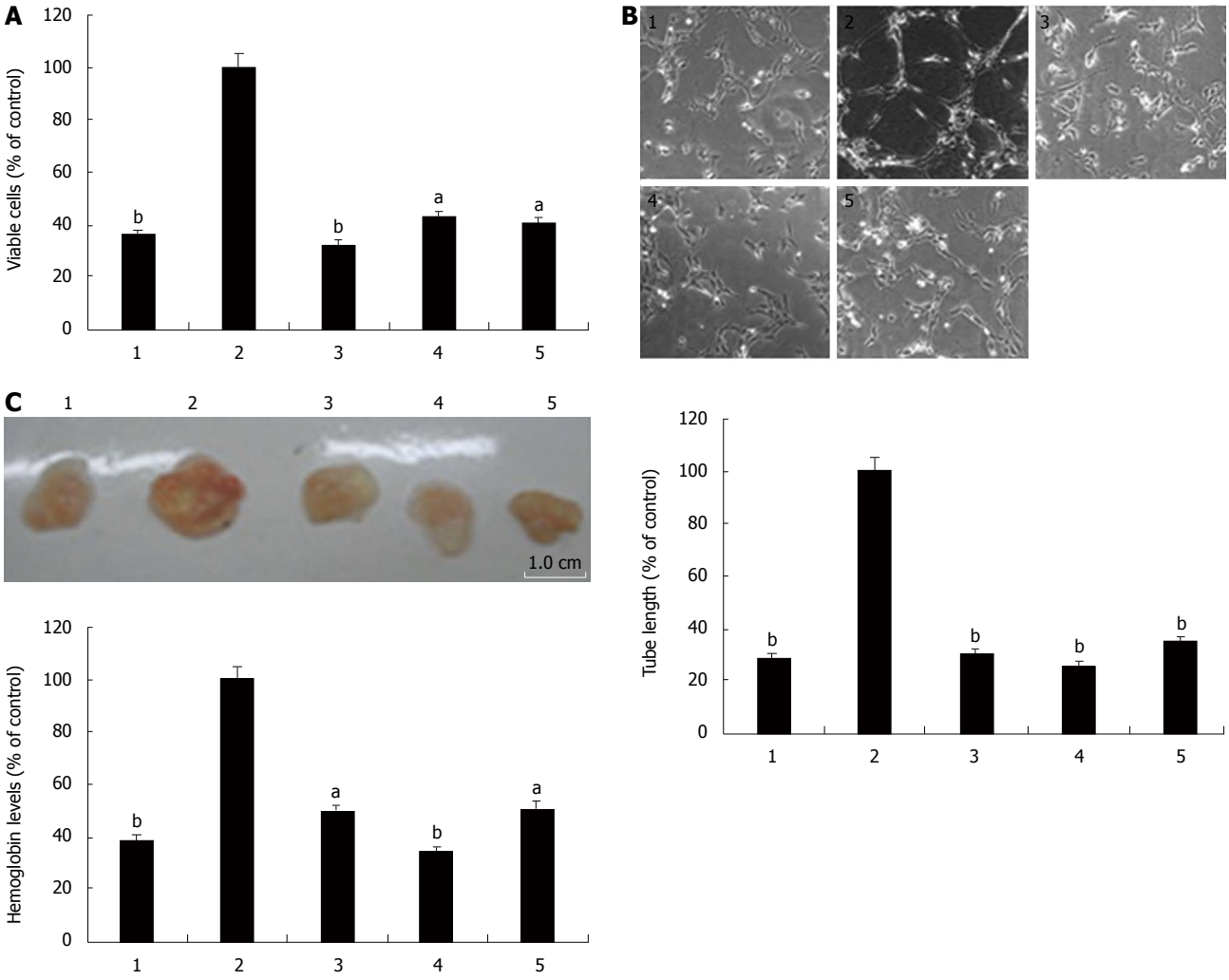

Figure 5 (-)-Epigallocatechin-3-gallate inhibits interleukin-6 induced angiogenesis *in vitro* and in *vivo*. The *in vitro* angiogenesis was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) and tube formation assay. Human umbilical vein endothelial cells (HUVECs) were cultured with the conditioned media in the presence of vascular endothelial growth factor (VEGF) neutralizing antibody, (-)-epigallocatechin-3-gallate (EGCG) or AG490. After 48 h incubation, the viable cells were quantified by MTT assay (A), and the formed tubes were fixed, stained, photographed and analyzed (B). The *in vivo* angiogenesis was determined by Matrigel plug assay. Matrigel plugs containing the conditioned media with VEGF neutralizing antibody, EGCG or AG490 were subcutaneously injected into nude mice. One week later, the Matrigel plugs were harvested and examined by measuring the density of hemoglobin (C). interleukin-6 (IL-6) apparently promoted vascular endothelial cell proliferation and tube formation *in vitro* and vascularization of Matrigel plugs *in vivo*. VEGF neutralizing antibody, EGCG and AG490 all markedly decreased these effects. 1: Without IL-6 stimulation as a negative control; 2: With IL-6 stimulation as a control; 3-5: With IL-6 stimulation and VEGF neutralizing antibody, EGCG or AG490. Values are expressed as percent of control (means ± SE, $n = 3$, $^{a}P < 0.05$, $^{b}P < 0.01$).

VEGF expression and angiogenesis *via* Stat3 remains to be elucidated. In this study, we demonstrated that EGCG inhibited IL-6-induced VEGF expression and angiogenesis in gastric cancer *via* suppressing Stat3 activity.

EGCG has shown to inhibit VEGF expression and angiogenesis in a variety of tumors[8-11], and is most effective in inhibiting VEGF expression and angiogenesis among the four main catechins of green tea[7]. However, the exact mechanism for the inhibitory effect of EGCG on VEGF expression and angiogenesis is not well understood. IL-6 is reported to induce VEGF expression and tumor vasculature in gastric cancer[27,28]. To evaluate the effect of EGCG on VEGF expression induced by IL-6 in gastric cancer, we examined VEGF expression in AGS cells treated with EGCG and IL-6. As shown in Figure 1A and B, after stimulated with 50 ng/mL of IL-6, a 2.4-fold increase of VEGF protein in tumor cells and a 2.8-fold increase in the conditioned media were observed. When treated with EGCG, VEGF expression and secretion were decreased in a dose-dependent manner. EGCG also dose-dependently inhibited VEGF mRNA expression in AGS cells (Figure 1C). Here we demonstrated that EGCG inhibited IL-6-induced VEGF expression in gastric cancer cells, and this inhibitory effect was at transcriptional level.

The important role of VEGF in angiogenesis has been well established. Therefore, we further evaluated the effect of EGCG on IL-6-induced angiogenesis both *in vitro* and *in vivo*. As shown in Figure 5, the conditioned media promoted vascular endothelial cell proliferation and tube formation *in vitro* and vascularization of Matrigel plugs *in vivo*. VEGF neutralizing antibody effectively

blocked these effects, confirming that IL-6-induced angiogenesis is VEGF-dependent. EGCG treatment also abrogated IL-6-induced angiogenesis *in vitro* and *in vivo*. These findings suggested that EGCG inhibited IL-6-induced angiogenesis *via* down-regulation of VEGF production in gastric cancer.

Several mechanisms have been proposed for the inhibitory effect of EGCG on VEGF expression[3-11]. In the present study, we found that EGCG inhibited IL-6-induced VEGF expression in AGS cells. IL-6 might act through several classic protein kinase cascades, such as MAPK, PI3K and Stat3[31,32]. To elucidate the signaling pathway that EGCG inhibited VEGF expression induced by IL-6 in AGS cells, we tested the effect of several signaling pathway inhibitors, and found that PD98059 and LY294002 did not affect IL-6-induced VEGF expression, suggesting that IL-6 did not induce VEGF expression in AGS cells through MAPK or PI3K signaling pathway, which was consistent with a previous study[32]. In contrast, EGCG and AG490 effectively inhibited VEGF expression induced by IL-6. In addition, EGCG and AG490 also effectively inhibited IL-6-induced vascular endothelial cell growth and tube formation *in vitro* and vascularization of Matrigel plug *in vivo*, indicating that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* Stat3 signaling pathway. A previous study also demonstrated that Stat3 pathway was predominantly involved in the signaling of IL-6 stimulation in VEGF expression in gastric cancer[32]. These data suggested that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* Stat3 signaling pathway in gastric cancer, and provided a novel mechanistic insight into the effect of EGCG on VEGF expression.

Activation by tyrosine phosphorylation is an indispensable prerequisite for the activity of Stat3. Compared with normal cells and tissues, abnormally activated Stat3 has been detected in a wide variety of human cancer cells and tissues, and associate with VEGF expression and tumor angiogenesis[14-23]. Previous studies have shown that EGCG inhibits activation of Stat3 in various cancer cells[9,24,25]. Our study also demonstrated that EGCG inhibited activation of Stat3 and VEGF expression in gastric cancer[11]. In this study, we found that Stat3 was constitutively activated in AGC cells. IL-6 induced a remarkable increase in Stat3 expression and activation. When treated with EGCG, Stat3 activation was inhibited in a dose-dependent manner, but the total Stat3 expression remained unchanged when compared with the control. EGCG treatment did not affect Stat3 mRNA expression, either (data not shown). These findings suggested that EGCG reduced VEGF expression in gastric cancer by suppressing Stat3 activation.

Once activated, Stat3 translocates into the nucleus, binds to specific DNA promoter sequence and induces downstream gene expression[15]. Stat3-binding site in VEGF promoter has been identified, providing evidence that VEGF is a direct target gene of Stat3. The activated Stat3 acts as a transcriptional activator and is capable of binding directly to the Stat3 consensus sequence in VEGF promoter region, thereby promoting the VEGF expression[18-20]. Previous studies showed that IL-6 up-regulated VEGF expression by promoting Stat3 binding to VEGF promoter[29-31]. Furthermore, blockage of Stat3 activation was associated with a decline in Stat3-DNA binding activity and VEGF mRNA expression in gastric cancer[32]. In this study, we found that IL-6 stimulation apparently increased Stat3 translocation into nucleus and Stat3-DNA binding activity. When treated with EGCG, Stat3 nuclear translocation and Stat3-DNA binding activity was markedly decreased. Masuda *et al*[9] also found that inhibition of Stat3 by EGCG significantly decreased VEGF promoter activity. Taken together, these data provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity.

Increasing evidences have suggested that cytokine/Stat3 signaling pathway plays an important role in tumor development and progression. Stat3 represents a point of convergence for these cytokine signaling pathways and has become a novel promising molecular target for intervention in cancer treatment. Great efforts have been made to disrupt Stat3 signaling pathway for inhibiting angiogenesis and tumor growth[37]. However, the toxicity with these approaches might be increased because multiple down-stream targets are affected. Recently, pharmacological approaches to Stat3 inhibition aim to identify natural products as inhibitors of Stat3 signaling pathway[38,39]. In this study, we demonstrated that EGCG inhibited IL-6-induced VEGF expression and angiogenesis in gastric cancer by the suppression of Stat3 activation, nuclear translocation and Stat3-DNA binding activity. As a natural, low cost and non-toxic product, EGCG has drawn special attention, and may become a promising inhibitor of Stat3 and an angiogenic inhibitor.

COMMENTS

Background

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and active component of green tea, has shown to have chemopreventive and chemotherapeutic properties for a variety of cancers, especially gastrointestinal cancers. Anti-angiogenic activity is one of its main effects against cancer. However, the detailed molecular mechanism is not fully understood.

Research frontiers

Angiogenesis is necessary for solid tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor, and EGCG has shown to inhibit angiogenesis and tumor growth *via* suppressing VEGF expression. However, the molecular mechanism remains unclear.

Innovations and breakthroughs

Increasing evidences suggest that cytokine/Stat3 signaling pathway plays an important role in tumor development and progression. Signal transducer and activator of transcription 3 (Stat3) represents a point of convergence for cytokine signaling pathways and has become a novel promising molecular target for intervention in cancer therapy. In this study, the authors demonstrate that EGCG inhibited IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity in gastric cancer, and provided further evidence of the molecular mechanism underlying the anti-angiogenic activity of EGCG.

Applications

By understanding how EGCG inhibits IL-6-induced VEGF expression and

angiogenesis *via* suppressing Stat3 activity, this study may represent a future strategy for therapeutic intervention in the treatment of gastric cancer.

Terminology

Stat3 represents a point of convergence for cytokine signaling pathways. IL-6 induces VEGF expression and angiogenesis *via* Stat3 in multiple tumors. Non-surprisingly, EGCG down-regulates VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity.

Peer review

The authors for the first time examined how EGCG inhibits IL-6-induced VEGF expression and angiogenesis *via* suppressing Stat3 activity. It provided direct evidence that EGCG down-regulated VEGF expression induced by IL-6 *via* suppressing Stat3 activation, nuclear translocation and Stat3-DNA binding activity. The results may represent a new molecular mechanism of anti-angiogenic activity of EGCG, and identified a new natural product as an inhibitor of Stat3 signaling pathway.

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ORIGINAL ARTICLE

LBP and CD14 polymorphisms correlate with increased colorectal carcinoma risk in Han Chinese

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Abstract

AIM: To explore the associations of polymorphisms of lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), toll-like receptor 4 (TLR-4), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) with the colorectal carcinoma (CRC) risk in Han Chinese.

METHODS: Polymorphisms of LBP (rs1739654, rs2232596, rs2232618), CD14 (rs77083413, rs4914), TLR-4 (rs5030719), IL-6 (rs13306435) and TNF-α (rs35131721) were genotyped in 479 cases of sporadic colorectal carcinoma and 486 healthy controls of Han Chinese in a case-control study. Single-nucleotide polymorphisms (SNPs) between cases and controls were analyzed by unconditional logistic regression.

RESULTS: GA and GG genotypes of LBP rs2232596 were associated with a significantly increased risk of CRC [odds ratio (OR) = 1.51, 95% confidence interval (CI) 1.15-1.99, *P* = 0.003; OR = 2.49, 95% CI 1.16-5.38, *P* = 0.016, respectively]. A similar association was also observed for the CG genotype of CD14 rs4914 (OR= 1.69, 95% CI 1.20-2.36, *P* = 0.002). In addition, a combination of polymorphisms in LBP rs2232596 and CD14 rs4914 led to a 3.4-fold increased risk of CRC (OR = 3.44, 95% CI 1.94-6.10, *P* = 0.000).

CONCLUSION: This study highlights the LBP rs2232596 and CD14 rs4914 polymorphisms as biomarkers for elevated CRC susceptibility in the Chinese Han population.

Key words: Colorectal carcinoma; Cluster of differentiation 14; Lipopolysaccharide binding protein; Single-nucleotide polymorphisms

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the major causes of cancer death throughout the world. In China, the incidence rate of newly diagnosed CRC cases is increasing rapidly[1]. Both environmental and genetic factors contribute to the tumorigenesis of CRC. The classical adenoma-carcinoma sequence model proposes that genetic mutations of K-ras, adenomatous polyposis coli (APC), the deleted in colorectal cancer (DCC), and p53 play important roles in the malignant transformation and cancer progression

of CRC[2]. Recent studies have demonstrated that chronic inflammation is also an important factor in the carcinogenesis of CRC[3,4]. In the tumor microenvironment, inflammatory cells, especially the so-called tumor-associated macrophages (TAMs), induce suppression of host antitumor activities, stimulate tumor cell growth, and promote malignant transformation, angiogenesis and metastasis[4-8].

