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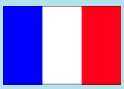

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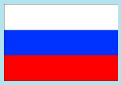

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• *GASTRIC CANCER* •

Effects of folic acid on epithelial apoptosis and expression of Bcl-2 and p53 in premalignant gastric lesions

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Abstract

AIM: To evaluate the effects of folic acid on epithelial apoptosis and expression of Bcl-2 and p53 in the tissues of premalignant gastric lesions.

METHODS: Thirty-eight patients, with premalignant gastric lesions including 18 colonic-type intestinal metaplasia (IM) and 20 mild or moderate dysplasia, were randomly divided into a treatment group (n = 19) receiving folic acid 10 mg thrice daily and a control group (n = 19) receiving sucralfate 1 000 mg thrice daily for 3 mo. All patients underwent endoscopies and four biopsies were taken prior to treatment and repeated after concluding therapy. Folate concentrations in gastric mucosa were measured with chemiluminescent enzyme immunoassay. Epithelial apoptosis and the expression of Bcl-2 and p53 protein in gastric mucosa were detected with flow cytometric assay.

RESULTS: The mean of folate concentration in gastric mucosa was 9.03±3.37 μg/g wet wt in the folic acid treatment group, which was significantly higher than 6.83±3.02 μg/g wet wt in the control group. Both the epithelial apoptosis rate and the tumor suppressor p53 expression in gastric mucosa significantly increased after folic acid treatment. In contrast, the expression of Bcl-2 oncogene protein decreased after folic acid therapy.

CONCLUSION: These data indicate that folic acid may play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in the patients with premalignant lesions.

Key words: Folic acid; Bcl-2; p53; Premalignant gastric lesions

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INTRODUCTION

Gastric cancer is one of the most common malignant diseases and remains the leading cause of cancer-related deaths in China[1]. Epidemiological analysis has revealed that individuals with a family history of gastric cancer have a 3-fold increased risk of developing gastric cancer as compared to the unaffected population[2,3], suggesting the fact that there is familial aggregation may reflect not only environmental but also genetic traits. Furthermore, nutritional studies have indicated that low consumption of fruits and vegetables is consistently related to an increased incidence of cancer[4]. Many components of fruits and vegetables such as folate and vitamin C may be responsible for the reduced risk of cancer[5-7]. It has been reported that diminished folate status is associated with cancer of the cervix, colorectum, lung, esophagus, brain, pancreas, and breast[8]. In a rat model of colonic carcinogenesis, folate deficiency enhances the development of colonic dysplasia and cancer, providing convincing evidence for the cause and effective relationship between diminished folate status and colon cancer[9]. Xiao *et al*[10] recently demonstrated that high-dose folic acid supplementation protected beagles against the development of gastric carcinogenesis induced by a chemical carcinogen N-ethyl-N-nitrosoguanidine.

Although the pathogenesis of gastric cancer still remains unclear, it has been proposed that the alteration of the balance between apoptosis and proliferation of gastric epithelial cells contributes to carcinogenesis[11]. The early indicator for the subject predisposed to gastric cancer is abnormal hyperproliferation of gastric epithelial cells, such as chronic atrophic gastritis, intestinal metaplasia (IM) and dysplasia, which have been considered as premalignant gastric lesions[12,13]. Generally, accumulation of genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes plays an important role in the process from premalignant lesions to malignant transformation. However, little is known about the effects of folic acid on the expression of *bcl-2* oncogene and tumor suppressor *p53* in the premalignant gastric lesions. Therefore, the purpose of this study was to examine whether folic acid affects the apoptosis of epithelial cells and the expression

of Bcl-2 and p53 protein in the patients with IM and dysplasia.

MATERIALS AND METHODS

Patients

Patients with an endoscopic screening and histologically confirmed colonic type (grade III) IM and mild or moderate dysphasia by two or more expert pathologists on biopsy specimens were studied for this prospective, randomized, investigator-blind trial. Exclusion criteria were: (1) previous history of gastric surgery; (2) alcoholics; (3) current metabolic or life threatening disease; (4) recent use of vitamin supplements, methotrexate, triamterene, phenobarbital, pyrimethamine, trimethoprim, cholestyramine, sulfasalazine, or non-steroidal anti-inflammatory drugs; (5) pregnancy; and (6) anemia or vitamin B_{12} deficiency. A total of 38 patients (18 IM and 20, dysplasia) who were recruited in the digestive unit at Affiliated Zhongda Hospital of Southeast University fulfilled the criteria for admission to the study. All patients gave their fully informed written consent before entering the study. The study also received the approval of the Medical Ethics Committee of Southeast University and Nanjing Medical University.

Groups

The patients were randomly divided into a treatment group (n = 19) receiving folic acid tablet (Changzhou Pharmaceutical Factory, Changzhou, China) 10 mg thrice daily for 3 mo, and a control group (n = 19) receiving a gastric mucosal protective reagent, sucralfate tablet 1 000 mg thrice daily for 3 mo. Each group involves 9 IM and 10, dysplasia. Treatment group consisted of 13 men and 6 women with an average age of 50±7 years. Control group was made up of 14 men and 5 women with an average age of 49±9 years. No significant differences of sex ratio and average age were found between the two groups.

