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EDITORIAL

Biofeedback therapy for dyssynergic defecation

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Abstract

Dyssynergic defecation is one of the most common forms of functional constipation both in children and adults; it is defined by incomplete evacuation of fecal material from the rectum due to paradoxical contraction or failure to relax pelvic floor muscles when straining to defecate. This is believed to be a behavioral disorder because there are no associated morphological or neurological abnormalities, and consequently biofeedback training has been recommended for treatment. Biofeedback involves the use of pressure measurements or averaged electromyographic activity within the anal canal to teach patients how to relax pelvic floor muscles when straining to defecate. This is often combined with teaching the patient more appropriate techniques for straining (increasing intra-abdominal pressure) and having the patient practice defecating a water filled balloon. In adults, randomized controlled trials show that this form of biofeedback is more effective than laxatives, general muscle relaxation exercises (described as sham biofeedback), and drugs to relax skeletal muscles. Moreover, its effectiveness is specific to patients who have dyssynergic defecation and not slow transit constipation. However, in children, no clear superiority for biofeedback compared to laxatives has been demonstrated. Based on three randomized controlled studies in the last two years, biofeedback appears to be the preferred treatment for dyssynergic defecation in adults.

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Key words: Biofeedback; Constipation; Pelvic floor dyssynergia; Dyssynergic defecation; Functional defecation disorders; Randomized controlled trials

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INTRODUCTION

Chronic constipation is a common self-reported bowel symptom that affects 2%-30% of people in Western countries and has considerable impact on health expenses and quality of life[1]. Most patients respond either to fiber-fluid supplementation or to judicious use of laxatives[1]. Among the non-responders, outlet dysfunction type constipation seems particularly common; it affects up to 50% of referrals to a tertiary care center[2].

Patients with outlet dysfunction can be divided into those with structural causes for obstructed defecation and patients with a functional defecation disorders[3,4]. Possible structural causes for obstructed defecation include stricture, neoplasia, rectocele, enterocele, and Hirschprung's disease. Functional defecation disorders include dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor and anal muscles during defecation) and inadequate defecatory propulsion (i.e., insufficient intra-rectal pressure due to inadequate contraction of abdominal wall muscles during defecation); both may lead to inadequate emptying of the rectum[4]. It is unclear whether idiopathic megarectum is associated with dyssynergic defecation. Functional defecation disorders are believed to be more common than obstructed defecation and approximately as common as slow transit constipation; however, the true prevalence of these subtypes of constipation has not been documented. Functional defecation disorders may coexist with slowed transit through the colon. Dyssynergic defecation is commonly considered to be a form of maladaptive behavior because there is no discernable neurological or anatomical defect and because it can be eliminated by behavioral training[4].

Diagnostic criteria for functional defecation disorders[4] include those for functional constipation[5], namely two or more of 6 symptoms present for the last 3 mo with an onset more than 6 mo in the past; the symptoms are straining, lumpy or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction/blockage, or manual maneuvers to facilitate defecation on more than 1/4 of bowel movements, or less than 3 bowel movements per week. To meet criteria for functional defecation disorders, the patient must also undergo

objective diagnostic testing and demonstrate at least two of three abnormalities: impaired evacuation of the rectum, inappropriate contraction or less than 20% relaxation of the pelvic floor muscles, and inadequate propulsive forces during defecation[4].

An exhaustive explanation of the diagnostic work up of these patients is beyond the scope of this review. However, most normal subjects can easily evacuate a 50 mL water-filled balloon from the rectum. Additional anorectal testing includes anorectal manometry, anal electromyography (EMG) and evacuation proctography (defecography)[4]. Anorectal manometry provides a comprehensive assessment of anal pressures, rectoanal reflexes, rectal pressures, sensation and compliance. Several types of recording devices are available, but perfused catheters and balloon probes are among the most commonly used. A paradoxical increment in anal pressure on straining efforts is a distinctive feature of dyssynergic defecation[4]. An increment in muscle motor activity on straining may be demonstrated by means of EMG either by intra-anal electrodes or by electrodes taped to the peri-anal skin.

Defecography is a radiographic test providing morphological and functional information on the ano-rectum. Several parameters may be assessed, such as pelvic floor descent, anorectal angle, rectocele, and rectal prolapse. Failure of the anorectal angle to become more oblique on straining provides indirect evidence of defective pelvic floor relaxation and impaired evacuation of contrast material is also suggestive of dyssynergia[4]. There must be manometric, EMG, or radiologic evidence for inappropriate contraction or failure to relax the pelvic floor muscles on straining to diagnose dyssynergic defecation according to Rome Ⅲ criteria[4].

Evaluation of colon transit by means of radiopaque markers is not relevant to the diagnosis, but it may be performed to additionally test for slow transit constipation.

Patients with functional defecation disorders are often unresponsive to conservative medical management, and the surgical division of the pubo-rectalis muscle (which has been proposed for the treatment of dyssynergic defecation) has resulted in poor benefit and an unacceptable risk of anal incontinence[1,6]. Treatment with botulinum toxin injection may provide temporary improvement, but it remains an investigational treatment. Therefore, behavioral treatment is a logical choice for these disabled patients[4].

Biofeedback is a conditioning treatment where information about a physiologic process (contraction and relaxation of a muscle) is converted to a simple visual or auditory signal to enable the patient to learn to control the disordered function. Biofeedback is considered appropriate when specific pathophysiological mechanisms are known and the voluntary control of responses can be learned with the aid of systematic information about functions not usually monitored at a conscious level[7]. As early as 1979, Thomas Almy and John Corson, in an enthusiastic editorial about the biofeedback treatment of fecal incontinence, pioneered the extension of behavior therapy to functional defecation disorders[8]. However, the first paper dealing with the subject included only a small number of subjects and was not published until 1987 due to the preference for conservative, drug-oriented therapy[9].

BIOFEEDBACK TECHNIQUES FOR TREATING DYSSYNERGIC DEFECATION

Paradoxical increases in anal pressure and electromyographic (EMG) activity during straining is easily detected[10,11]. Anal pressure may be measured by means of water-perfused catheters, solid state transducers or balloon catheters[10]. No single technique seems superior to the others, and the choice relies on the researcher's training and experience.

Anal EMG may be recorded either by intra-anal probes or by peri-anal EMG electrodes stuck to the skin[10,11]. The EMG activity used in biofeedback training is the averaged activity of large numbers of muscle cells rather than the activity of small groups of muscle cells innervated by a single axon. This averaged EMG activity is recorded with large electrodes on the skin or the mucosa of the anal canal rather than with needle electrodes. Averaged EMG recorded in this way is proportional to the strength of contraction of the underlying muscles.

Defective expulsion is commonly investigated by asking the patient to defecate a 50-mL water-filled rectal balloon; patients with functional defecation disorders usually fail this test[11]. Some patients also have a higher threshold for perceiving the urge to defecate[10], but the clinical significance of this sensory dysfunction is ill-defined, in contrast to the relevance of rectal sensory impairment in fecal incontinence[12]. Ano-rectal imaging studies (defecography, ultrasonography, and pelvic floor MRI) may also help to characterize the physiological dysfunction responsible for outlet dysfunction, but they do not seem to influence treatment outcome[4,13].

Biofeedback training protocols vary among different centers[10,11]. In the next paragraph, a standard biofeedback protocol is described and differences in biofeedback procedures are outlined. A mainstay of behavior therapy is to first explain the anorectal dysfunction and discuss its relevance with the patient before approaching the treatment[3,11]. Most protocols would then include training the patients on a more effective use of the abdominal muscles to improve pushing effort. Patients are next shown anal manometry or EMG recordings displaying their anal function and are taught through trial and error to relax the pelvic floor and anal muscles during straining[10,13]. This objective is first pursued with the help of visual feedback on pelvic floor muscle contraction, accompanied by continuous encouragement from the therapist. When the patient has learned to relax the pelvic floor muscles during straining, the visual and auditory help are gradually withdrawn[10,13]. Another retraining option is to simulate defecation by means of an air-filled balloon attached to a catheter, which is slowly withdrawn from the rectum while the patient concentrates on the evoked sensation and tries to facilitate its passage[3,11]. In the next phase of training, the patient is taught to defecate the balloon by bearing down, without the assistance of the therapist. Some centers also add a balloon sensory retraining to lower the urge perception threshold[14]. The number of

training sessions is not standardized, but 4 to 6 sessions are frequently provided. Individual training sessions last 30 to 60 min.

Therapeutic sessions are professionally demanding and a highly trained and motivated therapist is essential. No study has addressed the necessary training required for an individual to administer biofeedback therapy. Particularly, it is unclear whether the adequate provider should be either physician, psychologist, or nurse. Experience varies among centers, but the low cost reimbursement provided for behavior therapy is likely to influence future choices.

Controlled studies systematically comparing different biofeedback protocols to each other are lacking. However, a recent meta-analysis showed that in open label studies, the mean success rate with pressure biofeedback was slightly greater than with EMG biofeedback (78% *vs* 70%)[13]. No differences were found between anal *vs* peri-anal EMG recording. In addition, adding balloon feedback did not seem to influence the therapeutic outcome[13]. However, the majority of studies in the last ten years have utilized EMG biofeedback rather than pressure feedback even in the absence of scientific evidence[13]. There are no standardized protocols, and centers use different combinations of laboratory EMG training, home EMG training, and balloon feedback, depending on the researcher's experience.

EFFICACY OF BIOFEEDBACK TREATMENT FOR DYSSYNERGIC DEFECATION

Uncontrolled studies

In 1987 Bleijenberg and Kuijpers[9] were the first to report the efficacy of EMG biofeedback treatment combined with oatmeal porridge defecation in 10 patients affected by spastic pelvic floor syndrome, later redefined as functional defecation disorder[4]. Treatment was a complete success in 7 patients and a partial success in two others. This open label trial stimulated a number of uncontrolled studies to investigate the efficacy of behavior therapy in functional defecation disorder[7]. Therapeutic outcome varied greatly among centers with success rates ranging from 18% to 100% of patients studied[15,16].

A major drawback to assessing this literature was the huge variance in inclusion criteria, outcome criteria, follow-up intervals, and therapeutic protocols[13,17]. Additional limitations were small sample size (often no more than 30 subjects studied) and lack of any control group[17]. However, the majority of uncontrolled studies in adults reported a favorable outcome in about two thirds of patients, without side effects[7,11,13,17]. Coexisting morphological abnormalities of the pelvic floor, namely rectocele, intussusception and abnormal perineal descent, seemed not to influence behavior treatment outcomes[18]. Researchers were unable to identify any functional variable that could predict treatment outcome, but anxious patients appear to be less likely to succeed[13,17].

Although the majority of published uncontrolled studies reported beneficial effects of treatment, a series of studies from the St. Mark's group cast doubts on whether biofeedback training has specific value in the treatment of functional defecation disorder: these investigators reported similar benefits of biofeedback therapy in patients, irrespective of whether they had slow whole gut transit or functional defecation disorder[19,20] (See below for contrasting views). They also suggested that the autonomic innervation of the colon may influence the outcome of biofeedback treatment[21].

Controlled studies

Randomized, controlled trials were first performed in the pediatric population. In 1987 Wald *et al*[22] compared pressure biofeedback therapy with mineral oil in a group of 55 encopretic children; 16 of whom showed evidence of functional defecation disorder. Although a trend toward greater improvement in the biofeedback group was evident, the difference in success rate did not reach significance. In another controlled study[23], a well-defined pediatric population of 43 children with functional defecation disorder was randomized to receive either biofeedback therapy plus conventional care (laxatives) or conventional treatment only. All children had fecal impaction and encopresis. The biofeedback group did significantly better than the conventional one, with about half of patients showing successful symptoms resolution at one year follow-up compared to 16% in the conventional-care-only group. The clinical benefit was correlated with normalization of defecation dynamics. Similar benefits were reported in another controlled study[24] in the pediatric population, but the follow-up was too short (3 mo) to draw firm conclusions.

In contrast to the successful studies described above, the largest randomized, controlled study in pediatric constipation (192 children), which compared laxatives plus EMG biofeedback therapy to laxatives alone, failed to show any benefit from biofeedback[25]. A criticism of this study was that not all the subjects had functional defecation disorder. However, a similar failure to show superior efficacy with biofeedback was reported in a controlled study considering a smaller sample of children with both functional defecation disorder and encopresis[26]. In both studies, improved defecation dynamics were reported in biofeedback-treated patients, but this did not translate into greater symptom improvement.

In the adult population, four controlled studies were published prior to 2005. Two of these studies compared different biofeedback techniques to each other[27,28] and two studies compared EMG biofeedback to simulated defecation[19,29].

Heymen *et al*[27] compared intra-anal EMG biofeedback to (1) a combination of EMG and intra-rectal balloon distension training, (2) EMG and home trainers, and (3) a combination of all three techniques. All groups showed significant improvement from pretreatment, but no significant differences were found among treatment strategies. Glia *et al*[28] found peri-anal EMG biofeedback to be superior to pressure biofeedback combined with balloon defecation training. However, neither Glia *et al* nor Heymen *et al* had sufficient sample size to provide a meaningful analysis.

Bleijenberg *et al*[29] found an intra-anal EMG biofeedback to be superior to balloon defecation training (90% *vs*

60% improved). Although the sample size was too small to draw reliable conclusions, subjects who failed balloon defecation training were then given biofeedback training, yielding an 80% success rate.

The St. Marks group[19] studied a series of 60 adults with functional constipation unresponsive to conservative management and randomized them either to EMG and rectal balloon biofeedback or to abdomino-pelvic muscular coordination training and balloon feedback. After only two unsatisfactory sessions, patients who were judged unable to respond, were switched to the alternative treatment. At the end of treatment, approximately 50% of patients in both groups rated their symptoms as significantly improved. The outcome did not correlate with colon transit time, the presence of functional defecation disorder, or other functional and clinical variables. In addition, the St. Marks group recently reported biofeedback to be no more effective than bowel training and education for fecal incontinence in a large, controlled, randomized study[30]. These results challenge both the effectiveness of behavior therapy and the claim that retraining makes a specific contribution to the treatment of constipation other than education and/or psychotherapy.

To determine whether biofeedback is equally effective in slow transit constipation and dyssynergic defecation and also whether the benefits are due to education alone, we conducted an open study on 52 patients with slow transit constipation (objectively documented) who were unresponsive to conservative measurements[3]. Thirty-two of them showed evidence of dyssynergic defecation, 6 formed a mixed group who satisfied some but all criteria for dyssynergic defecation, and 12 had slow transit only. All patients received 5 weekly sessions of a biofeedback protocol, including improved use of the abdominal muscles to strain, anal EMG and balloon biofeedback to teach relaxation of the pelvic floor on straining, and simulated defecation.

Functional ano-rectal and clinical parameters were evaluated both before and after behavior therapy. After six months, 71% of patients with functional defecation disorder and slow transit reported satisfaction with treatment *versus* 8% in the slow transit only group. The results were well maintained at follow-up 2 years later[3]. Patients' satisfaction was correlated with improved rectal emptying as demonstrated by successful balloon expulsion and reductions in dyssynergia at manometry. A significant increase in rectal pressure on straining was also evident. Interestingly, biofeedback training resulted in a significant decrement in the threshold volume of balloon distention required to produce a sensation of urge to defecate, although no specific sensory retraining had been provided. Treatment success was predicted by pelvic floor dyssynergia, milder constipation, and less frequent abdominal pain at baseline.

This study allowed us to conclude that biofeedback therapy is specifically indicated for dyssynergic defecation and that retraining works through teaching patients to relax the pelvic floor and anal muscles during straining. Since biofeedback therapy is time- consuming, dedicated trained personnel are not easily found, and drug treatments (laxatives, muscle relaxants) are cheaper and more easily available, we were in strong need of a randomized, controlled study to prove that biofeedback is more effective than laxatives or placebo. This need was reinforced by the recent statement of the American College of Gastroenterology's Chronic Constipation Task Force that osmotic laxatives, namely polyethylene glycol and lactulose, are effective in improving stool frequency and consistency in all patients with chronic constipation[31].

Recently, three randomized, controlled studies coming from different centers have provided satisfactory answers to this question. The first of them[32] compared 5 weekly sessions of biofeedback to a commonly prescribed osmotic laxative (polyethylene glycol [PEG] in incremental dosage (14.6-29.2 g/d) given in combination with 5 weekly counseling sessions. Patients with normal transit constipation secondary to dyssynergic defecation were randomized either to the biofeedback (54 patients) or to the laxative group (55 patients). Follow-up assessment extended up to 12 mo in the laxative group and to 24 mo in the biofeedback group. Satisfaction with treatment, symptoms of constipation, and pelvic floor physiology were assessed at pretreatment, every six months in the first year, and at 24 mo. At six months, major clinical improvement was reported by 80% of patients in the behavior group *versus* only 20% in the PEG group. Biofeedback benefits were well sustained for the whole two-year follow-up interval. Clinical benefits correlated well with objective evidence of a reduction or elimination of paradoxical contractions of the pelvic floor during straining. The only clinical variable that correlated with treatment outcome was digital facilitation of defecation, which predicted failure; anorectal physiology could not predict outcome. Interestingly, laxatives consumption other than PEG was significantly decreased in the biofeedback group compared to the PEG group at 6-12 mo follow-up, while bowel frequency was significantly increased in both group compared to baseline.

Rao *et al*[33] conducted a randomized trial comparing biofeedback to sham feedback (relaxation therapy) and to standard medical care (diet, exercise, and laxatives). A significantly greater proportion of subjects receiving biofeedback (88%) reported more than a 20% improvement in global satisfaction and stool frequency on a visual analog scale compared to subjects receiving sham biofeedback (48%), but not when compared to standard care (70%). The authors also reported a significant improvement in favor of the biofeedback group to normalize the dyssynergic pattern and improve on a defecation index, with trends in favor of biofeedback subjects reducing balloon expulsion time and decreasing colonic marker retention compared to alternative treatment groups.

In a third randomized controlled trial, Heymen *et al*[34] randomly assigned 84 constipated subjects with dyssynergic defecation to receive either biofeedback (n = 30), diazepam (n = 30), or placebo (n = 24). An important feature of this study was that all subjects were trained to do pelvic floor muscle exercises to correct pelvic floor dyssynergia during 6 biweekly 1-h sessions, but only the

biofeedback patients received EMG feedback. All other patients received pills (muscle relaxant or placebo) 1-2 h before attempting defecation. Biofeedback was superior to diazepam by intention-to-treat analysis (70% *vs* 23% reported adequate relief of constipation), and also superior to placebo (38% successful). In addition, biofeedback patients had significantly more unassisted BMs compared to placebo, with a trend favoring biofeedback over diazepam. Biofeedback patients also reduced pelvic floor EMG during straining significantly more than diazepam patients.

Limitations of biofeedback training

The negative outcomes reported in controlled studies have been in the pediatric population. These poorer outcomes may be due to the inclusion of children whose constipation was not due to functional defecation disorder, since it is known that patients with other etiologies for their constipation respond poorly to biofeedback. In addition, biofeedback training requires complex cognitive processing and sustained attention that may be beyond the abilities of younger children. Finally, the quality of the therapist-patient relationship and the skills and experience of the therapist seem to influence the success of behavior therapy, and there is currently a shortage of trained personnel to provide this form of treatment.

In conclusion, a series of controlled studies have now shown that functional defecation disorder, one of the most frequent and disabling subtypes of adult constipation, can be treated effectively with biofeedback training. This form of treatment is more effective than laxatives, and it has no known adverse effects. Although this training is relatively expensive to provide, it produces improvements that are sustained for up to two years. For these reasons, we may conclude that biofeedback training is the treatment of choice for functional defecation disorder.

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EDITORIAL

Insulin resistance and hepatitis C

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Abstract

Insulin resistance is the major feature of the metabolic syndrome and depends on insulin secretion and insulin sensitivity. In chronic hepatitis C, insulin resistance and type 2 diabetes mellitus are more often seen than in healthy controls or chronic hepatitis B patients. Hepatitis C virus (HCV) infection promotes insulin resistance, mainly by increased TNF production together with enhancement of suppressor of cytokine (SOC-3); both events block PI3K and Akt phosphorylation. Two types of insulin resistance could be found in chronic hepatitis C patients: "viral" and "metabolic" insulin resistance. Insulin resistance in chronic hepatitis C is relevant because it promotes steatosis and fibrosis. The mechanisms by which insulin resistance promotes fibrosis progression include: (1) steatosis, (2) hyperleptinemia, (3) increased TNF production, (4) impaired expression of PPARγ receptors. Lastly, insulin resistance has been found as a common denominator in patients difficult-to-treat like cirrhotics, overweight, HIV coinfected and Afro-American. Insulin resistance together with fibrosis and genotype has been found to be independently associated with impaired response rate to peginterferon plus ribavirin. Indeed, in genotype 1, the sustained response rate was twice (60%) in patients with HOMA $\leqslant$ 2 than patients with HOMA > 2. In experiments carried out on Huh-7 cells transfected by full length HCVRNA, interferon alpha blocks HCV replication. However, when insulin (at doses of 128 μU/mL, similar that seen in the hyperinsulinemic state) was added to interferon, the ability to block HCV replication disappeared, and the PKR synthesis was abolished. In summary, hepatitis C promotes insulin resistance and insulin resistance induces interferon resistance, steatosis and fibrosis progression.

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Key words: Diabetes mellitus; Homeostasis model of assessment; Fibrosis; Steatosis; Sustained response; Peginterferon; Ribavirin

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INTRODUCTION

Insulin resistance emerges as a very important host factor in patients with chronic hepatitis C, mainly because it has been related to steatosis development, fibrosis progression and non-response to peginterferon plus ribavirin. IR appears as a consequence of the inability of insulin to induce the effect on glucose metabolism and an abnormally large amount of insulin is secreted to achieve a biological response. The hyperinsulinemic state induces several abnormalities in the liver, endothelium, and kidneys, and is the main feature in the metabolic syndrome. Obesity and metabolic syndrome are frequently found in western countries. In Spain, the prevalence of obesity has been found in around 13% and more than half of the population showed increased body mass index. In chronic hepatitis C, overweight and obesity showed a distribution similar than healthy people. Although insulin resistance has been found to be strongly associated with body mass index, in some circumstances it depends on the virus C. Two types of insulin resistance could be defined in patients with chronic hepatitis C: "metabolic" insulin resistance and "viral" insulin resistance.

MATERIALS AND METHODS

Insulin, after binding with it's receptor, induces the phosphorylation of receptor substrates in the liver and muscles and induces several steps toward the transactivation of GLUT-4 that increases glucose uptake by cells and the storage as glycogen and inhibits the net production of glucose by the liver, blocking glycogenolysis and neoglycogenesis. Moreover, insulin promotes lipids storage inhibiting lipolysis. When insulin is not able to induce glucose uptake, pancreatic beta-cells increase insulin production and the hyperinsulinemic state avoids hyperglycemia. Insulin secretion increases when insulin sensitivity decreases until a threshold in which insulin secretion did not induce improvement in insulin sensitivity, and diabetic state emerges. Several factors have been proposed as mediators in the islet adaptation: glucose, glucagon-like peptide 1, autonomic nervous system, and

free fatty acids[1].

The best method to measure insulin sensitivity is the hyperinsulinemic euglycemic clamp. This method measures the quantity of glucose required to maintain normal glycemic levels during insulin infusion. Briefly, a perfusion of 60 mU/kg per minute of insulin was started and every 5 minutes glucose levels were measured. To avoid hypoglycaemia, glucose (20%) was infused to maintain levels between 90 mg/dL and 100 mg/dL. The whole-body glucose uptake (M value) depends on the glucose infused in the last 30 min of the test. Lower M value (lower glucose requirements) are associated with insulin resistance. However, the clamp method is expensive, labor-intensive and uncomfortable for practical use in clinical medicine. A simple mathematical model named Homeostasis Model for Assessment [HOMA = fasting insulin (mUI/mL) * fasting glucose (mmol/L)/22.5] has been proved useful in the measurement of insulin sensitivity in euglycemic patients. However, this measurement is not useful once the blood glucose level begins to increase because it lacks accuracy. Normal plasma insulin concentrations are not standardized in healthy controls. Thus, HOMA has not been standardized yet. In previous reports HOMA < 2 has been considered "completely" normal, and higher than 4 as prediabetic state.

INSULIN RESISTANCE IN CHRONIC HEPATITIS C

The mechanisms by which hepatitis C induces increased insulin resistance and the risk for development of diabetes has not been completely understood. Liver fibrosis progression has been considered for long-time responsible of the appearance of insulin resistance and type 2 diabetes in patients with chronic liver diseases. Taking together available data from several studies about the prevalence of type 2 diabetes mellitus in hepatitis C, we found that in cirrhotic patients (n = 2152 cases) the prevalence of type 2 diabetes was 27.3%, higher than the 17.5% in non-cirrhotic hepatitis C patients (n = 1016 cases). Hyperinsulinemia in liver cirrhosis has been reported to be due to diminished hepatic insulin extraction by liver dysfunction and not to pancreatic hypersecretion. C-peptide and insulin are secreted in equimolar quantities. More than 50% of insulin is degraded in the liver at first pass, whereas C-peptide is degraded in the kidneys[2]. Simultaneous measurements of C-peptide and insulin revealed that both insulin resistance and insulin secretion contribute to glucose intolerance in patients with chronic hepatitis C[3]. In spite of insulin resistance correlated with liver fibrosis, it has been found higher in patients with chronic hepatitis C and mild fibrosis (F0-F1) than in healthy controls matched by age, sex, visceral obesity and body mass index[4]. In a cohort of patients with chronic hepatitis C and chronic hepatitis B matched by age, sex, body mass index and fibrosis stage HOMA index was found statistically higher in hepatitis C[5], this result confirms previous reports showing higher insulin resistance in chronic hepatitis C than in others hepatopathies[6]. Thus, hepatitis C virus induces insulin resistance independently of body mass index or fibrosis stage. Moreover, in a transgenic animal model, HCV core protein has been found able to induce insulin resistance, steatosis and type 2 diabetes mellitus. The main mechanism seems to be the over production of TNF. This cytokine phosphorylates serine residues of insulin-receptor substrates 1 and 2 and enhances the production of suppressor of cytokines (SOC3). The SOC-3 substance inhibits the phosphorylation of Akt and phosphatidyl inositol 3 kinase (PI3K). All these impairments in the intracellular signaling of insulin could block the transactivation of GLUT-4, avoiding the uptake of glucose by cells. Moreover, in transgenic animal models unable to express SOC-3, in spite of core HCV protein expression, insulin resistance does not develop. Indeed, in transgenic mice, TNF correlates with hyperinsulinemic state and the blockade of TNF production by anti-TNF drugs as infliximab also avoid insulin resistance appearance. Therefore, mechanisms inducing insulin resistance by hepatitis C virus include: TNF production, serine phosphorylation of IRS, and over expression of SOCs. Furthermore, over production of TNF in patients with chronic hepatitis C has frequently been seen[7], and correlated with higher fibrosis progression and impaired antiviral response to interferon alpha. Besides, in humans the risk for the development of insulin resistance and further appearance of DM is greater in patients with higher TNF production.

The development of type 2 diabetes mellitus depends on environmental, genetic and diet-related factors. Type 2 diabetes mellitus is more often seen in patients with chronic liver diseases than in general population[8]. Data supporting an association between diabetes mellitus and hepatitis C include: (1) Cross-sectional studies that found increased prevalence of diabetes mellitus in patients with chronic hepatitis C, or higher prevalence of hepatitis C in patients with diabetes mellitus. In a recent meta-analysis taking together available data about the prevalence of DM in patients with chronic liver diseases, the prevalence was higher in chronic hepatitis C than chronic hepatitis B. In non-cirrhotic chronic hepatitis C, DM was found in 17.5% and in chronic hepatitis B in 8.7% while in cirrhotics the prevalence was 27.3% in hepatitis C versus 15.2% in cirrhotics B (Figure 1). Besides, anti-HCV was more often detected in patients with diabetes mellitus (4.9%) than in the general population (0.77%)[9]. (2) case-cohort studies to analyze the development of diabetes mellitus during follow-up in patients with hepatitis C against uninfected patients, including follow-up after orthotopic liver transplantation. In a cross-sectional survey including 9841 persons, Mehta *et al* found that HCV-positive persons who were older than 40 years had an increased risk for type 2 diabetes mellitus higher than 3 times compared with persons without HCV-infection, while no difference was seen in HBV infection[10]. In a cohort of 1084 patients followed-up for 9 years 548 cases developed diabetes mellitus. The presence of hepatitis C was associated with a greater development of diabetes, but solely in high-risk diabetes patients[11]. Besides, in a cohort of 2327 cases, hepatitis C infection increased three times the rate of diabetes mellitus appearance during follow-up in patients aged between 35 and 49 years[12]. Thus, HCV infection could promote type 2 diabetes in high-risk populations.

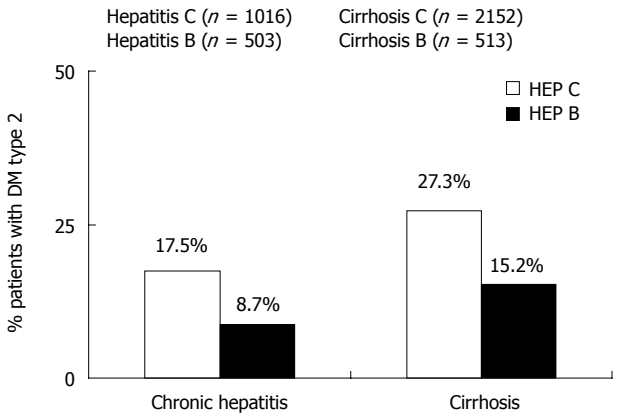

Figure 1 Prevalence of type 2 diabetes mellitus in patients with chronic hepatitis C and chronic hepatitis B.

Recently, in a Spanish study including 525 chronic hepatitis C patients treated with peginterferon plus ribavirin were followed-up after treatment and the incidence of altered baseline glucose and the appearance of diabetes type 2 was greater in non-responders than in sustained responders even after multivariate analysis including confounding variables as previous diabetes type 2 in relatives, older than 40 years and male sex. Thus, hepatitis C virus clearance induces a decrease in insulin resistance index during a short-time follow-up and decrease the incidence of diabetes type 2 in long-term follow-up[13].

Hepatocyte steatosis is a common feature found in the liver of patients with chronic hepatitis C. The mechanisms implied in steatosis appearance seem to be genotype-dependent. In patients infected by genotype 3, steatosis emerges as a cytopathic effect of the virus while in genotype 1 steatosis seems to be an expression of the metabolic syndrome. In genotype 3, steatosis degree correlated with liver and serum HCV load[14]. In genotype 1, steatosis depends on leptin levels and insulin resistance. Recently, insulin resistance has been found implied in the development of steatosis in 331 non-diabetic genotype 1 patients. Insulin resistance together with gender and gammaglutamyltranspeptidase (a surrogate marker of TNF levels) were independently associated with moderate/severe steatosis in this cohort[15]. Insulin resistance is the main pathogenic factor in the development of steatosis in chronic hepatitis C, both viral insulin resistance and metabolic insulin resistance could be implied in the development of steatosis.

The main deleterious effect of insulin resistance in chronic hepatitis C is the ability to promote fibrosis progression. High serum glucose levels have been found associated with an increased rate of fibrosis progression, greater even than overweight[16]. Mean HOMA index increases with the stage of fibrosis[17] and could help to differentiate stages of fibrosis. Recently, Sud *et al*[18], proposed an index to predict fibrosis containing age, cholesterol, gammaglutamyl transpeptidase and alcohol consumption together with HOMA. The mechanisms by which insulin resistance promotes fibrosis progression include: (a) steatosis, (b) hyperleptinemia, (c) increased TNF production, and (d) impaired expression of PPARγ receptors. (a) hepatocyte steatosis induces fibrosis progression. In a cohort of patients with chronic hepatitis C, the presence of steatosis in the first biopsy was associated with higher fibrosis progression rate, irrespective of genotype[19]. Besides, steatosis is more often seen in patients with advanced fibrosis[20]. (b) Hyperleptinemia has been usually found in patients with insulin resistance, also hepatic stellate cell showed specific leptin receptors, thus leptin could play a role in the activation of HSC and fibrosis progression[21,22]. (c) TNF production is enhanced in hepatitis C and has been implicated in the development of insulin resistance, also, TNF levels were related to fibrosis progression, owing to the ability to activate HSC and promote collagen deposits. Moreover, TNF could inhibit PPARs activity[23]. (d) In patients with hepatitis C an impaired expression of PPARγ receptors has been found[24]. PPARs agonist inhibits inflammation and fibrosis progression by blocking the activation of redox-sensitive transcription factor NFκB and TGFβ1[25].

In HIV-infected patients insulin resistance depends on host, virus and drugs-related factors. Adverse metabolic effects have been found in all antiretroviral drug classes. Protease inhibitors (PI) and nucleoside reverse transcriptase inhibitor (NRTI) therapy induces insulin resistance during treatment[26]. Insulin resistance has been found increased in HIV+/HCV+ co-infected patients. Factors implied in the development of insulin resistance in HIV infected patients include: HCV infection, PI and/or NRTI based-therapy, age, HIV and cytokine dysregulation induced by chronic infection and genetic predisposition[27]. Moreover, the development of type 2 diabetes mellitus was twice in co-infected patients versus HIV non-HCV infected[28]. Duong *et al*[29] reported a higher HOMA in co-infected patients than in HIV-infected cases without hepatitis C. However, in a large study no differences were seen between co-infected or not co-infected patients. In a recent study, including 127 co-infected patients that underwent antiviral therapy (peginterferon plus ribavirin) and 85 hepatitis C, insulin resistance index was higher in co-infected patients, in spite of lower body mass index and lower baseline glucose levels. Besides, in a genotype-dependent manner, insulin resistance was strongly associated with steatosis development and impaired the sustained response rate in genotype 1 co-infected patients. No association between insulin resistance and steatosis or sustained response was seen in genotype 2 or 3 coinfected patients[30]. Lastly, history of diabetes in relatives, increased body mass index and HCV-infection have been found as independent variables associated with diabetes development in HIV patients, supporting the hypothesis that HCV infection promotes diabetes in high-risk patients.

INSULIN RESISTANCE AND RESPONSE TO PEGINTERFERON PLUS RIBAVIRIN

In healthy volunteers, after the first injection of interferon alpha insulin resistance could be detected[31]. Besides in patients with chronic hepatitis C, interferon alpha induces insulin resistance in the first two weeks, mainly owing to a decrease of hepatic glucose uptake[32]. This effect has been related to the pro-inflammatory repertoire of cytokines induced by interferon. However it's a transitory effect because insulin resistance was not found at month three

of therapy[33] or at the end of therapy[34]. In patients with chronic hepatitis C receiving peginterferon plus ribavirin insulin resistance measured by HOMA, decreased in patient with HCV RNA clearance at mo 6, but not in non-responders. At the end of follow-up sustained responders showed a significantly lower HOMA in comparison with baseline insulin resistance index. However, in relapser patients, the HOMA index increased and the levels at the end of follow-up were not different from the baseline. These data support a connection between HCV replication and insulin resistance, and HOMA decreased when the virus was eradicated. Besides, the incidence of diabetes type 2 is different in cured patients than in non-responders, supporting a better control of insulin resistance after hepatitis C virus clearance[13].

Genotype and viral load have been found as the most important viral factor influencing peginterferon plus ribavirin response. Host factors include genes as HLA, weight, body mass index, hepatocyte steatosis, age, Afro-American ethnicity and fibrosis. Several genes and polymorphisms as HLA-B44 has been found related to the possibility of curing the disease[35]. On the other hand, features of metabolic syndrome as overweight, steatosis and fibrosis have been reported as independently associated with response. The influence of steatosis in the chance of curing hepatitis C has been controversial for long time. Steatosis impairs SVR rate in patients with genotype 1, but not in genotype 3. Thus, the possibility of finding steatosis as independent variable depends on the balance between genotypes included in the cohort. In fact, in a multivariate analyses including steatosis together with fibrosis, body mass index, genotype and insulin resistance, either steatosis or body mass index were not found independently associated with response but insulin resistance, genotype and fibrosis[32]. In genotype 1, sustained response rate was 32% in patients with insulin resistance (HOMA > 2) against 60% in patients with HOMA lower than 2[36]. However, in a recent cohort including 331 non-diabetic genotype 1 chronic hepatitis C patients, steatosis but not insulin resistance was found independently associated with response. Steatosis and insulin resistance showed a great colineality in genotype 1 patients and could be the expression of the same mechanisms. Thus, measure insulin resistance seems to be easier and comfortable than study steatosis in a liver biopsy. Besides, in 52 patients from UK also treated with peginterferon plus ribavirin, HOMA index was significantly higher in non-responders than patients with sustained response[37]. Thus, insulin resistance emerges as the most important host factors in the prediction of response in non-diabetic patients treated with the best available option peginterferon plus ribavirin. Interestingly, insulin resistance has been found as a common denominator to the majority of features associated with difficult-to-treat patients: patients with cirrhosis, obesity, co-infected by HIV and Afro-American. Unresolved question is whether insulin resistance is a marker of very difficult-to-cure or a pathogenic mechanism able to block antiviral activity of the interferon. Peginterferons induce their antiviral activity via extracellular receptor binding. The interferon alpha signalling pathway involves the activation of Janus kinase (Jak1) and tyrosine kinase (Tyk2), initiated by the binding of peginterferon alpha-2 to the interferon heterodimeric receptor complex (IFNAR1/IFNAR2), which leads to activation of their downstream substrates, signal transducers and activators of transcription (STAT 1 and STAT2). Activated STAT then assemble as a multimeric complex and translocate into the nucleus where they bind to interferon alpha-2-stimulated response elements in the promoters of interferon alpha-2-stimulated genes[38]. Recently, in a replicon model using Huh-7 cells transfected by full length HCVRNA, interferon alpha blocks HCV replication. However, when insulin (at doses of 128 μU/mL, similar that seen in the hyperinsulinemic state in patients with metabolic syndrome) was added to interferon, the ability to block HCV replication disappeared, and the PKR synthesis was abolished[39]. In this experiment, a blockage of PI3K by LY294002 avoided the interference of insulin, supporting that interferon resistance induced by insulin is mediated by PI3K (Figure 2). Moreover, in genotype 1 patients obesity has been found able to increase intrahepatic synthesis of SOC-3, inhibiting the interferon signalling and promoting interferon resistance[40]. Thus, hyperinsulinemia and obesity induce interferon resistance blocking intracellular signalling.

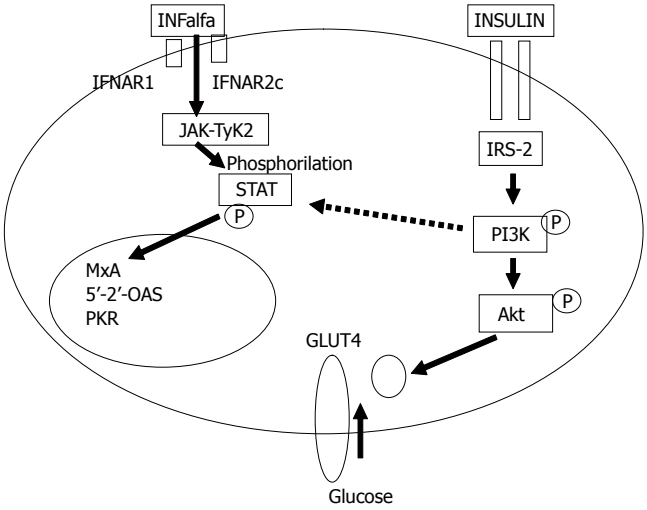

Figure 2 Interaction between insulin and peginterferon alpha signalling pathway. The phosphatidil-inositol-3-kinase (PI3K) activated by insulin seems to be responsible for the block of STAT 1 translocation avoiding antiviral effect of interferon. Dotted lines represent inhibition, continuous lines represent activation.

CONCLUSIONS

In summary, hepatitis C promotes insulin resistance and insulin resistance induces interferon resistance, steatosis and fibrosis progression in a genotype-dependent manner. In genotype 1 insulin resistance decreases sustained response rate, and increase the risk for the development of steatosis and fibrosis progression, in both, coinfected HCV+/HIV+ and in hepatitis C. However, the impact of insulin resistance in non-1 genotype seems not achieve enough importance to impair sustained response, probably due to the high sensitivity to peginterferon. The treatment of insulin resistance, decreasing hyperinsulinemia, could improve sustained response rate in genotype 1 patients with chronic hepatitis C when treated with peginterferon

plus ribavirin. A multicenter Spanish trial (TRIC-1) is ongoing to analyze the effect of triple therapy with peginterferon alfa-2a plus ribavirin and metformin versus standard therapy and placebo in patients with chronic hepatitis C infected by genotype 1 and showing insulin resistance.

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EDITORIAL

The "return" of hepatitis B

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Abstract

There has been a significant advance in the treatment of chronic Hepatitis B virus (HBV) infection and the following drugs were approved for therapy: Conventional interferon (IFN), pegylated interferon alfa-2a (PEG IFN α2a), lamivudine, adefovir and entecavir. Compared to nucleoside analogues IFN induces higher rates of sustained remission and HBsAg loss. Conventional IFN in lower doses (1, 5-3 MIU) tiw for 4-6 mo has similar efficacy in comparison to "standard IFN therapy". Longer IFN treatment is a significant factor for long-term remission in HBeAg-negative CHB, but the higher actual IFN dose is not such a factor. PEG IFN is superior to conventional IFN. There is no significant difference between PEG IFN α2a at doses 90 mcg/wk and 180 mcg/wk in HBeAg-positive patients. These results provide a rational for further clinical trials with lower doses PEG IFN α2a given in prolonged course as maintenance or intermittent treatment. Serious new problems arose after the introduction of nucleoside/nucleotide analogues in clinical practice. The most important ones are drug-resistance and the high rates of relapse after treatment discontinuation. Therapy should only be recommended if the expected benefit exceeds significantly the abstain from treatment. The choice of therapy should take into account the patient's age, co-morbidity, severity of liver disease and the risk of drug-resistance. New antivirals significantly suppress HBV-replication, but have no effect on cccDNA in hepatocytes, and after the treatment discontinuation viral relapses occurs. At the present level of knowledge it is impossible "to eradicate the virus" The realistic treatment goal is to achieve durable response by clearance of HBeAg, sustained decrease of serum HBV DNA levels, normalization of ALT, improvement of liver histology and stopping of liver fibrogenesis. The competition between IFN based therapy and nucleoside or nucleotide analogues still remains. IFN can cure the liver disease while nucleotide analogues only suppress the viral replication during therapy and can reduce the liver fibrosis. Treatment should be prolonged for 24-mo or longer by using maintenance or intermittent treatment course with the lowest effective IFN and PEG IFN doses. Nucleoside/nucleotide analogues are a promising treatment option, but additional data for treatment duration and long-term post-treatment outcome are necessary.

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Key words: Chronic Hepatitis B virus infection; Interferon; Pegylated interferon; Low-dose therapy; Cyclic treatment

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. This infection is especially endemic in Asia, South Pacific Region, sub-Saharan Africa and South America[1]. It is estimated that over 350 million people worldwide are chronically infected with HBV and up to one million die annually due to HBV-related complications, including hepatocellular carcinoma (HCC)[2]. In China and sub-Saharan Africa HCC associated with HBV is one of the leading causes of cancer in men[1].

In the mid-1980s, IFN became the first approved therapy for CHB. In addition effective vaccines against HBV became available and there was a flicker of hope that the problem can be solved. Over the years it became clear that the chronic HBV infection is much more severe and difficult to treat disease than previously supposed. The positive impact of routine infant HBV-immunization programs is beyond doubt, but chronic HBV-infection still remains an important public health issue.

During the last decade much was learned about the HBV genome organization, viral replication cycle, role of host immune response and natural course of chronic HBV infection[3]. The emergence of human immunodeficiency virus (HIV) infection on the other hand facilitated the search for effective and safe antiviral agents. Several new drugs were found to be quite promising candidates for the treatment of CHB. All these findings led to the 'return' of research interest in the treatment of CHB. A large number of clinical trials with new long-acting pegylated interferons (PEG IFN) and antiviral agents were performed. As a result of this intensive clinical research now we have five approved drugs for the treatment of CHB: Conventional IFN, PEG IFN α2a as well as three nucleoside or

Table 1 Treatment response at the end of 24-wk follow-up after 24-wk therapy with IFN α2a 4.5 MIU tiw, 90 mcg/wk, 180 mcg/wk and 270 mcg/wk PEG IFN α2a (Adapted from W Cooksley *et al*[12])

Response at the end of the 24-wk follow-up	4,5 MU IFN (*n* = 51)	90 μg PEG IFN α2a (*n* = 49)	180 μg PEG IFN α2a (*n* = 46)	270 μg PEG IFN α2a (*n* = 48)
HBV DNA < 500 000 cp/mL	13 (25%)	21 (43%)	18 (39%)	13 (27%)
Loss of HBeAg	13 (25%)	18 (37%)	16 (35%)	14 (29%)
HBeAg seroconversion	13 (25%)	18 (37%)	15 (33%)	13 (27%)
Normal ALT	13 (25%)	21 (43%)	16 (35%)	15 (31%)
Combined response[1]	6 (12%)	13 (27%)	13 (28%)	9 (19%)

[1]HBeAg-negative, HBV DNA < 500 000 cp/mL, Normal ALT.

nucleotide analogues (lamivudine, adefovir and entecavir). Furthermore, several new drugs are under investigation in phase Ⅱ and Ⅲ clinical trials: emtricitabine, tenofovir, clevudine and telbivudine. Nevertheless, the treatment of chronic HBV infection is still a challenge due to the low rates of durable response with currently available therapies, especially in HBeAg-negative patients. The main problems with current treatments now are associated with suboptimal efficacy, poor tolerability, and/or emergence of resistance.

There are no clinical trials comparing directly the efficacy of all discussed therapies. Furthermore, it is difficult to compare the results of clinical trials in CHB due to the different sensitivity of the assays used for detection of serum HBV DNA over the years. In the early 1980's the virological response was measured only by serological assays (HBeAg and anti-HBe Ab). Later it became clear that the presence and absence of HBeAg is not an accurate indicator of replicative and nonreplicative infection and low-sensitive hybridization assays (detection limit of approximately 10^6 copies/mL) were developed for the measurement of serum HBV DNA[3]. In the last years the majority of laboratories moved from hybridization techniques, through bDNA, to high-sensitive PCR (Roche Amplicor monitor) and RT PCR assays with lower detection limits of 10^3 copies/mL and < 100 copies/mL, respectively.

CURRENTLY APPROVED THERAPIES FOR CHRONIC HEPATITIS B

In spite of the mentioned difficulties, the treatment results of the approved therapies for CHB have been summarized by M Osborn and A Lok[4,5]. Available data suggest that higher rates of sustained remission and HBsAg loss can be achieved with interferon based treatment in comparison to nucleoside analogue therapy.

IFN and pegylated interferons in HBeAg-positive CHB: Standard and low-dose treatment schedules

Conventional IFN has been used in CHB patients for 20 years. IFN has both antiviral and immunomodulatory effects[6], but only 1/3 of HBeAg-positive patients achieve HBV DNA and HBeAg loss after 4-6 mo course of IFN at a dose of 5 MIU daily or 9-10 MIU thrice weekly[7]. Subsequently, 6 to 12 mo post therapy HBsAg loss was found only in 8% of HBeAg-positive patients[7]. These treatment doses and durations have been validated as a "standard IFN therapy" for HBeAg-positive CHB by both EASL and AASLD treatment guidelines[8,9]. However, some studies in HBeAg-positive patients suggested that conventional IFN, given in lower doses (1, 5-3 MIU thrice weekly) for 4-6 mo, is with similar efficacy compared to "standard IFN therapy"[10,11]. This low-dose therapy is much better tolerated than higher IFN doses. Although treatment with low-dose IFN has not been generally accepted, these results are quite interesting especially in the light of new-coming data for the efficacy of PEG IFNs. A phase Ⅱ clinical trail showed that PEG IFN α2a is superior to conventional IFN in HBeAg-positive CHB[12]. In addition no significant difference was found in PEG IFN α2a efficacy given in dose of 90 mcg/wk in comparison to dose of 180 mcg/wk (Table 1)[12].

Having in mind that the majority of the side effects of PEG IFN α2a are dose-related, these results provide a rational for further clinical evaluation of lower dose of PEG IFN α2a as well as for new clinical studies with individualization of treatment schedule by PEG IFN tapering therapy. This might be a possibility to achieve better tolerability and lower treatment costs without losing the treatment efficacy. Further investigations are needed to confirm or reject this hypothesis.

IFN and pegylated interferons in HBeAg-negative CHB: standard and low-dose treatment schedules

Early clinical trials in HBeAg-negative CHB, with 5-10 MIU of conventional IFN for 4-6 mo reported high (60%-90%) end-of-therapy response (ETR), but only 10% sustained response (SR) due to frequent relapses after the therapy[13]. Similarly to HBeAg-positive patients, HBeAg-negative subjects are sensitive to lower doses (1,5-3 MIU) of conventional IFN, but the relapse rate after 6-mo treatment course with low dose was also high[14,15].

Mathematical models of chronic HBV infection indicate that, although the half-life of HBV is short (< 1 d), the half-life of hepatocytes is relatively long (10-100 d) or even higher for the infected hepatocytes[16]. Therefore viral suppressive therapy might be continued for 1-10 years for viral elimination[16]. Subsequently, a longer course with conventional IFN was reported to improve the SR, which was found to be 11% and 22% after 6 and 12 mo of conventional IFN therapy, respectively[17]. Only the longer IFN course was identified as a significant factor of long-term remission, while the higher actual IFN dose was

not[17]. An Italian study showed a 30% SR after a 24-mo maintenance course with 6 MIU IFN tiw[18]. However, this prolonged treatment was associated with poor tolerability of IFN and high incidence of dose-related adverse events. In a recent pilot study we found similar SR by using approximately 2-year cyclic therapy with low-dose (1,5 MIU) conventional IFN therapy[19]. Taken together, this findings suggest that prolonged treatment (24-mo or longer) with conventional IFN in low-dose (1, 5-3 MIU) given as a maintenance or intermittent treatment course is appropriate therapeutic approach for HBeAg-negative CHB.

Recently, the efficacy of IFN-based therapy in CHB was improved by the introduction of PEG IFNs. In HBeAg-negative patients a 36% SR (combined biochemical and virological) was found at 6 mo after the 12-mo therapy with PEG IFN α2a[20]. However, 24-mo after the end of the treatment only 41% of patients with normal ALT levels 6-mo post therapy remained with combined biochemical and virological response[21]. Our results with cyclic (re-induction) treatment[19] provide a rational for further clinical trials with low-dose intermittent PEG INF therapy in responders of 12-mo PEG IFN α2a treatment. We have just initiated such a study aiming to test the potential of this treatment approach for reducing the relapse rate in end-of-treatment PEG IFN responders.

Lamivudine

Lamivudine was the first introduced nucleoside analogue in clinical practice. Twelve-month course with lamivudine induces HBeAg-seroconvertion in 17% to 22% of the patients[22-24]. HBeAg-seroconvertion rates increased to 50% after continuous treatment for 5 years, but this was associated with increasing rates of drug-resistant mutations (up to 70%) after 5-year therapy[25]. One-year lamivudine therapy suppressed viral replication in 65% to 90% of HBeAg-negative patients[26,27]. However more than 90% of HBeAg-negative responders at the end of 12-mo course relapsed after treatment discontinuation[27]. Extended treatment duration was associated with decreased response rate due to drug-resistance[28]. The main concerns with lamivudine treatment are the selection of drug-resistant mutations and the very high relapse rate after treatment discontinuation. Both drug-resistance and relapse are associated with risk of hepatic flare and liver failure. Due to these reasons it is not recommended to use lamivudine as a first line therapy, especially in young patients. It should be also stressed that recent studies show PEG IFN α2a monotherapy to induce higher SR rate in both HBeAg-negative and HBeAg-positive subjects in comparison to 1-year course with lamivudine[20,29]. Combined therapy with lamivudine plus PEG IFN α2a is with efficacy, similar to that of PEG IFN α2a monotherapy[20,29]. According to the current knowledge there is no biological rational for further use of this combination, but the consequent treatment with antivirals and IFN-based treatment is a possible treatment approach.

Adefovir

Recently, adefovir and entecavir were approved in the United States. Adefovir is effective in both HBeAg-positive and HBeAg-negative patients[30,31]. However, only 8% of the patients had a SR one year after treatment discontinuation[32]. Adefovir effectively suppress lamivudine resistant mutants which is a significant advantage of this drug. Because of the high rates of drug-resistance, patients on prolonged lamivudine therapy need to be closely monitored. If virological breakthrough occurs due to drug-resistance, adefovir can be administrated in addition to lamivudine. Resistance to adefovir is uncommon during 2-year therapy, but emerge later in the course of treatment to 30% after 5-year therapy. Thus drug-resistance will be an increasing concern with longer adefovir treatment duration. Nephrotoxicity is another disadvantage of this drug.

Entecavir

Entecavir is more potent than lamivudine in suppressing HBV replication with significantly higher rates of biochemical and histological responses both in HBeAg-negative and HBeAg-positive patients[33,34]. No entecavir-resistance was observed after 2 year therapy among nucleoside analogue naïve patients[35]. However, the presence of lamivudine resistance increases the likelihood of entecavir resistance[35,36]. Long-term data with entecavir still are not available.

NEW PROMISING ANTI-VIRAL AGENTS

Many new antivirals are under evaluation for the treatment of CHB. Emtricitabine (FTC) and tenofovir are licensed for use in HIV infection.

Emtricitabine

FTC is closely related structurally to lamivudine and therefore they share similar mutational sites[37]. A recent study in HBeAg-positive and HBeAg-negative patients found that 48 wk of treatment with emtricitabine 200 mg daily resulted in significant histological, virological, and biochemical improvement[38]. The results of emtricitabine treatment were quite similar to published data for lamivudine. The incidence of YMDD mutations in patients, receiving emtricitabine 200 mg daily, is 12% and 19% at treatment wk 48 and 96, respectively[37,38]. The role of emtricitabine as a monotherapy may be limited due to its structural similarity to lamivudine and the risk of development of drug resistance.

Tenofovir

Tenofovir is an acyclic nucleotide inhibitor of HBV polymerase and HIV reverse transcriptase with close chemical similarity to adefovir[37]. Antiviral activity of tenofovir against HBV is found to be greater than the one of adefovir 10 mg in lamivudine resistant patients[37,39]. Furthermore, the N236T mutation that confers resistance to adefovir is sensitive to tenofovir[37]. Phase Ⅲ clinical trials are under way to determinate the long-term safety and efficacy of tenofovir.

Clevudine

Clevudine is a nucleoside analog of the unnatural β-L configuration. A recent randomized, double-blind study

found no significant difference between 24-wk therapy with emtricitabine plus clevudine and emtricitabine alone[40]. However, there was a significantly greater virologic and biochemical response at wk 24 after the end of treatment in the emtricitabine plus clevudine arm[40]. Further studies are needed to assess the long-term efficacy and safety of this drug.

Telbivudine

Telbivudine (LdT) is another antiviral agent, which is under clinical investigation in CHB. A phase Ⅱ study in 104 HBeAg-positive patients compared different therapeutic schedules for 52 wk[41]. The telbivudine-treated patients exhibited significantly greater virological and biochemical response in comparison to lamivudine[41]. Results from the combined regimens (LdT plus lamivudine) were similar to those obtained with LdT alone[41]. These data support the ongoing phase Ⅲ evaluation of telbivudine for the treatment of CHB.

ADVANTAGES, DISADVANTAGES AND TREATMENT LIMITATIONS OF CURRENT THERAPIES

Substantial progress has been made in the treatment of chronic hepatitis B, but quite serious new problems arose after the introduction of nucleoside/nucleotide analogues in clinical practice. The most important of them is the selection of drug-resistant mutations. Drug-resistance usually is accompanied by virologic breakthrough during therapy and increased ALT levels after initial normalization[35]. In some patients this may cause severe hepatic flare with liver failure and death[35]. In addition, resistance to one antiviral drug may confer resistance to other agents and may limit future treatment options[35]. Another important problem is the high rate of relapse after therapy.

With regards to the limited long-term efficacies of the approved therapeutic regimens, therapy should only be recommended if the expected benefit exceeds significantly abstain from treatment[35]. The choice of therapy should take into account the patient's age, co-morbid medical conditions, the severity of the liver disease and the risk of drug-resistance[4].

Interferon based therapy is associated with more durable response and the absence of drug-resistant mutations[42]. However, IFN is effective mainly in a subgroup of young patients with high ALT levels and low viral load[7,37]. At present it is generally accepted that subjects with normal or slightly elevated ALT (< 2x ULN) are not indicated for antiviral therapy[8,9]. These patients should be strictly monitored at 3-mo intervals. Low serum HBV DNA levels (HBV DNA < 30 000 000 copies/mL) are an important predictor of response to both conventional IFN and PEG IFN therapy[37]. With regards to this the initiation of IFN should be avoided in patients with very high viral load and especially if ALT is not markedly elevated. If possible the start of IFN therapy should be postponed for the moment, when serum HBV DNA and ALT levels predict higher likelihood of treatment response. High baseline ALT levels are the most important predictor of response to lamivudine and adefovir as well[42]. However, it should be mentioned that the high levels of ALT also are a predictor of spontaneous remission in HBeAg-positive CHB. So the decision to start nucleoside analogue should balance between the benefits of this treatment and the risk of drug resistance and hepatic flare.

Interferon based treatment is also limited by significant disadvantages in terms of injection-based application, poor tolerability, potentially severe side-effects, contraindications and relatively high cost. Flu-like symptoms, fatigue, bone marrow suppression thyroid disorders, irritability and depression are the most common adverse effects[5]. Patients should be closely monitored with monthly clinical and laboratory examinations. Approximately one-third of subjects may require dose reduction and 5% may discontinue therapy prematurely due to the adverse events.[5] Furthermore, IFN and PEG IFN are contraindicated in decompensated cirrhosis as well as in subjects with autoimmune disorders. They are also ineffective in immunosupressed patients. In fact nucleoside or nucleotide analogues are the only available treatment option in decompensated cirrhosis and in immunosupressed subjects.

TREATMENT GOALS

Sensitive HBV DNA assays revealed that HBV replication might persist even after HBsAg seroconvertion and this finding changed our treatment concept. Although the new antiviral agents significantly suppress HBV-replication, there is a pool of covalently closed circular DNA (cccDNA), resistant to the available antiviral treatment. This cccDNA serves as a template for viral transcription, so viral relapses occurs once antiviral medications are discontinued[4]. At the present level of knowledge it is impossible "to eradicate the virus" The realistic treatment goal now is to achieve durable response by clearance of HBeAg, sustained decrease in serum HBV DNA levels with normalization of ALT, improvement of liver histology and stopping of liver fibgogenesis[4].

In conclusion the competition between IFN based therapy and nucleoside or nucleotide analogues treatment still remains. IFN can cure the liver disease while nucleotide analogues only suppress the viral replication and can reduce the liver fibrosis. In the future IFN treatment in HBeAg-negative CHB might need to be prolonged for 24-mo or even longer by using maintenance or intermittent treatment course with the lowest effective IFN or PEG IFN doses. Nucleoside/nucleotide analogues are a promising treatment option, but additional data for treatment duration and long-term post-treatment outcome are necessary.

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REVIEW

Pathophysiology of pulmonary complications of acute pancreatitis

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Abstract

Acute pancreatitis in its severe form is complicated by multiple organ system dysfunction, most importantly by pulmonary complications which include hypoxia, acute respiratory distress syndrome, atelectasis, and pleural effusion. The pathogenesis of some of the above complications is attributed to the production of noxious cytokines. Clinically significant is the early onset of pleural effusion, which heralds a poor outcome of acute pancreatitis. The role of circulating trypsin, phospholipase A2, platelet activating factor, release of free fatty acids, chemoattractants such as tumor necrsosis factor (TNF)-alpha, interleukin (IL)-1, IL-6, IL-8, fMet-leu-phe (a bacterial wall product), nitric oxide, substance P, and macrophage inhibitor factor is currently studied. The hope is that future management of acute pancreatitis with a better understanding of the pathogenesis of lung injury will be directed against the production of noxious cytokines.

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Key words: Acute pancreatitis; Cytokines; Acute respiratory distress syndrome; Complications of pancreatitis; Pleural effusion; Interleukins

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INTRODUCTION

Acute pancreatitis (AP), an acute inflammatory process of the pancreas in its severe form, is complicated by the development of multi-organ dysfunction syndrome (MODS) with a mortality of 15%-20%. Regardless of etiology, once AP is initiated the inflammatory events within the acinar cells will progress to a generalized systemic inflammatory response syndrome (SIRS). Amongst the systemic complications, pulmonary complications are the most frequent and potentially the most serious. Recognition of these complications and their pathology may lead to more rapid diagnosis and better therapies. The following is a brief summary of the current researches on each of the possible pulmonary complications ranging from hypoxemia to acute respiratory distress syndrome (ARDS). Hypoxemia may occur without radiological abnormalities in 75% of cases. There is a direct correlation between hypoxemia noted early in the course of AP and mortality. Pleural effusion, once thought to be a marker of AP, is now a noted poor prognostic sign. Atelectasis, a frequent radiological complication, is attributed to a decrease in the quantity of pulmonary surfactant. The most dangerous complication of the pulmonary system is ARDS. The pathophysiology of ARDS and most of the other pulmonary complications is multifactorial. Activated trypsin causes damage to pulmonary vasculature and increases endothelial permeability. Active circulating phospholipase A_2 (PLA_2) is known to remove fatty acids from phospholipids. One of the main components of surfactant is the phospholipid, dipalmitoylphosphatidylcholine. Many recent studies have assessed the role of platelet activating factor (PAF) which stimulates polymorphonuclear cells (PMNs) regulating the interaction between PMNs and endothelial cells facilitating migration of activated WBC into interstitial spaces. Based on the observation a PAF inhibitor Lexipafant has undergone double blind randomized studies. Another major advance in the understanding of the pathophysiology of pulmonary complications is the explosion of knowledge of cytokines. There are pro-inflammatory cytokines released from the pancreas such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6, and IL-8. PMNs also contribute to release of cytokines. Our aim was to help the clinician understand the importance of the many studies looking at the effect of inflammatory modulators in decreasing the severity of AP. Today's experimental medicine is likely to become a therapeutic modality in the near future.

PATHOPHYSIOLOGY OF PULMONARY COMPLICATIONS OF ACUTE PANCREATITIS

AP is an acute inflammatory process of the pancreas with

variable involvement of peripancreatic organs and/or remote organ systems in different degrees. In 10%-20% of AP, it presents in its severe form, which is frequently complicated by the development of MODS with a mortality rate of 15%-20% or more[1,2]. Recent studies have documented two peaks of mortality in patients with AP, an early mortality due to the effects of SIRS and MODS, and a late mortality caused by the effects of MODS combined with pancreatic sepsis following pancreatic necrosis[3,4]. About 30% of patients with fatal AP have been diagnosed first at autopsy[2,5], indicating how often we miss the diagnosis and how fast the disease can progress after the onset. Regardless of its etiology, the clinical course follows a pattern of interaction between inflammatory events within the pancreas which initiate a generalized systemic inflammatory response. The mortality and severity of the disease appear to be influenced by events occurring subsequently to the pancreatic injury as a result of release of cytokines and other mediators.

Severe AP is associated with MODS and/or local complications such as necrosis, abscess, pseudocyst, or pancreatic ascites. Fortunately, AP is usually mild.

Pulmonary complications of AP occur in almost 75% of cases, ranging from hypoxemia to ARDS[6,7]. The local and systemic complications are listed in Table 1. Of the systemic complications, acute respiratory failure is perhaps the most serious one. In the last two decades, our understanding and management of severe AP have substantially improved. The traditional concept that AP is entirely due to the release of activated proteases from an injured pancreas has been considerably changed. The present view is that the activation of trypsin by intracellular zymogens, trypsin activating chymotrypsinogen, proelastase and prophospholipase A plays an initiating role, but other chains of events predominate in precipitating complications[6-8].

Recent studies using secretagogue-induced models of pancreatitis, caerulein- induced AP in rats, sodium taurocholate- induced AP, choline deficient ethionine-supplemented diet- induced AP in mice and free fatty acid- induced AP in dogs, as well as a number of other models have suggested that pancreatitis is a disease that evolves in 3 phases. The initial phase is characterized by intrapancreatic digestive enzyme activation and acinar cell injury which involves the first few hours of AP. The second phase characterized by an intrapancreatic inflammatory reaction and varying degrees of acinar cell necrosis involves approximately 12-72 h. Finally, the third phase which involves the rest of the progression of AP is characterized by further progression of the pancreatic injury and the appearance of extrapancreatic changes including SIRS and ARDS[9].

It is during phase three that pulmonary insult occurs. In this review, we chiefly focus on the many pulmonary complications of AP, their pathogenesis, and clinical significance. This review is an extension of our previous review and other excellent recent reports on the topic[10-13]. The literature search was done by Medline search using the terms 'pancreatitis', 'pleural effusion', 'hypoxemia', and 'ARDS' in combination searches in the English language.

Pulmonary complications of AP for ease of explanation are divided into three stages. Stage 1 deals with pulmonary manifestations without any noticeable changes radiologically, stage 2 emphasizes radiologic changes observed and stage 3 discusses ARDS. Following these stages is a discussion on the players in the pathophysiology of the pulmonary complications of AP.

Table 1 Complications of acute pancreatitis

I	Pancreatic
	1 Necrosis-sterile *vs* infected
	2 Pseudocyst-infection/rupture/hemorrhage
	3 Abscess
II	Local-extrapancreatic
	1 Involvement of contiguous organs (intraperitoneal hemorrhage, GI bleeding, thrombosis of splenic vein, bowel infarction)
	2 Pancreatic ascites
	3 Obstructive jaundice
III	Systemic
	1 Pulmonary
	a Early arterial hypoxia
	b Atelectasis, pneumonia, pleural effusion, mediastinal abscess
	c ARDS
	2 Cardiac: shock, pericardial effusion, EKG changes, arrhythmias
	3 Hematologic: DIC, TTP/HUS
	4 Gastrointestinal: GI bleeding (portal-splenic vein thrombosis, colonic infarction)
	5 Renal: azotemia, oliguria
	6 Metabolic: hypocalcemia, hyperglycemia, hypertriglyceridemia, acidosis, elevation of free fatty acids
	7 CNS: psychosis, pancreatic encephalopathy, Purtscher's retinopathy
	8 Peripheral: fat necrosis (skin and bones), arthritis
	9 Miscelaneous: rhabdomyolysis

STAGE 1: HYPOXEMIA WITH NO RADIOLOGICAL ABNORMALITIES

Tachypnea, mild respiratory alkalosis, and hypoxemia are seen in almost two thirds of patients with AP during the first 2 d of admission to the hospital[14]. In these patients, physical examination is essentially normal, chest radiographs rarely demonstrate abnormalities (11%), and clinical signs if present, seldom indicate the severity of the hypoxemia[6]. Ranson and colleagues[15] observed that 52% of patients have arterial oxygen tensions (PaO2) < 71 mmHg. Imrie and co-workers[6] showed that 45% of patients have severe arterial hypoxemia (PaO2 < 60 mmHg) and indicated that a PaO2 of < 52.5 mmHg is associated with a mortality of more than 30%. A study conducted several years ago identified that the incidence of pulmonary insufficiency in acute edematous pancreatitis is < 10%, increasing to 47% for acute sterile necrosis and 74% for infected necrosis[16,17]. Ranson *et al*[18] reported that respiratory insufficiency characterized by an arterial pO2 < 75 mmHg and drawn within 48 h of admission is observed in 58%. Recently Lankisch and associates[19] studied a group of 204 patients with AP and demonstrated that 63% of the patients have an arterial pO2 < 70 mmHg, and 30% have a pO2 < 60 mmHg, showing that hypoxemia is present early in AP patients. The major cause of hypoxia is ventilation and perfusion mismatch,

which results in a right to left intrapulmonary shunting of up to 30% of cardiac output[20] The most important precipitating factor for MODS during the first week of AP is perhaps the failure to promptly recognize and treat hypoxia and hypovolemia[21]. The incidence of respiratory insufficiency is not related to the etiological factors of AP, severity of the disease, age of the patient, admission serum amylase, serum calcium, the amount or the nature of fluid administered (colloid solutions or blood), or the estimated fluid sequestration, but is seen more often in patients experiencing their first attack of AP.

Clinical significance

If pulmonary infiltrates and severe hypoxemia develop concurrently, mortality rate may be as high as 56%[16]. In two studies, Imrie and coworkers[6,22] correlated the overall mortality with the degree of hypoxemia. They found that patients with arterial pO2 below 70 mmHg have a mortality of 3% to 5.9% and when the arterial pO2 decreases below 60 mmHg the mortality rate increases to about 14%. Marked respiratory insufficiency in 22% of the cases of AP is associated with a 60% mortality from uncontrollable hypoxemia[23]. These studies suggest that respiratory insufficiency early in AP represents a risk for future ARDS especially if it is persistent and refractory to oxygen therapy.

STAGE 2: HYPOXEMIA WITH RADIOLOGICAL ABNORMALITIES

Pleural effusion

In 33% of all patients with AP, respiratory complications are clinically or radiographically detectable[16]. Pulmonary infiltrates or atelectasis (15%), pleural effusions (4%-17%), and pulmonary edema (8%-50%) are the pulmonary manifestations of AP in this category. Mortality and morbidity are significantly higher as compared to Stage 1. Some of these patients require ventilatory assistance. A direct relationship to prognostic factors is observed in this stage.

Presence of pleural effusion is currently considered an indication of severe pancreatitis and not just a marker of the disease[24]. Pancreatic ascites and pleural effusion are rare complications of both chronic and acute pancreatitis, and are associated with a mortality rate of 20% to 30%[25]. Mediastinal pancreatic pseudocysts and acute fluid collections are rare complications of pancreatitis resulting in an increase in morbidity and mortality[26]. Pleural effusions in AP are usually small, occasionally bloody, and are characterized by high amylase (up to 30 times greater than corresponding serum value), protein (> 30 gm/L), and lactic acid dehydrogenase (ratio) (> 0.6 serum value) levels[27]. The majority of pleural effusions (68%) are left-sided, 22% are bilateral and 10% are right-sided only. Two main causes of pleural effusion are transdiaphragmatic lymphatic blockage or pancreaticopleural fistulae secondary to leak and disruption of the pancreatic duct or pseudocyst caused by an episode of acute pancreatitis. The leak or disruption is more likely to lead to a pleural effusion if the duct disruption is posteriorly into the retroperitoneum. The pancreatic enzymes can track up into the mediastinum and then rupture into the pleural cavity either left side or bilaterally and so create a connection between the pancreatic duct and the pleural cavity. Pancreatic pleural effusions may be massive and require treatment[28].

Close to 1/3 of all patients with an internal pancreatic fistula present with pleural effusion[29]. Formation of a posterior mediastinal cyst indicates that a patient is at increased risk of developing pleuropulmonary complications including acute and chronic pleural effusions which can be sympathetic in origin or result from the development of a fistulous tract into the pleural space[30]. Dyspnea and hypoxia, chest pain, atelectasis, cough, and ARDS can develop, worsening the prognosis[26].

Treatment of pleural effusion is usually at first conservative. Pleural effusions which become symptomatic often require thoracentesis, tube thoracotomy, endotracheal intubation, ICU admission, parenteral alimentation, and administration of octreotide. When the intraabdominal etiology is resolved, pleural effusions often resolve as well. Chronic effusions often require drainage of the pseudocyst or abscess or excision of the fistulous tract[27].

Atelectasis

Consolidation of lung tissue and atelectasis are frequent radiological observations. In cerulein-induced experimental pancreatitis-associated lung injury, it has been observed that there is a decrease in the production of pulmonary surfactant[31]. A decrease in the quantity of pulmonary surfactant may be a significant cause of atelectasis formation in dependent lung regions. Cerulein-induced pancreatitis appears to cause a decrease in the strength of the muscles of the diaphragm as well as a decrease in the endurance of these muscles[32]. Because the primary lesion is found at the alveolar interstitial level, it is probable that this decreases lung compliance and increases the work of breathing, leading to fatigue of the diaphragm more quickly[32].