TAMs are key regulatory components of cancer-related inflammation. TAMs mainly derive from monocytic precursors in blood circulation, and are recruited to the tumor sites by tumor-derived chemokines such as C-C motif ligand 2 (CCL2) as well as cytokines in the tumor microenvironment, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) and macrophage colony stimulating factor (M-CSF). These chemokines and cytokines also regulate the survival and differentiation of TAMs[7,9]. Further studies have demonstrated that TAMs are defective in IFN-γ/lipopolysaccharide (LPS) responsiveness to bacterial invasion[10,11]. In solid tumors, TAMs are reprogrammed to have pro-tumor properties and therefore fail to respond to LPS stimulation that should have killed the cancer cells[12]. Whether LPS-induced signaling pathways play important roles in tumor progression warrants further investigation.

Lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), and toll-like receptor 4 (TLR-4) are pattern-recognition receptors (PRRs) that mediate innate immune response to LPS challenge[13,14]. LBP is a secretory class I acute phase protein, which can drastically increase LPS-induced activation of immune cells by binding with LPS and transferring it to CD14. CD14 is a glycosylphosphatidylinositol (GPI)-linked LPS receptor which exists as either membrane-bound forms on the surface of immune cells or soluble forms in the serum. TLR4 belongs to a family of innate immune receptors expressed on the surface of monocytes and macrophages, recognizing pathogen-associated molecular patterns (PAMPs) such as LPS. In the LPS signaling pathway, TLR4 can specifically recognize LPS with the aid of LBP, CD14 and MD-2 molecular complex and activate macrophages in response to LPS-induced inflammation.

Genetic variations of inflammatory factor genes are correlated with increased risk in several malignant tumors. Previous studies have demonstrated a strong association between IL-1beta, IL-8 polymorphisms and gastric carcinoma[15,16], IL-6 polymorphism and cervical carcinoma[17], IL-8, IL-10, TLR4 polymorphisms and prostate carcinoma[18,19], TNF-α polymorphism and non-small cell lung cancer[20]. However, the association between polymorphisms of LPS-signaling-related genes and CRC susceptibility in the Chinese Han population remains elusive. In this study, we directly addressed this issue and investigated the association between polymorphisms of LBP, CD14, TLR4, IL-6 and TNF-α genes and the CRC risk in a case-control study.

MATERIALS AND METHODS

Study population

All subjects were genetically unrelated Chinese Han people living in the southwest region of China. The characteristics of CRC cancer patients and controls included in this study are summarized in Table 1. Patients were chosen from Chongqing Xinqiao Hospital, the second affiliated hospital of Third Military Medical University who were treated from 2008 to 2010. The diagnosis of CRC was confirmed histologically. Patients with histories of previous cancers other than CRC and radiotherapy or chemotherapy were excluded. The controls were healthy people matched to cases by age, sex and dietary habits. Informed consent was obtained from all subjects and the study was approved by the Ethical Committee of Third Military Medical University. Individuals who had smoked over 100 cigarettes were classified as smokers, including current smokers and former smokers who had stopped smoking for at least one year. Individuals who had been drinking alcohol at least once a week for more than 6 months were labeled as drinkers, including current drinkers and former drinkers. Former drinkers were those who had abstained from drinking for more than one year.

Table 1 Characteristics of colorectal carcinoma cases and controls *n* (%)

Parameters	Cases	Controls	*P* value
Age (mean ± SD, yr)	57.85 ± 10.05	58.10 ± 13.47	0.751
Sex			
Male	259 (54.1)	254 (52.3)	
Female	220 (45.9)	232 (47.7)	
Total	479	486	0.574
Smoking status			
Never	334 (69.7)	357 (73.5)	
Former	15 (3.1)	39 (8.0)	
Present	130 (27.1)	90 (18.5)	
Total	479	486	0.199
Drinking status			
Never	10 (2.1)	16 (3.3)	
Former	280 (58.5)	207 (42.6)	
Present	189 (39.5)	263 (54.1)	
Total	479	486	0.248
Histological grade			
High	11 (2.3)		
Intermediate	296 (61.8)		
Low	172 (35.9)		
Location			
Colon	233 (48.6)		
Rectum	242 (50.5)		
Colon and rectum	4 (0.8)		
Stage			
Ⅰ	142 (29.7)		
Ⅱ	110 (23.0)		
Ⅲ	142 (29.6)		
Ⅳ	85 (17.7)		

DNA extraction, polymorphism selection and genotyping

Genomic DNA was extracted from whole blood samples of subjects by the TIANamp Blood DNA Kit (Tiangen, China) according to the manufacturer's instructions. Eight polymorphisms in 5 genes (rs1739654, rs2232596 and rs2232618 in LBP; rs4914 and rs77083413 in CD14; rs5030719 in TLR4; rs13306435 in IL-6; and rs35131721 in TNF-α) examined in our study have been reported with

the minor allele frequency over 1%[21]. SNP genotyping was carried out by the two-step SNaPshot assay. The first step was amplification of gene fragments containing these polymorphic sites. The polymerase chain reaction (PCR) was performed in 25 μL reaction mixture containing 1 × master mix (Tiangen, China), 30 ng genomic DNA templates and 0.4 μmol/L primer sets. Primer sequences (5' to 3') were presented in the order of forward, reverse and SNaPshot sequences, including LBP rs1739654: ACAGAAATGCAGGGACACCTCT, CCTGAGGCTCTCTCTTCCTCAC, GCGGCCCAGGAGGGGCTATT; LBP rs2232596: TCCAACTGGACCCTAGTGGAGT, CGCCTGGCCCTTAATTTTTACT, CCATGTTTTTCAGATTTGCGAAATGATCCAGAAATC; LBP rs2232618: TATGTTGGCACACACAGAACCA, CACTTCCATGTGTCCCTCTGTC, GCTCCTCAACTATTACATCCTTAACACC; CD14 rs77083413: TGAATTGGTCGAAAAGTCCTCA, CCCTGAACTCCCTCAATCTGTC, TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACCACGCCGGAGTTCATTGAGCCCTCGTG; CD14 rs4914: ACACGGACCCGTTGTTTAAGAT, TGGAACAGGTGCCTAAAGGACT, TTTTTTTTTTTTTTCTTGGATCTTAGGCAAAGCCCCGGGCCCCTTGGAG; TLR4 rs5030719: CAGAGTTGCTTTCAATGGCATC, TGCAGGAAACTCTGGTGTTCA, TTTTTTTTTTTTTTTTTTTTTTTTTCCTGGACCTCTCTCAGTGTCAACTGGAGCA; IL-6 rs13306435: ATGGAAGGGTCCTACTCAGAGC, CATAAGTTCTGTGCCCAGTGGA, CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCACTTTCATTTTCCTTCAGGCAAAGAATCTAGA; TNF-α rs35131721: GCGGGAAATATGACAGCTAAGG, CTCCCCCAAGACCAAAACTTTA, TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAACCATTCTCCTTCTCCCCAACAGTTCC. A similar amplification condition was used for all genes: one cycle of denaturation at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 30 s, annealing at 68℃ for 30 s, elongation at 72℃ for 40 s; and an eventual elongation at 72℃ for 5 min. The size of PCR products were confirmed by 1.5% agarose gel electrophoresis. The second step was genotyping using the SNaPshot Multiplex Kit (Applied Biosystems, USA). Three μL PCR amplification products were mixed with 5 μL SNaPshot Multiplex Kit and 1 μL 10 μmo/L SNaPshot primer. The SNaPshot PCR condition was 25 cycles of 10 s at 96℃, 5s at 50℃ and 30 s at 60℃. Subsequently, samples were mixed with Liz120 (Applied Biosystems, USA) and were electrophoresed using Genetic Analyzer 3130 instrument (Applied Biosystems, USA). The data were analyzed using the 4.0 Genemapper software (Applied Biosystems, USA).

Statistical analysis

Differences in demographic variables, smoking status, drinking status, grouped genotypic frequencies between cases and controls were evaluated by Student's *t* test and χ^2 test. Two-sided *P* values were considered significant at levels less than 0.05. The associations between polymorphisms of LPS-signaling-related genes and CRC risk were estimated from unconditional regression analysis using the SPSS 13.0 software (PASW, USA). All the eight SNPs were tested for the Hardy-Weinberg equilibrium.

RESULTS

The characteristics of 479 CRC cases and 486 healthy controls are summarized in Table 1. In this case-control study, eight polymorphisms of five genes involved in the LPS-signaling pathway were assayed, in which TLR4 rs5030719 and TNF-α rs35131721 SNPs were excluded due to data bias. All the other six polymorphisms satisfied the Hardy-Weinberg equilibrium ($P > 0.05$).

The effects of the polymorphisms of LPS-signaling-related genes on the risk of colorectal cancer are shown in Table 2. In the genetic model, the G allele of LBP rs2232596 SNP was significantly associated with CRC (GA genotype: odds ratio (OR) = 1.51, 95% confidence interval (CI) 1.15-1.99, $P = 0.003$; GG genotype: OR = 2.49, 95% CI 1.16-5.38, $P = 0.016$). Similarly, the G allele of CD14 rs4914 SNP showed a strong association with the risk of CRC (CG genotype: OR = 1.69, 95 %CI 1.20-2.36, $P = 0.002$). To examine the interaction between epidemiological factors and genetic variances, stratified analysis using logistic regression was performed and no significant difference was found in the genotype distribution of LBP rs2232596 and CD14 rs4914 with respect to age, sex, tumor location and stages (data not shown).

Gene-tobacco exposure interactions and gene-alcohol exposure interactions were also evaluated by stratification analysis using logistic regression (Tables 3 and 4). Smokers with GA genotype of LBP rs2232596 SNP had a significant association with the CRC risk (OR = 1.68, 95% CI 1.17-2.40, $P = 0.005$), whereas in non-smokers, an increased CRC risk with CG genotype of CD14 rs4914 SNP (OR = 2.82, 95% CI 1.64-4.85, $P = 0.000$) was observed. In alcohol drinkers, the presence of GA and GG genotypes of LBP rs2232596 SNP (OR = 1.61, 95% CI 1.23-2.11, $P = 0.001$) and CG genotype of CD14 rs4914 SNP (OR = 1.80, 95% CI 1.28-2.55, $P = 0.001$) was associated with increased risk of CRC.

Further evaluations of the combinatory effects of LBP rs2232596 and CD14 rs4914 SNPs were conducted using logistic regression analysis (Table 5). Subjects carrying risk genotypes in both LBP and CD14 (GA and GG in LBP rs2232596, CG in CD14 rs4914) showed synergistic effects on the associations with the CRC risk (OR = 1.46, 95% CI 1.11-1.92, $P = 0.007$ and OR = 3.44, 95% CI 1.93-6.10, $P = 0.000$).

DISCUSSION

Our study aimed to investigate the association of SNPs in LPS-signaling-related genes and the risk of CRC in the Chinese Han population. We found that the G alleles of both LBP rs2232596 and CD14 rs4914 were significantly associated with CRC. In addition, a combination of these two polymorphisms dramatically increased the CRC risk. To our knowledge, this is the first report on the relationship of these two polymorphisms with gastrointestinal malignancies.

Table 2 Genes, polymorphism and frequencies in colorectal carcinoma cases and controls

SNP	Genotype	Cases (n = 479)	Controls (n = 486)	Odds ratio (95% CI)	[1]P value
LBP					
rs1739654	GG	377 (78.7%)	360 (74.1%)		
	GA	93 (19.4%)	118 (24.3%)	0.75 (0.55-1.02)	0.07
	AA	9 (1.9%)	8 (1.6%)	1.07 (0.41-2.82)	0.88
	GA+AA	102 (21.3%)	126 (25.9%)	0.77 (0.57-1.04)	0.09
rs2232596	AA	289 (60.3%)	343 (70.6%)		
	GA	169 (35.3%)	133 (27.4%)	1.51 (1.15-1.99)	0.003
	GG	21 (4.4%)	10 (2%)	2.49 (1.16-5.38)	0.016
	GA+GG	190 (39.7%)	143 (29.4%)	1.58 (1.21-2.06)	0.001
rs2232618	TT	385 (80.4%)	396 (81.5%)		
	CT	93 (19.4%)	88 (18.1%)	1.09 (0.79-1.50)	0.613
	CC	1 (0.2%)	2 (0.4%)	0.51 (0.05-5.70)	0.581
	CT+CC	94 (19.6%)	90 (18.5%)	1.07 (0.78-1.48)	0.662
CD14					
rs77083413	GG	403 (84.1%)	400 (82.3%)		
	GC	70 (14.6%)	81 (16.7%)	0.858 (0.605-1.215)	0.388
	CC	6 (1.3%)	5 (1%)	1.19 (0.36-3.93)	0.774
	GC+CC	76 (15.9%)	86 (17.7%)	0.88 (0.63-1.23)	0.447
rs4914	CC	369 (77%)	415 (85.4%)		
	CG	102 (21.3%)	68 (14%)	1.69 (1.20-2.36)	0.002
	GG	8 (1.7%)	3 (0.6%)	3.00 (0.79-11.39)	0.091
	CG+GG	110 (23%)	71 (14.6%)	1.74 (1.25-2.42)	0.001
IL-6					
rs13306435	TT	415 (86.6%)	420 (86.4%)		
	AT	60 (12.5%)	65 (13.4%)	0.93 (0.64-1.36)	0.723
	AA	4 (0.9%)	1 (0.2%)	4.05 (0.45-36.37)	0.177
	AT+AA	64 (13.4%)	66 (13.6%)	0.98 (0.68-1.42)	0.921

[1]Adjusted for age, sex, smoking and drinking status.

Table 3 Stratification analyses for rs2232596 by smoking or drinking status

Genotype frequencies (%)	Status	Cases (n = 479)	Controls (n = 486)	[1]P value	Odds ratio (95% CI)
	Smoking				
AA	No	131 (27.3%)	155 (31.9%)		
GA	No	59 (12.3%)	55 (11.3%)	0.303	1.26 (0.81-1.95)
GG	No	10 (2.1%)	5 (1.0%)	0.292	1.86 (0.59-5.87)
GA/GG	No	69 (14.4%)	60 (12.3%)	0.190	1.32 (0.87-2.02)
AA	Yes	158 (33.0%)	188 (38.7%)		
GA	Yes	100 (20.9%)	78 (16.0%)	0.005	1.68 (1.17-2.40)
GG	Yes	11 (2.3%)	5 (1.0%)	0.084	2.59 (0.88-7.63)
GA/GG	Yes	111 (23.2%)	85 (17.0%)	0.002	1.73 (1.22-2.46)
	Drinking				
AA	No	6 (1.3%)	9 (1.9%)		
GA	No	4 (0.8%)	6 (1.2%)	0.892	0.89 (0.16-5.07)
GG	No	0 (0%)	1 (0.2%)	0.998	
GA/GG	No	4 (0.8%)	7 (1.4%)	0.739	0.75 (0.14-4.03)
AA	Yes	283 (59.1%)	334 (69.7%)		
GA	Yes	165 (34.4%)	127 (26.1%)	0.003	1.53 (1.16-2.03)
GG	Yes	21 (4.4%)	9 (1.9%)	0.015	2.68 (1.21-5.97)
GA/GG	Yes	186 (38.8%)	136 (28.0%)	0.001	1.61 (1.23-2.11)

[1]Adjusted for age, sex, and drinking or smoking status.

The LPS-signaling pathway is a crucial player in the innate immunity regulatory system, which includes LBP, CD14, TLR4/MD2 and other molecules involved in LPS-induced NF-κB activation such as MyD88, TIR, IRAK and TRAF6[22]. In intestinal mucosa, continuous exposure to LPS activates M1 type macrophages to perform tumoricidal tasks. TAMs in the cancer micro-environment belong to M2 macrophages and, on the contrary, promote tumor growth. In the presence of M2 macrophages, the LPS signaling pathway is down-regulated but the mechanisms remain unclear[10-12].