Endoscopies and biopsy specimens

All endoscopic examinations were performed under local anesthesia with lidocaine. Four biopsy specimens (each 2 from the lesser and greater curvature of the antrum about 2 cm away from the pylorus respectively) were taken prior to folic acid treatment and repeated after concluding therapy.

Measurement of folate concentrations in the gastric mucosa

Biopsy specimens were weighed and then homogenized in 0.2 mol/L acetic acid in a volume of 1 μL/μg. The homogenate was centrifuged at 1 500 g for 15 min and the supernatants stored at -20 ℃ for the folate assay. The folate concentrations in the gastric mucosa were measured with chemiluminescent enzyme immunoassay kit according to the procedure indicated by the manufacturer (the IMMULITE 2000 system, DPC, Los Angeles, CA, USA). In brief, an aliquot of pretreated samples, ligand-labeled folate and folate binding protein (FBP) were added to the reaction tube containing a bead coated with a monoclonal antibody against FBP. Folate from the sample competed with the ligand-labeled folate for the FBP, and ligand-labeled folate-FPB complex bound to the anti-FBP antibody on the bead. Alkaline phosphatase-labeled anti-ligand was added and bound to the ligand-labeled folate in the complex immobilized on the bead. Substrate addition initiated the chemiluminescent reaction, yielding a result that relates inversely to the folate concentrations in the samples. The concentrations of folate in gastric mucosa were expressed as micrograms of folate per gram tissue wet weight (μg/g wet wt).

Assessment of cell cycle and apoptosis by flow cytometry analysis

For the preparation of gastric epithelial cell suspensions, biopsy specimens were treated for 30 min at 37 ℃ water bath with digesting solution containing 1% porcine pepsin A (by weight of substrate, and adjusted to pH 1.5 with 1 mol/L HCl). The reactions were terminated by adding 10 mL physiological saline. The cell solution was centrifuged at 750 r/min for 10 min and then pressed through a nylon cell strainer (Spectrum Laboratories Inc.) to isolate single-cell suspensions. Flow cytometry assay followed routine procedures by using 1×10^6 cells per sample.

The cell pellets were fixed with 200 μL of 70% ethanol at -20 ℃ for at least 4 h. After the ethanol was removed, the cells were washed once in PBS and then resuspended in 1 mL of propidium iodide/Triton X-100 staining solution [PBS containing 0.1% Triton X-100 (Sigma), 0.2 mg/mL RNAse A (Sigma), and 50 μg/mL propidium iodide (PI, Sigma)] and incubated for 30 min in the dark at room temperature. The DNA contents of the cells were analyzed by using a FACScan flow cytometer in combination with Cell Quest and ModFit LF software (Becton Dickinson, CA, USA). Apoptosis rates were based on the proportion of the peak area of the sub-G1 phase.

To confirm the type of cell death induced by folic acid, aliquots of 1×10^6 cells were washed in ice-cold PBS and suspended in binding buffer (Annexin V FITC kit, Immunotech, France). Annexin V fluorescein isothiocyanate (FITC) and PI were added to the cell suspension, and the mixture was incubated for 10 min on ice in the dark. The stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson). A minimum of 10 000 cells was counted in each sample; apoptosis rate represents the percentage ratio of Annexin V+/PI- cells to the total cell population.

Flow cytometry analysis of Bcl-2 and p53 protein expression

For Bcl-2 and p53 expression, cells were stained with FITC-labeled monoclonal anti-Bcl-2 and anti-p53 antibodies (PharMingen, San Diego, CA, USA), washed, and stained with secondary antibodies. The samples were then run on a FACScan flow cytometer (Becton Dickinson), equipped with an argon laser emitting at 488 nm. A minimum of 10 000 cells were acquired in a list mode file format and analyses were performed with CELLQuest software (Becton Dickinson). The results were obtained as mean fluorescence index (MFI), calculated as the ratio of sample mean channel: control mean channel.

Statistical analysis

Data were shown as mean±SD. Statistical analyses of matched data were performed using the Student's t-test. A

$P \leqslant 0.05$ was considered statistically significant.

RESULTS

Folate concentrations in the gastric mucosa

Three months after folic acid treatment, the mean of folate concentrations in gastric mucosa was (9.03±3.37) μg/g wet wt in the treatment group, which were significantly higher than (6.83±3.02) μg/g wet wt in the control group ($P<0.01$ Table 1).

Table 1 Changes in the gastric mucosal folate concentrations after folate treatment

Group	*n*	Pre-T	Post-T
Treatment	19	7.16±1.88	9.03±3.37[b]
Control	19	6.90±2.72	6.83±3.02

n: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD) μg/g wet wt. [b]$P<0.01$ *vs* control group and before treatment in the same group.

Cell cycle analysis and apoptosis

Figure 1 shows the distributions of the DNA contents in gastric epithelial cells after folic acid treatment. A high peak in the sub-G1 phase as an induction of apoptosis was detected after folic acid therapy. However, the effect was not observed in the control group treated with sucralfate (data not shown).