There is mounting evidence that the proinflammatory cytokines are the agents behind the systemic complications of AP[33-35]. Cytokines are low molecular weight proteins, secreted by various cell types, that regulate the intensity and duration of immune responses and mediate cell-to-cell communication[36]. During AP IL-1, IL-6, and TNF-α are formed within the pancreas[32]. There is growing evidence that cytokines, mainly TNF-α and IL-1, could be the orchestrators of skeletal muscle dysfunction during sepsis. An experimental study showed that TNF-α at a lower concentration (50-400 ng/mL) causes no diaphragmatic fatigue in rats[37]. However at a higher concentration (10 μg/mL) of the cytokine, fatigue occurs in hamsters[38]. It has been noticed that TNF-α and IL-1 have a synergistic negative inotropic effect on the contractility of the diaphragm[39]. According to Matuszczak *et al*[32], cytokine levels need to be at a very high concentration to have a physiologic effect. Since the concentrations of cytokines in muscle during sepsis have not yet been quantified, the higher concentration of cytokines used in the study may be achieved in the natural physiologic setting of sepsis[32]. According to Norman *et al*[35], the intrapancreatic concentration of cytokines achieved in AP is much

higher than the systemic level and this argues in favor of the observation by Matuszczak *et al*[32] who used higher concentrations of cytokines to achieve the end result of diaphragmatic fatigue. According to these studies, it is possible that if IL-1, IL-6, TNF-α are present at high enough concentrations in the pancreatic juice, they could cause diaphragmatic fatigue. Diaphragmatic impairment may be the pathophysiologic cause of atelectasis in the lower regions of the lung via decreased ventilation in these regions[32].

STAGE 3: ARDS

The most dangerous complication of the pulmonary system is ARDS. ARDS, a syndrome first described in 1967[40], is observed in association with AP[16]. Of the patients who develop AP, 15% to 20% develop ARDS with an associated mortality of 56%[41]. ARDS usually manifests itself between two to seven days following the onset of AP, but may have a much more rapid course. Clinical features include severe dyspnea and extreme hypoxemia refractory to a high inspired oxygen concentration. Multilobar pulmonary infiltrates in patients with previous normal radiographs and a relatively normal pulmonary capillary wedge pressure (< 18 mmHg) are noted[42,43]. Autopsy studies in patients with AP have shown that morphological changes in the lungs are indistinguishable from those in patients with ARDS caused by other conditions, including shock, sepsis, and severe trauma[44]. The lungs are characterized by increased alveolar capillary permeability with interstitial edema. The complication of ARDS parallels the presence of other poor prognostic signs of AP[15]. Although less often than necrotizing pancreatitis, patients with interstitial edematous pancreatitis are also at risk (10%) of developing respiratory failure[45].

There are three clinical features that must be present in ARDS: widespread bilateral radiographic infiltrates, a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO2/FiO2) less than or equal to 200 regardless of the positive end-expiratory pressure (PEEP), and no clinical evidence for an elevated left atrial pressure (less than or equal to 18 mmHg)[21,46]. Hypoxia is worse in ARDS. ARDS accounts for 50%-90% of all deaths from pancreatitis[47]. Improved ventilatory and supportive care, has improved the outcome in ARDS. However the underlying etiology remains unclear, keeping the mortality still unacceptably high.

Pathophysiology

The pathophysiology of ARDS in AP is poorly understood. Actions of pancreatic enzymes as well as inflammatory mediators released as a result of pancreatic injury play a key role in the pulmonary complications. The pathophysiology of ARDS is described as increased pulmonary vasculature leaking protein- rich transudate into the alveolar space and decreased lung compliance manifested clinically as refractory hypoxemia, and radiologically as diffuse infiltration in the lungs.

In the pathogenesis of systemic complications of AP, the role of active enzymes in circulation, liberation of proinflammatory cytokines, leukoagglutination, migration of neutrophils, activation of leukocytes, complement-mediated injury, and platelet activating factor decreasing the normal defense mechanisms and increased production of Nitric oxide (NO) are studied. The agents which are suspected to play a role in the pathogenesis of AP are noted in Table 2. A number of experimental and clinical studies have helped to elucidate the mechanisms involved in the pathogenesis of lung injury secondary to AP.

Table 2 Pathogenic players in respiratory insufficiency

1	Phospholipase A2
2	Trypsin inhibitor
3	FFA/Lipoprotein lipase
4	Complement activation (C5a)
5	MIF
6	IL-6, IL-8, IL-1β
7	NO
8	TNF-α
9	fMet-Leu-Phe (a bacterial wall product)
10	ICAM-1
11	β-2-integrin (CD11b/CD18)
12	Trypsin
13	NF-κB
14	Substance P

Activated trypsin: It is a possible source for the cause of pulmonary insufficiency. Trypsin causes damage to the pulmonary vasculature and increases endothelial permeability[48]. In laboratory experiments trypsin has been shown to cause leukostasis in the pulmonary vasculature and thus activated trypsin could intensify intravascular coagulation in the pulmonary microcirculation[49]. In post mortem studies, patients with acute pancreatitis have been shown to have intravascular fibrin thrombi in different tissues, including the lungs[16]. Trypsin is capable of activating different complement factors directly, which can stimulate cytolysis and chemotactic leukocytes. Complement has been shown to produce ARDS raising the possibility of developing respiratory insufficiency.

Phospholipase A: As an incriminating factor in pulmonary complications of AP phospholipase A_2 (PLA_2) has been investigated by many authors. PLA_2, which is activated by trypsin in the duodenum, is known for its ability to remove fatty acids from phospholipids. Pulmonary surfactant lines the surface of the alveoli and prevents the alveoli from collapsing by maintaining the surface tension. One of the main components of surfactant is phospholipid dipalmitoy lphosphatidylcholine, which is a perfect substrate for PLA_2. The basic reason for pulmonary insufficiency and ARDS in AP is due to the destruction of the surfactant. Büchler *et al*[45,50] showed that there is a strong correlation between serum-activated PLA_2 and pulmonary insufficiency. In their prospective study, patients with pulmonary failure demonstrated a notably higher catalytic PLA_2 activity during the first week of AP than patients without arterial hypoxemia. Büchler *et al*[45] and Kortesuo *et al*[51] observed that there are many isoenzymes of PLA_2. The source of IR-PLA_2 and group I PLA_2 is the pancreas, but has no correlation with the severity of pancreatitis or likelihood of development of pulmonary insufficiency. Group II PLA_2, which has an extra-pancreatic source, correlates

well with the severity of AP and is probably responsible in part for the pulmonary insufficiency occurring as an extra-pancreatic complication of AP[45,51,52]. The role of other isoenzymes of PLA_2 in AP has yet to be elucidated. Inflammatory cells do not seem to be the source of Group II PLA_2, but they cause the pulmonary insufficiency and ARDS in AP[53]. The low molecular weight PLA_2 inhibitor has been shown to decrease the level of group II PLA_2 activity and has no effect on group I PLA_2 activity. The inhibitor also decreases tissue destruction and protects pancreatic acinar cells[54]. This is an area which needs further investigation to improve the outcome of AP.

Platelet activating factor: PAF is a potent biological mediator whose effect is manifested throughout the body. PAF stimulates PMN white cells and regulates the interaction between PMN cells and endothelial cells, facilitating migration of activated white cells into tissue spaces. PAF is a structural component of membrane lipids and is released upon the action of PLA2[13,55]. In induced experimental pancreatitis (whether induced by immune complex, caerulein, or taurocholate) PAF is released into the pancreas, ascitic fluid, lung and blood[56]. PAF can be controlled via the PAF receptor and PAF antagonists decrease the severity of experimental AP by reducing oxidative injury, morphological changes, white cell infiltration, vascular permeability in pancreas and lungs, pulmonary damage, blood, and peritoneal PAF exudative concentrations[56]. Lexipafant, a computer image analysis generated imidazolyl derivative of Sp^2 nitrogen compounds, is a powerful PAF receptor antagonist having an affinity for the receptor seven times more avid than PAF itself[57]. Lexipafant has undergone a randomized double blind phase II clinical trial in human pancreatitis and is very successful in reducing organ failure[58]. However, during phase III clinical trial, Lexipafant is unsuccessful in reducing new organ failure or mortality. In the largest systematic prospective study of severe AP ever undertaken, Johnson *et al*[59] found that 58% of patients in the placebo group and 57% of patients in the Lexipafant group develop one or more organ failures, indicating that there is no difference between these groups. Systemic sepsis affects fewer patients in the Lexipafant group, the development of pseudocysts is 14% in the placebo group and 5% in the Lexipafant group. IL-8, a marker for neutrophil activation and E-selectin, a marker for endothelial damage, decrease more rapidly in the Lexipafant group than in the placebo group. This adequately powered study showed that antagonism of PAF activity on its own is not sufficient to ameliorate SIRS in severe AP or change mortality rates[59].

Free fatty acid: Another possible cause of pulmonary insufficiency is the release of free fatty acid (FFA). During the inflammatory process, pulmonary lipoprotein lipase is activated and releases FFA from albumin, to which they are physiologically bound. The free fatty acids released from triglycerides (TG), have been shown to damage capillary alveolar wall membranes[60].

Digestive enzymes: In a normal pancreas, potentially harmful digestive enzymes (i.e. protease and phospholipase) are synthesized as inactive proenzyme forms which are only activated after pancreatic secretions reach the duodenum. Enterokinase, a brush border enzyme, cleaves trypsinogen into 2 products, namely N-terminal portion termed trypsinogen-activation peptide (TAP) and active trypsin. Active trypsin then catalyzes the conversion of other proenzymes secreted by the pancreas into their active forms[61]. Gukovskaya *et al*[62] demonstrated that neutrophils infiltrate into the pancreas facilitating intrapancreatic trypsin activation during cerulean (cholecystokinin analogue)-induced experimental pancreatitis. The immunocytochemical localization experiments using specific antibodies to TAP indicate that the trypsin activation mediated by neutrophils occurs within the pancreatic acinar cells[62]. Steer[63], in an excellent hypothesis, suggested that intrapancreatic (i.e., intra-acinar cell) activation of trypsinogen during pancreatitis is itself a 2-phase event. According to this interpretation, the initial phase of trypsinogen activation would be neutrophil-independent and solely dependent on acinar cell events. This initial phase of trypsinogen activation can lead to early acinar cell injury that triggers the pancreatic inflammatory response[63].

Activated neutrophils throughout the body, are attracted to the pulmonary microvascular network by various factors such as complement activation, cytokine production, alveolar macrophages and the upregulation of adhesion molecules[64]. Chemoattractants such as TNF-α, IL-1, IL-6, IL-8, fMet-Leu-Phe (a bacterial wall product) and complement factor C5a upregulate the beta-2-integrin expression on neutrophils and the ICAM-1 receptor on endothelial cells[65,66]. The binding of ICAM-1 to beta-2-integrin (CD11b/CD18) can increase the permeability of the pulmonary vasculature, allowing neutrophils to enter the lung parenchyma. Upon entry into the parenchyma, neutrophils release compounds which have been implicated in the damage caused by ARDS. This theory is supported by studies demonstrating that mice deficient in ICAM-1, develop a much less severe form of pancreatitis, and that ICAM-1 expression is increased in mice with pancreatitis[67,68]. Several compounds released by neutrophils once within the lung parenchyma are discussed in the following paragraphs.

TNF-α: High concentrations of TNF-α are found in a variety of locations throughout the body during an episode of AP, and pancreatic parenchyma, ascitic fluid, lymphatic drainage and serum levels can predict disease severity and possible mortality[35,69-72]. TNF-α activates neutrophils and increases lung damage levels at higher concentration in the lung[73,74]. A tetravalent guanylhydrazone compound CNI-1493 has been developed in a research program to prevent the production of macrophage-derived NO. It was then discovered that CNI-1493 blocks the production of inflammatory mediators such as TNF-α, IL-1, IL-6, macroinflammatory peptide (MIP)-1, by preventing the phosphorylation of p38 mitogen- activated (MAP) kinase[75]. If p38 MAP kinase is prevented from being phosphorylated, then TNF-α is not produced or is produced at a significantly lower level[76]. Denham *et al*[77] reported that CNI-1493 significantly attenuates the increase in pulmonary TNF-α resulting in decreased lung injury, confirming that CNI-1493 inhibits its pancreatic and pulmonary TNF-α gene induction in two models

of murine pancreatitis. These results also support that pulmonary congestion is decreased when anti-TNF-α antibody is administered after pancreatitis is induced and overall morbidity and mortality are decreased[78,79]. Several recent studies have suggested that TNF-α is one of the agents orchestrating the early stages of AP, especially in recruitment of inflammatory cells, regulation of cytokine production, and promotion of pancreatic acinar cell death by apoptosis[61,69,79]. TNF-α also plays a protective role in preventing the release of proinflammatory cytokines[76,80]. TNF-α induces concomitantly proapoptotic and antiapoptotic mechanisms[81]. Antiapoptotic mechanisms are controlled by NF-B and MAP kinases. Pancreatitis-associated protein (PAP) prevents apoptosis[81,82]. Apoptosis is possibly a more favorable demise for acinar cells than necrosis because it results in a quick removal of damaged cells without loss of plasma membrane integrity as well as decreased recruitment of leukocytes[81]. According to Kaiser *et al*[81] severe forms of AP demonstrate little apoptosis while edematous pancreatitis shows a higher number of apoptotic cells. There is an interaction between apoptosis and necrosis, with inflammatory cells infiltrating the pancreas and causing necrosis of the apoptotic cells[81,83]. Apoptosis appears to be advantageous over necrosis, but needs to be controlled to prevent excessive tissue loss[81].

Nitric oxide: The role of nitric oxide (NO) in vascular collapse during AP is highly controversial[77,84]. CNI-1493 blocks the production of NO by macrophages, subsequently the level of NO decreases lessening the damage to the lungs[84]. However, Tsukahara *et al*[85] demonstrated that alveolar macrophages during experimental pancreatitis mediate endothelial injury as well as increase microvascular permeability resulting in lung injury. In contrast, O'Donovan *et al*[86] showed that administration of sodium nitroprusside, a NO donor, to rats with caerulein-induced pancreatitis decreases lung myeloperoxidase activity, bronchoalveolar lavage protein concentration, and wet-to-dry lung weight, suggesting that NO may be protective against lung injury during pancreatitis. A possible explanation for the contradictory findings between Tsukahara *et al*[85] and O'Donovan *et al*[86] is the different experimental models or is possibly attributable to the paradoxic nature of NO[77].

Substance P: Substance P is a neuropeptide which is located in the nerve endings throughout the body and is released into the gap where it acts via the NK1 receptor to mediate pain[12]. It has also been implicated in many inflammatory states, such as immune complex- mediated lung injury, asthma and inflammatory bowel disease[87,88]. Once pancreatitis occurs, there is an increase in substance P levels as well as NK1 receptors on pancreatic acinar cells[12]. Interestingly, mice which have been genetically engineered to be deficient in NK1 receptors are protected from AP[89]. This gives clinical medicine another possibility for preventing AP and thus also for protecting patients against pulmonary complications of AP.

Chemokines: Chemokines are a family of cytokines which are distinguished by the fact that they act on the superfamily of G-protein–coupled serpentine receptors[12]. There are two subsets of chemokines based on the orientation of the first two cysteines: CC chemokines and C-x-C chemokines. The CC chemokines stimulate monocytes and C-x-C chemokines stimulate neutrophils. IL-8 is a C-x-C chemokine[90]. There is no direct homologue of IL-8, but there is a homologue of growth-related oncogene-alpha (GRO-α) which is cytokine-induced neutrophil chemoattractant (CINC)[90]. CINC (also a C-x-C chemokine) is a specific neutrophil attractant. A recent study reported that anti-CINC antibody reduces lung damage. During the study, anti-CINC was administered to Caerulein- induced pancreatitis mice prophylactically and during Caerulein administration. The results demonstrate that both the prophylactic group and the therapeutic group have a reduction in lung injury, but not a reduction in AP[90].

IL-8: IL-8 is a strong attractant of neutrophils in the lungs and its high concentrations have been observed in the lungs of patients with ARDS[91,92]. Donnelly *et al*[93] showed that bronchoalveolar lavage (BAL) levels of IL-8 are significantly higher in patients who develop ARDS than in those who do not develop ARDS. However they have not found a relationship between blood levels of IL-8 and patients with or without ARDS, but found that IL-8 may be a prognostic factor for ARDS. Kurdowska *et al*[94] took the IL-8 issue one step further to determine if the anti-interleukin 8 and interleukin-8 complex is a marker of patients who are at risk of developing ARDS, and found that anti-interleukin 8 and interleukin-8 complexes may be a marker of patients who may progress to ARDS.

Macrophage migration inhibitory factor (MIF): MIF is a cytokine discovered in 1966 and produced by T lymphocytes and plays a role in preventing random migration by macrophages[95,96]. It is now known that MIF is released not only by T lymphocytes but also by monocytes, macrophages, pituitary corticotrophic cells, and epithelial cells. MIF is currently viewed as an important proinflammatory cytokine[97,98]. MIF has a critical role in regulating the immune system by overriding the anti-inflammatory and immunosuppressive effect of glucocorticoids on macrophages and T cells[98,99]. MIF has been shown to be elevated in several inflammatory diseases such as sepsis, rheumatoid arthritis, bronchial asthma and also in the bronchoalveolar lavage samples of adults with ARDS[100-103]. It was reported that MIF levels in the lungs of taurocholate (TCA) pancreatitis rats are significantly higher than those in the lungs of normal rats, whereas MIF levels in the pancreas and liver have no differences between the normal and TCA pancreatitis rats[103,104]. There is a possible connection between TNF-α and MIF[103,105]. Pulmonary TNF-α levels are increased significantly in TCA- induced pancreatitis and the increase can be attenuated by treatment with anti-MIF antibody, but not with the control antibody[103]. Anti-MIF antibody also improves the survival rate in both TCA pancreatitis rats and choline deficient, ethionine-supplemented (CDE) pancreatitis mice[103]. Serum MIF levels are also found to be significantly higher in severe AP than in mild AP or in healthy controls[103]. Combining the increased survivability with anti-MIF antibody, induction of IL-8, stimulus of TNF-α production, ability to override the effects of glucocorticoids and the increased levels of MIF found

Table 3 Pro-inflammatory cytokines

IL-1β -major inflammatory mediator, major activator of macrophages and enhances B and T cell activation
IL-6 -acute phase reactant, stimulates B cell differentiation
IL-8 -stimulates the upregulation of adhesion molecules, chemotactic factor for neutrophils and lymphocytes
TNF-α -recruitment of inflammatory cells, regulation of cytokine production, and promotion of pancreatic acinar cell death by apoptosis

IL-10 is an anti-inflammatory cytokine by inhibiting macrophages releasing inflammator mediators.

in severe AP, suggests that MIF plays strongly a vital role in APand lung injury[103]. It also raises the possibility of treatment with anti-MIF antibody to prevent lung injury in AP in the future.

IL-10: IL-10 is one of the few known anti-inflammatory cytokines in human body which prevents the production of pro-inflammatory mediators particularly TNF-α by macrophages and T cells[106-109]. IL-10 also inhibits the release of IL-6, and IL-1-beta[12,108,110-112] as well as stimulates the synthesis of IL-1 receptor antagonist (IL-1ra) and the release of soluble p75 TNF receptor which inhibits the action of pro-inflammatory cytokines[113]. (Table 3) In particular IL-10 has been shown to inhibit the release of TNF-α from alveolar macrophages, thus implicating that it can decrease the likelihood of developing ARDS[114]. In another study, IL-10 was administered to one group of mice prior to the development of AP. IL-10 was administered to another group of mice 33 hafter the induction of AP. The study showed that mortality is decreased due to AP in both groups[115]. Two other studies showed that both IL-10 and IL-1ra significantly decrease the severity of AP when given after the induction of AP[113,115]. Several studies have shown that IL-10 when given after AP can decrease the severity of AP, which raises the possibility of IL-10 used in clinical treatment of AP[35,115].

CONCLUSION

Mortality in AP occurs in two peaks and is usually associated with MODS. Pulmonary dysfunction is perhaps the most critical for all extra-pancreatic manifestations of AP[3,4]. The pathogenesis of pulmonary complications in AP has been the topic of intense research in the past two decades. In this review, we have evaluated the different types of respiratory complications, with their clinical significance assessed and their pathogenesis discussed. Although the data are predominantly from experimental studies, their clinical relevance is of increasing importance. Hypoxemia occurs in nearly 50%-60% of patients with AP[6,18,19]. The major cause of hypoxemia is ventilation/perfusion (V/Q) mismatch which results in right to left intrapulmonary shunting. Failure to promptly recognize hypoxemia and hypovolemia may precipitate MODS. Pleural effusion on the left side, once considered just a marker of AP, is indeed a sign of poor prognosis[24]. Atelectasis is noted to be secondary to a decrease in pulmonary surfactant[31]. The role of cytokines has become quite evident in a number of experimental studies of atelectasis[33-36]. The most dangerous pulmonary complication is ARDS[33-35]. Of the patients who develop ARDS nearly 50% would die[16]. The incidence is much less in edematous pancreatitis. The clinical features of ARDS include widespread bilateral infiltrates in CXR and a ratio of partial pressure of arterial O2 to fraction of inspired oxygen (PaO2/FiO2) ⩽ 200 regardless of PEEP with no evidence of elevated left atrial pressure[21,46]. The pathophysiology of ARDS is complex. The action of enzymes in circulation (trypsin, and phospholipase A2, proinflammatory cytokines, leukoagglutination, migration of neutrophils, activation of leukocytes, complement mediated injury, PAF, NO) is noted to play a different role in the pathogenesis.

The future management of AP that currently carries an overall mortality of 5% and 15%-20% in necrotizing pancreatitis includes measures to counteract the proinflammatory agents[1,2]. With the exception of platelet activating inhibitor agents, such as Lexipafant, nothing seems to be clinically relevant at this time[57]. However, a better understanding of the pathogenesis of lung injury and other organ dysfunction is expected to revolutionize the treatment of AP.

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REVIEW

Associations between NOD2/CARD15 genotype and phenotype in Crohn's disease-Are we there yet?

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Abstract

There have been multiple NOD2/CARD15 genotype-phenotype analyses undertaken in patients with Crohn's disease since the gene's discovery in 2001. This review focuses on the major published series based upon their size and on the presence of specific clinical and genetic information provided in the published material from 2001 to 2005. Twelve studies provided raw data to carry out comparisons of disease location while ten studies included analysis of NOD2/CARD15 genotypes. NOD2/CARD15 variant frequency in ileal disease did not differ significantly among studies, whereas a comparison of disease location demonstrated highly significant differences among studies. Meta-analysis confirmed significant associations between NOD2/CARD15 variants and both ileal and ileocolonic disease locations, and with both stricturing and penetrating forms of disease behavior. This review underlines the significant phenotypic differences that exist among populations, including similar ethnic groups, and has demonstrated the need for further studies of patients with long-term "inflammatory" Crohn's disease.

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Key words: Crohn's disease; Phenotypic heterogeneity; Genotype; Inter-observer agreement; Disease location; Disease behavior

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INTRODUCTION

Chronic idiopathic inflammatory bowel diseases (IBD), made up predominantly of Crohn's disease (CD) and ulcerative colitis, remain fertile ground for both clinical and basic science research. These conditions have been held up as classic examples of complex polygenic disorders with the working hypothesis of a genetically susceptible host manifesting the disease phenotype after exposure to a single or a series of environmental triggers. Study of the genetics of IBD has expanded rapidly over the past 10 years since publication of the first genome-wide scan in 1996[1]. Technical advances have aided this advance with the culmination being the discovery of the NOD2/CARD15 gene within IBD1 on chromosome 16[2,3]. The case for further identification of IBD susceptibility genes has been put forward, specifically regarding the organic cationic transporters, OCTN1 and OCTN2, on chromosome 5 (IBD5) and DLG5 on chromosome 10[4,5]. These genes have not received universal support from independent studies and remain topics for discussion and research, specifically including further functional studies.

These rapid advances in IBD genetics, fuelled by the interest and enthusiasm of both clinicians and geneticists, has set the pace of IBD research over the past 8-10 years, which has succeeded the "IBD immunology" era of the 1990's. While the latter gave us a plethora of cytokine data, including the important role of TNF-α leading to anti-TNF strategies in CD, the discovery of NOD2 highlighted the significance of the gut's innate immune system including pathogen-associated molecular patterns (PAMPs)[6]. This progress has been particularly challenging for clinician researchers as they are expected to translate these critical discoveries into meaningful changes and advances in clinical practice, including assessment and treatment of IBD patients. In this review, we will assess the clinical interpretations of major breakthroughs in CD genetics focusing on the NOD2/CARD15 gene, including both the strengths and limitations of such studies and an analysis of significant differences in phenotypes and genotypes, and we will offer suggestions for future studies[7,8].

CLASSIFICATION OF PHENOTYPE

Clinical classifications of CD seem to be used synonymously with terms for phenotypes, and a number of papers have described "genotype-phenotype

correlations". However, genotypes and phenotypes are derived in fundamentally different ways. While genotype is based upon a limited number of options that can be determined mechanically and thus objectively, derivation of phenotype is subjective and influenced by potentially multiple variables, with many yet to be identified. A number of variables may not be included in multivariate analysis when genotype-phenotype analyses are performed.

The clinical classification systems that have been put forward have been purposefully kept simple. This may have been done to encourage individuals and groups to adopt them and hence maximize opportunities to compare and combine datasets[9-11]. However, given this intention, it does not appear to have been successful for genotype-phenotype studies in CD. The updated Vienna classification for Crohn's disease, now known as the Montreal classification, has maintained a simple approach including age at diagnosis, location, and behavior as the major parameters, with perianal disease as a subgroup of behavior[11]. It has appropriately split age at diagnosis and perianal penetrating from internal penetrating, but has not clarified the issue of stricturing disease working independently of penetrating disease[12]. There is no mention of the need to demonstrate the presence of transmural inflammation, assuming that this is a prerequisite for the diagnosis of CD. This is relevant to subsequent study of those patients with inflammatory CD who do not evolve into a stricturing and/or penetrating pattern. Do these patients fit into the definition of CD, given that they do not appear to show any association with NOD2/CARD15 after correction for disease duration?[13] Other variables that may well influence natural history, including smoking, prior appendectomy, and body mass index, have thus far not been included in this classification[14-16]. The latter may influence development of penetrating disease, the level of inflammation and hence CRP and the risk of metabolic bone disease[17,18].

Clinical classification systems for ulcerative colitis are few. There was no such system included in the Vienna initiative. The Montreal paper presented a simple classification based upon distribution and activity, but both of these parameters are subjective, particularly when the former is based upon macroscopic and not microscopic observations. There is again no inclusion of smoking or prior appendectomy despite strong evidence supporting a role for appendectomy in (re)shaping the natural history of ulcerative colitis.

No classification system or phenotypic approach has attempted to embrace the microscopic pattern of inflammation, damage and repair seen in both CD and ulcerative colitis. Limited work has been done on granulomata and their association with both clinical outcomes and with some genetic polymorphisms[19-21]. This is in contrast to several other fields of research, including liver disease, where histological appearances are used as important aids in determining the risk of fibrosis, and colorectal cancer, in which significant heterogeneity has been identified among cancers based both upon histological appearances and molecular markers[22,23].

STRENGTHS AND LIMITATIONS OF STUDIES TO DATE

Disease susceptibility and phenotype-genotype analyses have arrived in two "waves" since the seminal publications on NOD2/CARD15 and CD in 2001. The first wave of papers identified the key CD-causing variants in this gene and went on to establish an association with ileal location[12,24-29]. Some studies in this group supported a stronger association with stricturing disease behavior and not ileal location, after multivariate analysis[12,24,29]. Two studies carried out haplotype analysis[27,28]. The second wave included studies from smaller centers, predominantly across Europe, focusing on phenotype-genotype correlations[30,40]. There have been a limited number of studies that have investigated response to therapy[41,42].

The limitations of these studies center around two points: case-control ascertainment and phenotypic classification. Thus far, all of these studies have been sourced from specialist-based cohorts and often from large, tertiary referral centers[12,24-29]. Ascertainment of cases has also been influenced by earlier genetic studies where the primary aim has been "gene discovery" and hence there have been an exaggerated number of CD multiplex families[12,24]. Many of the studies combine the resources of multiple sites, which have contributed to collection of these multiplex families[12,24,25]. The method of ascertainment of controls is not always clear, and in the majority of cases they do not represent true population controls. Specifically, some studies have excluded controls with a family history of IBD thus biasing the population even further.

Since the majority of studies lack power to carry out detailed phenotype-genotype analyses, it is tempting to combine datasets in a meta-analysis. However, this has been hampered by a plethora of different approaches to clinical classification of the case population. Disease location is variously described as "any ileal" and "any colonic", or "ileal and right colon" with "left colon and rectum", without prior validation of these systems[24,26,28]. Similarly, disease behavior, which clearly evolves with time, was modified from the Vienna classification in several studies, again making it difficult to combine datasets. Disease duration has often been omitted. There have been inconsistencies in defining disease location, including the inclusion or exclusion of perianal abscess, combining ileal and ileocolonic locations without giving raw data on these locations individually and providing data on very specific subgroups, such as UC-like CD, where the number in this group (n = 56) is greater than the "colon-only" location as a whole (n = 29). More recent studies have concentrated on using the Vienna classification despite its known limitations, but have in some cases tried to address these limitations by carrying out supplemental analyses including investigation of an association between NOD2/CARD15 and stricturing disease independent of penetrating disease and internal penetrating disease independent of perianal disease.

In summary, studies thus far have provided us with

some important information on the strength of the NOD2/CARD15 association with CD as a susceptibility gene but these have not been based on population-based cohorts. Population-based studies are awaited and may be best coordinated through an international effort using agreed upon methods of case-control ascertainment, genotyping and clinical classification. Associations with ileal location have provided us with important clues to disease pathogenesis, including the role of Paneth cells and defensins. However, extensive further work is required using much larger datasets that include other key variables, such as treatment received, to determine the mechanisms by which NOD2/CARD15 variants may increase the risk of stricturing disease, and whether these variants are also implicated in the development of internal penetrating disease.

RESULTS OF STUDIES ON NOD2/CARD15 TO DATE

Multiple studies have now investigated the contribution of variants in the LRR domain of the NOD2/CARD15 gene to development of CD. This has been summarized in a recent meta-analysis. Individuals carrying only one high-risk allele had 2.39-fold (95% CI: 2.00-2.86) increased risk of the disease, while those with 2 or more high risk alleles carried a 17.1-fold (10.7-27.2) increased risk of CD compared to individuals without any high-risk alleles. The greatest relative risk was identified for the SNP13 variant (OR 3.76, 95% CI: 3.22-4.38 for one variant) but significant heterogeneity existed among studies ($P = 0.01$)[40].

For the purposes of the rest of this paper, we have selected 15 studies that each provide both genotype and (some) phenotype data on a minimum dataset of 200 CD cases[12,24-36,38]. We will discuss the evidence for associations between NOD2/CARD15 and key clinical variables including age at diagnosis, disease location and behavior, need for surgery and the presence of granulomata. We have carried out further analyses on these studies as a combined dataset and as individual series, where sufficient clinical and genetic data are available in the original publication. We focus specifically on differences in phenotype and genotype among studies, and have carried out meta-analysis for NOD2 associations with disease location and behavior.

DISEASE LOCATION

Of the 15 studies selected, 13 provide clearly interpretable data on disease location. Of these, 10/13 support a significant association with ileal disease (L1)[12,25-27,31-34,36,38], while some also demonstrate an association with absence of colonic location (L2)[24]. As indicated above, some studies have combined ileal (L1) with ileocolonic (L3) patients to investigate this association. This may be due to a lack of power in subgroup analysis. Clinically, this may be inappropriate. There are limited data on the natural history of ileocolonic disease compared with ileal disease but associations have been made between L3 and younger age at diagnosis and an increased risk of surgical recurrence compared to other disease locations[43,44]. In addition, some patients with L3 may have their major disease burden in the colon, thus weakening the association with NOD2/CARD15 compared to pure ileal disease or L1. Once again, the interpretation of results depends heavily on the clarity of describing phenotypes, and in this case how the presence or absence of ileal disease was determined, whether by endoscopy, histology, radiology or a combination of these.

DISEASE BEHAVIOUR

Of 13 studies that provided data for the variable disease behaviour, 7 showed a significant association with stricturing behaviour[12,24,29,30,34,36,38]. Two studies show significant positive associations with both stricturing and penetrating disease behaviour[12,34], but one of these excluded perianal penetrating disease from the analysis. If included, this association with NOD2 variants is lost[12]. One study described a negative association with penetrating behaviour[26]. Penetrating disease is defined according to the Vienna classification in 8/13 studies and in another study it is defined as "internal penetrating" behaviour, separating it from perianal penetrating disease[12]. The rest of the studies describe their own "in house" definition of penetrating disease. Given the overlap between these forms of disease behaviour and the length of time some patients may take to develop "complex" CD, supplementary analyses seem necessary to clarify these observations. Specifically, stricturing CD will often occur in combination with penetrating disease, both being hallmarks of transmural inflammation. One may therefore predict that NOD2/CARD15 should be associated with both complications. If this is not the case, it may relate to a lack of association of NOD2/CARD15 with perianal penetrating disease that influences the relationship with penetrating disease overall, as suggested by Brant *et al*[12]. Alternatively, NOD2/CARD15 variants may promote the development of fibrosis over fissuring ulceration. These questions clearly need to be addressed in future studies.

AGE AT DIAGNOSIS

Five of thirteen studies that looked for an association between NOD2/CARD15 and age of diagnosis found a significant association with early onset of the disease[12,24,26,32,38]. Of these, 4 found association only for NOD2/CARD15 homozygotes and compound heterozygotes and 1 found association with these groups as well as with the frameshift variant alone. Importantly, these findings remained significant after multivariate analysis when important confounders such as disease location and stricturing behaviour were included. These data support the results of previous linkage studies that demonstrated an association between IBD1 and earlier age at diagnosis[45]. They also support the concept of pediatric CD being a "more genetic" disease, consistent with other polygenic disease models.

OTHER CLINICAL ASSOCIATIONS

Eleven studies provided data on surgery for CD and 8 of

these provided NOD2/CARD15 genotype data on the "surgical" cohort. Two groups have identified an association between NOD2/CARD15 and increased risk of surgery. Lakatos *et al* demonstrated this to have an independent effect on need for surgery after logistic regression (OR 1.71, 95% CI: 1.13-2.62, $P = 0.01$) together with presence of stricturing disease behaviour. This cohort had the largest complete dataset of 527 patients and provided the most comprehensive analysis of NOD2/CARD15 genotype-phenotype relation[38]. The second study showing this association also carried out similar statistical analysis, finding a higher risk for surgery with those carrying two variant alleles (OR 17.8, 95% CI: 4.9-64.3)[36].

FURTHER ANALYSIS OF CLINICAL CHARACTERISTICS AND GENETIC ASSOCIATIONS

Statistical methods

Differences in proportions among different studies were tested using simple χ^2 distribution obtained as the sum of [(Observed – Expected)^2/Expected] with (R-1)*(C-1) degrees of freedom where R is the number of rows (studies) and C is the number of columns (locations, genotypes). The higher contribution to the χ^2 from different studies suggested a higher degree of variation in the distribution of reported location.

Meta-analysis was performed by calculating the odds ratio and confidence interval using the frequency distribution of NOD2 variants and the variable of interest presented by studies involved in the analysis. Fixed analysis used an inverse variance method to calculate weight, and weighted average was calculated as a pooled estimate. Heterogeneity among studies was calculated using Cochran's Q (sum of squared difference between individual study effect and pooled effect across studies). Analysis was performed using Meta command in STATA 9[46].

Differences among studies

Ten studies gave clear data on numbers of patients with pure ileal CD and their NOD2 variant frequencies. The majority of studies had small numbers in this important subgroup of CD (mean, 85 patients, range 23-136) with an overall NOD2 variant frequency of 41.1% (23.4%-53.6%). Comparisons between studies just failed to show a significant difference ($P = 0.06$). However, exclusion of one study from Finland, which is known to have lower NOD2 variant frequencies compared to the majority of other European populations, demonstrated similar proportions in the majority of these studies ($P = 0.53$).

In contrast, a comparison of disease location among 12 studies that provided data on this variable as per the Vienna and Montreal systems, demonstrated highly significant differences ($P < 0.0001$). Unlike the genotype data for NOD2, exclusion of any one study from this analysis did not influence heterogeneity. Proportions among studies did not appear to correlate with geographical region, but two groups emerged from the data; i.e., those with high rates of ileal CD (40%-50%), and those with lower rates of ileal disease (20%-30%). Does disease location for CD vary substantially among Caucasian populations? There are no population-based data to answer this question. Clearly, other factors may play a role, including rate of familial disease, smoking, type of center (medical or surgical bias), and methods of ascertainment. Interestingly, of the four studies with high rates of ileal disease, two had a high rate of familial CD (0.47, 0.86), one had a relatively low rate (0.11), and the fourth did not include data on familiality. Similarly, figures for surgery did not show consistency within this subgroup, ranging from 0.33 to 0.67, and rate of stricturing disease was surprisingly low (0.17-0.23). Alternatively, are there major differences in clinical assessment of patients and a corresponding reduction in interobserver agreement among centers? Studies have recently indicated that this may be so for both CD behavior and location[47,48]. The NOD2 analysis given above indicates that, at least for NOD2 variant ileal CD, there are limited differences among populations. As indicated above at the outset of this review, clinical characterization of CD, and IBD in general, remains a significant challenge.

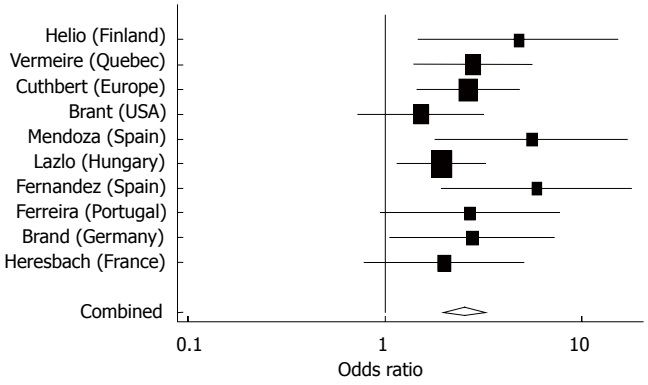

Figure 1 Meta-analysis comparing odds of having a NOD2 variant (SNPs 8,12 and/or 13) among Ileal versus Colonic CD patients in 10 studies. Pooled estimate = 2.50 (95% CI 1.97-3.25), $P < 0.0001$), test of heterogeneity among studies, $P = 0.49$.

META-ANALYSIS OF NOD2 ASSOCIATIONS WITH DISEASE LOCATION AND BEHAVIOUR

Location

These analyses confirmed the strong association between NOD2 variants and pure ileal CD compared to pure colonic disease, which was used as the reference group (Figure 1). Similar results were obtained in comparing patients with ileocolonic disease and the reference group (Figure 2). Both meta analyses achieved significance ($P < 0.0001$), while there was no significant heterogeneity demonstrated between studies.

Behaviour

Nine studies provided adequate data for inclusion in these meta analyses, of which four provided data on disease duration (range, 8.2-17.6 years). A strong association was confirmed between NOD2 variants and stricturing CD

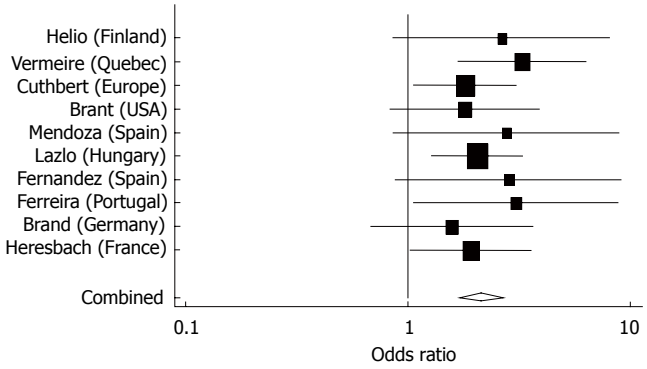

Figure 2 Meta-analysis comparing odds of having a NOD2 variant among Ileocolonic *versus* Colonic CD patients in 10 studies. Pooled estimate = 2.13 (95% CI 1.7-2.7), $P < 0.0001$, test of heterogeneity among studies, $P = 0.927$.

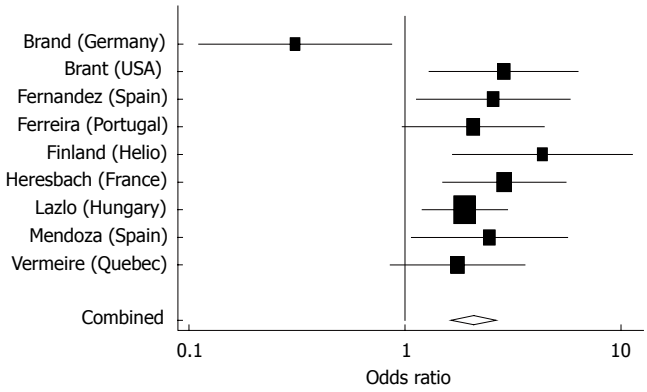

Figure 3 Meta-analysis comparing odds of having a NOD2 variant among Stricturing *versus* Inflammatory CD patients in nine studies. Pooled estimate = 2.06 (95% CI 1.42-2.98), $P < 0.0001$, test of heterogeneity among studies, $P = 0.02$.

(Figure 3) with inflammatory disease (non-stricturing, non-penetrating) as the reference group. Significant heterogeneity was detected among studies ($P = 0.02$) related to one "outlier". Importantly, and not surprisingly, the other form of complex disease behavior also showed significant association with NOD2 variants (Figure 4). This association is likely to be strengthened if data were available for internal penetrating disease independent of perianal penetrating disease for all studies.

CONCLUSION

The discovery of NOD2 as the first susceptibility gene for CD has resulted in a leap forward in our understanding of disease pathogenesis. It has also highlighted the heterogeneity of the disease. NOD2 represents the "low-lying fruit" of CD genetics-other susceptibility genes for this disease and for ulcerative colitis may not carry the same relative risk. The phenotype-genotype studies carried out to date have provided us with the critical observations that link NOD2 variants to ileal location, and this has highlighted the role of Paneth cells and antimicrobial peptides. However, there remain a number of questions. All these studies have been drawn from specialist cohorts leaving no accurate figure for NOD2 population attributable risk. There is significant heterogeneity in

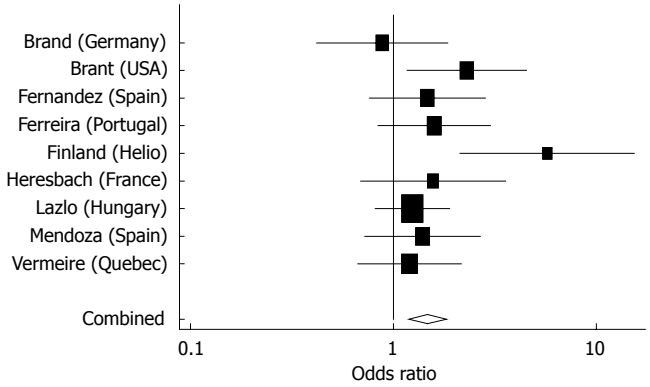

Figure 4 Meta-analysis comparing odds of having a NOD2 variant among Penetrating versus Inflammatory CD patients in nine studies. Pooled estimate = 1.47 (95% CI 1.19-1.82), $P < 0.0001$, test of heterogeneity among studies, $P = 0.15$.

phenotype among studies, raising concerns with respect to clinical characterization of patients at different centers and thus the utility of current classification systems and interobserver agreement in this field. Our meta-analysis has demonstrated a highly significant association between penetrating CD and NOD2 variants, and confirms the associations with stricturing behaviour and with both pure ileal and ileocolonic locations. These observations highlight the need for further clinical and genetic characterization of phenotypes not associated with NOD2, including inflammatory CD, perianal (penetrating) disease, and pure colonic disease.

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ESOPHAGEAL CANCER

Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer

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Abstract

AIM: To assess the proteome of normal versus tumor tissue in squamous cell carcinoma of the esophagus (SCCE) in Iranian patients and compare our results with former reports by using proteomics.

METHODS: Protein was extracted from normal and tumor tissues. Two dimensional electrophoresis was carried out and spots with differential expression were identified with mass spectrometry. RNA extraction and RT-PCR along with immunodetection were performed.

RESULTS: Fourteen proteins were found whose expression levels differed in tumor compared to normal tissues. Mass spectrometric analysis resulted in the identification of β-tropomyosin (TMβ), myosin light chain 2 (and its isoform), myosin regulatory light chain 2, peroxyredoxin 2, annexin Ⅰ and an unknown polypeptide as the down regulated polypeptides in tumor tissue. Heat shock protein 70 (HSP70), TPM4-ALK fusion oncoprotein 2, myosin light polypeptide 6, keratin Ⅰ, GH16431p and calreticulin were the up-regulated polypeptides found in tumor tissue. Several of these proteins, such as TMβ, HSP70, annexin Ⅰ, calreticulin, TPM4-ALK and isoforms of myosins, have been well recognized in tumorigenesis of esophageal or other types of cancers.

CONCLUSION: Our study not only supports the involvement of some of the formerly reported proteins in SCCE but also introduces additional proteins found to be lost in SCCE, including TMβ.

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Key words: Squamous Cell Carcinoma; Esophagus; Esophageal; Proteomics; Two dimensional electrophoresis; Polypeptide marker

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INTRODUCTION

As a complex disease, cancer arises from a range of genetic alterations that result in both structural and functional cellular changes. Such alterations, including up and down regulation of certain genes, mutations, chromosomal alterations, and suppression or activation of specific genes, confer cells with growth advantage and clonal expansion[1,2]. As a result, cells may acquire the ability to produce new proteins and other proteins that normally are present may be modified, reduced, augmented or even eliminated[3,4]. Quantitative variations in protein abundance represent alteration in functional gene expression, which demands analysis. Proteomics has provided simultaneous analysis of a large number of cellular protein constituents and is the most powerful direct analytical method for protein detection and evaluation[5,6].

Squamous cell carcinoma of the esophagus (SCCE) is the sixth most common cancer in the world. However, in developing countries it ranks fourth and occurs with higher frequencies in certain regions of the world, such as Iran, China, South Africa and France. The last epidemiologic

report indicated the highest incidence rate of this cancer from Iran[8]. So far, many molecular studies have been carried out on this cancer and the role of oncogenes, tumor suppressors, chromosomal abnormalities and other molecular events have been investigated. Noteworthy among these molecular alterations is the down regulation of important tumor suppressor genes and consequently their protein products, such as P53, Retinoblastoma (RB), Mutated in Colon Carcinoma (MCC), and Deleted in Colon Carcinoma (DCC)[9], which further indicates the importance of Tumor Suppressors (TSs) in the carcinogenesis of this type of cancer.

The present report represents further extension of our earlier work on esophageal cancer[10]. Here we applied proteomics as a method of comprehensive analysis of differential protein abundances present in esophageal cancer. Among the fourteen differentially expressed proteins identified in this study, we observed loss of ß-tropomyosin (TMβ) in all tumor samples, a protein that has been suggested to function as a tumor suppressor. Furthermore, this study additionally supports the involvement of TSs in the etiology of SCCE.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma unless otherwise specified.

Patients and tissue sampling

Cancerous and normal tissue specimens were collected from 45 patients with SCCE whom underwent surgery. Tissue samples were collected immediately after surgery, wrapped in aluminum foil, snap frozen in liquid nitrogen and maintained at -70℃. The age of the patients at the time of diagnosis ranged from 27 to 86 years (63% males, 37% females) with a mean of 55 years.

Protein preparation

100-150 mg of tissue was sliced on ice and pulverized under liquid nitrogen using a microdismemberator (Braun, Germany). Subsequently 600 μL homogenization buffer (10 mmol/L Tris-HCL (BioRad), 5 mmol/L $MgCl_2$ PH 7.4) was added to the pulverized tissues, mixed and 10 μL of the following protease inhibitors were added: Pepestatin (1 mg/mL in isopropanol), benzamidine (16 mg/mL in H_2O), phenylmethylsulphonyl fluoride (PMSF at 25 mg/mL in isopropanol). To this homogenate, 10 μL of RNaseA (10 mg/mL in homogenization buffer) and DNase Ⅰ (1 mg/mL in homogenization buffer) were added and incubated on ice for 20 min. Subsequently, urea at 7 mol/L, thiourea at 2 mol/L, 5% ß-mercaptoethanol and 0.5% SDS were gradually added and the volume of solution was adjusted to 1.5 mL with the homogenization buffer. Samples were centrifuged at high speed for removal of insoluble particles and five μL of each was used for protein concentration assessment using the Bradford assay.

Two-dimensional electrophoresis

Samples were subjected to isoelectrofocusing (IEF) following to adaptations and slight modification[11]. The first dimension gel was composed of 4.2% acrylamide, 0.22% N, N'methylenebisacrylamide, 8.5 mol/L urea, 0.27% (V/V) Nonidet P-40 (NP-40), 5% sucrose, and 6% ampholytes (pH 4-6, 5-7 and 6-8 at a ratio of 2:1:2, respectively) and 0.05% TEMED (N, N, N', N'-Tetamethylethylene diamine). The solution was degassed and 0.04% ammonium persulfate was added, mixed and poured to a height of 130 mm in cylindrical glass tubes with a 1.5 mm internal diameter. A volume of sample equal to 75 μg total protein was mixed with 0.33 volume of neutralizing buffer (9 mol/L urea, 8% NP-40 and 5% ampholytes pH 3.5-10), loaded on IEF gel, overlaid with 10 μL sample buffer (4 mol/L urea, 1% ampholytes pH 3.5-10) and filled with catholyte. The upper chamber buffer or catholyte was composed of extensively degassed 0.02 mol/L NaOH and the lower chamber buffer or anolyte; 0.01 mol/L phosphoric acid. Isoelectric focusing was applied, without prefocusing, at 300 V for 1 h, afterward at 600 V for 10 h and 800 V for 1 h in order to final focusing. Gels were removed and equilibrated for 20 min at room temperature in equilibration solution (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% β- mercaptoethanol (V/V), 10% glycerol (V/V), and 0.002% bromophenol blue). The second dimension gel consisted of 33.3 mL of 30% stock acrylamide solution and N, N'-methylene bisacrylamide (29.2% and 0.8% W/V respectively), 41.7 mL deionized water and 25 mL separation gel buffer (1.5 mol/L Tris-HCl, pH 8.8, 0.4% SDS [Sodium Dodecyl Sulfate]), 0.034% (W/V) ammonium persulfate and 0.05% TEMED. The equilibrated first dimension gel was layered on the second dimension gel and fixed in place with 1% agarose and electrophoresis was carried out at 30 mA/plate at 10℃ constant temperature by applying a cooling system.

Protein detection

Proteins were detected using a slight modification of the previously reported method[11]. The gel was fixed (methanol, water, acetic acid and formaldehyde: 50/38/12/0.05 per volume) for at least 1h with constant shaking followed by 3 times 20 min washes with 50% ethanol, pretreated with sodium thiosulfate ($Na_2S_2O_3.5H_2O$; 0.2 g/L) for 1 min, and washed three times each for 20 s with ddH_2O. Impregnation of the gel with $AgNO_3$ (1.9 g/L and 0.075% (V/V) of 37% formaldehyde) was carried out and the residual $AgNO_3$ was removed by 3 × 20 s successive washes with de-ionized water. Successively the gel was developed by soaking in developing solution containing Na_2CO_3 (60 g/L), 0.05% (V/V) of 37% formaldehyde and 4 mg/L of $Na_2S_2O_3 \cdot 5H_2O$ for 10 min up to the appearance of yellowish brown spots. The gel was then rinsed twice, each for 2 min, with ddH_2O. Further development was stopped by immersing the gel in stop solution (50% methanol and 12% acetic acid) and stored in 30% ethanol at 4℃ until scanning.

Isoelectric point determination

The isoelectric point (pI) of polypeptides was determined by either application of protein PI markers (Sigma) or by determining PI change along the entire length of the first dimension gel[12]. Gels were cut into 0.5 cm long pieces. Each piece was placed in a separate tube containing 0.5

mL of distilled H_2O, crushed and incubated at room temperature for 2 h. The pH of each tube was then measured.

Image analysis

Silver-stained gels were scanned using a calibrated GS-800 densitometer (Bio-Rad) at resolution of 600 dots and 12-bits per inch. Spot detection and gel matching were done according to Melanie-4 default settings and spot pairs were investigated visually (GeneBio, Geneva, Switzerland). Pairs of Two dimensional electrophoresis (2DE) gels composed of tumor and normal gels from each patient were used and the percent volume of each spot was estimated and analyzed for image analysis. The polypeptide variants of each pair of gels (tumor versus normal) were recorded and the most common changes in the polypeptide expression pattern (70% or higher) in all 2DE gels were used as a reference for comparison between tumor and normal gels.

Immunological detection and localization of actin

Following to the 2DE, gels were equilibrated for 30 min in transfer buffer (25 mmol/L Tris-base, 192 mmol/L glycine, 20% methanol and 0.1% SDS) and polypeptides were electrophoretically transferred to nitrocellulose membranes at 14 V overnight. Membranes were blocked for 2 h in blocking solution (TBST; 100 mmol/L Tris-base or HCl, pH7.5, 0.9% NaCl and 0.05% Tween 20) and exposed to biotinylated anti-actin antibody at 1/3000 dilution for 1 h with constant shaking. Membranes were subsequently washed 3X with TBST, incubated with strptavidin conjugated alkaline phosphatase in TBST at 1/4000 dilution and further incubated (1 h) with constant shaking. Membranes were washed two times with TBST, once with TBS and exposed to color solution (33 μL NBT from 50 mg/mL stock solution in 70% dimethylformamide and 17 μL BCIP from 50 mg/mL stock solution in 100% dimethylformamide). To stop excess color formation, membranes were washed with distilled water[13].

RNA extraction and RT-PCR of TMβ and beta actin

Total RNA was extracted by acid phenol method and the first strand cDNA was synthesized applying oligo dT 18 (Roche). Subsequently amplification was carried out using the forward primer 5'-GGC TGA TGA GAG CGA GAG AG-3' and the reverse primer 5'-GCA CTG GCC AAG GTC TCT TC-3' for amplification of TMβ and 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' as forward and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' as reverse primers for amplification of beta actin. The PCR condition was composed of primary denaturation at 95℃ 1 min followed by 30 cycles of amplification according to the subsequent scheme; denaturation 1 min at 95℃, annealing at 56℃ 1 min and extension at 72℃ 1 min and final extension at 72℃ for 10 min. Subsequently 4 μL of the PCR product was used for agarose gel electrophoresis.

Mass spectrometry

Silver stained protein spots containing the proteins of interest were destained thoroughly with 1% H_2O_2 (typically 1 min) and lyophilized to dryness[14]. Silver stain removal using H_2O_2 was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI)[14]. The dehydrated gel bands were hydrated with 15 μg/L (Promega, Madison, WI) of porcine trypsin in 25 mmol/L NH_4HCO_3, pH8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/L NH_4HCO_3, pH8.2 and incubated at 37℃ overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifluoroacetic acid. Samples were desalted with C18 Zip Tips (Millipore, Bedford, MA) as per manufacturer's protocols. 0.5 μL of sample was co-crystallized with 0.5 μL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid and spotted directly on a stainless steel MALDI target plate. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). MALDI-TOF/TOF spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

RESULTS

Protein extraction, 2DE and image analysis

Before doing 2DE, the concentrations of extracted proteins from tissue samples were determined. The protein extraction efficiencies were similar for tumor tissues and their adjacent normal nonmalignant tissues. The yields of extractions were approximately 80 μg/mg for both tumor and normal tissues. Subsequently seventy five micrograms of proteins from either tissue type were used in the first dimension. Because of the epithelial origin of squamous cell carcinoma, the mucosa of the normal non malignant tissues were separated from the rest of the esophageal tract for protein extraction and proteome comparison with corresponding tumors. For further verification that an equal amount of protein from either type of tissue was applied, immunological detection of actin was carried out following to 2DE. Following electrophoresis, the resolved proteins were recorded for each of the 2DE gel pairs. The densitometry map contained almost 800 features ranging in the molecular masses from 10 to 220 kDa and pIs from 4.8 to 8.0. For deciphering tumor-associated polypeptides, 2DE gels of tumor tissues were compared with their corresponding matched normal tissues. Compared with the matched normal tissues, 92 definite proteins (spots) in 2DE gels from 45 tumor tissues belonging to 45 patients were found indicating lower levels of expression. In contrast, 88 spots were identified in tumors that indicated higher levels of expression with respect to the 2DE gels of their corresponding matched normal tissues. To eliminate the possibility of artifacts, pairs of 2DE gels (tumor versus normal for each patient) were matched and compared and spot variants that occurred most often, or at least in 70% or higher of all 2DE patterns, were considered to be significant and were used for further comparisons. There were 7 out of 92 spots that indicated lower levels

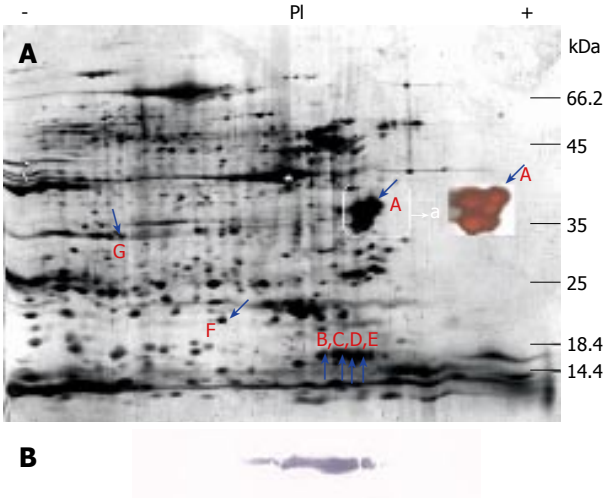

Figure 1 **A**: A representative 2DE gel of a normal tissue. Proteins that become down-regulated in corresponding tumor (Figure 2A) are shown with arrows and capital letters. For a better visualization of spots within the box, silver stained image of another gel is shown; **B**: Immunodection of actin as an internal control of protein loading, the * represents the location of actin.

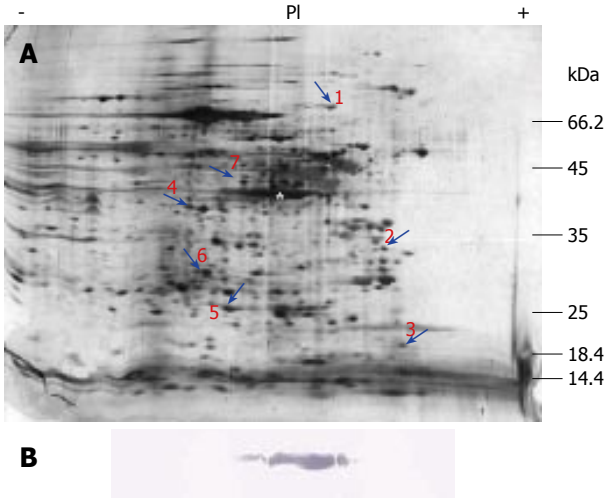

Figure 2 **A**: A representative 2DE gel of tumor tissue. Arrows and numbers indicate up-regulated proteins in comparison with their matched normal tissue (Figure 1A); **B**: Immunodetection of actin and the * represents the location of actin.

Table 1 Mass spectrometric identification and characteristics of the 14 proteins whose expression were subjected to change in SCCE

Polypeptide	Tissue specification	Protein name	Accession number based on NCBI	Chromosome	PI/MW	Expression profiling or level
A	Normal	Beta tropomyosin	NM-003289	9p13.2-p13.1	5.5/40.3	Disappearance
B	Normal	Myosin regulatory light chain 2	XM-027060	18p11.31	5.45/17.6	Down Regulation
C	Normal	Myosin light chain 2	NM-000432	16p11.2	5.6/17.6	Down Regulation
D	Normal	Unknown	NC-000963	Map No	5.8/17.6	Down Regulation
E	Normal	Myosin light chain 2	NM-000432	16p11.2	6/17.6	Down Regulation
F	Normal	Peroxiredoxin 2	NM-005809	19p13/2	6.6/26	Down Regulation
G	Normal	Annexin 1	NM-000700	9	7.3/35.5	Down Regulation
1	Tumor	Heat shock protein 70 kD	XM-044201	Unpublish	6/64	Up Regulation
2	Tumor	TPM4-ALK fusion oncoprotein 2	A186109	19p13/1	5.5/32.5	Up Regulation
3	Tumor	Myosin light polypeptide 6	NM-0211019	12p13/3	5.4/20.1	Up Regulation
4	Tumor	Keratin 1	AF304164	12q13	6.9/42.5	Up Regulation
5	Tumor	DNA directed RNA polymerase B (ropB)	NC-000915		6.1/27.2	Up Regulation
6	Tumor	GH16431P	Ay051511		6.6/30.1	Up Regulation
7	Tumor	Calreticulin	NM-004343	19p13/3	5.2/50	Up Regulation

of expression and 7 out of 88 spots that indicated higher levels of expression respectively. Figures 1A and 2A represent the resulting 2DE gels of the normal and the corresponding tumor tissues of one patient.

To identify whether an equal amount of protein was applied in 2DE, immunodetection of actin was carried out as an internal control of loading (Figures 1B and 2B). As Figures 1B and 2B show, equal amount of proteins were applied in the first dimension. The 14 observed differentially abundant proteins are indicated by arrows (Figures 1A and 2A). The proteins that were observed with decreased abundance in tumors, compared with their corresponding normal tissues, are labeled alphabetically as A, B, C, D, E, F and G. Spots B, C and E are observed in a chain of spots and likely indicate that they are isoforms or modified forms of a specific protein. On the other hand, those proteins that were observed to have an increased abundance in tumors were numbered as 1, 2, 3, 4, 5, 6 and 7. The apparent molecular weight of polypeptides, approximate isoelectric points and changes in expression level, is presented in Table 1. It was found that spot A was absent in tumor samples whereas, spots B, C, D and E were considerably down regulated and spots F and G were shown to be more than two-fold down regulated. It suggests that down regulated proteins were involved in the maintenance of normal phenotype whereas the other up-regulated polypeptides were involved in the development of malignancy.

Mass spectrometric analysis, identification of polypeptides and RT-PCR

Following to 2DE, spots of interest were excised, in-gel

Table 2 An example of MALDI/TOF/TOF mass spectrometry. The identified protein was β tropomyosin, one of the fourteen identified proteins as designated by letter A in Figure 1A

Calculated mass	Observed mass	Start Seq.	End Seq.	Sequence	Modification
846.4679	846.433	232	238	LKEAETR	
894.4679	894.4463	162	168	YEEVARK	
916.4734	916.4508	192	198	QLEEELR	
1107.5792	1107.5211	239	248	AEFAERSVAK	
1143.6116	1143.5856	190	198	ARQLEEELR	
1170.6727	1170.6464	169	178	LVILEGELER	
1243.6528	1243.6317	92	101	IQLVEEELDR	
1262.597	1262.5764	179	189	SEERAEVAESR	
1298.7677	1298.7423	168	178	KLVILEGELER	
1332.6389	1332.6127	78	90	ATDAEADVASLNR	
1343.6801	1343.6615	38	48	QLEEEQQALQK	
1399.7539	1399.7324	91	101	RIQLVEEELDR	
1443.8053	1443.7762	106	118	LATALQKLEEAEK	
1460.7339	1460.7119	77	90	KATDAEADVASLNR	
1471.775	1471.7477	38	49	QLEEEQQALQKK	
1488.74	1488.715	78	91	ATDAEADVASLNRR	
1493.7338	1493.7158	141	152	MELQEMQLKEAK	Oxidation (M)
1616.835	1616.8109	77	91	KATDAEADVASLNRR	
1671.8911	1671.8682	169	182	LVILEGELERSEER	
1702.8792	1702.8088	36	49	CKQLEEEQQALQKK	
1719.8582	1719.834	192	205	QLEEELRTMDQAL	Oxidation (M)
1727.8922	1727.8687	92	105	IQLVEEELDRAQER	
1799.9861	1799.9597	168	182	KLVILEGELERSEER	
1817.8776	1817.8594	153	167	HIAEDSDRKYEEVAR	
1883.9933	1883.9731	91	105	RIQLVEEELDRAQER	
1946.9963	1946.9734	190	205	ARQLEEELRTMDQALK	Oxidation (M)
2202.1248	2202.1091	106	125	LATALQKLEEAEKAADESER	
2414.252	2414.2375	169	189	LVILEGELERSEERAEVAESR	
2534.1636	2534.271	199	220	TMDQALKSLMASEEEYSTKEDK	
3751.876	3751.9631	252	284	TIDDLEETLASAKEENVEIHQTLDQTLLELNNL	

digested and analyzed with a MALDI/TOF/TOF (Matrix-assisted laser desorption/Ionization/ Time of Flight/ Time of Flight) mass spectrometer. The observed peptide mass spectra were analyzed for protein identification by peptide mass fingerprinting that resulted in the identification of the 14 proteins that are listed in Table 1. The identified protein corresponding to spot A was found to be β-tropomyosin as shown in Table 2. As mentioned above, this protein was found to be lost in tumors thus, for further validation of results achieved regarding with TMβ, RT-PCR was conducted by applying TMβ specific primers. As Figure 3A indicates, the 431 bp amplification product is only limited to the cDNA synthesized from RNA extracted from normal tissue. Applying beta actin as an internal control of RT-PCR, which gives rise to a 670 bp amplification product (Figure 3B), and running the extracted RNA (Figure 3C) indicate that lack of amplification in tumor tissues should be due to the loss of expression of TMβ. These observations further point out that down regulation of TMβ occurs at transcriptional level in SCCE. As discussed later in the Discussion section, TMβ is a down stream target for several oncogenes and functions as a tumor suppressor gene.

DISCUSSION

Former studies on SCCE have led to the identification of Annexin I [15,16], tumor rejection antigen (gp96)[16], clustrin[17], tropomyosin 3, retinoblastoma binding like protein and K506 binding protein[18] as potential markers of esophageal and other types of cancers, some of which were also identified in the present study for instance loss of TMβ in breast cancer[19], myosin light chain 2 isoforms in transformed osteosarcoma[20], peroxiredoxin Ⅱ in pancreatic adenocarcinoma[21], calreticulin in bladder[22] and colon cancers[23], HSP70 in hepatocellular[4] and keratin 1 in gastric carcinoma cell lines[24]. In addition to those proteins previously introduced for esophageal cancer, we identified new proteins, among which TMβ was found to be completely absent in all tumor samples. Therefore we suggest this protein as a potential marker for SCCE. Loss or down regulation of TMβ in cancer is not restricted to our study. Studies on other types of cancers such as oral tongue squamous carcinoma[25] and carcinomatous breast lesions[26] have also led to this observation.

Different studies have indicated that TMβ functions as a tumor suppressor. For example, reversion of neoplastic phenotype, anti-angiogenic activity[27,28], induction of slow growth rate, anchorage, cytoskeleton organization in breast cancer MCF7 cell lines[29] and anoikis[19] are all the consequence of TMβ expression. TMβ is required for cytoskeleton establishment and mediation of TGF-β mediated stress fiber formation. Alternatively, the Ras-ERK pathway antagonizes with TGF-β induced stress fiber formation by suppressing TMβ expression[30]. Indeed TMβ is a down stream target for the ras oncogene[31]. To the best of our knowledge, no strong evidence has been documented regarding with the involvement of the

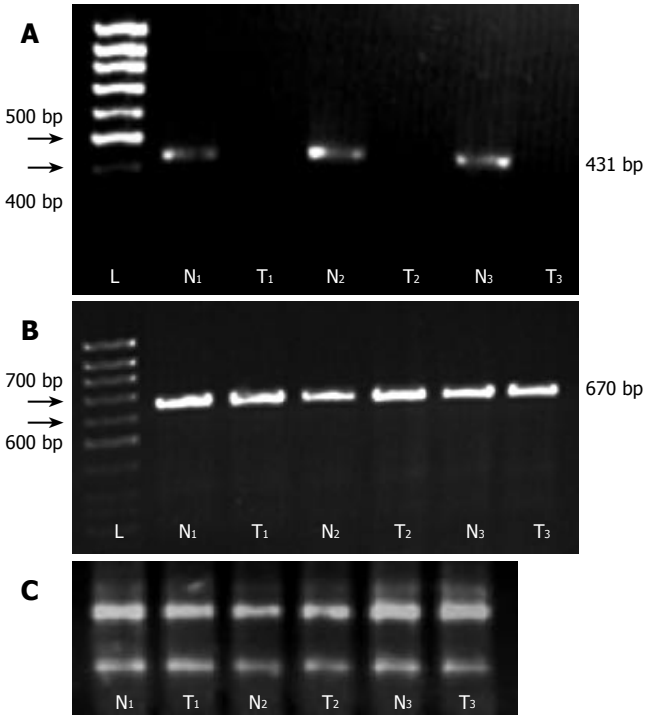

Figure 3 **A**: Verifying differential expression pattern of ß tropomyosin by RT-PCR in three separate experiments as indicated by numbers (1 to 3), normal versus tumor tissues. The amplification product (431bp) is limited to normal tissue which indicates loss or strong down regulation of this protein as observed by 2DE; **B**: RT-PCR amplification of ß-actin as an internal control of RT-PCR; **C**: Electrophoresis of the total RNA from normal and tumor tissues used for cDNA synthesis and RT-PCR. L: DNA marker; N: normal tissue; T: tumor tissue.

ras family of oncogenes in esophageal carcinogenesis, therefore it is likely that other oncogenes may be involved in the down regulation of TMβ, such as TGF-α and activation of EGFR, both of which participate in the down regulation of TMα and β[32]. Interestingly high levels of TGF-α were reported in all gastrointestinal cancers, including the late stages of esophageal cancer[33], along with amplification of EGFR in esophageal cancer[34]. Loss of TMβ in tumors suggests that this protein may play an important role in tumor suppression of esophageal carcinogenesis.

In addition to TMβ, down regulation of several other proteins was also observed in this study, including annexin Ⅰ. As we mentioned above, reports on the loss of annexins Ⅰ or its isoforms was not limited to our study but other research groups[15,16,37] have also observed this event. It was suggested that such a loss of expression in esophageal and prostate tumors is an early event[15] of carcinogenesis. Hypermethylation of annexin Ⅰ was suggested as another mechanism leading to inactivation and loss of expression in cell lines such as B cell non-Hodgkin's lymphoma[35]. Annexin Ⅰ induces both spontaneous and caspase-3 activated apoptosis[36]. Furthermore, mutations in p53 were also found to result to the down regulation of annexin 10, another member of the annexins family in hepatocellular carcinoma, along with vascular invasion, early recurrence and poor prognosis[38]. We formerly showed mutations in the p53 tumor suppressor gene in SCCE[10], thus it is possible that such mutations correlate with down regulation of annexin 10 as well as annexin Ⅰ. It is well-demonstrated that EGFR amplification occurs in SCCE[34]. On the other hand, annexin Ⅰ undergoes phosphorylation and inactivation by EGFR[39]. Thus our result additionally supports former report[16] regarding with loss of annexin Ⅰ in SCCE.

To our knowledge this is the first report introducing down regulation of peroxyredoxin Ⅱ in SCCE. Peroxiredoxins are members of the phase Ⅱ detoxifying enzymes involved in cytoprotection and cellular defense against oxidants by detoxifying xenobiotics and oxidants, especially H_2O_2. By reducing H_2O_2, peroxyredoxins protect cells from oxidative stress induced apoptosis and proapoptotic signals. Peroxyredoxins inhibit H_2O_2 induced signal cascade in response to PDGF and EGF. They also inhibit NFκB and tumor necrosis factor α in response to external H_2O_2 and H_2O_2 induced expression of proapoptotic protein; BAX[40,41]. Thus, it is possible that loss or down regulation of this protein or other members of detoxifying enzymes in esophageal epithelium, which normally could be exposed to stresses, contribute to the carcinogenesis by cellular failure in detoxification of oxidative agents affecting this tissue.