Previous studies in this field mainly focused on the effects of genetic variations of aforementioned genes in non-tumoral diseases such as bacterial infections[23,24], sepsis[25] and myocardial infarction[26]. Recently, how these genetic variations contribute to the risk of developing var-

Table 4 Stratification analyses for rs4914 by smoking or drinking status

Genotype frequencies (%)	Status	Cases (n = 479)	Controls (n = 486)	[1]P value	Odds ratio (95% CI)
	Smoking				
CC	No	146 (30.5%)	191 (39.3%)		
CG	No	50 (10.4%)	23 (4.7%)	0.000	2.820 (1.64-4.85)
GG	No	4 (0.8%)	1 (0.2%)	0.144	5.210 (0.57-47.55)
CG/GG	No	54 (11.2%)	24 (4.9%)	0.000	2.920 (1.72-4.96)
CC	Yes	223 (46.6%)	224 (46.1%)		
CG	Yes	52 (10.9%)	45 (9.3%)	0.524	1.155 (0.74-1.80)
GG	Yes	4 (0.8%)	2 (0.4%)	0.430	1.993 (0.36-11.05)
CG/GG	Yes	56 (11.7%)	47 (9.7%)	0.429	1.190 (0.77-1.83)
	Drinking				
CC	No	9 (1.9%)	10 (2.1%)		
CG	No	1 (0.2%)	5 (1.0%)	0.215	0.227 (0.02-2.37)
GG	No	0 (0%)	1 (0.2%)	0.998	
CG/GG	No	1 (0.2%)	6 (1.2%)	0.154	0.190 (0.02-1.88)
CC	Yes	360 (75.2%)	405 (83.3%)		
CG	Yes	101 (21.1%)	63 (13.0%)	0.001	1.800 (1.28-2.55)
GG	Yes	8 (1.7%)	2 (0.4%)	0.060	4.470 (0.94-21.18)
CG/GG	Yes	109 (22.8%)	65 (13.4%)	0.000	1.890 (1.34-2.64)

[1]Adjusted for age, sex and drinking or smoking status.

Table 5 Colorectal carcinoma risk with combined lipopolysaccharide binding protein rs2232596 and CD14 rs4914 SNPs

No. of risk genotype	Cases (n = 479)	Controls (n = 486)	[1]P value	Odds ratio (95% CI)
"0"	235 (49.1%)	296 (60.9%)		
"1"	194 (40.5%)	172 (35.4%)	0.007	1.46 (1.11-1.92)
"2"	50 (10.4%)	18 (3.7%)	0.000	3.44 (1.94-6.10)
"1+2"	244 (50.9%)	190 (39.1%)	0.000	1.66 (1.28-2.15)

[1]Adjusted for age, sex, smoking and drinking status.

ious cancers has drawn much attention. Polymorphisms in the CD14 promoter can affect the susceptibility to CRC[27] and *Helicobacter pylori* infection–related gastric carcinoma[28] in Chinese patients, and prostate cancer in African American men[29]. Effects of polymorphisms of TLR4 and other PRRs on cancer risk have also been reported[30,31].

Our study of the genetic variances in LBP rs2232596 and CD14 rs4914 provided strong evidence of interactions between LPS-signaling-related genes and the risk of CRC, indicating that the genetic modulation of LPS-induced inflammation may contribute to CRC development and progression. TAMs with defective LPS responsiveness are common components of the micro-environment of different cancers. In addition, the current study and several previous studies revealed that functional polymorphisms in LPS-signaling-related genes are associated with various cancer risks. More studies are needed to shed light on the underlying genetic mechanisms.

Tobacco and alcohol exposure have been identified as high-risk factors for CRC[32, 33]. However, our data failed to show any significant associations of tobacco and/or alcohol exposure with CRC susceptibility. We found that smokers and drinkers carrying LBP rs2232596 polymorphisms had a higher risk of CRC. But only drinkers carrying CD14 rs4914 polymorphism showed modest risk of CRC. One possible explanation is that different mechanisms regulate tobacco-gene and alcohol-gene interactions. This study lacked detailed information on the smoking and drinking status of the subjects. Further stratification analysis is needed to evaluate the risk of lifestyle factors.

What mediates the observed association between gene polymorphisms and CRC susceptibility still remains unknown. It would be interesting to compare the serum levels of LBP and CD14 from different genotypes to examine the relationship between gene polymorphisms and their expression levels.

In conclusion, the functional G alleles in both LBP rs2232596 and CD14 rs4914 SNPs showed significant associations with a high CRC risk. Further studies are needed to elucidate the effects of these genotypes on gene transcription, expression and functions in CRC and other types of malignancies.

COMMENTS

Background

Colorectal carcinoma (CRC) is a leading cause of cancer death in China and throughout the world. Chronic inflammation is considered to be important in the carcinogenesis of CRC. In this study, the authors examined the association between the gene polymorphisms of several lipopolysaccharide (LPS)-signaling factors and the risk of CRC to better elucidate the mechanism of inflammation in tumorigenesis.

Research frontiers

LPS-induced signaling is an important innate immune response that involves many different molecules, such as lipopolysaccharide binding protein (LBP), cluster of differentiation 14 (CD14), and toll-like receptor 4 (TLR-4). Recently, it has become a hot area to employ polymorphism analysis to identify genetic mutations in the immune system that are significantly correlated with tumor development.

Innovations and breakthroughs

To date, there has been no study on the polymorphisms of LPS-signaling-related genes and CRC susceptibility in the Chinese Han population. In this study, the authors directly addressed this issue and performed genetic analysis to screen for polymorphisms that are associated with increased CRC risk. The authors also explored the gene-environment interactions by studying the effects of smoking or drinking exposure on CRC susceptibility.

Applications

By demonstrating the association of LBP rs2232596 and CD14 rs4914 polymorphisms with increased CRC risk, the authors identified two important biomarkers for predicting CRC and further improved the understanding of the inflammation-related mechanisms in CRC development.

Terminology

Single-nucleotide polymorphism (SNP): a DNA sequence variation occurring when a single nucleotide in the genome differs between members of the same biological species or paired chromosomes in an individual. SNP analysis can shed light on how genetic variations affect disease development in humans.

Peer review

This is an interesting well-conducted and well-written study.

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BRIEF ARTICLE

Utility of pancreatography for diagnosing autoimmune pancreatitis

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Abstract

AIM: To identify pancreatographic findings that facilitate differentiating between autoimmune pancreatitis (AIP) and pancreatic cancer (PC) on endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP).

METHODS: ERCP findings of 48 AIP and 143 PC patients were compared. Diagnostic accuracies for AIP by ERCP and MRCP were compared in 30 AIP patients.

RESULTS: The following ERCP findings suggested a diagnosis of AIP rather than PC. Obstruction of the main pancreatic duct (MPD) was more frequently detected in PC ($P < 0.001$). Skipped MPD lesions were detected only in AIP ($P < 0.001$). Side branch derivation from the narrowed MPD was more frequent in AIP ($P < 0.001$). The narrowed MPD was longer in AIP ($P < 0.001$), and a narrowed MPD longer than 3 cm was more frequent in AIP ($P < 0.001$). Maximal diameter of the upstream MPD was smaller in AIP ($P < 0.001$), and upstream dilatation of the MPD less than 5 mm was more frequent in AIP ($P < 0.001$). Stenosis of the lower bile duct was smooth in 87% of AIP and irregular in 65% of PC patients ($P < 0.001$). Stenosis of the intrahepatic or hilar bile duct was detected only in AIP ($P = 0.001$). On MRCP, diffuse narrowing of the MPD on ERCP was shown as a skipped non-visualized lesion in 50% and faint visualization in 19%, but segmental narrowing of the MPD was visualized faintly in only 14%.

CONCLUSION: Several ERCP findings are useful for differentiating AIP from PC. Although MRCP cannot replace ERCP for the diagnostic evaluation of AIP, some MRCP findings support the diagnosis of AIP.

Key words: Autoimmune pancreatitis; Pancreatic cancer; Endoscopic retrograde cholangiopancreatography, Magnetic resonance cholangiopancreatography

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a newly described entity of pancreatitis, the pathogenesis of which appears to involve autoimmune mechanisms[1-4]. Clinically, AIP patients and patients with pancreatic cancer (PC) share many features, such as preponderance of elderly males,

frequent initial symptom of painless jaundice, development of new-onset diabetes mellitus, and elevated levels of serum tumor markers. Radiologically, focal swelling of the pancreas, the "double-duct sign", representing strictures in both biliary and pancreatic ducts, and encasement of peripancreatic arteries and portal veins are sometimes detected in both AIP and PC[4-6]. AIP often mimics PC, and 2.4% of 1808 pancreatic resections in the USA were reported to have AIP on histological examination[7]. AIP responds dramatically to steroid therapy; therefore, accurate diagnosis of AIP can avoid unnecessary laparotomy or pancreatic resection.

Serum IgG4 levels were elevated in 77%[8]-81%[9] of AIP patients, but they were also elevated in 4%[8]-10%[10] of PC patients. There is no definite serological marker for AIP; therefore, AIP is currently diagnosed using a combination of characteristic radiological, serological, and pathological findings. Irregular narrowing of the main pancreatic duct (MPD) on endoscopic retrograde cholangiopancreatography (ERCP) is a characteristic radiological feature of AIP, and this pancreatographic finding on ERCP is mandatory in the Japanese diagnostic criteria for AIP[11]. We previously compared the pancreatograms of 17 AIP patients having a mass-forming lesion in the pancreatic head and 40 patients with pancreatic head cancer[6]. In the present study, we further compared pancreatograms of 48 AIP patients and 143 PC patients, and evaluated more accurately by further date.

Although pancreatographic findings are useful to differentiate AIP and PC, ERCP can cause adverse effects, such as pancreatitis. Since magnetic resonance cholangiopancreatography (MRCP) has become popular as a non-invasive method for obtaining high quality images of the pancreaticobiliary tree, MRCP is replacing diagnostic ERCP in many pancreatobiliary diseases. In the new Korean diagnostic criteria, AIP can be diagnosed by MRCP without the need for ERCP[12]. In our previously study of the utility of MRCP for diagnosing AIP in 20 AIP patients who were examined before 2008, MRCP could not replace ERCP, because narrowing of the MPD in AIP was not visualized on MRCP[13]. However, with development of MRCP models, spatial resolution of the pancreatic duct has improved on MRCP. We again studied the usefulness of MRCP for diagnosing AIP in 30 AIP patients and assessed whether MRCP could replace ERCP for diagnosing AIP.

MATERIALS AND METHODS

From 1992 to August 2010, pancreatograms on ERCP were obtained in 48 AIP patients [34 males, 14 females; age, 64.1 ± 12.1 (mean ± SD) years; age range, 27-83 years]. They were diagnosed as having AIP according to the Asian Diagnostic Criteria for AIP[12]: pancreatic enlargement on computed tomography (CT) and ultrasonography (US) (n = 48), irregular narrowing of the MPD on ERCP (n = 48), elevated serum IgG4 (n = 42), presence of autoantibodies (n = 26), histological findings of lymphoplasmacytic sclerosing pancreatitis (LPSP, n = 18), and steroid responsiveness (n = 38). Overall, 23 patients had diffuse enlargement of the pancreas, and 25 patients had segmental enlargement of the pancreas (head, n = 17; body and/or tail, n = 8). An initial dose of oral prednisolone of 30-40 mg/d was administered for 2-3 wk. It was then tapered by 5 mg every 1-3 wk until it reached 5 mg/d. After about three months of induction, maintenance therapy of 2.5-5 mg/d was administered for six months to 143 mo. Malignant diseases, such as pancreatic or biliary cancers, were excluded by long follow up in the 30 patients without histological examination. During the same period as the AIP cases, ERCP pancreatograms were examined in 143 PC patients (81 males, 62 females; age, 63.5 ± 10.9 years; range, 42-79 years). These PCs were resected or histologically confirmed after adequate imaging studies, such as US, CT, and magnetic resonance imaging (MRI). The locations of PC were confirmed to include the head (n = 88), body (n = 37), and tail (n = 18) on these imaging examinations.

The pancreatographic findings were evaluated in the 44 AIP patients and the 143 PC patients to measure the length of the narrowed MPD and the diameter of the upstream MPD (dilated MPD above the narrowed part). It was also determined whether obstruction and skipped lesions in the MPD (discrete narrowed lesions scattered in the almost normal MPD) or side branch derivation from the narrowed portion of the MPD were present. The length of the narrowed MPD and the maximal diameter of the dilated upstream MPD were measured using measuring function on computerized images or a manual goniometer. The cholangiographic findings were evaluated to identify morphological modifications in the 38 AIP patients and 77 PC patients with bile duct involvement.

Furthermore, 30 of the 48 AIP patients also underwent MRCP within three weeks, and MRCP and ERCP findings were compared. MRCP was performed using a 1.5-T magnetic resonance imaging machine (INTERA, Philips Co. Ltd., The Netherlands) by two-dimensional (up to 2005) and three-dimensional (2006-2010), coronal, heavily T2-weighted, signal-shot, rapid acquisition with relaxation enhancement. Since 2006, a 1.5-T magnetic resonance imaging machine (MAGNETOM Avanto, Siemens Co. Ltd., Germany) was also used. Diagnostic accuracies for AIP on ERCP and MRCP were compared separately in diffuse-type AIP patients (n = 16) and segmental-type AIP patients (n = 14). Two endoscopists and radiologists, who were blind to clinical information, reviewed the radiological findings.

Statistical analyses were performed with the Mann-Whitney's U test and Fisher's exact probability test. P values of less than 0.05 were considered significant.