To confirm the type of cell death induced by folic acid, gastric epithelial cells were stained twice with PI and FITC-labeled Annexin V, and then analyzed by flow cytometry (Table 2). As Annexin V detects phosphatidylserine exposure in the plasma membrane of apoptotic cells, this technique can differentiate among normal (double negative), early apoptotic (Annexin V-FITC single positive), and necrotic (Annexin V-FITC and PI double positive) cells. In Figure 2, the lower left population of cells that have low level of FITC and PI signals in each plot indicates normal cells. Those in the upper left, which have low FITC and high PI signals, indicate necrotic cells. The lower right and upper right populations correspond to apoptotic and secondary necrotic (late apoptotic), which have high FITC and low PI, and high FITC and high PI signals respectively. More than 80% of

Table 2 Effects of folic acid on the apoptosis rate in gastric epithelial cells

Group	*n*	Staining with PI (%)		Staining with annexin V FITC/PI (%)	
		Pre-T	Post-T	Pre-T	Post-T
Treatment	19	6.19±2.82	6.78±2.15[b]	16.22±3.80	21.00±4.61[a]
Control	19	6.27±3.24	6.23±2.70	15.10±2.52	15.70±2.56

Apoptosis rate represents the ratio of Annexin V+/PI- cells to the total cell population. *n*: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD)%. [a]$P<0.05$ and [b]$P<0.01$ *vs* control group and before treatment in the same group.

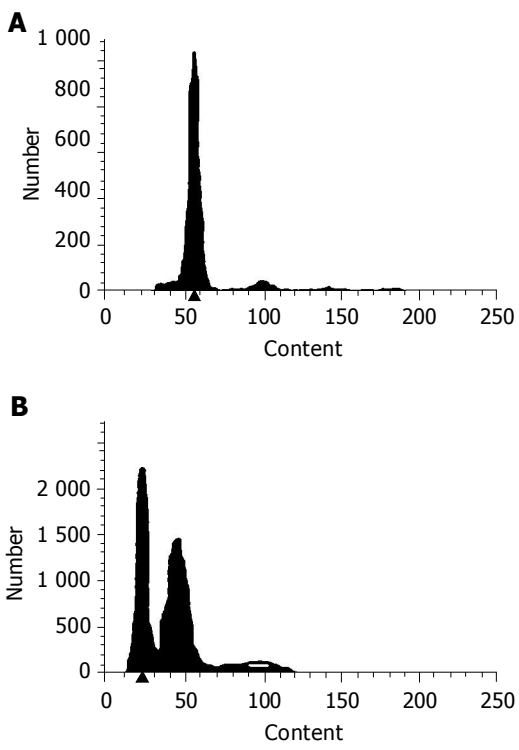

Figure 1 Effect of folic acid on the cell cycle and the induction of apoptosis DNA content was determined by flow cytometry after ethanol fixation, permeabilization and PI staining. A: before; B: after folic acid treatment. Representative results of one of the subjects are shown.

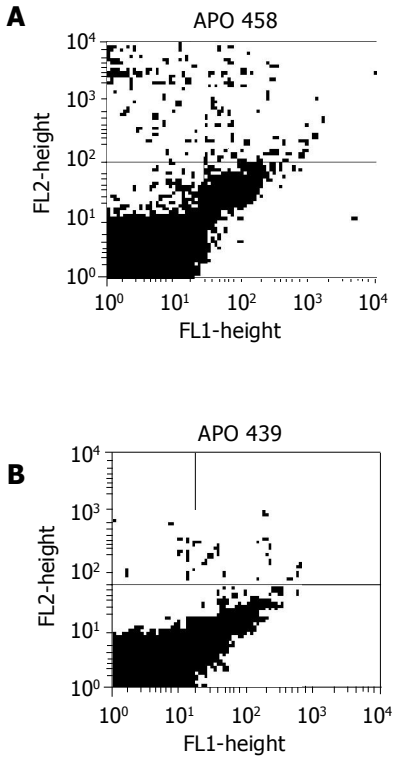

Figure 2 Annexin V-FITC/propidium iodide double staining of gastric epithelium before and after treatment with folic acid The proportion of apoptosis was measured by flow cytometry using Annexin V-FITC and PI. In the figures, X-axis (FL1-H) indicates Annexin V, Y-axis (FL2-H) indicates PI. A: before; B: after folic acid treatment. Representative results of one of the subjects are shown.

the untreated cells were Annexin and PI double negative (Figure 2A), whereas an increase in the lower right population was observed (Figure 2B) after folic acid treatment.

Expression of Bcl-2 and p53 protein

We also investigated intracellular expression of molecules known to be involved in apoptosis such as Bcl-2 and p53. Representative distributions of Bcl-2 and P53 in the gastric epithelial cells were shown in Figure 3 (A-D). Bcl-2 MFI was weaker whereas P53 MFI was higher after folic acid treatment (B and D) when compared with treatment before (A and C). Therefore, after treatment with folic acid for 3 mo, expression of Bcl-2 decreased and that of p53 increased significantly (Table 3).