Loss of muscle forming proteins is an important cause of body weight loss or cachexia in cancers primarily due to loss of the myosin heavy chain[42]. Myosin light chain 2 is an indispensable component of cytoskeleton, migration and apoptosis[43]. Down regulation of myosin light chain 1 and myosin regulatory light chain 2 proteins was shown in oral tongue squamous cell carcinoma[25]. Due to a common embryonic origin of oral and esophagus tissues, strong down regulation of myosin regulatory chain 2 indicates an important role for this protein in both organs. Formerly Kumar and Chang have shown that transformation of osteosarcoma cells is associated with complete loss of myosin light chain 2 isoforms, which indicates myosin light chain 2 as a target for cellular transformation and malignancy[20]. Our report is the first demonstrating down regulation of myosin light chain isoforms in esophageal cancer. Alternatively, up regulation of myosin light polypeptide 6 in tumors could possibly be due to the selective down regulation of myosin heavy chain and other isoforms of myosin light chain. Supporting our results is the report of Samoszuk *et al* that have shown up regulation of this protein in clonogenic human breast cancer[44].

We also found several up regulated proteins in tumors, among which is heat shock protein 70. Investigations on gastric, hepatocellular and colonic adenocarcinoma cancers have indicated higher levels of expression of HSP70 along with other stress associated proteins[4,45]. HSP70 exerts antiapoptotic activity by suppressing tumor necrosis factor induced apoptosis and caspase 3 down stream events[46], interfering with SAPK/JNK and ceramide induced apoptosis and cleavage of caspase 3 substrate enzyme PARP, which is involved in DNA repair[47]. Theses findings have raised the idea that HSP70 not only should be considered as a tumor marker for HCV-caused hepatocellular carcinoma[48] but also as a target for cancer treatment[49]. Thus, as an organ of the digestive system, esophagus carcinogenesis might follow the same molecular path as other organs of this system.

Calreticulin and keratin 1 were among other up

regulated proteins in tumors. The involvement of calreticulin in tumors has been reported by several groups. Rendon Huerta *et al*[50] have shown that protein kinase C isoforms bind and phosphorylate calreticulin. This would suggest a possible role for calreticulin in signal transduction and involvement in cell division. It was also found that calreticulin regulates p53 function by affecting its rate of degradation and nuclear localization[51]. Thus, association of p53 mutations with esophageal carcinogenesis[10] as well as p53 degradation could explain the role of calreticulin in the development of esophageal tumor. In addition to calreticulin we also found overexpression of Keratin 1, a member of keratin family in SCCE. Formerly Trask and colleagues have shown different expression patterns of keratin isoforms in normal versus tumor tissues[52]. They proposed the expression pattern of keratin isoforms as biomarkers for differentiating normal from malignant cells. Furthermore, Kinjo and coworkers have shown overexpression of keratin 1 in colonic adenocarcinoma cells[53] and Nishikawa *et al*[24] have found up to 17 folds of truncated keratin 1 in Epstein-Barr virus transfected gastric carcinoma cell lines. In esophageal cancer, several groups have reported an increased level of cytokeratin 19 (keratin 19) in serum and secretion by cell lines[54]. The later groups have proposed cytokeratin 19 to have the best specificity and sensitivity as a prognostic marker for evaluating esophageal cancer. Thus involvement of another member of the keratin family in SCCE not only supports former results but also introduces a new marker from this family.

Activation of anaplastic leukemia kinase (ALK) is an example of chromosomal abnormalities, such as translocations, that lead to fusion proteins. ALK was first identified in anaplastic large cell lymphomas (ALCL) and subsequently in inflammatory myofibroblastic tumors (ITM). The aberrantly expressed protein activates several protein kinases, such as IP3 kinase, STAT5 and transcription factors, which end up to mitogenic effects[55]. The fusion of ALK with nucleoplasmin is frequently observed in ALCL. Walking on chromosome 2, Lamant *et al* have shown the fusion of tropomyosin 3 and ALK as a result of t (1; 2) (q25; p23)[56]. They suggested that such a fusion provides an active promoter for ALK, because nucleoplasmin is a housekeeping gene and TM3 is constitutively expressed. Further studies led to the identification of not only TM3-ALK but also TM4-AKL fusion proteins in patients with inflammatory myofibroblastic tumors[57] and as a result perturbation of normal tumor suppressor activity of TM isoforms. It was also suggested that the extent of such rearrangements and fusions could possibly engage other proteins. Here we demonstrated that expression of ALK is not restricted only to the neural but also to other tumors. TPM4-ALK fusion protein is also among proteins being introduced for the first time in SCCE.

There were also three other proteins found in SCCE as homologues of formerly identified proteins in other organisms. Thus, we interpret them to be newly found proteins in SCCE. Among these proteins, two were up regulated in tumors that were identified to be DNA-directed RNA polymerase Beta subunit (ropβ) with almost 100% (99.998) homology with that of *H pylori*. The other protein, spot 6 had a mass spectrum that matched with a high score (99.925) with GH16431P of Drosophila melanogaster (NCBI ACCESSION; AAK92935). Looking into patients pathology files indicated that almost all were *H Pylori* positive, thus we think that up regulation of this protein in tumors, to some extent, may be due to contamination with this bacterium. However, the question that remains unanswered is why such contamination was not observed in normal tissues. A possible explanation could be a preferentially higher growth of bacteria in tumors than in normal tissues. The other protein was the spot D in normal tissue whose mass spectrum matched with high score (99.963) to an unknown protein (NCBI Accession; NC 000963). This protein is highly expressed in normal tissue while loss of it was observed in tumor specimens. Being an unknown protein, further studies are required to reveal its true biological activity in tumor suppression.

In conclusion, this is the first report on the proteomics of SCCE from Iran, a country with the highest incidence rate of SCCE[8]. We were able to identify fourteen differentially expressed proteins in esophageal tumors. Our study further showed that several proteins, which are commonly affected in other digestive organs such as liver, are also affected in SCCE, which may indicate a common molecular mechanism for digestive system tumorigenesis and putative candidate biomarkers. It should also be mentioned that our study brings further evidence for the importance and requirement of a deeper investigation of the molecular events down stream to the EGFR activation in SCCE. The expression levels of several proteins identified in our study is affected by the activation of this receptor. To our knowledge a general molecular marker has not been introduced for cancers, however, finding the disappearance of TMβ in SCCE could introduce a useful tumor marker for SCCE diagnosis, evaluation and follow up.

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Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor

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Abstract

AIM: To investigate the functions of promoter hypermethylation of secreted frizzled-related proteins (sFRPs) genes in colorectal tumorigenesis and progression.

METHODS: The promoter hypermethylation and expression of *sFRP* genes in 72 sporadic colorectal carcinomas, 33 adenomas, 18 aberrant crypt foci (ACF) and colorectal cancer cell lines RKO, HCT116 and SW480 were detected by methylation-specific PCR and reverse transcription PCR, respectively.

RESULTS: None of the normal colorectal mucosa tissues showed methylated bands of any of four *sFRP* genes. *sFRP1*, *2*, *4* and *5* were frequently methylated in colorectal carcinoma, adenoma and ACF (*sFRP1* > 85%, *sFRP2* >75%, *sFRP5* > 50%), and the differences between three colorectal tissues were not significant (P > 0.05). Methylation in colorectal tumors was more frequent than in normal mucosa and adjacent normal mucosa. The mRNA of *sFRP1-5* genes was expressed in all normal colorectal mucosa samples. Expression of *sFRP1, 2, 4* and *5* and *sFRP1*, *2* and *5* was downregulated in carcinoma and adenoma, respectively. The downregulation of sFRP2, 4 and 5 was more frequent in carcinoma than in adenoma. Expression of *sFRP3* which promoter has no CpG island was downregulated in only a few of colorectal tumor samples (7/105). The downregulation of *sFRP1*, *2*, *4* and *5* expression was significantly associated with promoter hypermethylation in colorectal tumor. After cells were treated by DAC/TSA combination, the silenced *sFRP* mRNA expression could be effectively re-expressed in colorectal cancer cell lines.

CONCLUSION: Hypermethylation of *sFRP* genes is a common early event in the evolution of colorectal tumor, occurring frequently in ACF, which is regarded as the earliest lesion of multistage colorectal carcinogenesis. It appears to functionally silence *sFRP* genes expression. Methylation of *sFRP1*, *2* and *5* genes might serve as indicators for colorectal tumor.

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Key words: Colorectal tumor; *Secreted frizzled-related protein* genes; Methylation; Indicator; Re-expression

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INTRODUCTION

Secreted frizzled-related proteins (sFRPs) comprise a family of five secreted glycoproteins that antagonize Wnt canonical and noncanonical signaling by different mechanisms directly or indirectly[1]. Wnt signaling regulates cell growth, motility and differentiation in animal development and has increasingly been implicated in tissue homeostasis in adult organisms. Aberrant Wnt signaling pathway is an early progression event in 90% of colorectal cancers, contributing to the growth, proliferation and loss of apoptosis of tumor cells[2]. Thus, role of sFRP as a negative regulator of Wnt signaling may have important implications in tumorigenesis, and its downregulation has been correlated with human cancers. To date, no mutation in *sFRP* genes has been associated with their dysregulation in tumors. Aberrant hypermethylation of CpG islands in gene promoter has been found to be a primary mechanism in the inactivation of several tumor suppressor genes. As we know, colorectal tumor is one of the tumors with high frequencies of gene methylation[3,4]. Epigenetic gene silencing plays an extremely important role in the early stage of colorectal tumorigenesis. In this study, we examined hypermethylation and expression of *sFRP* genes in different stages of colorectal tumor and colorectal cancer cell lines in order to investigate the functions of promoter hypermethylation of *sFRP* genes in colorectal tumorigenesis and progression, and whether it can serve as an indicator for colorectal tumor.

MATERIALS AND METHODS

Materials

Tissue samples: Tissue samples, including 72 sporadic colorectal carcinomas, 33 sporadic colorectal adenomas (male 56, female 49, age 27-76 years), 38 adjacent normal mucosa from 28 colorectal carcinomas and 15 colorectal adenomas, and 20 normal colorectal mucosa tissues (male 11, female 9, age 20-54 years) were obtained from surgical resection of patients receiving endoscopy examination at the clinic of Zhongnan Hospital of Wuhan University and The Tumor Hospital of Hubei Province between October 2003 and April 2006. Eighteen ACFs from 15 colorectal carcinomas were also collected[5]. Samples were stored at -70℃ until processing. None of the patients had received chemotherapy or radiation therapy prior to surgery. All patients gave informed consent for their participation in the study which had been approved by the Ethical Committee of our university.

Cell culture and treatment: Three colorectal carcinoma cell lines, including RKO, HCT116, and SW480 (cell lines were purchased from China Center for Type Culture Collection) were used in this study. Cell lines were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum and treated with 5-aza-2'-deoxycytidine (DAC), a DNA methyltransferase (DNMT) inhibitor, and trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. Three cell lines all received four different treatments: low-dose DAC (200 nmol/L, 48 h), high-dose DAC (5 μmol/L, 72 h), TSA (300 nmol/L from a 1.5 mmol/L ethanol-dissolved stock, 24 h), and DAC (200 nmol/L, 48 h) followed by addition of TSA (300 nmol/L, 24 h), the drugs and medium being replaced every 24 h.

Methods

Methylation analysis: Methylation-specific PCR (MSP) was used to examine the promoter hypermethylation of *sFRP1, 2, 4* and *5* genes. Genomic DNA was extracted by the phenol-chloroform standard methods. Bisulfite genomic DNA modification and purification was performed as previously reported[6]. The modified DNA was amplified by methylated and unmethylated primers (exact primers are available upon request). Products were visualized by 12% polyacrylamide gel electrophoresis. MSP was not performed for *sFRP3*, which has no CpG island in promoter region.

Reverse transcription PCR (RT-PCR) analysis: RT-PCR was employed to detect *sFRP1-5* genes mRNA expression. TRIZOL Reagent (Invitrogen) was used to extract total RNA from tissues and cells. The integrity of RNA was identified by methanal denatured agarose electrophoresis. Ultraviolet-visible light spectrophotometer (Beckman Coulter DU530) was used to determine $A_{260/280}$ of total RNA. Reverse transcription was performed on 1 μg of total RNA in a reaction volume of 20 μL. PCR assays were performed in 50 μL volume using 100 ng cDNA. Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was employed as an internal reference gene to ensure cDNA quality and loading accuracy. Products were visualized by 2.5% agarose electrophoresis, and then analyzed by gel imaging system (SynGene GGM) and GeneTools software. RT-PCR was performed three times for the tumor samples with detectable mRNA, and then the gene expression index (density Lum of samples mRNA/density Lum of *GAPDH* mRNA) of these samples was compared with the normal samples (*t* test). Those with reduced mRNA expression and $P < 0.01$ were considered as significantly downregulated.

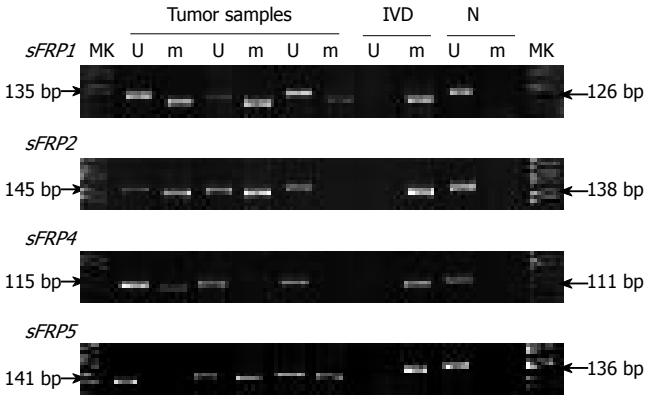

Figure 1 Hypermethylation of *sFRP* genes in colorectal tissues. Tumor samples showed unmethylated (U) and methylted (m) bands, but normal tissues (N) showed only unmethylated bands. The *in vitro* methylated DNA (IVD) serves as a positive control of methylated *sFRP* genes.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. Associations between the discrete variables were assessed using the two-sided Fisher's exact test or Pearson's chi square tests. *P* value less than 0.05 was regarded as statistically significant.

RESULTS

Methylation of sFRP genes

None of normal mucosa showed methylated bands. *SFRP1, 2, 4* and *5* were methylated in 93.1% (67/72), 83.3% (60/72), 36.1% (26/72) and 52.8% (38/72) of colorectal carcinoma; 87.9% (29/33), 81.8% (27/33), 24.2% (8/33) and 57.6% (19/33) of colorectal adenoma (Figure 1); 52.6% (20/38), 28.9% (11/38), 2.6% (1/38) and 18.4% (7/38) of adjacent normal mucosa to colorectal carcinomas and adenomas, respectively. Most of *sFRP* genes were methylated in tumor samples when methylation was presented in the adjacent normal mucosa from the same sample, *sFRP1* (20/20), *sFRP2* (9/11), *sFRP4* (1/1) and *sFRP5* (6/7). The tumors showed stronger methylation signals than the adjacent normal tissues (Figure 2).

The methylation of each of the *sFRP1, 2, 4* and *5* genes was significantly different between carcinoma and normal mucosa ($P < 0.001$ for *sFRP1, 2* and *5*, $P = 0.002$ for *sFRP4*), between carcinoma and adjacent normal mucosa ($P < 0.001$ for *sFRP1, 2, 4* and *5*), between adenoma and normal mucosa ($P < 0.001$ for *sFRP1, 2* and *5*, $P = 0.0462$ for *sFRP4*), and between adenoma and adjacent normal mucosa ($P = 0.001$ for *sFRP1*, $P < 0.001$ for *sFRP2* and *5*, $P = 0.018$ for *sFRP4*). While no significant difference was found between carcinoma and adenoma ($P = 0.614$ for

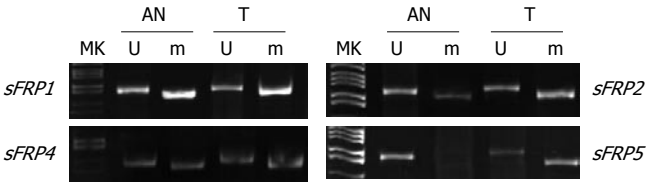

Figure 2 Hypermethylation of *sFRP* genes in colorectal tumor samples (T) and the corresponding normal tissues adjacent to them (AN). The tumors showed stronger methylation signals than the adjacent normal tissues.

Table 1 Correlation of sFRP expression with methylation status

Gene		Colorectal tumor		
		Unmethylated	Methylated	χ^2, *P*
sFRP1	+	6	9	$\chi^2 = 17.627$
	-	3	87	$P < 0.001$
sFRP2	+	11	28	$\chi^2 = 5.345$
	-	7	59	$P = 0.021$
sFRP4	+	68	15	$\chi^2 = 37.041$
	-	3	19	$P < 0.001$
sFRP5	+	38	10	$\chi^2 = 39.872$
	-	10	47	$P < 0.001$

Expression (+) and reduced expression (-).

sFRP1, $P = 0.848$ for *sFRP2*, $P = 0.228$ for *sFRP4* and $P = 0.647$ for *sFRP5*).

SFRP1, 2, 4 and *5* were methylated in 94.4% (17/18), 77.8% (14/18), 27.8% (5/18) and 55.6% (10/18) of 18 ACF samples. *SFRP1, 2, 4* and *5* methylation differed significantly between ACF and normal mucosa ($P < 0.001$ for *sFRP1*, *2* and *5* and $P = 0.017$ for *sFRP4, Fisher's* exact test). While no significant difference was found between ACF and carcinoma ($P = 1.000$ for *sFRP1*, $P = 0.836$ for *sFRP2*, $P = 0.506$ for *sFRP4* and $P = 0.833$ for *sFRP5*), and between ACF and adenoma ($P = 0.794$ for *sFRP1*, $P = 1.000$ for *sFRP2*, $P = 1.000$ for *sFRP4* and $P = 0.059$ for *sFRP5*). At least one of the four *sFRP* genes was methylated in 18 ACF samples.

Expression of sFRP genes

The 20 normal colorectal mucosa specimens expressed considerable levels of sFRP1-5. Compared with the normal mucosa, *sFRP1, 2, 4* and *5* expression was silenced or significantly reduced in 90.3% (65/72), 70.8% (51/72), 26.4% (19/72) and 61.1% (44/72) of colorectal carcinomas, respectively ($P < 0.001$ for *sFRP1*, *2* and *5* and $P = 0.023$ for *sFRP4*); *sFRP1, 2,* and *5* were markedly downregulated or silenced in 75.8% (25/33), 45.5% (15/33) and 39.4% (13/33) of colorectal adenomas ($P < 0.001$ for *sFRP1, 2* and *5*). *SFRP4* was downregulated or silenced in 9.1% (3/33) of colorectal adenomas relative to the normal mucosa ($P = 0.438$). The downregulation of *sFRP 2, 4* and *5* expression was more frequent in carcinoma than in adenoma ($P = 0.012$, $P = 0.043$ and $P = 0.038$). *SFRP1* expression had no significant difference between carcinoma and adenoma ($P = 0.094$). A minority of adjacent normal mucosa samples had reduced expression

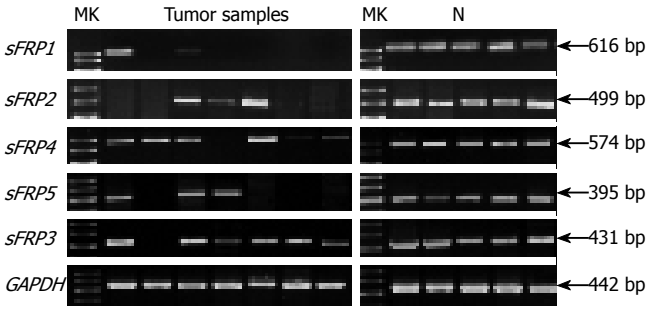

Figure 3 Expression of *sFRP* gene family in colorectal tumor and normal tissues (N). The gene *GAPDH* serves as a positive indicator for RNA quality and loading.

of *sFRP1-5* genes, and the ratio was 13.2% (5/38), 5.3% (2/38), 0% (0/38), 7.9% (3/38) and 7.9% (3/38), respectively. *SFRP3* that has no CpG island in promoter region was downregulated in only 7 of 105 colorectal tumor samples (Figure 3). Reduced expression of *sFRP1, 2, 4* and *5* genes was significantly associated with aberrant hypermethylation of these genes (Table 1).

Re-expression of silenced sFRP genes in colorectal carcinoma cell lines

SFRP1, *2*, *4* and *5* genes were methylated in RKO and HCT116 cell lines. *SFRP1* and *2* but not *sFRP 4* and *5* were also methylated in SW480 cell line (Figure 4A). When *sFRP* genes were methylated, their corresponding mRNA expression was absent in three colorectal carcinoma cell lines. SW480 cell line expressed *sFRP 4* and *5* mRNA, in which methylation was not detected in *sFRP 4* and *5* promoter. *SFRP3* was expressed in three colorectal carcinoma cell lines.

However, after cells were treated by low-dose DAC, the silenced *sFRP1, 2* and *5* mRNA expression could be re-expressed in RKO and HCT116 cell lines; and *sFRP1* expression could be re-expressed in SW480 cell line. High-dose DAC treatment could strengthen the effects of low-dose DAC treatment, and result in reexpression of *sFRP 4* (RKO and HCT116) and *2* (SW480) mRNA, which could not be induced by low-dose DAC. The addition of TSA to DAC rendered the silenced *sFRP* genes mRNA to be re-expressed effectively, and markedly elevated the mRNA expression level that had been re-expressed or increased by high-dose DAC. A single application of TSA could not induce re-expression of *sFRP* genes mRNA. The influence of demethylation treatment on *sFRP3* expression was minimal (Figure 4B and 4C).

DISCUSSION

Aberrant activation of the Wnt canonical signaling pathway is associated with a variety of human cancers, such as head and neck carcinoma, lung cancer, melanoma, and mesothelioma, and particularly colorectal cancer, as well as the growth, proliferation and loss of apoptosis of tumor cells. The tumor suppressor adenomatous polyposis coli (APC) mutates in a high proportion of colorectal carcinomas; meanwhile it is an important member of Wnt canonical pathway involved in the degradation of

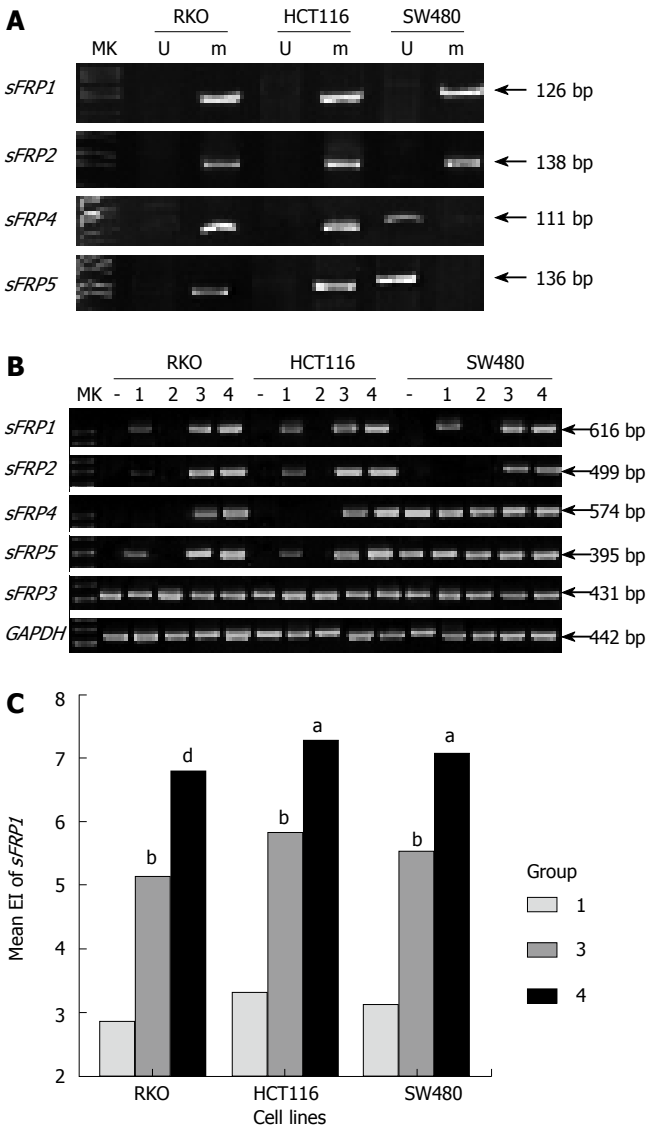

Figure 4 **A**: Hypermethylation of *sFRP* genes in colorectal cancer cell lines; **B**: The mRNA expression level of *sFRP* genes in colorectal cancer cell lines before and after demethylation treatment. Three cell lines all received 4 different treatments: never treated (-), low-dose DAC (1), TSA (2), high-dose DAC (3), DAC + TSA (4); **C**: The *sFRP1* gene expression index (EI) in three cell lines after demethylation treatment, low-dose DAC (1), high-dose DAC (3), DAC+TSA (4). $^{b}P < 0.01$ *vs* 1, $^{d}P < 0.01$ *vs* 3, $^{a}P < 0.05$ *vs* 3.

β-catenin. Wnt signaling has been identified as one of the key signaling pathways in cancer, regulating cell growth, motility and differentiation. It has been reported that the noncanonical pathway is also involved in tumorigenesis, being associated with inhibition of apoptosis[7].

SFRPs contain a characteristic cysteine-rich domain (CRD) that shares homology with the CRD of frizzled (Fz) receptor of Wnt in the N-terminal half of the proteins. Thus, sFRPs may block Wnt signaling either by interacting with Wnt proteins to prevent them from binding to Fz proteins or by forming nonfunctional complexes with Fz[1]. It has been reported that sFRPs can potentially inhibit the entire canonical Wnt pathway even in the presence of activating mutations of *APC* or β-*catenin* downstream of the Fz receptor in colon cancer cell[8]. Therefore, silence of *sFRP* genes may be essential for the aberrant activation of the Wnt canonical pathway in colorectal tumorigenesis.

To date, no mutation in *sFRP* genes has been associated with their dysregulation in tumors. Aberrant hypermethylation of CpG islands in gene promoters has been found to be a primary mechanism in the inactivation of several tumor suppressor genes. However, the presence of gene silencing by hypermethylation is an important character of colorectal tumor[3,4]. This study demonstrated that genes of the sFRP family were methylated in most cases of colorectal carcinoma, adenoma and ACF, especially *sFRP1* and *2*. The proportion of *sFRP1* and *2* methylation was more than 85% and 75%, respectively. However, *sFRP* genes methylation was much less common in normal-appearing epithelia adjacent to colorectal tumor and never detected in normal mucosa. These data suggest that methylation of *sFRP* genes occurs as an early event in the evolution of ACF-adenoma-carcinoma sequence and is increased through carcinogenic transformation. Notably, 80.6% of colorectal carcinomas showed methylation of both *sFRP1* and *2*, and at least one of the four *sFRP* genes with CpG islands was methylated in 97.2% of the colorectal carcinomas and all ACF samples. *SFRP1*, *2 and 5* appeared to be more specific for colorectal carcinoma, adenoma and ACF, and may be more suitable candidate markers for these lesions.

Based on our study, methylation represents a likely mechanism of *sFRP* gene silencing in colorectal tumor: (1) *sFRP* expression is absent or markedly decreased in colorectal carcinoma and adenoma but generally high in normal mucosa, which is associated with *sFRP* gene methylation; (2) mRNA expression of *sFRP* genes is absent in cancer cell lines with corresponding *sFRP* gene methylation but high in cell lines with unmethylated *sFRP* genes; and (3) *sFRP* gene expression is reactivated in methylated cancer cell lines treated by demethylation; (4) s*FRP3* that has no CpG island in promoter region is expressed in cancer cell lines and a majority of tumor and normal samples. These data suggest *sFRP* gene silencing induced by promoter hypermethylation plays a key role in colorectal tumorigenesis by permitting aberrant activation of Wnt canonical signaling. Furthermore, we propose that the inhibiting effect of s*FRP3* on Wnt signaling is weak. Although sFRP3 is still expressed in tumor cells, it is not sufficient to block Wnt signaling. To confirm that, further studies are needed on the action of Wnt signaling in colorectal tumor cells.

We also found promoter hypermethylation of *sFRP* genes was present at equal frequency in colorectal carcinoma and adenoma, but the downregulation of *sFRP 2*, *4* and *5* expression was more frequent in carcinoma than in adenoma. These suggest that *sFRP* genes are not completely silenced by promoter hypermethylation in the stage of adenoma, although methylation has been presented in adenoma and even early stage ACF. The silence of *sFRP* genes tends to increase with the colorectal tumor progression. Thus, the downregulation of *sFRP* genes may be associated with the progression and malignant potential of colorectal tumor. Our data showed *sFRP1* methylation and downregulation were both extremely frequent in colorectal carcinomas and adenomas. It suggests the methylation and downregulation of *sFRP1* occur more frequently and earlier than other *sFRP* genes

in colorectal tumor, and perhaps play more important role in the progression of colorectal tumorigenesis. However, the methylation and downregulation of *sFRP4* were less common than *sFRP1, 2* and *5* genes in colorectal tumor, though they were both high in mesothelioma and esophageal adenocarcinoma as previously reported[9,10]. It has been reported *sFRP4* has the least homology with other family members[11]. These suggest the Wnt signaling is regulated by different sFRP molecules in different histiocytes. In colorectal tumor cells, the role of sFRP1, 2 and 5, especially the sFRP1, is more important than sFRP3 and 4.

Two key epigenetic pathways are involved in gene transcription regulation: DNA methylation and histone acetylation. DNA methylation is carried out by three DNA methyltransferases (DNMT), DNMT1, DNMT3A, and DNMT3B. DNA methylation represses transcription directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activities[12]. Deacetylation catalyzed by histone deacetylases is important for turning off genes and maintaining some genes in a repressed state[13]. DAC is a DNMT inhibitor that induces DNA demethylation by metabolic incorporation into genomic DNA, thereby covalently arresting DNMT. TSA is an HDAC inhibitor that causes hyperacetylation on histones H3 and H4. Our data showed *sFRP* genes were effectively demethylated by the high-dose DAC or combination of DAC and TSA treatment and re-expressed in colorectal cancer cell lines. The effect of combination of DAC and TSA treatment was better than high-dose DAC, even though the DAC concentration was low in the combination. A single application of TSA could not induce re-expression of *sFRP* genes mRNA, although some genes were upregulated in expression by TSA alone in the previous report[14]. These data indicate DNA methylation and histone deacetylation are synergetic in the inhibition of gene transcription, and in terms of *sFRP* genes, DNA methylation plays the dominant role.

Our study shows that hypermethylation of *sFRP* genes is a common early event in the evolution of colorectal tumor, and hypermethylation patterns of *sFRP* genes may provide a potentially useful marker system for predicting the risk of colonic neoplasia.

Silence of *sFRP* genes induced by promoter hypermethylation plays a key role in colorectal tumorigenesis. As DNA sequence is not altered by epigenetic modification, and gene silenced by promoter hypermethylation could normally transcript after demethylation, modulation of Wnt protein-driven cell growth, through reversal of *sFRP* genes silencing, may represent potential targets for colorectal cancer prevention and treatment.

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VIRAL HEPATITIS

Genetic vaccination with Flt3-L and GM-CSF as adjuvants: Enhancement of cellular and humoral immune responses that results in protective immunity in a murine model of hepatitis C virus infection

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Abstract

AIM: To investigate whether transfection of plasmid DNA encoding these cytokines enhances both humoral and cellular immune responses to hepatitis C virus (HCV) in a murine model.

METHODS: We established a tumor model of HCV infection using syngenic mouse myeloma cells stably transfected with NS5. Co-vaccination of DNA encoding granulocyte macrophage colony-stimulating factor (GM-CSF) and Flt-3 ligand together with a plasmid encoding for the HCV NS5 protein was carried out. Mice were sacrificed 14 d after the last immunization event with collection of spleen cells and serum to determine humoral and cellular immune responses.

RESULTS: Co-vaccination of DNA encoding GM-CSF and Flt-3 ligand together with a plasmid encoding for the HCV NS5 protein induced increased antibody responses and CD4+ T cell proliferation to this protein. Vaccination with DNA encoding GM-CSF and Flt-3L promoted protection against tumor formation and/or reduction in mice co-immunized with cytokine-encoding DNA constructs. This suggests this strategy is capable of generating cytotoxic T lymphocyte activity *in vivo*. Following inoculation with plasmid DNA encoding Flt-3L, no increase in spleen size or in dendritic cell (DC) and natural killer cell numbers was observed. This was in contrast to a dramatic increase of both cell types after administration of recombinant Flt3-L *in vivo*. This suggests that vaccination with plasmid DNA encoding cytokines that regulate DC generation and mobilization may not promote unwanted side effects, such as autoimmunity, splenic fibrosis or hematopoietic malignancies that may occur with administration of recombinant forms of these proteins.

CONCLUSION: Our data support the view that plasmid DNA vaccination is a promising approach for HCV immunization, and may provide a general adjuvant vaccination strategy against malignancies and other pathogens.

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Key words: DNA-vaccination; Dendritic cells; Flt3-L; granulocyte macrophage colony-stimulating factor; Hepatitis C virus

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INTRODUCTION

Dendritic cells (DCs) play a pivotal role in the initiation of immune responses and are considered as important targets for effective immunotherapeutic strategies against cancer and infectious diseases[1,2]. DCs acquire antigen in peripheral tissues, migrate to peripheral lymphoid tissue and subsequently present MHC-peptide complexes to naive T cells for the induction of immune responses. In this scheme, DCs are also believed to control the type of immune response that is generated through the secretion of cytokines such IFN-γ or IL-4 which regulate Th1 or Th2 responses, respectively. The development of DC

and their activation state is dependent upon growth factors, such as fms-like tyrosine kinase 3 ligand (Flt-3L) and granulocyte macrophage colony-stimulating factor (GM-CSF). When administered *in vivo* in the form of recombinant proteins, these cytokines can dramatically alter the absolute number of DC and the subset composition of lymphoid tissue and blood-borne DC[3,4]. This has important implications for the use of these cytokines as adjuvants, since recent evidence suggests that distinct DC subsets are responsible for the generation of different classes of immune responses. Monocyte-derived CD11c+ DCs induce T cells to produce Th1 cytokines *in vitro*, whereas the CD11c- plasmacytoid T cell-derived DCs elicit the production of Th2 cytokines. Flt-3L can increase the number of cells in both subsets of DC, but GM-CSF only increases the CD11c- precursors[3,4]. A number of studies have shown that recombinant Flt-3L protein can augment *in vivo* immune responses and that this cytokine has immunotherapeutic potential[5-7]. It is important to establish if the adjuvant effects of Flt-3L can be achieved by plasmid DNA immunization, which would provide a more versatile delivery system for antigen delivery in vaccination protocols that involve this cytokine[8].

Direct intramuscular or intradermal injection of plasmid DNA generates potent cell-mediated and humoral immune responses against a variety of pathogens, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), ebola virus, influenza virus, mycobacteria and malaria in animal models of these diseases[9-11]. Genetic vaccines induce antigenic expression that resembles a response to native viral epitopes more closely than do other conventional vaccines. Live attenuated or killed vaccines are often altered in protein structure and antigenicity and may be rendered less efficient at stimulating protective immunity. An important feature of DNA-based immunization is the induction of cytotoxic T cells (CTL) that recognize and lyse virus-infected cells, thereby limiting viral spread. Recent DNA-based immunization studies have reported the induction of anti-HIV CTL activity and anti-plasmodium T cell responses in chronically infected individuals who were immunological naïve for these pathogens[12,13]. Several lines of evidence implicate DC as the principal antigen-presenting cell (APC) in mediating effective immune responses that involve plasmid DNA vaccination. Firstly, bone marrow-chimeric mice have shown that bone marrow-derived APCs are responsible for the induction of the immune response after DNA vaccination[14,15]. Secondly, DCs but not B cells or keratinocytes, isolated from DNA-vaccinated mice were able to present the immunizing antigen to T cells *in vitro*[17]. Thirdly, injection of DNA leads to the direct transfection of DCs *in vivo*[1]. Finally, direct *in vivo* visualization of antigen-expressing DCs from draining lymph nodes after gene-gun vaccination has been demonstrated[18]. Collectively, these reports indicate plasmid DNA immunization with a DC-specific adjuvant is likely to lead to a potent immune response.

Hepatitis C virus (HCV) infection is the major causative agent of transfusion-associated and community-acquired non-A or non-B hepatitis. An estimated 170 million individuals are infected with HCV worldwide and this represents a viral pandemic[19]. Infection with HCV is associated with high morbidity and mortality, since 75%-85% of individuals infected with HCV develop chronic infection and hepatitis whilst approximately one third progress to cirrhosis and eventual liver failure[20]. Moreover, HCV-infected individuals are at increased risk for hepatocellular carcinoma (HCC) within 10-20 years of infection[21]. An effective therapeutic and/or prophylactic anti-HCV vaccine is, therefore, of considerable clinical importance[22]. We have previously identified various HCV structural and non-structural proteins that may be used for vaccination studies designed to limit HCV pathogenesis[23-25]. Here we have shown that co-immunization of DNA encoding GM-CSF and Flt-3L, together with DNA encoding HCV viral antigen leads to enhanced humoral and cellular immune responses that collectively provide protective immunity against HCV-induced pathogenesis in mice.

MATERIALS AND METHODS

Construction of DNA expression vectors

A plasmid coding for HCV NS5 was cloned as described in detail previously[25]. pcDNA3-Flt3-L was cloned from a plasmid PCRblunt-mFlt3-L encoding the leader sequence and the extracellular domain of mouse Flt3-L. PCR amplification was performed with the following primer pair: in sense direction 5´-CGG*GGTACC***ATG**ACAGTGCTGGCGCCAGCCTGG-3´ (*Kpn* I *site* and **engineered start codon**) and in antisense direction 5´-GC*TCTAGAAAGCTT***TTATTA**ATGGTGATGATGGTGATGCTGCCTGGGCCGAGGCTC-3´ (*Xba* I *and Hind* Ⅲ *site*, **engineered stop codons** and 6xHis Tag) with a proof reading high fidelity system (Roche, Germany) and a standard PCR protocol as follows: Pre-denaturation at 95℃ for 2 min; 30 amplification cycles, each cycle consisting of denaturation at 95℃ for 1 min, primers annealing at 57℃ for i min and extension at 72℃ for 1 min; and finally an extra incubation at 72℃ for 7 min to ensure full extension of the products. After restriction enzyme digest with *Kpn* I and *Xba* I for 20 h, the insert was cloned into a standard mammalian transfection vector with a CMV promoter and a given number of CpG motifs (pcDNA3; Invitrogen, Groningen, Netherlands)). Standard sequencing was performed to verify correct nucleotide sequence (Sequence Laboratories, Göttingen, Germany). Cloning and characterization of pGM-CSF was described elsewhere[25]. Plasmids were grown in DH5α cells, and plasmid DNA was subsequently purified by Qiagen Giga Kit using the endofree buffer system (Hilden, Germany).

HCV infection model

In the absence of a small animal model for HCV infection, we used a tumor model expressing a target HCV antigen (HCV-NS5). A stable HCV NS5-expressing mouse myeloma cell line SP2-NS5-21 (H-2^d background) syngenic to BALB/c mice was established by electroporation and limiting dilution, followed by Western blot analysis for HCV NS5 expression. BALB/c mice inoculated with 2×10^6 syngenic myeloma cells (SP2-NS5-21) into the flank

develop visible tumors within 7 d and succumb to terminal tumor burden within 15-25 d. The tumors constitutively express HCV NS5 protein and are an established model of HCV viral challenge that has been characterized previously[25].

In vitro expression

A human hepatoma cell line (HuH-7) was transiently transfected with the various plasmids pcDNA-Flt3-L, pcDNA3-NS5 and pcGM-CSF to verify protein and antigen expression as determined by Western blot and ELISA. Detail protocols for immunoblot for NS5 and for Western blot and ELISA for GM-CSF are described previously[23,25]. Flt3-L expression was confirmed by Western blot technique with an anti-His-Tag antibody and a mouse Flt3-L-specific ELISA (R&D, Minneapolis, MN). Cell supernatants were collected 48 h after standard calcium phosphate transfection and cell lysates were prepared in a RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 10 mL/L Nonidet P-40, 5 g/L sodium deoxycholate, 10 g/L SDS). Proteins were lysed and supernatants were loaded onto a 150 g/L-SDS-PAGE gel and blotted onto nitrocellulose membranes. After blocking with 30 g/L BSA in TBS for 2 h, membranes were incubated for 1 h with an anti-His-Tag antibody (Quiagen, Hilden, Germany) in TBS at room temperature (dilution 1:1000), followed by an incubation with sheep-anti-mouse Ig horseradish peroxidase antibody (Amersham Pharmacia, Freiburg, Germany) at 1:1000 dilution. Chemiluminescence detection was performed with the ECL system following the manufacturer's protocol (Amersham Pharmacia). Flt3-L ELISA was performed with a commercially available ELISA kit (R&D, Minneapolis, MN). Lysates and supernatants were used undiluted. To analyze Flt3-L serum levels of immunized mice, a 1:50 dilution of serum collected through tail vein bleeding was used as first antibody in this assay.

Plasmid DNA Immunization

BALB/c (H-2^d) and C57/BL6 (H-2^b) mice were purchased from Charles River Laboratories and maintained under standard pathogen-free conditions in the animal facility (Zentrales Tierlabor) at our institution (University of Heidelberg). Five days prior to plasmid immunization, 100 μL of 50 μg/mL Flt3-L or mock plasmid DNA was injected into the quadriceps muscle over five different sites. Plasmid immunization was performed three times thereafter (100 μg plasmid DNA in 100 μL 9 g/L NaCl) at bi-weekly intervals (administration into the opposite leg). Empty plasmid vector (mock) immunization was employed as a negative control. Animals (n = 5) were injected with pcDNA3-NS5 (50 μg) alone or in combination with an Flt3-L (50 μg) or GM-CSF (50 μg) plasmid. Furthermore, one group was immunized with 50 μg of HCV NS5 and Flt3-L combined with GM-CSF (both 25 μg) to study the combined effect of both cytokines. One group was immunized subcutaneously into the footpad. Mice were sacrificed 14 d after the last immunization event with collection of spleen cells and serum to determine humoral and cellular immune responses. The immunization experiments were once repeated; each immunization group contained at least 5 animals.

Measurement of humoral immune responses

Anti-HCV NS5 antibody level in the serum of each immunized animal was determined by ELISA. In brief, microtiter plates (Nunc Maxisorp) were coated with 0.5 μg/well recombinant HCV NS5-4 protein (Mikrogen, Munich, Germany), incubated overnight at 4℃ and blocked with foetal bovine serum (FBS) for 2 h at 20℃. A 1:50 dilution of mouse serum was added to the plates, incubated for an additional 1 h at 20℃, and washed 4 times with phosphate buffered saline (PBS) containing 5 mL/L Tween-20. A peroxidase-conjugated AffinePure goat anti-mouse IgG (Dianova, Hamburg, Germany) was applied to the plates at a 1:2000 dilution, incubated for 1 h, washed, and substrate (OPD) was added for color development (Abbott).

Lymphoproliferation and cytokine release assays

Mice were anesthetized with diethylether for harvesting of spleen cells. Erythrocytes were removed by incubation in 8.3 g/L NH_4Cl / 0.17 mol/L Tris (pH 7.4) for 5 min at 25℃. Spleen cells were washed 2 times and cultured in triplicate using 96-well round bottom plates at 5 × 10^5 cells/well in 200 μL DMEM (Cellgro Mediatech, Washington, DC) containing 100 mL/L FBS and 2-mercaptoethanol (5 × 10^{-5} mol/L). Cells were stimulated for 3 d with recombinant HCV NS5-4 protein (aa 2622–2868) (Mikrogen, Munich, Germany) at various concentrations (0, 0.01, 0.1 and 1 μg/mL). Following the addition of radioactive ^{3}H-thymidine (1 μCi/well), cells were incubated for an additional 18 h, and ^{3}H-thymidine uptake into DNA was measured after harvesting; incorporation of radioactivity was corrected for background activity (Δ cpm). To determine cytokine secretion levels of lymphoproliferative cells stimulated with recombinant HCV NS5-4 protein, cells were cultured as described, and mIFN-γ and mIL-4 levels were measured by commercial kits according to manufacturer's instructions (Pharmingen, San Diego, CA).

ELISPOT assay

To assess the number of IFN-γ-secreting cells at the individual cell level, single cell suspensions from spleens harvested from immunized mice mouse were analyzed in an IFN-γ ELISPOT assay. Cells were directly assessed in this assay without prior *in vitro* expansion in the presence of 1 μg/mL recombinant NS5-4 protein at 37℃ (50 mL/L CO_2) in IFN-γ-bound microtiter plates to measure IFN-γ cytokine secretion as means of CD8+-T-cell function (AID, Straßberg, Germany). After washing with PBS-Tween buffer, cells were incubated with a secondary antibody suspended in DMEM supplemented with 100 mL/l FCS and 2-mercaptoethanol.

Assessment of CTL activity in vivo

BALB/c mice were immunized im three times with either mock DNA or NS5 vector and co-immunized with Flt3-L or Flt3-L and GM-CSF. One week after the last immunization event with 2 × 10^6 syngenic SP2/0-derived cells stably expressing NS5 were washed (Sp2-NS5-21), resuspended in 200 μL of PBS, and inoculated sc into

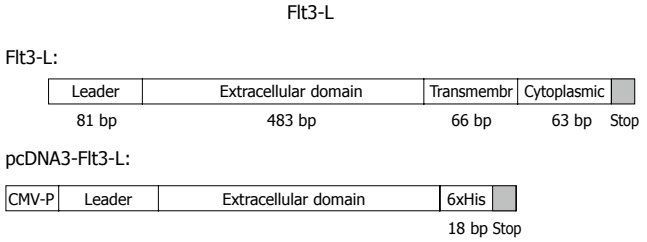

Figure 1 Cloning of the soluble form of mouse Flt3-L containing the leader and the extracellular domain into a mammalian expression vector with CMV-promoter and RSV-enhancer. The C-terminus was tagged with a 6xHis. The soluble form of mFlt3-L is fully active.

the right flank. SP2/0 cells that stably expressed HCV core protein (SP2/19) were used as a control in selected animals. Tumor formation, tumor size, mouse weight and mouse survival was assessed at distinct intervals post inoculation.

RESULTS

In vitro expression of Flt-3L from Flt-3L-pcDNA3

Flt-3L is type I transmembrane, non-disulphide linked, homodimer glycoprotein of approximately 30 ku. This cytokine may also be found as a soluble form in serum that comprises only the extracellular domain of approximately 160 amino acids that retains full biological activity[26,27]. We, using PCR, have cloned Flt-3L DNA encoding the leader sequence and extracellular domain, together with a hexahistidine C-terminal tag, into the eukaryotic expression vector pcDNA-3 for use as a Flt-3L delivery system. Figure 1 shows a schematic representation of the major domains of full-length and truncated forms of Flt-3L. DNA encoding the leader and extracellular domains of Flt-3L was generated by PCR and ligated into the eukaryotic expression vector pcDNA3 to generate Flt-3L-pcDNA3. Restriction digest and DNA sequence analysis confirmed the presence of the predicted insert size and its correct orientation (data not shown).

Figure 2 shows that Flt-3L-pcDNA3 was authentically expressed after transient transfection into HuH-7 cells. Figure 2A shows a Western blot of Flt-3L-pcDNA3-transfected HuH-7 cell lysate and culture supernatant probed with an anti-His monoclonal antibody. Anti-His reactive protein bands of 20 – 30 ku were present in both cell lysate and supernatant of cells transfected with Flt-3L-pcDNA3. This protein band pattern most likely represents the different glycosylation forms of Flt-3L which have been described previously[27]. Additional bands of 40 – 42 ku were detected in the cell culture supernatants that may represent the dimeric form of Flt-3L. No reactivity with the anti-His monoclonal antibody was seen in either cell lysate or culture supernatants from HuH-7 cells transfected with control vector. Further confirmation that Flt-3L was expressed from Flt-3L-pcDNA3 was provided by ELISA using an anti-Flt-3L specific monoclonal antibody with samples from Flt-3L-pcDNA3-transfected HuH-7 cells as substrate. Figure 2B shows that Flt-3L could be detected in cell lysate and culture supernatant from Flt-3L-pcDNA3-transfected cells, whereas no reactivity was detected in equivalent samples from control transfected cells. This

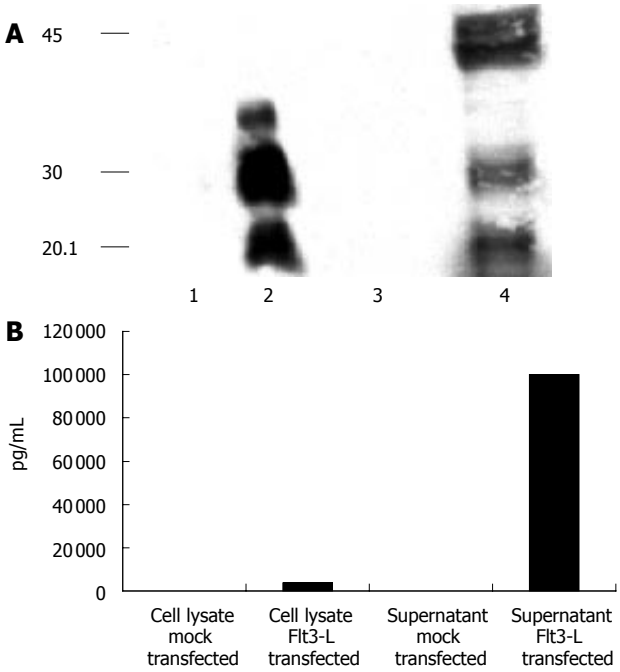

Figure 2 Expression of Flt3-L in transiently transfected HuH-7 cells: (**A**) Detection of mouse Flt3-L on a 150 g/L SDS-PAGE using an anti-His-Tag antibody. Different glycosylation forms of Flt3-L in cell were detected in cell lysates and supernatants. Lane 1 and 3: mock-transfected cells; lane 2 and 4: Flt3-L-transfected cells; lane 1 and 2: supernatants after transient transfection; lane 3 and 4: cell lysates. (**B**) To confirm these results, supernatants and cell lysates were incubated by an Flt3-L-specific ELISA. Again, secretion levels into the supernatant were more than 20-fold higher compared to levels seen in the cell compartment.

data demonstrates that Flt-3L protein could be generated from Flt-3L-pcDNA3.

Flt-3L- or GM-CSF-pcDNA3 enhances anti-HCV immune responses

Immune responses in mice to the HVC structural protein NS5 in the presence or absence cytokines that regulate dendritic cells were examined. Mice were immunized intramuscularly three times, at bi-weekly intervals, with pcDNA3-NS5 in combination with either Flt-3L-pcDNA3 or GM-CSF-pcDNA3. Serum samples from vaccinated mice were investigated for the presence of anti-HCV antibodies by ELISA using as substrate, the peptide NS5-4 which comprises amino acids 2622-2868 of the protein NS5. The data in Figure 3 shows that co-immunization of pcDNA3-NS5 with Flt-3L-pcDNA3 led to elevated levels of anti-HCV antibody compared to that found when pcDNA3-NS5 was administered alone. The level of anti-HCV antibody was further increased when pcDNA3-NS5 was co-immunized with GM-CSF-pcDNA3. Similar results were obtained using either BALB/c or C57/BL6 mice (data not shown). These experiments showed that administration of plasmid DNA encoding cytokines that regulate the development and maturation of dendritic cells, at the same time as inoculation with DNA encoding HCV protein, can produce elevated anti-HCV humoral immune responses. The adjuvant effect of Flt-3L or GM-CSF did not appear to affect the level of anti-HCV-specific T cell proliferation not readily evident at the level of T cell activation and proliferation. Spleen T cells from NS5 plasmid DNA-immunized mice were stimulated *in vitro* with recombinant

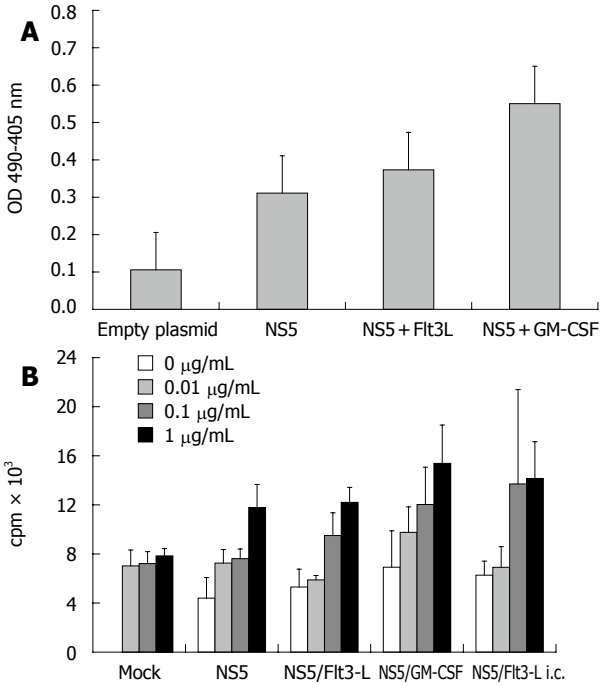

Figure 3 **A:** Anti-HCV NS5 ELISA showing mean levels of specific antibodies after genetic immunization with HCV-NS5-expressing plasmid. Controls included wells coated with BSA and sera derived from mock-immunized mice. Each group comprised 10 BALB/c mice and mice sera were pooled before the assay. ELISA plates were coated with HCV-NS5-4, therefore, antibody levels may be underestimated; **B:** T-cell proliferation assay (n = 10 mice/group) with different amounts of stimulating recombinant HCV NS5-4 protein (0.01-1 μg/mL). An increase of thymidine incorporation was seen after stimulation with 0.1 and 1 μg/mL recombinant protein. However, levels of T-cell proliferation after co-immunization with Flt3-L were only slightly higher than in NS5-immunized mice. Note that stimulation with a non-relevant protein (HBsAg) induced only background activity, demonstrating the antigen specificity (data not shown).

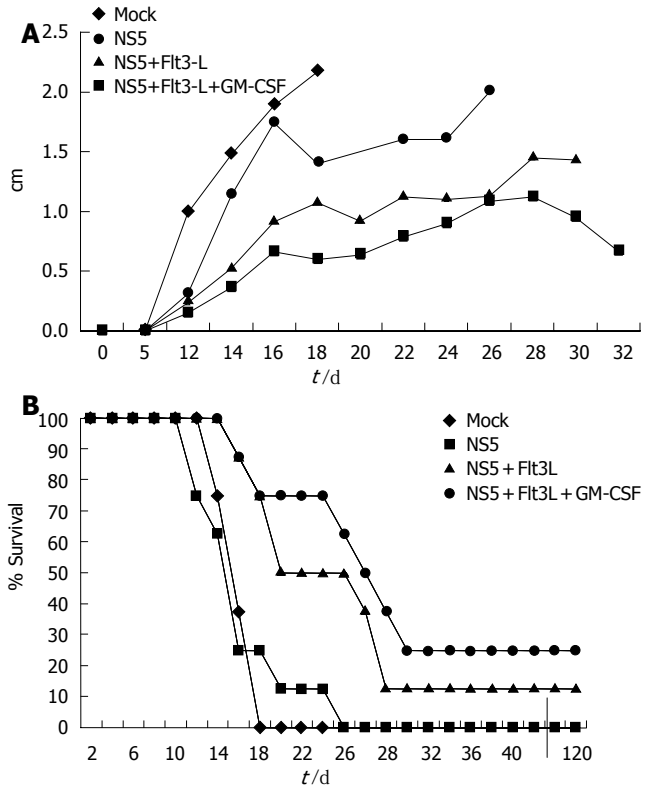

Figure 4 Tumor model to assess CTL activity generated *in vivo* after genetic vaccination. Mice were immunized three times with a total of 100 μg of plasmid DNA. Each vaccination group (n = 8) was challenged with 2 × 10^6 syngenic mouse myeloma cells stably expressing HCV-NS5 protein in the right flank. Mice developed notable tumors around d 8-10. (**A**) Tumor size in different immunization groups. Note that mice had to be sacrificed at maximum tumor diameter size of 2 cm. Sacrificed mice were then taken out from the measurement. (**B**) Mouse survival after genetic vaccination in each group. Three mice were long-term survivor and never developed significant tumor burden.

HCV NS5-4 peptide and the subsequent proliferative response measured by 3-H-thymidine uptake. The data in Figure 3 shows that there was no significant difference in anti-NS5 HCV T lymphocyte proliferation between cells isolated from mice immunized with NS5-pcDNA3 alone or in combination with either Flt-3L pcDN3 or GM-CSF-pcDNA3. In addition, when IFN-γ and IL-4 levels in the supernatant of stimulated T cells were measured by ELISA, there were no significant differences between any of the immunization groups (data not shown).

Protective immunity generated by Flt-3L and GM-CSF

In vivo CTL activity was assessed by a tumor model. Ten days after the last immunization event, animals were challenged in the right flank with a HCV-NS5 mouse myeloma cell line. Eighteen days after challenge, all mock plasmid-immunized animals were dead. Animals immunized with 50 μg of HCV-NS5 plasmid lived slightly longer, but all animals died by d 26. Co-vaccination with Flt3-L or Flt3-L and GM-CSF lead to survival and complete protection in some animals: 25% and 10%, respectively. Tumor size was also significantly reduced in co-immunized animals (Flt3-L and combination of Flt3-L and GM-CSF). Mice immunized with the same syngenic SP2/0 cell line expressing a different HCV structural protein (HCV core) as a control died as well by d 17, like mock-immunized animals. Indeed, 100% of mice immunized with mock DNA or challenged with SP2/19 cells demonstrated tumor formation, confirming the specificity of the cytotoxic activity in this tumor model (Figure 4).

In vitro and in vivo CTL activity: ELISPOT and tumor model

We used IFN-γ-bound microtiter plates to measure IFN-γ cytokine secretion as means of CD8+-T cell function in an ELISPOT on an individual cell level. Spleen cells were harvested and cultured overnight in the presence or absence of 1 mg/L recombinant NS5-4 protein. Flt3-L, GM-CSF, the combination of both cytokines injected each with smaller amounts of DNA (25 μg per vaccination) and the subcutaneous injection with Flt3-L resulted in comparable levels of NS5-4-specific IFN-γ-producing T cells. The number of cells was significantly higher compared to NS5-immunized animals alone and the mock-injected animals (Figure 5).

In vivo expression of Flt3-L from Flt3-L- pcDNA3

In studies by He *et al* where Flt-3L DNA has been administered by hypodynamic needle, levels of serum Flt-3L have been raised 10-fold compared to control values. To determine whether Flt3-L-pcDNA3 administration *in vivo* also led to elevated levels of serum Flt-3L, mice were inoculated with this vector by intramuscular injection and serum samples harvested at 3, 6, 12, 24 h and at 3 d and 7

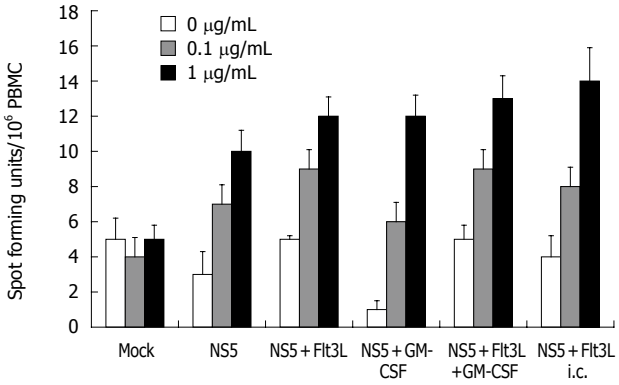

Figure 5 ELISPOT assay: Assessment at the individual single cell level of the number of mIFN-γ-secreting cells as means of CD8 T cell function. Spleen cells were stimulated with recombinant HCV-NS5 protein at 0.1 and 1 μg/mL, and the number of spot forming units/10^6 PBMC was counted after 24 h incubation period (*n* = 10 mice/group). Note that lymphocytes were not restimulated in NS5-expressing cells.

d post-inoculation. Serum samples were assessed for Flt-3 L by Flt-3L-specific ELISA. On no occasion, using either BALB/c or C57/BL6 mice, were the levels of serum Flt-3L significantly increased compared to control mice (data not shown). In addition, there was no increase in the size of lymphoid tissue, such as the spleen (data not shown), as has been reported following administration of recombinant Flt-3L protein or Flt-3L DNA by hypodynamic needle.

DISCUSSION

DNA vaccination is an efficient and versatile mechanism for the delivery of antigen to the immune system. The mechanism of antigen delivery is believed to operate by direct transfection of dendritic cells with the inoculated DNA construct; plasmid expression within these cells and the subsequent processing and presentation of antigen to T cells[17]. Dendritic cells are potent antigen-presenting cells for naïve antigen-specific T cells and, therefore, have the capacity to initiate humoral and cellular immune responses, which is an essential requisite of any vaccination strategy[28,29]. A feature of plasmid DNA vaccination has been the reported longevity of expression of the DNA construct which may lead to evidence of immunity for periods greater than 12 mo[14]. This may represent continuous expression of the plasmid DNA construct within lymphoid tissue where antigen-presenting cells, such as dendritic cells and antigen-specific T cells, interact in the appropriate environment to allow the necessary cellular interactions that lead to an effective immune response[30]. Because of these features, plasmid DNA vaccination has been considered as an effective means to provide prophylactic or therapeutic immune responses against HCV infection[31]. However, several studies to date that have investigated plasmid DNA vaccination in animal models of human HCV infection have so far only demonstrated low antibody titers against the immunizing HCV proteins[23-25]. It is clear if plasmid DNA vaccination is to provide effective immunization against HCV infection that enhanced immune responses will need to be generated. In order to address this issue, we considered it appropriate to target dendritic cells in an attempt to promote enhanced plasmid DNA-induced anti-HCV immunity in mice.

In this study, we have successfully generated an enhanced immune response against a structural HCV protein by co-immunization with plasmid DNA-encoding cytokines that regulate development and maturation of dendritic cells. When HCV plasmid and Flt-3L DNA were co-injected, the resultant anti-viral antibody titer was significantly increased, and was further enhanced by co-immunization with GM-CSF plasmid DNA. These phenomena parallel what has been seen upon administration of recombinant Flt-3L and GM-CSF protein during immunization with protein antigens. Importantly, we have demonstrated that this enhanced plasmid DNA-based immunization is effective at providing protective immunity against HCV-based tumor formation. We used an *in vivo* tumor model that expressed a HCV non-structural protein. Control mice died after 18 d, whereas co-immunized mice survived to some extend, especially after co-administration of an Flt3-L and a GM-CSF plasmid underlining synergistic effects of both cytokines seen in *in vitro* studies[8]. This underlines the local and antigen-specific immune induction, because models of recombinant administration (up to 500 μg/kg per day) or virally transduction with Flt3-L DNA have also demonstrated inhibition of the growth of B16 melanoma, EL-4 lymphoma, murine leukemia, and C3L5 breast tumors in mice[32-35]. However, these models used large amounts of recombinant FLT3-L and repeated injections are required to induce these effects, unlike the relatively small amounts of plasmid DNA injection used in this study.

A feature of our study is that protective immunity against HCV-induced tumor formation was achieved in the apparent absence of any detectable increase in serum cytokine level from the inoculated plasmids. In humans, serum levels of Flt3-L are relatively low (< 100 pg/mL). In mice we measured serum levels around 300 pg/mL and did not find an increase after genetic vaccination with Flt3-L plasmid. These results are in contrast to data that have reported an initial increase in serum Flt3-L levels after a single injection of hydrodynamic human Flt3-L gene delivery when serum levels up to 40 μg/mL were observed and remained above 1 μg/mL for 6 d in mice[36]. In addition, elevated levels of serum Flt-3L, such as those following *in vivo* administration, resulted in an increase in the size of peripheral lymphoid tissues like spleen and lymph nodes. Furthermore, these elevated levels of Flt3-L were associated with significant increases in the number of DCs and NK-cells in various tissues, such as spleen and liver[36]. Our observations on the absence of any change in serum Flt3-L following plasmid DNA vaccination with this cytokine are in agreement with reports by others using other cytokines, such as IL-2, GM-CSF or IL-12, for plasmid DNA co-vaccination studies[23,37,38]. High levels of DC in a number of different organs may, under certain conditions, be associated with adverse side effects, including induction of haematopoietic malignant disease and the induction of autoimmunity[39]. Interestingly, a very recent study demonstrated the successful local recruitment of DC to the side of injection and the induction of CD4+ proliferative

responses in a large animal model of a veterinary disease (calves) after co-vaccination with Flt3-L and GM-CSF plasmids, which has important implications of translating results from the small animal model mouse to men[40]. In this regard, a local increase in DC numbers with an accompanied enhancement of the immune response would be distinct clinical advantage and is demonstrated against HCV in our study. This approach of co-immunization with Flt3-L and GM-CSF has, therefore, important implications for the development of an effective antiviral HCV vaccine.

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Elicitation of strong immune responses by a DNA vaccine expressing a secreted form of hepatitis C virus envelope protein E2 in murine and porcine animal models

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Abstract

AIM: To characterize the immunogenicity of a hepatitis C virus (HCV) E2 DNA vaccine alone or with a protein vaccine boost in murine and porcine animal models.

METHODS: A DNA vaccine expressing a secreted form of HCV E2 protein was constructed and used to vaccinate mice and piglets with or without boosting with a recombinant E2 protein vaccine formulated with CpG ODN and 10% Emulsigen. The immunogenicity of HCV E2 vaccines was analyzed by ELISA for antibody responses, MTT assay for lymphocyte proliferation, ELISPOT for the number of interferon-γ secreting cells, and cytotoxic T lymphocyte assays.

RESULTS: Intradermal injection of E2 DNA vaccine induced strong Th1-like immune responses in mice. In piglets, E2 DNA vaccine elicited moderate and more balanced immune responses. A DNA vaccine prime and protein boost vaccination strategy induced significantly higher E2-specific antibody levels and shifted the immune response towards Th2-like ones in piglets.

CONCLUSION: A DNA vaccine expressing a secreted form of HCV E2 protein elicited E2-specific immune responses in mice and piglets. Recombinant E2 protein vaccination following DNA immunization significantly increased the antibody response in piglets. These HCV E2 vaccines may represent promising hepatitis C vaccine candidates for further investigations.

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Key words: Hepatitis C virus E2; DNA vaccine; DNA vaccine prime-protein boost

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INTRODUCTION

Hepatitis C continues to be a severe health threat to a large population with about 123 million people being affected globally[1]. The etiologic agent, hepatitis C virus (HCV)[2] is able to establish persistent infections in up to 85% of infected individuals with severe clinical consequences[3]. Current therapy with pegylated interferon and ribavirin is only effective in about 50% of the patients[4]. Although vaccines represent one of the most effective means to combat infectious diseases, there is no vaccine available for hepatitis C[5]. Hence, evaluating different vaccination strategies that can induce HCV-specific immunity is critical for the development of effective vaccines to reduce HCV-related mortality and morbidity.

HCV is the only member of the *Hepacivirus* genus in the *Flaviviridae* family[6]. The positive-sense, single-stranded RNA genome encodes a polyprotein of about 3100 amino acids in length[7]. Processing of the polyprotein by cellular or viral proteases generates up to 11 viral proteins, including three structural proteins (core, envelope proteins E1 and E2) and six non-structural proteins (NS-2, -3, -4A, -4B, -5A, and -5B). As the major envelope protein in HCV particles[8,9], the E2 protein is likely to be critical for inducing antibody responses against HCV infections. In line with this notion, anti-E2 antibodies have been consistently detected in hepatitis C patients[10-13]. Furthermore, there is evidence to suggest that anti-E2 antibodies can inhibit HCV infections in both *in vivo* and *in vitro* settings[14-17]. However, generation of E2-specific antibodies in hepatitis C patients is usually delayed and of low magnitude, which may be one of the reasons for such a high rate of persistent HCV infections[12,13]. These findings indicate that E2-specific antibodies are beneficial and induction of these antibodies should be taken into

consideration when designing a vaccine against hepatitis C.

Although the correlations for a successful immune response that can resolve HCV infections have not been well characterized, previous studies suggest a rapid, vigorous, and broadly targeted cell-mediated immune response tends to be associated with HCV clearance[18-22]. In line with this notion, E2-specific cellular immune responses have been detected in hepatitis C patients as demonstrated by E2-specific lymphocyte proliferation, cytotoxic T lymphocyte, and ELISPOT assays[23-25]. More importantly, it has been demonstrated that a stronger E2-specific cell-mediated response is associated with better response to interferon therapy and viral clearance[25]. These findings indicate that it is desirable for a candidate HCV E2 vaccine to induce cell-mediated immune responses.

Delivery of transgenes by plasmid DNA is a novel platform technology for vaccine development. DNA vaccines tend to induce a Th1-biased response in the host[26-30]. Manipulation of protein subcellular localization may enhance antibody responses to the antigen. For instance, directing antigenic expression to secretion pathways by adding a signal peptide sequence may increase B-cell mediated responses[31]. In addition, boosting with a protein subunit vaccine following DNA vaccination is another feasible means for inducing strong antigen-specific humoral responses[32-36].

In this study, a DNA vaccine was designed to induce expression of a secreted form of HCV E2 protein. Immunogenicity studies using inbred mice showed that this HCV E2 DNA vaccine elicited strong Th1-like immune responses. In piglets, the E2 DNA vaccine elicited a moderate and more Th1-Th2 balanced response. E2 protein vaccination after DNA immunization had a more pronounced boosting effect in piglets than in mice by significantly increasing E2-specific antibody response, causing a shift of the immune response towards a Th2-type.

MATERIALS AND METHODS

HCV E2 DNA vaccine construction

To generate a DNA vaccine encoding a secreted form of HCV E2 protein, a portion of the E2 coding sequence (amino acid residues 412-661 of HCV polyprotein) without the hypervariable region 1 (HVR1) and the hydrophobic region was amplified by polymerase chain reaction (PCR). The template was plasmid pDM22, a cDNA clone of the BK isolate (genotype 1b; kindly provided by Dr. A. Takamizawa)[37]. The primers were E2-412-Nhe-Sense (5'-AATTGCTAGCCAGCTTATAAACACCAATGGG-3', *Nhe*I site is underlined) and E2-661-Bgl-AS (5'-AATTAGATCTTCACTCCGGCCTATCCCTGTC-3', a stop codon TGA was added followed by a *Bgl*Ⅱ site which is underlined). The PCR product was cloned into a plasmid vector pSLIA-tPAs[38] with *Nhe*I and *Bgl*Ⅱ (New England Biolabs), allowing the addition of the signal peptide sequence of the tissue plasminogen activator (tPA) to the amino-terminus of the truncated E2 protein. The identity of PCR amplified E2 gene was confirmed by DNA sequencing experiments. Subsequently, the *Hin*dⅢ-*Bgl*Ⅱ fragment containing the tPAs-tE2 fusion gene was cloned into a CpG enriched DNA vaccine vector pBISIA24[39], which contains 24 copies of a Th1-promoting CpG motif (GTCGTT). An unexpected extension of the open reading frame resulted from the *Nde*I recognition sequence in the multiple cloning site of plasmid pBISIA24 was removed by deleting the small fragment after *Pst*I digestion and subsequent ligation of the large fragment. The final DNA vaccine construct was designated pBISIA24-tPAs-tE2 (Figure 1). Plasmid prepared by endotoxin-free plasmid purification reagents (Qiagen) was used for immunization.

Transfection and immunoblotting analysis

To determine whether the constructed DNA vaccine plasmid expressed the E2 protein, TE671 cells, a human rhabdomyosarcoma cell line, were transfected with plasmids pBISIA24-tPAs-tE2 and pBISIA24 using the calcium phosphate method[40]. At 24 h after transfection, culture medium was collected and cells were lyzed in a lysis buffer (1% SDS, 10 mmol/L Tris-HCl, pH8.0). The protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin (BSA) (Sigma) as a standard. Twenty-five micrograms of protein were loaded on a 10% SDS protein gel and transferred to a PVDF membrane (GE Healthcare). The membrane was incubated with a monoclonal antibody against HCV E2 protein (H52, kindly provided by Dr. J. Dubuisson). After incubation with an HRP-conjugated secondary antibody, immuno-reactive protein bands were visualized by the enhanced chemiluminescence system (ECL^{Plus}, GE Healthcare).