RESULTS

Pancreatographic differences between AIP and PC

Whereas obstruction of the MPD was more frequently detected in PC (AIP: 4% *vs* PC: 69%, $P < 0.001$), skipped lesions of the MPD were detected only in AIP (27% *vs* 0%,

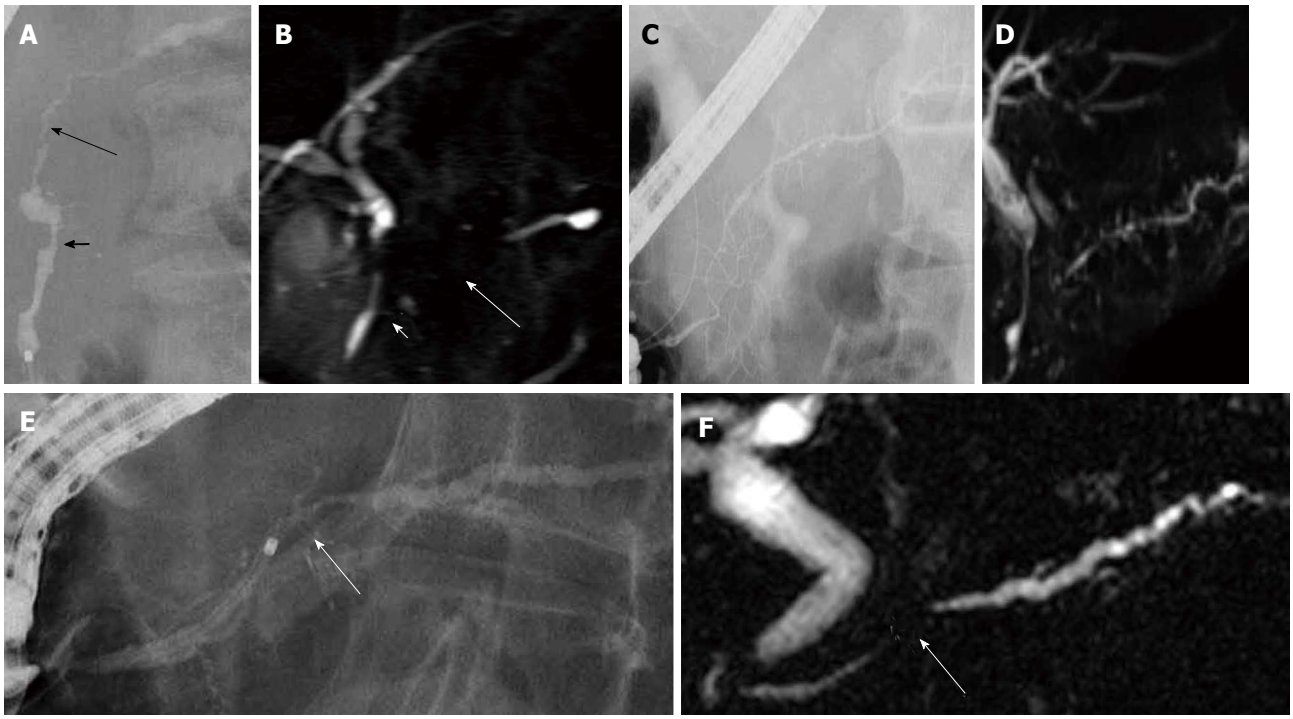

Figure 1 Pancreatography of autoimmune pancreatitis. A: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing skipped lesions of the main pancreatic duct (short and long arrows); B: On magnetic resonance cholangiopancreatography, skipped lesions (short and long arrows) on endoscopic retrograde cholangiopancreatography were not visualized; C: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing side branch derivation from the narrowed portion of the main pancreatic duct; D: Recent magnetic resonance cholangiopancreatography could show diffuse narrowing of the main pancreatic duct fairly well; E: Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing a short narrowed main pancreatic duct (arrow) with upstream dilatation less than 5 mm; F: On magnetic resonance cholangiopancreatography, the narrowed portion (arrow) was not visualized.

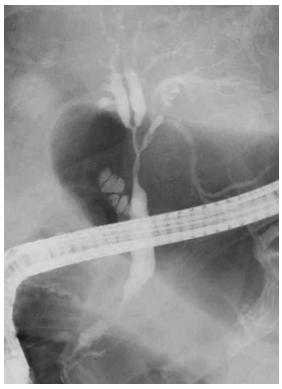

Figure 2 Endoscopic retrograde cholangiopancreatography finding of autoimmune pancreatitis showing stenosis of the hilar and intrahepatic bile duct.

$P < 0.001$) (Figure 1A), and side branch derivation from the narrowed portion of the MPD was more frequent in AIP (81% *vs* 22%, $P < 0.001$) (Figure 1C). The narrowed MPD was longer in AIP (7.6 ± 4.3 cm *vs* 2.5 ± 0.9 cm, $P < 0.001$), and a narrowed MPD longer than 3 cm was more frequent in AIP (90% *vs* 27%, $P < 0.001$). The maximal diameter of the upstream MPD was smaller in AIP (2.9 ± 0.8 mm *vs* 6.8 ± 2.1 mm, $P < 0.001$), and upstream dilatation of the MPD less than 5 mm was more frequent in AIP (95% *vs* 27%, $P < 0.001$) (Figure 1E) (Table 1). A short narrowed MPD was difficult to differentiate from stenosis of the MPD in PC (Figure 1E).

Cholangiographic differences between AIP and PC

Stenosis of the lower bile duct was smooth in 33 (87%) AIP patients, but irregular in 50 (65%) PC patients ($P < 0.001$). Left-side deviation of the lower bile duct was detected in both groups. Stenosis of the intrahepatic or hilar bile duct was detected in only six AIP patients ($P = 0.001$) (Figure 2) (Table 2).

Diagnostic accuracy for AIP by ERCP and MRCP

In diffuse-type AIP, diffuse narrowing of the MPD on ERCP was shown as skipped non-visualized lesions in 50% (Figure 1B), faint visualization in 19%, and non-visualization in 31% on MRCP. Diffuse narrowing of the MPD could be fairly well visualized in two recent patients (Figure 1D). Side branches from the narrowed portion of the MPD shown on ERCP were visualized faintly only in 21% on MRCP. Stenosis of the bile duct was also detected on MRCP. After steroid therapy, the MPD was visualized in 100%, and the branches were visualized in 50% on MRCP. Resolution of bile duct lesions was seen completely in 53% and incompletely in 47% on MRCP (Table 3).

In segmental-type AIP, segmental narrowing of the MPD on ERCP was shown as faint visualization in 14% and non-visualization in 86% on MRCP (Figure 1F). Side branches from the narrowed MPD shown on ERCP were visualized faintly only in 18% on MRCP. After steroid therapy, the MPD was visualized in 100%, and the branches were visualized in 60% on MRCP. Resolution of bile duct lesions was seen completely in 50% and incompletely in 50% on MRCP (Figure 3A and B) (Table 4).

Table 1 Pancreatographic differences between autoimmune pancreatitis and pancreatic cancer *n* (%)

	Autoimmune pancreatitis (*n* = 48)	Pancreatic cancer (*n* = 143)	*P*-value
Obstruction of the MPD +/-	2/46 (4)	98/45 (69)	< 0.001
Skipped lesions of the MPD +/-	13/35 (27)	0/143 (0)	< 0.001
Side branch derivation from the narrowed MPD +/-	39/9 (81)	10/35 (22)	< 0.001
Length of the narrowed MPD (cm)	7.6 ± 4.3	2.5 ± 0.9	< 0.001
Length of the narrowed MPD > 3 cm +/-	43/5 (90)	12/33 (27)	< 0.001
Diameter of upstream MPD (mm)	2.9 ± 0.8	6.8 ± 2.1	< 0.001
Diameter of upstream MPD < 5 mm +/-	19/1 (95)	12/33 (27)	< 0.001

MPD: Main pancreatic duct.

Table 2 Cholangiographic differences between autoimmune pancreatitis and pancreatic cancer *n* (%)

	Autoimmune pancreatitis	Pancreatic carcinoma	*P*-value
Stenosis of the lower bile duct	*n* = 38	*n* = 77	
Smooth stenosis	33 (87)	27 (35)	< 0.001
Irregular stenosis	5 (13)	50 (65)	
Left-side deviation of the lower bile duct +/-	20 (53)	49 (64)	NS
Stenosis of the intra/hilar bile duct	6 (16)	0 (0)	0.001

NS: Not significant.

Table 3 Diagnostic accuracy for diffuse-type autoimmune pancreatitis by endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography

ERCP before steroid	MRCP	
	Before steroid	After steroid
MPD narrowing (*n* = 16)	Skipped non-visualization 8 (50%) Faint visualization 3 (19%) Non-visualization 5 (31%)	Visualization 16/16 (100%)
Branches from the narrowed MPD (*n* = 14)	Faint visualization 3 (21%) Non-visualization 11 (79%)	Visualization 7/14 (50%)
Bile duct stenosis (*n* = 15)	Stenosis 15 (100%)	Resolution Complete 8/15 (53%) Incomplete 7/15 (47%)

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct.

DISCUSSION

AIP responds dramatically to steroid therapy; therefore, it is of the utmost importance that AIP be differentiated from PC to avoid unnecessary laparotomy or pancreatic resection. Histopathological findings of AIP in Japan are characterized by dense infiltration of T lymphocytes and IgG4-positive plasma cells, storiform fibrosis, and obliterative phlebitis in the pancreas; this is called LPSP. Abundant lymphoplasmacytic cells infiltrate around interlobular ducts with fibrosis, especially the medium and large ducts, including the MPD. The periductal inflammation is usually extensive and distributed throughout the entire pancreas. However, the degree and extent of periductal inflammation differ from duct to duct according to the location of the involved pancreas. The infiltrate is primarily subepithelial, with the epithelium only rarely being infiltrated by lymphocytes. It encompasses the ducts and narrows their lumen by infolding of the epithelium. If the inflammatory process affects the pancreatic head, it usually also involves the lower common bile duct, where it leads to a marked thickening of the bile duct wall due to fibrosis with lymphoplasmacytic infiltration. The epithelium of the bile duct is also well preserved. The thickening of the bile duct wall sometimes spreads extensively to the bile duct, where stenosis is not apparent on cholangiography[4,14-16]. On the other hand, PC cells infiltrate scirrhously, destroy the epithelium of the pancreatic and bile ducts, and frequently obstruct the main and branch pancreatic ducts. These histopathological differences around the ducts represent the different cholangiopancreatographic findings between AIP and PC. Pancreatographic findings, such as no obstruction of the MPD, skipped lesions of the MPD, side branch derivation from the narrowed portion of the MPD, narrowed portion of the MPD > 3-cm-long, a maximal diameter < 5 mm of the upstream MPD, and smooth stenosis of the lower bile duct are highly suggestive of AIP rather than PC. Stenosis of the intrahepatic or hilar bile duct is recognized as other

Table 4 Diagnostic accuracy for segmental-type autoimmune pancreatitis by endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography

ERCP before steroid	MRCP	
	Before steroid	After steroid
MPD narrowing (n = 14)	Faint visualization 2 (14%) Non-visualization 12 (86%)	Visualization 10/10 (100%)
Branches from the narrowed MPD (n = 11)	Faint visualization 2 (18%) Non-visualization 9 (82%)	Visualization 6/10 (60%)
Bile duct stenosis (n = 8)	Stenosis 8 (100%)	Resolution Complete 4/8 (50%) Incomplete 4/8 (50%)

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct.

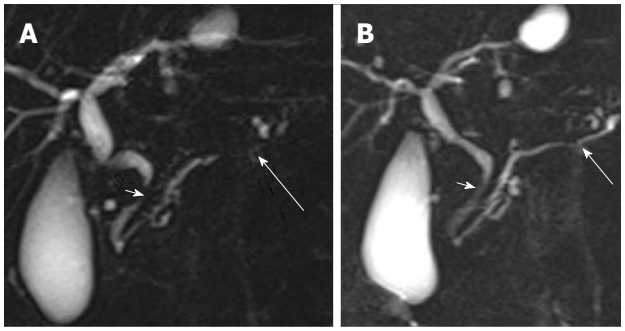

Figure 3 Magnetic resonance cholangiopancreatography finding of autoimmune pancreatitis. Stenosis of the lower bile duct (short arrow) and the narrowing of the main pancreatic duct (long arrow) (A) improved after steroid therapy (B).

organ involvement of AIP and supports the diagnosis of AIP rather than PC, although it should be distinguished from cholangiocarcinoma and primary sclerosing cholangitis[17,18]. Wakabayashi *et al*[19] compared pancreatograms of nine segmental-type AIP patients and 80 PC patients. They reported that obstruction of the MPD was frequent in PC [11% (1/9) *vs* 60% (48/80)]. A narrowed portion of the MPD ≥ 3-cm-long [100% (8/8) *vs* 22% (6/27)] and a maximal diameter < 4 mm of the upstream MPD [(67% (4/6) *vs* 4% (1/23)] were frequently detected in AIP, and side branch derivation from the narrowed portion of the MPD was detected in 50% (4/8) of AIP patients[19]. In Nakazawa's cholangiopancreatographic study of 37 AIP patients, skipped lesions of the MPD were detected in six (16%) patients, and stenosis of the intrahepatic or hilar bile duct was detected in 16 (43%) patients[20].

The major problem with MRCP for diagnosing AIP is that the narrowed MPD seen on ERCP cannot be visualized on MRCP, because of the inferior resolution of MRCP compared with ERCP. Segmental narrowing of the MPD seen on ERCP was not visualized in 86% on MRCP, and distinguishing between AIP and PC was quite difficult on MRCP. However, in these cases, less upstream dilatation of the MPD on MRCP may suggest AIP rather than PC. In diffuse-type-, diffuse narrowing of the MPD on ERCP was shown as skipped, non-visualized lesions in 50% and faintly visualized in 19% on MRCP. With the development of MRCP models, diffuse narrowing of the MPD could be visualized fairly well on MRCP in two recent patients. Skipped, non-visualized lesions and a faintly visualized, narrowed MPD can suggest AIP with typical diffuse enlargement of the pancreas on CT or MRI. Side branch derivation from the narrowed portion of the MPD is a useful pancreatographic finding suggesting AIP, but the finding can rarely be seen on MRCP. However, stenosis of the bile duct was also detected on MRCP and resolution of the pancreatic and bile ducts after steroid therapy can be fully evaluated on MRCP. Park *et al*[21] also reported that skipped MPD narrowing and less upstream MPD dilatation on MRCP suggest AIP. Although secretin is not available in Japan currently, secretin-MRCP is reported to enable demonstration of the integrity of the MPD and lead to a correct diagnosis of AIP[22].

In conclusion, the following ERCP findings are fairly specific for AIP and are useful to differentiate AIP from PC: less obstruction of the MPD, skipped lesions of the MPD, side branch derivation from the narrowed portion of the MPD, length of the narrowed MPD > 3 cm, maximal diameter of the upstream < 5 mm, smooth stricture of lower the bile duct, and stenosis of intra or hilar bile duct. Although MRCP cannot fully replace ERCP for the diagnostic evaluation of AIP, MRCP may deserve to be used in some diffuse-type AIP cases and is useful for follow-up of AIP.

COMMENTS

Background

Autoimmune pancreatitis (AIP) is a peculiar type of pancreatitis of presumed autoimmune etiology. Since AIP responds dramatically to steroid therapy, it is most important that AIP be differentiated from pancreatic cancer (PC) to avoid unnecessary laparotomy or pancreatic resection. However, some cases were still difficult to distinguish between AIP and PC.

Research frontiers

Pancreatographic findings to facilitate differentiating between AIP and PC on endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP) were investigated.

Innovations and breakthroughs

Obstruction of the main pancreatic duct (MPD) was more frequently detected in PC. Skipped MPD lesions and side branch derivation from the narrowed MPD were detected in AIP. The narrowed MPD was longer in AIP. Maximal diameter of the upstream MPD was smaller in AIP. Stenosis of the lower bile duct was smooth in AIP and irregular in PC patients. Stenosis of the intrahepatic or hilar

bile duct was detected only in AIP. Diffuse narrowing of the MPD on ERCP was shown as a skipped non-visualized lesion in 50% and faint visualization in 19% on MRCP.

Applications

Several ERCP findings are useful to differentiate AIP from PC. MRCP cannot replace ERCP for the diagnostic evaluation of AIP, but some MRCP findings support the diagnosis of AIP.

Peer review

This is a well-written manuscript about pancreatography for diagnosing autoimmune pancreatitis and is of some interest.

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BRIEF ARTICLE

Prospective randomized controlled trial investigating the type of sutures used during hepatectomy

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Abstract

AIM: To determine whether absorbable sutures or non-absorbable sutures are better in preventing surgical site infection (SSI), in this paper we discuss the results of a randomized clinical trial which examined the type of sutures used during hepatectomy.

METHODS: All hepatic resections performed from January 2007 to November 2008 at the Department of Surgery at Iizuka Hospital in Japan were included in this study. There were 125 patients randomly assigned to an absorbable sutures (Vicryl) group or non-absorbable sutures (Silk) group.