Table 3 Effects of folic acid on the expression of Bcl-2 and p53 in gastric epithelial cells

Group	*n*	Bcl-2 expression		p53 expression	
		Pre-T	Post-T	Pre-T	Post-T
Treatment	19	8.17±2.06	6.22±1.26[a]	2.03±0.35	2.56±0.47[b]
Control	19	6.78±2.57	6.98±2.55	1.75±0.65	1.70±0.39

n: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD)%. [a]$P<0.05$ and [b]$P<0.01$ *vs* control group and before treatment in the same group.

DISCUSSION

Folate is a member of water-soluble B vitamin family; its principal biochemical function is the mediation of one-carbon transfer or methylation reactions. These reactions include (1) purine and pyrimidine nucleotide biosynthesis; (2) amino acid conversions-the interconversion of serine and glycine, catabolism of histidine to glutamic acid and conversion of homocysteine to methionine; (3) generation and utilization of formate and (4) methylation of small amounts of transfer RNA. Folate deficiency has been shown to be common in various regions of China including Beijing[14,15], and a number of epidemiological studies conducted in the Chinese population have consistently indicated an inverse association between consumption of vegetables and fruits, a major source of folate, and the risk of gastroesophageal cancer[16-18]. Folic acid is the synthetic form of folate, which is used for nutritional supplements and food fortification. The present study demonstrated that folic acid, which was administered to the patients for 3 mo, markedly increased the concentrations of folate in the gastric mucosa. A higher intake of the micronutrient folate was first proposed by Freudenheim[19] to reduce the risk of colorectal cancer. Thereafter, the evidence from epidemiological, animal and human studies strongly suggests that folate status modulates the risk of several malignancies, the most notable of which is the colorectum[8,9,20-22]. Recent animal study indicated that high dose of folic acid also played a prevention role in gastric carcinogenesis in beagles[10].

Although the incidence rates for gastric cancer have been declining in many countries[23], it remains the leading cause of cancer-related deaths in China[1,24]. The incidence and mortality of gastric cancer varies from province to province, generally very high in the north but relatively low in the south of China[25], suggesting that environmental factors, particularly those associated with the diet, may play an important role in gastric carcinogenesis. More than 90% of gastric cancers are adenocarcinomas, which are divided into intestinal and diffuse histological types. In the view of

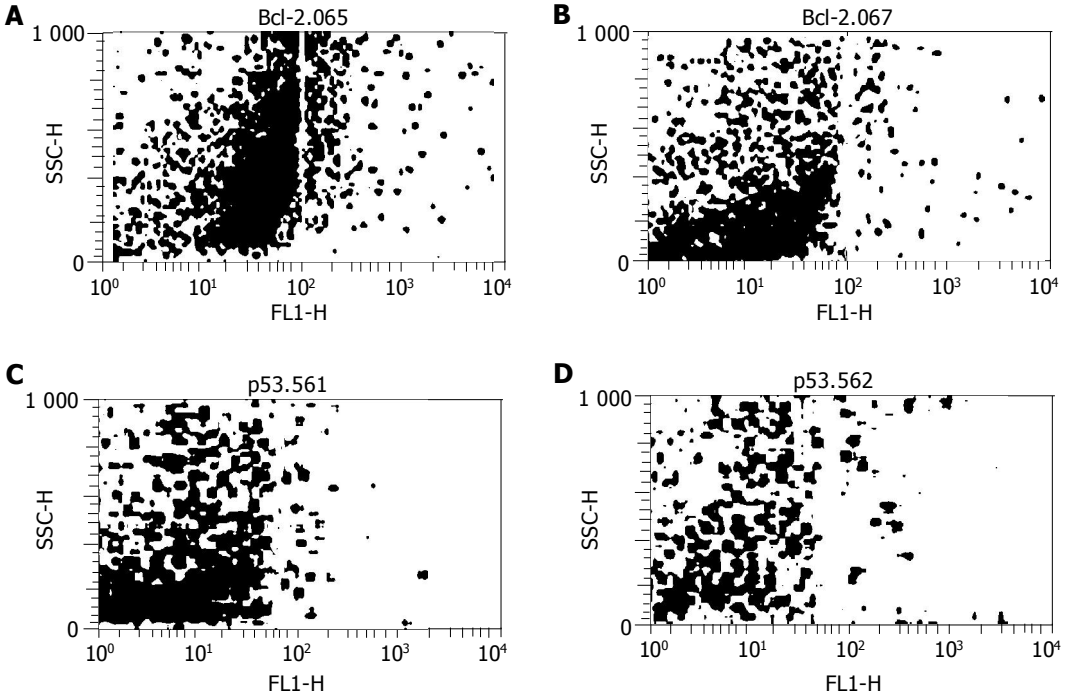

Figure 3 Flow cytometry analysis of Bcl-2 and p53 expression in a patient before and after folic acid treatment For Bcl-2 and p53 expression, cells were stained with FITC-labeled monoclonal anti-Bcl-2 (A and B) or anti-p53 antibodies (C and D); and then measured by flow cytometry. A and C: before; B and D: after folic acid treatment. Representative results of one of the subjects are shown.