Recombinant E2 protein expression and purification

To generate a recombinant E2 protein vaccine, HCV E2 protein was expressed and purified as a fusion protein with a poly-histidine tag. For this purpose, the coding sequence of HCV E2 was amplified by PCR using plasmid pDM22 as the template. The primers for the PCR reaction were E2-Bam-Nhe-Sense (5'-GGGGGGGGATCCGCTAGCGATACCCACGTGACAGGGG-3', *Bam*HI and *Nhe*I sites are underlined) and E2-746-Bgl-AS (5'-GGGGGGAGATCTCAGGCCTCGGCCTGGGCTA-3', a stop codon TGA was added followed by a *Bgl*Ⅱ site which is underlined). The PCR fragment was cloned into an expression vector pRSETA (Invitrogen) using restriction enzymes *Bam*HI and *Bgl*Ⅱ, allowing the addition of a poly-histidine tag at the amino-terminus of the E2 protein. The resultant plasmid, designated pRSET-E2, was confirmed by restriction analysis and DNA sequencing. The *Not*I-*Bgl*Ⅱ fragment of plasmid pRSET-E2 was substituted by the *Not*I-*Bgl*Ⅱ fragment of pBISIA24-tPAs-tE2, generating plasmid pRSET-tE2 encoding a poly-histidine-tagged E2 protein without the carboxyl hydrophobic region. Plasmid pRSET-tE2 was then transformed into *E. coli* BL21 (pLysS) and expression of E2 protein was induced by isopropylthio-β-galactoside (IPTG, Invitrogen). The induced poly-histidine-tagged tE2 protein was purified under denaturing conditions in the presence of 8 mol/L urea using Ni-NTA agarose (Qiagen) *as per* manufacturer's instructions. The urea was removed from the protein preparation after dialysis against phosphate buffered saline (PBS). The level of endotoxin was determined by the

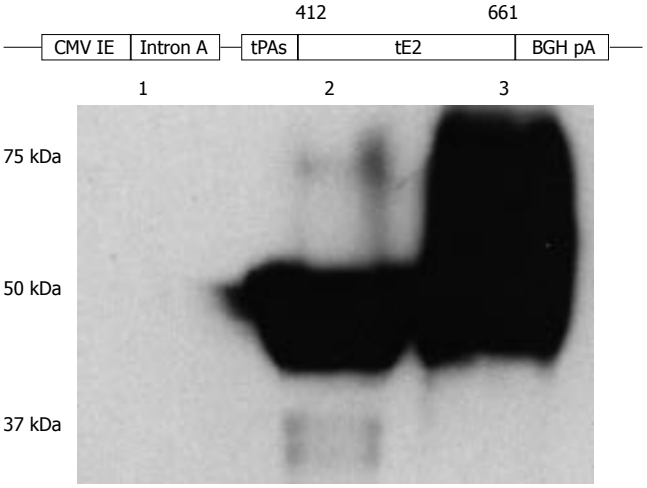

Figure 1 Construction and characterization of HCV E2 DNA vaccine. A secreted form of HCV E2 protein generated by removing the carboxyl-terminal hydrophobic region and replacing the hypervariable region 1 with the signal peptide sequence of the tissue plasminogen activator (tPAs) was cloned into a DNA vaccine vector pBISIA24. The construction of E2 DNA vaccine, pBISIA24-tPAs-tE2, is schematically presented. TE 671 cells were transfected with pBISIA24 (lane 1) or pBISIA24-tPAs-tE2 (lanes 2 and 3). Cell lysates (lanes 1 and 2) and culture medium (lane 3) were analyzed in immunoblotting using an E2-specific antibody.

Limulus amoebocyte test (QCL-1000 Chromogenic *Limulus* amebocyte lysate kit, Cambrex).

Mouse and piglet immunization

The experimental protocols were approved by the Committee of Animal Care and Supply, University of Saskatchewan. In the mouse trial, 24 six-week old B6C3F1 (H-2^d) female mice were randomly divided into three groups. Groups of mice were immunized either three times with 40 μL saline subcutaneously (s.c.), three times with 50 μg of DNA vaccine pBISIA24-tPAs-tE2 intradermally (i.d.), or twice with 50 μg of DNA vaccine pBISIA24-tPAs-tE2 i.d. followed by one subcutaneous vaccination with 5 μg of recombinant tE2 protein. The recombinant truncated E2 protein vaccine was formulated with 10 μg of CpG oligonucleotide (ODN) 1826 (5'-TCCATGACGTTCCTGACGTT-3', CpG motifs are underlined; kindly provided by Merial Limited) and 10% Emulsigen (MVP Laboratories) per dose. This formulation has been shown to elicit strong immune responses with a superior safety profile[41]. The vaccines were given three weeks apart. Blood was sampled two weeks after each immunization and spleens were collected two weeks after final immunization for analyzing immune responses.

In the piglet trial, 24 out-bred piglets (Landrace cross, 4-5 wk old, Prairie Swine Center) were randomly allocated into three groups with eight piglets in each group. The vaccination schedule was the same as that in the mouse trial but the doses were 10-fold of those used for mice. In addition, CpG ODN 2007 (5'-TCGTCGTTGTCGTTTTGGTCGTT-3', kindly provided by Merial Limited), which can stimulate porcine peripheral blood mononuclear cells (PBMCs)[42], was used for protein vaccine formulation.

Enzyme-linked immunosorbent assay (ELISA)

To analyze antibody levels in murine or porcine sera after immunization, 96-well polystyrene plates (Immulon 2, Dynatech Laboratories) were coated with purified E2 protein at 100 ng/well in a carbonate buffer (pH9.6) overnight at 4℃. Serially diluted murine or porcine sera were added to each well and incubated for 2 h at room temperature (RT). To determine the total IgG levels, biotinylated goat anti-mouse IgG antibody (Caltag Laboratories) at a dilution of 1:10000 or alkaline phosphatase labeled goat anti-porcine IgG (KPL) at a dilution of 1:2500 was applied to detect bound IgG. To determine E2-specific IgG1 and IgG2a antibody levels in murine sera, bound antibodies were incubated with biotinylated goat anti-mouse IgG1 or IgG2a antibodies (Caltag Laboratories) at a dilution of 1:10000, respectively, followed by streptavidin-AP (Jackson ImmunoResearch Laboratories) at a dilution of 1:5000. To determine E2-specific IgG1 and IgG2 antibody levels in porcine sera, bound antibodies were incubated with mouse anti-porcine IgG1 or IgG2 antibodies (Serotec) at a dilution of 1:100, respectively, followed by biotinylated goat anti-mouse IgG antibody (Caltag Laboratories) at a dilution of 1:10000. The reactions were developed by adding *p*-nitrophenyl phosphate (PNPP) (Sigma) at 100 ng/well and the optical density was recorded at 405 nm.

Enzyme-linked immunospot (ELISPOT) assay

ELISPOT assay was performed to determine the frequency of interferon-γ (IFN-γ) secreting cells in mouse splenocytes or porcine PBMCs after vaccination. Microplate Devices Unifilter 96-well plates (Waterman) were coated with anti-mouse IFN-γ antibody (125 ng/well) (BioSource International) or anti-porcine IFN-γ monoclonal antibody (500 ng/well) (Endogen) overnight at 4℃. After washing and blocking, 1×10^6 murine splenocytes or porcine PBMCs were added along with 200 ng of purified E2 protein into each well. The plates were incubated at 37℃ and 5% CO_2 for 40 h. For mouse sera, biotinylated anti-mouse IFN-γ antibody (125 ng/well) was added and incubated at 37℃ and 5% CO_2 for 3 h. For porcine sera, rabbit anti-porcine IFN-γ antibody (200 ng/well) (Endogen) was added and incubated at RT for 4 h followed by the addition of biotinylated goat rabbit IgG (Zymed) at a dilution of 1:5000 for 2 h at RT. All the plates were then incubated for 1.5 h at RT with streptavidin-AP (Jackson ImmunoResearch Laboratories) at a dilution of 1:500 and developed with 5-bromo-4-chloro-3-indolylphospohate and nitroblue tetrazolium substrate tablets (Sigma). The plates were dried and the spots were recorded.

Lymphocyte proliferation-MTT assay

Splenocytes (3×10^5 cells) isolated from each group of mice were seeded into each well of round bottom tissue culture 96-well plates (Nunc). Purified E2 protein (100 ng) was added to each well and incubated at 37℃ and 5% CO_2 for 72 h. Eighty ng of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37℃ and 5% CO_2 for 3 h. The plates were centrifuged at 1000 × g for 10 min. One hundred μL of the supernatant was mixed with 100 μL of acidified isopropanol (0.375% HCl in isopropanol) and the optical density was recorded at 590 nm.

Cytotoxic T lymphocyte (CTL) assay

Effector cell preparation and stimulation: To prepare effector cells, splenocytes were isolated from each group of mice and pooled 14 d after vaccination. To generate stimulating cells, splenocytes isolated from naïve, syngenic mice were infected with a recombinant vaccinia virus expressing HCV BK E2 protein (VP1478, kindly provided by Sanofi Pasteur MSD) at an m.o.i. of 10 for 1 h at 37℃ followed by an irradiation at 3000 rads. The splenocytes from experimental groups were cultured with irradiated stimulating cells for four days at 37℃ and 5% CO_2 before they were used as effector cells in CTL assays.

Target cell preparation: To generate target cells, a syngeneic mastocytoma cell line P1-HTR-TK^+[43] (H-2^d, a highly transfectable variant of P815 cells, kindly provided by Dr. T. Boon) was transfected with a plasmid expressing HCV BK E2. Stable transfectants, designated P1-E2 cells, were selected with 800 μg/mL of Geneticin (Invitrogen). The expression of HCV E2 protein was confirmed by immunohistochemical staining using an E2-specific polyclonal antibody as previously described[44]. In brief, after fixation and blocking, P1-E2 cells or plasmid vector-transfected P1 cells were incubated with an anti-E2 antibody at a dilution of 1:1000 in PBS for 30 min. Cells were then washed and incubated with a biotinylated secondary antibody for 30 min. The reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories).

CTL assay: CTL assays were performed based on the evaluation of the cytoplasmic lactate dehydrogenase (LDH) after cell lysis as described previously[45]. In brief, effector cells were harvested by centrifugation at 800 × g for 10 min and washed twice with medium. The cells were then adjusted to 2.0×10^7 cells/mL in AIM-V medium and incubated with target cells at standard effector:target (E:T) ratios in triplicates in a 96-well round bottom plate. After 4 h incubation, the plate was centrifuged at 250 × g for 4 min. Fifty μL of the supernatant was transferred to a 96-well flat bottom plate and mixed with the Substrate mix provided in the Cytotox 96 Non-radioactive Cytotoxicity Assay kit (Promega). After 30 min, the reaction was terminated by adding 50 μL of the Stop Solution into each well and the optical density was determined at 490 nm. Spontaneous and maximal LDH release by target cells was determined by incubating the target cells with medium alone or with medium plus the lysis buffer containing 0.9% Triton X-100 (Promega). HCV E2-specific lysis was calculated as [(experimental release-spontaneous release)/(maximal release-spontaneous release)] × 100.

Statistical analysis

The experimental data were analyzed by software programs Prism 4 (GraphPad) or Excel (Microsoft) and were expressed as mean ± SE. A P value of $\leqslant 0.05$ determined by Student's t test was considered statistically significant.

RESULTS

Construction and characterization of a DNA vaccine expressing a secreted form of HCV E2 protein

We designed a DNA vaccine encoding a secreted form of HCV E2 protein by removing the transmembrane domain and replacing the hypervariable region 1 (HVR1) of the E2 protein with the tissue plasminogen activator signal peptide sequence (tPAs). The corresponding coding sequence was amplified by PCR and cloned into a DNA vaccine vector pBISIA24[39], resulting in the plasmid pBISIA24-tPAs-tE2 (Figure 1). To determine the expression of E2 protein, TE671 cells were transfected with pBISIA24 (vector control) and pBISIS24-tPAs-tE2. The presence of E2 protein in cell lysates and culture medium was analyzed by immunoblotting. As shown in Figure 1, an E2-specific antibody recognized proteins of about 50 kDa in cell lysates and of 50-75 kDa in culture medium after pBISIA24-tPAs-tE2 transfection, whereas these proteins were not detected after pBISIA24 vector transfection, indicating the expression and secretion of the E2 protein by pBISIA24-tPAs-tE2. The apparent molecular masses (50-75 kDa) of tE2 protein are larger than the calculated one (31 kDa) and the extracellular form contained larger protein species than the intracellular form, suggesting that E2 protein may have been modified post-translationally by glycosylation.

Recombinant tE2 protein expression and purification

The E2 protein used in immunization was produced in *E. coli*. The expression of poly-histidine tagged tE2 was induced by IPTG and purified by affinity chromatography (not shown). The purified poly-histidine tagged tE2 protein was dialyzed into phosphate-buffered saline before it was used in mouse and piglet immunization. The protein vaccine contained endotoxin at a concentration of 80 ng/mg of protein as determined by the *Limulus* amoebocyte test.

Antibody responses to HCV E2 DNA vaccine in mice

To determine whether the DNA vaccine expressing a secreted E2 protein elicited antibody responses in mice, mouse sera collected after vaccination were analyzed for E2-specific IgG titers by ELISA assays. As shown in Figure 2A, vaccinated-mice all developed E2-specific IgG antibody after intradermal DNA vaccination with the average IgG titer being 1.6×10^5. To test whether a protein vaccine could enhance the IgG response, mice were injected twice with the DNA vaccine followed by recombinant E2 protein boosting. Mice that received the protein boosting vaccination had about two-fold higher titers of E2-specific IgG (3.0×10^5) (Figure 2A). These results indicate that the DNA vaccine expressing a secreted form of HCV E2 protein induced strong antibody responses in mice, which were further increased by a protein vaccine.

Since the relative levels of IgG subclasses are an indicator for evaluating the quality of the immune response[46,47], the titers of IgG1 and IgG2a were determined. E2 DNA vaccine induced higher IgG2a titers than IgG1 (Figure 2), indicating a Th1-biased response. Boosting with E2 protein formulated with CpG ODN and 10% Emulsigen did not change this pattern, although the IgG1 and IgG2a levels were elevated (Figure 2).

Cell-mediated responses to HCV E2 DNA vaccine in mice

To determine whether the HCV E2 vaccine induced cell-mediated immune responses in mice, we analyzed the

responses of mouse splenocytes after vaccination by measuring cell proliferation and interferon-γ secretion upon antigen re-stimulation *in vitro*.

In lymphocyte proliferation assay, splenocytes from saline vaccine group showed detectable proliferation after antigen stimulation (Figure 3A). Splenocytes isolated from mice after DNA vaccination expanded to a significantly higher degree after E2 antigen re-stimulation than medium control ($P < 0.001$, Figure 3A). Protein boost immunization did not change E2-specific lymphocyte proliferation (DNA *vs* DNA/Protein, $P = 0.47$, Figure 3A). The results of IFN-γ ELISPOT assay demonstrated that DNA vaccination elicited a strong IFN-γ response (DNA *vs* saline, $P < 0.001$, Figure 3B), whereas a protein boost immunization induced fewer IFN-γ secreting spots (DNA *vs* DNA/Protein, $P = 0.03$, Figure 3B). These results indicate that stronger E2-specific Th1-type immune responses were induced in mice that received the DNA vaccine three times than in mice that received twice DNA vaccination followed by a protein boosting injection.

Since cytotoxic T lymphocytes are one of the major effectors in cell-mediated immune responses, we also determined the presence of E2-specific cytotoxic T lymphocytes after vaccination. For this purpose, a syngeneic cell line expressing HCV E2 protein was generated. Immunohistochemistry staining using an anti-E2 antibody detected specific signal in P1-HTR cells stably transfected by the E2-expressing plasmid, but not in vector-transfected cells, demonstrating the expression of the E2 protein by the stable transfectant (not shown). When the E2-expressing cell line was used as target cells in the CTL assay, effector cells from DNA vaccine- or DNA and protein vaccine-immunized mice showed 36% or 31% specific lysis at an effector to target ratio of 100:1, respectively (Figure 4). No CTL activity was detected after saline immunization (Figure 4). These results demonstrate the elicitation of E2-specific cytotoxic T lymphocytes in mice immunized with E2 DNA vaccine or DNA vaccine followed by a protein boost.

Immune responses to HCV E2 vaccines in piglets

Since vaccine efficacy can be different in various test species[31] and pigs represent a promising model for human biology because of its body size and physiology[48], we evaluated our E2 vaccines in piglets. As shown in Figure 5A, all the piglets receiving the E2 DNA vaccine three times intradermally developed E2-specific IgG titers with the average titer being 1×10^3. Vaccination with the E2 protein after two DNA vaccine priming injections significantly boosted the E2-specific IgG titers (DNA vaccine *vs* DNA/Protein, $P = 0.0156$, Figure 5A). These results indicate that E2 DNA vaccine was also effective in inducing antigen-specific antibody response in piglets, which was boosted significantly by a protein vaccine.

To analyze the type of the immune responses, we determined the ratios of E2-specific IgG1 to IgG2 levels in the porcine serum samples after vaccination. As illustrated in Figure 5B, DNA vaccination induced a slightly Th1-biased immune response with the IgG2 to IgG1 ratio being 1.4, whereas protein boosting after DNA vaccination shifted the immune response towards Th2 direction as demonstrated by a reduced IgG2/IgG1 ratio (0.58).

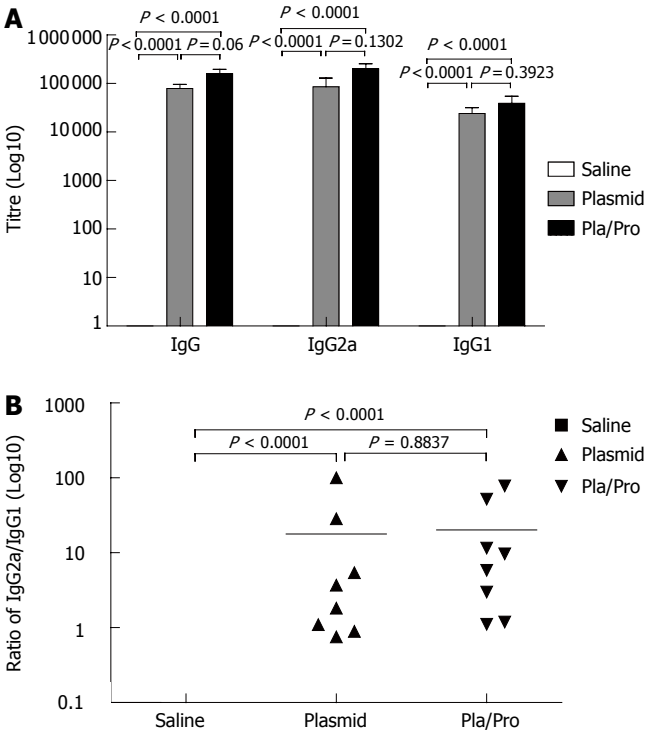

Figure 2 Antibody responses to HCV E2 vaccines in mice. B6C3F1 mice were intradermally injected with pBISIA24-tPAs-tE2 three times or pBISIA24-tPAs-tE2 twice followed by subcutaneous vaccination with E2 protein formulated with CpG ODN 1826 and 10% Emulsigen at 3-wk intervals. E2-specific IgG, IgG1, and IgG2a levels were determined by ELISA assays (**A**). The ratios of IgG2a to IgG1 were determined (**B**) ($P < 0.0001$, *vs* saline).

To further characterize the immune responses of piglets to E2 vaccines, the number of IFN-γ secreting cells in porcine PBMCs was determined after vaccination. As shown in Figure 6, PBMCs from E2 vaccinated piglets contained significantly higher number of IFN-γ secreting cells than saline treated piglets after *in vitro* antigen re-stimulation in ELISPOT assay (DNA *vs* saline, $P < 0.0001$, DNA/Protein *vs* saline, $P < 0.0001$). No difference was detected between the DNA vaccine alone group and the DNA prime and protein boost group (Figure 6, DNA *vs* DNA/Protein, $P = 0.4531$).

DISCUSSION

Numerous research groups have evaluated the potential of using E2 protein as a hepatitis C vaccine candidate through different strategies[5,36,49-74]. These studies have demonstrated E2-specific immune responses, mostly in mouse trials, when E2 alone or as part of the entire HCV structural region was delivered as a DNA vaccine, viral vectored vaccine, or subunit protein vaccine. The most important finding of these studies is that the vaccine-induced E2-specific immune responses were able to delay HCV infections in chimpanzees or humans[55,61,62,64]. While these studies have demonstrated the feasibility of inducing E2-specific immune responses through vaccination, the efficacy of E2 vaccines is less satisfactory in inducing sterilizing immunity. It is thus obvious that further

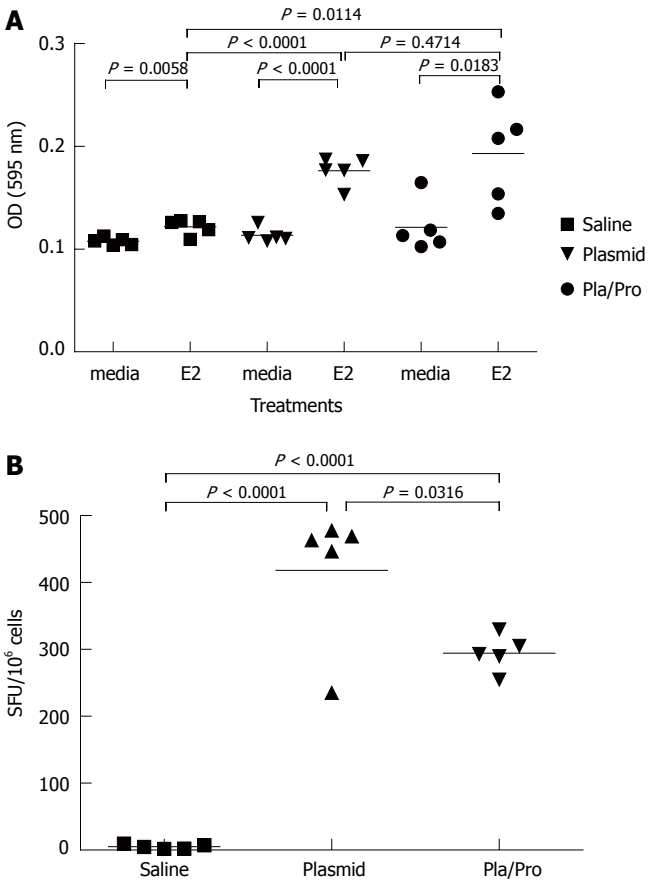

Figure 3 Cell-mediated immune responses to HCV E2 vaccines in mice. Murine splenocytes were isolated 2 wk after last immunization and were re-stimulated with E2 protein. Cell proliferation was analyzed by MTT assay (**A**). ($P < 0.0001$ *vs* saline; $P < 0.05$, $P < 0.01$ *vs* media). The number of interferon-γ secreting cells was determined by ELISPOT assay (**B**). ($P < 0.05$ *vs* plasmid; $P < 0.0001$ *vs* saline).

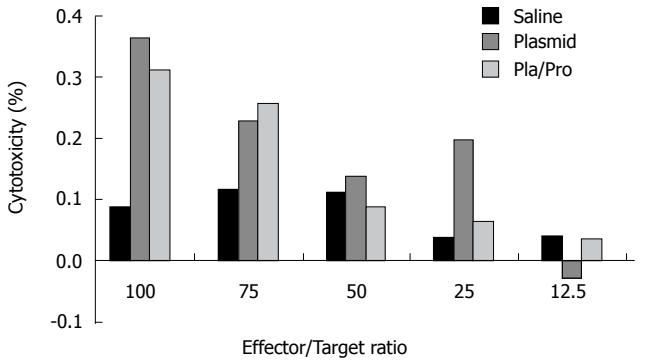

Figure 4 Cytotoxic T lymphocyte (CTL) response to HCV E2 vaccines in mice. Murine splenocytes isolated from groups of mice two weeks after final immunization were pooled and stimulated with feeder cells infected with recombinant vaccinia virus expressing HCV E2 protein. The cytotoxic activity was determined by measuring the released lactate dehydrogenase (LDH) by the target cells after incubation with the effectors at different ratios.

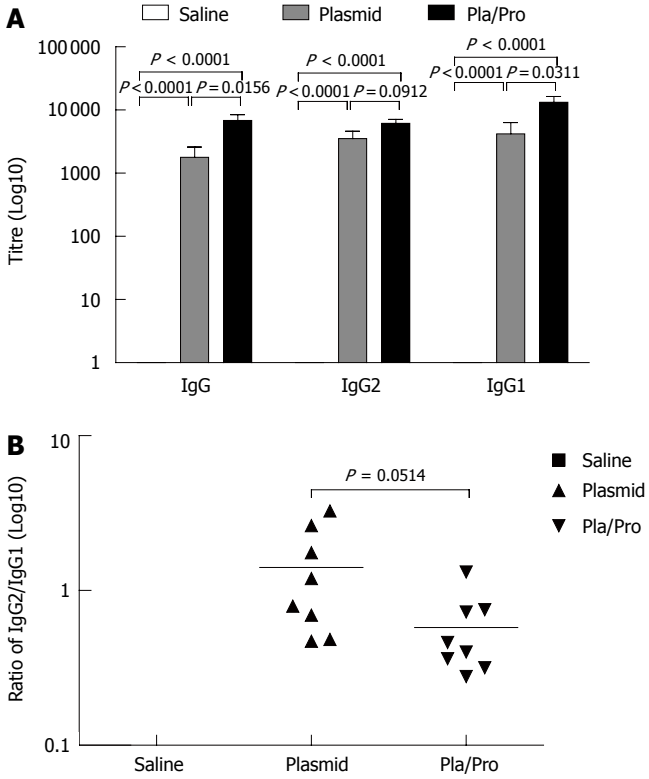

Figure 5 Antibody responses of piglets to HCV E2 vaccines. Piglets were immunized by saline, E2 DNA vaccine three times, or E2 DNA vaccine twice followed by subcutaneous vaccination with E2 protein formulated with CpG ODN 2007 and 10% Emulsigen at three-week intervals. ELISA assays were performed to determine E2-specific IgG, IgG1, and IgG2 levels (**A**), as well as the ratios of IgG2 to IgG1 (**B**) ($P < 0.05$ *vs* plasmid; $P < 0.0001$ *vs* saline).

improvement of E2 vaccines is required by evaluating additional vaccination strategies.

Given the importance of E2 protein in inducing host immune responses, we have chosen E2 as a vaccine candidate against hepatitis C. It has been established that HCV E2 expressed by a number of systems is an intracellular protein modified by high-mannose type oligosaccharides[75-78]. However, the envelope proteins on the hepatitis C virions have been shown to be modified by complex type oligosaccharides[79], suggesting secretion of E2 protein has occurred in HCV life cycle. In agreement with this notion, the secreted, complex form E2 protein possesses greater immunoreactivity against hepatitis C patient sera than the intracellular E2 protein when it is expressed in cell culture[78]. The retention of the expressed E2 protein is due to the presence of a membrane anchor domain (amino acids 718-746) at its carboxyl-terminus[80-82] and deletion of the transmembrane domain leads to E2 protein secretion[83]. However, a comparative study of a series of carboxyl-terminally truncated E2 proteins has demonstrated that truncation to amino acid residue 661 is necessary to achieve proper folding of the secreted E2 protein[83]. Hepatitis C virus is known to be able to quickly develop multiple sequence variants after infection within the host due to the lack of proof-reading activity of RNA-dependent RNA polymerase[84]. A short stretch of coding sequence (amino acids 384-411, the hypervariable region 1) at the very amino-terminus of E2 has shown extremely high variability[85,86] and may contribute to the generation of immune escape HCV variants. Although E2 HVR-1 may contain antigenic epitopes[15,16], rapid appearance of variable mutants and their interference with the development of cellular immunity in the host[87] make it a less favorable component in an E2 DNA vaccine. Based on

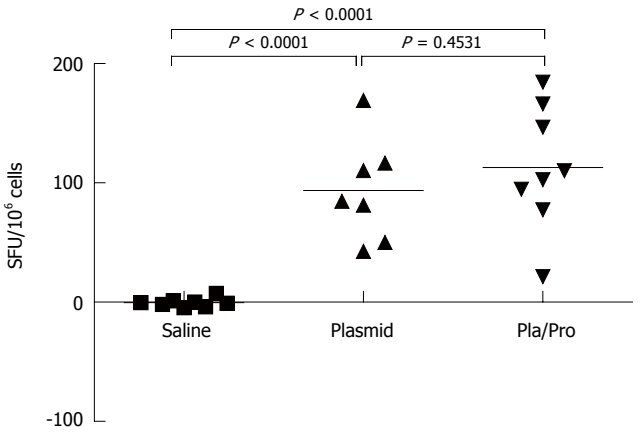

Figure 6 Cell-mediated immune response of piglets to HCV E2 vaccines. Porcine peripheral blood mononuclear cells (PBMCs) were isolated three weeks after final immunization. After re-stimulation with E2 antigen, the number of interferon-γ secreting cells was determined by ELISPOT assay ($P < 0.0001$ *vs* saline).

the above findings and analysis, we designed an E2 DNA vaccine by deleting the carboxyl-terminal hydrophobic region after amino acid residue 661 and replacing HVR1 by a strong signal peptide sequence derived from tissue plasminogen activator[88]. Indeed, we showed that the engineered E2 protein was efficiently secreted into the culture medium (Figure 1). In addition, in agreement with previous studies[83], the apparent molecular masses of the intracellular as well as secreted tE2 proteins are larger than that calculated from the amino acid sequence and the secreted tE2 proteins contain species of even higher molecular mass than the intracellular tE2 protein, suggesting that intracellular and extracellular E2 proteins may have been modified by different glycosylation.

Intradermal injection of this E2 DNA vaccine elicited strong E2-specific antibody responses in mice as demonstrated by high IgG levels (Figure 2). IgG isotyping, IFN-γ ELISPOT, lymphocyte proliferation, and CTL assays (Figures 2-4) indicated that both Th1 lymphocyte and cytotoxic T lymphocyte responses were induced by E2 DNA vaccine. Interestingly, DNA vaccine alone or DNA vaccine followed by an E2 protein formulated with CpG ODN and 10% Emulsigen induced comparable E2-specific IgG2a to IgG1 ratios (Figure 2B) and CTL responses (Figure 4). This is in agreement with the proven roles of immuno-stimulatory CpG ODN in promoting Th1 immune responses[41,89-91]. However, although DNA prime and protein boost strategy induced a relatively strong IFN-γ response, the number of IFN-γ secreting cells in the splenocytes derived from mice immunized with DNA prime and protein boost was significantly lower than that of DNA vaccine group (Figure 3B).

DNA vaccines are often less effective in large model animals and in humans than in mice[31,92]. Therefore, it is critical to test the vaccine efficacy in an out-bred, large animal model. Our piglet trial indicated that E2 DNA vaccine induced E2-specific IgG titers (Figure 5A) that were about 100-fold lower than those detected in mice (Figure 2A). As for the type of vaccine-induced immune response, E2 DNA vaccine induced a more balanced response in piglets (Figure 5B), in contrast to a strongly Th1-biased response in mice (Figure 2B). The effect of protein boost injection in modulating the type of the immune response was also different: while protein boost had little effect in mice (Figure 2B), a shift towards Th2 direction was detected in piglets (Figure 5B). Taken together, while these results demonstrate that E2 vaccines were also effective in inducing antigen-specific immune responses in piglets, inter-species difference in immune responses to different vaccine strategies requires further investigation.

In conclusion, our results demonstrate that a DNA vaccine expressing the secreted form of HCV E2 protein induces E2-specific immune responses in mice and piglets. An E2 protein vaccine formulated with CpG ODN and 10% Emulsigen further increases the antibody responses. In addition, our results highlight the importance of testing the magnitude and type of vaccine-induced immune responses in multiple model species before primate or human trials are initiated.

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COMMENTS

Background

Hepatitis C is a devastating liver disease worldwide. Current interferon and ribavirin therapy is far from satisfactory. An effective vaccine for hepatitis C is urgently needed.

Research frontiers

To develop an effective vaccine for hepatitis C, a rational design based on the understanding of the magnitude and the quality of the immune responses is critical for a success. The application of appropriate adjuvants and vaccine delivery means also play a major role.

Innovations and breakthroughs

The novelty of this article lies in the design of the HCV E2 vaccine, namely the hypervariable region-1 was replaced by the signal peptide sequence of the tissue plasminogen activator and the hydrophobic region at the carboxyl-terminus was removed.

Applications

The HCV E2 DNA vaccine developed in this study should be further tested in primate models with hepatitis C virus challenge to demonstrate protective activity of the vaccine.

Peer review

HCV infection is a big burden worldwide. Numerous studies have evaluated the potential of using E2 protein as a hepatitis C vaccine candidate through different strategies, but the efficacy of E2 vaccines is less satisfactory in inducing sterilizing immunity. It is interesting to investigate further improvement of E2 vaccines by evaluating additional vaccination strategies. The authors characterized the immunogenicity of a HCV E2 DNA vaccine alone or with a protein vaccine boost in murine and porcine animal models. They found that this E2 DNA vaccine elicited E2-specific immune responses in mice and piglets and recombinant E2 protein vaccination boosting significantly increased the antibody response in piglets. The study is well designed and results are convincing. The presentation and readability of the manuscript is satisfactory.

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H pylori

Antimicrobial activity of *Sapindus mukorossi* and *Rheum emodi* extracts against *H pylori*: *In vitro* and *in vivo* studies

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Abstract

AIM: To evaluate the antibacterial activity of *Sapindus mukorossi* (*S. mukorossi*) and *Rheum emodi* (*R. emodi*).

METHODS: Powders of *S. mukorossi* and *R. emodi* were extracted successively with petroleum ether, benzene, chloroform and ethanol and were concentrated in vacuum. The disk diffusion method was used for *in vitro* studies and *in vivo* studies were performed on male Wister rats. Thirty resistant clinical isolates of *H pylori*, as determined by their antibiotic sensitivity patterns by *E*-test, along with two Gram +ve (*S. aureus*, *B. subtilis*) and two Gram -ve (*E. coli*, *P. vugaris*) organisms were screened for their susceptibility patterns against these extracts.

RESULTS: In our screening, all 30 resistant isolates and the other four organisms (two Gram +ve *S. aureus*, *B. subtilis* and two Gram -ve, *E. coli*, *P. vugaris*) were sensitive to the test compounds. It was found that ethanol and chloroform extracts of *S. mukorossi* and ethanol and benzene extracts of *R. emodi* inhibited *H pylori* at very low concentrations. In the *in vitro* study, the isolates showed a considerable zone of inhibition at very low concentrations (10 μg/mL) for both the extracts. In the *in vivo* study, the *H pylori* infection was cleared with minimal doses of extracts of *S. mukorossi* (2.5 mg/mL) and *R. emodi* (3.0 mg/mL) given orally for seven days.

CONCLUSION: We can conclude from this study that the extracts of *S. mukorossi* and *R. emodi* inhibited the growth of *pylori in vitro* and, in *in vivo* studies, the *H pylori* infection cleared within seven days at very low concentrations. We also found that *H pylori* did not acquire resistance against these herbal extracts even after 10 consecutive passages.

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INTRODUCTION

H pylori has infected more than half the population of the world. Most people are unaware that they are infected because they remain asymptomatic throughout life and survive without any harmful infection-related clinical sequelae. However, some develop duodenal or gastric ulcers and a small proportion are diagnosed with MALT lymphoma or gastric malignancy[1]. Others suffer from non-specific dyspeptic symptoms with no obvious cause other than *H pylori* infection and its associated gastritis.

Global elimination of *H pylori* is a noble goal that would have a major impact on present and future world health. During the past decade effective treatment therapies have been developed to cure *H pylori* infection, following the work of Bazzoli *et al*[2] and Lamouliatte *et al*[3]. Current treatment is based on a combination of a proton pump inhibitor (PPI) with either amoxycillin or metronidazole. Large multicentric studies, especially MACH 1[4] and MACH 2[5], have confirmed the excellent results of pilot studies leading to eradication rates higher than 90%. Unfortunately, in subsequent years such good results have not been achieved, especially in South East Asia and Southern Europe, where resistance to antibiotics has become more prevalent. Complete eradication of *H pylori* is still in the initial stage. Resistance to amoxycillin, clarithromycin and metronidazole is widespread, and probably increasing as a result of the constant use of these drugs against *H pylori* infection. In our recent multicentric study, undertaken by the Indian *H pylori* study group, we assessed the Indian scenario of resistance[6]. The study showed higher resistance to antibiotics than in developed countries[7]. In this study, 100% resistance of *H pylori* towards metronidazole was reported in Hyderabad, which was much higher compared to other regions of India. If this trend continues the treatment with clarithromycin and metronidazole may become ineffective.

In view of the incomplete cure achieved with the

triple therapy described above and its possible side effects, alternative medicine cures are gaining much prominence and are found to be safe and effective eliminators of *H pylori* infection. Thus, in an endeavor to overcome increasing resistance, we continued to look for some selected herbal extracts that are capable of inhibiting the growth of *H pylori* with minimal or no side effects. In the present study, we selected two plants, namely *Sapindus mukorossi* and *Rheum emodi,* and investigated the inhibitory effect of these plant extracts against *H pylori in vitro* and *in vivo.*

S. mukorossi Gaerten (Sapindaceae), commonly known as Ritha or Aritha is found throughout India. The major constituents of its fruit are saponins (10%-11.5%), sugars (10%) and mucilage[8]. The fruit of the plant is reported to have expectorant, emetic, alexipharmic and abortificiant effects. It is also used for excessive salivation, epilepsy and chlorosis[9,10]. Saponins from this plant are known to be spermicidal *in vitro*[11]. This spermicidal property has been used in contraceptive cream[12]. The alcoholic extract of (*Sapindus trifoliatus Linn*) is reported to possess anti-implantation activity[11].

R. emodi (Polygonaceae), commonly known as Indian or Himalayan Rhubarb, is found in India. The major constituents of rhubarb rhizomes are anthraquinones. Rhubarb is used as a laxative and diuretic to treat kidney stones and it is used for gout and liver diseases characterized by jaundice. Externally, it is used to heal skin sores and scabs. Paradoxically, larger doses are used as a laxative, although small doses are used to treat dysenteric diarrhea[13]. The Chinese use rhubarb as an ulcer remedy, anti-helminthic and to treat cancer, upper intestinal bleeding (ulcers), fever, and headache. They consider it to be a bitter, cold dry herb used to "clear heat" from the liver, stomach and blood[14,15]. It is also used to treat toothache[16]. In Europe, rhubarb is a component of spring tonics or blood cleansing cures, including Swedish bitter[17]. Turkish or medicinal rhubarb is also one of the four major ingredients in the herbal cancer remedy.

We isolated extracts from both plants and carried out antimicrobial screening for various micro-organisms. Based on the above results, a study was designed using the obtained products of *S. mukorossi* and *R. emodi* to assess the antibacterial activity against *H pylori in vitro* and *in vivo.*

MATERIALS AND METHODS

Plant Materials

Authentic samples of *S. mukorossi* and *R. emodi* were obtained from an authorized supplier (M/s Munnalal Dawasas and Co. Hyderabad, Andhra Pradesh, India). The plants were identified and authenticated by experts in the Post Graduate and Research Department of Botany, Anwar-ul-loom College, Hyderabad, Andhra Pradesh, India.

Bacterial strains

Thirty *H pylori* strains along with two gram +ve (*Staphylococcus aureus, Bacillus subtilis*) and two gram -ve (*Escerichia coli*, *Proteus vulgaris*) pathogenic bacteria were used in the study. The *H pylori* strains were isolated from gastric biopsy specimens [15 from duodenal ulcer (DU), 8 from gastric ulcer (GU), 4 from non-ulcer dyspepsia (NUD), 3 from gastric carcinoma (GC)], after informed consents were obtained from patients who underwent upper gastrointestinal endoscopy at Deccan College of Medical Sciences, Hyderabad, India. The other four pathogens were obtained in pure culture form from the Department of Microbiology, Deccan College of Medical Sciences, Hyderabad, A. P, India.

E-test strips

E test strips were obtained from AB Biodisk, Solna, Sweden.

Animals

Male Wister rats (175-200 g) were acclimated to the housing facilities for 5 d before initiation of the study. Free access to standard pellet chow was allowed throughout the experimental protocol, with the exception of overnight fasting before induction of the ulcer. All protocols were approved by the Animal Care and Use Committee of the Deccan College of Medical Sciences and Research Centre, Hyderabad, India where the study was conducted.

Extraction, separation and purification of the compounds

For phytochemical analysis, approximately 100 g of fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* were collected and the materials were chopped, air dried at 35-40℃ and pulverized in an electric grinder. The powder obtained was successively extracted in petroleum ether (60-80℃), benzene, chloroform and ethanol.

The extracts were then powdered by using a rotary evaporator under reduced pressure. Fruit pericarp of *S. mukorossi* yielded 38g, 28g, 34g and 35g of powdered extracts with petroleum ether, benzene, chloroform and ethanol respectively. Rhizomes of *R. emodi* yielded 19 g, 17 g, 21 g and 22 g of powdered extracts. Extracts obtained by percolation using 70% of ethanol as a solvent at room temperature were processed according to process A of Farmacopeia dos Eastados Unidos do Brasil (1959) (AOAC 1990).

The extracts were evaporated at 40℃ under vacuum and the residue was freeze-dried. The dry extracts of the fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* were tested for the presence of saponins and anthraquinones. Each extract of the fruit pericarp of *S. mukorossi* (SM) and rhizomes of *R. emodi* (RE) were column chromatographed over a silica gel (200 mesh), eluted with $CHCl_3$-MeOH (70:30, 60:40, 50:50, 25:75) and compound fractions of (250 mL each) were collected and monitored by TLC. These column chromatographed compound fractions were further filtered to yield saponins and anthraquinones, which were separated by paper chromatography and preparative TLC to yield saponins [(SM-A (petroleum ether), SM-B (benzene), SM-C (chloroform) and SM-D (ethanol)] and anthraquinones [(RE-A (petroleum ether), RE-B (benzene), RE-C (chloroform) and RE-D (ethanol)] respectively.

All the filtrates obtained were dried by evaporation

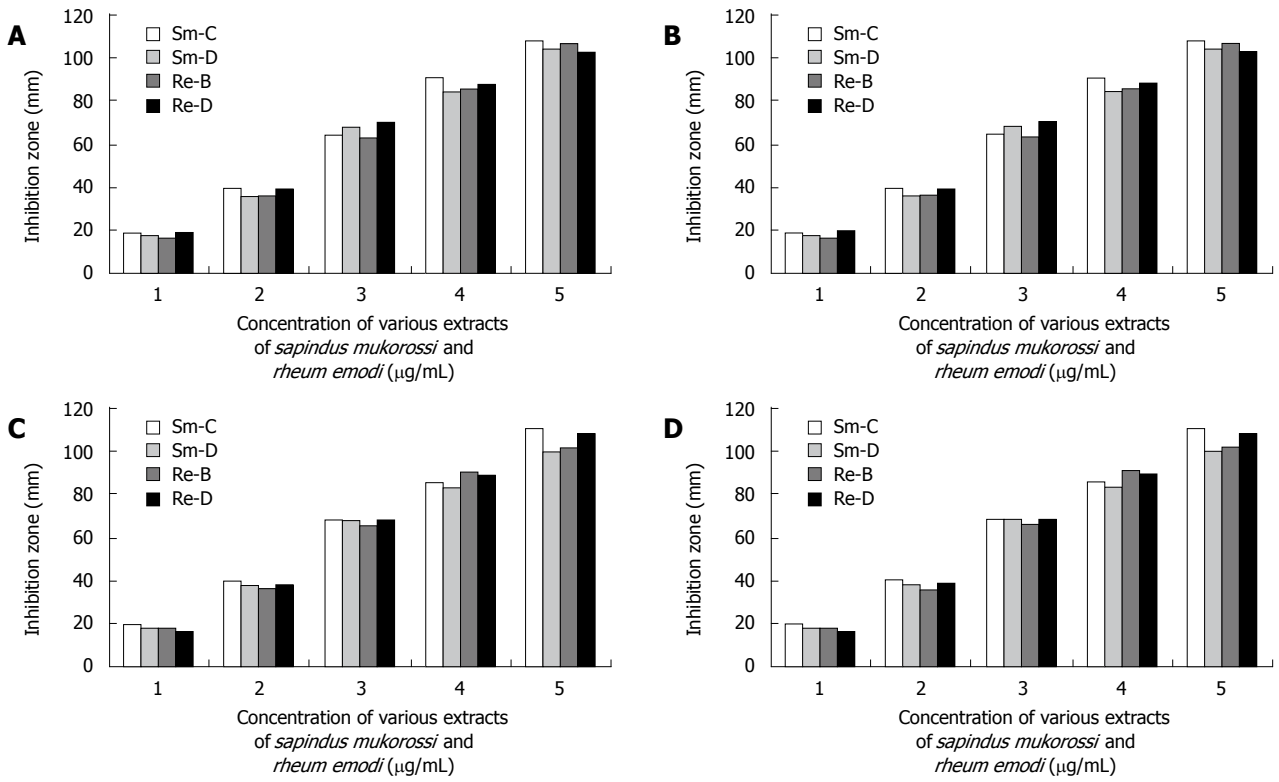

Figure 1 Antibacterial activity of various extracts *Sapindus mukorossi* and *Rheum emodi* against *S. Aureus* (**A**), *B. Subtilis* (**B**),*E.Coli* (**C**), *P vulgaris* (**D**).

(Rotometer, 40℃) and the dried extracts were individually once again dissolved in 10 mL ethanol (95%). Then subjected to a complete drying process and weighed according to the AOAC (1990) method[18]. The products obtained were tested initially for antimicrobial activity against different gram +ve organisms (*S.aureus, B.subtilis*) and gram -ve organisms (*E.coli*, *P.vulgaris*). Effective antibacterial activity was noted (Figure 1A-1D).

Isolation of H pylori

From every patient a total of three-biopsy specimens were collected, 1 in urea solution and two biopsies (1 from the antrum and the other from the corpus) were collected in Brucella broth supplemented with 2% fetal calf serum [FCS] (Gibco BRL, Germany) and 10% glycerol. The biopsy specimens were smeared directly onto Brucella agar (Difco Chemicals, Detroit, USA) supplemented with 7% (v/v) sheep blood, Vancomycin- 6 mg/mL, Amphoteracin B- 2mg/mL and Polymixin B- 2500 IU/mL (Sigma Chemicals Inc, Bangalore).

The agar plates were incubated at 37℃ for 3-5 d under microaerobic conditions (4% Oxygen, 5% Hydrogen, 5% Carbon-di-oxide, and 86% Nitrogen) in a candle jar dessicator. *H pylori* were identified based on the culture characteristics, such as small translucent colonies (2-4 mm) under a dissected microscope, gram -ve spiral organisms, positive for catalase, cytochrome oxidase and urease tests. Stocks of pure culture were preserved in Brucella broth (Difco Chemicals, Detroit, USA) supplemented with 10% FCS and 15% glycerol and stored in -80℃ and revived when required.

Antibiotic sensitivity tests

The antibiotic sensitivity patterns of all the 30 *H pylori* strains towards commonly used antibiotics such as metronidazole, amoxycillin, tetracycline and clarithromycin were evaluated by the *E*-test method. All the strains were seeded on Brucella agar supplemented with 7% sheep blood and grown for 48 h under microaerobic conditions. Bacterial growth was scraped from the plates and resuspended in sterile saline. The inoculum was prepared to contain 10^8 CFU/mL by adjusting the suspension to match the McFarland No-0.5 turbidity standard for MIC studies[19] and 2×10^8 CFU/mL of Mc Farland no- 1 standard for antibiotic sensitivity studies[20,21] (Table 1).

Determination of MIC of the herbal extracts on H pylori by disc diffusion method

Growth inhibition was performed by the filter paper disc diffusion method[22] on brucella agar plates supplemented with 7% sheep blood under micro-aerobic conditions at 37℃. To check the antimicrobial activity of the extracts and to read the antibiotic sensitivity of the isolates, inoculum was prepared as described above and the culture was spread over the plates with the help of a sterile cotton swab.

Various concentration disks (10-200 μg/mL) of the plant extracts were prepared and the latter were placed on brucella agar plates with 7% sheep blood earlier inoculated with 50 μL bacterial suspension in brucella broth with 5% FCS (10^8-10^9 CFU/mL). This was done to evaluate the minimum concentration of the extracts at which *H pylori* growth was inhibited effectively. The MIC was determined

Table 1 Antibiotic sensitivity patterns of *H pylori* strains as determined by *E*-test (n = 30)

Antibiotic	Sensitive n (%)	Resistance n (%)	Range (μg/mL)	MIC[1] (μg/mL)
Metronidazole	7 (23.4)	23 (76.6)	0.0125 - > 256	> 8
Clarithromycin	28 (93.3)	2 (6.7)	< 0.016 - > 256	> 2
Amoxicillin	28 (93.3)	2 (6.7)	< 0.016 - > 256	> 0.5
Tetracycline	28 (93.3)	2 (6.7)	< 0.016 - 2	> 4

[1]MIC- Minimum Inhibitory Concentration. (Values in parentheses are percentages).

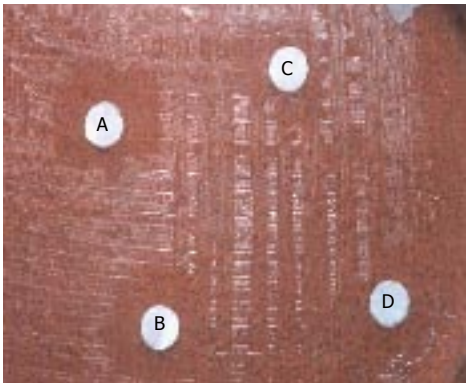

Figure 2 Photograph showing inhibitory activity of *S. mukorossi* and *R. emodi* on *H pylori*. 'A' and 'C' represents ethanolic and chloroform extracts of *S. mukorossi*. 'B' and 'D' represents ethanolic and benzene extracts of *R. emodi*.

by measuring the zone of inhibition at each concentration after incubation at 37℃ for 3-5 d (Figure 2).

Determination of antimicrobial activity of the herbal extracts in an in vivo model

Induction of ulcers: Ulcers were induced using a model modified from that described by Okabe and Pfeiffer[23]. Briefly, rats were given acetic acid (0.5 mL of 80% vol/vol) with a syringe instilled into the stomach and allowed to remain in contact with the stomach for 1 min, then it was aspirated and the area rinsed with sterile saline. The area exposed to acetic acid was 60 mm^2. The rats were killed by cervical dislocation and the stomach was removed and pinned out on a wax block. A paper grid with an area of 25 mm^2 was placed alongside the ulcer, which was then photographed (Figure 3). We then determined the ulcer area by planimetry, using × 5 enlargements of the photographs.

The area of ulceration in pixels was converted to square millimeters using the paper grid as a reference. Previous work using this ulcer model has revealed that ulcers induced by acetic acid are characterized by a thick layer of granulation tissue at the base and glandular disorganization at the ulcer margins. The ulcers involved the full thickness of the mucosa and penetrated into the muscularis mucosa. Perforation was not observed with this model. The rats were infected by giving the *H pylori* inoculum orally every alternate day for seven days.

Bacterial content of ulcers: To determine the bacterial content in gastric ulcers, animals were killed under aseptic

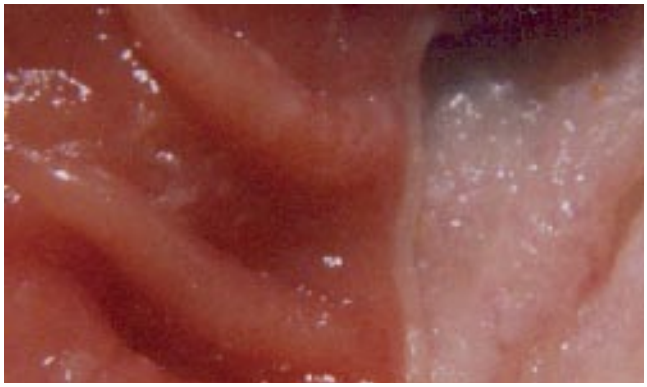

Figure 3 Control Ulcer.

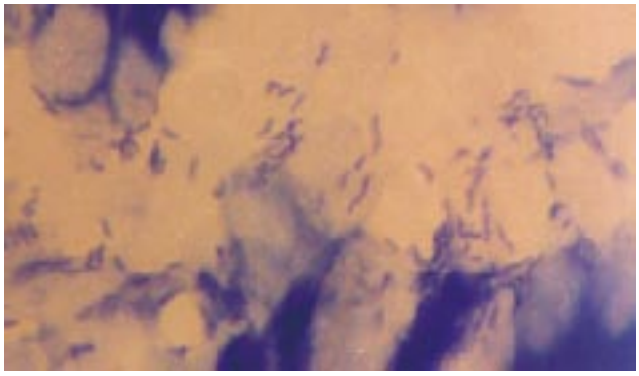

Figure 4 Typical photograph showing gastric biopsy stained with Giemsa Stain showing tufts of spiral shaped *H pylori*.

conditions. Tissue (150 mg) was washed in sterile PBS and transferred to sterile, pre-weighed containers (to determine sample weight), sterile PBS was added, and then the sample was homogenized. Serial dilutions were then plated on Mc-Conkey agar and tryptic soy agar plates and incubated for 18-24 h under aerobic conditions. The colony-forming units (CFU) were determined using a Leica colony counter. Results are expressed as CFU per gram of tissue. Tissue samples of the ulcers and healthy tissues were embedded in plastic using a commercially available kit (JB-4 embedding kit; Polysciences, Warrington, PA). Thin sections (1-1.5 μm) were stained with methylene blue-fuchsin (basic) and examined under a light microscope (Figures 4 and 5).

Further, the stomach tissue was collected for determination of ulcer size, histopathology and molecular confirmation of *H pylori* using Polymerase Chain Reaction (PCR) (Figure 6).

The rats were then divided into two treatment groups, Group Ⅰ of 6 rats who orally received methanolic extract of *S. mukorossi* (Figure 7) and Group Ⅱ of 6 rats who orally received ethanolic extract of *R. emodi* (Figure 8). Group Ⅲ of 6 rats received saline or dextrose. The total daily intake of dried *Smukorossi* and *Remodi* was 2.5 mg/d and 3.0 mg/d respectively for seven days.

Effect of treatment

The effect of treatment with the extracts of *S. mukorossi* and *R. emodi* was assessed. Ulcers were induced in the rats

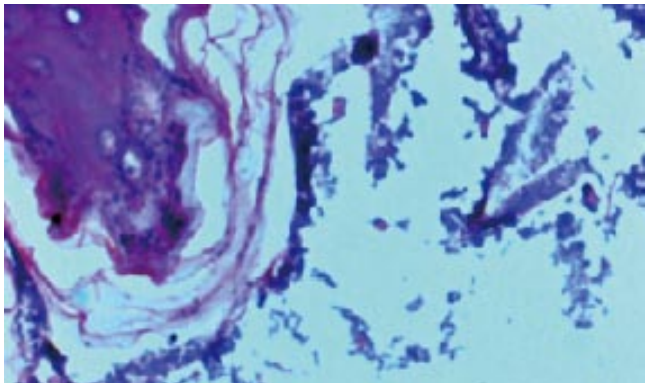

Figure 5 Photograph showing colonization of *H pylori* in the upper gastric and esophageal region.

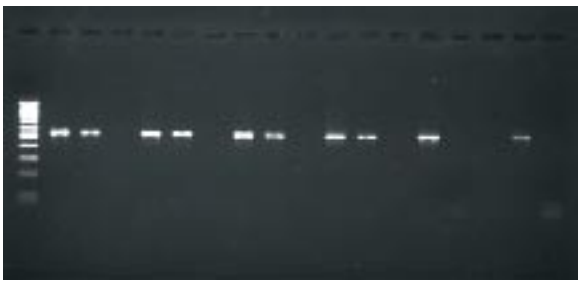

Figure 6 Typical gel image showing 16S rRNA amplification of *H pylori*. Lane 1 represents 100bp Molecular weight marker, Lane 2 & 3, 5 & 6, 8 & 9, 11 & 12, 14 & 15 represents 16S rRNA amplification of *H pylori* DNA isolated from the culture. Lane 17 represents positive control of band at 534 bp. (ATCC26695), Lane 18 represents negative control.

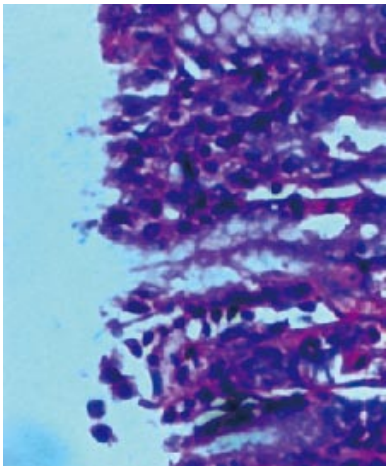

Figure 7 Photograph of gastric mucosal section showing surface erosion and mucous depletion, *H pylori* can not be seen. Photograph of Post-infection treatment with the extract of *Sapindus mukorossi*.

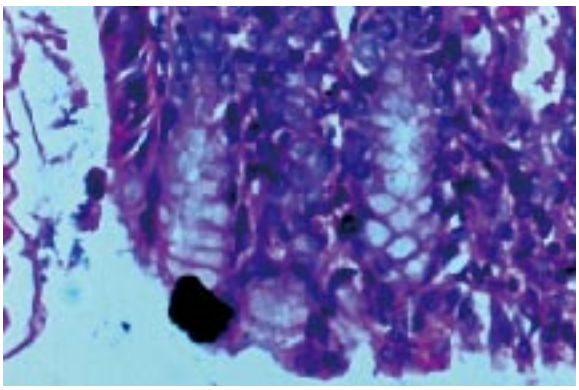

Figure 8 Photograph of gastric mucosal section showing small foci surface erosion mucin secreting cells in pits are seen, No *H pylori* detected. Photograph of Post-infection treatment with the extract of *Rheum emodi*.

as described above. Seven days after ulcer induction and *H pylori* infection, both groups of rats were orally given the prescribed dose of both the extracts of 2.5 mg/mL and 3.0 mg/mL for seven days. The effect of treatment was checked by sacrificing the rats by cervical dislocation, removing the stomach and photographing it for ulcer determination, and taking tissue samples for bacterial culturing.

Statistical analysis

The data were analyzed with a chi-square (χ^2) test.

RESULTS

In the *in vitro* study, we found that *S. mukorossi* and *R. emodi* extracts exert an inhibitory effect on *H pylori* (Figure 9). Ethanol and chloroform extracts of *S. mukorossi* and benzene and ethanol extracts of *R. emodi* with various concentrations ranging from 10-200 μg/mL were tested for their effect on *H pylori* growth by the disk diffusion method. In the *in vivo* study, we found that the extracts of *S. mukorossi and R. emodi* were able to clear *H pylori* infections in the rat models at concentrations of 2.5 mg/mL and 3.0 mg/mL post-infection (Figures 7 and 8).

MIC of the extracts as determined by Kirby Bauer's disc diffusion method

It was found that, in the 30 isolates tested, the strains showed approximately 20 mm and 19 mm zones of inhibition with very low (10 μg/mL) concentrations of the ethanol extract of *S. mukorossi* and the benzene extract of *R. emodi*. The chloroform extract of *S. mukorossi* and the ethanol extract of *R. emodi* showed approximately 18 mm and 15 mm zones of inhibition at 10 μg/mL concentration. Maximum inhibition zones of approximately 98 mm and 94 mm were attained with the extracts of *S. mukorossi,* and maximum inhibitions zones of 92 mm and 90 mm were attained with the extracts of *R. emodi* at a concentration of 200 μg/mL. The details of the MIC of both the extracts against *H pylori* are listed in (Figure 9). *In vivo,* we found that the extracts of *S. mukorossi and R. emodi* were able to clear *H pylori* infections at the concentrations of 2.5 mg/mL and 3.0 mg/mL post-infection (Figures 7 and 8).

DISCUSSION

Elimination of *H pylori* has been a major objective of treatment strategies worldwide. Unfortunately, none of them have been able to achieve 100% eradication rates[24]. The main reason underlying this failure could be the rapidly emerging resistance among many strains of *H pylori* towards various antibiotics. In addition to increased acquired resistance with the majority of

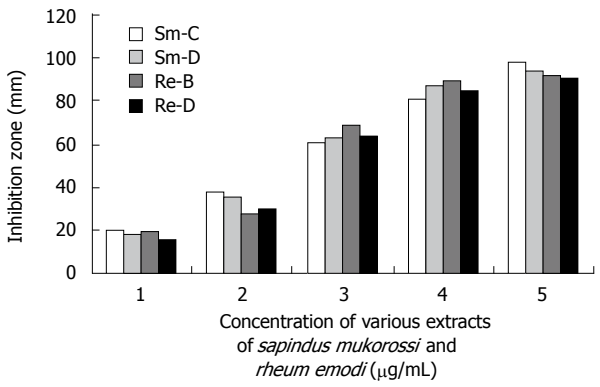

Figure 9 Antibacterial activity of various extracts *Sapindus mukorossi* and *Rheum emodi* against *H pylori*.

antibiotic therapies, which are considered highly efficient for long-term treatment, studies have shown non-compliance among patients.

Apparently related to the incomplete cures achieved with the triple therapies and their possible side effects, herbal systems of medicine have become increasingly popular in recent years. According to one estimate, nearly 70% of synthetic drugs are derived from medicinal herbs and medicinal herbs have figured high in pharmaceutical industry research because of their high therapeutic activity. Herbal therapies have shown better efficiency and efficacy in inhibiting *H pylori* at both *in vitro* and *in vivo* levels[25,26]. Based on the above, we decided to look for plant extracts capable of inhibiting the growth of *H pylori*. The reason for selecting *S. mukorossi* and *R. emodi* to assess antibiotic activity against *H pylori* is their chemical nature. The chemistry of these herbs is very complex and involves large amounts of physiologically active compounds. Not all the constituents present in the plant have therapeutic activity. Physiological activity is largely dependent on the complete structure, including the glycosidic moiety[27]. This activity is enhanced several-fold due to the presence of sugars in these compounds[28]. *S. mukorossi* and *R. emodi* are saponins and anthraquinones in nature and it is because of this that these plants exhibit antibacterial activity.

Among various extracts of both these plants, we found chloroform and ethanolic extracts of *S. mukorossi* exerted strong antibacterial effects on all the gram +ve and gram -ve organisms, including *H pylori,* while the petroleum ether and benzene extracts did not show any effect. Similarly, benzene and ethanolic extracts of *R. emodi* were found to exhibit a strong inhibitory effect on *H pylori* and other organisms while the petroleum ether and chloroform extracts showed no potential affect either on *H pylori* or other gram +ve and -ve microbes. Our study is the first to report the antibacterial effect of these plant extracts on *H pylori*. In our screening, we found that all 30 *H pylori* isolates were sensitive to the test compounds. As evident from the results, the compounds tested were found to possess antibacterial activities on *H pylori* and were not affected by the resistance of the organism to antibiotics.

The sensitivity of *H pylori* to commonly used antibiotics by (*E*-test) was carried out to assess susceptibility patterns and the sensitivity of the compounds was studied to evaluate the anti-*H pylori* activity of our extracted compounds (10-200 μg/mL by disk diffusion method). We assessed the minimum inhibitory dose of the compounds at which the survival of *H pylori* was in jeopardy. As evident from Figure 9, the extracts of *S. mukorossi* and *R. emodi* are highly effective even at a very minimal dose of 10 μg/disk.

Further, we found that none of the isolates acquired resistance even after 10 passages at sub-inhibitory concentrations. We found that *S. mukorossi* extracts and *R. emodi* extracts of 10 μg/mL concentration proved to be the MIC for almost all the isolates (except MS-24 and MS-28, which were found to be completely inhibited at 8 μg/mL). The reason behind this could be strain variation (data not shown).

S mukorossi and R. emodi extracts were found to inhibit all strains of *H pylori* (both sensitive and resistant) at a concentration of 10 μg/mL. This inhibitory concentration is comparable with the previously reported allicins 6-12 μg/mL, garlic oil 8-32 μg/mL[29] and ajoenes 10-25 μg/mL of oil macerated garlic extract. Our compounds proved to be more potent than thiosulfinate 40 μg/mL, vinyldithiins < 100 μg/mL, epigallocatechin gallate in Chinese lung chen tea 50-100 μg/mL and garlic powder 250 μg/mL[29]. Further more, the organism did not develop any resistance to the test compound even after 10 subsequent passages, grown at sub-inhibitory concentrations, whereas *H pylori* strains acquired resistance to amoxycillin and clarithromycin after 10 sequential passages[30,31]. In addition, we found that our extract had almost equivalent bacterial activities against both antibiotic susceptible and resistant *H pylori* strains. Animal studies suggest that an extract of *S. mukorossi and R. emodi* taken orally may help prevent stomach ulcers and it is of interest to note that *S. mukorossi and R. emodi* appear to be both anti-inflammatory and anti-ulcerogenic. However, future studies concerning these properties must be carried out to justify their anti-ulcerogenic properties.

In conclusion, the results presented here indicate that the extracts of *S. mukorossi and R. emodi*, which were screened for their anti-bacterial activity against *H pylori,* are active both *in vitro* and *in vivo*. In addition, the *in vivo* studies also proved to be highly efficient in terms of dosage, tolerability and curing active *H pylori* infection. Future studies will assess the mechanism by which these extracts effect the survival of *H pylori*.

ACKNOWLEDGMENTS

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BASIC RESEARCH

Elenoside increases intestinal motility

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Abstract

AIM: To study the effects of elenoside, an arylnaphthalene lignan from *Justicia hyssopifolia*, on gastrointestinal motility *in vivo* and *in vitro* in rats.

METHODS: Routine *in vivo* experimental assessments were catharsis index, water percentage of boluses, intestinal transit, and codeine antagonism. The groups included were vehicle control (propylene glycol-ethanol-plant oil-tween 80), elenoside (i.p. 25 and 50 mg/kg), cisapride (i.p. 10 mg/kg), and codeine phosphate (intragastric route, 50 mg/kg). *In vitro* approaches used isolated rat intestinal tissues (duodenum, jejunum, and ileum). The effects of elenoside at concentrations of 3.2 x 10^{-4}, 6.4 x 10^{-4} and 1.2 x 10^{-3} mol/L, and cisapride at 10^{-6} mol/L were investigated.

RESULTS: Elenoside *in vivo* produced an increase in the catharsis index and water percentage of boluses and in the percentage of distance traveled by a suspension of activated charcoal. Codeine phosphate antagonized the effect of 25 mg/kg of elenoside. *In vitro*, elenoside in duodenum, jejunum and ileum produced an initial decrease in the contraction force followed by an increase. Elenoside resulted in decreased intestinal frequency in duodenum, jejunum, and ileum. The *in vitro* and *in vivo* effects of elenoside were similar to those produced by cisapride.

CONCLUSION: Elenoside is a lignan with an action similar to that of purgative and prokinetics drugs. Elenoside, could be an alternative to cisapride in treatment of gastrointestinal diseases as well as a preventive therapy for the undesirable gastrointestinal effects produced by opioids used for mild to moderate pain.

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Key words: Elenoside; Gastrointestinal motility; Small intestine preparation

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INTRODUCTION

A great number of arylnaphthalene lignans have been isolated from different species of *Justicia*, many of them exhibiting diverse biological activities including antitumoral[1-4], antiviral[5-7], insecticidal[8], cardiotonic[9,10], antiulcerogenic[11] and anti-inflammatory properties[12,13], and an ability to inhibit lipid peroxidation[14]. Interesting activities also include platelet activating factor antagonism and central nervous system action[15]. Recently it has been observed that magnolol and honokinol, two neolignans, obtained from *Magnolia officinalis* Rehd. et Wils, inhibited the contractility of isolated gastric fundus strips from rats treated with Ach or 5-HT and of isolated ileum from guinea pigs treated with Ach or $CaCl_2$; in each case, the neolignans behaved as non-competitive muscarinic antagonists[16]. However, clinical or experimental studies on the effects of the gastrointestinal activity of lignans obtained from *Justicia* species have not, to our knowledge, been performed.

In a previous paper[17] we reported the isolation from *Justicia hyssopifolia L.* of an arylnaphthalene lignan and its aglycone, now called elenoside and elenin respectively, the former being a β-D-glucoside. Elenoside is a cytotoxic arylnapthalene lignan (NSC 644013-W/1) that displayed cytotoxic activity in the human tumor cell line panel of the US National Cancer Institute[18]. Preliminary screening results suggest that elenoside exerts a sedative, hypnotic barbiturate-type effect[18,19]. In addition, we have found that elenoside has digitalis-like activity similar to that of mammalian lignans[20]. Recently we have also observed that elenoside has central sedative effects and a possible application in anxiety conditions[21]. The current study was designed to investigate the effects of elenoside on gastrointestinal motility.

MATERIALS AND METHODS

Plant material and chemicals

Justicia hyssopifolia L. belongs to the family *Acanthaceae* and is an endemic species in the Canary Islands. Leaves of *J. hyssopifolia* were collected in April 2002 at Punta Cangrejo,

Adeje, Tenerife. A voucher specimen was deposited at the Herbarium of the Department of Botany, Faculty of Biology, University of La Laguna (TFC-28938). The elenoside was extracted and identified in *Centro de Productos Naturales y Agrobiologia, CSIC,* of Tenerife. It was suspended in a mixture of propylene glycol-ethanol-plant oil-Tween80 (40:10:50:2) and administered intraperitoneally in a total volume of 0.5 mL. The control group received vehicle only. An activated charcoal suspension indicator was prepared with 10 g activated charcoal (Panreac, Barcelona, Spain) and 2.5 g Arabic gum dissolved in 100 mL of distilled water. Cisapride was also used (Janssen-Cilag, S.A. Madrid, Spain).

Animals and treatment

Male Sprague Dawley rats weighing 200-250 g were used. The rats were housed under normal laboratory conditions at 22℃ on a standard light-dark schedule (12:12; lighted from 8 am-8 pm) and had free access to standard laboratory chow and water. Animal care complied with the Guide for the Care and Use of Laboratory Animals in accordance with the Guiding Principles in the Use of Animals in Pharmacology. The study protocol was approved by the Local Ethical Committee for animal experimentation at the University of La Laguna.

The standard dosage treatment protocol for each set of experiments described is as follows: rats assigned to randomized groups of 10 each receiving intraperitoneally (i.p.) a solution of propylene glycol-ethanol-plant oil-Tween 80 (40:10:50:2) (vehicle control group), 25 or 50 mg/kg of elenoside in vehicle, or 10 mg/kg cisapride.

Catharsis index

The established index of catharsis is the number of humid boluses produced in 24 h. Masri *et al* found that the majority of humid boluses are produced in the first hours following administration of the substance[22]. Thus, we used their method to study the effect of elenoside. Rats were fasted for 24 h prior to the experiment. Following this period, the rats received the standard dosage treatment protocol (n = 10 per dosage group). Rats were then individually placed in metabolism cages, and the boluses were collected for a 6 h period above blotting paper to facilitate counting.

Water percentage of boluses

Using the standard dosage protocol described above, we tested the water percentage of the boluses. Rats (n = 10 per dosage group) were individually placed in metabolism cages, and the boluses were collected in a methacrylate container for a period of 6 h and deposited in a watch glass. Afterward, the samples were weighed and placed in a heater at 100℃ for 3 h, followed by another weighing to establish the percentage of water in each.