RESULTS: SSI was observed in 13.6% (17/125) patients participating in this study, 11.3% in the Vicryl group and 15.8% in the Silk group. Incisional SSI including superficial and deep SSI, was observed in 8% of the Vicryl group and 9.5% of the Silk group. Organ/space SSI was observed in 3.2% of the Vicryl group and 6.0% of the Silk group. There were no significant differences, but among the patients with SSI, the period for recovery was significantly shorter for the Vicryl group compared to the Silk group.

CONCLUSION: The incidence of SSI in patients receiving absorbable sutures and silk sutures is not significantly different in this randomized controlled study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures.

Key words: Hepatectomy; Absorbable suture; Surgical site infection

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INTRODUCTION

Despite recent developments in surgery and patient management during the perioperative period, critical complications still develop in a few patients who undergo hepatic resection[1,2]. Surgical site infection (SSI) is one of the most important morbidities of surgery and leads to prolonged hospital stays. Previous studies revealed absorbable suture material in gastrointestinal surgical procedures to reduce the risk of SSI[3,4] although most of those studies have been limited to skin closures. Absorbable sutures are used in most hospitals in the United States and Europe based on the available clinical studies. On the other hand, silk sutures are still used in most hospitals in Japan. A

study by Kobayashi *et al*[5] revealed patients with intraoperative bowel injury, blood loss > 2000 mL, and age > 65 years are at risk of developing SSI after hepatectomy for liver cancers and then all blood vessels and bile ducts were ligated with silk or vessel clips during parenchymal resection. Absorbable sutures are now widely used for abdominal closure in Japan according to the CDC guidelines[6]. During the resection of the liver, silk sutures are generally used for the vessels in Japan, because silk sutures are easier to handle and less expensive than absorbable sutures. However, foreign materials, especially silk, are known to accelerate infection[6,7] and thus lead to a prolonged hospital stay. Togo *et al*[8] reported the usefulness of absorbable sutures to prevent SSI with a historical control study using an animal model. There has been no known randomized clinical trial for suture material used in the ligation of the cut surface of the liver. In this paper the authors provide the outcome of a randomized clinical trial investigating the type of sutures used during hepatectomy to determine which is better in preventing SSI, absorbable sutures or non-absorbable sutures.

MATERIALS AND METHODS

Study design

All hepatic resections without biliary reconstruction performed from January 2007 to November 2008 at the Department of Surgery at Iizuka Hospital in Japan were included in this study. Only patients who met the following criteria were enrolled: (1) liver resection without biliary reconstruction; (2) without other malignancy; and (3) until compensated cirrhosis with Child-Pugh class A or B. At the author's department, the rate of SSI during hepatectomy was 25% according to previous data. The sample size required to detect a difference by chi-square test with 80% power at the 5% significance level was 124 patients. There were 130 patients enrolled in this study. One patient refused to participate in the study. The authors obtained approval from the local ethics committee and obtained informed consent from the other patients. Finally 125 patients were randomly assigned to an absorbable sutures (Vicryl, Johnson & Johnson Corp., Tokyo, Japan) or a non-absorbable sutures (Silk) group. Because the operative procedure used on 4 patients was either conversion to radiofrequency ablation or biliary reconstruction (Figure 1) they were not included in the study. Randomization was achieved by providing sealed envelopes to be opened by the operator before laparotomy. Data analysis was performed for 125 patients.

Liver function was evaluated preoperatively using Child classification and indocyanine green retention test at 15 min (ICGR15) in patients with underlying liver disease. The presence of ascites and ICGR15 test value of more than 40% were considered an absolute contraindication for resection. Hepatitis B surface antigen (HBs Ag) and hepatitis C antibody (HCV Ab) were routinely measured preoperatively.

Surgical technique and intra-operative care were standardized by the same team in this study. These included a J-shaped incision for routine abdominal access, a slow and gentle hepatic dissection using an ultrasonic dissector with coagulator (CUSA Excel, Integra Co., USA), with systematic ligation of all sizable vessels, and close ultrasonographic guidance along the transection line. Cholecystectomy was performed in all patients if the gallbladder was present. Intraoperative bile leakage test was routinely performed to identify bile leakage[9]. With this procedure, we recognized small bile leakage sites on the cut liver surface and could repair these sites by Z-suturing using 6-0PDSII (Johnson & Johnson Corp., Tokyo, Japan). Intraoperative vascular control was achieved using the Pringle maneuver[10]. In order to prevent backflow bleeding central venous pressure was decreased to as low as 5 mm Hg when possible with careful circulating volume and respiratory assistance. When central venous pressure control was insufficient, outflow control was achieved by selective vascular exclusion, or by clamping of the infrahepatic inferior vena cava[11]. One or two closed drains were inserted close to the cut surface of liver parenchyma. Before the subcutaneous tissue was closed, the wound was washed with 1 L saline. Vicryl (Johnson & Johnson Corp., Tokyo, Japan), an absorbable suture material, was used during abdominal wound closure. Drains were removed when no bleeding or bile leakage were observed within 3 d. Bile leakage was defined as the drainage of macroscopic bile from the surgical drains for more than 7 d after surgery[9]. If bile leakage is clinically suspected after the removal of drains, percutaneous drainage is performed. Intravenous ampicillin/sulbactam (ABPC/SBT) 1.5 g was administered 30 min before surgery and additional ABPC/SBT was administered every 3 h. Systemic antibiotics were used for only two days after surgery. During the operation gloves were changed every 3 h.

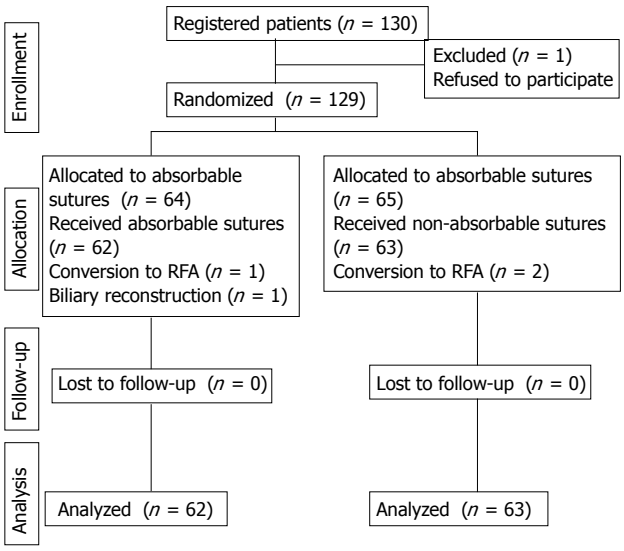

Figure 1 The trial profile of the patients in this study. RFA: Radiofrequency ablation.

Postoperative infection

SSI was defined as a condition in which purulent dis-

Table 1 Comparison of clinicopathological data of the groups

Variables	Vicryl group (n = 62)	Silk group (n = 63)	P-value
Age	68 ± 10	67 ± 12	0.684
Male/female	37/25	41/22	0.720
Body mass index	22.6 ± 3.0	23.7 ± 12	0.159
Diabetes mellitus (%)	19.4	33.0	0.104
HBV (%)	17.7	20.6	0.560
HCV (%)	50.0	39.7	0.666
Albumin (g/dL)	3.6 ± 0.5	3.6 ± 0.5	0.694
Total bilirubin (mg/dL)	0.7 ± 0.4	0.7 ± 0.4	0.813
AST (IU/L)	46 ± 31	43 ± 27	0.592
Platelet count (x 10^4/μL)	21.0 ± 11.0	19.7 ± 10.1	0.460
ICGR15 (%)	15.4 ± 9.0	16.4 ± 11.9	0.589
Child-Pugh A/B	58/4	59/4	0.999
HCC/non-HCC	46/16	47/16	0.826
Liver cirrhosis (ch/lf/lc)	15/20/27	15/22/26	0.948
Anatomical/nonanatomical	20/42	20/43	0.618
Operation time (min)	273 ± 88	276 ± 105	0.876
Blood loss (g)	698 ± 894	763 ± 1329	0.749
Transfusion (%)	12.9	17.4	0.620
Bile leakage (%)	3.2	6.3	0.680
Total SSI (%)	11.3	15.8	0.603
Incisional SSI (%)	8.0	9.5	0.999
Organ/space SSI (%)	3.2	6.3	0.680
Remote infection (%)	3.2	1.6	0.546
Hospital stay (d)	16 ± 15	16 ± 14	0.880

SSI: Surgical site infection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AST: Aspartate aminotransferase.

Table 2 Comparison of clinicopathological data of the groups

Variables	SSI(+) (n = 17)	SSI(-) (n = 108)	P-value
Age	68 ± 10	67 ± 11	0.667
Male/female	13 /4	65/43	0.287
Body mass index	24.3 ± 5.2	23.0 ± 3.9	0.208
Diabetes mellitus (%)	35.3	25.0	0.384
HBV (%)	23.5	18.5	0.703
HCV (%)	64.7	41.7	0.560
Albumin (g/dL)	3.6 ± 0.4	3.6 ± 0.5	0.977
Total bilirubin (mg/dL)	0.7 ± 0.3	0.7 ± 0.4	0.938
AST (IU/L)	56 ± 22	43 ± 29	0.070
Platelet count (x 10^4/μL)	20.7 ± 7.3	20.4 ± 11.0	0.904
ICGR15 (%)	19.9 ± 8.3	15.3 ± 10.7	0.101
Child-Pugh A/B	17/0	100/8	0.597
HCC/non-HCC	15/2	78/30	0.638
Liver cirrhosis (ch/lf/lc)	2/7/8	28/35/45	0.435
Anatomical/nonanatomical	8/9	32/76	0.228
Operation time > 265 min (%)	76.5	44.4	< 0.050
Blood loss > 1000 g (%)	41.2	18.5	< 0.050
Transfusion (%)	11.8	15.7	0.999
Bile leakage (%)	35.3	0	< 0.010
Vicryl/silk	7/10	55/53	0.603
Hospital stay (d)	29 ± 23	15 ± 12	< 0.010

SSI: Surgical site infection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AST: Aspartate aminotransferase.

charge was observed from any incision or space that was manipulated during an operation with or without microbiological evidence as in the guideline issued by CDC[6] and it was identified prospectively by direct observation of the surgical site**.** Patients were followed up 30 d after hospital discharge. SSI occurring after hospital discharge was included in this study. Remote infection was defined as a condition in which fever and leukocytosis were present with bacteria in sputum, urine, catheter-tip, blood, or other body fluid/space with or without microbiological evidence.

Statistical analysis

We analyzed associations between the continuous and categorical clinico-pathologic variables using the Student's t tests and χ^2 tests, respectively. Multivariate analysis was performed with a logistic regression test. A P value less than 0.05 was considered statistically significant.

RESULTS

Incidence of postoperative infection

The clinicopathological factors between the Vicryl and Silk groups were compared (Table 1). No significant differences were recognized during the evaluation of the host factors and the operative factors. SSI was observed in 13.6% (17/125) of patients in this study, 11.3% in the Vicryl group and 15.8% in the Silk group (P = 0.620). Incisional SSI including superficial and deep SSI, were observed in 8% of the Vicryl group and 9.5% of the Silk group (P = 0.999). Organ/space SSI was observed in 3.2% of the Vicryl group and 6.0% of the Silk group (P = 0.680). Organ/space SSI was namely abdominal abscess and percutaneous drainage was needed. Remote infection was observed with an incidence of 3.2% in the Vicryl group and 1.6% in the Silk group (P = 0.546). Pneumonia and urinary tract infection were observed in the Vicryl group and pneumonia in the Silk group. There were no significant differences. There was no hospital mortality in this study.

Risk factor for SSI

In the univariate analyses, operation time > 265 min, blood loss > 1000 g, and bile leakage differed significantly as predictive factors of SSI (Table 2). The type of sutures was not significant. Five factors including operation time > 265 min, blood loss > 1000 g, bile leakage and the type of sutures were analyzed with multivariate logistic regression. Results are given in Table 3. Blood loss > 1000 g was the only independent variable among the five factors.

Comparison of duration to recovery in the patients with SSI

Among the patients with SSI, the period for recovery in the Vicryl group was significantly shorter compared to the Silk group (Table 4).

DISCUSSION

Togo *et al*[8] reported the usefulness of absorbable sutures to prevent SSI with a historical control study using an animal model. This is the only report that the use of

Table 3 Multivariate logistic stepwise regression analysis for predictive factors of surgical site infection

	Adjusted odds ratios (95% CI)	*P* value
Blood loss > 1000 g	7.598 (1.396-41.364)	< 0.01

Table 4 Comparison of the period for recovery in patients with surgical site infection

Vicryl	28 ± 11 d
Silk	54 ± 30 d

$P < 0.05$.

absorbable sutures contributed significantly to the prevention of development of SSI. In our prospective study, the incidence of SSI in the Vicryl group was lower compared to the Silk group for both incisional SSI and organ/space SSI, however there were no other significant differences. Watanabe *et al*[12] reported the use of absorbable sutures in seromuscular suturing or intra-abdominal ligation was associated with a significantly lower incidence of SSI than non-absorbable sutures in the lower alimentary tract procedure, but not in the upper. The incidence of SSI is low so any significant difference is difficult to observe. The duration for recovery from SSI was significantly shorter in the Vicryl group than in the Silk group. Since SSI increases treatment costs and diminishes patient satisfaction markedly, the use of an absorbable suture is recommended in view of the medical economy and patient's quality of life.

The incidence of SSI as a form of postoperative infection was previously reported to be 20%-25%[8,13,14]. In our clinical experience, the incidence of SSI was observed to be 13.1% in this study and the rate of occurrence is decreasing every year because of SSI surveillance and development of surgical techniques such as closed suction drain and the prevention of bile leakage. Togo *et al*[8] reported the incidence of postoperative infection gradually decreased significantly with additional countermeasures. A larger sample size as well as a multi-institutional study is required for a randomized controlled study.

Intraoperative blood loss, long operation time and bile leakage were associated with SSI in our study. Both transfusion and operation time are reported to be risk factors of SSI in hepatectomy or other surgery[5,15]. Transfusion could induce immunosuppression in postoperative patients by reduction of the natural killer cell and cytotoxic T-cell populations[16,17]. Operation time could influence the concentration of systemic antibiotics, leading to the incidence of SSI. Bile leakage was observed at 4.8% (6/125) in this study. Generally the incidence of bile leakage was 4.0%-7.2% in recently reported large series[9,18-20]. The presence of bile, blood, and devitalized tissues in the dead space after hepatectomy may provide the ideal environment for bacterial growth and development of organ/space SSI, which frequently results in liver failure. To reduce organ /space SSI, it is most important to prevent bile leakage.

In conclusion, there were no significant differences between absorbable sutures and silk sutures on the incidence of SSI during the resection of the liver in this randomized control study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures. Further study is needed to provide evidence on the use of absorbable or non-absorbable suture materials in a multi-institutional randomized clinical trial.

COMMENTS

Background

Surgical site infection (SSI) is one of the most important morbidities of surgery and leads to prolonged hospital stays. In this paper the authors provide the outcome of a randomized clinical trial investigating the type of sutures used during hepatectomy to determine which is better in preventing SSI, absorbable sutures or non-absorbable sutures.

Research frontiers

During the resection of the liver, silk sutures are generally used for the vessels in Japan, because silk sutures are easier to handle and less expensive than absorbable sutures.

Innovations and breakthroughs

This is a randomized clinical trial investigating the type of sutures used during hepatectomy.

Applications

The incidence of SSI was not significantly different between absorbable sutures and silk sutures in this randomized controlled study; however, the period for recovery in patients with SSI was significantly shorter for absorbable sutures.

Terminology

Vicryl is an absorbable suture material and silk is a non-absorbable suture material, which is widely used in Japan.