Correa's multistep model of gastric carcinogenesis[13,26], intestinal-type gastric cancer develops from chronic active gastritis, premalignant lesions (chronic atrophic gastritis, IM and dysplasia) and finally to gastric cancer. Therefore, studying the effects of interventions on the progression of these premalignant lesions is the most viable option for decreasing the risk of gastric cancer. It has been demonstrated that folic acid supplementation reverses IM and dysplasia and thus delays the progression of gastric carcinogenesis[27]. Mark and colleagues[28] have also revealed the reversion effect of vitamin and mineral supplement including folate on esophageal dysplasia. However, the mechanisms for these phenomena have not yet been clarified.

Folate is an important factor in DNA synthesis, stability and integrity, repair and methylation, aberrations all of which are implicated in carcinogenesis[8,29,30]. A growing body of *in vivo* and *in vitro* evidence suggests that folate deficiency is associated with DNA damage, impaired DNA repair, abnormal DNA methylation, and increased susceptibility to mutagenesis, which can be overcome by folate supplementation[8,30]. An alteration in DNA methylation has been suggested to be an important factor in causing genetic instability[31,32] and is thought to contribute to carcinogenesis by affecting the expression of proto-oncogenes and/or tumor suppressor genes[33]. The present study clearly shows that folic acid significantly increases the epithelial apoptosis and p53 expression in the gastric mucosa. The tumor suppressor gene *p53* may directly induce apoptosis through several pathways and plays a major role in the protection of cells from DNA damage[34,35]. The balance between cell proliferation and apoptosis results in a disturbance of tissue homeostasis and this may promote the development of cancer. Recent insight into the *p53*-mediated biochemical pathways of cell-cycle arrest and apoptosis has provided further understanding of the mechanisms related to *p53*-mediated tumor suppression[36]. The transformation of gastrointestinal epithelial tissue to carcinomas has been shown to be associated with the progressive inhibition of apoptosis[37,38]. In the present study, high levels of p53 protein from intestinal metaplastic and dysplastic epithelium were induced by folic acid, and overexpression of p53 could arrest the cell cycle and induce apoptosis in high-risk patients for gastric cancer.

Apoptosis is an essential and highly conserved mode of cell death that is important for normal development, host defense and suppression of oncogenesis. Among the numerous proteins and genes involved, members of the Bcl-2 family play a central role to inhibit or promote apoptosis[39]. Levels of Bcl-2 within cells are critical to antiapoptotic activity, decreasing Bcl-2 could be a mechanism to sensitize cells to apoptosis[39,40]. The present study has demonstrated that folic acid was able to induce the apoptosis in premalignant gastric lesions; this apoptosis may be mediated by down-regulating the expression of apoptosis-associated gene *bcl-2* and up-regulating the expression of tumor suppressor gene *p53*. Apoptosis is distinguished from necrosis because it is highly regulated, requires new gene expression, and leads to changes in nuclear morphology, DNA laddering and membrane blabbing[41]. The changes in membrane composition lead to extracellular exposure of phosphatidylserine (PS) residues and occur early in the apoptotic cycle, regardless of the initiating signal[42]. Exposed PS residues avidly bind Annexin V, a natural ligand, in a calcium-dependent manner[43]. Membrane changes leading to PS exposure occur rapidly in apoptotic cells, while the cell loses membrane integrity later in the apoptotic process. Necrotic cells expose PS and lose membrane function simultaneously soon after cell injury[43]. Using a DNA binding dye such as PI in tandem with fluorochrome-conjugated Annexin V, apoptotic cells are identified and discriminated from necrotic cells[42]. Because the extracellular exposure of PS occurs earlier than DNA fragmentation in the apoptotic cycle, Annexin V-FITC/PI double staining is superior to other apoptosis detection methods, such as PI staining and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling) assay.

Furthermore, folic acid is important for the production of S-adenosylmethionine, the primary methyl donor for DNA methylation[29,44]. Decreased DNA methylation is associated with an increased risk of human gastric cancer[45]. Folate deficiency may deplete cellular S-adenosylmethionine levels causing DNA hypomethylation and inappropriate activation of proto-oncogenes[46].

In conclusion, this study demonstrates that both the epithelial apoptosis rate and the tumor suppressor p53 expression in gastric mucosa were significantly increased, while the expression of *Bcl-2* oncogene protein decreased after folic acid treatment. These findings indicate that folic acid may play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in patients with premalignant lesions.

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• *GASTRIC CANCER* •

Effects of 7.5% hypertonic saline on fluid balance after radical surgery for gastrointestinal carcinoma

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Abstract

AIM: To investigate the effects of 7.5% hypertonic saline on positive fluid balance and negative fluid balance, after radical surgery for gastrointestinal carcinoma.

METHODS: Fifty-two patients with gastrointestinal carcinoma undergoing radical surgery were studied. The patients were assigned to receive either Ringer lactate solution following 4 mL/kg of 7.5% hypertonic saline (the experimental group, n = 26) or Ringer lactate solution (the control group, n = 26) during the early postoperative period in SICU. Fluid infusion volumes, urine outputs, fluid balance, body weight change, PaO_2/FiO_2 ratio, anal exhaust time as well as the incidence of complication and mortality were compared between the two groups.