Intestinal transit

This experimental method allows the evaluation of drug action on intestinal transit speed, by measuring the distance travelled by a suspension of activated charcoal when it has been administered intragastrically (po). Rats (n = 10 per dosage group) were fasted for 24 h prior to the experiment. At 15 min following treatment with the standard dosage protocol, the rats received 2 mL of a suspension of activated charcoal po. After 20 min, the rats were anesthetized and sacrificed; their intestines were removed from the pylorus through the ileocecal junction. The migration of activated charcoal from the pylorus to the most distal point of migration was expressed as distance (cm) migration using the stain. The percentage of distance travelled by the activated charcoal suspension established the intestinal transit. This percentage was expressed as % = 100 × l/L, in which *"l"* is the migration distance of the activated charcoal and *"L"* is the distance between the pylorus and the ileocecal junction.

Intestinal transit and codeine antagonism

Using the standard treatment protocol, we studied the effect of elenoside in the presence of codeine phosphate, an opioid with inhibitory activity on intestinal motility. Following administration of the standard dosage treatment, the rats immediately received 50 mg/kg of codeine phosphate po. The method used for the measurement of intestinal transit was the same as that described in the preceding section.

Isolated rat intestine

Rats were anesthetized and sacrificed. The abdomen was opened and a length of duodenum, jejunum and ileum was removed and placed in Tyrode´s solution. Pieces of smooth muscle (1-2 cm) were dissected free from surrounding tissues and mounted in an organ bath with Tyrode´s solution at 37℃, pH 7.4, through which a mixture of 50 mL/L CO_2 and 950 mL/L O_2 bubbled continuously[23]. The isotonic contractions of the preparation were recorded on a Grass Model 7D Polygraph through a Statham force displacement transducer. The load applied to the lever was 1 g. After stabilization, the effect of elenoside was studied for 5 min at the following concentrations: 3.2×10^{-4}, 6.4×10^{-4} and 1.2×10^{-3} mol/L, and cisapride at 10^{-6} mol/L. Ten samples each of duodenum, jejunum, and ileum were used for each concentration.

Statistical analysis

Statistical analysis was performed using the Prism program with two-way analysis of variance (ANOVA) (between group-factor: dose repeated measures factor: time), followed by Tukey´s Multiple Comparison *post-hoc* test. For nonparametric analysis, the Kruskal-Wallis test, was followed by the Mann-Whitney test for catharsis index, water of boluses, intestinal transit, and intestinal transit and codeine antagonism. A probability of 5% or less was considered to indicate a significant difference.

RESULTS

Catharsis index

Table 1 shows the results of cathartic activity for the vehicle control group and groups receiving elenoside at doses of 25 or 50 mg/kg or cisapride at dose of 10 mg/kg. Elenoside produced an increase in the catharsis index compared to the control group. The results of 50 mg/kg group and controls (P < 0.01) and of the 50 mg/kg and 25

Table 1 Effect of vehicle control, elenoside (E) and cisapride (C) on catharsis index in rats (n = 10) per treatment group

	Rat weight	Catharsis index
Control	239 ± 4	0.1
E-25 mg/kg	240 ± 3	1.5
E-50 mg/kg	238 ± 3	2.16[a]
C-10 mg/kg	236 ± 2	2.04[a]

Values are mean ± SE for 10 rats. [a]P < 0.05 *vs* control.

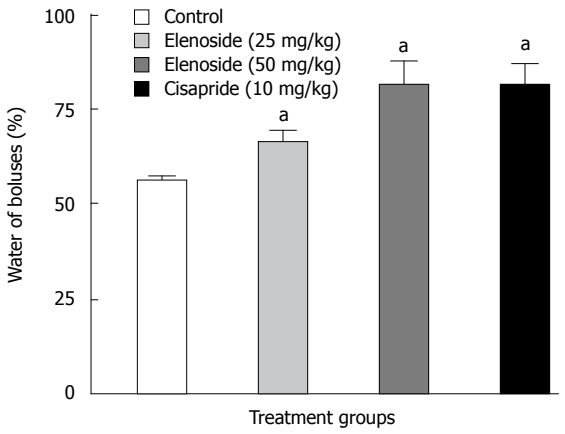

Figure 1 Percentage increase in water of boluses in vehicle control, elenoside, and cisapride. Values are mean ± SE for 10 rats/group. [a]P < 0.05 *vs* control.

mg/kg elenoside treatments, respectively, were significantly different from one another. Cisapride produced an increase in the catharsis index compared to the control group (P < 0.05), but it did not differ significantly from 50 mg/kg of elenoside in its effects.

Water percentage of boluses

Figure 1 shows the results of the water percentage of boluses analysis for control group, elenoside at doses of 25 and 50 mg/kg, and cisapride at 10 mg/kg. Both concentrations of elenoside produced an increase in water percentage *vs* vehicle control (*vs* 25 mg/kg elenoside, P < 0.01; *vs* 50 mg/kg elenoside, P < 0.01). Cisapride caused a significant increase in the water percentage versus the control group (P < 0.01), an effect similar to that produced by 50 mg/kg elenoside.

Intestinal transit

Figure 2 shows the results of the analysis of the effects of elenoside and cisapride on small intestinal transit (SIT) speed. Both concentrations of elenoside produced an increase in the percentage of distance the suspension of activated charcoal travelled *vs* the vehicle control (*vs* 25 mg/kg elenoside, P < 0.01; *vs* 50 mg/kg elenoside, P < 0.01). Cisapride produced a significant increase in SIT (control *vs* cisapride 10 mg/kg, P < 0.05). There was no difference between cisapride and the 50 mg/kg of elenoside.

Intestinal transit and codeine antagonism

Figure 3 shows the results of SIT analysis for elenoside with codeine phosphate. Codeine phosphate antagonized

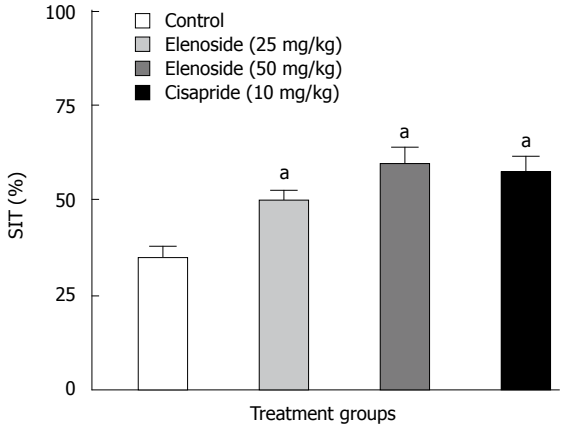

Figure 2 Small intestinal transit (SIT) in vehicle control and elenoside-treated and cisapride-treated animals. Values are mean ± SE for 10 rats/group. [a]P < 0.05 *vs* control.

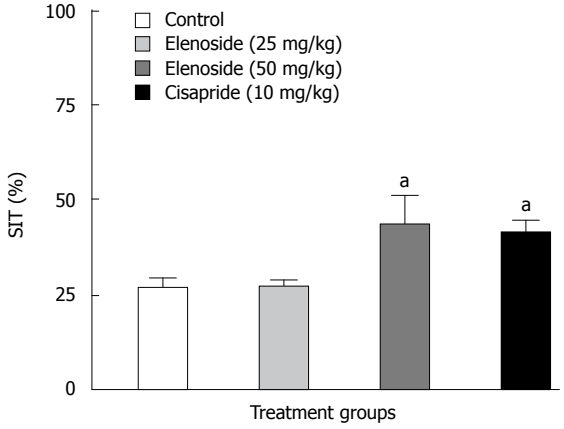

Figure 3 Codeine phosphate induced delay in small intestinal transit (SIT) in vehicle control, elenoside, and cisapride groups. Values are mean ± SE for 10 rats/group. [a]P < 0.05 *vs* control.

the effect of 25 mg/kg of elenoside. Moreover, codeine phosphate at 50 mg/kg produced an antagonistic effect on 50 mg/kg of elenoside and 10 mg/kg of cisapride, although this effect was not complete (control *vs* 50 mg/kg elenoside, P < 0.05; control *vs* 10 mg/kg cisapride, P < 0.05)

Isolated rat intestine

Figure 4 A-C show the results of the concentration force analysis; effects of elenoside and cisapride on duodenum, jejunum and ileum are depicted in Figures 4A, 4B and 4C, respectively. There was a clear dose-effect relationship over the three concentrations of elenoside.

At one minute, elenoside produced a decrease in the contraction force, which continued with increasing concentration from 3.2×10^{-4} mol/L (P < 0.01) to 6.4×10^{-4} mol/L (P < 0.01) to 1.2×10^{-3} mol/L (P < 0.01) during a five-minute assessment. Cisapride produced an effect similar to that of the 6.4×10^{-4} mol/L of elenoside. Elenoside at a concentration of 1.2×10^{-3} mol/L produced a significant increase in contraction force *vs* 3.2×10^{-4} mol/L at 2 (P < 0.01), 4 (P < 0.01) and 5 (P < 0.01) min respectively (Figure 4A).

Elenoside applied to jejunum produced a decrease in the contraction force at one minute, followed by an

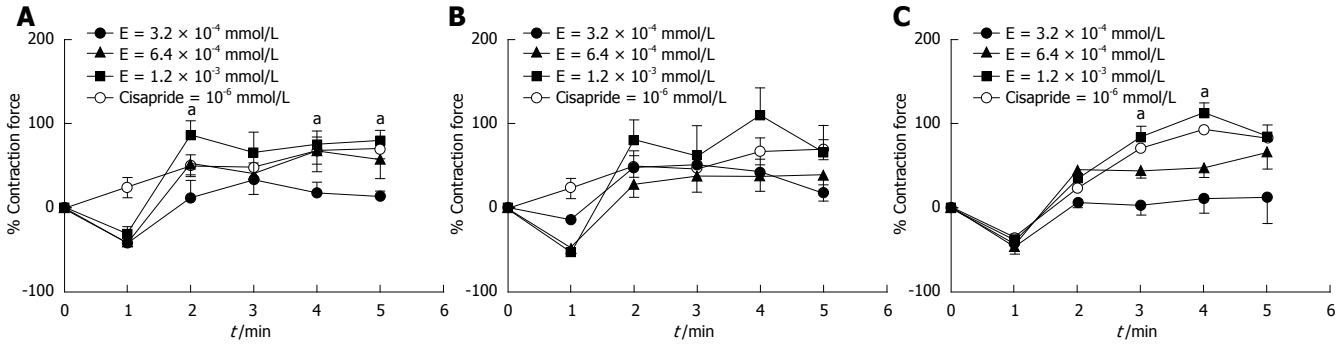

Figure 4 **A**: Time-course of the effects of elenoside and cisapride on contraction force in rat duodenum vs baseline. Values are mean ± SE for 10 duodenum samples during 6 min; $^aP < 0.05$. **B**: Time-course of the effects of elenoside and cisapride on contraction force in rat jejunum vs baseline. Values are mean ± SE for 10 jejunum samples during 6 min; $^aP < 0.05$. **C**: Time-course of the effects of elenoside and cisapride on contraction force in rat ileum vs baseline. Values are mean ± SE for 10 ileum samples during 6 min; $^aP < 0.05$.

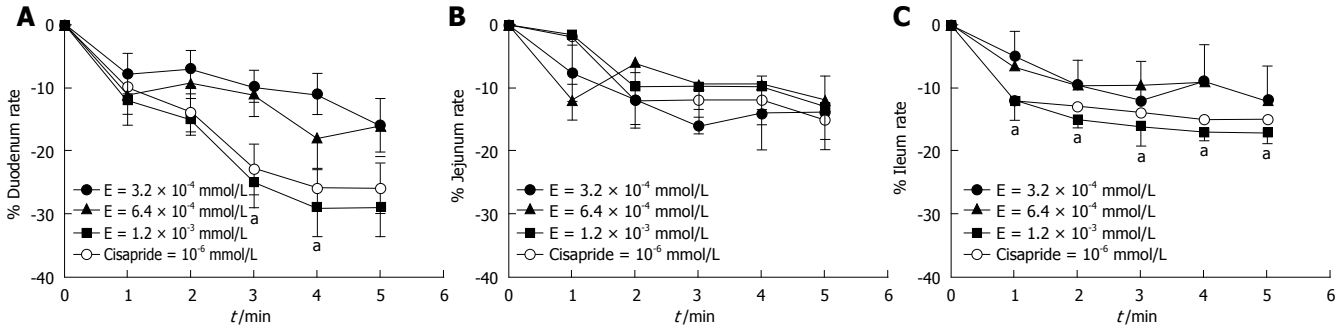

Figure 5 **A**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat duodenum vs baseline. Values are mean ± SE for 10 duodenum samples during 6 min; $^aP < 0.05$. **B**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat jejunum vs baseline. Values are mean ± SE for 10 jejunum samples during 6 min; $^aP < 0.05$. **C**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat ileum vs baseline. Values are mean ± SE for 10 ileum samples during 6 min; $^aP < 0.05$.

increase at concentrations of 3.2×10^{-4} mol/L ($P < 0.01$), 6.4×10^{-4} mol/L ($P < 0.05$), and 1.2×10^{-3} mol/L ($P < 0.01$) at 5 min. Cisapride produced an effect similar to that obtained with elenoside at a concentration of 3.2×10^{-4} mmol/L (Figure 4B).

When elenoside was applied to ileum, a decrease in the contraction force was produced a minute later. In the next minute, the contraction force increased and continued to do so up to five minutes with elenoside concentrations of 6.4×10^{-4} mol/L ($P < 0.01$) and 1.2×10^{-3} mol/L ($P < 0.01$). There was no effect on contraction force with the mid-range concentration of elenoside. Cisapride induced an effect similar to that of elenoside at a concentration of 1.2×10^{-3} mol/L. Elenoside at a concentration of 3.2×10^{-4} mol/L produced a significant increase in the contraction force *vs* 1.2×10^{-3} mol/L elenoside at 3 ($P < 0.05$) and 4 ($P < 0.05$) min, respectively (Figure 4C).

Figure 5A-5C depicts results from the time-course analysis of the effects of elenoside on intestinal frequency of duodenum, jejunum, and ileum respectively. Elenoside produced a decrease in the intestinal frequency of duodenum at concentrations of 6.4×10^{-4} mo/L ($P < 0.05$) and 1.2×10^{-3} mo/L ($P < 0.01$) during the time-course. Cisapride produced an effect similar to that of elenoside at a concentration of 1.2×10^{-3} mo/L. Elenoside at a concentration of 1.2×10^{-3} mo/L produced a significant decrease in the intestinal frequency *vs* 3.2×10^{-4} mo/L at 3 ($P < 0.01$) and 4 ($P < 0.01$) min, respectively (Figure 5A).

Elenoside decreased intestinal frequency in jejunum at all concentrations used during the time-course. No statistical differences among the concentrations of elenoside were observed (Figure 5B).

Elenoside produced a decrease in intestinal frequency in ileum at all concentrations, but only the concentration of 1.2×10^{-3} mo/L ($P < 0.01$) produced a statistically significant decrease (Figure 5C).

DISCUSSION

In this study, elenoside, has been shown to act as a stimulant of gastrointestinal motility. It produced an increase in the catharsis index and in the number of humid boluses at a dose of 50 mg/kg. The water volume of the boluses was also enhanced after treatment with elenoside at the two doses used. These effects of elenoside on the gastrointestinal tract suggest its actions similar to that of purgative drugs.

Another effect of elenoside was the increase in the distance traveled by the charcoal suspension at doses of 25 and 50 mg/kg. The distance traveled by the charcoal suspension has been used in the evaluation of drugs to determine their effects on gastrointestinal motility[24-26]. Elenoside elicited gastrointestinal activity similar to that effected by cisapride at a dose of 10 mg/kg. This effect exerted by elenoside suggested an action similar to that of cisapride, a commonly used prokinetic drug

that acts *via* a mechanism that facilitates cholinergic neurotransmission[27], and of other prokinetic drugs, such as metoclopramide, domperidone, erythromycin, and mosapride[28,29], ghrelin and GHRP-6[30], betanecol[31], and magnolol and honokiol[16]. Other lignans can exert similar effects: Podophyllotoxin and picropodophyllotoxin, lignans isolated from *Podophyllum* species, and produce diarrhea when administered to animals[32]. Herbal remedies with prokinetic activity similar to elenoside are peppermint oil[33], *Cocculus hirsutus*[34], *Zingiber officinale*[35], *Indigofera dendroides*[25], Shundao granules[36], and Banxia-houpo-tang HKT[37].

Codeine phosphate totally antagonized this prokinetic action at the lowest dose of elenoside and partially antagonized it at the higher elenoside dose. In addition, it antagonized the prokinetic action of cisapride. Codeine phosphate is an opium alkaloid with an activity similar to but weaker than that of morphine. It is given mainly orally in the treatment of mild to moderate pain[38]. However, although opioids are given to relieve pain, they exert undesirable gastrointestinal side effects such as nausea, vomiting, and a decreased gastrointestinal transit[39].

Elenoside could be an alternative together with cisapride in the treatment of the undesirable gastrointestinal effects produced by opioid drugs. Increased peristaltic and cathartic activity are parameters of irritant cathartics, according to Fling classification[40].

In isolated rat duodenum, jejunum, and ileum, elenoside produced a decrease in the contraction force followed by an increase. Elenoside applied to duodenum and ileum decreased intestinal frequency, but no significant changes in jejunum were found. Other lignans have been shown to affect contraction force and intestinal frequency[41-43]. Lignans obtained from *Podophylum* species caused a decrease in the initial rate and amplitude of contractions of isolated intestinal preparations followed by an increase in the force of contraction[32]. 2, 3-dibenzil-butirolactone, a lignan obtained from *Carthamus tinctorus* L. (Compositae), was responsible for the cathartic activity of this plant[44]. *Curcuma longa* relaxed spontaneous contractions in isolated rabbit jejunum[45]. On the other hand, some lignans used in the treatment of diarrhea, produced an inhibition in the normal intestine propellant movement[46]. Magnolol and honokiol obtained from *M. officinalis* induced a decrease in contractility of guinea pig ileum[16].

Traditional herbal remedies are used because they can improve the symptoms of gastrointestinal diseases, such as dyspepsia, nausea, vomiting, and abdominal distension. Elenoside obtained from *J. hyssopifolia* could be used to treat these symptoms of gastrointestinal diseases. It produced an increase in the catharsis index or number of humid boluses; an enhancement of the water volume of boluses; and an increase in the distance traveled by the charcoal suspension. In addition, codeine phosphate totally antagonized its prokinetic action; in isolated rat duodenum, jejunum, and ileum, elenoside elicited a decrease in contraction force followed by an increase; and elenoside caused a decrease in intestinal frequency in duodenum and ileum, but not in jejunum.

In conclusion, elenoside in the gastrointestinal tract exhibits activities similar to that of purgative and prokinetics drugs. Elenoside could be an alternative to cisapride in the treatment of gastrointestinal diseases as well as a preventive of the undesirable gastrointestinal effects produced by opioids used for mild to moderate pain.

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BASIC RESEARCH

Effect of Kaiyu Qingwei Jianji on the morphometry and residual strain distribution of small intestine in experimental diabetic rats

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Abstract

AIM: To investigate the effect of a Chinese medicine, Kaiyu Qingwei Jianji (KYQWJJ) used for diabetic treatment, on the morphometry and residual strain distribution of the small intestine in streptozotocin (STZ) -induced diabetic rats. Correlation analysis was also performed between the opening angle and residual strain with the blood glucose level.

METHODS: Forty-two male Wistar rats weighing 220-240 g were included in this study. Thirty-two STZ-induced diabetic rats were subdivided into four groups (n = 8 in each group), i.e. diabetic control group (DM); high dose of KYQWJJ (T1, 36g/kg per day); low dose of KYQWJJ (T2, 17 g/kg per day) and Gliclazide (T3, 50 mg/kg per day). Another ten rats were used as non-diabetic control (CON). The medicines were poured directly into stomach lumen by gastric lavage twice daily. The rats of CON and DM groups were only poured the physiological saline. Blood glucose and plasma insulin levels were measured. Experimental period was 35 d. At the end of experiment, three 5-cm long segments were harvested from the duodenum, jejunum and ileum. Three rings of 1-2 mm in length for no-load and zero-stress state tests were cut from the middle of different segments. The morphometric data, such as the circumferential length, the wall thickness and the opening angle were measured from the digitized images of intestinal segments in the no-load state and zero-stress state. The residual strain was computed from the morphometry data. Furthermore, the linear regression analysis was performed between blood glucose level with morphometric and biomechanical data in the different intestinal segments.

RESULTS: The blood glucose level of DM group was consistent 4-fold to 5-fold higher than those in CON group during the experiment (16.89 ± 1.11 *vs* 3.44 ± 0.15 mmol/L, P < 0.001). The blood glucose level in the T1 (16.89 ± 1.11 *vs* 11.08 ± 2.67 mmol/L, P < 0.01) and T3 groups (16.89 ± 1.11 *vs* 13.54 ± 1.73 mmol/L, P < 0.05), but not in T2 group (P > 0.05) was significantly lower than those in DM group. The plasma insulin levels of DM, T1, T2 and T3 groups were significantly lower than those in CON group (10.98 ± 1.02, 12.52 ± 1.42,13.54 ± 1.56,10.96 ± 0.96 vs 17.84 ± 2.34 pmol/L respectively, P < 0.05), but no significantly difference among the groups with exception of CON group. The wet weight/cm and total wall thickness of duodenum, jejunum and ileum in DM group were significantly higher than those in CON group (wet weight (g/cm): duodenum 0.209 ± 0.012 *vs* 0.166 ± 0.010, jejunum 0.149 ± 0.008 *vs* 0.121 ± 0.004, ileum 0.134 ± 0.013 *vs* 0.112 ± 0.007; Wall thickness (mm): duodenum 0.849 ± 0.027 *vs* 0.710 ± 0.026, jejunum 0.7259 ± 0.034 *vs* 0.627 ± 0.025, ileum 0.532 ± 0.023 *vs* 0.470 ± 0.010, all P < 0.05), T1 and T3 treatment could partly restore change of wall thickness, but T2 could not. The opening angle and absolute value of inner and outer residual stain were significantly smaller in duodenal segment (188 ± 11 degrees, -0.31 ± 0.02 and 0.35 ± 0.03 *vs* 259 ± 15 degrees, -0.40 ± 0.02 and 0.43 ± 0.05) and larger in jejunal (215 ± 20 degrees, -0.30 ± 0.03 and 0.36 ± 0.06 *vs* 172 ± 19 degrees, -0.25 ± 0.02 and 0.27 ± 0.02) and ileal segments (183 ± 20 degrees, -0.28 ± 0.01 and 0.34 ± 0.05 *vs* 153 ± 14 degrees, -0.23 ± 0.03 and 0.29 ± 0.04) in DM group than in CON group (P < 0.01). T1 and T3 treatment could partly restore this biomechanical alteration, but strong effect was found in T1 treatment (duodenum 243 ± 14 degrees, -0.36 ± 0.02 and 0.42 ± 0.06, jejunum 180 ± 15 degrees, -0.26 ± 0.03 and 0.30 ± 0.06 and ileum 163 ± 17 degrees, -0.23 ± 0.03 and 0.30 ± 0.05, compared with DM, P < 0.05). The linear association was found between the glucose level with most morphometric and biomechanical data.

CONCLUSION: KYQWJJ (high dose) treatment could partly restore the changes of blood glucose level and the remodeling of morphometry and residual strain of small intestine in diabetic rats. The linear regression analysis

demonstrated that the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level.

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Key words: Diabetes; Intestine; Kaiyu Qingwei Jianji; Residual strain; Rat

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INTRODUCTION

Dysfunction of upper gastrointestinal tract (GI) is common among diabetic patients[1]. As many as 75% of patients visiting diabetes clinics report significant GI Symptoms[2,3]. Common complaints include dysphagia, early satiety, reflux, abdominal pain, nausea, vomiting, and diarrhea[2,3]. As with other complications of diabetes, the duration of the disorder and poor glycemic control seem to be associated with more severe GI problems[2,3]. Histologically, many experiments have demonstrated prominent proliferation of GI wall layers, especially mucosa in the small intestine and esophagus of diabetes[4-9]. Many studies have shown that DM causes morphological changes and biomechanical remodeling in the esophagus[10,11], stomach[12] and small intestine[13,14]. Recently, Frøkjær et al in a human study found an increase in esophageal wall thickness and altered deformation to the distension with reduced longitudinal shortening and the radial stretch in insulin dependent diabetes mellitus (IDDM) patients (unpublished data). The biomechanical remodeling of the GI tract likely plays an important role in the GI disorder of diabetic patients[1]. Therefore, it is important to study the biomechanical properties of the small intestine in diabetic animals. Remodeling of the structural and biomechanical properties can be measured as changes in the residual strain and stress-strain distributions. Residual strain is defined as the strain in the no-load state (where external forces are zero) in reference to the zero-stress state.

To the best of our knowledge, only few studies related to the residual strain in diabetic small intestine have been studied[13,15]. Previously we have demonstrated that gliclazide treatment could partially restore the changes of biomechanical parameters of small intestine in the diabetic rats[15]. Regarding the Kaiyu Qingwei Jianji (KYQWJJ), several previous studies have demonstrated that it could decrease blood glucose level, decrease blood lipid level, increase sensitivity of insulin and decrease resistance of insulin[16-18]. However, no study has been performed about the effect of KYQWJJ on the morphometric and biomechanical properties of small intestine in diabetes. Therefore, the goal of the present study was to investigate the effect of KYQWJJ on the remodeling of the zero-stress state of the small intestine. The gliclazide is serving as positive control in the present study.

MATERIALS AND METHODS

Animal model and groups

Fifty male Wistar rats weighing 220-240 g were included in this study. Forty rats were made diabetic by a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, Sigma Company). This dose resulted in a fasting serum glucose level greater than 11.1 mmoL/L in 80% of rats after 4 d of injection; the remaining 20% were finally not used in this study. Another 10 rats of similar age and body weight from the same vendor were used as non-diabetic control (CON). Thirty-two diabetic rats were subdivided into four groups (n = 8 in each group), i.e. diabetes mellitus control group (DM); high dose of KYQWJJ (T1); low dose of KYQWJJ (T2) and Gliclazide (T3).

Drugs and administration methods

KYQWJJ was composed of Radix Bupleuri, Radix et Rhizoma Rhei, Rhizoma Pinelliae and Rhizoma Coptidis, and provided by China-Japan Friendship Hospital. Gliclazide was purchased from Hua Ju pharmaceutical factory (Tanjun, China). The medicines were injected directly into stomach lumen by gastric lavage twice daily and the dosage was 36 g/kg for T1, 17 g/kg for T2 and 50 mg/kg for T3 respectively. The rats of CON and DM groups were only poured the physiological saline.

Experimental procedures

Weight and blood glucose levels were measured 5 d, three weeks and five weeks after initiating the experiment. The plasma insulin level was measured when the experiment finished. Experimental period was 35 d.

After 35 d, the animals were anesthetized with sodium pentobarbital (50 mg/kg ip). Following laparatomy, the calcium antagonist, papaverine (60 mg/kg) was injected into the lower thoracic aorta through a cannula (22 G/25 mm) in order to abolish contractile activity in the GI tract. After obtaining smooth muscle relaxation, the whole small intestine was harvested. The most proximal segment was close to the pylorus whereas the most distal segment was close to the ileo-cacal valve. The length of duodenum and whole jejunum and ileum in vitro was measured. Within a short time, the residual contents in the lumen were gently cleared using saline and the wet weight of duodenum, jejunum and ileum was measured. Three 6 cm-long segments from duodenum, jejunum and ileum were harvested. The duodenum was taken from the descending part, 1 cm down the pylorus; the jejunum from 5 cm distal to the ligament of Treitz, and the ileum from 5 cm proximal to the ileo-cecal valve. Then the segments were placed immediately into cold aerated Krebs solution containing 6% dextran. Three rings, 1-2 mm in length, were cut from the middle part of each segment for no-load and zero-stress state tests. The rings were photographed in the no-load state using a video camera (SONY CCD Camera, Japan) and then cut radially on the anti-mesenteric side to obtain their zero-stress state. A 60-min-period

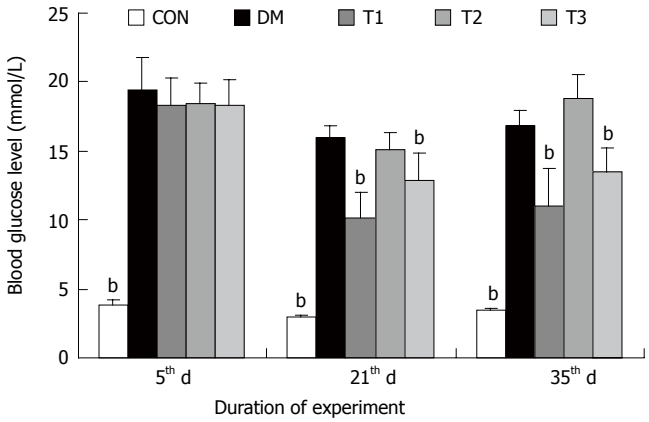

Figure 1 The blood glucose levels in different groups, compared with DM group ($^bP < 0.01$).

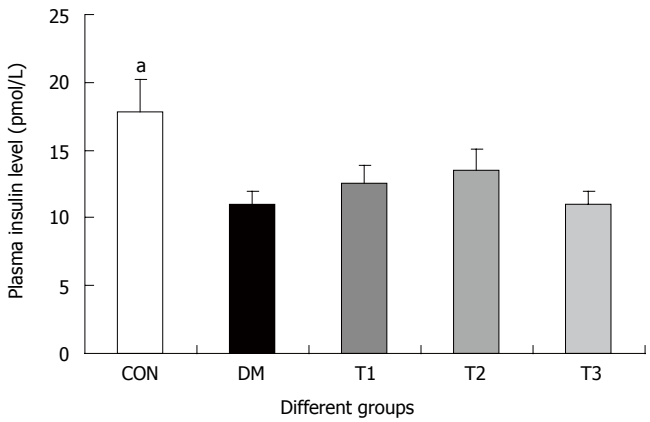

Figure 2 The serum insulin levels in different groups, compared with DM group ($^aP < 0.05$).

was allowed for equilibration and the specimens were photographed again. The selection of this time period was based on pilot experiments.

Data analysis

The morphometric data were obtained from the digitalized images of the photographs of the segments in the zero-stress and no-load states. Measurements were done using SigmaScan software (Jandel Scientific, Germany). The following data were measured from each specimen: the circumferential length (C), the wall thickness (h), and the opening angle at the zero-stress state. The subscripts i, o, n, and z refer to the inner (mucosal) surface, outer (serosal) surface, no-load state and zero-stress state condition. The data from triplet of rings were averaged before further analysis.

The measured data was used for computation of residual strains defined as:

Residual Green's strain at the mucosal surface:

$$e_i = \frac{(\frac{C_{i-n}}{C_{i-z}})^2 - 1}{2} \quad (1)$$

Residual Green's strain at the serosal surface:

$$e_o = \frac{(\frac{C_{o-n}}{C_{o-z}})^2 - 1}{2} \quad (2)$$

Thus, from the circumferential lengths at the no-load and zero-stress state, we can compute the circumferential residual strain, at the mucosal and serosal surfaces in the sense of Green strain. Green strain is used when large deformations are encountered as in this study. Negative strain implies that the tissue is in compression whereas positive strain implies extension.

The association between the glucose level with opening angle and residual strain in different groups with referenced to the DM group were performed using the linear regression analysis.

Statistical analysis

The data were representative of a normal distribution and accordingly the results were expressed as mean ± SD unless otherwise stated. Analysis of variance was used to detect difference among the different groups (Sigmastat 2.0TM). Linear regression analysis was used to demonstrate possible association between the blood glucose levels and opening angle and residual strain (Sigmastat 2.0TM). The results were regarded as significant when $P < 0.05$.

RESULTS

The blood glucose and plasma insulin level

The changes of the blood glucose level during the development of diabetes are shown in Figure 1. The blood glucose level of DM group was consistent 4-fold to 5-fold higher than those in CON group during the experiment ($F = 19.4$, $P < 0.001$). Compared with DM group, significant lower of blood glucose level was found in the T1 and T3 groups ($F = 16.1$, $P < 0.01$ and $F = 5.8$, $P < 0.05$), but not in T2 group ($F = 0.8$, $P > 0.05$). The plasma insulin level was shown in Figure 2. The plasma insulin levels of DM, T1, T2 and T3 groups were significantly lower than those in CON group ($P < 0.05$), but no significantly difference was found among the groups with exception of CON group ($P > 0.05$).

The intestinal weight and wall thickness (Figures 3 and 4)

The wet weight per centimeter and the wall thickness were increased during diabetes in the duodenum, jejunum and ileum ($P < 0.01$). After treatment with T1, T2 and T3, the wet weight per centimeter of different segments did not change significantly. However, T1 and T3 partly inhibited the wall thickness increase, in which the effect of T1 was stronger ($P < 0.05$).

Biomechanical data

The opening angles and residual strains of the different intestinal segments were shown in Figures 5 and 6. In brief, the opening angles and absolute value of residual strain were smaller in duodenum and larger in jejunum and ileum in the DM group when compared to those in CON group ($P < 0.01$). T1 and T3 treatment could partly restore this biomechanical alteration, in which the effect of T1 was stronger ($P < 0.05$). No significant effect in T2 treatment was found.

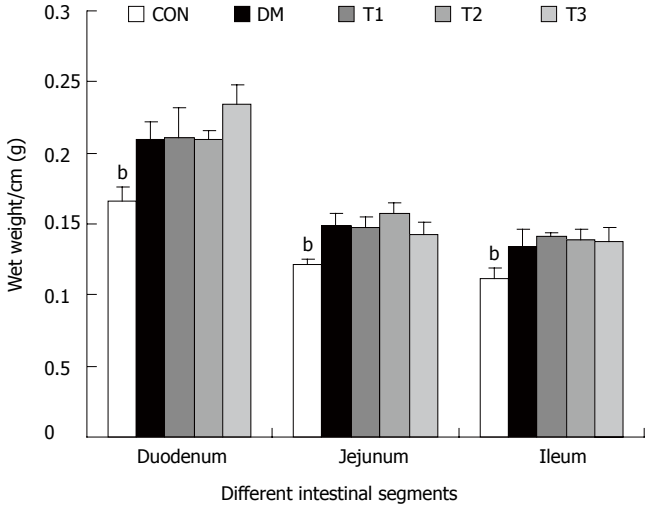

Figure 3 The intestinal wet weight of different segments per centimeter long in different groups, compared with DM group ($^bP < 0.01$).

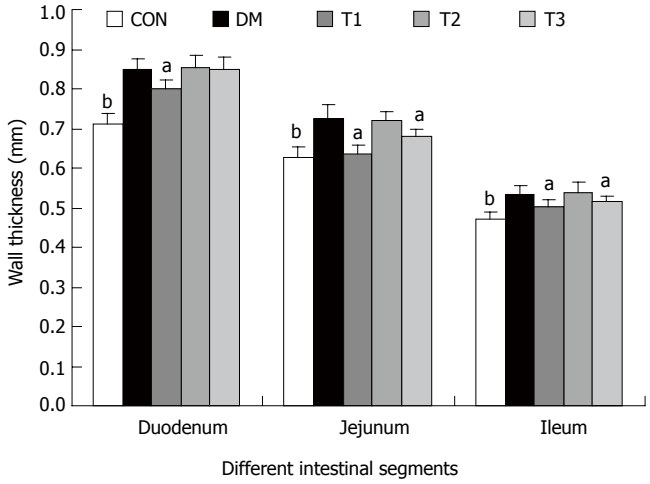

Figure 4 The intestinal wall thickness of different segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

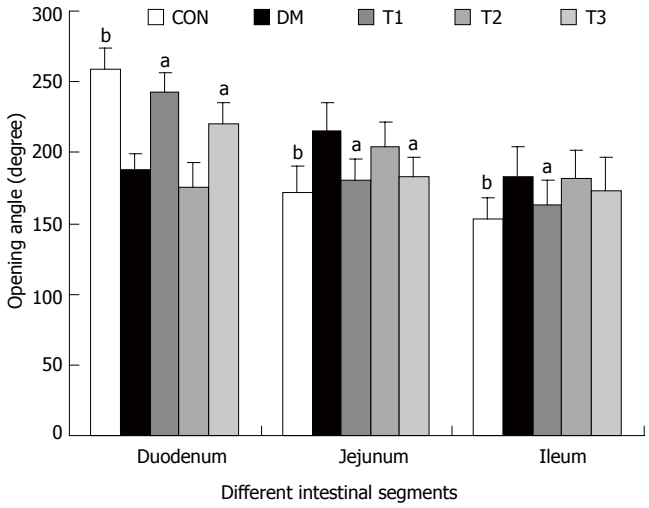

Figure 5 The opening angles of different intestinal segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

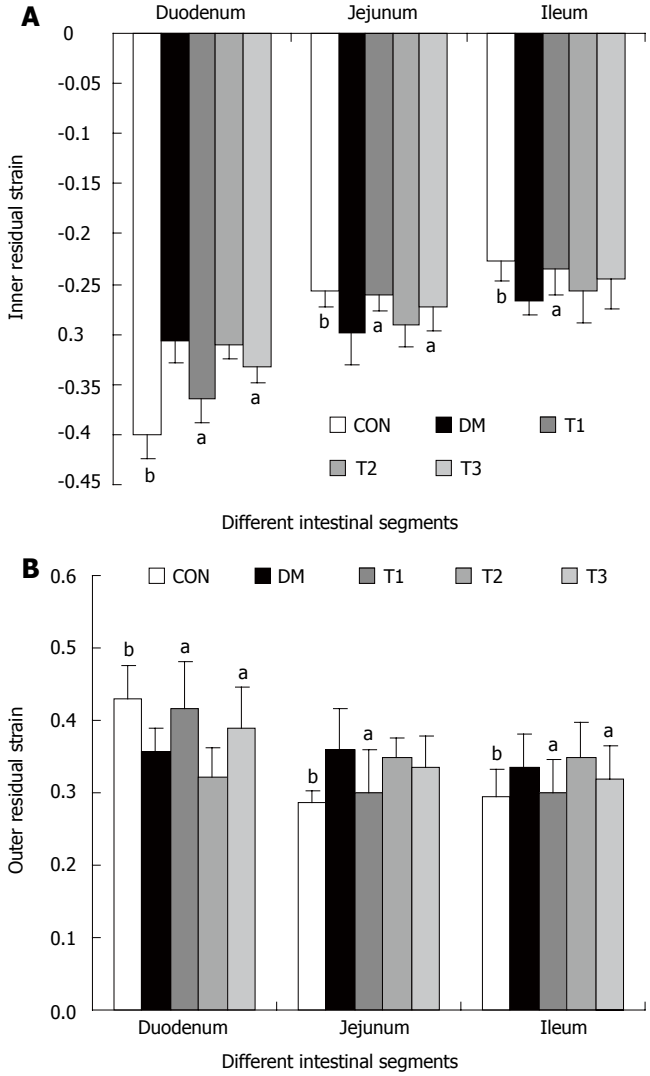

Figure 6 The inner (**A**) and outer (**B**) residual strain distribution of different intestinal segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

Association between blood glucose level with morphometric and biomechanical data

Linear regression analysis indicated that the linear association existed between blood glucose level with most morphometric and biomechanical data (Table 1). The examples of association between the glucose with opening angle and inner residual strain in duodenal segment were shown in the Figure 7. The high linear association existed between blood glucose level with opening angle ($r = 0.657$, $P < 0.001$, Figure 7A) and inner residual strain ($r = 0.653$, $P < 0.001$, Figure 7B).

DISCUSSION

Studies of the gut in the streptozotocin model of diabetes have almost entirely involved use of the rat. The form of diabetes mellitus induced by parenteral streptozotocin administration in rats is insulin-dependent (type I). Without treatment with insulin, STZ-induced diabetic rats become hyperglycaemic, polyphagic, polidipsic, poliuric and undernourished. In our studies, the blood glucose level was 4-5 times higher in diabetic rats than in non-

Table 1 Association between blood glucose level with morphometric and biomechanical data

	Duodenum			Jejunum			Ileum		
		F	*P*		*F*	*P*		*F*	*P*
Opening angle	y = -4.937x + 281.6 R = 0.656	28.7	< 0.001	y = 4.253x + 131.7 R = 0.591	20.4	< 0.001	y = 2.244x + 142.6 R = 0.33	4.7	0.038
Inner residual strain	y = 0.007x - 0.422 R = 0.653	28.2	< 0.001	y = -0.003x - 0.236 R = 0.447	9.5	0.004	y = 0.001x - 0.286 R = 0.13	0.7	0.418
No-load wall thickness	y = 0.007x + 0.707 R = 0.575	18.3	< 0.001	y = 0.004x + 0.617 R = 0.37	5.9	0.02	y = 0.003x + 0.485 R = 0.32	4.4	0.042

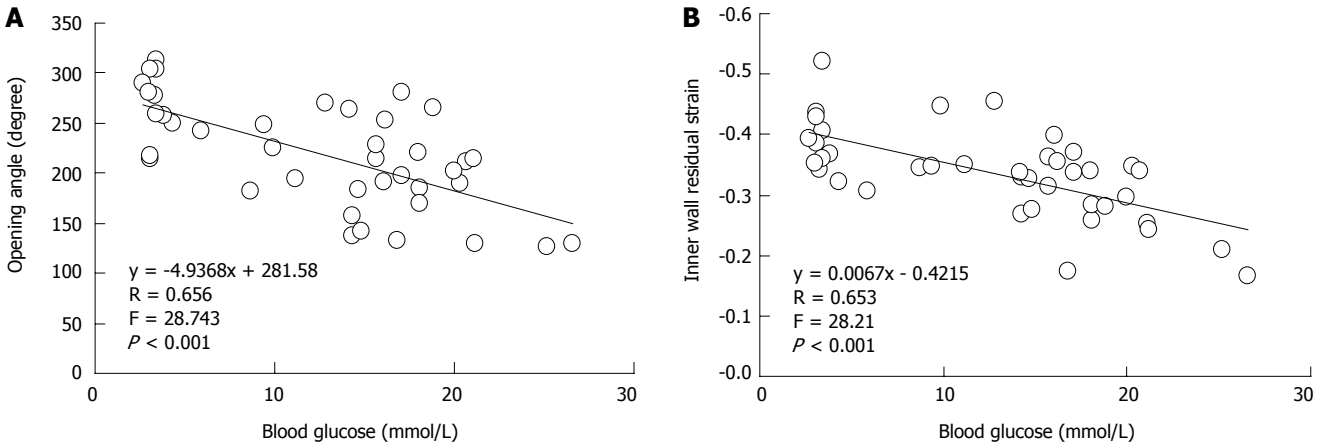

Figure 7 The examples of association between the glucose with opening angle (**A**) and inner residual strain (**B**) in duodenal segment.

diabetic rats. The major findings in our studies were that the opening angle and residual strain were smaller in the duodenum and larger in the jejunum and ileum in diabetic rats compared to normal rats. After gliclazide and high dose KYQWJJ treatment, these biomechanical parameters trend to back to normal state. Furthermore, we confirmed previous data that the intestinal weight per-unit length prominently increased in the diabetic rats[9,12-14], gliclazide and high dose KYQWJJ treatment can partly reverse the remodeling of intestine. High dose KYQWJJ seems better than gliclazide to reverse the morphometric and biomechanical remodeling of intestine in STZ -induced diabetic rats.

The small intestine is the longest section of the digestive tract and consists of three segments, namely the duodenum, jejunum and ileum, forming a passage from the pylorus to the large intestine. The main functions of the small intestine can be roughly divided into transport, absorption and secretion. The movement of luminal contents is facilitated by changes in the geometry of the wall and lumen due to the motility. It is well known that a bolus propelled in front of a peristaltic contraction distends the gut wall[19]. Therefore, it is important to know the passive mechanical wall properties devoid of any neurohumoral regulatory mechanisms. The biomechanical properties of the small intestine depend on its structure. Because significant structural remodeling of the intestine is occurred in the diabetes[3-9,12], therefore, the biomechanical properties of the small intestine may be also changed. To our understanding of knowledge, we consider the zero-stress state and residual strain as the most relevant quantitative aspect of remodeling because it is a measure of the non-uniformity of growth or resorption in different parts of the intestinal wall. Furthermore, the structural components of the intestinal wall must be measured at the zero-stress state because in this state the morphology and sizes of the cells and extracellular matrix are not distorted by stress and strain. In our present experiment, during the development of diabetes, the opening angles and absolute value of residual strains were decreased in the duodenum and increased in the jejunum and ileum when compared to those in the controls. High dose KYQWJJ and Gliclazide treatment partly restored this biomechanical alteration, the effect of the former is stronger. In the previous study we have discussed the possible mechanism of effect of Gliclazide on the diabetic intestinal remodeling[15]. Regarding the KYQWJJ, several previous studies have demonstrated that it could decrease blood glucose level, decrease blood lipid level, increase sensitivity of insulin and decrease resistance of insulin[16-18]. We confirmed in the present study that KYQWJJ could decrease blood glucose level in the diabetic rats. The linear regression analysis demonstrated that the linear association existed between blood glucose level with most morphometric and biomechanical data. Therefore, the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level. These seem to be the part mechanism for the effect of KYQWJJ on the diabetic intestinal remodeling. However, regarding more detail mechanisms of effect of KYQWJJ on the diabetic intestinal remodeling, further studies should be done.

The changes in the opening angle of intestine during the development of diabetes shown in this study suggest that the morphological and biomechanical remodeling of different layers are an important determinant of growth and remodeling of the zero-stress state. Fung's hypothesis of non-uniform remodeling suggested that if the inner wall grows more than the outer wall, the opening angle will increase; whereas if the outer wall grows more than the inner wall, the opening angle will decrease[20]. In this experiment, since the changes in jejunum and ileum primarily are in the mucosa layers, the inner wall grows more than the outer wall and hence the opening angle increased. Correspondingly, the outer residual strain became more tensile whereas the inner residual strain became more compressive in these two segments. But in the duodenal segment, the opening angle and absolute value of residual strain were decreased during the diabetes. We have demonstrated that the all layers of duodenum increased after experimental diabetes and the wall became stiffer[13], at this condition the biomechanical properties of layers may determine the remodeling of the zero-stress state. Because the wall becomes stiffer, the opening angle may become smaller.

The GI wall structure or deformation changes in the diabetes may alter the relative positions of the mechano-sensitive afferents (zero setting of the mechanosensitive afferents)[21]. The biomechanical remo-deling in the diabetes such as alterations of residual strain and stress distribution[15] and increase the wall stiffness[13,14] will alter the tension and stress distribution of the mechanosensitive afferents[22,23]. As results, the perception and motility of the GI tract will change as well. Hence, the morphological changes and biomechanical remodeling of GI tract in the diabetes is likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory function. After KYQWJJ treatment, the structural remodeling of different segments was partly improved, accordingly the biomechanical remodeling of these segments was partly reversed. Therefore, the KYQWJJ should have the effect to improve motor and sensory dysfunction of diabetic small intestine.

In conclusion, Chinese medicine of KYQWJJ treatment could partly restore the changes of blood glucose and insulin levels and remodeling of morphometry and residual strain of small intestine in STZ-induced diabetic rats. The linear regression analysis demonstrated that the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level. Therefore, it is possible to develop some Chinese herbs, such as KYQWJJ, to improve the intestinal dysfunction and further may be applicable in the clinics.

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CLINICAL RESEARCH

Noninvasive evaluation of hepatic fibrosis in children with infant hepatitis syndrome

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Abstract

AIM: To elucidate the impact of hemodynamic parameters on ultrasonography and serum fibrosis markers for the assessment of liver fibrosis in the children with infant hepatitis syndrome (IHS).

METHODS: Forty-one children with IHS and 46 healthy infants were examined by ultrasonography, and several hemodynamic indices such as peak systolic velocity (PSV) and resistant index (RI) of proper hepatic artery (PHA) were measured. Serum fibrosis markers including hyaluronic acid (HA), pre-collagen type-Ⅲ (PC-Ⅲ), collagen type Ⅳ (C-Ⅳ), and laminin (LN) were assayed by radioimmunoassays. In children with IHS, liver tissues were obtained either by ultrasound-guided liver biopsy (n = 35) or in the course of operation (n = 6). The stages of hepatic fibrosis were scored as mild (S_1 and S_2), moderate (S_3), or severe (S_4) according to liver histological diagnosis. Multiple groups comparative and Spearman correlative analyses were carried out.

RESULTS: Histopathologically, 39 children (95.1%) were found to have hepatic fibrosis, 12 of them stage S1 or S2, 12 stage S3, and 15 stage S4. PSV, RI of the PHA, and serum HA showed a consecutive increase from mild to severe hepatic fibrosis and a close positive correlation with hepatic fibrosis in IHS group (r = 0.717, 0.745 and 0.712, respectively, P = 0.001). The Doppler waveform of HV was also positively correlated with the degree of hepatic fibrosis in IHS group (r = 0.783, P < 0.001).

CONCLUSION: Combination of ultrasonic studies on the hepatic hemodynamics with the evaluation of serum HA may provide an indicator for hepatic fibrosis in patients with IHS. This may be a useful noninvasive method for the diagnosis and evaluation of the prognosis of IHS.

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Key words: Infant hepatitis syndrome; Hepatic fibrosis; Doppler ultrasonography; Hemodynamics; Hyaluronic acid

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INTRODUCTION

Infant hepatitis syndrome (IHS)[1] is defined as elevation of serum levels of conjugated bilirubin in the first 12 mo of life, may be due to infectious, genetic, metabolic, or undefined abnormalities, giving rise either to mechanical obstruction of bile flow or to functional impairment of hepatic excretory function and bile secretion. Main symptoms are prolonged jaundice, hepatomegaly or hepatosplenomegaly, and impairment of the liver focused. Although IHS has multiple etiologies, hepatic fibrosis is one of the main pathologic features. Histology is the gold standard for assessing the degree of hepatic fibrosis and for estimating prognosis[2-6]; however, there are several limitations in the usage of liver biopsy in general clinical practice due to the invasive nature of the examination, especially in infant age group. So there is a need for a non-invasive examination, which is clinically useful and accurately reproducible.

In adults, two-dimensional ultrasonography (2DUS)[2-5,7,8], Doppler blood stream[7-10,11-14,26], and determination of serum fibrosis markers[15-19,21-23,25,26] have been performed widely for the noninvasive diagnosis of hepatic fibrosis and therapeutic surveillance[20,24], and they were also useful in the assessment of cirrhosis in the pediatric age group[27-30]. But until recently, only a few studies[31,32] focussed on infants, especially on infants with IHS.

This prospective study investigated the association

between the hepatic fibrosis in infants with IHS and hepatic hemodynamic parameters and serum fibrosis markers, correlated the results with the histological findings, and evaluated the clinical relevance of the noninvasive methods.

MATERIALS AND METHODS

Patients

Forty-one infants (27 boys and 14 girls; mean age 5.2 ± 3.6 mo) who were admitted in the Department of pediatrics in our hospital from January 2000 to December 2003 were included. The patients were diagnosed as infant hepatitis syndrome (IHS) according to the diagnosis criteria of the national infant virus hepatitis prevention and cure symposium[1]. Infants presented with jaundice, hepatomegaly or hepatosplenomegaly, and impairment of the liver function. They didn't have any contraindication for liver biopsy. Those with clinically overt heart failure were excluded. Etiologies included viral infection, metabolic disorders, familial cholestasis, or idiopathic neonatal hepatitis. Etiologies were categorized on the basis of their clinic and pathologic diagnosis (Table 1). Forty-six infants (34 boys and 12 girls; mean age 4.6 ± 3.6 mo) who presented with normal liver size and function, negative HBV status, and without any history of clinical signs of liver disease served as healthy controls.

Table 1 Etiology of IHS

Etiology	*n*	%
Cytomegalovirus hepatitis (CMVH)	18	43.90
CMVH + Glucose-6-phosphate dehydrogenase	3	7.32
CMVH + congenital choledochocele	2	4.88
CMVH + intrahepatic biliary atresia	1	2.44
CMVH + hemangioma	1	2.44
Glycogen storage disease	5	12.20
Infant Hepatitis Syndrome (undefined)	8	19.51
Congenital deformity of biliary tract (Extrahepatic biliary atresia)	1	2.44
Congenital choledochocele	1	2.44
Drug-induced hepatitis	1	2.44
Total	41	100.00

Ultrasonography

Ultrasonography was performed on all children (healthy and those with IHS) by two physicians, who were unaware of the results of the liver biopsy and any laboratory finding at the time of the examinations. All children were held in the arms of their mothers and studied after a fasting period of 2-4 h. Some of them were examined during normal sleep, some were helped by use of a sugar nipple, if necessary, and some were given a sedative drug before the examination. GE-LOGIQ9 ultrasound scanner (GE, Milwaukee, and American) equipped with an 8-MHz electric curved scanning transducer or HP 8500 ultrasound scanner (HP, Andover, American) with a 7.5-MHz electric curved scanning transducer were used for the examination. Evaluation of liver parenchyma includes the assessment of the size, configuration, homogeneity, contour, and the vascular structure. Spleen and visibility of the gallbladder were also evaluated and compared with normal data. Color Flow Mapping(CFM) was used to determine the direction of flow of the hepatic blood vessels, and Pulse Wave Doppler (PW) was used to measure the Doppler waveforms of the PHA, portal vein (PV), and hepatic vein (HV). During the PW examination, the angle between the Doppler beam and longitudinal axis of the blood vessels was kept at 30°, and Doppler sample volume was smaller than the inner diameter of vessels. In each individual, the Doppler waveforms of the PHA, PV, and HV were measured. Doppler waveform analysis was calculated from 3-5 consecutive waveforms on the Doppler tracing in each subject. Flow in the middle hepatic vein (MHV) was evaluated at subcostal or intercostals, a distance of at least 2 cm from the inferior vena cava (IVC). This distance was important to rule out the possibility that the MHV flow pattern was influenced more by the flow pattern of the IVC than by the surrounding liver parenchyma.The waveforms of the MHV were classified into 3 types[11,14]: Type 1 (HV0), regular triphasic waveforms (with a short phase of reversed flow) which were considered to be normal flow, whereas type 2 (HV1), biphasic waveforms (without reversed flow) and type 3 (HV2), flat waveforms (with continuous blood flow) were considered to be abnormal.

Laboratory tests

Serum total bilirubin (T-Bil), indirect bilirubin (I-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (AKP), total protein, albumin, globulin, the ratio of albumin to globulin (A/G), and prothrombin time were measured by routine methods. The etiologic differential diagnosis was made according to the presence of serological markers for viral hepatitis type B, toxoplasma, rubella, cytomegalovirus, herpes simplex, and syphilis (TORCHS) (positive IgM or more than a fourfold rise in IgG titer), and urine cytomegalovirus (CMV) culture. The serum specimens for fibrosis indices, including HA, PC-Ⅲ, C-Ⅳ and LN, were determined by commercially available radioimmunoassays (RIA kits; Shanghai Navy Medical Institution, Shanghai, China). Samples of peripheral venous blood of the subjects were collected, immediately separated, and preserved at -40℃ until they could be assayed.

Liver biopsy and histological analysis

Liver biopsy was performed in 41 children with IHS, 35 cases underwent US-guidance liver biopsies and 6 cases underwent operation biopsies. Intercostals or subcostal ultrasound-guided liver biopsies were performed during the scanning with an 18G biopsy-cut (CR Bard, Inc., Covington, GA, USA) needle driven by the spring-loaded automatic biopsy gun (CR Bard, Inc., MAGNER, USA). The Bard biopsy gun consists of a hand-held device that triggers rapid firing of an 18G cutting needle. When the gun is fired, an inner trocar with its 1.9 cm sample notch thrusts forward, followed by its outer cannula which shears a core of tissue with minimum crushing of the specimen. For a satisfactory sampling, an average of two specimens

was obtained in each ultrasound-guided core-needle biopsy case. All biopsy specimens were 0.8-1.9 cm in length and were fixed in 10% buffered formalin. Sections were stained with hematoxylin-eosin; Masson's trichrome stain was used specially for staining of fibrous tissue components.

The stage of hepatic fibrosis was scored according to the diagnostic criteria of viral hepatitis in Xian, China, 2000[33]. S0 corresponds to the absence of fibrosis, S1 to fibrosis within portal tract areas, S2 to fibrosis around the portal tract areas with formation of fibrosis segregation while maintain lobular structure, S3 to the formation of fibrosis segregation and disorder of lobular structure without hepatic cirrhosis, and S4 to early or late stage cirrhosis. Then these patients were divided into three groups: mild (S1-S2 grade), moderate (S3 grade) and severe (S4 grade).

Statistical analysis

The one-way ANOVA was used to compare numerical variables between multiple groups. The chi-square test and Fisher exact test were used to compare categorical variables. Association between the ranked values was evaluated using Spearman rank correlation. $P < 0.05$ was considered statistically significant. All statistical analyses were done with SPSS for windows, version 10.0 (SPSS Inc. American).

RESULTS

In the IHS group, there was an increase in T-Bil which reached up to 810 μmol/L. D-Bil increased in almost all except for 5 children (I-Bil was higher than D-Bil). There was an increase in serum ALT activity in all cases, the highest value was 598 U/L, mean 126.9 U/L. Serum AST activity increased in all except for 3 cases, and the highest value was 2816 U/L, mean 282.4 U/L. The AST/ALT ratio was 3.5 (normal 1.15), the ratio of albumin to globulin (A/G) decreased in 29 cases, and inversed in 5 cases. Pathology showed liver fibrosis in 39 cases (95.1%), 12 cases with S1 or S2, 12 cases with S3, and 15 cases with S4. Further findings were ballooning change (n = 35, 85.4%), hepatocyte necrosis (n = 30, 73.2%), cholestasis in liver cells (n = 26, 63.4%), cholestasis in the hepatic lobules (n = 21, 51.2%), cholestasis in portal area (n = 7, 17.1%), and cytomorphosis of giant corpuscle (n = 20, 48.8%).

2DUS findings

In the 39 cases of liver fibrosis, 15 cases with S4 stage liver fibrosis (confirmed by histological diagnosis) showed that the liver parenchyma was more echogenic and coarse than normal, among which 6 severe cases with obvious echogenic changes showed nodularity of the liver edge and ascites by two-dimensional ultrasonography (2DUS). The other 24 cases with S1-S3 stage liver fibrosis (confirmed by histological diagnosis) were all hepatomegaly but the liver parenchyma was normal, except for 5 cases of Glycogen Storage Disease (1 case in S0 stage, 2 in S2 stage and 2 in S3 stage) presented with significant hepatomegaly, increased echogenicity, and slightly increased attenuation. Additionally, we found that proper hepatic arteries (PHA) in the IHS group were enlarged and even were easy to display on the 2DUS.

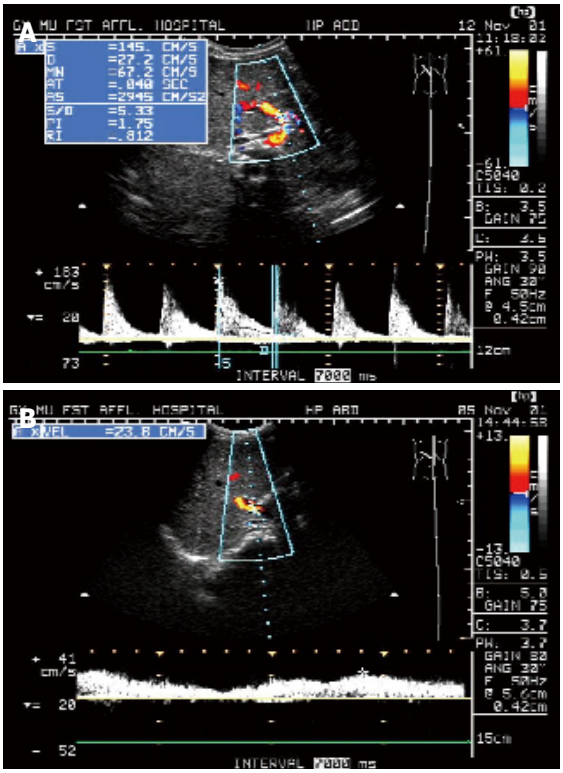

Figure 1 **A**: Duplex Doppler Sonogram of case with IHS showed the PHA was easy to see as well as PSV and RI of the PHA increased significantly; **B**: Duplex Doppler Sonogram of the normal subject showed normal waveform of PV, and PHA cannot display clearly.

CDUS findings

We found that PHA in the IHS group can be displayed easily on the B-Mode, as well as on the Color Flow Mappings and even better (Figure 1A). PHA can be seen in 100% of patients in IHS group but only in 58.7% of the control subjects, there was significant difference between IHS group and control group ($P < 0.001$). And then, PW parameters showed an increase in PSV, EDV, and RI (Figure 1A). PSV and RI in IHS group were significantly higher than in the control group ($P < 0.05$), as well as, with development of hepatic fibrosis from mild to severe, PSV and RI of the PHA increase gradually (Figure 2A and 2C), and EDV of the PHA decreased gradually (Figure 2B), there was a significant difference between groups ($P < 0.05$). Furthermore, PW waveform of the MHV developed from HV1 (damped biphasic waveform without reversed flow) to HV2 (flat waveform) with the severity of the hepatic fibrosis (Table 2).

Serum fibrosis index

Serum concentration of fibrosis markers was higher in the IHS than in the control group ($P < 0.05$). Especially serum HA increased gradually from the mild to the severe group along with the severity of the hepatic fibrosis (Figure 2D), and it was significantly higher in IHS group than in the control group ($P < 0.05$). For PC-Ⅲ, C-Ⅳ and LN, there were also significant differences between infants with severe hepatic fibrosis and the control group ($P < 0.05$). When infants of the IHS group with mild or moderate

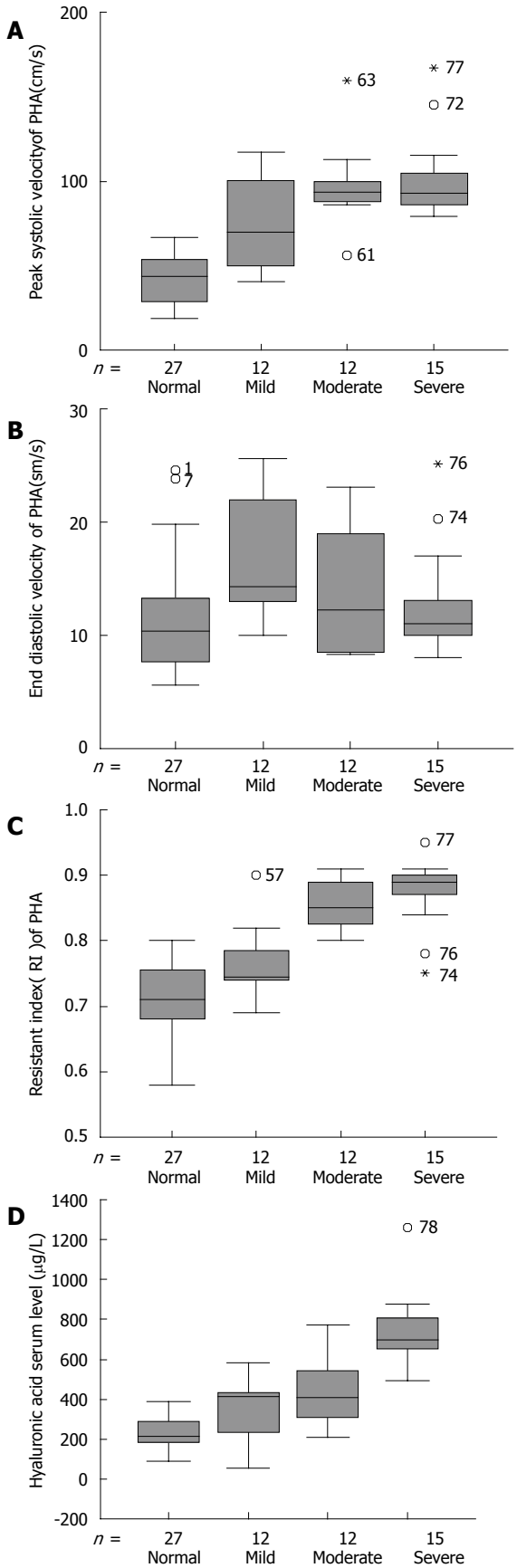

Figure 2 Boxplots showing **A**: Significantly higher PSV of PHA in patients with advanced liver fibrosis; **B**: Decreased gradually EDV of PHA in patients with advanced liver fibrosis; **C**: Significantly higher RI of PHA in patients with advanced liver fibrosis; **D**: Significantly higher serum HA level in patients with advanced liver fibrosis.

Table 2 Doppler sonograms in hepatic veins in patients with different fibrosis stage of IHS *n* (%)

Group	Patients (*n*)	HV0	HV1	HV2	Total
Control group	46	43 (93.50)	3 (6.50)	0 (0.00)	46 (100)
Mild group	12	2 (16.67)	10 (83.33)	0 (0.00)	12 (100)
Moderate group	12	1 (8.33)	10 (83.4)	1 (8.33)	12 (100)
Severe group	15	1 (6.67)	10 (66.67)	4 (26.67)	15 (100)

Chi-square test, $\chi^2 = 37.67$, $P < 0.001$.

fibrosis were compared to the healthy group, differences did not reach statistical significance (Table 3).

Correlative analyses

We found close positive correlations between the serum HA and hepatic fibrosis ($r = 0.712$, $P < 0.001$), between PSV of PHA and hepatic fibrosis ($r = 0.717$, $P = 0.001$), and between RI of PHA and hepatic fibrosis ($r = 0.745$, $P = 0.001$). The changes of hepatic vein waveforms were also positively related to liver fibrosis ($r = 0.783$, $P < 0.001$). In contrast, liver size, the size of the spleen and hepatic capsule, the echogenicity of the liver parenchyma, the inner diameter of the PV, nor the velocity of the PV were found to be useful for the diagnosis of hepatic fibrosis in IHS patients.

DISCUSSION

The basic pathological changes of chronic liver disease in the adults have been recognized more accurately and profoundly[33-36]. According to a new pathological classification method which was carried out in the adults in our country[33], staging of hepatic fibrosis can help us to recognize the development of the hepatic fibrosis in infants with IHS in our study. In the IHS group, 39 cases (95.1%) showed to have hepatic fibrosis by pathology. Further findings were ballooning change ($n = 35$, 85.4%), hepatocyte necrosis ($n = 30$, 73.2%), cholestasis in liver cells ($n = 26$, 63.4%), cholestasis in the hepatic lobules ($n = 21$, 51.2%), cholestasis in portal area ($n = 7$, 17.1%), and cytomorphosis of giant corpuscle ($n = 20$, 48.8%) of. Our findings showed that hepatic fibrosis was also one of the main pathologic features in the patients of this age group. We found no significant changes between the mild and the moderate hepatic fibrosis in 2DUS examination, it is compatible with other reports in the literature about adults[2,3,5-8].

PSV and RI of the PHA were significantly higher in infants with IHS than in the control group ($P < 0.05$). This phenomenon might be due to shrunken intrahepatic blood vessel space and the correspondingly increased peripheral resistance of the artery microvasscular bed, which increased the PSV and RI. This might also be the result of the severity of diffuse liver parenchyma impairment, hepatocellular degeneration and necrosis, inflammatory cell infiltration and fibrous connective tissue development[9,10]. Our findings also showed that PSV, RI of

Table 3 Serum fibrosis indexes in patients with different fibrosis stage of IHS (mean ± SD, μg/L)

Group	Patients (*n*)	HA	PC- Ⅲ	C Ⅳ	LN
Control group	46	225.18 ± 80.58	432.32 ± 166.68	129.69 ± 33.08	141.63 ± 34.63
Mild group	12	352.11 ± 157.33[a]	445.73 ± 191.11	149.85 ± 49.27	151.22 ± 36.57
Moderate group	12	447.48 ± 175.57[b]	480.77 ± 217.15	141.91 ± 47.51	132.56 ± 41.29
Severe group	15	745.32 ± 231.12[b,d]	580.36 ± 147.79[a]	185.00 ± 45.82[b]	167.90 ± 29.41[a]

HA, Serum hyaluronic acid; PC- Ⅲ: Pre-Collagen type Ⅲ; C-Ⅳ: Collagen type Ⅳ; LN: Laminin. [a]$P < 0.05$, and [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$, *vs* mild and moderate group.

the PHA increased gradually and EDV decreased gradually with advanced liver fibrosis, and there were significant differences between their groups ($P < 0.05$). PSV and RI of the PHA were positively correlated with the severity of liver fibrosis ($r = 0.717$ and 0.745, respectively $P = 0.001$). Hemodynamic changes of chronic liver disease in adults[7, 9,10] and in the pediatric age group[29] have been described by other researchers. Some results of our study were inconsistent with the findings of Xue *et al*[10] whose work was focused on chronic liver disease and confirmed cirrhosis in adults. One point was EDV of PHA elevating in IHS group instead of declining, another was velocity of PHA increasing without deficiency of PV. These may be concerned with a relatively short course of disease, abundant hepatic blood flow, active liver fibrosis and inflammation, or vasodilatation induced by inflammatory substances in patients with IHS.

In our findings, the changes in the waveforms of the hepatic vein were positively correlated with the severity of liver fibrosis ($r = 0.783$, $P < 0.001$). Other groups described a damped waveform in the hepatic veins of patients with liver cirrhosis, and our results were consistent with the studies of Bolondi *et al*[14]. This may be due to increased parenchyma stiffness, which impaired the compliance of the hepatic veins and caused the waveforms to change from HV0 to HV1. With the development of the hepatic fibrosis, diffuse fibrosis can cause disorganization of lobular architecture. Therefore, the hepatic veins were stenosis by pressure of the fibrosis tissues, pseudo lobule and the hepatic capsule. Furthermore, a damped waveform (HV2) in the hepatic veins was associated with focal hepatic vein stenosis[11-14].

Many groups addressed serum indexes such as HA, PC-Ⅲ, LN and C-Ⅳ, which reflected the stage of hepatic fibrosis. There is agreement that the four serum fibrosis indexes are relevant for the serodiagnosis of hepatic fibrosis, either alone[2,5,12,15,17-19,21,24,31,32] or in combination[4,16,20,22,23,25-28]. In our study, although we found some cases were inconsistent with pathological diagnosis, even with a high stage of hepatic fibrosis, the four serum fibrosis indexes were still around normal range, but statistical results indicated that the serum fibrosis index HA increased gradually from the mild to severe group as severity of liver fibrosis increased, and was significantly higher in each of fibrosis groups than in the control group. And then, the serum fibrosis index HA has a high positive correlation with the severity of hepatic fibrosis in IHS group. For the other serum fibrosis indices, PC-Ⅲ, C-Ⅳ and LN, there was only a significant difference between the severe hepatic fibrosis group and control group. And PC-Ⅲ, C-Ⅳ had a low positive correlation with the severity of hepatic fibrosis in IHS group. There was no correlation between LN and the severity of hepatic fibrosis in IHS group. One researcher's result showed the rate of inconsistency between the four serum fibrosis indexes and stage of hepatic fibrosis: HA was 29.2%, C-Ⅳ 31.9%, PC-Ⅲ 34.5% and LN 38.1%, the rate of consistency between serum HA and stage of hepatic fibrosis was the highest one, which was followed by C-Ⅳ. This suggested that among the four serum fibrosis indexes, HA was the most ideal index for the diagnosis of hepatic fibrosis[15]. Our results were also consistent with other reports in the literature[5,15-17,22]. Furthermore, infants less than one year old belong to a special group in the mostly active developing phase, the concentration of serum fibrosis indices was affected by many factors. In particular, serum PC-Ⅲ is very high in neonates, but quickly decreases by one year of age showing a marked negative correlation with age[30].

Liver biopsy guided by ultrasound in infants in 35 cases were performed during the scanning with an 18 G biopsy-cut needle driven by spring-loaded automatic biopsy gun, satisfactory sampling were get in all cases without any complication. A level of serum total bilirubin ≤ 200 μmol/L(former 136 μmol/ L) was indicated for liver biopsy in IHS group. According to our results, we consider that in clinical application, ultrasound-guided liver biopsy is also a valuable technology, secure, rapid, with high achievement ratio and no complication in the infant age group.