Peer review

Authors report prospective randomized study of surgical site infection comparing silk to vicryl ties in liver resection.

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BRIEF ARTICLE

XPD Lys751Gln polymorphism and esophageal cancer risk: A meta-analysis involving 2288 cases and 4096 controls

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Abstract

AIM: To evaluate the association between xeroderma pigmentosum group D (XPD), genetic polymorphism Lys751Gln and esophageal cancer risk.

METHODS: We searched PubMed up to September 1, 2010 to identify eligible studies. A total of 10 case-control studies including 2288 cases and 4096 controls were included in the meta-analysis. Statistical analysis was performed with Review Manage version 4.2. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the strength of the association.

RESULTS: The results suggested that there is no significant association between XPD Lys751Gln polymorphism and esophageal cancer susceptibility in the overall population. However, in subgroup analysis by histology type, a significant association was found between XPD Lys751Gln polymorphism and esophageal adenocarcinoma (for CC *vs* AA: OR = 1.25, 95% CI = 1.01-1.55, P = 0.05 for heterogeneity).

CONCLUSION: Our meta-analysis suggested that XPD Lys751Gln polymorphism may be associated with increased risk of esophageal adenocarcinoma.

Key words: Xeroderma pigmentosum group D; Polymorphism; Esophageal cancer; Mwta-analysis

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Yuan L, Cui D, Zhao EJ, Jia CZ, Wang LD, Lu WQ. XPD Lys751Gln polymorphism and esophageal cancer risk: A meta-analysis involving 2288 cases and 4096 controls. *World J Gastroenterol* 2011; 17(18): 2343-2348 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i18/2343.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i18.2343

INTRODUCTION

Esophageal cancer, with a 5-year survival rate of < 20%, is considered as one of the most deadly malignancies[1,2]. It has already been identified that cigarette smoking, alcohol drinking, obesity, dietary factors, history of Barrett's esophagus, and esophageal reflux disease can contribute to the development of esophageal cancer[3-6]. However, only a fraction of exposed individuals develop esophageal carcinoma, which suggests that genetic variations in sensitivity to carcinogen exposure and DNA repair capacity

might be important inherited risk components in carcinogenesis[7,8]. DNA damage caused by exogenous, endogenous carcinogens or mutants is viewed as a crucial event in carcinogenesis. It can be repaired through activation of various pathways such as the nucleotide excision repair pathway (NER), base excision repair pathway (BER) and double-strand break pathway. The xeroderma pigmentosum group D (XPD) enzyme is involved in the NER pathway which plays an important role in the repair of bulky DNA adducts, such as pyrimidine dimmers, photoproducts and cross-links[9]. Several single-nucleotide polymorphisms (SNPs) have been identified in the XPD gene. Among them, a polymorphism in the XPD gene, codon 751 A to C, resulting in an amino acid alteration from lysine (Lys) to glycine (Gln) has been reported to be associated with an increased susceptibility to lung cancer, and head and neck carcinoma[10-12]. Other malignancies such as esophageal cancer have also been investigated.

To date, many molecular epidemiological studies have explored the association between XPD Lys751Gln polymorphism and esophageal cancer risk[12-23]. However, results of these studies are controversial, which may be caused by the limitation of individual studies. Therefore, we performed a meta-analysis of 10 published case-control studies covering 6384 subjects in order to get a more precise evaluation of the relationship between the XPD Lys751Gln polymorphism and esophageal cancer risk.

MATERIALS AND METHODS

Search strategy

We conducted a comprehensive search in the US National Library of Medicine's PubMed database (as of September 1, 2010) using search terms including "XPD", "xeroderma pigmentosum group D", "ERCC2", "excision repair cross-complementing rodent repair deficiency", "polymorphism", "esophageal", "esophagus" and the combined phrases for all genetic studies on the relationship between XPD polymorphism and esophageal cancer. Moreover, we reviewed the references from original articles to search for more studies. No language restrictions were imposed. Two investigators conducted all searches independently. Studies were absorbed in this meta-analysis if they met the following criteria: (1) a case-control study of the XPD Lys751Gln polymorphism and esophageal cancer risk; and (2) the authors must offer the size of the sample, odds ratios (ORs) with 95% confidence intervals (CIs) or the information that can help infer the results in the articles. If data were reported in more than one study, the most recent and complete study was chosen for this analysis.

Data extraction

Two investigators independently extracted data and reached a consensus on all of the items. Information was collected from each article, including the first author's name, year of publication, country of origin, racial descent of the subjects (categorized as Asian, European and mixed populations), sources of controls, genotyping method, histological type (categorized as esophageal adenocarcinoma and squamous cell carcinoma), number of different genotypes in cases and controls, Hardy-Weinberg equilibrium (HWE), and minor allele frequency in controls.

Statistical analysis

We assessed the strength of association between XPD Lys751Gln polymorphism and esophageal cancer risk by using ORs with 95% CIs which were obtained from the data given in the eligible studies. We evaluated the risk of codominant model (CC *vs* AA, CA *vs* AA), the dominant model (CA/CC *vs* AA), and recessive model (CC *vs* AA/CA), respectively. The between-study heterogeneity was investigated by Chi-square based Q-test[24], and it was considered significant if $P < 0.05$. The random-effects model (DerSimonian and Laird method) was then selected to pool the data[25]. Otherwise, the fixed-effects model (Mantel-Haenszel method) was used[26]. If heterogeneity was absent, these two models provided similar results. We used the funnel plot and the Egger weighted regression method ($P < 0.05$ was considered representative of statistical significance) to test possible publication bias in this meta-analysis[27]. All statistical analyses were performed in Statistical Analysis System software (v.9.13; SAS Institute, Cary, NC), and Review Manage (v.4.2; Oxford, England). All the tests were two-sided and the significant level was 0.05.

RESULTS

Eligible studies

A total of 12 potential relevant studies that described the association between the XPD genetic polymorphisms and esophageal cancer were retrieved through PubMed. After reading the full articles, one study by Liu *et al*[17] was excluded since the subjects had also been included in a study by Tse *et al*[20]. One other study was excluded because it did not list data clearly enough for further analysis[23]. Finally, we identified 10 eligible studies including 2288 cases and 4096 controls in total. As summarized in Table 1, four studies were conducted in Asians, four studies in Europeans, and two in mixed subjects. In terms of histology type, there were 4 studies of esophageal adenocarcinoma (EADC), 4 studies of esophageal squamous cell carcinoma (ESCC) and 2 of both EADC and ESCC. Diverse genotyping methods including PCR-RFLP, TaqMan and iPLEX™ were used. The classic PCR-RFLP assay was used in 60% (6/10) studies. Six studies mentioned the quality control. The genotype distributions in the controls of all the included studies were in accordance with HWE.

Meta-analysis

The main results of the meta-analysis on the association between XPD Lys751Gln polymorphism and esophageal cancer risk are shown in Table 2. Overall, no significant association was found between XPD Lys751Gln polymorphism and esophageal cancer risk (for CC *vs* AA: OR = 1.19, 95% CI = 0.84-1.69, $P = 0.01$ for heterogeneity, Figure 1; for CA *vs* AA: OR = 1.03, 95% CI = 0.83-1.27, $P =$

Table 1 Characteristics of case-control studies included in the meta-analysis

First author	Year	Country	Racial descent	Source of controls	Genotyping method	Histological type	Genotype distribution						*P* for HWE[1]	C
							Case			Control				
							AA	AC	CC	AA	AC	CC		
Xing	2002	China	Asian	Age matched	PCR-RFLP	ESCC[3]	367	63	3	451	70	3	0.87	0.07
Yu	2004	China	Asian	Age matched	PCR-RFLP	ESCC	108	16	11	133	17	2	0.11	0.07
Casson	2005	Canada	European	Randomly selected	PCR-RFLP	EADC[4]	31	21	4	34	46	15	0.93	0.40
Ye[2]	2006	Sweden	European	Age matched	PCR-RFLP	EADC	27	51	18	198	203	71	0.11	0.37
Ye[2]	2006	Sweden	European	Age matched	PCR-RFLP	ESCC	23	44	14	198	203	71	0.11	0.37
Sobti	2007	Indian	Asian	Age matched	PCR-RFLP	ESCC	52	61	7	63	77	20	0.64	0.37
Doecke	2008	Australia	Mixed	Age matched	iPLEXTM	EADC	108	124	31	575	588	174	0.22	0.35
Ferguson	2008	Ireland	European	Randomly selected	TaqMan	EADC	80	94	34	91	121	35	0.61	0.39
Tse	2008	America	Mixed	Age matched	TaqMan	EADC	104	159	49	193	208	52	0.72	0.34
Pan[2]	2009	America	European	Age matched	TaqMan	EADC	137	153	56	187	216	53	0.43	0.24
Pan[2]	2009	America	European	Age matched	TaqMan	ESCC	17	18	3	187	216	53	0.43	0.24
Zhai	2009	China	Asian	Age matched	PCR-RFLP	ESCC	167	31	2	148	51	1	0.12	0.13

[1]Hardy-Weinberg equilibrium (HWE) in controls; [2]The studies included esophageal adenocarcinoma (EADC), and esophageal squamous cell carcinoma (ESCC) for cases group, but controls were same; [3]Esophageal squamous cell carcinoma; [4]Esophageal adenocarcinoma. PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism.

Table 2 Summary odds ratios and 95% confidence interval of xeroderma pigmentosum group D Lys751Gln polymorphism and esophageal cancer risk

	CC *vs* AA		CA *vs* AA		CA/CC *vs* AA		CC *vs* CA/AA	
	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]
Total	1.19 (0.84-1.69)	0.01	1.03 (0.83-1.27)	0.01	1.05 (0.85-1.32)	0.01	1.16 (0.97-1.39)	0.06[2]
Ethnicity								
European	1.26 (0.95-1.65)	0.05[2]	1.00 (0.64-1.54)	0.01	1.01 (0.65-1.56)	0.01	1.20 (0.94-1.55)	0.28[2]
Asian	1.44 (0.37-5.66)	0.02	0.91 (0.71-1.15)	0.12[2]	0.96 (0.63-1.45)	0.03	1.47 (0.38-5.71)	0.02
Histological type								
EADC	1.25 (1.01-1.55)	0.05[2]	1.13 (0.85-1.52)	0.02	0.98 (0.68-1.41)	0.01	1.18 (0.97-1.44)	0.21[2]
ESCC	1.24 (0.56-2.70)	0.04	1.02 (0.73-1.41)	0.04	1.05 (0.74-1.50)	0.01	1.06 (0.71-1.58)	0.06[2]

[1]*P* value for heterogeneity; [2]Estimates for fixed-effects model. OR: Odds ratio; CI: Confidence interval.

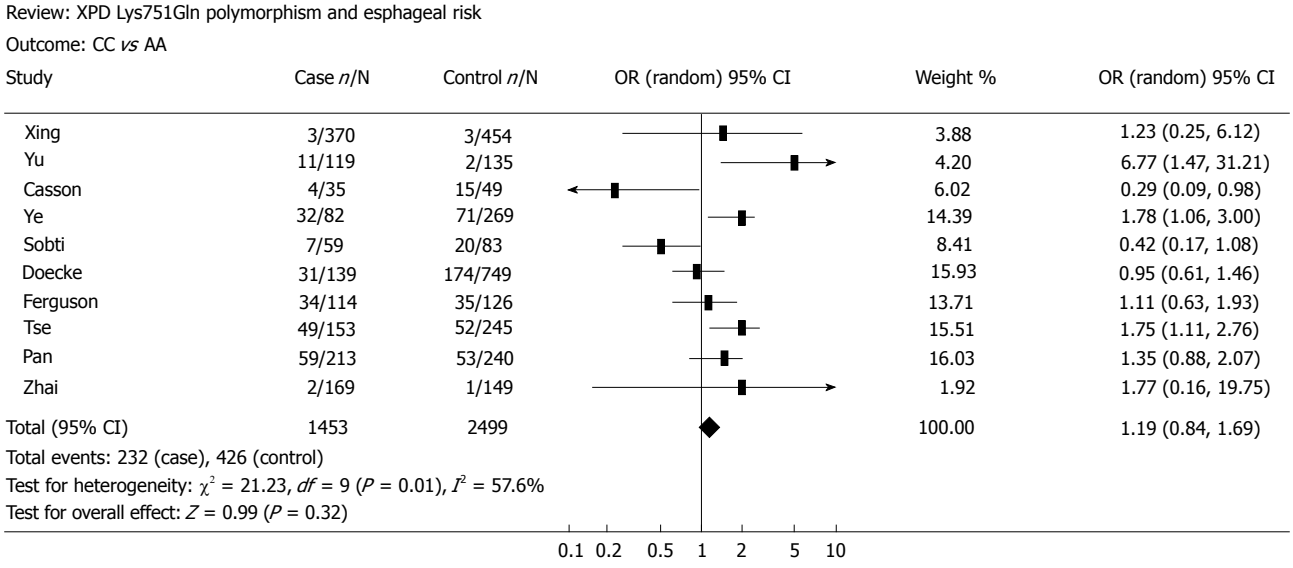

Figure 1 Odds ratio of esophageal cancer associated with xeroderma pigmentosum group D Lys751Gln polymorphism for the CC genotype compared with the AA genotype. XPD: Xeroderma pigmentosum group D; OR: Odds ratio.

0.01 for heterogeneity; for the dominant model CA/CC *vs* AA: OR = 1.05, 95% CI = 0.85-1.32, *P* = 0.01 for heterogeneity; for the recessive model CC *vs* CA/AA: OR = 1.16, 95% CI = 0.97-1.39, *P* = 0.06 for heterogeneity, Figure 2). In subgroup analysis by ethnicity, we also did not detect any significant association in all genetic models. However,

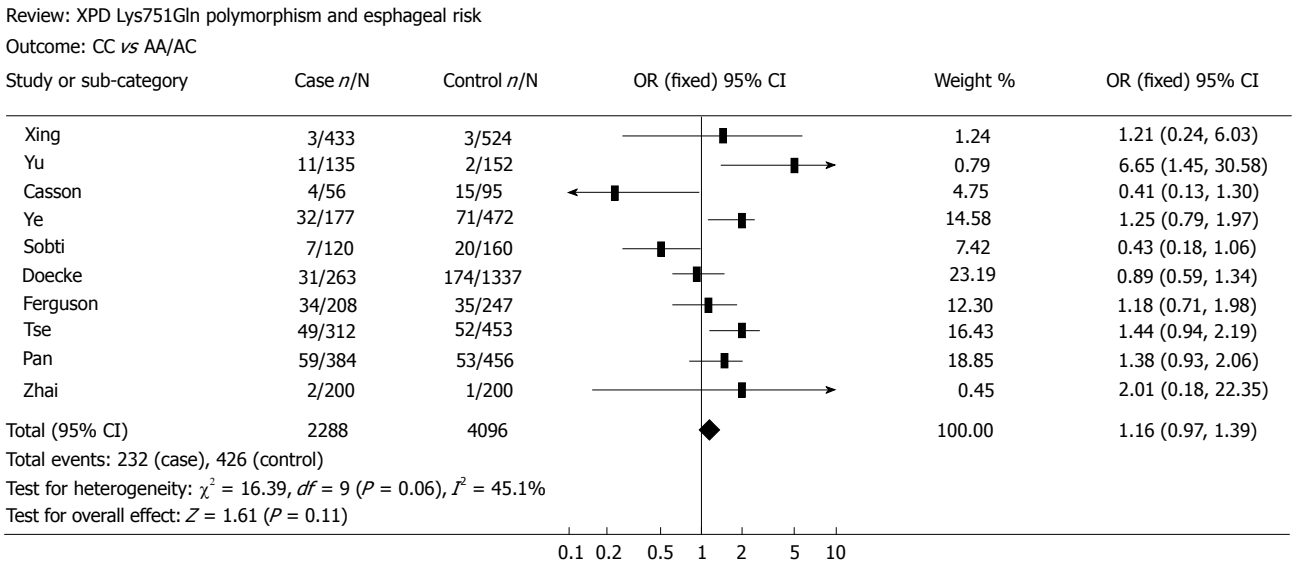

Figure 2 Odds ratio of esophageal cancer associated with xeroderma pigmentosum group D Lys751Gln polymorphism for the CC genotype compared with the AA/AC genotypes. XPD: Xeroderma pigmentosum group D; OR: Odds ratio.