RESULTS: Urine outputs on the operative day and the first postoperative day in experimental group were significantly more than in control group (P<0.000001, P = 0.000114). Fluid infusion volumes on the operative day and the first postoperative day were significantly less in experimental group than in control group (P = 0.000042, P = 0.000415). The volumes of the positive fluid balance on the operative day and during the first 48 h after surgery, in experimental group, were significantly less than in control group (P<0.000001). Body weight gain post-surgery was significantly lower in experimental group than in control group (P<0.000001). The body weight fall in experimental group occurred earlier than in control group (P<0.000001). PaO_2/FiO_2 ratio after surgery was higher in experimental group than in control group (P = 0.000111). The postoperative anal exhaust time in experimental group was earlier than in control group (P = 0.000006). The overall incidence of complications and the incidence of pulmonary infection were lower in experimental group than in control group (P = 0.0175, P = 0.0374).

CONCLUSION: 7.5% hypertonic saline has an intense diuretic effect and causes mobilization of the retained fluid, which could reduce fluid infusion volumes and positive fluid balance after radical surgery for gastrointestinal carcinoma, as well as, accelerate the early appearance of negative fluid balance after the surgery, improve the oxygen diffusing capacity of the patients' alveoli, and lower the overall incidence of complications and pulmonary infection after the surgery.

Key words: Hypertonic saline; Fluid balance; Positive fluid balance; Negative fluid balance; Abdominal surgery; Gastrointestinal carcinoma

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INTRODUCTION

The practice of perioperative fluid therapy is variable. In the perioperative fluid therapy of radical surgery for gastrointestinal carcinoma, the positive fluid balance significantly exists after which the negative fluid balance appears[1]. The volume of positive fluid balance is the important risk factor of the postoperative complications and in-patient mortality. In the nonsurvivors group a much more positive fluid balance was found, together with increasing the extent of the organ dysfunction. The stronger correlation between fluid intake and fluid balance in nonsurviving patients compared with survivors points to the need for careful fluid management in critically -ill patients[2,3]. Appearance of the negative fluid balance indicates surgical stress response subsiding and resuming of the internal environment stability, and portends a good prognosis. The delaying of the negative fluid balance indicates that the stability of the internal environment has not been restored and complications may occur[4].

In recent years, attention has been directed towards the problem of larger volume of positive fluid balance after abdominal aortic aneurysm resection and coronary artery bypass grafting surgery, which seriously impacts the resuming of the cardiopulmonary function. Hypertonic saline is being applied abroad to stimulate excretion of excess body fluid, accumulated during and after surgery, to decrease the volumes of positive fluid balance[5-10], and to accelerate the early appearance of negative fluid balance after surgery[6].

Hence, we applied 7.5% hypertonic saline to the patients with gastrointestinal carcinoma undergoing radical surgery to observe the effects on the positive and negative fluid balance after the surgery.

MATERIALS AND METHODS

Case selection

Fifty-two patients with gastrointestinal carcinoma undergoing radical surgery in our hospital from February 2003 to March 2004 were divided into the experimental group (n = 26) and the control group (n = 26) according to their diseases and surgery modes. Diseases and surgery modes of the two groups are shown in Table 1. General information of the two groups are shown in Table 2, of which POSSUM (Physiological and Operative Severity Score for the EnUmeration of Mortality and Morbidity) scoring is accounted by Copeland method[11].

Table 1 Diseases and surgery modes of experimental group and control group

	Experimental group	Control group
Gastric carcinoma		
Distal gastrectomy D_2	8	8
Proximal gastrectomy D_2	2	2
Total gastrectomy D_3	2	2
Carcinoma of the colon		
Sigmoid colon D_3 excision	4	4
Right-half colon D_3 excision	2	2
Left-half colon D_3 excision	1	1
Transverse colon D_3 excision	1	1
Rectal carcinoma		
Dixon procedure	4	4
Miles procedure	2	2

Table 2 Comparison of general data between experimental group and control group

	Experimental group	Control group	t or χ^2	P
Sex (male/female cases)	12/14	15/11	χ^2 = 0.69	0.4050
Age (yr)	57.19±9.58	55.19±13.48	t = 0.567821	0.575221
Body weight (kg) [1]	55.58±9.44	58.21±9.72	t = 1.074640	0.292797
Serum sodium (mmol/L) [1]	139.92±2.46	139.31±3.87	t = 0.813147	0.423811
HCT [1]	0.33±0.07	0.34±0.07	t = 0.773980	0.446200
Serum albumin (g/L) [1]	38.35±3.53	39.42±3.73	t = 1.001330	0.326262
PaO_2/FiO_2 ratio [1]	395.88±45.55	410.43±60.33	t = 1.021750	0.316684
MAP (mmHg) [2]	91.88±11.31	96.17±13.35	t = 1.145300	0.263864
Heart rate (times/min) [2]	89.38±12.85	88.54±11.49	t = 0.223948	0.824775
POSSUM score	34.54±4.83	33.46±4.12	t = 0.951963	0.350230
Operative period (min)	252.42±73.35	248.85±73.19	t = 0.208407	0.836599
Blood transfusion (cases)	8	5	χ^2 = 0.92	0.3367

[1]Examination results before surgery. [2]Result at time of entering SICU.