Our findings showed that young age, short course of disease, incompatibility with age and degree of liver fibrosis were the features of IHS group, parameters such as serum HA, PSV, and RI of PHA as well as the changes in the waveforms of the hepatic veins were more sensitive and reliable indicators in evaluating hepatic fibrosis in patients with IHS. Combination of ultrasonic studies on the hepatic hemodynamics with the evaluation of serum HA may provide an indicator for revealing hepatic fibrosis in children with IHS. This may be a useful noninvasive method for the diagnosis and estimation of the prognosis of IHS.

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RAPID COMMUNICATION

Relationship between Crohn's disease, infection with *Mycobacterium avium* subspecies *paratuberculosis* and *SLC11A1* gene polymorphisms in Sardinian patients

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Abstract

AIM: To study the association between Crohn's disease (CD), *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and genetic factors by examining the role of natural resistance-associated macrophage protein 1 (*NRAMP1*) gene polymorphisms (now *SLC11A1*) in Sardinian patients with CD and controls.

METHODS: Thirty-seven CD patients and 34 controls with no inflammatory bowel disease (IBD) were recruited at the University of Sassari after giving written consent. Six *SCL11A1* polymorphisms previously reported to be the most significantly associated with IBD were searched. *M. paratuberculosis* was identified by IS900 PCR and sequencing. Logistic regression was used to calculate odds ratios (OR) for the associations among CD, presence of MAP, and 6 loci described above.

RESULTS: For the first time, a strong association was observed between polymorphisms at *NRAMP1* locus 823C/T and CD. While CD was strongly associated with both *NRAMP1* and MAP, *NRAMP1* polymorphisms and MAP themselves were not correlated.

CONCLUSION: Combined with previous work on the *NOD2/CARD15* gene, it is clear that the interplay of genetic, infectious, and immunologic factors in the etiology of CD is complex.

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Key words: *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's disease; *SCL11A1* polymorphisms

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INTRODUCTION

Natural resistance-associated macrophage protein 1 (NRAMP1), now strictly referred to as SLC11A1 (Solute carrier 11a1) and the gene which encodes for it is recognized as having a role in the susceptibility of men and animals to a number of mycobacterial infections. In human beings, the *NRAMP1* gene is located on human chromosome region 2q35. It is composed of 15 exons and covers at least 16 kb of DNA. It encodes an integral membrane protein of 550 amino acids that is expressed exclusively in the lysosomal compartment of monocytes and macrophages[1]. The promoter region of the *NRAMP1* gene possesses a polymorphism within a possible enhancer element containing a Z-DNA-forming dinucleotide repeat[2,3].

It has been proposed that *NRAMP1* polymorphisms play a role in susceptibility to mycobacterial infections[4,5]. To date, studies have yielded contradictory results[2]. In a West African population, NRAMP1 variants have been associated with susceptibility to *Mycobacterium tuberculosis*[6]. Malik *et al*[7] reported that variants of NRAMP1 are associated with tuberculosis (TB) in children. *NRAMP1*

gene polymorphisms have been linked with genetic susceptibility to infection with *M. tuberculosis* and progression of TB into severe clinical forms in eastern China[8]. Abe *et al*[9] reported that genetic variation in the *NRAMP1* gene is associated with tuberculous cavitation of the lungs in Japanese patients. Kim *et al*[10] found that *NRAMP1* polymorphisms are associated with tuberculous pleurisy.

Various studies have been carried out to establish a connection between *NRAMP1* variants and diseases caused by mycobacteria other than the TB bacilli, such as *Mycobacterium ulcerans* causing Buruli ulcers[11] and *Mycobacterium leprae*[12]. A recent study[5] showed that the *NRAMP1* promoter region polymorphism are positively associated with leprosy but not with the Mitsuda reaction (intradermal injection of lepromin), and that variants of the *NRAMP1* gene favour microbial survival inside the macrophages by blocking the efficient transport of iron. It was reported that the *NRAMP1* gene may have a role in some autoimmune diseases, including rheumatoid arthritis (RA)[13]. It was also reported that *NRAMP1* 823 C/C prevents the development of rheumatoid nodules in RA patients[14], type 1 diabetes[15] and multiple sclerosis[16]. Variants of the *NRAMP1* gene have been associated with improved response to Bacillus Calmette-Guerin immunotherapy for superficial bladder cancer[17]. Hofmeister *et al* showed that *NRAMP1* variants are specifically associated with Crohn's disease (CD)[18].

In a previous study, we found that CD is associated with polymorphisms of the *NOD2* gene and the pathogen MAP in Sardinian patients[19]. NOD2 protein is an intracellular protein that activates NFkB upon binding to microbial peptidoglycan. MAP is the etiological agent of Johne's disease, a granulomatous enteritis of ruminants and other monogastric animals[20]. Although the association between MAP and CD (a human equivalent of paratuberculosis) has been postulated for a long time, only recent studies have made improvements in isolation and genomic techniques have allowed this link to be firmly established in a number of different populations[21-24].

As a part of our ongoing studies with *NOD2/CARD15*, we also looked at the role of other genes, especially *NRAMP1*, which may be associated with the survival of intracellular pathogens such as MAP. The role of NRAMP1 in regulating microbial survival inside phagosomes is related to iron transport, although the mechanism has not yet been completely elucidated[5,25].

MATERIALS AND METHODS

Patients were recruited at the University of Sassari after giving written consent. Using a case-control design, we analyzed 37 CD patients and 34 controls with no inflammatory bowel disease (IBD).

We searched for polymorphisms in 6 loci previously reported to be the most significantly associated with IBD[13]. The *SCL11A1* polymorphisms that we analyzed included a (GT)n microsatellite in the promoter region, (-)237 C/T; 469 + 14G/C, INT4; a non-conservative base substitution at codon 543 (D543N); 823 C/T; and a 4-bp TGTG deletion locating 55 nucleotides downstream of the last codon in exon 15 (1729 + del55del4).

Table 1 Full and reduced logistic regression main effects models for the association between Crohn's disease, MAP infection, and the presence of a number of genetic polymorphisms of the NRAMP1 gene

		Full model		Reduced model[2]	
		Odds Ratio	*P*	Odds Ratio	*P*
MAP	Negative	1.0[1]		1.0[1]	
	Positive	45.5	< 0.001	42.4	< 0.001
1729 + 55del4	+/+	1.0[1]		1.0[1]	
	+/DEL, DEL/DEL	5.6	0.014	5.6	0.011
823 C/T	CC	1.0[1]		1.0[1]	
	CT	75.6	0.001	50.8	0.001
D534R	GG	1.0[1]			
	GC	1.1	0.913		
(-)237C/T	CC	1.0[1]			
	CT, TT	0.5	0.350		
INT4	GG	1.0[1]			
	GC, CC	2.5	0.224		
GT (*n*)	Allele1	1.0[1]			
	Allele2	1.2	0.807		
	Allele3	0.6	0.673		

[1]Reference category; [2]Model statistics: Likelihood ratio test for equivalence with full model: $P = 0.436$; Pearson goodness-of-fit test: $P = 0.407$; Area under Receiver-Operating Characteristic curve: 0.886.

Amplification was performed as previously reported[13] using 100 ng of template genomic DNA previously extracted from intestinal tissues. The primer sequences that we used were previously reported[13]. Detection of MAP also was performed as previously reported[24].

Logistic regression was used to calculate odds ratios (OR) for the associations among CD, presence of MAP, and the 6 loci described above. Saturated and reduced models were computed and their comparability was assessed using the likelihood ratio test. The reduced model was assessed for goodness of fit using the covariate patterns as groups. In addition, the actual and model-predicted probabilities of CD according to levels of the independent variables in the final model were calculated.

RESULTS

Table 1 shows the full and reduced logistic regression models computed for this study. The full model showed significant associations between CD and the presence of MAP, the 1729 + 55del4 deletion polymorphism and the 823 C/T CT polymorphism. No significant associations were found between CD and the INT4, D534R, GT (n) and (-) 237 C/T loci. These non-significant loci were dropped in the reduced model and likelihood ratio testing showed no difference between the full and reduced models (likelihood ratio test: $P = 0.436$). Furthermore, the reduced model showed good fit (Pearson goodness-of-fit test: $P = 0.407$) and predictive power. We tested for significant interactions between MAP infection and each of the genetic loci, none of which was statistically significant. We therefore selected the reduced main effect model as our final model. No association was found between MAP and the 823 C/T or 1729 + 55del4 polymorphisms.

Figure 1 shows the actual and final model-predicted probabilities of CD by MAP infection and the two statistically significant loci, 823 C/T and 1729 + 55del4. The close similarity between the actual and predicted probabilities reflected the good fit of our final model to the data. Strong independent effects of MAP infection and each of the loci on the probability of CD were observed (Figure 1). Even with no mutant polymorphism, MAP infection was highly predictive of CD. Having the CT polymorphism at the 823 locus, the probability of CD was greatly increased both among patients infected with MAP and among patients not infected with MAP. The effect of deletions at the 1729 locus was more moderate. It should be noted that these probabilities were sensitive to the ratio of cases to controls in the study, which were shown here to illustrate the particularly strong association among CD, MAP infection and the 823 C/T locus as well as the close fit between the experimental data and our chosen statistical model.

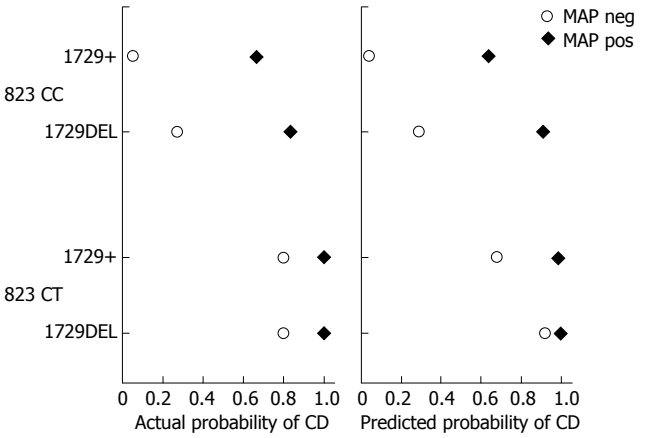

Figure 1 Actual probability of CD and predicted probability of CD showing strong independent effects of MAP infection and each of the loci on the probability of CD.

DISCUSSION

For the first time, a strong association has been observed between CD and polymorphisms at the 823C/T and 1729 + 55del4 loci in the *NRAMP1* gene in Sardinians.

Although the combination of MAP infection and 823 CT mutation could perfectly predict CD, generalizations were limited by the small sample size in our study. Only 8 patients in this study simultaneously had MAP infection, 823 CT mutation, and CD. It would be worthwhile to follow up this finding in a larger study to determine if these two factors are a useful prognostic index for the development of CD.

It was reported that polymorphisms in the *NOD2* gene and the presence of MAP are strongly associated with CD[19]. Both *NOD2* polymorphisms and MAP infection had a strong independent association with CD, and are also associated with one another, suggesting that susceptibility to MAP infection may be influenced by *NOD2* polymorphisms[26]. NOD2 protein is involved in the activation of NFkB factor and failure of its priming signal causes failure of pathogen clearance, possibly explaining the abnormal adaptive immune response to pathogens[27].

Our findings agree and disagree with some previous genetic studies of various *NRAMP1* loci and CD. Our finding which is lacking of association between GT (*n*) alleles and CD is consistent with the failure to find an association between any of these alleles and IBD in Americans[28]. Stokkers *et al*[29] found that mutations at the 823C/T or (-) 274C/T loci mutations are not associated with CD, which is in agreement with our findings at (-) 274C/T locus in this study. However, we have found a very strong association between CD and the 823CT polymorphism.

Although previous studies have suggested that NRAMP1 mutations may favour microbial survival, the fact that this study failed to find any association between *NRAMP1* polymorphisms and MAP infection does not support that viewpoint. Interestingly, although a previous study[6] showed allele 2 of this polymorphism is associated with mycobacterial infections, we did not find such an association between this allele and MAP infection.

The lack of association between *NRAMP1* polymorphisms and MAP infection is particularly curious because NRAMP1 is thought to directly impact microbial survival inside of macrophages. NRAMP1 is thought to either deny needed iron in intraphagosomal microbes, or to increase transphagosomal iron needed to create bactericidal hydroxyl radicals via the Haber-Weiss/Fenton reaction[25].

The 823CT and 1729 + 55del4 polymorphisms have been associated with autoimmune disease[14], but not with another mycobacterial disease, tuberculosis[30], which probably does not have an autoimmune etiology. In the present study, these polymorphisms were found to be associated with CD which probably does have an autoimmune component, but not associated with MAP infection. These findings, combined with the strong association between MAP infection and CD, suggest that *NRAMP1* polymorphisms may not cause CD by affecting the survival of MAP in intestinal tissue, but rather work together with MAP infection to cause CD *via* an autoimmune mechanism. In other words, one possible interpretation may be that these data support the aetiology of CD with both infectious and autoimmune components. At least, one study has elucidated a mechanism by which NRAMP1 affects the survival of intracellular pathogens and is simultaneously involved in autoimmune responses[25].

A further step of the study is to test the derived model on a large cohort of patients and controls.

In conclusion, CD may be strongly influenced by both NRAMP1 and MAP, although *NRAMP1* polymorphisms and MAP infection are not themselves correlated. Combined with previous work on the *NOD2/CARD15* gene, a complex interplay of genetic, immunologic and infectious factors may play a role in the aetiology of CD.

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RAPID COMMUNICATION

Trocar-related abdominal wall bleeding in 200 patients after laparoscopic cholecistectomy: Personal experience

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Abstract

AIM: To determine the complications and incidence of the first and second access-related vascular injuries induced by videolaparoscopic cholecistectomy.

METHODS: We retrospectively reviewed vascular injuries in 200 consecutive patients who underwent videolaparoscopic cholecistectomy from 2003 to 2005. One hundred and one patients with placement of radial expanding trocars were assigned into group A and 99 patients with placement of pyramidal tipped trocars into group B. All the patients were submitted to open access according to Hasson for the first trocar.

RESULTS: Bleeding did not occur at the intraoperative cannula-site in group A. However, it occurred at the intraoperative cannula-site of 7 patients (7.1%) in group B, with a statistically significant difference ($P < 0.01$). No mortality was registered. More vascular lesions were found in group B.

CONCLUSION: The advantage of Hasson technique is that peritoneal cavity access is gained under direct vision, preventing most severe injuries. The open technique with radial expanding trocars is recommended for secure access to the abdominal cavity in videolaparoscopy. Great care should be taken to avoid major complications and understanding the abdominal wall anatomy is important for reducing bleeding during or after s placement of trocars.

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Key words: Videolaparoscopy; Minor vascular complications; Trocars; Prevention

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INTRODUCTION

As minimally invasive techniques become more popular in modern surgery, laparoscope is now an instrument used by almost all surgical disciplines[1].

Primary and secondary accesses to the peritoneal cavity are the most crucial phases of laparoscopy. In fact vascular and visceral injuries (0.003%-6%) have been reported secondary to incisions made for positioning the laparoscopic cannulae or ports[1-3].

These injuries usually occur during the initial phase of operation or during placement of secondary cannulae, which should be placed under direct vision and with prior transillumination of the abdominal wall[4]. The technique of open laparoscopy developed by Harrith M. Hasson in 1970[4] combines safety of minilaparotomy with versatility of laparoscopy. This open access technique has also been introduced with the aim of reducing such injuries[3].

The aim of this study was to evaluate our laparoscopic experience with a focus on access-related vascular injuries and compare the effectiveness of two different types of trocar.

MATERIALS AND METHODS

We retrospectively reviewed the vascular injuries in 200 consecutive patients who underwent videolaparoscopic cholecystectomy in our university hospital from 2003 to 2005, with the approval of the ethics committee of our hospital.

One hundred and one patients with placement of radial expanding trocars (Ethicon Endo-Path Excel Endo-Surgery©) were assigned into group A and 99 patients with placement of pyramidal tipped trocars (Ethicon Endo-Path Tristar Endo-Surgery©) into group B. All the patients were submitted to open access according to Hasson for the first trocar, and then the following trocars were introduced under direct vision, after abdominal wall puncture and transillumination. We even used "open access" as previously described[4].

Carbon dioxide was insufflated into the intra-abdominal cavity with a pressure of 10-12 mmHg. We did not formally evaluate the time needed to achieve

abdominal entry and distension during open laparoscopy, but we noted that it generally varied from 3 to 10 min. Time variations depended on patient weight, abdominal wall strength, a well-developed Richet's fascia and post-operative fibrosis.

The diameter of Hasson, subxyphoid, and hypocondriac trocars was 10, 5, and 10 mm, respectively. The "primary access-related complications" were defined as those caused by the introduction of the first trocar and the "secondary access-related complications" as those caused by the introduction of the second or following trocars in the open access using Hasson's technique.

RESULTS

The age, gender, clinical history, comorbidity and American Society of Anesthesiology (ASA) classification of the patients were similar in both groups (Table 1).

Primary access-related complications occurred in 7 cases (3.5%), all in group B. No first-trocar lesions (Hasson-related) were registered in the two groups. Secondary access-related complications occurred in 6 cases of epigastric vessels injuries, all of which were treated with direct suture by the end of general anaesthesia (in all cases the bleeding started at the disinflation of pneumoperitoneum). One case (0.5%) of large abdominal wall haematomas was managed conservatively. No bleeding occurred at the intra-operative cannula-site in group A. However, bleeding occurred in 7 patients (7.1%) of group B, with a statistically significant difference ($P < 0.01$). Statistical analysis was performed by χ^2 test and $P < 0.01$ was considered statistically significant. No intraoperative morbidity or mortality was registered in our study.

Table 1 Personal experience

	Group A	Group B	*P*
Equipe position	French	French	-
ASA class Ⅰ / Ⅱ / Ⅲ	10/64/27	8/72/19	NS
Bleeding comorbidities	4%	1%	NS
Type of trocar	Conical	Pyramidal	-
Patients	101	99	NS
M:F ratio	0.6:1	0.6:1	NS
Age (mean)	62	64	NS
Body weight (mean, Kg)	72.7	70.8	NS
Bleeding (7 cases = 3.5%)	0	7 (7.1%)	< 0.01
Relaparotomy	0	0	0
Mortality	0	0	-

DISCUSSION

Between 1997 and mid-2002, FDA received more than 1300 trocar-associated injury reports, including 30 deaths. Hemorrhage due to vessel injury and infection secondary to bowel injury, especially when diagnosis is delayed, are the most serious complications which most likely result in death. Most data suggest that the rate of trocar-related complications is about 0.003%-6% and the average incidence of trocar-related vascular injuries is approximately 0.1%[5,6].

Minor vascular injuries are referred to the injuries to vessels of lesser importance than the aorta, inferior vena cava and iliac vessels. By far, the most common minor vascular injury of the inferior epigastric vessels occurs in up to 2.5% of laparoscopic cholecystectomies[3,7].

The major advantage of the open access technique using a step-by-step entrance to all layers of the abdominal wall is that peritoneal cavity access is gained under direct vision, preventing severe injuries[5].

The "11 secured steps" for the safe insertion of trocars using conical disposable trocars are recommended[8]. A randomized study comparing the conventional cutting with radial expanding trocars at all port sites reported that there is a significant difference in cannula-site bleeding and post-operative wound complications, which is in favour of using radial expanding trocars[9].

In a rabbit model, the relative risk of vessel injury induced by pyramidal tipped trocars is significantly increased compared with that induced by conical tipped trocars, especially if larger diameter trocars are used. The smooth contour of the conical tip stretches and separates the tissue layers with little damage, whereas the sharp-edged pyramidal tip lacerates any tissue it encounters, including vessels, even if the trocar is inserted directly over (in 88% of cases, partial interruption of vessel) or at 1-2 mm from the vessel (no injuries)[10,11].

In 1999, Balzer KM *et al*[11] dissected the abdominal walls of 21 cadavers transversally and measured the morphometric distances as well as determined the distances of the epigastric arteries and the ascending branch of the deep circumflex iliac artery from the midline and lateral edge of rectus muscle. The results showed that In order to minimize the danger of lesions of abdominal vessels, trocars should be placed in the ventral midline or in a 5 cm wide zone to the lateral border of the rectus sheath.

If prevention fails, it is possible to try to tamponade by applying pressure to the abdominal wall with a balloon trocar or a Foley catheter pulled against the abdominal wall and attached to a clamp (the temporary pressure usually stops bleeding from small vessels or veins), which should not be used to stop the bleeding at the trocar site.

If significant bleeding occurs, ligation is needed using Keith or Reverdin needle and large suture that can be removed in the recovery room when the patient awakens. Occasionally, in catastrophic bleeding from the abdominal wall, an incision should be made to dissect down the bleeding vessels and control the bleeding by ligation[12].

In conclusion, the risk of vessel injury varies markedly with the different trocars used. The open technique with radial expanding trocars is recommended for access to the abdominal cavity during videolaparoscopy. However, open laparoscopy cannot prevent small bowel injury or post-operative herniation, but can eliminate failed laparoscopy attempts, inappropriate insufflation, gas embolism, peritoneal, stomach, colon and vessel injury.

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RAPID COMMUNICATION

Protective effects of amphetamine on gastric ulcerations induced by indomethacin in rats

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Abstract

AIM: To study the effects of amphetamine, an indirect-acting adrenomimetic compound on the indomethacin-induced gastric ulcerations in rats.

METHODS: Male Wistar-Bratislava rats were randomly divided into four groups: Group 1 (control), received an ulcerogenic dose of indomethacin (50 μmol/kg) and Groups 2, 3 and 4, treated with amphetamine (10, 25 and 50 μmol/kg). The drug was administered simultaneously with indomethacin and once again 4 h later. The animals were sacrificed 8 h after indomethacin treatment. The stomachs were opened and the incidence, the number of lesions and their severity were evaluated. The results were expressed as percentage and as mean ± standard error (mean ± SE).

RESULTS: The incidence of ulceration in the control group was 100%. Amphetamine, at doses of 10, 25 and 50 μmol/kg, lowered the incidence to 88.89%, 77.78% and 37.5% respectively. The protection ratio was positive: 24.14%, 55.17% and 80.6% respectively. The total number of ulcerations/rat was 12.44 ± 3.69 in the control group. It decreased to 7.33 ± 1.89, 5.33 ± 2.38 and 2.25 ± 1.97 under the effects of the above-mentioned doses of amphetamine.

CONCLUSION: Amphetamine affords a significant dose-dependent protection against the indomethacin-induced gastric ulcerations in rats. It is suggested that the adrenergic system is involved in the gastric mucosa protection.

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Key words: Amphetamine; Gastric ulceration; Indomethacin; Rats

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INTRODUCTION

The involvement of the adrenergic system in some models of gastric ulcerations is well documented[1-4]. Our previous investigations have shown that amphetamine, an indirectly acting sympathomimetic amine has a protective activity on gastric ulcerations induced by reserpine and immobilization in rats[5]. These findings were interpreted as an evidence of a protective activity of the adrenergic mechanisms on the integrity of the gastric mucosa[3,4,6].

Other models of gastric ulcers imply the administration of some nonsteroidal anti-inflammatory agents such as acetylsalicylic acid[7], phenylbutazone[8] or indomethacin[9]. The genesis of these ulcerations arises from both the local depression of prostanoid biosynthesis[10-12] and a local injury[13]. However, the participation of the adrenergic system in this experimental setting is not excluded[14,15].

In this paper, we studied the effect of amphetamine on the indomethacin-induced gastric ulcerations in albino rats.

MATERIALS AND METHODS

Animals and ulcer model

The experiments were carried out on male Wistar albino rats, weighing 144-255 g (from the Animal Center of University of Medicine and Pharmacy Iuliu Haţieganu, Cluj-Napoca). The animals were kept in a room with a constant temperature (20-22℃) and humidity, under a natural light-dark regime. They were fed common rat chow and received water ad libitum. The food was withdrawn 12 h prior to administration of the ulcerogenic agent. The rats were randomly divided in four groups.

Control group (Group Ⅰ) was administered intraperitoneally (ip) with indomethacin 50 μmol/kg. Groups Ⅱ, Ⅲ and Ⅳ were simultaneously ip injected with

Table 1 Effect of amphetamine on incidence and severity of indomethacin-induced gastric ulcerations (%)

Groups		TU	LU	PR
I	I_{50}	100	100	
II	$I_{50} + A_{10}$	88.89	77.778	(+) 24.138
III	$I_{50} + A_{25}$	77.78	66.667	(+) 55.172[1]
V	$I_{50} + A_{50}$	37.5[a]	12.5[a]	(+) 80.603[1]

The subscript indices represent the doses expressed as μmol/kg. I : Indomethacin; A: Amphetamine; TU: Total number of ulcerations; LU: Large ulcerations (> 1 mm); PR: Protection ratio. [a]$P < 0.05$ *vs* group I (Fischer's exact probability and median's test); [1]Significant at PR > (+) 33.33%.

Table 2 Effect of amphetamine on number and severity of indomethacin-induced gastric ulcerations (mean ± SE)

Groups		Number of ulcerations/stomach		Severity
		TU	LU	UI
I (9)	I_{50}	12.44 ± 3.69	10.11 ± 3.74	1.61 ± 0.31
II (9)	$I_{50} + A_{10}$	7.33 ± 1.89	5.44 ± 1.52	1.22 ± 0.25
III (9)	$I_{50} + A_{25}$	5.33 ± 2.38	3.11 ± 1.9	0.72 ± 0.21[a]
IV (8)	$I_{50} + A_{50}$	2.25 ± 1.91[c]	1.0 ± 1.0[c]	0.31 ± 0.19[b]

The subscript indices represent the doses expressed as μmol/kg. I: Indomethacin; A: Amphetamine. TU: Total number of ulcerations; LU: Large ulcerations (> 1 mm); UI: Ulcer index. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.05$ *vs* group I .

indomethacin and amphetamine sulphate, and 4 h later, only with amphetamine. The doses of amphetamine were 10, 25 and 50 μmol/kg , respectively.

During the ulcerogenesis period, the rats were fasted, but water was allowed ad libitum. Eight hours after indomethacin administration, the animals were sacrificed; the stomachs were removed, opened along the curvatura maior and rinsed with saline. They were examined immediately, with a magnifying glass. The following parameters were recorded: the body weight (g), the incidence of ulcerations/group, the total number of ulcerations (TU), the number of large ulcerations (LU) i.e. ulcerations larger than 1 mm and the severity of ulcerations estimated according to a scale between 0 and 4 (ulcer index-UI)[3].

Drugs

Indomethacin: Powder supplied by Terapia SA, Cluj-Napoca, Romania, was suspended in a mixture of 2% methylcellulose and 1% glycerin, ten times diluted with saline. The concentration was 0.025 mol/L (8.945 g/L) and the dose, 50 μmol (17.89 mg)/kg.

Amphetamine sulphate (racemate): It was prepared as an aqueous solution 0.025 mol/L (9.21 g/L) for the dose of 50 μmol (18.425 mg)/kg. Doses of 10 μmol (3.685 mg)/kg and 25 μmol (9.2105 mg)/kg were adapted after dilution. All drugs were ip administered in a volume of 2 mL/kg.

Ethical issues

The study was conducted in accordance with the Helsinki's Declaration on Animal's Studies and approved by the local ethics committee.

Statistical analysis

All values are expressed as means and standard errors (mean ± SE). The indicators of ulcerogenesis were analyzed initially by a one-way ANOVA test. The significance of differences between groups was observed by the bilateral Student's *t* test and by multiple comparison tests of Newman-Keuls and Scheffé. This last test allows comparisons between blocks of groups. These procedures were followed by non-parametric Kruskal-Wallis, Mann-Whitney, median's and Fischer's exact probability tests[16,17]. The statistical significance of the differences was admitted if $P < 0.05$.

The protection ratio (PR) was calculated according to the formula:

$$PR\% = (UI_{control} - UI_{treated}) / UI_{control} \times 100$$

Statistical significance for PR was considered for values outside (-) 33% → (+) 33% range[3].

RESULTS

The body weight of the rats was a homogenous parameter, i.e. there were no significant differences between groups (data not included in Tables). The doses of 25 and 50 μmol/kg of amphetamine induced a stereotyped behavior. It was not affected by indomethacin.

Indomethacin induced ulcerations in 100% of the rats. The total number of ulcerations was 12.4 ± 3.69, most of them (10.1 ± 3.74) were larger than 1 mm. The UI was 1.61. When amphetamine was administered, all the parameters diminished in a dose-dependent manner (Tables 1 and 2). In addition, a dose-dependent increase of the PR was found (Table 1).

Significant differences between the control group and the groups treated with 25.0 μmol/kg and 50.0 μmol/kg of amphetamine were found in most tests. Some tests showed significant differences between the 10.0 μmol/kg amphetamine administered group and the group that received 50.0 μmol/kg (Tables 1 and 2). For the parameters of ulcerogenesis (TU, LU, and UI), an increase of significance was found in the order: total ulcerations (TU) < ulcerations larger than 1 mm (LU) < ulcer index (UI).

DISCUSSION

These results seem at first glance rather surprising, since, according to most authors, the ulcerations produced by nonsteroidal anti-inflammatory agents are due to an impaired prostanoids biosynthesis in the gastric mucosa[10,18,19], and to a local injury as well[13].

It is possible that other mechanisms are involved in the ulcerogenesis caused by indomethacin: the direct cytotoxic effect[20], the promotion of apoptosis of gastric mucosal cells[21-23] as well as the induction of interactions between polymorphonuclear leukocytes and endothelium of gastric mucosa vessels[24-28]. The effects of amphetamine may be related to any of these mechanisms. However, only the links between prostaglandins and adrenergic system are firmly established[29]. Prostaglandins, particularly those

from E series inhibit catecholamines release[30,31]. On the other hand, adrenergic stimulation evokes an increase of prostaglandin release[32,33].

It is well known that amphetamine acts mainly via the release of dopamine and noradrenaline from both central and peripheral sites[34,35]. The question arises whether dopamine or noradrenaline or both are involved in the protection of gastric ulcerations afforded by amphetamine. Our previous results have shown that dopaminergic mechanisms were not involved in the protective activity of amphetamine and catecholamines in restraint and reserpine-induced gastric ulcerations[3,5,7]. We did not investigate the involvement of dopamine receptors in indomethacin-ulcerations.

On the other hand, there are data in the literature indicating that the dopaminergic system may be also involved in the genesis of the ulcerations induced by nonsteroidal anti-inflammatory agents[36,37]. So, dopaminergic agonists, such as apomorphine and bromocriptine, diminished significantly the incidence and the severity of the ulcerations evoked by indomethacin in albino rats. The protective activity of apomorphine was antagonized by haloperidol[36]. This agent and other dopaminergic antagonists aggravated gastric ulcerations induced by indomethacin[36], reserpine[37] and stress[38,39]. These findings suggest that dopamine would have a protective activity in various models of experimental ulcers[37,40-42]. It was also shown that gastric pentadecapeptide BPC 157 markedly affects the central and peripheral dopaminergic system. It blocks the acute stereotype??? elicited by amphetamine[43]. At the same time, it completely prevents the gastric ulcerations induced by haloperidol[37,44].

More recently, a cocaine- and amphetamine-regulated transcript (CART) messenger RNA was isolated from the brain[45,46], peripheral nerves[47] and tissues[48,49]. Its corresponding polypeptide inhibits after central injection, food intake, the gastric emptying and gastric acid secretion in 24 h fasted rats. However, CART polypeptide, ip injection does not have the same effect. It was also shown that the intracisternal injection of CART greatly reduces the gastric acid output elevated by subcutaneous indomethacin administration[50]. These data are consonant with the view that the adrenergic system has a protective action on gastric mucosa.

In conclusion, amphetamine protects gastric mucosa against the damaging effect of indomethacin. The mechanism of action is still obscure, it might involve both the central and peripheral segments of the adrenergic system.

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RAPID COMMUNICATION

Non-invasive diagnosis of gastric mucosal atrophy in an asymptomatic population with high prevalence of gastric cancer

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Abstract

AIM: To validate a non-invasive method to detect gastric mucosal atrophy in a Chilean population with high prevalence of gastric cancer and a poor survival rate.

METHODS: We first determined the optimal cut-off level of serum pepsinogen (PG)-1, PG-1/PG-2 ratio and 17-gastrin in 31 voluntary symptomatic patients (mean age: 66.1 years), of them 61% had histologically confirmed gastric atrophy. Then, in a population-based sample of 536 healthy individuals (209 residents in counties with higher relative risk and 327 residents in counties with lower relative risk for gastric cancer), we measured serum anti-*H pylori* antibodies, PG and 17-gastrin and estimated their risk of gastric cancer.

RESULTS: We found that serum PG-1 < 61.5 μg/L, PG-1/PG-2 ratio < 2.2 and 17-gastrin > 13.3 pmol/L had a high specificity (91%-100%) and a fair sensitivity (56%-78%) to detect corpus-predominant atrophy. Based on low serum PG-1 and PG-1/PG-2 ratio together as diagnostic criteria, 12.5% of the asymptomatic subjects had corpus-predominant atrophy (0% of those under 25 years and 20.2% over 65 years old). The frequency of gastric atrophy was similar (12% *vs* 13%) but *H pylori* infection rate was slightly higher (77% *vs* 71%) in the high-risk compared to the low-risk counties. Based on their estimated gastric cancer risk, individuals were classified as: low-risk group (no *H pylori* infection and no atrophy; *n* = 115; 21.4%); moderate-risk group (*H pylori* infection but no atrophy; *n* = 354, 66.0%); and high-risk group (gastric atrophy, with or without *H pylori* infection; *n* = 67, 12.5%). The high-risk group was significantly older (mean age: 61.9 ± 13.3 years), more frequently men and less educated as compared with the low-risk group.

CONCLUSION: We propose to concentrate on an upper gastrointestinal endoscopy for detection of early gastric cancer in the high-risk group. This intervention model could improve the poor prognosis of gastric cancer in Chile.

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Key words: Gastric cancer; *H pylori*; Gastric atrophy; Non-invasive diagnosis; Pepsinogen; Gastrin

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INTRODUCTION

Gastric cancer (GC) is still highly prevalent in Chile and the world[1]. With the exception of Japan, which has implemented early detection strategies through the population screening, the diagnosis of GC is usually late and its prognosis is very poor. In Chile, a study showed that a global survival rate at 5 years after diagnosis was less than 15%, although it surpassed 90% when early stage tumors were treated with curative resection[2].

In spite of the identification of *H pylori* infection as a relevant etiological factor, this has not yet led to significant changes in the diagnostic or therapeutic strategy. The initial pathogenic role of *H pylori* infection is currently undisputed, explaining between 70% and 90% of GC risk in a given population[3,4]. As first postulated by Correa[5], *H pylori* produces an inflammation of the gastric mucosa (gastritis) that, evolving asymptomatically through many years, progresses in a decreasing proportion to gastric atrophy (GA), intestinal metaplasia, dysplasia, and finally to well differentiated adenocarcinoma. Other diverse environmental and genetic factors modulate this chain of

events. Atrophy and intestinal metaplasia of the gastric mucosa are doubtfully reversible lesions that determine a significant increase in the risk of developing GC[5,6].

The "gold standard" for the diagnosis of gastric atrophy is the histological study of biopsies obtained during an upper gastrointestinal (GI) endoscopy, an invasive method hardly suitable for population screening. Non-invasive strategies, applicable to an asymptomatic population, would allow having better knowledge of the frequency and natural history of gastric mucosal atrophy, testing prevention strategies and diagnosing more effectively the progression to (early) gastric cancer.

The possibility to detect gastric atrophy by means of some serum markers, such as pepsinogen (PG)-1, PG-1/PG-2 ratio and 17-gastrin, has recently been described[7-9]. However, the apparent geographic or even racial variability in the diagnostic performance of these tests precludes its simple extrapolation to our milieu.

The general objective of this study was to validate a non-invasive diagnostic tool for gastric mucosal atrophy and then to apply it to an asymptomatic population in order to identify the population groups at greater risk for developing GC. The specific objectives were: (1) to determine the diagnostic performance of PG-1, PG-1/PG-2 and 17-gastrin in serum for the diagnosis of gastric atrophy in a sample of symptomatic patients, compared to the histology as a gold standard; (2) to determine serologically the frequency of gastric atrophy in a sample of asymptomatic subjects residing in counties with different risks of developing GC; and (3) to non-invasively categorize the estimated risk of developing GC in the same sample.

MATERIALS AND METHODS

Subjects

For the diagnostic validation study, we included voluntary patients older than 40 years old, with clinical indications for upper GI endoscopy. Exclusion criteria were: use of proton pump inhibitors within the last month, gastric cancer, active peptic ulcer or previous gastric resection. The Ethics Committee of our institution approved the study.

For the population study, we used the sera of 536 subjects selected from the 3600 subjects that constituted a random Chilean population sample in which a National Health Survey was performed[10]. This sub-sample was obtained by ordering the subjects from the original sample according to mortality risk for GC in their county of residence (described below) and then selecting subjects from the extreme counties on the list (those with greater and lower relative risk of GC, respectively) until reaching the number of available determinations.

Diagnostic validation study

After obtaining informed consent from the patients, an upper GI endoscopy was performed. Two sets of biopsies from the gastric corpus and the antrum (both the gastric wall and greater curve) were sent separately for histological analysis. An additional antral biopsy was destined to a rapid urease test (ProntoDry®, Medical Instrument Corp, Brignais, France). Serum from a 10-mL blood sample was separated and stored at -20℃ until processing.

Population study

Blood samples were obtained from all over the country during the 2003 National Health Survey, sent to regional hospitals and then to our center, where the sera were stored at -80℃ until processing. The categorization of relative risk (RR) of GC for each subject was calculated according to the mortality rates for GC registered in his/her county of residence during 1985-2002, corrected for age, sex and population size, and adjusted through a hierarchical Poisson regression model, considering the extra-Poisson structural variability, estimated by Bayesian analysis[11,12]. The counties with a RR of mortality for GC > 1 were considered high-risk counties.

Analytical determinations

For anti-*H pylori* serology, the commercial bioelisa assay HELICOBACTER IgG (BioKit®, Barcelona, Spain) was used. In previous studies, we determined that the most appropriate cut-off level for the diagnosis of *H pylori* infection in the Chilean adult population was 72.8 arbitrary units (AU)/mL.

Determination of serum levels of PG-1, PG-2 and 17-gastrin was performed using a commercial ELISA assay (Gastropanel®; (BioKit®, Helsinski, Finland), following the manufacturer's instructions.

Histological study

The formalin-fixed and paraffin-embedded samples were cut into thin sections and stained with hematoxylin-eosin (H&E). The modified Sydney classification[13] was used to classify the histological findings in the gastric corpus and the antrum separately. We used previously described criteria for histological categorization[14]. Non-atrophic chronic gastritis was defined as the presence of chronic inflammation (score ⩾ 1), with or without acute inflammation, with no atrophy (score = 0) in the corpus or the antrum. Chronic atrophic gastritis was diagnosed in the presence of atrophy (Sydney score ⩾ 1), with or without intestinal metaplasia, associated with chronic inflammation (score ⩾ 1). The patients with atrophic gastritis were classified as antrum predominant when the atrophy score was greater in the antrum than in the corpus, corpus predominant when the score was greater in the corpus and multifocal when the score was the same in the both sites.

Statistical analysis

The Student's *t* test, ANOVA or Kruskal-Wallis test were used for univariate analysis of the discrete variables (age and serum levels of PG-1, PG-1/PG-2, 17-gastrin) and the chi-square test or the Fisher exact test for categorical variables (sex, endoscopy variables and levels of PG-1, PG-1/PG-2 and 17-gastrin above or below the respective cut-off levels) associated with the presence of histological atrophy. Through ROC (receiving operator characteristic) curves, the best cut-off levels were determined for the serum levels of PG-1, PG-1/PG-2 and 17-gastrin for the detection of the antrum and corpus atrophy (Table 1). To evaluate the diagnostic performance of the serologic

Table 1 Correlation between the serum levels of pepsinogen and gastrin with the type and topography of histological gastritis (median)

	Chronic non-atrophic gastritis (n = 10)	Antrum-predominant atrophic gastritis (n = 10)	Corpus-predominant atrophic gastritis[1] (n = 9)
PG-1 (μg/L)[a]	115.6	103.3	40.9
PG-1/PG-2[a]	4.39	5.62	2.22
17-gastrin[b] (pmol/L)	6.2	3.7	36.8

[1]Including 1 patient with multifocal atrophic gastritis; [a]$P < 0.05$ or [b]$P < 0.01$ between the three groups (Kruskal-Wallis Test).

Table 2 Diagnostic performance of serology (pepsinogen and 17-gastrin) to diagnose gastric atrophy

	Cut-off level	Sensitivity % (95% CI)	Specificity % (95% CI)	LR[5]+	LR-	Youden's J index
PG-1 (μg/L)[3]	< 25[1]	44 (12-77)	95 (87-100)	8.8	0.59	0.40 ± 0.17
	⩽ 61.5[2]	78 (40-97)	91 (71-99)	8.6	0.24	0.69 ± 0.15
PG-1/PG-2[3]	< 2.5[1] ⩽ 2.2[2]	56 (21-86)	100 (84-100)	∞	0.44	0.56 ± 0.17
17-gastrin (pmol/L)	< 2[1,4]	30 (16-58)	86 (70-100)	2.1	0.81	0.16 ± 0.16
	⩽ 7.5[2,4]	90 (56-98)	52 (30-74)	1.89	0.19	0.42 ± 0.14
	> 13.3[2,3]	67 (30-92)	96 (77-99)	14.7	0.35	0.62 ± 0.16

[1]Suggested by the manufacturer; [2]Determined by ROC curves; [3]To detect corpus-predominant atrophic gastritis; [4]To detect antrum-predominant atrophic gastritis; [5]LR = likelihood ratio.

determinations, we used the likelihood ratio (LR) and Youden's J index[15] (Table 2). Nominal logistic regression was used to perform a multivariate analysis of variables associated to the groups with different estimated risks of GC (Table 3). A P value less than 0.05 was considered statistically significant. The statistical analyses were performed using Epi Info version 3.2 (Epidemiology Program Office, CDC, Atlanta, GA, USA) and SPSS version 14 (SPSS Inc, Chicago, Illinois, USA) computer programs.

RESULTS

Diagnostic validation of the serologic methods

Thirty-one patients (21 women, 68%) with an average age of 66.1 (range: 42-90) years were included. Endoscopy was normal in 12 (39%) patients, compatible with gastric atrophy in 8 (26%) patients, showed erosive esophagitis in 2 (6.45%) patients and erosive antropathy in 1 (3.22%) patient. Using histology, serology and rapid urease test, *H pylori* infection was found in 13/31 (42%) patients. The serum level of PG-1 was significantly greater in the *H pylori*-infected patients (136.7 ± 60.8 μg/L) as compared with the non-infected patients (78 ± 68.2 μg/L) ($P < 0.05$). The serum levels of PG-2 and 17-gastrin and the PG-1/PG-2 ratio were similar in the both groups.

Histological features of the gastric specimens

According to the Sydney classification[13], chronic gastritis was found in 29 (94%) patients, 19 (61%) of them were atrophic. The atrophy was antrum-predominant in 10 (53%) patients, corpus-predominant in 8 (42%) and multifocal in 1 (5%) patient. Glandular atrophy was scored as moderate or severe (score ⩾ 2) in 47% of the cases. The frequency of *H pylori* infection was higher in the patients with chronic non-atrophic or antrum-predominant atrophic gastritis compared to the patients with corpus-predominant atrophic gastritis (60% and 11%, respectively; $P = 0.054$).

Correlation between histology and serum levels of pepsinogen and gastrin

The results are summarized in Table 1. The serum level of PG-1 was significantly lower in the group with the corpus atrophy, and the PG-1/PG-2 ratio was also lower in this group, although without attaining statistical significance. In comparison with non-atrophic gastritis, the average serum level of 17-gastrin was significantly lower in the patients with the antrum atrophy and significantly higher in those with the corpus atrophy. Table 2 shows the diagnostic performance of the different determinations for the detection of the corpus or antrum gastric atrophy, using the cut-off levels recommended by the manufacturer and those determined through ROC curves. The latter slightly improves sensitivity for the diagnosis of the corpus atrophy, conserving high levels of specificity, which determines LR+ in ranges close to those considered clinically useful (> 10)[16] and a slight improvement in the Youden's J index. The diagnosis of the antrum atrophy through the 17-gastrin level was not reliable. In contrast, the 17-gastrin level over 13.3 pmol/L had a diagnostic performance comparable to PG-1 and the PG-1/PG-2 ratio to diagnose the corpus atrophy.

Population study

Of the 536 subjects, 209 resided in counties at high risk for GC (mean RR = 1.25) and 327 in counties at low risk for GC (mean RR = 0.8). The frequency of *H pylori* infection was 72.9% (95% CI: 70%-76%). The frequency

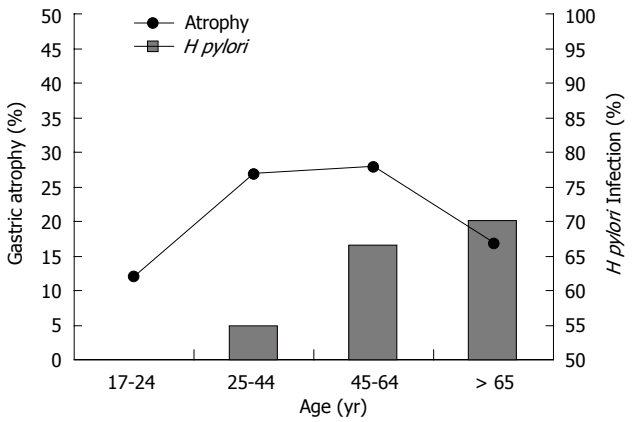

Figure 1 Frequency of gastric atrophy and *H pylori* infection in 536 asymptomatic subjects by age.

of the corpus atrophy was 26.5% when using serum level of PG-1 ⩽ 61.5 μg/L as a diagnostic criterion, and 12.5% when using the more restrictive combination of PG-1 ⩽ 61.5 μg/L and PG-1/PG-2 ⩽ 2.2[17], which was used for the following analyses.

The frequency of *H pylori* infection was higher in the counties with high RR for GC (76.6%) compared to those with low RR (70.6%), although did not reach a statistical significance. The frequency of gastric atrophy was similar between the both groups (11.5% and 13.1%, respectively). The gastric atrophy was significantly correlated with age (Figure 1). No atrophy was detected in the subjects younger than 25 years, while 20.2% of those older than 65 years had PG-1 level and PG-1/PG-2 ratio compatible with the corpus atrophy, which probably explained the reduction in the serological evidence of *H pylori* infection observed in this group.

Using serological data, we divided the samples into three groups: Group A = no *H pylori* infection and no gastric atrophy (low GC risk); Group B = *H pylori* infection but no gastric atrophy (moderate GC risk); and Group C = serological evidence of gastric atrophy, with or without *H pylori* infection (high GC risk). The demographic characteristics and relevant comparisons among the three groups are shown in Table 3. There were significant differences in the distribution of some variables classically related with GC risk, such as age and educational level. Similar to the samples of the symptomatic patients, *H pylori* infection was associated with a significant elevation of PG-1 (87.4 ± 53.2 in non-infected group *vs* 116.6 ± 51.2 μg/L infected group; $P < 0.05$). Similarly, the corpus atrophy was associated with a significant elevation in the serum level of 17-gastrin (Table 3). The proportion of the subjects residing in counties with a high RR of GC was not significantly different among the three groups, although due to the relatively low frequency of gastric atrophy, dispersion of this parameter was wide (95% CI: 25.3%-43.3% for Group A; 36.1%-46.6% for Group B; and 24.5%-48.5% for Group C). In a multivariate analysis, using Group A as a reference, the only variable associated with *H pylori* infection (Group B) was 17-gastrin (OR: 1.05 (95% CI: 1.005-1.10), $P < 0.05$). For the gastric atrophy, the most significant variable was 17-gastrin (OR: 1.13 (95% CI: 1.07-1.18), $P < 0.001$), and also male (OR: 2.08 (95% CI: 1.07-4.04), $P < 0.05$) and older age [OR: 1.02 (95% CI: 1.004-1.05), $P < 0.05$] were significant risk factors, while educational level was a protective factor [OR: 0.9 (95% CI: 0.84-0.99), $P < 0.05$].

Table 3 Characteristics of the asymptomatic population samples according to the estimated gastric cancer risk

Characteristics	Estimated gastric cancer risk			Total (n = 536)
	Low (A) (n = 115)	Medium (B) (n = 354)	High (C) (n = 67)	
Corpus atrophy[1]	Absent	Absent	Present	
H pylori infection[2]	Absent	Present	Present or absent	
Men, n (%)	46 (40.0)	168 (47.5)	35 (52.2)	249 (46.5)
Age (yr)[a], (mean ± SD)	49.6 ± 22.2	48.6 ± 18.0	61.9 ± 13.3	50.5 ± 19.0
Educational level (yr)[a], (mean ± SD)	8.8 ± 5.2	8.2 ± 4.3	5.8 ± 4.2	8.0 ± 4.6
Rurality, n (%)	27 (23.5)	99 (28.0)	22 (32.8)	148 (27.6)
Living in high-risk counties, n (%)	39 (33.9)	146 (41.2)	24 (35.8)	209 (39.0)
17-Gastrin[a] (pmol/L), (mean ± SD)	4.1 ± 6.8	5.6 ± 7.1	14 ± 14.3	6.3 ± 8.8

[1]As determined by serum level of PG-1 ⩽ 61.5 μg/L and PG-1/PG-2 ratio ⩽ 2.2. [2]Serology (+) for *H pylori* (ELISA). [a]Significant variables in the multivariate analysis ($P < 0.05$): B *vs* A: 17-gastrin (OR 1.05); C *vs* A: sex (OR 2.08); age (OR 1.02); education years (OR 0.9); 17-gastrin (OR 1.13).

DISCUSSION

In spite of the advances in the knowledge of gastric carcinogenesis, including the role of *H pylori* infection, early diagnosis and opportune time for surgical intervention will continue to be the basis of effective treatment. Digestive symptoms are late and non-specific events[18] such that in order to diagnose early stage tumors, it is necessary to identify high-risk asymptomatic subjects. This work intends to validate a non-invasive method to advance in this direction.

Even though the detection of *H pylori* infection identifies a group that concentrates virtually all the subjects at risk to develop GC in a population[4], the low incidence of GC in the infected and the generally high frequency of infection in countries with high GC frequency, such as Chile and other Andean countries of South America, determine a limited localizing effect. Additionally, the loss in serological evidence of infection detected in the old-aged group in this population, probably as a consequence of gastric atrophy, would imply missing the group with the highest GC risk. Moreover, the eradication of *H pylori* infection does not seem to prevent the development of GC in subjects that have already developed gastric atrophy or intestinal metaplasia[19].

Gastric atrophy is generally accepted the major risk factor for GC development. A prospective study by Uemura *et al*[4] showed that the patients with severe atrophy had 5 times greater RR than those without atrophy. Other Japanese studies have shown that in *H pylori*- infected subjects, gastric atrophy is associated with up to 90 times greater risk for GC[6,20,21].

The serological diagnosis of gastric atrophy by means of serum PG is based on the fact that a small proportion of the diverse isoforms of this digestive enzyme, produced in the mucosa of the upper digestive tract, enters the blood. The isoforms corresponding to PG-1 are synthesized only in the gastric corpus, while PG-2 isoforms are synthesized in the whole gastric mucosa and even in the proximal duodenum. In the presence of corpus atrophy, the serum level of PG-1 diminishes, while PG-2 is stable or diminishes very slightly. However, published studies are relatively heterogeneous. While most studies seem to validate the diagnostic performance of the method[22-27], other studies show a much less reliable performance[28,29] and even suggest the influence of racial factors[30]. A recent meta-analysis suggested that additional studies are required in diverse populations to determine its real value[17].

Our results showed that in this population, low level of serum PG-1 or low PG-1/PG-2 ratio were able to detect the presence of the corpus atrophy with moderate sensitivity (56%-78%) and high specificity (91%-100%). The latter minimizes the risk of false positives and makes the method especially appropriate to be used in a low prevalence setting, such as expected in an asymptomatic population. The diagnostic performance of low levels of 17-gastrin to detect the antrum atrophy was clearly unsatisfactory in these samples, probably due to the antagonistic effect of concurrent corpus atrophy, which determines a significant elevation of the gastrin level. In contrast, an elevated level of 17-gastrin was highly reliable for the diagnosis of corpus atrophy in the group with confirmed histological diagnosis (Table 1) and also showed a significant correlation with the gastric atrophy in the group with serological diagnosis (Table 3), which supports the validity of this finding. A recent multi-ethnic non-European study, including subjects from Japan, China, Tanzania and the Dominican Republic, and using diagnostic criteria very similar to ours, showed wide variation in serum 17-gastrin levels, related to sex, age and the country of origin, and also demonstrated a significant positive correlation between the level of 17-gastrin and the presence of gastric atrophy[31]. If these results are confirmed through correlation with histology, 17-gastrin may be as useful as PG-1 level to diagnose corpus-predominant gastric atrophy in some populations.

The serum level of PG-1 is also affected by antagonistic influences. Our study and other previous studies[32] confirm that *H pylori* infection, and also the use of proton pump inhibitors[33], raise the level of serum PG-1, which could mask the diminishment determined by gastric atrophy and thereby explain the limited sensitivity of the method.

It is pertinent to ask whether the diagnostic performance demonstrated in the initial sample, constituted of selected, symptomatic, old-aged patients with a high frequency of gastric atrophy (60%) and therefore a relatively low frequency of *H pylori* infection, can be extrapolated to an asymptomatic population sample. To dispel this doubt, it would be necessary to certify histologically the frequency of gastric atrophy in a representative sub-sample of the latter group, which we expect to do soon. In the meantime, we restricted the diagnosis of gastric atrophy only to those subjects who simultaneously had PG-1 levels and PG-1/PG-2 ratios below the previously determined cut-off levels, a more demanding and restrictive diagnostic criterion that has been suggested to minimize the risk of false positive[17]. There is no reliable data with respect to the frequency of gastric atrophy in the Chilean population. A recent study from Japan, a country with which we share an elevated risk for GC, using the same methodology, demonstrated the frequencies of *H pylori* infection and gastric atrophy for the age groups very similar to ours[34].

The demographic analysis of the three proposed risk groups, that were determined serologically, showed that they differed significantly in some variables recognized as associated with GC risk, such as age[35], gender and educational level[36], which indirectly supports the validity of this categorization. Our suggestion is that the group with evidence of gastric atrophy should be followed up and studied preferentially through upper GI endoscopy and biopsies. This non-invasive assessment of GC risk has been evaluated in a recent Japanese study[37], showing that the subjects with serologically detected gastric atrophy, either with or without *H pylori* infection, had annual incidence of gastric cancer significantly higher than those subjects without atrophy. As has been recommended[38], focusing invasive and more expensive diagnostic methods on high-risk groups significantly increases the chance of detecting early GC, that in our country comprised less than 10% of the diagnosed cases[2,39] and merely increased to 15% in the only population-based study performed by Llorens[40] between 1978 and 1986 in non-selected volunteers. As long as the precocity of the diagnosis is not modified, it will not be possible to change the disappointing prognosis of GC in Chile, with a 5-year survival rate after diagnosis, ranging between 3% and 12%[2,39]. In contrast, a recent Japanese population screening study, using serum PG and radiology as diagnostic the methods, detected 88% of early GC with a much lower cost than the conventional screening and concluded that this type of program would be very beneficial for high-risk populations[41].

It is known that Chile has one of the highest mortality rates for GC in the world[42]. Nevertheless, the RR is markedly heterogeneous at the regional and county level, ranging between 0.26 and 2.25. Apparently, the greater part of this difference is explained by the higher frequency of *H pylori* infection, mainly in young people, in high-risk counties (Ferreccio *et al* submitted). The eradication of *H pylori* in this stage, before the development of gastric atrophy, would probably reduce the incidence of GC, as has been demonstrated in a Chinese population[19], although it is a strategy of doubtful practical feasibility given the Chilean population's high frequency of infection (73%). The frequency of gastric atrophy, a late consequence of infection, was expected to be greater in the high-risk counties which could not be confirmed in this study, probably due to an insufficient sample size.

In summary, our results confirmed the diagnostic usefulness of the serum levels of pepsinogen and gastrin for the non-invasive detection of corpus-predominant gastric atrophy in the symptomatic patients, showed its

feasibility in an asymptomatic population and suggested that the method could be useful to identify the groups with higher risks of developing GC, in which preventive and control measures can be focused. It is probable that successive PG and/or 17-gastrin determinations (annually or biannually), confirming low levels or showing a decreasing tendency, would lead to even better diagnostic performance. The incorporation of this method to preventive health examination in a selected population (i.e., men, older than 50, residents in high-risk counties) could probably increase the proportion of GC diagnosed in the early or incipient phase, the only method to improve the prognosis of this serious and frequent disease.

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RAPID COMMUNICATION

Ultrasound guided percutaneous cholecystostomy in high-risk patients for surgical intervention

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Abstract

AIM: To assess the efficacy and safety of ultrasound guided percutaneous cholecystostomy (PC) in the treatment of acute cholecystitis in a well-defined high risk patients under general anesthesia.

METHODS: The data of 27 consecutive patients who underwent percutaneous transhepatic cholecystostomy for the management of acute cholecystitis from January 1999 to June 2003 was retrospectively evaluated. All of the patients had both clinical and sonographic signs of acute cholecystitis and had comorbid diseases.

RESULTS: Ultrasound revealed gallbladder stones in 25 patients and acalculous cholecystitis in two patients. Cholecystostomy catheters were removed 14-32 d (mean 23 d) after the procedure in cases where complete regression of all symptoms was achieved. There were statistically significant reductions in leukocytosis, ($13.7 \times 10^3 \pm 1.3 \times 10^3$ μg/L *vs* $13 \times 10^3 \pm 1 \times 10^3$ μg/L, $P < 0.05$ for 24 h after PC; $13.7 \times 10^3 \pm 1.3 \times 10^3$ μg/L *vs* $8.3 \times 10^3 \pm 1.2 \times 10^3$ μg/L, $P < 0.0001$ for 72 h after PC), C -reactive protein (51.2 ± 18.5 mg/L *vs* 27.3 ± 10.4 mg/L, $P < 0.05$ for 24 h after PC; 51.2 ± 18.5 mg/L *vs* 5.4 ± 1.5 mg/L, $P < 0.0001$ for 72 h after PC), and fever (38 ± 0.35℃ *vs* 37.3 ± 0.32℃, P < 0.05 for 24 h after PC; 38 ± 0.35℃ vs 36.9 ± 0.15℃, $P < 0.0001$ for 72 h after PC). Sphincterotomy and stone extraction was performed successfully with endoscopic retrograde cholangio-pancreatography (ERCP) in three patients. After cholecystostomy, 5 (18%) patients underwent delayed cholecystectomy without any complications. Three out of 22 patients were admitted with recurrent acute cholecystitis during the follow-up and recovered with medical treatment. Catheter dislodgement occurred in three patients spontaneously, and two of them were managed by reinsertion of the catheter.

CONCLUSION: As an alternative to surgery, percutaneous cholecystostomy seems to be a safe method in critically ill patients with acute cholecystitis and can be performed with low mortality and morbidity. Delayed cholecystectomy and ERCP, if needed, can be performed after the acute period has been resolved by percutaneous cholecystostomy.

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Key words: Percutaneous cholecystostomy; Acute cholecystitis; Ultrasound; High risk; Elderly

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INTRODUCTION

Cholecystectomy is the appropriate treatment of acute calculous and acalculous cholecystitis, and it has a mortality rate of 0%-0.8%[1,2]. However, the mortality rate of surgical treatment may be as high as 14%-30% in elderly or critically ill patients with comorbid diseases[3,4].

Percutaneous cholecystostomy (PC) has been introduced as an alternative method to treat acute cholecystitis in patients with significant comorbid diseases[5-8]. PC can be achieved with the guidance of either computed tomography (CT) or ultrasonography (USG). PC, which was initially described by Radder in 1980, has proved to be an effective treatment for acute cholecystitis in critically ill patients, and it has low morbidity and mortality rates[9].

In this study, we have retrospectively evaluated the clinical data of patients treated with PC for acute cholecystitis. The aims of the study were to investigate the efficiency of PC in the treatment of acute cholecystitis in critically ill patients, the ratio of delayed cholecystectomy, and the clinical outcome of patients who had no further interventions following PC.

MATERIALS AND METHODS

Between January 1999 and June 2003, percutaneous transhepatic cholecystostomy was performed on 27 elderly patients with acute cholecystitis and comorbid diseases. All patients received physical examination, whole blood count, biochemical analysis, and ultrasonography. Age, gender, comorbid diseases, etiology of cholecystitis, clinical and laboratory findings, morbidity associated with PC, and the clinical outcome were recorded. The diagnostic criteria of acute cholecystitis included clinical signs and symptoms (right upper quadrant pain or tenderness), leukocytosis or positive C - reactive protein (CRP) (> 5 mg/L) value, and at least one of the ultrasonographic criteria including gallbladder stones (for calculous cholecystitis), ultrasonographic Murphy's sign, gallbladder wall thickening (> 3 mm), pericholecystic fluid, and gallbladder distension. All patients were given empiric intravenous Ampicillin-sulbactam (1 g/d four times) treatment starting immediately after the diagnosis, and subsequently antibiotic therapy was managed according to the culture results of bile samples. All of the procedures were performed in the Department of Radiology. None of the patients were premedicated and the procedure was performed under local anesthesia.

Risk classification

The American Society of Anesthesiologists (ASA) physical status classification was used to define the risk for cholecystectomy. Twenty (74.1%) patients were classified as ASA Ⅲ and 7 (25.9%) as ASA Ⅳ.

Procedure

Under aseptic conditions, a USG guided transhepatic approach through the right lobe was used to access the gallbladder in all patients. After puncture of the bile duct with an 18 GA Seldinger needle, a guide-wire (Amplatz, 0.035 inch, 75 mm) was inserted into the gallbladder via the lumen of the Seldinger needle. The route was then dilated using 8F and 10F dilatators. Finally a 10F locking pigtail catheter was inserted into and locked within the gallbladder. Catheter localization was controlled by fluoroscopy (by injecting a small amount of contrast) and USG. Bile samples were obtained for cultures. The catheter was flushed with 10 mL of a saline-gentamycin (40 mg) mixture every 6-8 h to avoid obstruction, and the catheter was left for gravity drainage.

On the second day of the PC, USG was performed to check the site of the catheter, look for bile or blood leakage, and to observe the ultrasonographic findings following PC. Positive clinical response was defined as the normalization of at least two of the three clinical parameters of acute cholecystitis (abdominal pain, fever, and leukocytosis) and a decrease in CRP value within 72 h.

Statistical analysis

Demographic data are given as medians (range), whereas the other data are expressed as means ± SE. Differences were evaluated using the Wilcoxon signed ranks test. $P < 0.05$ was accepted as significant. Data were analyzed using statistical software (SPSS for windows 10.0; SPSS, Chicago, IL, USA).

Table 1 Comorbid diseases of the patients

Comorbid diseases	*n*
Cardiac disorders[1]	19
Haematological diseases[2]	2
Chronic obstructive pulmonary failure	1
Polytrauma	1
Diabetes mellitus and chronic renal failure	4
Total	27

[1]Congestive heart failure, hypertension, ischemic heart diseases, valve replacement; [2]Chronic myeloid leukemia, thrombotic thrombocytopenic purpura.

Table 2 White blood cell (WBC), C-reactive protein (CRP), and axillary temperature of the patients before and after PC

	Before PC	24 h after PC	72 h after PC
WBC × 10^3/μL	13.7 ± 1.3	13 ± 1	8.3 ± 1.2
CRP mg/L	51.2 ± 18.5	27.3 ± 10.4	5.4 ± 1.5
Body temp. (℃)	38 ± 0.35	37.3 ± 0.32	36.9 ± 0.15
		$P < 0.05$	$P < 0.0001$

RESULTS

The median age of the patients was 71.4 years (range 64-93 years) and the female/male ratio was 4.4/1 (22/5). Two (7.4%) patients had acalculous and 25 (92.5%) had calculous cholecystitis. Comorbid diseases included congestive heart failure or severe ischemic heart disease in 19 (70.3%) patients, haematologic diseases [chronic myeloid leukemia (CML) and thrombotic thrombocytopenic purpura (TTP)] in 2 (7.4%) patients, renal failure in 4 (14.8%), chronic obstructive respiratory failure in one (3.7%), and polytrauma in one (3.7%) patient (Table 1). The majority (96.3%, $n = 26/27$) of the patients had pain and tenderness in the right upper quadrant.

The mean white blood cell counts (WBC) upon admission ($13.7 \times 10^3 \pm 1.3 \times 10^3$/μL), axillary temperatures (38 ± 0.35℃), and CRP (51.2 ± 18.5 mg/L) values were significantly decreased in the 72 h following PC [$8.3 \times 10^3 \pm 1.2 \times 10^3$/μL ($P < 0.0001$), 36.9 ± 0.15℃ ($P < 0.0001$), 5.4 ± 1.5 mg/L ($P < 0.0001$), respectively] (Table 2).

The clinical and ultrasonographic findings of acute cholecystitis decreased in all patients. Bile cultures were negative in 5 (18.5%) patients. *E.coli* was the only bacteria in 17 (63%) patients and *E.coli, Enterobacter,* and *Enterococci* were identified in the remaining 5 (18.5%) patients.

The median hospitalization time was 8 d (1-20 d) and the median catheterization period was 23 d (14-32 d). Catheter dislodgement occurred in three patients on the 6th, 8th, and 14th d. Catheters were reinserted in two patients due to prompt recurrent cholecystitis but the other patient did not need recatheterization. Unexpected bleeding from the liver parenchyma occurred in only one (3.7%) patient following catheter removal. USG guided percutaneous drainage of a hematoma in the subhepatic region was performed in this patient and no further complications occurred.

Cholecystocholangiography was performed prior to the removal of the catheter to visualize the bile tree and gallbladder in 24 (88.8%) patients. This procedure could not be performed in 2 patients due to noncompliance and difficult mobilization (polytrauma) in one patient with acalculous cholecystitis. The cystic duct was patent in 16 (66.7%) and occluded in 8 (33.3%) patients. Common bile duct stones were detected in 3 (11.1%) patients.

These 3 patients underwent sphincterotomy and stone extraction from the common bile duct with ERCP. Delayed cholecystectomy was performed in 5 (18.5%) patients within a mean period of 2 mo following PC. The postoperative outcome was uneventful in these patients. The remaining 22 patients were followed up by physical examination and USG monthly for a median of 11 (6-18) mo after the removal of the catheter. Three of these 22 patients experienced recurrent cholecystitis and recovered with medical therapy (Table 3).

The morbidity rate of PC was 25.9% [catheter dislodgement (n = 3), recurrent cholecystitis (n = 3), and bleeding (n = 1)] and there was no mortality associated with PC.

Table 3 Follow-up of the patients after PC

	Calculous cholecystitis (n = 25)	Acalculous cholecystitis (n = 2)
No further treatment	18 (72%)	2
Recurrence	3 (12%)	0
Reinsertion of the catheter	2 (8%)	0
Medical treatment	1 (4%)	0
ERCP	3 (12%)	0
Delayed surgery	5 (20%)	0

DISCUSSION

PC was defined as an alternative treatment method in patients with acute cholecystitis who were at high risk during surgery due to comorbid diseases. The morbidity and mortality associated with emergent cholecystectomy is considerably higher in such patients, 55%-66% and 14%-30% respectively[3,4]. The morbidity and mortality rates of PC were found to be much lower, less than 10% and 2% respectively[10,11]. PC resulted in successful treatment in 56%-100% of patients with acute cholecystitis and significant comorbid diseases[12-17]. The therapeutic rate of PC was significantly higher in patients with clinical signs and symptoms referred to the right upper abdomen compared to patients without clinical signs and symptoms (intensive care patients)[12]. Although unexplained sepsis regressed dramatically after PC in approximately 60% of intensive care patients, the therapeutic response to PC is lower in patients with symptoms and signs in the right upper abdomen who received open cholecystectomy[11,12]. In this study, morbidities were seen in 7 (25.9%) patients, but 3 of these complications were due to catheter squeezing as a result of patient carelessness. There was no procedure-related mortality.

PC can be easily performed under local anesthesia with USG or CT guidance. Two ways of accessing the gall bladder were defined for the procedure. Either a transhepatic or transperitoneal approach can be used. The complications related to the procedure include bile duct injury, bile leakage and peritonitis, portal or parenchymal vessel injury and bleeding, catheter dislodgement, colon injury, and vagal reactions. The transhepatic approach decreases the risk of bile leak, portal vessel injury, and colon injury but it carries a risk of pneumothorax and bleeding from the liver parenchyma[18-20]. Although the transperitoneal approach decreases the risk of bleeding and secondary liver contamination by infected bile, it increases the risk of bile peritonitis, colon perforation, portal vessel injury, and displacement of the catheter after decompression of the gall bladder[21-23]. In addition to other complications, van Sonnenberg *et al* also reported vagal reactions in the transperitoneal approach due to vagal innervation of the gall bladder wall[24]. Therefore, the transhepatic approach seems to be the best approach for PC except in the presence of severe liver disease and coagulopathy[25-27]. In this study the transhepatic approach was preferred to access the gall bladder. All procedures were completed under local anesthesia without any complications related to the procedure.

We performed cholecystocholangiography in 24 patients. The cystic duct was patent in 16 (66.7%) patients. Common bile duct stones which could not be visualized by USG, were detected in three (11.1%) of these 16 patients. These three patients underwent sphincterotomy and stone extraction with ERCP. Bleeding from the liver parenchyma was detected unexpectedly in only one (3.7%) patient following removal of the catheter. No bile leakage was detected in any patient.

The management of patients after the acute cholecystitis period is still controversial. Lebigot *et al* followed up 90% of patients for 12 mo after PC and reported only one (6.25%) endoscopic sphincterotomy and one (6.25%) delayed cholecystectomy[28]. No additional therapy was needed in the remaining 87.5% of the patients. The risk of recurrent cholecystitis was reported as 12% at 1 year in a study by Welch *et al*, and the authors mentioned PC as a possible definitive treatment in patients with acalculous cholecystitis[29]. In a recent study, the authors were able to apply delayed surgery in 56.4% of patients after the acute period[6]. In this study, we reevaluated the patients after regular medical treatment of the comorbid diseases. Five (18.5%) patients were then selected for delayed elective surgery and the remaining 22 were followed. Nineteen (86.4%) of these 22 patients required no additional therapy. Recurrent cholecystitis was detected in 3 (13.6%) patients and the symptoms regressed with medical therapy.

In conclusion, PC is a fast, easy, effective, and safe treatment method for the acute phase of cholecystitis in elderly and critically ill patients. Procedure-related morbidity and mortality is very low compared to surgery. Patient management after the acute phase of cholecystitis is still controversial. Conservative treatment for patients who are not suitable for surgery is acceptable. Delayed elective cholecystectomy is another option in patients who respond well to medical treatment of comorbid diseases.

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RAPID COMMUNICATION

Efficacy of leukocyte esterase dipstick test as a rapid test in diagnosis of spontaneous bacterial peritonitis

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Abstract

AIM: To evaluate the efficacy of dipstick test in diagnosis of spontaneous bacterial peritonitis (SBP) in cirrhotic patients who underwent abdominal paracentesis based on the locally available dipstick test.

METHODS: There were 200 consecutive samples from cirrhotic patients who underwent abdominal paracentesis. Urine dipstick (Combur[10] Test®M, Roche, Mannheim, Germany) was used as a screening test. A manual cell count with differential study was done in all samples by experienced technicians. The polymorphonuclear (PMN) cell count more than 250 cells/mm^3 was used as a diagnostic cut off level. One to three plus dipstick results were used as cut off levels for a positive result. The dipstick test results had to be agreed by three experienced readers. The sensitivity, specificity, positive and negative predictive values and accuracy of two different colorimetric cut off scales (1+ and 2+) were calculated and compared.

RESULTS: The prevalence of SBP diagnosed by manual cell count was 21.0%. There were 128 specimens that had a true negative result by dipstick. The sensitivity, specificity, positive and negative predictive values and accuracy of 1+ and 2+ cut off scale to diagnose SBP were 88%, 81%, 55%, 96% and 83% respectively, and 63%, 96%, 82%, 81% and 89% respectively.