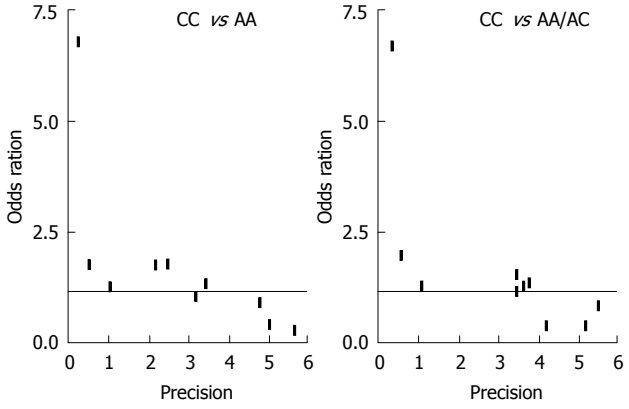

Figure 3 Funnel plot analysis to detect publication bias. Each point represents a separate study for the indicated association. The Odds ration is plotted on a logarithmic scale against the precision (the reciprocal of the SE).

further analysis by histological type revealed that individuals carrying the variant homozygote CC genotype showed an elevated risk to EADC compared to those with the wild-type AA genotype (OR = 1.25, 95% CI = 1.01-1.55, $P = 0.05$ for heterogeneity).

Publication bias

Funnel plot and the Egger's test were performed to assess possible publication bias. As shown in Figure 3, no publication bias was revealed by the funnel plots, which was approximately symmetrical for the codominant model CC *vs* AA and the recessive model CC *vs* CA/AA. Statistical evidence from the results of Egger's test confirmed the funnel plot symmetry (for CC *vs* AA: $t = 2.23$, $P = 0.06$; for CA *vs* AA: $t = 2.03$, $P = 0.08$; for CA/CC *vs* AA: $t = 1.48$, $P = 0.18$; for CC *vs* CA/AA: $t = 2.33$, $P = 0.05$).

DISCUSSION

Through analyzing data from the 10 eligible studies on relationship between XPD Lys751Gln polymorphism and esophageal cancer risk, we found no significant association between XPD Lys751Gln polymorphism and esophageal cancer risk in overall population. However, in the stratified analysis according to histological type, positive association were observed between XPD Lys751Gln polymorphism and elevated susceptibility to EADC.

The XPD gene has been mapped in chromosome 19q13.3. It spans over 20 kb, contains 23 exons and encodes the 761-amino acid protein. The XPD protein possesses both single-strand DNA-dependant ATPase and 5'-3' DNA helicase activities, which is essential for NER pathway and transcription[28]. The NER pathway generally removes bulky adducts caused by exogenous carcinogens, especially from cigarette smoking which is a well defined risk factor for EADC[29]. Any functional variation in NER pathway such as SNPs of key repair genes may lead to a deficiency in the DNA repair capacity (DRC) which is associated with a higher risk of cancer[28,30-32]. Benhamou *et al*[33] found that the single nucleotide substitution from A to C at codon 751 in the XPD gene leads to a complete change in the electronic configuration of the resulting amino acid, and reduces DNA repair efficiency. Many epidemiological studies have investigated the association between XPD Lys751Gln polymorphism and esophageal cancer, but the results were inconclusive. Xing *et al*[12] first explored the polymorphisms of DNA repair gene XPD and their associations with risk of esophageal squamous cell carcinoma in a Chinese population, but a Lys751Gln polymorphism in the XPD gene did not influence risk of ESCC in this study. However, two other studies on the relationship between XPD Lys751Gln polymorphism and ESCC revealed a contradictive result which suggested an increased risk of ESCC in association with the XPD 751 Gln/Gln genotype[13,15]. The more interesting finding revealed by Zhai *et al*[22] suggested an inverse association, which indicated that the XPD codon 751Gln allele was a protective factor rather than a risk factor to ESCC (OR = 0.628, 95% CI = 0.400-0.986). The first study

on the association between XPD 751 codon polymorphism and EADC was conducted by Casson, who observed the protective effect of the homozygous variant of XPD Lys-751Gln for EADC (OR = 0.24, 95% CI = 0.07-0.88)[14]. However, this result has not been supported by more studies. Both studies of Ye *et al* and Tse *et al* suggested that the XPD 751Gln allele was associated with an elevated risk for esophageal adenocarcinoma which is consistent with the result of our meta-analysis.

Some limitations of our meta-analysis should be acknowledged. Firstly, though it is known that the XPD gene has more polymorphisms than just Lys751Gln, we focused our meta-analysis on the most studied Lys-751Gln polymorphism due to limited evidence on others. Secondly, some studies on this relationship were modified by some other potentially suspected factors such as BMI, smoking status, alcohol consumption, history of gastroesophageal reflux disease and lifestyle; however, our results were based on unadjusted estimates due to a lack of the original data. Finally, the XPD gene may influence susceptibility to esophageal cancer with other genes, but we did not conduct the gene-gene interactions analysis in our study.

In conclusion, our meta-analysis suggested that XPD Lys751Gln polymorphism may be a risk factor for esophageal adenocarcinoma. Large and well designed epidemiological studies will be necessary to combine genetic factors together with other potential risk factor such as smoking status, alcohol consumption and history of gastroesophageal reflux disease in order to validate the relationship between XPD Lys751Gln polymorphism and esophageal cancer risk.

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COMMENTS

Background

Esophageal cancer is one of the most deadly malignancies. Many studies have explored the association between the Xeroderma pigmentosum group D (XPD) genetic polymorphism Lys751Gln and esophageal cancer risk, but the results are inconclusive and even controversial. It is, therefore, necessary to perform a meta-analysis in order to get a more precise evaluation of the relationship between the XPD Lys751Gln polymorphism and esophageal cancer risk.

Research frontiers

The XPD gene is responsible for bulky adducts and repair of UV-induced DNA damage. To date, there have been many case-control studies on the association between XPD Lys751Gln and esophageal cancer risk, but few meta-analyses were conducted on this topic.

Innovations and breakthroughs

Our meta-analysis suggested that XPD Lys751Gln polymorphism might alter individuals' susceptibility to esophageal adenocarcinoma. Further studies are needed to confirm it.

Applications

The finding that individuals carrying the variant homozygote CC genotype showed an elevated risk to esophageal adenocarcinoma indicates that genetic variations in the DNA repair protein may contribute to the risk of EADC. This meta-analysis gave a structured and systematic integration of information on the etiology of esophageal cancer, and the result may provide valuable information for researchers and clinicians.

Peer review

This is a review of the ten previously published association studies and extracted the importance of esophageal adenocarcinoma. This information is worthwhile.

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CASE REPORT

Lapatinib-induced hepatitis: A case report

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Abstract

Lapatinib is an inhibitor of the tyrosine kinases of human epidermal growth factor receptor type 2 (HER2) and epidermal growth factor receptor type 1, with clinical activity in HER2-positive metastatic breast cancer. We present here a 60 year-old patient with metastatic breast cancer who presented with jaundice and increased serum aminotransferase levels and who had been treated with lapatinib for the previous 14 days. Laboratory tests excluded other causes of acute liver injury. Liver biopsy revealed lesions compatible with drug-induced hepatotoxicity. Bilirubin and liver enzymes returned to normal within three months of lapatinib discontinuation. Lapatinib should be included among the causes of drug-induced hepatitis.

Key words: Lapatinib; Hepatitis; Hepatotoxicity; Breast cancer; Human epidermal growth factor receptor type 2

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INTRODUCTION

Metastatic breast cancer is the leading cause of death from cancer among women worldwide[1]. The overexpression of human epidermal growth factor receptor type 2 (HER2) predisposes patients to a greater risk for disease progression and death than women whose tumors do not overexpress HER2[2]. Therapeutic approaches to block HER2 signaling pathways include both trastuzumab (a recombinant, humanized, monoclonal antibody that binds to the extracellular domain of the HER2) and lapatinib. Lapatinib is an orally administered small molecule that inhibits the tyrosine kinases of HER2 and epidermal growth factor receptor type 1 (EGFR). A number of studies have shown that lapatinib has clinical activity in patients with HER2-positive breast cancer, with a significant reduction in the risk of disease progression[3]. Lapatinib is generally well tolerated and the most common treatment-related adverse events include rash, diarrhea, and nausea[4].

We report here a case of advanced breast cancer treated with lapatinib with drug induced hepatitis. Upon discontinuation of lapatinib, levels of serum aspartate amino transferase (SGOT) and serum alanine amino transferase (SGPT) declined progressively.

CASE REPORT

A 60 year-old woman presented with metastatic breast cancer in the lung. Three years earlier she was diagnosed with invasive ductal adenocarcinoma of the right breast

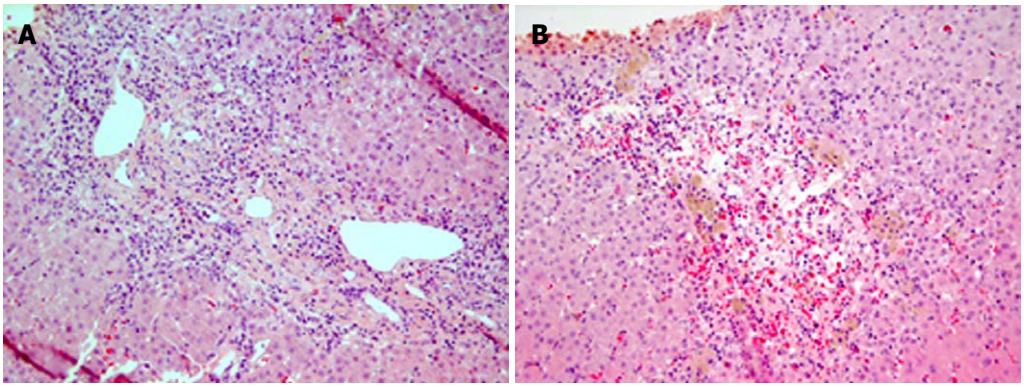

Figure 1 Photomicrograph. A: Areas of bridging necrosis (HE x 200); B: Areas of centrilobular hepatocellular dropout, hemorrhage and macrophages (HE x 200).

(pT1cN0M0). Immunohistochemistry was positive for estrogen (70% positive cells), progesterone (2% positive cells) receptors and for HER-2. She was treated with lumpectomy and axillary lymph node dissection. Subsequently she received adjuvant chemotherapy (four cycles of epirubicin/cyclophosphamide followed by 4 cycles of paclitaxel) and treatment with the monoclonal antibody trastuzumab for one year. Furthermore, radiotherapy was given to the entire breast as an adjunct to breast conservation treatment and hormonal therapy with the aromatase inhibitor exemestane was started. She remained free of disease for three years.

In terms of periodic reassessment, the patient had a computed tomography (CT) of the chest in January 2010 and was found to have three nodules in the right lung compatible with metastatic sites. A CT of the brain and abdomen showed no abnormalities and a mammogram of both breasts and a bone scan were also normal. According to laboratory data, there were no abnormal values.

The patient commenced therapy with capecitabine (1000 mg/m^2 twice daily, day 1-14) and lapatinib (1250 mg/d), while exemestane was discontinued after two and a half years of continuous administration. After ten days, capecitabine was discontinued due to grade 2 diarrhea and the patient continued to receive lapatinib only.

Two weeks later, the patient developed jaundice without any other clinical signs such as asthenia or pruritus and she was evaluated in the Department of Clinical Oncology.

Physical examination was unremarkable except for jaundice and mild hepatomegaly. Laboratory results showed: SGPT 583 U/L units (normal range: 5-45), SGOT 457 U/L (normal range: 5-40), ALP 348 U/L (normal limits < 270), g-glutamyl transpeptidase 213 U/L (normal range: 10-55), total bilirubin 4.1 mg/dL (normal range: 0-1.5), LDH 305 units (normal range: 100-240), total protein 6.4 g/dL (normal range: 6-8.4), albumin 3.7 g/dL (normal range: 3.4-5), white blood cells 7500/mm^3, Hb 12.8 g/dL, Hct 38.8%, platelets 118000/mm^3 and international normalized ratio-1.14 (normal range: 0.8-1.2). Other blood chemistry results, including glucose, cholesterol, triglycerides, serum amylase, uric acid, creatinine, BUN, Na, K, Ca as well as urinalysis were normal.

Serology tests for hepatitis A, B, and C viruses were negative. Immune serology was also negative for ANA, anti-DNA, c-ANCA, p-ANCA, antismooth muscle, and antimitochondrial antibodies. Furthermore, thyroid hormone tests, a1-antitrypsin, IgG, IgA, IgM and blood ceruloplasmin levels were within normal limits.

Abdominal ultrasound demonstrated an increased-sized liver with non-homogeneous and diffuse echogenicity, without biliary tract abnormalities. Normal blood flow was seen in the portal vein, hepatic artery, hepatic veins, and inferior vena cava. A new abdominal CT showed hepatomegaly without biliary tract obstruction.

Fifteen days after the clinical evolution, a liver biopsy was performed and showed necrosis of contiguous hepatocytes in portal-to-portal and portal-to-central fashion (bridging necrosis). Foci of severe hemorrhage and hepatocellular dropout around the centrilobular areas were also demonstrated (Figure 1). Eosinophils were observed in more than twelve portal spaces without granulomas.

The patient was not taking other concomitant hepatotoxic medications, herbal or dietary supplements and did not have any underlying liver dysfunction, chronic hepatitis or alcohol use history.

Lapatinib was discontinued and both the patient's jaundice and liver function tests gradually improved and returned to normal range within 3 mo (Table 1).

DISCUSSION

Lapatinib combined with capecitabine has been approved for the treatment of patients with HER-2 positive metastatic breast cancer improving median time to disease progression[3].

Furthermore, recent studies suggest that single-agent lapatinib has clinical activity with manageable toxicity in HER2-overexpressing breast cancer that progressed on trastuzumab-containing therapy[4,5]. The fact that lapatinib inhibits EGFR signaling may also contribute to its activity in the context of refractory HER2-positive breast cancer.

No significant liver dysfunction has been recorded to lapatinib administration in any daily dose (500-1600 mg) in many phase I and II studies[5,6]. Hence, grade 3 and 4 liver toxicity with elevations in transaminases are uncommon after single agent lapatinib administration and only one out

Table 1 Evolution of laboratory tests after lapatinib discontinuation

	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	GGT (U/L)	TBIL (mg/dL)
Baseline (before initiation of capecitabine/lapatinib)	24	21	120	11	0.8
Ten days later (capecitabine discontinuation due to diarrhea)	30	22	110	20	0.7
Two weeks after capecitabine discontinuation (lapatinib discontinuation)	583	457	348	213	4.1
Two weeks after lapatinib discontinuation	481	589	310	294	11.8
1 mo after lapatinib discontinuation	260	238	318	160	2.7
2 mo after lapatinib discontinuation	229	180	298	140	2.3
3 mo after lapatinib discontinuation	44	36	246	42	0.9

SGPT: Serum glutamic pyruvic transaminase; SGOT: Serum glutamic oxaloacetic transaminase; ALP: Alcaline phosphatase; GGT: Gamma-glutamyl transpeptidase; TBIL: Total Bilirubin.

of 37 patients experienced grade 3 elevation of transaminases in a phase II trial in patients with brain metastases from HER-2 positive breast cancer[7].