Fluid therapy after surgery

After surgery, the patients were immediately sent into SICU. Patients in the experimental group firstly received 4 mL/kg of 7.5% hypertonic saline and were continuously transfused with Ringer lactate solution. Non-visible dehydration was calculated according to 400 mL/(m^2·d), transfusing 12% more when the body temperature rose by 1 ℃ and supplied 5% glucose solution. Patients in the control group that did not receive 7.5% hypertonic saline and other fluid therapies were same as that of the experimental group. Transfusion of the whole process was measured with the transfusion pump and the transfusion speed was adjusted according to urine outputs of every hour, which referred to blood pressure, pulse and HCT value. The objective was to keep the hemodynamics stable and enough urine outputs. When urine outputs <0.5 mL/(kg·h) and/or pulse speed and HCT rose, the transfusion was accelerated; when urine outputs >1.0 mL/(kg·h), transfusion was slower.

The fluid volumes transfused and discharged and the urine outputs of every hour were recorded. Fluid balance of unit time was calculated. After surgery, body -weight of each hour was measured with the hydraulic weighing sickbed. Body weight increase value = postoperative maximum body weight-preoperative basic value of the body weight (kg). Bodyweight falling time refers to the hour difference between time of starting of body weight falling and time of entering SICU (h). Serum electrolyte and arterial blood gas analysis were checked every 6 h until negative fluid balance disappeared.

Data analysis

Curves of body weight and urine outputs of each hour after surgery were drawn. Fluid infusion volumes, urine outputs, volumes of positive fluid balance, body weight gain value, body weight falling time, PaO_2/FiO_2 ratio, anal exhaust time, complication incidence and mortality of two groups were compared. Data mean value was expressed with mean±SD. Comparison of statistics difference was inspected with χ^2 test and t test. Statistical analysis was performed with software SPSS10.0.

RESULTS

Statistic comparability

Difference of surgery modes (Table 1), patient conditions and POSSUM scores (Table 2), fluid infusion volumes (Table 3) and urine outputs (Table 4) during surgery between the two groups are not significant but are statistically comparable.

Fluid infusion volumes

Difference of fluid infusion volumes between the two groups are summarized in Table 3.

Urine outputs

Comparison of urine outputs of experimental group and control group are shown in Table 4.

Table 3 Fluid infusion volumes (mL) of experimental group and control group

	During surgery	Operative day	First postoperative day
Experimental	3 607.69±1 029.15	7 140.23±1 497.91	3 051.58±968.16
Control	3 657.69±1 086.34	8 888.04±1 389.93	4 458.85±1 415.86
t	0.192560	4.953730	4.068690
P	0.848859	0.000042	0.000415

Table 4 Comparison of urine outputs (mL) between experimental group and control group

	During surgery	Operation day	First postoperative day
Experimental	630.77±212.64	2 446.73±361.12	2 641.65±558.97
Control	723.08±316.93	1 735.31±381.04	1 998.65±482.76
t	1.081120	6.958838	4.569320
P	0.289961	<0.000001	0.000114

Fluid balance and body weight change

Differences of fluid balance and body-weight change between the two groups are summarized in Table 5. On the day of operation, patients of the two groups had significant positive fluid balance; on the first postoperative day, 19 patients of the experimental group showed negative fluid balance and only one in the control group showed negative fluid balance (χ^2 = 26.33, R67.8, 95% CI 58.3-77.2, P<0.0001; NNT1.44).

Table 5 Comparison of fluid balance and body weight between experimental group and control group

	VPFB on operative day (mL)	VPFB in 48 h after surgery (mL)	BW increase value (kg)	BW falling time (h)
Experimental	2 832.85±970.67	2 519.15±1 629.44	3.46±1.15	18.38±7.16
Control	4 944.46±1 289.67	6 887.31±2 115.84	6.96±2.41	29.58±7.80
t	7.475810	9.19234	7.096470	7.23379
P	<0.000001	<0.000001	<0.000001	<0.000001

Note: VPFF = volume of positive fluid balance; BW = body weight.

PaO_2/FiO_2 ratio

PaO_2/FiO_2 ratio of patients of two groups before surgery was not different (Table 2). Within 6 h after entering ICU, PaO_2/FiO_2 ratios of the experimental group and control group were respectively 463.03±136.23 and 339.79±54.42 (t = 4.578857, P = 0.000111).

Postoperative anal exhaust time

Postoperative anal exhaust time of the patients of the experimental group on an average was about 2.81±0.75 (1-4) d after surgery and that of the control group was about 4.27±1.04 (2-7) d after surgery (t = 5.718330, P = 0.000006).

Postoperative hypernatremia

After surgery, serum sodium concentration of five patients of the experimental group (within 6 h after entering SICU) was 145.2-153 mmol/L but had no clinical symptom; after 6 h (12 h after entering SICU) serum sodium concentration returned to the normal value. Patients of the control group had no hypernatremia.