CONCLUSION: Dipstick test can be used as a rapid test for screening of SBP. The higher cut off colorimetric scale has a better specificity and positive predictive value but a lower sensitivity.

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Key words: Spontaneous bacterial peritonitis; Diagnosis; Dipstick; Screening

INTRODUCTION

Apart from variceal bleeding, spontaneous bacterial peritonitis (SBP) is another serious complication that can develop in cirrhotic patients. Prompt diagnosis and treatment are essential for the survival of patients with SBP[1,2]. Unfortunately, symptoms of SBP including fever, abdominal pain, nausea, and vomiting are not presented in all cirrhotic patients who develop SBP[3,4]. In addition, many hospitalized cirrhotic patients develop SBP during their non-SBP related admissions especially those with gastrointestinal bleeding[5]. Therefore, routine diagnostic paracentesis is the recommended practice for patients with ascites who develop signs or risk factors for SBP. The standard criteria for diagnosis of SBP are an ascitic fluid polymorphoneuclear (PMN) cell count of $\geqslant$ 250/mm^3 and/or a positive ascitic fluid bacterial culture[6-8]. Due to the nature of bacterial culture, the result is not available within a day. Therefore, decision making for SBP treatment is mainly based on PMN cell count. However, manual ascitic fluid PMN cell count is not always available in some small patient care units especially in the out patient offices and sometimes it cannot be done as in emergency basis.

Recently, leukocyte esterase activity testing by dipstick has been used for a rapid diagnosis of infection in many body fluids such as urine, pleural fluid, and cerebrospinal fluid[9-11]. The leukocyte esterase released from PMN cells reacts with an esterified chemical compound in the reagent strip yielding a violet azo dye, the intensity of which correlates to leukocyte count[12]. Recently, many studies have shown the efficacy of dipstick in diagnosing SBP[13-19]. However, strip tests used in these studies are not the same and have different colorimetric scales. Therefore, the sensitivity and accuracy of different dipsticks may vary. To date, there has been no recommended calorimetric scale for each dipstick to diagnose SBP.

The aim of this study was to evaluate the usefulness of dipstick in rapid diagnosis of SBP in cirrhotic patients who underwent abdominal paracentesis based on the locally available dipstick test and to define the validity scores from 2 different thresholds of colorimetric scales.

MATERIALS AND METHODS

During the period between July 2004 and November 2005 at King Chulalongkorn Memorial Hospital, 200 consecutive ascitic fluid specimens (including 21 from out patient unit) were collected from 127 cirrhotic patients (Table 1). The clinical indications for paracentesis were routine paracentesis (n = 95), relief of patient's discomfort (n = 54), suspected SBP (n = 13), miscellaneous (n = 38). The diagnosis of cirrhosis was established according to the histologic criteria or analytical, clinical, and ultrasonographic findings of the liver. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the University.

Immediately after paracentesis, fresh ascitic fluid specimen was collected in a clean dry container and tested using a dipstick (Combur[10] Test®M, Roche, Mannheim, Germany) for granulocyte esterase designed for urine analysis. The strip has a colorimetric 4-grade scale (negative, 1+ to 3+) and is read at 90 s for granulocyte esterase activity. The procedure was the same as what the manufacturer described for urine.

Each of the colorimetric scale readings was graded by 2 well trained on-duty internists (the reading processes were scored separately). All the readings were confirmed by WR who is a house staff covering for this project. WR made the final decision for all discrepancy results. A correlation between PMN cell count and the 4-grade scale was suggested by the manufacturer as follows: negative, 0 PMN cell/mm^3; 1+, 10-25 PMN cells/mm^3; 2+, 75 PMN cells/mm^3; 3+, 500 PMN cells/mm^3.

Ascitic fluid was conventionally processed including cytology, PMN cell count and lymphocyte count, and appropriate biochemical tests (glucose, protein, albumin, lactic dehydrogenase, and sugar). The sample for PMN and total leukocyte count was collected into a heparin anticoagulant tube and analyzed within 4 h of extraction. Differential cell count and cytology were examined with a conventional optical microscope. A manual cell count with differential study was done in all samples by experienced technicians.

Ascitic fluid cultures were performed by inoculation of at least 10 mL fluid in VersaTrek® REDOX 1® (TREK Diagnostic Systems, Cleveland, OH). All bottles were processed in a standard fashion according to the manufacturer's guideline.

SBP was defined as PMN cell count in ascitic fluid $\geqslant$ 250/mm^3. The 1+ or 2+ result of the leukocyte esterase from dipstick was considered as a positive test. The negative result from dipstick was considered as a negative test. We also defined bacterascites as the combination of a positive ascitic fluid culture, PMN cell count in ascitic fluid < 250/mm^3, and no evident intra-abdominal surgically treatable source of infection. In patients with hemorrhagic ascites (i.e. ascites red blood cell count > 10000/mm^3), a subtraction of one PMN leukocyte per 250 red blood cells was made[6]. Secondary peritonitis was suspected when there was an abdominal source of infection, acute pancreatitis, or more than one organism in the ascitic fluid culture.

Table 1 Characteristics of 127 patients and 200 ascitic fluid specimens

Characteristics	n (%)
Male/Female	75/52
Age (mean + SD)	57.4 ± 13.3
Child-Pugh classification A/B/C	3/34/90
Etiology of cirrhosis	
Hepatitis B	34 (26.8)
Hepatitis C	8 (6.3)
Alcohol	25 (19.7)
Alcohol and hepatitis B	9 (7.1)
Alcohol and hepatitis C	9 (7.1)
Hepatitis B and hepatitis C	1 (0.8)
Autoimmune	2 (1.6)
Hemochromatosis	2 (1.6)
Cryptogenic	10 (7.9)
Others	27 (21.3)

Statistical analyses

Results of dipstick test were compared to PMN cell count, ascitic fluid culture and clinical data in all patients. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of dipstick in the diagnosis of SBP from two different colorimetric scales (1+ and 2+) were calculated and compared (Figure 1).

RESULTS

We diagnosed 42 episodes of SBP by PMN cell count, of which 37 (88.1%) was diagnosed using 1+ cut off scale and 27 (64.3%) 2+ cut off scale. Of the 158 specimens with PMN cell count < 250/mm^3, 128 had a true negative result by dipstick test (Table 2). Five patients were diagnosed as SBP by manual cell count but had a negative dipstick test result.

Sixteen specimens had a positive culture for bacteria, of which four were compatible with bacterascites. The dipstick test results in these bacterascites specimens were all negative. No secondary bacterial peritonitis was diagnosed in this series. The most common organism growing from these specimens was *E. coli* (Table 3).

The sensitivity, specificity, PPV, NPV and accuracy of 1+ and 2+ cut off scales to diagnose SBP were 88%, 81%, 55%, 96% and 83% respectively, and 63%, 96%, 82%, 91% and 89% respectively (Table 4).

DISCUSSION

Due to the high rates of morbidity and mortality from delayed treatment in cirrhotic patients who suffered from SBP, diagnostic tests for early diagnosis are very important[20,21]. Currently, ascitic fluid PMN count is the standard tool for SBP treatment decision making. However, several-hour delay in reporting the result may arise from the laboratory department. In addition, this test may not be available in a small out patient setting.

Leukocyte esterase has been shown as an important marker for PMN cell activity[22]. Originally, the purpose

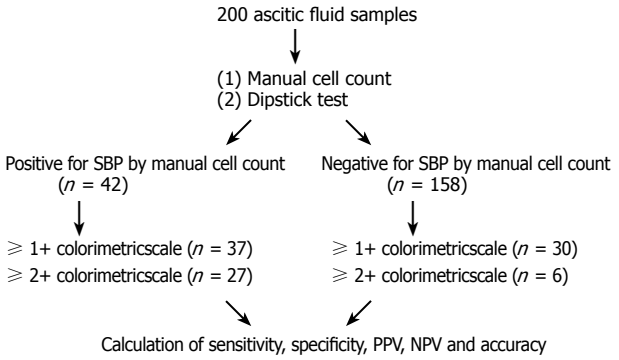

Figure 1 Flow diagram for SBP evaluation.

Table 2 Dipstick test results in SBP and non-SBP groups

Colorimetric scales	PMN cell count ⩾ 250/mm^3	PMN cell count < 250/mm^3	Total
1+	10	24	34
2+	19	6	25
3+	8	0	8
0	5	128	133
Total	42	158	200

of leukocyte esterase test was limited to urine analysis[23]. Recently, it has been found to be useful across the wide range of body fluid infections[9-11]. The efficacy of this test in detecting infection in the ascitic fluid has been confirmed from many centers[14-19]. The overall sensitivity, specificity, PPV and NPV of this test are approaching 90% (Table 5)[14-19]. However, the different commercial dipsticks used in previous studies had different colorimetric scales for PMN cell count. In addition, the cut off colorimetric scale for each dipstick has not been standardized yet.

To date, there has been no dipstick that was specifically designed to use in ascitic fluid. Moreover, many dipsticks do not have a precise colorimetric scale for 250 PMN cells/mm^3. In our study, we used the Combur^{10} Test®M that is widely available in Thailand as a screening test for SBP. According to the manufacturer's guideline, the 1+, 2+ and 3+ scales are equivalent to 10-25 PMN cells/mm^3, 75 PMN cells/mm^3 and ⩾ 500 PMN cells/mm^3 respectively. We chose the lower scales (1+ and 2+) as cut off levels for SBP diagnosis. The limitation of the Combur^{10} Test®M is an absence of precise calorimetric scale for the cut off level of PMN cell count at ⩾ 250/mm^3. Because of this limitation, we speculated that our specificity might be lower than others[17-19]. Although our sensitivity (88%) from 1+ cut off level was comparable to others, the specificity and PPV were lower (81% and 55% respectively) compared to other studies[17-19]. By using 2+ scale as a cut off level for SBP, our specificity and PPV increased (96% and 82% respectively), unfortunately the sensitivity decreased to only 63%. Anyhow, NPV and accuracy from these two cut off levels were comparable to other series[17-19].

Aution stick (A. Menarini Diagnostics, Firenze, Italy) is another dipstick that can be read at 90 s. The benefit of the Aution stick over the Combur^{10} Test®M is the precise colorimetric scale that correlates with ⩾ 250 PMN cells/mm^3. The correlation between PMN cell count and colorimetric scale suggested by the manufacturer are as follow: grade 0, 0 PMN cell/ mm^3; grade 1, 25 PMN cells/ mm^3; grade 2, 75 PMN cells/ mm^3; grade 3, 250 PMN cells/mm^3; and grade 4, 500 PMN cells/mm^3. Catellote *et al*[15] studied the use of Aution sticks for diagnosis of SBP in cirrhotic patients with ascites who underwent abdominal paracentesis at a university based hospital, and found that the sensitivity, specificity and PPV are 89%, 99% and 98%, respectively[15].

Table 3 Results of dipstick tests and PMN cell counts in positive culture specimens

Patient	PMN cells/mL	Calorimetric scale	Culture result
1	14	0	*A.Baumanii*
2	20	0	*S.agalactae*
3	21	0	*P.aeruginosa*
4	100	0	*K.pneumonia*
5	254	0	*E. coli*
6	271	0	*S.bovis*
7	257	0	*K.pneumoniae*
8	672	1	*E. coli*
9	3640	1	*P.aeruginosa*
10	13312	1	*E. coli*
11	2129	2	*Streptococcus gr.D*
12	3494	2	*Streptococcus gr.D*
13	12840	2	*P.aeruginosa*
14	25805	2	*E. coli*
15	41990	2	*E. coli*
16	3398	3	*E. coli*

Table 4 Validity scores of the Combur^{10} Test®M to diagnose SBP considering a positive test > 1 or > 2 in the 4-grade colorimetric scale

Variables	⩾ 1 grade, %	⩾ 2 grade, %
Sensitivity	88	63
Specificity	81	96
Positive predictive value	55	82
Negative predictive value	96	91
Accuracy	83	89

Multistix®10SG (Bayer Diagnostics Corporation, Puteaux, France) has colorimetric scales as follow: grade 0, 0 PMN cell/ mm^3; grade 1, 15 PMN cells/mm^3; grade 2, 70 PMN cells/ mm^3; grade 3, 125 PMN cells/ mm^3; and grade 4, 500 PMN cells/mm^3. Butani *et al*[24] used the Multistix®10SG to diagnose SBP in 136 specimens by using grade 2 as a cut off scale, and found the sensitivity, specificity, PPV and NPV of the Multistix®10SG are 83%, 99%, 91% and 98% respectively. Although, the Multistix®10SG has no precise colorimetric scale for 250 PMN cells/ mm^3, its specificity was still better than that of the Combur^{10} Test®M in our series. This may be due to a closer 250 PMN/mm^3 colorimetric scale of Multistix® 10SG (grade 3+ ⩾ 125 PMN cells/mm^3).

There are many factors that influence the accuracy of the dipsticks. False positive result can result from alteration of pH, osmolality and temperature of the specimens[25].

Table 5 Validity scores reported in previous studies

Authors	Castellote *et al*	Kim *et al*	Butani *et al*	Sapey *et al*	Thévenot *et al*
Dipsticks	Aution sticks	UriSCAN/Mutistix10SG	Multistix®10SG	Nephur test/ Multistix10SG	Multistix 8SG/Combur²test LN (combined result)
Inclusion scale	⩾ 3 (PMN ⩾ 250/mm³)	⩾ 3 (PMN ⩾ 500/mm³) / ⩾ 3 (PMN ⩾ 75/mm³)	⩾ 2 (PMN ⩾ 70/mL mm³)	N/A	⩾ 3 (PMN ⩾ 125/mm³) / ⩾ 2 (PMN ⩾ 75/mm³)
Sensitivity (%)	89	67/50	89	88/65	89
Specificity(%)	99	100/100	99	100/100	100
PPV (%)	98	100/100	89	94/92	100
NPV (%)	97	89/87	99	99/97	99

In addition, non-leukocyte cells can release esterase and produce a false positive result[26]. Moreover, antibiotics can produce both false positive and negative results[27]. The false negative result in the present series was found in 5 patients (12%), which is similar to the series presented by Sapey *et al*[19] None of our patients with false negative result received antibiotics prior to abdominal paracentesis. Interestingly, 3 specimens had a manual PMN cell count between 250/mm^3 and 300/mm^3 (data not presented), suggesting that the smaller number of PMN cells in these specimens may lead to a false negative result. Campillo *et al*[28] demonstrated that the sensitivity of dipsticks remains low with PMN cell count ⩽ 1000/mm^3 when they used Multistix 8 SG and Combur 2 LN as dipstick tests.The false positive rate in our series was 19% which is higher than that of Sapey *et al*[19], who found that only one patient form each test (Nephur-test® and MutistixSG®) had a false positive result. The possible explanation for a better result from their series is a much closer 250 PMN cells/mm^3 of their dipstick scales. Again this information helps to confirm that a precise colorimetric scale for 250 PMN/mm^3 is essential for an ideal dipstick to detect SBP.

Currently, there is no specific dipstick test for bacterial count in the ascitic fluid. In our series, it is not surprised that the correlation between the culture and dipstick results was very poor. There were 4 specimens that were compatible with a condition generally called "monomicrobial non-neutrocytic bacterascites (MNB)". All the MNB specimens had a negative dipstick result. Generally, the clinical outcome of asymptomatic MNB is much better than that of classic SBP[29]. Our 4 patients with MNB did not receive any treatment and were asymptomatic during 3-6 mo follow-up. In addition, repeat ascitic fluid analysis and culture were performed and reported unremarkable results in these 3 patients.

Patients with ⩾ 250 PMN cells/mm^3 in the absence of bacterial growth from the culture are classified as culture-negative neutrocytic ascites (CNNA). In the present series, only 12 patients (28.6%) had a classic SBP (positive culture and cell count ⩾ 250 PMN cells/mm^3). Of these culture negative SBP patients, at least 25 (83.3%) had CNNA (no history of prior antibiotics use), demonstrating that CNNA has the same clinical course as classic SBP[6]. Therefore, dipstick that determines mainly for PMN cell activity can be used for SBP screening.

In conclusion, dipstick test in ascitic fluid from cirrhotic patients is a good screening tool for SBP. However, not all dipsticks have a precise colorimetric scale for a measurement of ⩾ 250 PMN cells/mm^3, therefore the sensitivity of different dipsticks may not be similar. With a good NPV the decision making not to treat SBP based on the results of Combur^{10} Test®M can be made in the majority of cases. Further study is needed to improve the efficacy of the test in detecting PMN cells and bacteria in ascitic fluid.

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COMMENTS

Background

The decision making for SBP treatment is mainly based on PMN count. However, manual ascitic fluid PMN count is not always available in every patient care unit.

Research frontiers

Leukocyte esterase activity testing by dipstick has been used for a rapid diagnosis of infection in a variety of body fluids such as urine, pleural fluid, and cerebrospinal fluid. The efficacy of this test in detecting infection in the ascitic fluid has been confirmed from many centers. The overall sensitivity, specificity, PPV and NPV of these tests are approaching 90%.

Innovations and breakthroughs

The sensitivity and NPV of dipstick test is excellent in all different types of stick. However, some have a slightly better value due to their approximity to the 250 cell/mm^3 cut-off level.

Applications

The dipstick may be used as a screening tool for SBP. A better designed test is awaited.

Peer review

The study used Combur as a screening test. The sensitivity of this test is slightly poorer than others. However, the other validity scores are comparable. The author may need to continue to explore other dipsticks and compare their results using manual cell count as a gold standard.

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RAPID COMMUNICATION

Expression of altered retinoblastoma protein inversely correlates with tumor invasion in gastric carcinoma

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Abstract

AIM: To investigate the clinical and pathological significance of altered retinoblastoma (Rb) encoding protein (pRb) in gastric carcinoma.

METHODS: Expression of altered pRb was analyzed in 91 patients with gastric adenocarcinoma by immunohistochemistry.

RESULTS: Sixty-five percent (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Moreover, strong expression of altered pRb was found in 35% (6/17), 24% (5/21), 17% (8/46) and 0% (0/7) of T1, T2, T3 and T4 gastric carcinomas, respectively. Altered pRb expression was inversely correlated with the depth of tumor invasion (P = 0.047). Degree of immunoreactivity had no significant correlation with tumor grade, node metastasis and distant metastasis. In terms of prognostic significance, univariate analysis showed that poor differentiation [41 (66.1%) *vs* 34 (42.5%) P = 0.051], advanced tumor stage (P < 0.001) and weakly altered pRb expression [17 (80.5%) *vs* 58 (49.6%) P = 0.044] were associated with worse prognosis in these patients. However, multivariate analysis revealed that advanced tumor stage was the only independent poor prognostic factor (P < 0.001).

CONCLUSION: The mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma.

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Key words: Gastric adenocarcinoma; Altered retinoblastoma protein; Immunohistochemistry

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INTRODUCTION

Cellular proliferation normally follows an orderly progression through the cell cycle, which is governed by protein complexes encoding various genes. Malignant transformation results from a series of genetic alterations that lead to aberrant regulation of cell division (cell-cycle control), cell death (apoptosis), and maintenance of genomic integrity (DNA repair).

The retinoblastoma gene (Rb) encoding the 105kd nuclear phosphoprotein, maps to chromosome 13, band 13q14, and is a prototypical tumor-suppressor gene[1,2]. The retinoblastoma protein plays an important role in regulating the ability of cells to enter S phase, during which DNA is synthesized[3]. Although the alteration of the Rb gene has been shown to be critical for the development of retinoblastoma[4-6], dysregulation of the Rb gene is important in a wide array of human cancers, such as sarcoma[7], hepatoma[8], leukemia[9], and esophagus[10], breast[11], bladder[12], prostate[13] and lung cancers[14]. But studies concerning Rb gene change in gastric carcinoma are limited.

This study was to examine the correlation of altered pRb expression with the clinicopathological status and prognostic value of altered protein expression in patients with gastric carcinoma.

MATERIALS AND METHODS

Patients

The study population consisted of 91 patients (80 men, 11 women; mean age: 68 years; range: 32-86 years) who underwent surgery for primary gastric adenocarcinoma at our institute between 1992 and 2000. Eighteen (19.8%) tumors were located in the upper third, 19 (20.9%) in the middle third, and 54 (59.3%) in the lower third of the stomach, respectively. Following the international tumor node metastasis (TNM) staging system, 23 (25.3%)

tumors were considered stage Ⅰ, 20 (22.0%) stage Ⅱ, 28 (30.8%) stage Ⅲ, and 20 (22.0%) stage Ⅳ, respectively. Histological grade was assessed following the World Health Organization (WHO) criteria. Forty-nine (53.8%) tumors were well- or moderately- differentiated, and forty-two (46.2%) poorly- or undifferentiated.

Sixteen of the 20 stage Ⅳ patients had distant metastasis (5 peritoneal, 5 hepatic, 1 lung, 3 peritoneal and T4 lesion, 1 peritoneal and hepatic, 1 peritoneal and N3 lymph node status), while the other cases were classified as stage Ⅳ because of N3 lymph node status (n = 3) and/or T4 lesions (n = 1). Sixteen patients underwent palliative surgery (16 distant metastases). The other 75 patients underwent radical D2 resection. Of the patients 12 were treated with postoperative systemic chemotherapy (5 for 6 cycles of FEM, and 7 for 6 cycles of 5-FU). To rule out any confounding effect of adjuvant chemotherapy, this variable was entered in the multivariate analysis of survival. Follow-up evaluation consisted of physical examination, chest radiography, and tumor marker (CEA, and CA19.9) assay every 6 mo for the first 3 years and once a year thereafter, abdominal computed tomography (CT), and endoscopy once a year.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor samples taken during surgery were used for the study. pRb expression was detected immunochemically using the monoclonal antibody (M7131, DAKO, Denmark) generated from mouse anti-human Rb product. The 4-μm thick sections from their original histology blocks were eparaffinized in xylene and treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase. For immunohistochemical detection of the pRb protein, the sections were boiled for 30 min in 10 mmol/L citrate buffer solution (pH6.0) using a microwave heater for antigen retrieval. Mouse monoclonal anti-Rb (Clone Rb1, DAKO, Glostrup, Denmark) was used as primary antibody. The clone Rb1, which can be used on (archival) paraffin-embedded material, is a mouse monoclonal antibody against recombinant human retinoblastoma gene product representing amino acid 330-612 and reacts with the retinoblastoma gene product as indicated by immunoblotting SV40 transformed human tumor cell lines[15]. The sections were first incubated for 60 min at room temperature with the primary antibody. Biotinylated anti-mouse IgG was then applied as secondary antibody, followed by peroxidase-cojugated streptavidin. Demonstration of binding sites with the peroxidase reaction was achieved with 3, 3'-diaminobenzidine tetrahydrochloride (0.25 mg dissolved in 1 mL 0.02% hydrogen peroxide). Phosphate-buffered saline was used for rinsing between each step. Faint nuclear staining, sufficient to aid in orientation but not to influence the judgement of positivity, was performed with Mayer hematoxylin solution.

Evaluation of pRb protein expression

For each section, 10 high-power fields were chosen randomly, and a total of 1000 cells were evaluated by a pathologist (Tseng HH), who had no prior knowledge of the patients' outcomes or tumor characteristics. The pattern of immunoreactivity was scored on the basis of the percentage of tumor cell nuclei with positive staining. The parameter obtained was the mean relative nuclear positive area evaluated on at least 10 fields observed at a magnification of × 40.

Immunoreactivity was semiquantitatively graded. The grade scheme was: 0+ (no immunoreactive cells present), 1+ (1%-24%), 2+ (25%-49%), 3+ (50%-74%), 4+ (75%-100%) immunoreactivity cells, respectively.

In evaluation of the clinical significance of pRb expression in gastric carcinoma, tumors with grade "0+ - 2+" immunostaining were grouped as weak staining, and tumors with grade "3+ and 4+" immunostaining as strong staining.

Statistical analysis

Associations between categorical variables were analyzed using Fisher's exact test, χ^2 test, or Mann-Whitney U test. Univariate analysis of survival was carried out with a Kaplan-Meier estimator, and the difference between curves of subgroups was determined using the log-rank test. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. Independent survival variables were studied by Cox regression analysis. All data were calculated with SPSS for windows with P < 0.05 as the level of significance.

RESULTS

A total of 59 out of 91 (65%) tumors were positive for altered pRb, with 16 cases graded as "1+", 24 as "2+", 11 as "3+" and 8 as 4+". In 91 unselected primary gastric carcinomas, we compared the common prognostic markers, such as age, sex, tumor location, Lauren type, tumor grading, TNM system, and altered pRb expression. On examining the distribution of tumors with strong or weak altered pRb expressions (Table 1), no significant differences were found in sex, age, tumor site, Lauren type, and histologic grading. However, statistical analysis demonstrated a significant association between altered pRb status and tumor stage. The altered pRb protein-high tumors were more in earlier stage (stages Ⅰ and Ⅱ) than in more advanced stage (stages Ⅲ and Ⅳ) (P = 0.010). Moreover, the altered pRb status significantly correlated with depth of invasion (T status), but not with lymph node metastasis (N status), and distant metastasis (M status). The altered pRb expression was weak in 11 of the 17 (65%) T1 tumors, 16 of the 21 (76%) T2 tumors, 38 of the 46 (83%) T3 tumors, and 7 of the 7 (100%) T4 tumors. The altered pRb was inversely correlated with the depth of tumor invasion (P = 0.047).

Analysis of survival was carried out in 75 patients undergoing curative resection. The results of univariate analysis of survival in relation to clinicopathological findings are reported in Table 2. Tumor staging and altered pRb expression were correlated with 5-year survival. Kaplan-Meier curves showed that the 5-year survival was 80.5% in patients with strong altered pRb expression (n = 17) compared with 49.6% in patients with weak altered pRb expression (n = 58) (P = 0.0436). Only TNM

Table 1 Altered pRb expression and clinicopathological findings in 91 patients with gastric adenocarcinoma

Variables	*n*	Altered pRb expression, *n* (%)		*P*
		Strong	Weak	
Age				
< 70 yr	43	7 (16)	36 (84)	0.307
⩾ 70 yr	48	12 (25)	36 (75)	
Sex				
Male	80	17 (21)	63 (79)	1.000
Female	11	2 (18)	9 (82)	
Tumor location				
Upper third	18	2 (11)	16 (89)	0.553
Middle third	19	5 (26)	14 (74)	
Lower third	54	12 (22)	42 (78)	
Lauren type				
Intestinal	67	12 (18)	55 (82)	0.304
Diffuse	20	4 (20)	16 (80)	
Mixed	4	2 (50)	2 (50)	
Differentiation				
High or moderately	49	10 (20)	39 (80)	0.905
Low	42	9 (21)	33 (79)	
Tumor depth				
T1	17	6 (35)	11 (65)	0.047
T2	21	5 (24)	16 (76)	
T3	46	8 (17)	38 (83)	
T4	7	0 (0)	7 (100)	
Lymph node metastasis				
N0	28	9 (32)	19 (68)	0.109
N1	35	6 (17)	29 (83)	
N2	22	3 (14)	19 (86)	
N3	6	1 (17)	5 (83)	
Distant metastasis				
No	75	17 (23)	58 (77)	0.366
Yes	16	2 (13)	14 (87)	
TNM stage				
Ⅰ and Ⅱ	43	14 (33)	29 (67)	0.010
Ⅲ and Ⅳ	48	5 (10)	43 (90)	

Table 2 Univariate analysis of survival in 75 patients with curative resection for gastric adenocarcinoma

Variables	5-year survival, *n* (%)	*P*
Age		
< 70 yr	36 (54.0)	0.8036
⩾ 70 yr	39 (58.8)	
Sex		
Male	69 (57.2)	0.5069
Female	6 (50.0)	
Tumor location		
Upper third	14 (51.9)	0.4312
Middle third	14 (64.8)	
Lower third	47 (54.6)	
Differentiation		
Well + moderate	41 (66.1)	0.0509
Low	34 (42.5)	
TNM stage		
Ⅰ and Ⅱ	43 (88.6)	< 0.0001
Ⅲ and Ⅳ	32 (14.7)	
Altered pRb expression		
strong	17 (80.5)	0.0436
weak	58 (49.6)	

Table 3 Multivariate analysis of survival in 75 patients wirh curative resection for gastric adenocarcinoma

Variables	Category	Relative risk	95% CI	*P*
Histologic grade	Poorly *vs* well and moderately-differentiated	1.073	0.640-1.800	0.788
Altered pRB expression	Weak *vs* strong	0.891	0.443-1.791	0.746
TNM stages	Ⅲ & Ⅳ *vs* Ⅰ & Ⅱ	5.067	2.764-9.289	0.000

stage was found to be an independent prognostic factor for survival (Table 3) on multivariate analysis by Cox regression.

DISCUSSION

The half-life of mutant Rb protein is even longer than that of the wild Rb protein. This feature results in immunohistochemically detectable expression of the mutant Rb protein. It has been established that altered Rb protein expression pattern gives a fairly good estimate of the mutation frequency in the Rb gene[16,17].

In this study, 65% (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Constancia *et al*[18] reported that alterations affecting the Rb gene are rather infrequent in human gastric carcinomas, but our data indicate the altered pRb protein expression is frequent and widely variable in gastric carcinoma. We directly detected the altered Rb protein in tumor cells, which is different from the Constancia's study detecting the normal Rb protein[18]. The Rb gene mutation is frequent in various human malignancies including sarcoma[7], hepatoma[8], leukemia[9], and esophagus[10], breast[11], bladder[12], prostate[13] and lung cancers[14], being similar with that in gastric carcinoma in our study.

Rb mutations resulting in the loss of Rb function play a role in the initiation of retinoblastoma and other tumors that develop as second malignancies in individuals with the hereditary form of retinoblastoma[19]. The functional loss of the Rb gene has been implicated in a diverse group of human malignancies, including carcinoma of the breast, urinary bladder, liver, esophagus, prostate, and colon[8,10-12,20,21]. The significance of these mutations in other tumors is a potentially critical issue. A key issue is whether the changes reported within the Rb gene in other various cancers are causally related to the initiation of tumors or whether they are involved in tumor progression. Phillips *et al*[20] reported that loss of Rb gene function is an early event in prostatic tumorigenesis. Our findings demonstrate that the altered pRb protein expression is more frequent in gastric carcinomas with superficial invasion than in those with advanced invasion. This feature means that the Rb gene mutation is also an early event in the development of gastric carcinoma and then gradually gets lost during its progression. However, only 35% (6/17) of T1 lesions had strong staining in our study. The T1 lesions occurred earlier than T2, T3, and T4 lesions and might be a "late" event in the development of gastric carcinoma. Therefore, in-depth studies should be undertaken to clarify whether the Rb gene mutation is causally related to the initiation of gastric adenocarcinoma

as in the hereditary retinoblastoma.

The prognostic value of pRb protein expression in various carcinomas has been investigated. Some studies found that pRb protein expression is an important prognostic indicator of cancers, such as bladder cancer[22] and non-small-cell lung cancer[23]. Low pRb protein expression is associated with significantly poorer survival among them. In contrast, in colorectal cancer no prognostic significance of pRb protein expression has been reported[14]. Our findings demonstrate that there is a negative correlation between pRb protein expression and survival in gastric carcinoma at univariate analysis, but not significantly correlated between them at multivariate analysis. In the present study, however, the pRb protein-high tumors were more frequent in earlier stage (stages Ⅰ and Ⅱ) than in advanced stage (stages Ⅲ and Ⅳ), suggesting that the pRb protein expression is not an independent prognostic marker of gastric adenocarcinoma.

In conclusion, our data indicate that the mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb protein inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma

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RAPID COMMUNICATION

Comparison of amplicon-sequencing, pyrosequencing and real-time PCR for detection of YMDD mutants in patients with chronic hepatitis B

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Abstract

AIM: To compare the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of Tyrosine-methionine-aspartate-aspartate (YMDD) mutants in patients with chronic hepatitis B.

METHODS: Mixtures of plasmids and serum samples from 69 chronic hepatitis B patients treated with lamivudine were tested for YMDD mutations by sequencing of PCR products, pyrosequencing, and real-time PCR, respectively. Time required and reagent costs of the three assays were evaluated.

RESULTS: Real-time PCR detected 100%, 50%, 10%, 1% and 0.1% of YVDD plasmid in mixtures with 10^6 copies/mL of YMDD plasmid, whereas sequencing and pyrosequencing only detected 100% and 50% of YVDD plasmid in aliquots of the corresponding mixtures. Completely concordant results were obtained from 60 (87%) out of the 69 clinical serum samples by the three assays. Mutants were detected by real-time PCR in less than 20% of the total virus population, but no mutant was detected by sequencing and pyrosequencing. In addition, real-time PCR required less time and was more cost-effective than the other two assays. However, throughput of pyrosequencing was the highest.

CONCLUSION: Among the three assays compared, real-time PCR is the most sensitive, cost-effective, and time saving for monitoring YMDD mutants in patients with chronic hepatitis B on lamivudine therapy.

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Key words: Tyrosine-methionine-aspartate-aspartate mutant; Hepatitis B virus; Real-time PCR; Sequencing; Pyrosequencing

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INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) and that more than 1 million will die of liver cirrhosis and hepatocellular carcinoma (HCC) each year[1-3]. Lamivudine is an effective antiviral agent for patients with chronic hepatitis B and advanced liver diseases[4]. However, long-term lamivudine monotherapy induces emergence of lamivudine-resistant HBV mutants in some patients chronically infected with HBV[4,5]. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase, which is part of the catalytic site of HBV polymerase[6]. Virological breakthrough and alanine transaminase (ALT) flare have been observed in 2 and 3 mo after the emergence of YMDD mutants, respectively[7]. Several technologies developed can detect lamivudine-resistant mutants[8], but the number of studies actually comparing these assays is limited.

Nucleotide sequencing of PCR products is routinely used to detect lamivudine resistance. However, this method is expensive and laborious and only can detect mutant virus when it comprises at least 25% of the total virus population[9]. Pyrosequencing has a high throughput and can detect minor sequence variants[10]. Real-time PCR is a rapid and highly sensitive method for detection of mutant HBV[11-14].

In the present study, we compared the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutants in mixed plasmids and clinical samples from chronic hepatitis B patients treated with lamivudine. Time required and reagent costs were also analyzed to enable a comparison among these assays for their cost-effectiveness.

MATERIALS AND METHODS

Plasmids and controls

Plasmids and controls were prepared as previously described[14]. Briefly, three previously identified serum samples containing YMDD, YVDD and YIDD respectively were used as a template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, WI, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, CA, USA).

Patients, samples and extraction of HBV DNA

Serum samples were collected from 69 patients with chronic HBV infection. Real-time PCR showed that all the patients after treatment with lamivudine for three months to three years were HBV-DNA positive. HBV DNA was extracted from serum samples using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. HBV DNA was measured on Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China) approved by the State Food and Drug Administration of China for *in vitro* diagnostic use.

Sequencing of PCR products

HBV DNA samples were prepared for sequencing by PCR amplification as described by Allen *et al*[6]. HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, CA, USA) and eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration of DNA were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA).

Pyrosequencing

Pyrosequencing was carried out as described by Lindstrom *et al*[10]. In brief, the primer (5'-AGT GGG CCT CAG TCC GTT TC-3') was designed to anneal the adjacent YMDD motif. HBV DNA was extracted from serum samples and amplified by nested-PCR. One of the inner PCR primers was biotinylated to prepare single-stranded DNA with PCR products on the streptavidin-coated beads for pyrosequencing. Pyrosequencing was performed on the automated 96-well PSQ pyrosequencer (Pyrosequencing AB, Uppsala, Sweden) with the PSQ SNP 96 reagent kit (Pyrosequencing AB, Uppsala, Sweden). The nucleotide dispensation order was ATGTATGATG, which was designed on sequences of the interested YMDD motif of the wild-type and mutant HBV.

Real-time PCR

Real-time PCR for detection of YMDD mutants was performed as previously described[14]. In brief, parallel reactions C, V and I were used to detect total HBV, YVDD, and YIDD, respectively. The reactions differed only in the reverse primer they contained. The amplification was performed on Mx3000P PCR system (Stratagene, La Jolla, CA, USA) by incubating the reaction mixture (50 μL) at 50℃ for 2 min, at 95℃ for 5 min, followed by 40 cycles of PCR amplification at 94℃ for 20 s and at 53℃ for 30 s. The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The threshold cycle (Ct) is the cycle at which a significant increase in fluorescence occurs. The percentage of mutants in total viruses was calculated by the equations of Bernard (ΔCt = Ct of control-Ct of mutants)[15] and Shi (ratio of mutants to total viruses = $2^{\Delta Ct}$)[14].

Mixing experiments

Mixing experiments were used to evaluate the abilities of the sequencing, pyrosequencing and real-time PCR to accurately detect and quantify minor sequence variants. Mutant plasmid containing YVDD sequence and wild-type plasmid were mixed at a final concentration of 10^6 copies/mL, and the percentage of YVDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1% and 0.01% respectively. The mixtures were analyzed by the three methods, respectively. For real-time PCR, each mixture was analyzed five times, and the mean of the five runs was calculated.

Time study

Time analysis was carried out as described by Krafft and Lichy[16]. Three skilled technologists were selected to perform the assays. Time required for each assay was calculated by direct observation during the procedures.

Cost analysis

Costs for each assay were estimated based on the prices of reagents in China. The costs of instruments and labors were not included.

RESULTS

Detection of YVDD and YMDD mutants in plasmid mixture

YVDD and YMDD mutants in the plasmid mixture were detected at different ratios by sequencing, pyrosequencing and real-time PCR, respectively. YVDD, YVDD/YMDD mutants in the mixture containing 100% and 50% of YVDD plasmid and only YMDD in the mixture containing 10%, 1%, 0.1% and 0.01% YVDD plasmid were detected by sequencing and pyrosequencing. YVDD mutants in the mixture containing 100% YVDD plasmid, YVDD/YMDD mutants in the mixture containing 50%, 10%, 1% and 0.1% YVDD plasmid and only YMDD mutants in the mixture containing 0.01% YVDD plasmid were detected by real-time PCR. The ratio of YVDD to YMDD mutants in the real-time PCR assay was almost identical to the true ratio in the mixtures (Table 1).

Comparison of sequencing, pyrosequencing and real-time PCR for detection of YMDD and YVDD mutants in clinical samples

We detected YMDD and YVDD mutants in clinical serum samples from 69 chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were compared (Table 2). Completely concordant results were obtained from 60

Table 1 Results of sequencing, pyrosequencing and real-time PCR for detection of mixed plasmids containing YVDD and YMDD at a final concentration of 10^6 copies/mL

YVDD plasmid in the mixture	Sequencing	Pyrosequencing	Real-time PCR	ΔCt (mean ± SD)	Calculated percentage (mean ± SD) (%)
100%	YVDD	YVDD	YVDD	-0.024 ± 0.06	98 ± 4.0
50%	YVDD	YVDD	YVDD	-0.98 ± 0.07	51 ± 2.5
10%	YMDD	YMDD	YVDD	-3.12 ± 0.09	11 ± 0.7
1%	YMDD	YMDD	YVDD	-6.60 ± 0.14	1 ± 0.1
0.1%	YMDD	YMDD	YVDD	-10.12 ± 0.20	0.09 ± 0.01
0.01%	YMDD	YMDD	YMDD	ND	0

ND: Not detected.

(87%) samples by the three assays. YMDD mutants in 24 samples and YVDD mutants in one sample were detected by pyrosequencing, while YMDD mutants in 22 samples and YVDD mutants in three samples were detected by real-time PCR. The percentage of mutants in the virus population obtained by real-time PCR in the three YVDD samples was 20%, 7% and 6% respectively. Twenty-four types of YIDD mutant and two mixed types of YIDD and YVDD mutants were detected by pyrosequencing, while 21 types of YIDD mutants and 5 mixed types of YIDD and YVDD mutants were detected by real-time PCR. The results were identical in 18 YVDD samples obtained by pyrosequencing, but YVDD mutants were detected in one sample by sequencing and pyrosequencing while YMDD mutants were detected in one sample by real-time PCR.

Among the five samples containing mixed types of YIDD and YVDD mutants by real-time PCR, mixed mutants were detected by pyrosequencing whereas only dominant mutants were detected by sequencing in two samples.

Time required

The time required for each assay of a certain number of samples was dependent on the throughput of the instruments used for detection. We measured the time required for each assay based on a 4-sample run because the highest throughput of ABI 3130 sequencer used in this study was 4 samples per run. The total assay time for sequencing, porysequencing and real-time PCR was 5, 4.5 and 2 h, respectively.

Costs

The cost for each assay was calculated based on the prices of the reagents in China. The sequencing reagents were from Applied Biosystems (Applied System, Foster, CA, USA). Pyrosequencing reagents were from Pyrosequencing AB (Pyrosequencing AB, Uppsala, Sweden). Primers and probes were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost for each sequencing, pyrosequencing and real-time PCR was 120, 150 and 80 Yuan RMB, respectively.

Table 2 Comparison of results obtained by sequencing, pyrosequencing and real-time PCR *n* (%)

Type	Sequencing	Pyrosequencing	Real-time PCR
rtM204	25 (36)	24 (35)	23 (33)
rtM204I	26 (38)	24 (35)	21 (30)
rtM204V	18 (26)	19 (27)	20 (29)
rtM204I + rtM204V	0 (0)	2 (3)	5 (8)

DISCUSSION

Lamivudine is one of the first-line medicines for chronic hepatitis B. Mutations in the YMDD motif of polymerase gene have been detected in patients with chronic hepatitis B treated or untreated with lamivudine[14,17-19]. Clinical breakthrough can be observed 2 wk to 7 mo after the emergence of YMDD mutations[7,9,19-20]. Acute and severe exacerbation due to YMDD mutations can cause considerable morbidity and mortality[21-24]. Lamivudine therapy after the emergence of YMDD mutations has no effect on chronic hepatitis B patients[25]. Antiviral agents, such as adefovir and entecavir, are effective against lamivudine-resistant HBV[26-28]. Pegylated interferon is also one of the first-line therapeutic options for hepatitis B virus infection and can induce sustained responses in some of lamivudine-resistant patients[29-31]. Monitoring YMDD mutation during lamivudine therapy contributes to the clinical decision of treatment of chronic hepatitis B patients.

Many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B[8]. However, they are different in sensitivity, specificity, cost, and time required. Sequencing of PCR products is a conventional assay and pyrosequencing is a high throughput method that can detect minor sequence variants. In the present study, we compared sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutations in chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were completely concordant in 60 (87%) patients. YVDD mutants were detected by real-time PCR in three samples. The percentage of the mutants in total viruses in the three samples was 20%, 7%, and 6%, respectively, all of which were below the detection limit of sequencing[9]. YVDD mutants were detected by pyrosequencing in the sample with 20% of mutants. These results suggest that real-time PCR is more sensitive, cost-effective and time saving than sequencing and pyrosequencing. In one sample, YVDD mutants were detected by sequencing and pyrosequencing and YMDD mutants were detected by real-time PCR. Further study of the clinical data of the sample revealed that it contained 620 copies/mL of HBV DNA, which was below the limit of real-time PCR assay[14].

Pyrosequencing is a high throughput, non-gel-based DNA sequencing technique based on real-time detection of the released pyroghosphate during DNA synthesis[10]. Pyrosequencing is widely used in detection of single nucleotide polymorphisms (SNP) in the human genome[32].

However, pyrosequencing only can detect DNA sequence with a length of no more than 40 base-pair, while sequencing of PCR products can detect much longer DNA sequences. Real-time PCR detects specific point mutations recognized by the primers or probes.

The time required for each assay is dependent on the throughput of instruments used. In the present study, ABI 3130 was used for sequencing of PCR products. Its highest throughput was 4 samples per run. The instruments used for pyrosequencing and real-time PCR were 96-well plates and had the highest throughput of 96 samples and 32 samples, respectively. Although pyrosequencing has the highest throughput, relatively few laboratories have used pyrosequencing equipments[10]. Real-time PCR assay is well suited for routine screening of YMDD mutants because real-time PCR systems are routinely used in many laboratories.

For detection of mutants in virus population, real-time PCR can calculate the ratio of mutants to total viruses[14], while sequencing and pyrosequencing only can estimate the ratio by the signal intensity. The background noises affect the results. When serum HBV-DNA is below 10^8 copies/mL, no false positive results have been observed[14]. However, the accuracy of the ratios calculated in real-time PCR can be affected by variations in the ΔCt value, especially in cases where mutants are above 50% in the virus population.

In conclusion, among the three assays studied, real-time PCR is the most sensitive, cost-effective, and time saving method for monitoring YMDD mutations in patients with chronic hepatitis B on lamivudine therapy.

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CASE REPORT

Hypertriglyceridemia-induced pancreatitis: A case-based review

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Abstract

Hypertriglyceridemia is an established cause of pancreatitis. In a case-based approach, we present a review of hypertriglyceridemia and how it can cause pancreatitis. We outline how to investigate and manage such patients. A 35 year old man presented to the emergency department with abdominal pain and biochemical evidence of acute pancreatitis. There was no history of alcohol consumption and biliary imaging was normal. The only relevant past medical history was that of mild hyperlipidemia, treated with diet alone. Physical exam revealed epigastric tenderness, right lateral rectus palsy, lipemia retinalis, bitemporal hemianopsia and a delay in the relaxation phase of his ankle reflexes. Subsequent laboratory investigation revealed marked hypertriglyceridemia and panhypopituarism. An enhanced CT scan of the head revealed a large suprasellar mass impinging on the optic chiasm and hypothalamus. The patient was treated supportively; thyroid replacement and lipid lowering agents were started. He underwent a successful resection of a craniopharyngioma. Post-operatively, the patient did well on hormone replacement therapy. He has had no further attacks of pancreatitis. This case highlights many of the factors involved in the regulation of triglyceride metabolism. We review the common causes of hypertriglyceridemia and the proposed mechanisms resulting in pancreatitis. The incidence and management of hypertriglyceridemia-induced pancreatitis are also discussed.

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Key words: Hypertriglyceridemia; Pancreatitis; Hyperlipidemia

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INTRODUCTION

Acute pancreatitis is a common condition with several possible etiologies. The mortality rate may be up to 20%. While alcohol and gallstones are the most common etiologies, metabolic, structural and iatrogenic causes are responsible for 20%-25% of cases in the United States. Although hyperlipidemia can be associated with acute pancreatitis as an epiphenomenon, hypertriglyceridemia or chylomicronemia is the underlying cause in up to 7% of all cases of pancreatitis. It is the most common cause of acute pancreatitis not due to gallstones or alcohol[1-4]. Hypertriglyceridemia-induced pancreatitis rarely occurs unless triglyceride levels exceed 20 mmol/L[3,5]. For triglyceride levels to exceed 20 mmol/L, most patients will have some form of primary or genetic defect in lipid metabolism. Genetic factors determine over 60% of the variability in serum lipids. As most patients can be effectively treated with existing drug therapy, it is critical that the gastroenterologist recognize these patients and manage them appropriately. Since not all hyperlipidemia is familial in nature, an awareness of the secondary causes of hypertriglyceridemia and factors that can alter triglyceride metabolism is imperative.

In a case-based approach, we present a review of the common causes of hypertriglyceridemia, the proposed mechanisms resulting in pancreatitis as well as further details on the incidence and management of hypertriglyceridemia-induced pancreatitis (Table 1). Although the case presented is an uncommon scenario, it clearly demonstrates how a sequence of metabolic and endocrine abnormalities can lead to a common disease process- acute pancreatitis. The key to investigating, diagnosing and managing this patient was taking a detailed history and performing a thorough physical exam.

CASE PRPORT

A 35-year-old man presented to the emergency department with a 36-h history of severe epigastric pain and vomiting. He also complained of a four-month history of headaches, fatigue, cold intolerance, constipation, reduced libido, and erectile dysfunction. One year prior to presentation, he was

Table 1 Causes of hypertriglyceridemia

Primary
Familial combined hyperlipidemia
Familial hypertriglyceridemia
Type Ⅲ hyperlipoproteinemia
Chylomicronemia
Lipoprotein lipase deficiency
Apolipoprotein C-Ⅱ deficiency
Secondary
Insulin resistance
Diabetes mellitus
Obesity
Hypothyroidism
Alcohol
Diet (excessive carbohydrate intake)
Nephrotic syndrome
Chronic renal failure, uremia
Biliary obstruction/cholestasis
Acute hepatitis
Monoclonal gammopathy
Multiple myeloma
Systemic lupus erythematosus
Stress, Sepsis
Pregnancy
Ileal bypass surgery
Lipodystrophy
Glycogen storage disease
Drugs
Estrogen
Isotretinoin
B-blockers
Glucocorticoids
Cyclosporine
Bile acid-binding resins
Protease inhibitors
Thiazides
Tamoxifen

Modified from Fung and Frohlich[44].

told that he had mildly elevated "cholesterol" and was told to modify his diet. His past medical history was otherwise negative. He did not smoke, rarely consumed alcohol (none in the last 14 d) and was not taking any medications. Physical exam found a somnolent but easily rousable male with marked epigastric tenderness. No masses or organomegaly was noted on examination of his abdomen. A right lateral rectus palsy, lipemia retinalis and bitemporal hemianopsia were also noted on exam but there were no signs of increased intracranial pressure. The thyroid exam was normal but the relaxation phase of his ankle reflexes was delayed.

Electrolytes and complete blood count were normal. Serum lipase was 754 U/L (normal 20-190 U/L) and his liver transaminases, alkaline phosphatase and lactate dehydrogenase were normal. Ultrasound detected inflammatory changes in the pancreas but the gallbladder and biliary tree were normal. Serum triglyceride was 38.5 mmol/L (normal 0-2), cholesterol; 12.4 mmol/L (normal 0-5.2), thyroid stimulating hormone; 0.17 (normal 0.4-4.0), free T4; 3.8 pmol/L (normal 10.8-23.8), random cortisol; 62 mmol/L (normal 138-690), serum testosterone < 0.7 nmol/L (normal 10-33), and luteinizing hormone; 0.1 IU/L (normal 1.3-6.3). An enhanced CT scan of the head revealed a large suprasellar mass impinging on the hypothalamus and optic chiasm.

Figure 1 Enhanced CT scan showing a large suprasellar craniopharyngioma (arrow).

A diagnosis of acute pancreatitis was made. Chylomicronemia caused by central hypothyroidism was the most likely etiology. The patient was treated with intravenous fluids and narcotics for pain control. His epigastric pain resolved over three days. He was given dexamethasone 4 mg three times daily preoperatively and L-thyroxine 50 micrograms daily. The fibric acid derivative gemfibrozil was started at 600 mg twice daily. At craniotomy, a suprasellar craniopharyngioma displacing the optic chiasm and encasing the pituitary stalk was completely removed (Figure 1). He was started on glucocorticoid replacement therapy, L-thyroxine, DDAVP and testosterone for panhypopituitarism. His postoperative course was uncomplicated; he has subsequently remained asymptomatic over a three-year follow-up period.

DISCUSSION

Hypertriglyceridemia-induced pancreatitis

Epidemiology: It is well known that hyperlipidemia is associated with acute pancreatitis, both as a precipitant and as an associated epiphenomenon[1,2]. Hypertriglyceridemia or chylomicronemia may be responsible for 1%-7% of all cases of pancreatitis[3,4]. Failure to consider and investigate chylomicronemia as a cause of pancreatitis may lead to an underestimate of the incidence. Pregnancy or medical conditions such as diabetes (both known to precipitate marked hypertriglyceridemia) should prompt further work-up[6,7]. Chylomicronemia may be the cause of 20% of episodes of acute pancreatitis in non-drinkers free of biliary tract disease. Chang *et al* found that hypertriglyceridemia was the cause of 56% cases of gestational pancreatitis[7]. In many settings, determining the exact etiology of pancreatitis may be complicated by the role of ethanol in precipitating severe hypertriglyceridemia. The proportion of "alcoholic pancreatitis" caused by direct as opposed to secondary hyperlipidemic effects is unknown.

Hypertriglyceridemia-induced pancreatitis rarely occurs unless triglyceride levels exceed 20 mmol/L[3,5]. In contrast, mild to moderate elevations in triglycerides (2-10 mmol/L) are extremely common in the early phase of acute pancreatitis of any etiology. One study noted such elevations in 47% of randomly selected patients presenting with acute pancreatitis[2]. In the same study, mild to moderate elevations of triglycerides were felt more

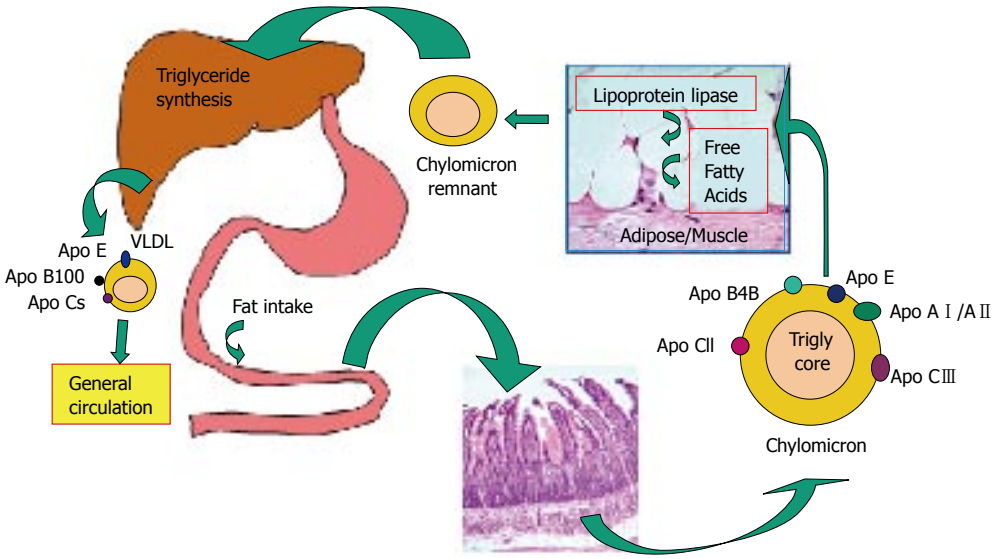

Figure 2 Triglyceride metabolism.

likely to be an epiphenomenon of the acute pancreatitis rather than a true causal precipitant[2]. It is important to remember that chylomicrons are the product of dietary fat absorption. Enforced abstinence from eating after a diagnosis of pancreatitis may allow rapid metabolism of the triglyceride-rich chylomicrons. Dominguez-Munoz *et al* found severe elevations (> 20 mmol/L) in 10% of patients but levels rapidly fell in the majority of patients within 72 h of presentation; therefore, a delay in the presentation or consideration of diagnosis can lead to false conclusions about etiology. Triglyceride levels remained mildly elevated for up to 15 d, probably reflecting an underlying lipid disorder[2].

Pathogenesis of hypertriglyceride-induced pancreatitis

The exact mechanisms involved in hypertriglyceridemia-induced pancreatitis are unclear. Chylomicrons are triglyceride-rich lipoprotein particles believed to be responsible for pancreatic inflammation. They usually present in the circulation when serum triglyceride levels exceed 10 mmol/L. These largest of lipoproteins might impair circulatory flow in capillary beds; if this occurs in the pancreas, the resulting ischemia might disturb the acinar structure and expose these triglyceride-rich particles to pancreatic lipase. The pro-inflammatory non-esterified free fatty acids generated from the enzymatic degradation of chylomicron-triglycerides may lead to further damage of pancreatic acinar cells and microvasculature. Subsequent amplification of the release of inflammatory mediators and free radicals may ultimately lead to necrosis, edema, and inflammation[8,9]. This hypothesized sequence of events was substantiated by studies showing both triglycerides and free fatty acids caused edema, hemorrhage and elevated amylase levels[9]. Hypertriglyceridemia has also been shown to exacerbate other experimental models of pancreatitis[10].

Studies using oral lipid-loading tolerance tests have documented elevated post-load plasma triglyceride levels in patients with previous pancreatitis as compared with controls[11,12]. To address whether hyperlipidemia is a pre-existing metabolic disorder or a consequence of acute pancreatitis, Dominguez-Munoz and co-workers addressed the hypothesis that mild to moderate elevation of serum triglyceride levels are likely to be an epiphenomenon of the pancreatic disease whereas the severe hyperchylomicronemia and hypertrygliceridemia required to trigger acute pancreatitis would require a relevant defect in the lipid catabolism and clearance[13]. They looked at a group of ten patients with a history of acute pancreatitis associated with severe elevations of serum triglycerides (> 20 mmol/L) and ten patients with acute pancreatitis and normal lipid values. Four to six months after recovery from the pancreatitis, they administered an infusion of intralipid of 20% and calculated the fractional removal rate (K2) and the maximal clearance capacity (K1) of exogenous triglycerides. All patients with a previous attack of "normo-lipidemic" acute pancreatitis had normal K2 and K1 values. However, five patients (50%) with previous hyperlipidemic acute pancreatitis had an abnormally low clearance capacity of exogenous triglycerides while the remaining five had normal removal values. They concluded that, even in the setting of a marked elevation of serum lipid levels, there may be other etiological factors involved[13]. These findings were supported by a similar earlier study[14].

Interestingly, mutations in the lipoprotein lipase (LPL) gene have been identified in patients with hypertriglyceridemia-induced pancreatitis[15-20]. Specifically, mutations in the LPL gene are thought to be a common cause of pregnancy-induced chylomicronemia. One group described 5 such patients all of whom had different mutations in the LPL gene leading to dramatic reductions in LPL activity[21]. Dysregulation or deficiency of key enzymes and substrates involved in triglyceride metabolism has also been described in patients with recurrent hypertriglyceridemia-induced pancreatitis (Figure 2). Lecithin-cholesterol acyltransferase (LCAT) deficiency is one example[22].

Hyperchylomicronemia and hypothyroidism

All patients that develop significant chylomicronemia are thought to have an underlying genetic disorder of lipoprotein metabolism. A disturbance in lipoprotein

lipase activity, the rate-limiting enzyme of triglyceride-rich particle metabolism, is often the culprit. Clinical problems rarely develop unless there is another precipitating condition affecting metabolism such as diabetes, ethanol excess, pregnancy, certain drugs (estrogen[23-26], furosemide[27], isotretinoin[28,29], tamoxifen[30], beta-blockers[31]) or, as in this case, hypothyroidism. Physical findings can include eruptive xanthomas produced by macrophage uptake of chylomicrons which accumulate in the skin over extensor surfaces and lipemia retinalis that is a result of high concentrations of circulating chylomicrons in retinal vessels[32].

Hypothyroidism has a well-established association with hyperlipidemia. Elevations of triglyceride levels are observed in up to 35% of hypothyroid patients[33]. Impaired low-density lipoprotein (LDL) receptor activity may result in decreased clearance and thus an accumulation of LDL particles. A low circulating free thyroid hormone level may also impair lipoprotein lipase activity in adipose tissue. Replacement therapy with L-thyroxine treatment may reverse both of these defects.

Treatment

In the acute setting, pancreatitis due to hyperlipidemia should be treated in much the same manner as other causes of pancreatitis. Currently there is no clear evidence that hyperlipidemia-induced pancreatitis differs from other types of pancreatitis in terms of frequency of necrosis[2], complications or outcomes[3]. A similar approach to medical and diagnostic management is thus indicated. Although chylomicron and triglyceride levels fall rapidly after oral fat intake ceases, efforts to accelerate the removal of the precipitating lipoproteins have been considered.

Direct removal of chylomicrons in the acute setting can be readily achieved by plasmapheresis and there are numerous documented reports[34-37]. Chronic plasmapheresis as prophylaxis has also been reported in patients with recurrent pancreatitis due to severe primary hypertriglyceridemia that was unresponsive to drug and dietary interventions[38]. Recognizing that decreased lipoprotein lipase (LPL) activity is a prominent cause of hypertriglyceridemia has fuelled attempts to enhance LPL activity. Although this is an interesting concept only one small study in the literature has addressed this in which intravenous insulin and heparin, both of which enhance LPL activity, were used to treat a small number of patients with hypertriglyceridemia-induced pancreatitis[39]. They found that the therapy reduced triglyceride levels and appeared to improve the pancreatitis[39]. Diabetic patients should be treated aggressively with intravenous insulin infusions to obtain and maintain euglycemia rapidly.

Patients with known rare defects in lipid metabolism have been treated with replacement therapies with some success. Purified apoC-Ⅱ infusion has yielded transient normalization of triglyceride levels and clinical improvement in pancreatitis patients with apoC-Ⅱ deficiency[40].

Therapeutic efforts following recovery from pancreatitis need to be directed at preventing recurrence by controlling triglyceride levels. As most cases of hyperlipidemia occur when a genetically-predisposed individual is exposed to a secondary condition, therapy should be targeted towards the concomitant disorder. Alcohol should be discontinued as should oral estrogen therapy or selective estrogen receptor modulators such as tamoxifen or raloxifene. Diabetes should be treated with oral hypoglycemics and/or insulin aiming for tight glycemic control. Obviously, as in our case, hypothyroidism should be treated with L-thyroxine.

Persistence of hyperlipidemia on a fat-reduced diet should prompt the institution of lipid-lowering agents. The fibric acid derivatives (fibrates), such as gemfibrozil, fenofibrate or bezafibrate, are the drugs of first choice. These agents are generally well tolerated and highly effective if taken regularly and diet restrictions are continued. Niacin is inexpensive and often effective alone or in combination with a fibrate. Side effects such as flushing, hyperuricemia and hepatic transaminasemia may limit the use of niacin as a first line agent. Controversy exists as to whether niacin may exacerbate glucose intolerance in patients with diabetes mellitus. Omega-3 fatty-acid products show promise as adjunctive agents in refractory cases. Medium-chain triglycerides have also been used to prevent acute hyperlipidemic pancreatitis during pregnancy[41] as their absorption doesn't require chylomicron formation.

Interestingly, in a study of 21 such patients with primary hypertriglyceridemia and a history of recurrent attacks of pancreatitis, lipid lowering toward normal was achieved in all patients with a program of combined dietary and drug therapy[42]. Five patients had recurrent episodes of pancreatitis during the treatment program all of whom were diagnosed subsequently with bulimia[42]. Anti-oxidant therapy (selenium, beta-carotene, vitamin C and α-tocopherol) has been used with success in the reduction of recurrent pancreatitis episodes in patients with familial lipoprotein lipase deficiency who remained markedly hypertriglyceridemic after medical therapy[43]. It is postulated that the antioxidants might protect the pancreatic acinar cells from free radical damage brought on by the ischemia of chylomicron-induced changes in pancreatic capillary circulation[43].

CONCLUSIONS

This case illustrates a sequence of clinical events, due to a combination of genetic and acquired disorders, which resulted in a common medical problem. The main clues to diagnosing this complex chain of events leading to pancreatitis became evident from a thorough history and physical exam. Identifying features of a possible intracranial lesion, hypothyroidism, hypogonadism and hypertriglyceridemia in the context of an understanding of triglyceride metabolism facilitated the diagnosis.

Hypertriglyceridemia is a common clinical problem that can be exacerbated by numerous medications and medical conditions. Markedly elevated triglyceride levels can lead to pancreatitis, a serious and potentially fatal complication. General and specific therapy is available to reduce triglyceride levels during the acute phase of pancreatitis, which may improve the outcome. Nutrition, pharmacologic therapy and avoiding agents that can

elevate triglycerides may be essential in preventing further attacks. There are numerous causes of pancreatitis. Various diagnostic tools can be very helpful in determining the cause of pancreatic inflammation; however, the most valuable approach remains a thorough history and physical exam.

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CASE REPORT

Endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for multiple intraductal papillary mucinous adenomas

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Abstract

In benign or low-grade malignant pancreatic tumors, complete removal of the lesion is sufficient for a cure, and thus minimal resection techniques with preservation of the pancreatic functional reserve have advantages over more extended pancreatic resections. However, a high incidence of postoperative pancreatic fistula in such procedures has been reported. Moreover, branch-type intraductal papillary mucinous neoplasms of the pancreas tend to locate in the head of the pancreas, and show less malignant potential. We describe an endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for branch-type multiple intraductal papillary mucinous adenomas, along with a gastric wall-covering method for the prevention of pancreatic leakage.

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Key words: Intraductal papillary mucinous neoplasm; Single-branch resection; Gastric wall-covering method

Kuroki T, Tajima Y, Tsutsumi R, Tsuneoka N, Kitasato A, Adachi T, Kanematsu T. Endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for multiple intraductal papillary mucinous adenomas. *World J Gastroenterol* 2006; 12(44): 7203-7205

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INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is characterized by dilatation of the main and/or branch pancreatic ducts, mucin-secretion, and intraductal papillary growth. IPMNs are classified into three types, a main pancreatic duct type, branch type, and mixed type. The branch type IPMN is more frequently located in the head of the pancreas, and shows less malignant potential[1,2]. Therefore, several new surgical procedures for minimal resection of the pancreas with pancreatic functional reserve have been proposed, including duodenum-preserving pancreatic head resection, segmental pancreatectomy and single-branch resection of the pancreas[3-5]. Single-branch resection is a minimally invasive surgical procedure for the resection of the pancreas because it requires no reconstruction for the main pancreatic duct or the common bile duct. In addition, single-branch resection should avoid injury to the main pancreatic duct which could cause a postoperative pancreatic fistula. Although several surgical techniques and devices have been advocated to avoid breaking the main pancreatic duct, the incidence of pancreatic fistula is still relatively high[6]. We report herein a case of successful single-branch resection for two IPMNs within the head of the pancreas under the guidance of intraoperative pancreatography by using an endoscopic naso-pancreatic drainage (ENPD) tube along with a gastric wall-covering technique for the prevention of pancreatic leakage.

CASE REPORT

A 59-year-old man was referred to our hospital for further evaluation of two cystic tumors in the head of the pancreas which were detected by routine ultrasonography (US). Upon admission, there were no abnormal findings in the laboratory data. The endocrine pancreatic function by a 75 g oral glucose tolerance test and the exocrine function by a pancreatic function diagnostic (PFD) test were within normal range. Abdominal US and computed tomography (CT) showed two multilobular cystic lesions, 15 mm and 40 mm in diameter, respectively, in the head of the pancreas. Endoscopic retrograde pancreatography (ERP) showed two cystic lesions communicating with the main pancreatic duct. Although mucous secretion was detected from the duodenal papilla, the main pancreatic duct showed no abnormality. Magnetic resonance cholangiopancreatography (MRCP) and ERP revealed a communicating branch to these two cystic lesions (Figure 1). No mural nodules were noted in the cysts. The cytology of

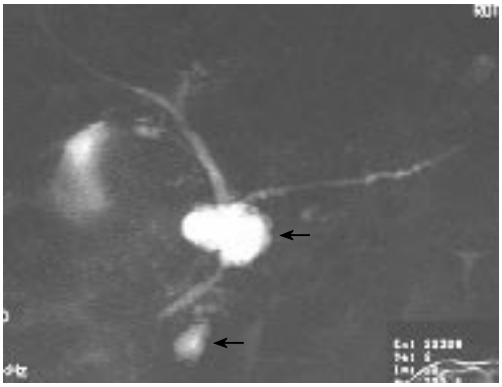

Figure 1 MRCP showing two cystic lesions in the head of the pancreas (arrows).

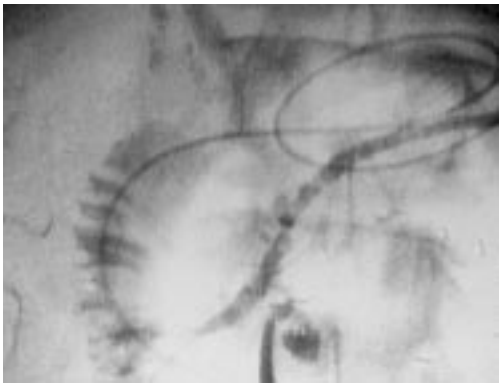

Figure 2 Intraoperative pancreatography using an ENPD tube demonstrates a communicating duct between the main pancreatic duct and cystic lesion.

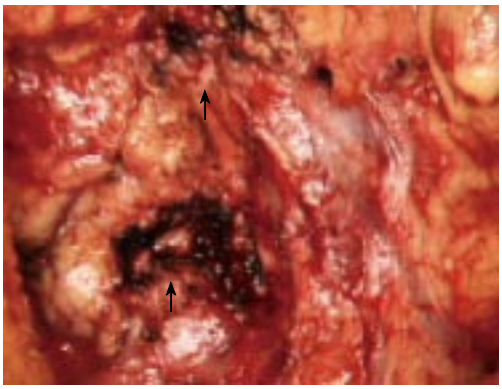

Figure 3 Operative photograph shows the pancreas after completion of the single-duct resection of the pancreas for multiple IPMNs. Arrows indicate the cut surface after pancreatic resection.

the pancreatic juice was negative for malignant cells. K-ras point mutation at codon12 was detected in the pancreatic juice. Consequently, the cystic lesions were diagnosed as a branch-type intraductal papillary mucinous neoplasm (IPMN) and the patient was considered a candidate for surgery.

Under general anesthesia, we placed a 5F-size ENPD tube (Olympus, Tokyo Japan) for intraoperative pancreatography prior to laparotomy. The ENPD tube was inserted deep into the main pancreatic duct for approximately 10 cm through the orifice. After an upper abdominal midline incision, the gastrocolic ligament was divided and the pancreas exposed. The duodenum was mobilized to facilitate access to the head of the pancreas. The communicating branches between the cystic lesions and main pancreatic duct were carefully evaluated by using intraoperative US and pancreatography (Figure 2), and a single-branch resection for both cystic tumors was then performed. During the resectioning of the cystic tumors, the ENPD tube in the main pancreatic duct was clearly and easily detected by intraoperative US, and thus both the direction and position of the main pancreatic duct and the correlation between the cystic tumors and main pancreatic duct were correctly identified. The two cystic tumors were dissected along the border of the tumor and the surrounding normal pancreatic parenchyma, and then the confluences of the pancreatic branch ducts and main pancreatic duct were ligated using a 4-0 non-absorbable monofilament suture. Intraoperative frozen section histological examination of the dissected pancreatic ducts revealed a disease-free margin. After removal of the tumors, pancreatography through the ENPD tube was performed and revealed no injury to the main pancreatic duct or leakage from the transected pancreatic branch ducts. Because a mixture of indigocarmine and contrast material was used for the pancreatography, we were able to detect any minor pancreatic leakage as a blue spot at the cut surface of the pancreas and close the leaking points appropriately with 4-0 absorbable monofilament sutures. After pancreatic resection, the ENPD tube was used for the drainage of the pancreatic duct. It was not necessary to perform any reconstruction of the digestive tract (Figure 3). To prevent pancreatic leakage from the small pancreatic branches at the cut surface of the pancreas, the "gastric wall-covering method [7]" was performed as follows: each cut surface after resection of the two cystic tumors was fixed to the posterior wall of the gastric body by suturing between the pancreatic parenchyma and the seromuscular layer of the stomach with 4-0 absorbable monofilament. The operating time was 225 min, and blood loss was 240 mL. Histological diagnosis was intraductal papillary mucinous adenoma of the pancreas. The ENPD tube was removed on the 14th postoperative day. The postoperative course was uneventful, and the patient was discharged home on the 25th postoperative day with no endocrine or exocrine pancreatic insufficiency.