Lapatinib is predominantly metabolized in the liver *via* the cytochrome P450 system, by the enzyme P450 3A4 and < 2% of the drug is excreted unchanged in urine.

Concurrent administration of the strong CYP3A4 inhibitor ketoconazole increases the lapatinib area under the curve and prolongs the $t_{1/2}$. For this reason, strong inhibitors of CYP3A4, including grapefruit juice, should be avoided, as they may increase plasma concentrations of lapatinib and thus lapatinib toxicity[8].

Furthermore, it can be assumed that polymorphisms of the CYP3A4 gene may affect lapatinib disposition[9]. However, pharmacogenomical studies on the same gene did not show any correlation between gene polymorphisms and commonly observed toxicities such as exanthema or diarrhea caused by the use of erlotinib, an epidermal growth factor receptor tyrosine-kinase inhibitor[10].

The patient described in our case developed severe hepatic enzyme disturbances during lapatinib treatment and liver biopsy showed acute drug-induced hepatitis.

The patient did not take any other drug except lapatinib and recovered when the treatment was discontinued. There was no evidence of viral or autoimmune hepatitis or any other cause of hepatitis. Hepatic enzymes normalized rapidly after discontinuation of lapatinib.

The time to onset of jaundice and laboratory test abnormalities as well as the time and course of recovery, while the patient did not receive other medications, led us to support the idea that lapatinib is the most likely cause of hepatic injury. Exemestane appears not to be an important risk factor for the development of hepatitis because this medication was administered for two and a half years, without any clinical symptoms or abnormal laboratory tests.

Based on the fact that capecitabine was stopped only two weeks before the onset of symptoms, it was necessary to assesss the likelihood of the agent being causative. Capecitabine has not been implicated in hepatocellular injury and hepatitis with the specific histological features. Furthermore in order to confirm that liver injury was due to lapatinib, we used the Roussel Uclaf Causality Assesment Method, a scoring system which assigns attribution for drug-induced liver injury[11]. The summed points grouped lapatinib into highly probable category, confirming our suggestion.

Our case demonstrates the potential risk of developing toxic hepatitis during treatment with lapatinib.

To conclude, to our knowledge this is the first case of acute drug-induced hepatitis secondary to lapatinib. Given that lapatinib is now often used in the treatment of HER-2 positive advanced breast cancer, it is mandatory for medical oncologists to be aware of this potential side effect in clinical practice.

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CASE REPORT

Transarterial injection of H101 in combination with chemoembolization overcomes recurrent hepatocellular carcinoma

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Abstract

Transcatheter arterial chemoembolization (TACE) has become the standard treatment modality for unresectable hepatocellular carcinoma (HCC). Nonetheless, the clinical outcomes in patients with unresectable HCC are often unsatisfactory, especially in those with recurrent HCC. H101, an E1B gene deleted adenovirus, is known to have a significant antitumor activity. In addition, local injection of H101 can enhance the effect of antitumor therapies (chemotherapy and radiotherapy). Transarterial H101 gene injection in combination with TACE may help to control refractory and recurrent HCC. In this study, we report a 55-year-old patient with recurrent HCC which was treated with transarterial injection of H101 in combination with TACE, leading to a good clinical prognosis of the patient.

Key words: Hepatocellular carcinoma; H101; Transcatheter arterial chemoembolization; Therapy

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INTRODUCTION

It is difficult to eradicate hepatocellular carcinoma (HCC) because of its repeated recurrence in the liver. Surgical resection and transcatheter arterial chemoembolization (TACE) are the mostly used effective modalities for HCC, but it still frequently recurs after treatment with such modalities[1,2]. Gene therapy for tumors is a new hope in the 21st century. It is coming to be widely used in clinic and has achieved relatively well results especially when it is directly injected into the tumor[3-9]. Since combined TACE and gene injection may help to manage recurrent HCC, we tried to use the H101 gene in combination with TACE to treat our patient with recurrent HCC and achieved a good clinical prognosis.

CASE REPORT

A 55-year-old man was diagnosed with HCC in the right liver lobe, which was histologically proven (Figure 1A) in June 2006. The patient underwent six rounds of TACE from June 2006 to September 2008 (Figure 1B). His α-fetoprotein (AFP) level was 595.4 ng/mL after the six rounds of TACE, and decreased to 10.99 ng/mL after a partial hepatectomy. In February 2009, a recurrent nodule was found in the remnant liver at a routine postoperative computed tomography (CT) scan. At that time, his AFP level was 724 ng/mL. Because the effect of TACE was poor on HCC (Figure 2A), we decided to treat the patient with combined TACE and H101, a recombinant human type-5 adenovirus (Ad5), in which the E1B-55 kDs gene

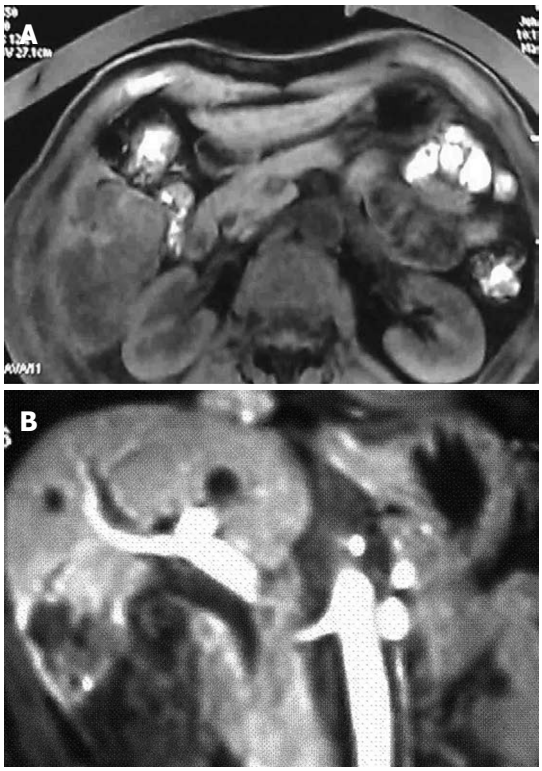

Figure 1 Magnetic resonance imaging. A: A hypodense nodule and hepatocellular carcinoma before therapy; B: A hypodense nodule with partial contrast enhancement before hepatectomy.

Table 1 Schedule of therapy and change in follow-up α-fetoprotein

Time (yr-mo)	APF (ng/mL)	TACE (yes/no)	H101 (yes/no)	Resection (yes/no)
2006-06	1210.00	Yes		
2006-07	143.20	Yes		
2007-09	70.00	Yes		
2007-11	21.67	No		
2008-03	355.60	Yes		
2008-04	491.16	No		
2008-06	1390.00	Yes		
2008-07	955.10	No		
2008-08	1072.00	Yes		
2008-09	595.40	No		Yes
2008-10	10.99	No		
2009-02	352.30	Yes		
2009-03	724.00	Yes	Yes	
2009-05	338.40	Yes	Yes	
2009-07	4.56	Yes	Yes	
2010-12	2.28	No		

APF: α-fetoprotein; TACE: Transcatheter arterial chemoembolization.

is totally deleted (Oncorine, Shanghai Sunway Biotech, China). First, we injected 5-fluoro-2-deoxyuridine (1.0 g), vinorelbine (40 mg), and cisplatin (80 mg) into the celiac trunk. Then, H101 and iodized oil were injected into the artery that supplies the tumor. A total of 1×10^{12} virus particles (VP) and 10 mL iodized oil were administered.

The patient had no discomfort after the procedure. Two months later, routine follow-up CT showed a fairly good result (Figure 2B) and the AFP level in the patient decreased to 338.4 ng/mL from 724 ng/mL before the treatment. We repeated this therapy 2 times at 2-mo interval. At the last admission, abdominal CT demonstrated complete deposit of oil with no signs of recurrence (Figure 2C). Furthermore, his AFP was within the normal reference range (4.56 ng/mL).

Eighteen months following the last H101/TACE treatment, the patient showed no evidence of recurrence and no abnormal liver function, but had a normal serum AFP level. The AFP levels in the patient during the therapy are listed in Table 1.

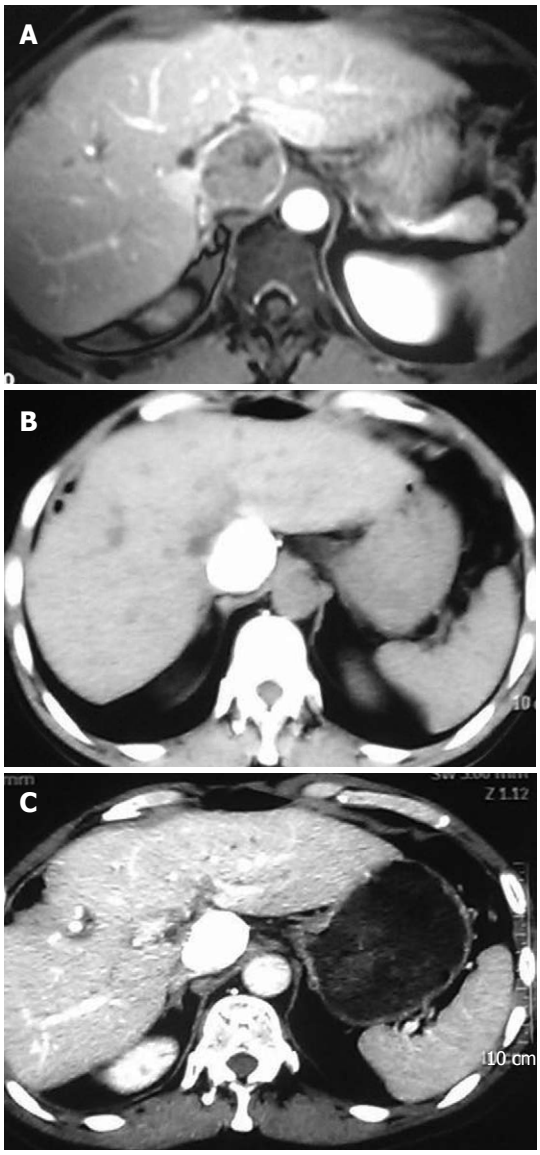

Figure 2 Contrast magnetic resonance imaging. A: A hypodense nodule with circle contrast manifestation after hepatectomy and the seventh Transcatheter arterial chemoembolization; B: Contrast computed tomography showing homogeneous dense retention of lipiodol within the entire tumor mass two months after treatment; C: Contrast computed tomography no recurrent mass 4 mo after treatment.

DISCUSSION

Although TACE has become the standard treatment modality for unresectable HCC, it is frequently unsuccessful[1,2].

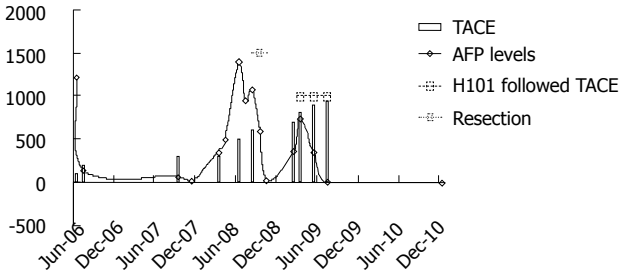

Figure 3 Changes in α-fetoprotein levels during and after treatment.

Similarly, HCC recurs frequently following its resection. Although TACE was effective in our patient, HCC recurred and failed the seventh TACE. The recurred HCC was unresectable because it occupied the entire caudate lobe and was close to the vena cava and abdominal aorta. Thus, H101 in combination with TACE was attempted for the patient with a fairly good outcome without any complications. From Table 1 and Figure 3, we can see that the AFP level in the patient decreased significantly following treatment with TACE plus H101. Both the initial series of TACE and tumor resection failed to prevent recurrent HCC although AFP was controlled, whereas combined H101 and TACE appeared to be effective and well-tolerated.

H101 is a recombinant human type-5 adenovirus in which E1B-55 kDs gene is totally deleted. The H101 virus produced by Shanghai Sunway Biotech also contains a deletion in the E3 region with a significant antitumor activity. This recombinant adenovirus has a replication-selective property and replicates only in tumor cells. Before modification, the E1B region of the wild adenovirus type 5 expresses early gene products that bind to and inhibit the function of p53, a tumor suppressor. Deletion or mutation of the E1B region confers p53-selective replication of oncolytic viruses which infect tumor cells and induce massive accumulation of normal p53. By this way, the adenovirus causes direct cytotoxicity only to tumor cells during replication. The E3 region is related to the inhibition of host immunity, which enhances the virus replication and spread in tumors[3,4]. The virus replication and spread can be enhanced by repeated injection of H101. By sacrificing the spread ability, the virus may activate the host immune response to virus-infected tumor cells and help the host immune system to recognize tumor cells, thus benefiting patients under such a therapy. Metastasis is prevalent in patients with malignant tumors, leading to treatment failure and death of patients. Moreover, patients may have more than one tumor lesion, and some of these lesions may be hard to reach in order to be injected with H101. Therefore, the ability of H101 to activate the host immune response seems crucial. Treatment with the E3 region deleted adenovirus, H101, may have additional benefits to patients.

H101 is formulated as a sterile viral solution in phosphate buffered saline and kept at -20℃. Each vial contains 0.5 mL virus solution with 5×10^{11} viral particles and titered less than 1/60 of 50% tissue culture infectious dose. Sterile and purified viruses were produced for clinical use by Shanghai Sunway Biotech (Shanghai, China), and tested for the titer, sterility, and general safety by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Its safety has been demonstrated in a number of clinical trials[5-8].

Although the anticancer activity of H101 has been proved in a wide type of advanced cancer by intra-tumor injection, its clinical efficacy against liver cancer has been rarely reported[9-11]. Transcatheter arterial injection of H101 was effective against liver cancer in our patient, suggesting that H101 in combination with TACE is useful for recurrent HCC. However, subsequent large, multi-center randomized, controlled studies are needed to facilitate the introduction of genetically engineered and reinforced viruses as novel therapeutic platforms for the treatment of cancers.

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Prophylactic antibiotics for variceal hemorrhage: Clostridium difficile infection still can be a risk

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Abstract

Bron *et al* presented a retrospective study regarding the prophylactic use of antibiotics for variceal hemorrhage. Antibiotics appeared to improve the survival rate of patients without increasing clostridium difficile infection (CDI). We argue against the conclusion of the authors and consider that this result may be simply due to concurrent use of metronidazole, a therapeutic agent against CDI.

Key words: Variceal hemorrhage; Prophylactic antibiotics; Clostridium difficile infection

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TO THE EDITOR

Brown *et al*[1] presented a retrospective study regarding prophylactic use of antibiotics in treatment of variceal hemorrhage. The data show that antibiotics appear to improve the survival rate of patients without increasing Clostridium difficile infection (CDI). However, 70.3% of the patients who were given antibiotics received metronidazole, a therapeutic agent against CDI. No apparent increase in CDI may be simply due to the suppression of the organism by metronidazole. As pointed out in the article, currently recommended antibiotic for this purpose is ceftriaxone[2,3], which is known to predispose to CDI[4]. The result of the article should not be interpreted as the currently recommended use of ceftriaxone posing a low risk of CDI.

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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

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

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

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

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