DISCUSSION

The branch type of IPMN of the pancreas shows a more favorable prognosis than invasive ductal adenocarcinoma of the pancreas. In the benign or low-grade malignant IPMN, complete tumor resection is sufficient for a cure[1,2]. Therefore, several organ-preserving techniques, including duodenum-preserving pancreatic head resection, have been advocated in the surgical treatment for IPMN in the head of the pancreas[3]. The advantage of duodenum-preserving pancreatic head resection is the complete preservation of the duodenum, including the duodenal papilla sphincter function, compared with a pylorus-preserving pancreaticoduodenectomy. However, the

duodenum-preserving pancreatic head resection requires a reconstruction of the main pancreatic duct and/or common bile duct. In addition, another challenge when performing the duodenum-preserving pancreatic head resection is preserving the blood supply to the duodenum. Therefore, single-branch resection of the pancreas for multiple IPMNs has been performed as an ultimate organ-preserving surgery[5]. In the present case, we developed single-branch resection of the pancreas using an ENPD tube for multiple branch-type IPMNs as an ideal pancreas-preserving surgery. The most important advantage of the single-branch resection is both the complete preservation of the main pancreatic duct and the complete removal of the lesions. The insertion of an ENPD tube was useful for identifying the pancreatic duct in order to prevent injury to the main pancreatic duct. The ENPD tube in the main pancreatic duct is easily detectable using intraoperative US, and it can demonstrate a repeated intraoperative pancreatography. The first benefit of intraoperative pancreatography using an ENPD tube is that the identification of the anatomical relationship between the cystic lesions and main pancreatic duct can be confirmed not only by a radiological image, but also by a direct palpation. In addition, we used a mixture of a dye and contrast medium for the intraoperative pancreatography, which allowed us to identify the pancreatic leakage directly as a visible blue point by detecting the dye leakage. Pancreatic leakage is the most frequent complication and is still responsible for most mortality after pancreatic surgery[6]. In particular, the incidence of pancreatic leakage following partial resection of the pancreas is high. In the present case, a novel surgical procedure, a "gastric wall-covering method," was performed to prevent pancreatic leakage[7]. The concept of the gastric wall-covering method is that the opening small pancreatic ducts at the cut surface as a cause of pancreatic leakage were covered completely with the gastric wall. As shown in our patient, the multiple partial resections of the pancreas had a wide cut surface. Therefore, the gastric wall-covering method is useful for the prevention of pancreatic leakage from the opening of the small pancreatic branch ducts at the cut surface.

In conclusion, single-branch resection of the pancreas, without reconstruction of the pancreatobiliary tract, is the most ideal, least invasive pancreatic resection for a low-grade malignant neoplasm, including multiple IPMNs. ENPD tube-guided pancreatectomy is an improved technique that allows the surgeon to more accurately determine the precise location and correlation of tumors and pancreatic ducts.

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CASE REPORT

Thrombotic microangiopathy involving the gallbladder as an unusual manifestation of systemic lupus erythematosus and antiphospholipid syndrome: Case report and review of the literature

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Abstract

Gallbladder disease is no more common in patients with systemic lupus erythematosus (SLE) than in the general population. We describe a 17-year-old patient with SLE, who developed nephritis that was well controlled with medications. Initial treatment consisted of azathioprine, aspirin and prednisone with stable control of her symptoms. Two years later she developed a right quadrant abdominal pain, and an abdominal ultrasound revealed microlithiasic cholecystitis. Open cholecystectomy was performed and the histopathological findings revealed vasculitis with thrombotic microangiopathy in the gallbladder. This case presentation illustrates that calculous or acalculous cholecystitis should be considered as a manifestation of active SLE and APS.

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Key words: Gallbladder; Cholecystitis; Systemic lupus erythematosus; Antiphospholipid syndrome

De-Leon-Bojorge B, Zaltzman-Girsevich S, Ortega-Salgado A, Prieto-Patron A, Córdoba-Córdoba R, Yamazaki-Nakashimada M. Thrombotic microangiopathy involving the gallbladder as an unusual manifestation of systemic lupus erythematosus and antiphospholipid syndrome: Case report and review of the literature. *World J Gastroenterol* 2006; 12(44): 7206-7209

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INTRODUCTION

Gallbladder disease is no more common in patients with systemic lupus erythematosus (SLE) than in the general population[1]. Scarce reports on acalculous cholecystitis (ACC) in SLE or in antiphospholipid syndrome (APS) have been described in the literature[2-12]. We describe a patient with SLE, nephritis, pancreatitis and microlithiatic cholecystitis. Open cholecystectomy was performed and the histopathological findings revealed vasculitis and thrombotic microangiopathy in the gallbladder. She was diagnosed with secondary APS and started on anticoagulation therapy. Coincidentally, her lupus nephritis also improved. This case illustrates that calculous or acalculous cholecystitis should be considered as a manifestation of SLE and APS.

CASE REPORT

In 2002, a 17-year-old female with arthritis and oral ulcers was referred to the Instituto Nacional de Pediatría in Mexico City. Laboratory results showed autoimmune hemolytic anemia, leucopenia, lymphopenia, hypocomplementemia. She also had positive anti-nuclear antibodies (ANA), anti-dsDNA, anti-β_2-glicoprotein-Ⅰ, and anti-cardiolipin antibodies. Based on her clinical presentation and laboratory studies, she was diagnosed with SLE. Initial treatment consisted of azathioprine, aspirin and prednisone with stabilization of symptoms. In 2003 she presented with renal complication including proteinuria and hematuria. Oral cyclophosphamide was started with improvement in her renal function. In 2004 she developed right quadrant abdominal pain, and an abdominal ultrasound revealed microlithiasic cholecystitis. The abdominal pain spontaneously resolved and dietary modifications were initiated. She was clinically well until July 2005 when she presented with a flare consisting of hypertension, autoimmune hemolytic anemia and marked proteinuria.

On admission the physical examination was unremarkable. Laboratory tests showed proteinuria: 7.2

g/d, serum Cr: 1.6 g/dL, Coombs: +, Hb: 9.3 g/dL, leucocyte count: 3300/mm^3, total lymphocyte count: 369, platelets: 218 000/mm^3. Immunological tests were positive for anti-dsDNA, anti-β2-glicoprotein- Ⅰ, and anti-cardiolipin antibodies, CH50: 49 IU/mL (normal 150-250 IU/mL), C3: 21 mg/dL (normal 86-184 mg/dL), C4: 5.3 mg/dL (normal 19-58 mg/dL). She was started on methylprednisolone and cyclophosphamide.

A renal biopsy showed a global, diffuse, proliferative and membranous glomerulonephritis (class Ⅳ-Ⅴ) without thrombosis. During her admission she developed acute abdominal pain, including a positive Murphy sign on exam. Liver and pancreatic function tests were elevated: gamma-glutamyltranspeptidase was 210 mU/mL (30-60 mU/mL), AST 339 mU/mL (11-33 mU/mL) ALT 154 mU/mL (4-30 mU/mL), total bilirubin 1.1 mg/dL (0.2-1.0 mg/dL) with direct bilirubin, 0.6 mg/dL,amylase 932 mU/mL (68-215 mU/mL) and lipase 649. Abdominal ultrasound revealed lithiasic cholecystitis and pancreatitis. The patient underwent open cholecystectomy and histopathological findings of the gallbladder showed segmental necrotizing vasculitis and vascular occlusion with fragmented erythrocytes (Figures 1A and 1B). Anticoagulation therapy was started with subsequent resolution of the proteinuria and normalization of the laboratory values including the creatinine level.

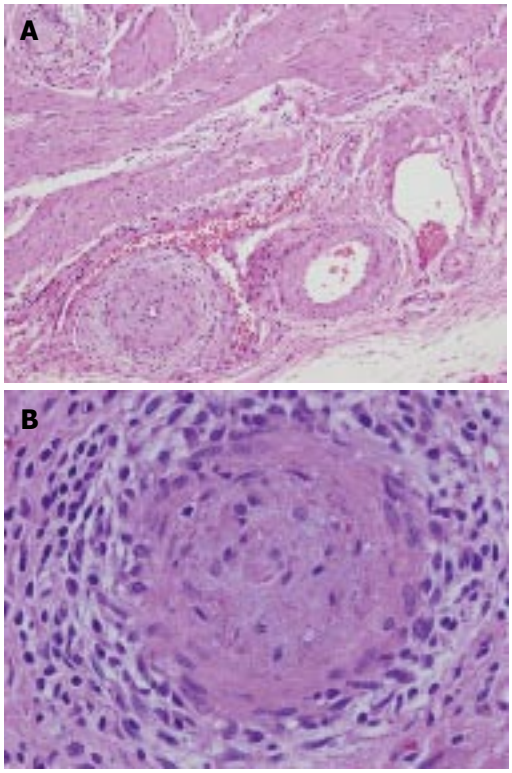

Figure 1 Occlusion with fibrosis in a gallbladder artery (HE x 1) (**A**); Total artery occlusion, fragmented erythrocytes and perivascular inflammation with mononuclear infiltration (**B**).

DISCUSSION

A variety of gastrointestinal complications have been reported in association with systemic lupus erythe-matosus (SLE), including mesenteric arteritis, intestinal perforation, and peritonitis[9]. Gallbladder disease is no more common in patients with systemic lupus erythematosus than in the general population[1]. Gallstone disease is a common condition in Western populations, and its etiology is multifactorial, including genetic and environmental factors. Hemolysis, obesity, aging, estrogen treatment, pregnancy and diabetes are consistently associated with a higher risk of developing gallstones[10].

Symptomatic gallbladder vasculitis requiring treatment by cholecystectomy is rare. Chen[13] has divided the gallbladder vasculitis into three groups: gallbladder vasculitis as a manifestation of polyarteritis nodosa (group 1), gallbladder vasculitis occurring in diseases known to be associated with vasculitis as in our SLE case (group 2) and isolated gallbladder vasculitis (group 3). In 1983, Swanepoel *et al*[3] described the first case of acute acalculous cholecystitis (ACC) in SLE. Since then, only a few SLE cases have been reported in the literature. Newbold *et al*[9] have reported two cases of gallbladder involvement in patients with SLE; histologically there was acute arteritis with periarterial fibrosis, changes similar to those found in polyarteritis nodosa. In fact, the development of gallbladder disease appears to be more common in polyarteritis nodosa than in SLE. Recently, Bando *et al*[12] have reported a case of acalculous cholecystitis induced by mesenteric inflammatory veno-oclusive disease in SLE. Although surgical removal of the gallbladder is the generally accepted treatment for SLE patients who develop acute cholecystitis successful treatment with corticosteroids alone has also been reported[11]. It is of note that, rarely, the symptoms of serositis can mimic those of acute cholecystitis[13,14].

Antiphospholipid syndrome (APS) was first described in patients who suffered from recurrent fetal loss and thromboses. Since then the spectrum of associated symptoms in APS has broadened considerably. The clinical criteria for diagnosis of APS include arterial, venous, or small vessel thrombosis in any tissue or organ. Thrombosis has to be confirmed by imaging, Doppler studies or histopathology. For laboratory diagnosis of APS, affected patients have antibodies to β2-glicoprotein- Ⅰ, cardiolipins or positive lupus anticoagulant in two separate occasions at least 6 wk apart. Gallbladder involvement secondary to APS has rarely been described in the literature. Dessailloud *et al*[2] reported a fatal case of a 29-year-old woman with acalculous cholecystitis secondary to catastrophic APS. The patient developed mesenteric ischemia, left limb ischemia and acute renal failure. In another report, Nolen *et al*[3] have described a 26-year-old female with primary APS who was treated successfully with medical management. Rhoton *et al*[8] described a 22-year-old woman with SLE who developed hemobilia, and also had high titers of anticardiolipin antibodies.

Our case presented with thrombotic and inflammatory complications involving the gallbladder, likely secondary to both SLE and APS. The vasculopathy showed thrombotic microangiopathy (TMA) and perivasculitis. Although microlithiasis could have contributed to the inflammatory process located in the gallbladder and pancreas, the presence of vasculitis reflects an autoimmune process.

Table 1 SLE and APS cases reported in the literature with gallbladder involvement

Author	Gender	Age(yr)	Diagnosis	Gallbladder pathology	Serology
Present report (2006)	F	17	SLE/APS	TMA Vasculitis	ANA Anti-DNA Anticardiolipin
Newbold *et al* (1987)	F	28	SLE	Acalculous Cholecystitis vasculitis and thrombosis	ANA
Newbold *et al* (1987)	F	38	SLE	Small vessel vasculitis	ANA Anti-DNA
Kamimura *et al* (1998)	F	27	SLE	ACC	ANA Anti-DNA Anti-Sm
Rhoton *et al* (1993)	F	22	SLE	Hemobilia	Not specified
Raijman *et al* (1989)	F	34	SLE	Hemorrhagic Acalculous Cholecystitis	ANA Anti-DNA Anticardiolipin Anti-platelet antibodies
Swanepoel *et al* (1983)	F	22	SLE	AAC	ANA
Bando *et al* (2003)	F	43	SLE	AAC	ANA Anti-DNA
Shin *et al* (2002)	F	39	SLE Sjogren syndrome	AAC	ANA Anti-dsDNA Anti-Ro
Nolen *et al* (1999)	F	26	APS	AAC	Anticardiolipin Lupus antcoagulant
Kara *et al* (2004)	M	65	APS	AAC	Not reported
Dessailloud *et al* (1998)	F	29	APS	Acalculous Ischemic Gallbladder Necrosis	ANA β_2-glycoprotein- I Antiphosphatidylserine

Eventually the histopathological findings helped us diagnose the APS and initiate anticoagulation therapy, which led to improvement of our patient's renal disease. Although presence of small gallstones is associated with increased risk of acute pancreatitis, our patient's pancreatitis could also be a manifestation of APS[15]. All the SLE and APS cases reported in the literature with gallbladder involvement are summarized in Table 1. Of note is that our patient is the youngest patient described to date, reflecting the rarity of this complication in the pediatric age group.

Renal thrombotic manifestations have been reported since the initial description of APS. TMA is the best known characteristic lesion of APS nephropathy, leading to hypertension, proteinuria and renal impairment. Recently, consideration of the APS nephropathy in every SLE case has been emphasized because successful treatment requires both anticoagulation in addition to immunosuppresion[16].

In summary, although rare, gallbladder involvement in patients with SLE should raise the suspicion of vasculitis or APS. We believe that both gallbladder vasculitis and thrombosis contributed in the clinical picture of our patients. Sometimes surgical cholecystectomy is needed for both therapeutic and diagnostic purposes. If thrombotic manifestations of APS are documented, anticoagulation therapy should be started.

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CASE REPORT

Subcutaneous extension of a large diaphragmatic hydatid cyst

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Abstract

A 53-year-old male patient with a large hydatid cyst of the left hemidiaphragm and smaller secondary cysts located in the left thoracic cavity and upper left abdominal quadrant presented with two progressively enlarging lipoma-like masses in the left hypochondrium and under the left scapulae respectively. Total excision of all the cysts was performed through a bilateral subcostal incision, with the left hemidiaphragm near totally excised and replaced by a synthetic bilayer mesh.

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Key words: Hydatid cyst; Diaphragm; Mesh reconstruction; Intrathoracic cavity; Subcutaneous extension

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INTRODUCTION

Hydatid disease is a parasitosis, known as hydatidosis or echinococcosis caused by the larval stage of the Echinococcus granulosus tapeworm. The disease was first described by Hippocrates as "cysts full of water" in a man's liver and by Aristotle in human lungs and liver[1]. The disease remains a considerable public health problem in endemic countries, including the Mediterranean and Middle East countries, New Zealand, Australia, South Africa and South American countries. The primary hosts are members of the Canidae family (dogs, wolves and coyotes); the intermediate hosts are sheep, cattle and deer[2]. Humans are accidental intermediates in the biologic cycle of taenia echinococcus granulosus, more frequently by direct contact with dogs than by ingestion of food or water contaminated with their feces[3]. The most commonly involved organs are the liver and lungs. Extrapulmonary intrathoracic location of a cyst involving the mediastinum, pleura, pericardium, chest wall and diaphragm is rare[4] and even rarer is the subcutaneous extension of echinococcus. Herein, we report a 53-year-old man with a primary large hydatid cyst of the diaphragm and smaller secondary hydatid cysts of the abdomen and thorax protruding subcutaneously.

CASE REPORT

A 53-year-old farmer with a past medical history of two car accidents at 16 and 18 years of age, which resulted in a left hemothorax evacuated with a chest tube in the first one and a fracture of the left femur internally fixated in the second one, was admitted to our surgical department. The patient's main complaint was the presence of a small soft lump in the left hypochondrium 15 years ago that was gradually increasing in size. A similar lump protruding under the left scapulae 1 mo before his admission presented without any symptoms indicating absence of compression of adjacent organs. Diminished breath sounds in the lower left hemithorax, the presence of a small (3-5 cm) subcutaneous lump between the posterior segments of the 7th and 8th ribs and a larger (13-15 cm) soft-tissue subcutaneous mass in the left hypochondrium were the clinical findings. Chest radiograph showed a left-sided opacity with scarce microcalcifications (Figure 1A). Pulmonary functional tests were normal. Thoraco-abdominal CT scan (Figure 2) revealed a hydatid cyst (14 cm in diameter) located in the left hemidiaphragm extending simultaneously to the thoracic cavity and the upper abdomen (cyst a), a cystic lesion (6 cm in diameter) in contact with the left side of the pericardium and the anterior surface of pleura anteriorly to the previous cyst (cyst b), a cystic lesion (9 cm in diameter) in the postero-lateral thoracic wall (cyst c) protruding intercostally to a small subcutaneous cyst under the scapulae (solid arrow) and an abdominal cystic lesion (13 cm in diameter, cyst d) protruding through the left costal cartilage to the left hypochondrium subcutaneously (broken arrow). CT scan showed a sand-glass appearance of the last two cysts. Laboratory tests were normal and serologic tests were

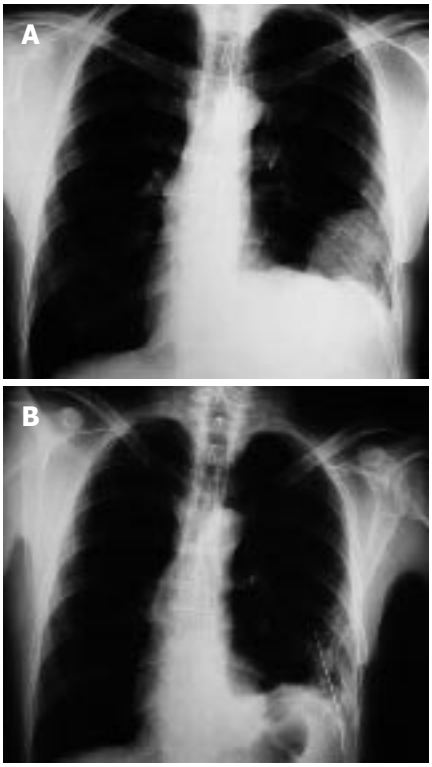

Figure 1 (**A**) Preoperative chest radiograph showing the left-sided opacity and (**B**) postoperative chest radiograph showing the chest tube placed in the left thoracic cavity.

positive for echinococcus granulosus (1/2048 antibodies, using enzyme-linked immunosorbent assay).

A bilateral subcostal incision was performed. Precautions were taken in order to avoid any spillage of cyst contents in the operation field including intact excision of all hydatid cysts and placement of sponges impregnated with hypertonic saline surrounding the involved organs. The subcutaneous hydatid cyst of the left hypochondrium was removed with its intra-abdominal portion and a partial resection of the left costal cartilage. The large hydatid cyst of the left hemidiaphragm embedded in the muscular layer was free of adhesions to the adjacent abdominal and thoracic organs. A total excision of this cyst with a nearly total excision of the left hemidiaphragm was performed. The hydatid cyst adjacent to the pericardium was completely removed. The hydatid cyst on the posterolateral thoracic wall was removed with concomitant resection of the subscapular protrusion and the eroded posterior segment of the 9th rib. The left hemidiaphragm was replaced by a bilayer synthetic mesh (Bard Composix ® Mesh 15.2 cm × 20.3 cm) and a chest tube was placed in the left thoracic cavity (Figure 1B). The patient was discharged on the 7th postoperative day and albendazole therapy was started at a dose of 400 mg bid. The postoperative course of our patient during the follow-up period (14 mo) was uneventful, with a good respiratory function.

DISCUSSION

Hydatid disease most frequently involves the liver (50%-70%) and lungs (20%-30%), which can be explained by a hypothesized novel dissemination theory via diaphragmatic lymphatic drainage proposed by Isitmangil *et al*[5]. However, other organs may be involved as well,

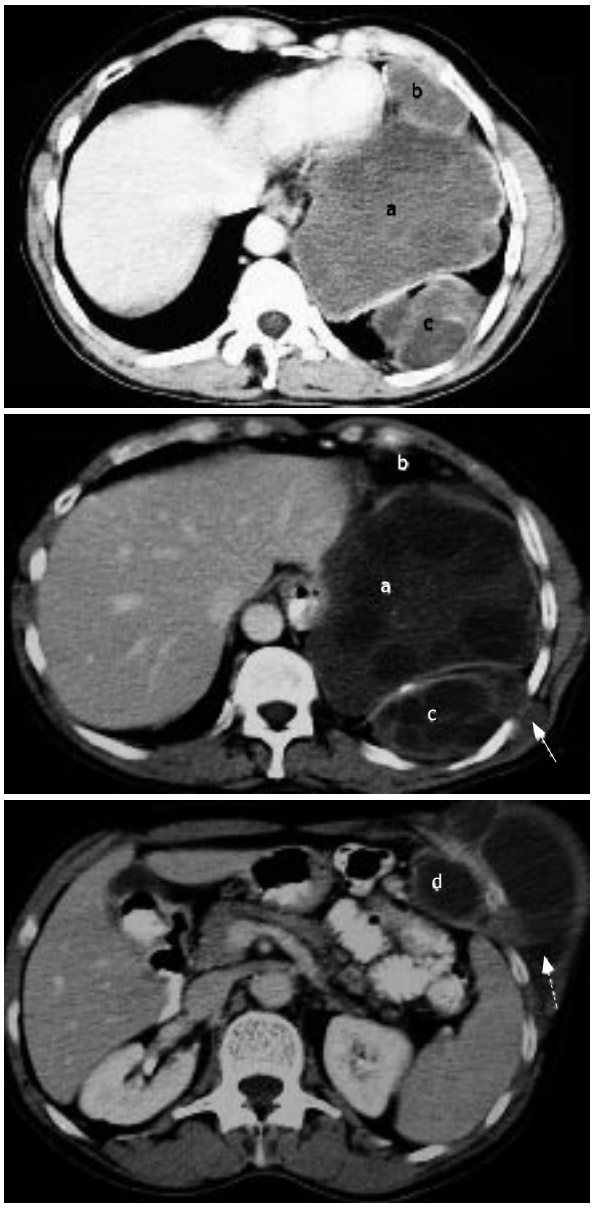

Figure 2 Thoraco-abdominal CT scan showing four separate hydatid cysts located in the left hemidiaphragm (a), anteriorly in contact with the left side of the pericardium (b) and the posterolateral wall of the left hemithorax (c) and intra-abdominally in contact with the left hypochondrium (d). Subcutaneous extension of hydatid cyst c protruding intercostally to a small subcutaneous cyst under the scapulae (solid arrow) and abdominal cyst d protruding through the left costal cartilage to the left hypochondrium subcutaneously (broken arrow) are demonstrated.

such as the brain, heart, bone and muscle (10%)[6]. Subcutaneous location or extension of a hydatid cyst is even more rare[7-9]. This rare localization of echinococcosis presents as painless soft tissue tumors or lipomas and the patient usually does not seek surgical advice.

Total excision of hydatid cysts is the definitive therapy. A thorough preoperative study of the involved organs combining the findings of chest radiograph, ultrasonography, computed tomography and magnetic resonance imaging is necessary. The bilateral subcostal incision performed in this case has the advantage of a better approach to both the abdominal and thoracic lesions, without necessitating the use of standard thoracotomy which is preferred to treat intrathoracic extrapleural

hydatid disease. However, repair or reconstruction of the diaphragm poses a technical challenge. If a synthetic mesh is used to close the defect, it must be properly applied and sutured under tension to avoid paradoxical movement and respiratory distress postoperatively. Moreover, septic complications related to mesh reconstruction during these procedures are rare and have not been reported in the literature.

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CASE REPORT

Esophageal perforation caused by fish vertebra ingestion in a seven-month-old infant demanded surgical intervention: A case report

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Abstract

A seven-month-old infant was admitted to our hospital with a 1-wk history of shortness of breath, dysphagia, and fever. Diagnosis of esophageal perforation following fish vertebra ingestion was made by history review, pneumomediastinum and an irregular hyperdense lesion noted in initial chest radiogram. Neck computed tomography (CT) confirmed that the foreign body located at the cricopharyngeal level and a small esophageal tracheal fistula was shown by esophagogram. The initial response to treatment of fish bone removal guided by panendoscopy and antibiotics administration was poor since pneumothorax plus empyema developed. Fortunately, the patient's condition finally improved after decortication, mediastinotomy and perforated esophagus repair. To our knowledge, this is the first case report of esophageal perforation due to fish bone ingestion in infancy. In addition to particular caution that has to be taken when feeding the innocent, young victim, it may indicate the importance of surgical intervention for complicated esophageal perforation in infancy.

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Key words: Fish bone; Esophageal perforation; Mediastinitis; Decortication; Mediastinotomy

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INTRODUCTION

In East Asia (China, Japan, Korea), fish bone ingestion is a common cause of emergency room visits and usually needs no invasive management other than prompt removal of the fish bone[1,2]. However, fish bone impaction complicated with esophageal perforation and pneumomediastinum is life-threatening and needs emergency surgical management. Most of the patients are above one year old since self-feeding has not begun at infancy. The mortality is extremely high if diagnosis is delayed and the sequelae might exhaust the patient and cause a catastrophic result. The outcome depends on the size of the rupture, the time elapsed between rupture, and diagnosis, as well as the underlying health of the patient[1]. Successful treatment is very difficult. Here, we report such a case of 7-mo-old infant of fish bone ingestion, and review the related literature.

CASE REPORT

A seven-mo -old male infant weighing 7.8 kg was brought to our pediatric emergency room for progressive shortness of breath and fever over the previous 1 wk. Associated symptoms included progressive irritability, poor sleep quality, fever off and on, drooling, and dysphagia. On review of his history, he was previously healthy and had no admission record. The patient's development and growth were within the normal range. He had been fed fish-rice soup as a nutrition supplement since the age of 5 mo. The initial chest radiogram showed pneumomediastinum (Figure 1A), and an irregular hyperdense lesion in the cricopharyngeal area was seen in the lateral view of the neck (Figure 1B). He was admitted to our pediatric Intensive Care Unit with unstable vital signs. Due to his feeding history, fish bone ingestion was strongly suspected. Emergency neck computed tomography (CT) and an esophagogram were arranged to confirm the diagnosis. The neck CT showed a hyperdense material at the cricopharyngeal level (Figure 2) and a small fistula tract at C3–C4 was noted on the esophagogram (Figure 3).

The day following admission, he was intubated via a bronchoscope to establish an accurate tube position. Panendoscopy was then performed after sufficient sedation

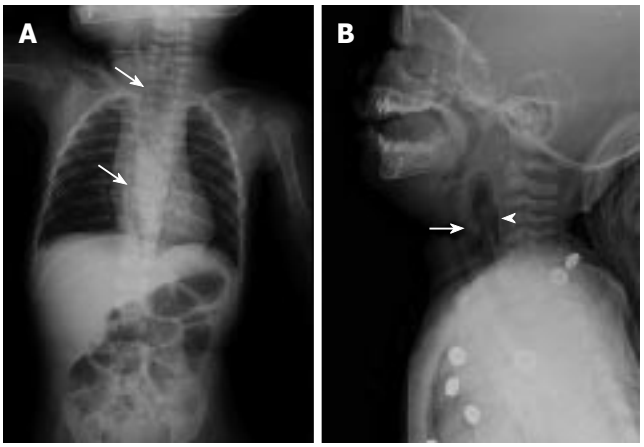

Figure 1 **A**: Extensive pneumomediastinum and air collections in retropharyngeal area (arrows), **B**: Irregular hyperdense lesion (arrow head) around cricopharyngeal area, and intra-esophageal opacification (arrow) was noted at C3 - C4 level.

Figure 3 Much air collection (arrow) was in the retropharyngeal space and mediastinum, contrast leakage (arrow head) into the retropharyngeal space with opacification of a small fistulous tract at C3-4 level (cricopharyngeal level), suggestive of esophageal perforation.

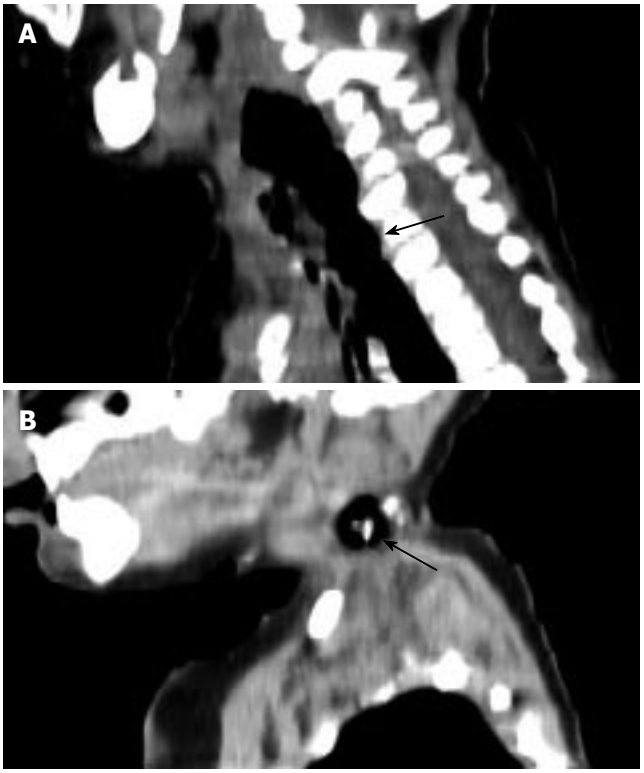

Figure 2 **A**: Esophageal rupture with retropharyngeal air collection (arrow), and **B**: Irregular foreign body impacted at upper esophagus (arrow).

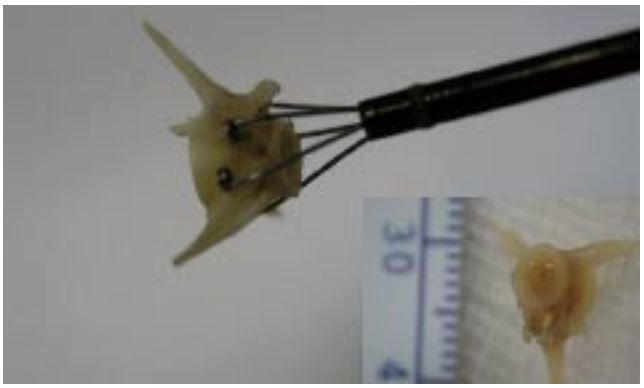

Figure 4 The fish vertebra with three sharp spines, which is about 1 cm x 1 cm in size, was removed by grasping forceps.

of the patient. A fish bone (part of a vertebra) measuring 1 cm × 1 cm was found near the esophageal inlet, which was removed smoothly with grasping forceps (Figure 4).

Although the fish bone was removed, antibiotics (ceftriaxone + amikin + metronidazole) were administrated empirically to prevent esophageal wound infection. The patient continued to drool and suffer from dyspnea on the third day of admission, and a subsequent chest radiogram showed persistent retropharyngeal air collection. Bronchoscopy and panendoscopy were repeated, but did not improve the dyspnea. CT-guided retropharyngeal aspiration was performed on the fifth day of admission and 200 mL of air was aspirated. A pneumothorax with desaturation developed on the seventh day of admission, and empyema was detected after a chest tube was inserted. A pediatric surgeon was consulted, and surgery was performed. At surgery, the findings included multilocular empyema with trapped lung, mediastinitis (abscess formation), and esophageal perforation in the cervical region. Decortication and mediastinotomy were performed to remove the inflamed tissues, and the perforated esophagus was repaired with Dexon sutures. After surgical management, the medical treatment became more effective. Coagulase-negative Staphylococcus aureus was cultured in the pus, and the fever disappeared after combination treatment with antibiotics (vancomycin + ceftriaxone + metronidazole). Total parenteral nutrition was instituted, and a nasogastric (NG) tube was inserted via panendoscopy for decompression and to prevent esophageal stricture or synechia. The right Horner's syndrome that occurred postoperatively resolved within 6 mo. Although the patient's weight had decreased to 5.8 kg, he regained body weight steadily after beginning NG feeding (15 d after the operation). Complete oral feeding was achieved after 1 mo of NG feeding. The patient was discharged from our hospital after 4 mo, without developing the obvious symptoms of esophageal stricture. On follow-up, he weighed 7.7 kg at 8 mo (20th percentile),

9.4 kg at 13 mo (25th percentile), and 11.3 kg at 21 mo (30th percentile). No serious respiratory complications were noted.

DISCUSSION

Foreign body ingestion is common in children and can be underestimated because there are no witnesses and 50% of cases are asymptomatic[3,4]. Less than 1% of foreign body ingestions result in serious morbidity; most cases need observation only. The peak age in children is from 6 mo to 3 years[5]. Based on previous experience with the pediatric population, fish bones are the second most commonly ingested foreign body (coins are the most common). In East Asia, fish bones are the most commonly ingested foreign body[2]. Nevertheless, fish bone ingestion is quite rare in infancy since self-feeding has not begun, and the caretaker screens the food before feeding.

The symptoms of foreign body ingestion are variable and depend on the size, shape, and material of the foreign body. Usually, large sharp objects cause greater morbidity[1]. The common signs and symptoms[6] in patients with a foreign body that has been retained for less than 24 h tend to be gastrointestinal and include dysphagia, drooling, vomiting, gagging, and anorexia. Major respiratory symptoms are more common weeks or months after ingestion, such as coughing, stridor, fever, chest pain, wheezing, chronic upper respiratory tract infections, pneumonia, and hemoptysis. Patients may also develop acute respiratory distress with choking and cyanosis. In our case, the patient's symptoms were compatible with typical foreign ingestion, and a lack of awareness delayed the diagnosis.

Only 32% of ingested fish bones can be identified radiographically[7], and most part of fish skeletons that cause impaction in esophagus is fish rib. Fish vertebra was seldom reported. Due to its prolonged course, pneumomediastinum was seen on the plain chest radiograph in our patient. The esophagogram confirmed the diagnosis of foreign body impaction and located the esophageal perforation. In fact, instrumental perforation and spontaneous perforation are the two major causes of esophageal injury in infants and children. Esophageal perforation due to fish bone ingestion in infancy has not been reported. Furthermore, the complications of mediastinitis, pneumothorax, and empyema were life-threatening in our patient. The following guidelines are suggested for selecting nonoperative treatment[8]: clinically stable patients; instrumental perforations detected before major mediastinal contamination has occurred, or perforations with such a long delay in diagnosis that the patient has already demonstrated tolerance for the perforation without the need for surgery; and esophageal disruptions well contained within the mediastinum or a pleural loculus. In a series of 12 children (3-7 years old) with esophageal perforation, Demirbag *et al*[9] reported that esophageal perforation could be treated safely by nonoperative means. Rivas *et al*[10] recommended conservative treatment as the best option for esophageal perforation in children. In contrast to adults, children have some important advantages in esophageal perforation with regard to complication severity, wound-healing rate, and mediastinal tissue resistance. In our case, however, the patient was younger (7 mo old), and the diagnosis delayed (about 1 wk). Although the fish bone was removed via panendoscopy soon after admission, the conservative management was ineffective, and surgery was required.

Total parenteral nutrition supplement played an important role in the postoperative therapy. It provided sufficient nutrition for the patient and allowed complete rest of the gastrointestinal tract. Treatment with broad-spectrum intravenous antibiotics was also essential for reversing the potentially fatal infection secondary to esophageal perforation. In this case, coagulase-negative S. aureus was identified on culturing of the pus. This organism is the predominant pathogen causing empyema in developing countries[5].

Two special techniques were applied in this case: bronchoscopy for intubation and CT-guided aspiration to reduce the retropharyngeal air collection. Both of these measures prevented deterioration in the patient's respiratory status. The excellent outcome in this patient was beyond our expectations. No major complications were noted one year later. We believe that the great support of his family contributed equally to the medical management.

To our knowledge, this is the first case report of esophageal perforation complicated by fish bone ingestion in infancy. Although current guidelines of therapy for fish bone ingestion in children remains conservative, this case illustrates the possibility of unpredictable and severe complications which demand surgical intervention.

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CASE REPORT

Synchronous occurrence of carcinoid, signet-ring cell carcinoma and heterotopic pancreatic tissue in stomach: A case report and literature review

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Abstract

We presented an unusual case with coexistence of carcinoid, signet-ring cell carcinoma (SRC) and heterotopic pancreatic tissue in stomach. Gastroscopic examination of this 63-year-old male patient showed multiple protrusions in gastric corpus near the greater curvature, identified by subsequent biopsy as carcinoid. Distal subtotal gastrectomy was performed. Histological and immunohistochemical examinations showed a carcinoid tumor in gastric corpus near the greater curvature, an intramucosal SRC at the lesser curvature of corpus and heterotopic pancreatic tissue in muscularis propria of the antrum at the lesser curvature with hyperplasia of peripheral endocrine cells producing multiple pancreatic hormones. We reviewed the literatures on clinicopathological characteristics and the differential diagnosis of the above three abnormalities, and concluded that the carcinoid in corpus near the greater curvature and SRC in the lesser curvature are independent lesions; the foci of endocrine cells in the muscularis propria and serosa are hyperplastic lesions from the heterotopic pancreatic tissue, rather than dissemination of carcinoid in corpus.

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Key words: Heterotopic pancreas; Carcinoid; Signet-ring cell carcinoma; Gastric tumour; Endocrine cell hyperplasia

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INTRODUCTION

Gastric carcinoid is rare, accounting for 0.4%-1.8% in tumors of the gastrointestinal tract[1], while signet-ring cell carcinoma (SRC) is frequent[2]. Both of them occur often in an occult way, with different behavior and prognosis. Pancreatic heterotopia in stomach is often incidentally encountered during surgery or autopsy, with its incidence ranging from 0.6% to 13.7%[3]. We presented a case with synchronous occurrence of these three lesions in stomach, and reviewed related literatures.

CASE REPORT

A 63-year-old male patient visited this hospital with the complaint of "cauterized discomfort in the upper abdomen for more than 2 years, and aggravated for 2 mo". By gastroscopy, multiple flat nodules (Lesion 1) were found in the front wall near the greater curvature, ranging from 0.2-0.8 cm in diameter. Mucosal biopsy was taken, and the lesions were considered a malignant neuroendocrine cell tumor with the help of immunohistochemistry. Findings from physical examination were unremarkable except for deep tenderness under xiphoid. Chest X-ray, upper alimentary canal contrast, abdominal ultrasonography failed to detect any abnormalities, while a slight increase in thickness of the antrum wall was observed by computed tomography (CT) scanning. Levels of circulating CA199, CEA, CA724 and CA242 were in normal ranges. Serum antibody against parietal cell was positive, and serum gastrin was 93.2 mg/L. No family history of cancer was recorded. Distal subtotal gastrectomy specimen was routinely resected. The samples were fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections of 5 μm in thickness were prepared and stained with hematoxylin and eosin (HE) and Alcin blue/periodic acid Schiff reaction (AB/PAS). Immunohistochemical staining was conducted using a polymer-peroxidase detection kit (PV-9000) following the instructions of the manufacturer. Polyclonal antibodies were used to detect glucagon, gastrin, somatostatin, vasoactive intestinal peptide (VIP), pancreatic polypeptide (PP), and monoclonal antibodies were used for demonstration of CK18, insulin, ACTH, synaptophysin (SY), chromogranin A (CgA), NSE, vimentin, p53 protein (DO-7) and Ki-67 antigen. All of the reagents were supplied by Zhongshan Golden Bridge Biotech Co. Ltd., Beijing. Samples of a gastric SRC and a

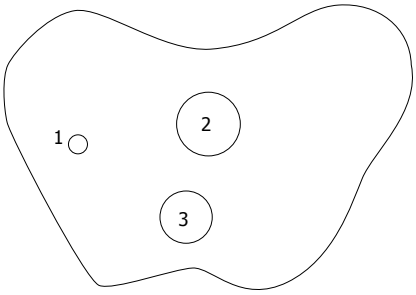

Figure 1 Diagrammatic representation of three lesions. 1: Carcinoid nodule of 0.7 cm in diameter near the greater curvature of gastric corpus; 2: SRC sizing 3.0 cm × 2.0 cm within mucosa at the lesser curvature of gastric corpus; 3: Ectopic pancreas nodule of 2.5 cm in diameter between submucosa and serosa in gastric corpus-antrum.

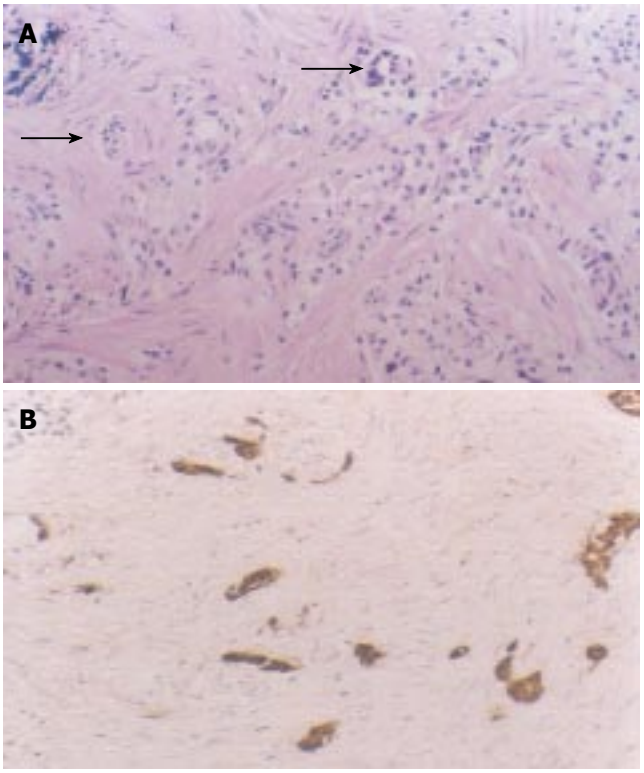

Figure 2 Lesion 1, intramucosal carcinoid with the muscularis mucosa infiltrated (**A**) (arrows, HE × 20), positively expresses CgA (**B**) (S-P method, conterstained with hematoxylin, × 20).

pancreatic endocrine tumor were used as positive controls, and the immunoglobins from a preimmune rabbit and mouse were used to substitute for the primary antibodies as negative controls. Slides were scored semi-quantitatively according to the percentage of positive cells in all counted cells from 5 randomly selected representative fields based on staining distribution and intensity as below: positive rate < 5% was defined as negative (-); weakly positive (+): 5%-25%; moderately positive (++): 25%-50%; strongly positive (+++): > 50%.

Three non-continuous lesions (Figure 1) with distinct histochemical phenotypes (Table 1) were identified: Lesion 1 (Figure 2), multiple nodules of 0.2-0.8 cm in diameter was seen in the anterior wall near the greater curvature of

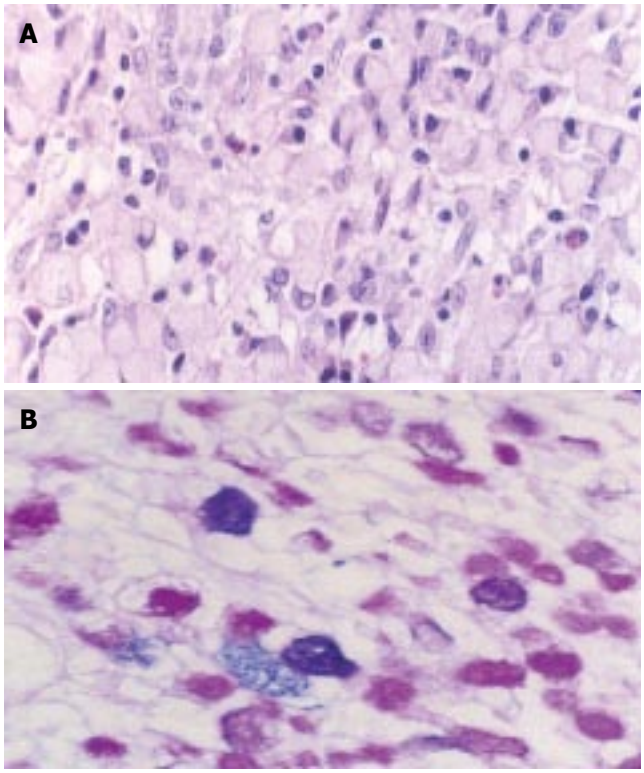

Figure 3 Lesion 2, intramucosal signet-ring cell carcinoma (**A**) (HE × 40) with cytoplasmic mixed mucin (**B**) (AB/PAS reaction, × 40).

gastric corpus, and multiple foci of hyperplastic endocrine cells were seen in the deeper part of lamina propria, infiltrating the muscularis mucosa. Immunoreactivities were seen in the cells for CK18, NSE, SY and CgA. Therefore, Lesion 1 was regarded as intramucosal endocrine cell hyperplasia and formation of a type I carcinoid, with muscularis mucosae involved. Lesion 2 was identified beside the lesser curvature of corpus and sized 3.0 cm × 2.0 cm. The cancer cells contained mixed mucin, positive for both Alcin blue and PAS, but were negative for the neuroendocrine markers (Figure 3). Therefore, Lesion 2 was diagnosed as an intramucosal SRC (superficially flat type), rather than a signet-ring-like carcinoid. Lesion 3 (Figure 4) was a yellowish mural mass sizing 3.0 cm × 2.0 cm on cut surfaces, located between the submucosa and lamina muscularis of the antrum. Histologically, the mass was composed of well developed pancreatic acini and ducts, intermingled with hyperplastic smooth muscle tissue, giving rise to the pattern of an adenomyoma. At the peripheral area, multiple nodules of 0.1-0.4 mm in diameter were found. They were composed of well circumscribed small, bland epithelial cells which arranged in solid nests. The nodules resembled normal pancreatic islets in shape, scattered within the muscularis propria and serosa of the gastric wall. No stromal reaction was observed. The epithelial cells were shown to be diffusely positive for CK18, CgA, SY, insulin, glucagons, somatostatin and gastrin, the pattern being similar to that of pancreatic islets. Neither mitosis nor necrosis was identified. Therefore, the mass was diagnosed as pancreatic heterotopia and those peripheral nodules were hyperplastic

Table 1 Immunohistochemical phenotypes of different mucosal lesions and various components of heterotopic pancreatic tissue in gastric wall

Markers	Mucosal lesions			Heterotopic pancreas (Lesion 3)			
	Carcinoid (Lesion 1)	NE cell hyperplasia	SRC (Lesion 2)	Endocrine cell hyperplasia	Islets	Acini	Ducts
CK18	+++	+++	+++	+	+	++	++
Vimentin	-	-	-	-	-	-	-
NSE	++	+-++	-	+++	+++	-	+
CgA	+++	+++	-	+++	+++	-	+
Synaptophysin	+++	+++	-	+++	+++	-	+
Insulin	-	-	-	+++	+++	-	±
Glucagon	-	-	-	++	++	-	+
Somatostatin	-	+	-	++	++	±	±
Gastrin	-	++	-	++	++	-	±
VIP	±	+	-	±	±	-	-
ACTH	-	-	-	-	-	-	-
PP	-	-	-	-	-	+	±
p53 protein	2%	-	-	-	-	-	-
Ki-67-LI	2%	< 1%	20%	1%	< 1%	3%	5%

ACTH: adrenocorticotropin; CK18: cytokeratin 18; CgA: chromogranin A; Ki-67-LI: Ki-67-lebelling index; PP: pancreatic peptide; NE: neuroendocrine; NSE: neuron-specific enolase; SRC: signet-ring cell carcinoma; VIP: vasoactive intestinal peptide.

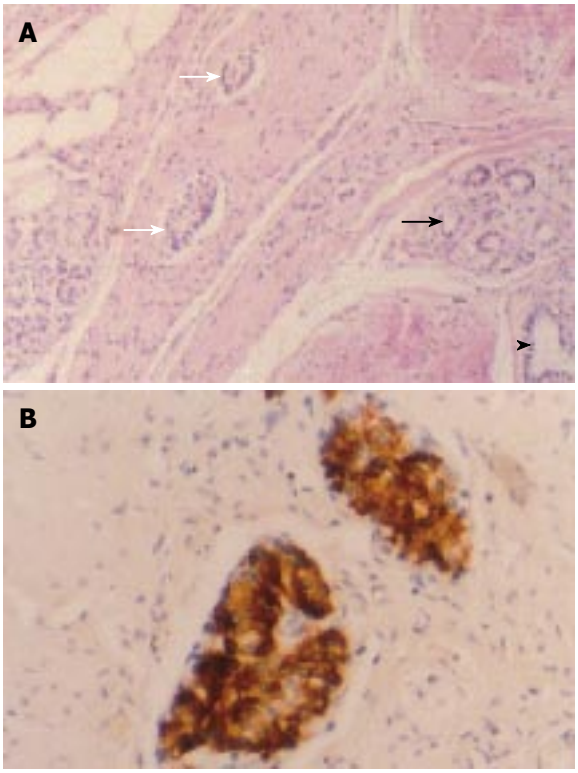

Figure 4 Lesion 3, heterotopic pancreatic tissue in gastric wall, which was composed of well developed pancreatic acini (darker arrow), ducts (arrowhead), and islets (lighter arrow) intermingled with the hyperplastic smooth muscle tissue, giving rise to the pattern of an adenomyoma (**A**) (HE × 20); Small-cell nodules of 0.1-0.4 mm in diameter scattered within the muscularis propria and serosa of the gastric wall and strongly positive for insulin (**B**) (S-P method, conterstained with hematoxylin).

tissues from endocrine pancreatic heterotopia rather than dissemination of Lesion 1.

Standard lymph node dissection was conducted and no metastasis was found in all of the twelve dissected lymph nodes.

The patient remains well 17 mo after surgery.

DISCUSSION

Gastric endocrine neoplasms are classified into carcinoid (well differentiated endocrine neoplasm), small cell carcinoma (poorly differentiated endocrine neoplasm) and tumor-like lesions (including hyperplasia and dysplasia of endocrine cells)[4]. Most endocrine tumors of the stomach are well differentiated, nonfunctioning enterochromaffin-like (ECL) cell carcinoids arising from lamina propria in the corpus or fundus[5]. Three distinct types have been recognized. Type I carcinoids are associated with autoimmune chronic atrophic gastritis. Type Ⅱ carcinoids are linked to multiple endocrine neoplasia type 1(MEN-I)) and Zollinger-Ellison syndrome (ZES). Type Ⅲ carcinoids are sporadic, without hypergastrinemia or autoimmune atrophic gastritis[6]. Types I and Ⅱ carcinoids develop through a sequence of "hyperplasia-dysplasia-neoplasia", and ECL-cell hyperplasia and dysplasia are identified as the precancerous lesions of ECL-cell carcinoid[7]. Lesions up to 0.5 cm in diameter or infiltrating submucosa are recognized as carcinoids[8]. In this case, mild to moderate atrophic gastritis was noted, coexisted with intestinal metaplasia and a high level of serum gastrin. A carcinoid of 0.7 cm in diameter was identified. All of the lesions listed above were in line with Type I carcinoid associated with endocrine cell hyperplasia.

In this case, superficial SRC (Lesion 2) was identified in corpus mucosa of the lesser curvature, which was failed to be detected by gastroscopy and biopsy before surgery. An unusual type of signet-ring-like carcinoid[9-11] was reported in literature, but the following three points in this case ruled out the possibility. First, cells in Lesions 1 and 2 were different from each other in morphology. Second, they occurred in two different sites, without anatomical continuity. Third, their histochemical and immunohistochemical phenotypes were different. Lesion 1 was negative for mucin, while positive for neuroendocrine markers (Table 1). Lesion 2, however, showed positive staining of mucin, while negative for neuroendocrine

markers. Therefore, the carcinoid and SRC in this case were two independent lesions. Up to date, 5 cases of gastric carcinoid coexisting with adenocarcinoma have been reported[12-16], and only one of them with SRC[16].

The incidence of gastric ectopic pancreas ranges from 0.6% to 13.7%, frequently in the antrum and prepyloric region on the greater curvature or posterior wall. It often locates in submucosa, lamina muscularis or subserosa. Histologically, heterotopic pancreas can be divided into 4 types: type 1, composed of all cell types, namely complete heterotopia; type 2, composed of ductal components only, the canalicular heterotopia; type 3, composed of acinar cells only, the exocrine heterotopia; and type 4, composed of islet cells only; the endocrine heterotopia[17]. In this case, the heterotopic pancreas belongs to the first type. Most of the patients with gastric ectopic pancreas are asymptomatic. When mucosa is involved, however, patients may complain of an upper abdominal pain, bleeding, and obstruction may occur. Malignant transformation has been encountered, albeit rarely, giving rise to an adenocarcinoma[18-25] or a neuroendocrine neoplasm[26]. Caution should be paid to establishing a diagnosis of a carcinoma from heterotopic pancreas. For this, 3 points were considered necessary. First, the carcinoma should be found within or close to the heterotopic pancreas. Second, the transitional area between pancreatic structures and carcinoma should be observed and a metastatic carcinoma or dissemination of carcinoma from the adjacent gastrointestinal tract must be excluded. In addition, the non-neoplastic heterotopic pancreatic tissue should contain at least fully developed acinar or ductal structures[3]. It is evident that SRC in this case is independent from Lesion 3. Besides, a functional pancreatic endocrine tumor is composed of neoplastic cells secreting the same hormone (insulin, glucagon or somatostatin), differing from the endocrine cell hyperplasia whose constituent cells produce multiple hormones. In this case, Lesion 3 belonged to the complete heterotopic pancreas, with endocrine cell proliferation, which scattered preferentially among bundles of smooth muscle fibers or along vascular structures, mimicking intramural infiltration or metastasis of the carcinoid. It is important to know whether the endocrine tissue is hyperplastic or neoplastic, in consideration of the presence of a carcinoid (Lesion 1) at the corpus of the stomach and their structural and morphologic similarities. The following data revealed that endocrine component in Lesion 3 is non-neoplastic. The endocrine tissue was accompanied by the exocrine components in most parts of the lesion. Functionally, the endocrine tissue was composed of cells separately producing insulin, glucagon or somatostatin, reflecting its polyclonal nature, the phenotypes being similar to that of islets and different from Lesion 1 (Table 1).

In summary, we presented a case with coexistence of carcinoid, SRC and heterotopic pancreas in stomach. Differential diagnosis of the three abnormalities was of great importance for prognosis evaluation of the patient.

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CASE REPORT

Hyperinsulinemic hypoglycemia due to adult nesidioblastosis in insulin-dependent diabetes

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Abstract

In neonates, persistent hyperinsulinemic hypoglycemia (PHH) is associated with nesidioblastosis. In adults, PHH is usually caused by solitary benign insulinomas. We report on an adult patient who suffered from insulin-dependent diabetes mellitus, and subsequently developed PHH caused by diffuse nesidioblastosis. Mutations of the MEN1 and Mody 2/3 genes were ruled out. Preoperative diagnostic procedures, the histopathological criteria and the surgical treatment options of adult nesidioblastosis are discussed. So far only one similar case of adult nesidioblastosis subsequent to diabetes mellitus Ⅱ has been reported in the literature. In case of conversion of diabetes into hyperinsulinemic hypoglycemia syndrome, nesidioblastosis in addition to insulinoma should be considered.

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Key words: Hyperinsulinemic hypoglycaemia; Adult nesidioblastosis; Diabetes; Multiple Endocrine Neoplasia Type 1; Insulinoma

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INTRODUCTION

Persistent hyperinsulinemic hypoglycemia (PHH) caused by functionally defectice β-cells in the setting of a nesidioblastosis is the most common pathological substrate in newborns, whereas in adults, PHH is usually caused by solitary insulinomas[1-3]. Several genetic abnormalities were identified as the causes of PHH in infancy. The most important mutations are in the β-cell sulfonylurea receptor (SUR1) gene and encoding proteins composing the ATP-sensitive potassium channel in the cell membrane of the β-cell (Kir6.2)[4,5]. Loss of function due to mutations of the glucokinase (GCK) and glutamat dehydrogenase (GLUD1) genes were also identified as possible causes of PHH of infancy[6-8]. The morphologic substrate of these molecular changes are either diffuse β-cell hypertrophy (diffuse nesidioblastosis) or focal β-cell hypertrophy and β-cell hyperplasia (focal nesidioblastosis)[4,9]. The term nesidioblastosis for describing the lesions of the endocrine pancreas in neonates was first used by Yakovac[10] in 1971, when he described 12 infants with PHH.

Establishing the diagnosis of nesidioblastosis in an adult is a great challenge in endocrinology. To date, it is not possible to diagnose diffuse nesidioblastosis in adults on clinical grounds, since imaging techniques or detection of a specific causative mutation are of no help. The fasten test for the diagnosis of nesidioblastosis has been controversially discussed. Up to now, the diagnosis is based on (1) exclusion of an insulinoma by all means of clinical diagnostic procedures and (2) pathological analysis of the pancreatic tissue specimens. A combination of various histopathological criteria, marked as major and minor criteria, has recently been published to establish the diagnosis of a diffuse adult nesidioblastosis[11].

Since the first reported case of an adult nesidioblastosis in 1975, less than 100 patients have been described in the literature. Most of them were reported in individual case studies. Reports of PHH with known diabetes mellitus are extremely rare[12-15]. In our hospital, diffuse nesidioblastosis has been diagnosed in 4 out of 137 patients suffering from PHH. A coincidence of PHH with diabetes was diagnosed in only one patient. The aim of our report is to describe this patient in detail concerning (1) clinical presentation, (2) mutational analysis of the MEN1 and Mody 2/3 genes, (3) pathology of the pancreatic specimens. The diagnostic procedures and the surgical treatment strategies are discussed on the basis of the presented case.

CASE REPORT

Clinical presentation

A 40-year-old man (170 cm in height; BMI: 24.9) diagnosed with organic hyperinsulinism was admitted to our

hospital. The previous medical history was marked by a primarily insulin-independent diabetes (type Ⅱ). Insulin treatment has been required over the past 6 years. At the age of 35, he developed an idiopathic facial nerve pareses and brainstem encepahlitis of unknown etiology. Family history showed no pathologic findings, except diabetes of a grandmother. On the onset of diabetes, his blood sugar rose to 3800 mg/L with elevated HbA1c (8.0%). The patient was started on insulin treatment. During three hospital admissions for hyperglycemia over a 5-year period, blood sugar levels were often higher than 4000 mg/L and he was treated with insulin. Starting in 2005, the insulin treatment began to reduce and two months later discontinued, because of normalized serum glucose levels. He was admitted to hospital for symptoms of hyperadrenergic reactions and neuroglycopenic symptoms of confusion and somnolence due to hypoglycemia with blood sugar levels of 250 mg/L.

Diagnosis with a pathological fasting test indicated hypoglycemia. Blood sugar levels were repeatedly reduced to 300 mg/L with typical neuroglycopenic symptoms. The insulin level was up to 93.3 mU/L with a raised insulin index of 2-3. Exogenous insulin intake could be excluded by C-peptide levels. Unfortunately, the real C-peptide level could not be specified because of insufficient terms of transport (external laboratory). Insulin antibodies were negative. Attempts to localize an insulinoma with computed tomography (CT), ultrasound (US) of the abdomen and endoscopic ultrasound (EUS) were unsuccessful.

Laparotomy was performed and the pancreas was completely exposed and investigated by bimanual palpation and intraoperative ultrasound. A spleen preserving pancreatic-tail resection was performed because of a suspected small tumor in the tail of the pancreas. The patient's postoperative course was uneventful. Postoperatively, he displayed no signs of recurrent hypoglycemia. The blood sugar levels measured rose up to 1660-2430 mg/L, therefore low dose insulin application was necessary. The patient was discharged on the seventh postoperative day.

The patient and his family doctor reported recurrent diabetes metabolism over the previous 5 mo during a nine month follow-up after surgery (blood sugar levels: 300-2420 mg/L depending on insulin intake). Unfortunately, a reassessment of the diabetes was declined by the patient at the time.

Mutational analysis

Genetic sequence analysis of the exons 2-10 of the MEN1 gene on chromosome 11q13 as previously described[16,17], was performed for differential diagnosis. The genetic make-up ruled out a molecular germline mutation in the MEN1 gene. Additional genetic sequence analyses of the glukokinase-gene of exon 1A-10 on chromosome 7p15-p13 were performed. Mutations pointing to maturity diabetes of the young Typ 2 were excluded. In addition, genetic sequence analyses of exons 1-10 of the hepatic nuclear factor 1 alpha gene for MODY Typ 3 were negative.

Histopathological analysis

Pathological examinations were done using serial sections from different regions of the specimens which were immunohistochemically stained, by the avidin-biotin-complex (ABC) method with a monoclonal antibody against insulin (BioGenex, San Ramon, Canada, primary dilution 1:40) and polyclonal antibodies against glucagon (BioGenex, dilution: 1:60), somatostatin (Dako, Glostrup, Denmark, dilution: 1:200) and pancreatic polypeptide (DAKO, dilution: 1:5000). Appropriate controls were performed in order to ascertain the staining specificity. Histological and immunostained pancreatic sections were analyzed by three different pathologists in a double-blind fashion. Histological criteria for nesidioblastosis in adults were used to evaluate the sections[11]. Age- and sex-matched control pancreatic specimens were used as references.

Gross examination and step sectioning of the pancreatic samples excluded the presence of an endocrine tumor. The lobular architecture of the exocrine parenchyma was preserved. The pancreatic duct was patent and of normal size. There was no evidence of amyloid deposits or a quantitative disturbance of beta-cells. A consistent finding was the presence of multiple enlarged islet cells, whose arrangement often produced a lobulated islet pattern. Cytologically, a considerable subpopulation of endocrine cells showed enlarged and hyperchromatic nuclei (Figure 1A). These cytological changes were seen in more than 2/3 of the islets. Using immunohistochemistry and subsequent staining with hematoxylin, the cytologically conspicuous endocrine cells were identified as beta-cells (Figure 1B). In contrast, glucagon, somatostatin and pancreatic polypeptide cells did not reveal any cytologic abnormalities (Figure 1C). The subcellular distribution of insulin was regular and apparently equal amounts of C-Peptide were seen. There was no evidence of an expression of the nuclear proliferation antigen ki-67 within the islets. According to this histopathological analysis the patient fulfilled the recently described "major criteria" of a diffuse nesidioblastosis in adults[11].

DISCUSSION

Nesidioblastosis was detected and diagnosed in 4 patients out of a series of 137 patients treated for organic hyperinsulinism at our hospital. One of them was diagnosed by clinically reversing a highly insulin-dependant diabetes. Diagnosis of nesidioblastosis was established by (1) exclusion of an insulinoma by all means of clinical diagnostic procedures and (2) by pathological examination revealing the characteristic signs of a diffuse nesidioblastosis.

Preoperative localization by CT-scan, abdominal ultrasound and endoscopic ultrasound (EUS) failed to detect an endocrine tumor. An indication for surgery was based on hypoglycemia in the presence of elevated insulin levels and excluding exogene insulin intake by C-peptide levels and screening for sulfonylureas.

After completely exposing the pancreas and investigation by bimanual palpation and intraoperative

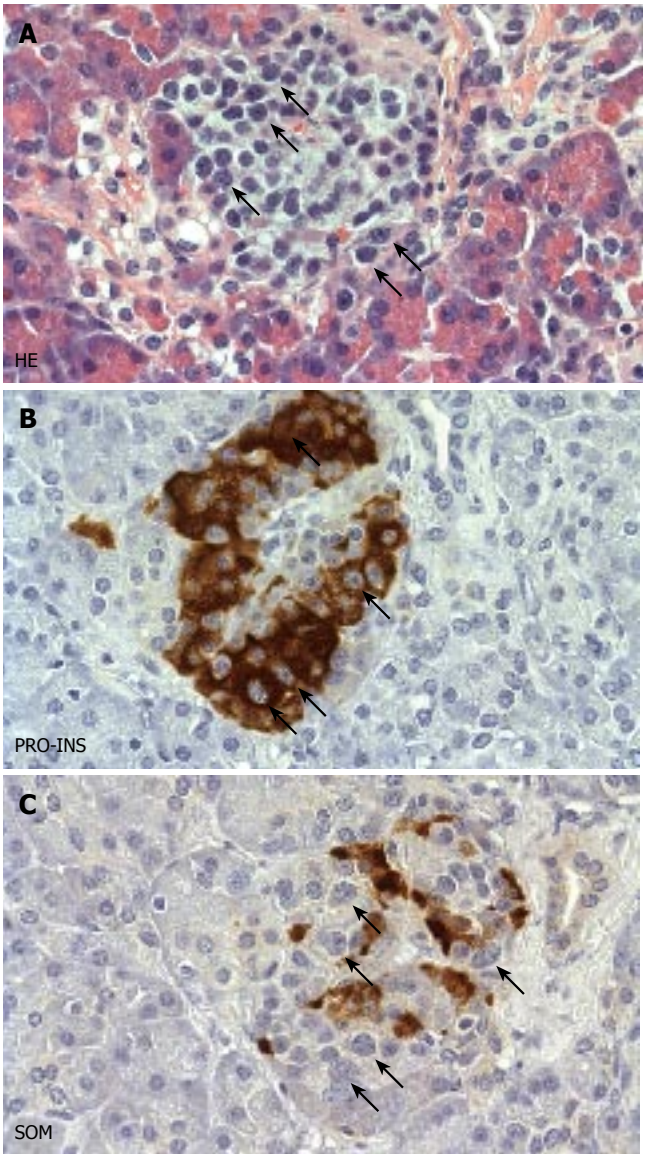

Figure 1 Histopathological features of diffuse nesidioblastosis. **A:** HE stained sections demonstrating a prominent lobulation of an islet. Some of the endocrine cells showing hyperchromatic and enlarged nuclei are labelled with arrows. **B**, **C**: Adjacent section analysis demonstrating cytoplasmic positivity for proinsulin (PRO-INS) in those endocrine cells with hyperchromatic nuclei (arrows in **B**). In contrast, these cells are negative for somatostatin (SOM) (arrows in **C**).

ultrasound, a small tumor in the tail of the pancreas was suspected and a spleen preserving pancreatic-tail resection was performed. Nesidioblastosis was diagnosed postoperatively by the pathologist according to the recently published major and minor criteria for nesidioblastosis in adults[11].

Our knowledge of the pathogenesis of PHH in infants has expanded in recent years. Some genetic abnormalities underlie the disease. The most important genetic defects are inactivating mutations on chromosome 11 (SUR1 and Kir6.2)[4,5]. Gain-of-function mutations of the glucokinase gene (GCK) and glutamate dehydrogenase genes are infrequent (GLUD1)[11]. Because of the patient's young age, a subgroup of diabetes mellitus, a maturity onset diabetes of the youth (MODY), should be discussed for differential diagnosis. So far 6 different types of MODY have been diagnosed. Glucokinase plays a critical role in the insulin secretion in β-cells. Heterozygous mutations in the glucokinase-encoding gene (GCK) results in the reduction of encymatic activity (MODY 2)[18,19]. Loss of function mutations in GCK could be ruled out as a possible reason for hyperglycemia in our patient.

The diagnosis and treatment of an adult nesidioblastosis still remain an open issue. There are no imaging techniques to differentiate focal from nonfocal (diffuse) organic hyperinsulinism. Frequently, insulin-producing tumors are less than 10 mm in diameter and may therefore escape preoperative detection. Additionally, preoperative imaging as EUS can yield misleading findings in the pancreas[20]. An intra-arterial calcium stimulation test may be able to differentiate focal hyperactive β-cells from diffuse hypertophic β-cells[21-23]. Due to the failure of the modern imaging techniques in these patients, the localization of pathologic increased insulin secretion would be a diagnostic tool for the surgeon to help avoid blind pancreatic resection. The treatment of adult nesidioblastosis is surgical resection. However, the line between 'too much' and 'too less' resection, with the risk of endocrine and exocrine pancreatic insufficiency and recurrent hypoglycemia is very narrow. Nesiodioblastosis has to be considered in all cases without localized insulinoma because it has been recently shown that this disease can be present in up to 4% of patients with PHH[11]. In the case of recurrence, a secondary surgical intervention with further pancreatic resection is necessary. In our case, hypoglycemia may be life threatening, but the risk of lifetime diabetes should be considered as well. Nowadays, recurrent pancreatic resections can be performed with low risk in specialized centers.

The present case illustrates a very rare coincidence of diabetes and adult nesidioblastosis. Up to now, only four cases with organic hyperinsulinism and diabetes have been reported[12-15]. Our case is the second description of a concomitant adult nesidioblastosis and diabetes type Ⅱ. In summary, diabetes can be reversed by functionally defective hypertrophic β-cells. In the case of reversing diabetes, an organic hyperinsulinemic hypoglycemia should be considered as a differential diagnosis.

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LETTERS TO THE EDITOR

Targeting intestinal microflora in inflammatory bowel disease

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TO THE EDITOR

In their recent review article[1], Andoh and Fujiyama examined the various therapeutic approaches targeting intestinal microflora in patients with inflammatory bowel disease (IBD). I would like to provide some additional data to complete and update their comments. First of all, when considering the role of probiotics in IBD treatment it must be emphasized that, in addition to *Bifidobacteria*, the *Nissle 1917 E. coli* strain and cocktails of microorganisms such as *VSL # 3* mentioned in the article, other probiotic agents have been tested in the short- and long-term treatment of either ulcerative colitis and Crohn's disease, the results of those studies being reported in major international scientific journals.

For instance, *Saccharomyces boulardii*, a non-pathogenic yeast, which is widely used in the treatment of diarrhea and is effective in preventing relapse of *Clostridium difficile* infection[2], has been employed in the maintenance treatment of patients with inactive Crohn's disease[3]. Thirty-two patients were randomly allocated to a six-month maintenance therapy either with mesalazine alone (500 mg of sustained-release microgranules, 3 times daily) or with the same mesalazine preparation 500 mg twice daily plus *Saccharomyces boulardii* 500 mg once daily. Clinical relapse, defined as CDAI > 150 with an increase of 100 points over the baseline values for more than 2 wk, was observed in 37.5% of patients receiving mesalazine alone and in only 6.25% of subjects in the group treated with mesalazine plus *Saccharomyces boulardii* ($P = 0.04$ by Fisher's exact test).

Furthermore, in 25 patients with a history of poor tolerance to corticosteroids, who had a clinical flare-up of mild to moderate left-sided ulcerative colitis while on maintenance with mesalazine, *Saccharomyces boulardii* (250 mg, 3 times daily) was added to the ongoing mesalazine treatment for 4 wk[4]. Clinical evaluation was performed before and after the treatment with Rachmilewitz's activity index. Clinical remission, -Endoscopically confirmed clinical emission was achieved in 68% of cases on an intention-to-treat basis.

Although the effect of each probiotic agent is different, placebo-controlled clinical trials employing *Lactobacillus GG* in the maintenance treatment of Crohn's disease have failed to show any significant effect in preventing recurrences at 6-24 mo[5,6]. However, the probiotic is able to maintain clinical remission of ulcerative colitis [7].

Finally, when considering the possible role of antibiotics in IBD, the recent data on rifaximin, a poorly absorbable antibacterial agent, must be quoted. A double-blind, placebo-controlled trial showed that addition of 800 mg rifaximin twice a day for 12 wk is effective in inducing clinical remission of active Crohn's disease[8]. It was reported that 400 mg rifaximin twice daily for 4 wk can achieve clinical remission in 76% of ulcerative colitis patients who had relapse while on mesalazine maintenance[9].

The virtual absence of systemic side-effects and the encouraging results reported so far suggest rifaximin can effectively inhibit the intestinal flora in IBD patients without severe side effects.

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AUTHOR'S FEEDBACK

Premature chomosome condensation technique: A very promising approach to radiotherapy for digestive system cancers

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Dear Editior-in-Chief,

I am grateful to the renowned World Journal of Gastroenterology for publishing our manuscript about hepatoma radiosensitivity in volume 11 issue 26, page 4098-4101[1]. Since this paper appeared online first, it was widely considered. Up to date, it has been cited four times in various journals covered by Science Citation Index[2-4], and the click count and download count added up to 459 and 163[1], respectively. Some scientists from Australia and Germany frequently sent me letters to ask for original data to be cited, but a fatal mistake made them arduous. The correspondence author's e-mail address should be yangjs@impcas.ac.cn, but not tuyangjs@impcas.ac.cnit. I sincerely expected your editors could rephrase and upload it to your online data base.

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net
6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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In press

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

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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

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

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

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

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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

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

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