Postoperative complications and mortality

Two patients of the experimental group had complications (7.69%) after surgery and nine patients of the control group had complications (34.62%) after surgery (χ^2 = 5.65, P = 0.0175). In the two groups, five patients had wound infection (9.62%) after surgery, who were cured through drainage therapy; four patients developed pulmonary infection (7.69%), who were cured through antibiotic therapy; one anastomotic leak case (1.92%), who was a patient for 18 d after right-half colon D_3 excision, clinically diagnosed as anastomotic leak because of the localized peritonitis accompanying high fever and cured through total parenteral nutritional support and antibiotic therapy; one ileus case (1.92%), who was cured through non-surgical therapy such as gastrointestinal decompression, spasmolysis and low pressure clysis. A patient of the control group died (0.04%), who was patient of right-half colon D_3 excision for carcinoma of cecum and died on the 21st postoperative day due to hepatic failure. Comparison results of the incidence of complication and mortality between the experimental group and the control group are shown in Table 6.

Table 6 Comparison of complications and mortality (cases) between the two groups

	Wound infection	Pulmonary infection	Ileus	Anastomotic leak	Death
Experimental	2	0	0	0	0
Control	3	4	1	1	1
χ^2	0.22	4.33	1.02	1.02	1.02
P	0.6381	0.0374	0.3126	0.3126	0.3126

DISCUSSION

The principles of perioperative fluid therapy were fostered in the late 1950s and early 1960s. Recommendations for restricted fluid regimen came primarily from Moore. In contrast, Shires postulated a decrease in extracellular volume after surgery, due to internal redistribution of fluids, the 'third space' losses, and advocated replacement of these losses by additional fluid infusion[12]. Clinical practice has largely been influenced by Shires' recommendations in elective surgical procedures. This is especially the case in major abdominal surgery[13].

Major surgical operation, similar to severe trauma, burn and sepsis, may cause systemic inflammatory response syndrome and systemic capillary leakage. In response to surgery, serum colloid osmotic pressure is decreased, which is primarily caused by increased capillary permeability, resulting in fluid shifts from the vascular bed to the interstitial fluid. In these cases, except local effusion and edema, increased systemic capillary permeability causes intravascular fluid loss into the extravascular compartment[14]. As a result, hypovolemia, hyposarca, less urine outputs and higher HCT value occur. Fluid resuscitation must cause positive fluid balance, worse hyposarca and body weight gain[1,8].

Administration of excess fluid may cause several problems after surgery. The resulting increased demands on cardiac function, due to an excessive shift to the right on the Starling myocardial performance curve, may potentially increase postoperative cardiac morbidity. Fluid accumulation in the lungs may predispose patients to pneumonia and respiratory failure. Gastrointestinal motility may be inhibited, prolonging postoperative ileus. Excess fluid may decrease tissue oxygenation with implications for wound and anastomotic healing[14]. Petrašovicová *et al*[2] found, through

observing 117 surgical critical patients, that patients with large volume positive fluid balance indicate poor prognosis and have higher organ dysfunction incidence and mortality. Result of the logic regression analysis of Moller *et al*[3] shows that the large volume of positive fluid balance is the most important risk factor of the postoperative complications and mortality. Alsous *et al*[4] found through analysis that appearance of negative fluid balance indicates the disease is turned back with good prognosis and delaying of negative fluid balance indicates poor prognosis. Thus, how to decrease fluid intake and the volume of positive fluid balance and to make negative fluid balance appear early as possible is the key to the postoperative fluid therapy.

In this research, diseases and surgery modes of two groups of patients are shown in Table 1. Dereference of two groups of patients on sex ratio (male/female patients), age, basic body- weight, preoperative biochemical factors (serum sodium concentration, serum albumin concentration, HCT and PaO_2/FiO_2 ratio), hemodynamic conditions when entering SICU (mean arterial pressure and heart rate) and number of blood-transfusing patients is insignificant (Table 2).

The documents of our department prove that POSSUM score (physiology score and surgery severity score) can exactly predict the sizes of the surgery for gastrointestinal cancer and their postoperative complication incidence and mortality[15]. Difference between the two groups of patients on POSSUM and surgery hours is insignificant (Table 2). It is obvious that clinical data of the cases of the experimental group and control group are statistically comparable.

The research results prove that 7.5% hypertonic saline has an intense diuretic effect, which is manifested by the urine outputs increase of the patients in the experimental group on the operative day and the first postoperative day. Such diuresis effect is different from diuretic, which can increase the plasma volume within a short time, keep stability of hemodynamics and cause mobilization of the retained fluid excess. As a result, the postoperative fluid infusion volumes were significantly decreased, also the postoperative positive fluid balance of the patients in the experimental group was reduced. This effect was confirmed by the lesser increase in body-weight measured after surgery in the experimental group. This is consistent with application result of 7.5% hypertonic saline after abdominal aortic aneurysm resection and coronary artery bypass graft surgery[5-10].

The body weight gain is the objective exhibition of positive fluid balance (input fluid volume >excreted fluid volume). Increased extent of the body weight indicates volume of the positive fluid balance. Falling of the body weight indicates appearance of the negative fluid balance (input fluid volume <excreted fluid volume). Research shows that 7.5% hypertonic saline increasingly makes the extent of postoperative body weight, of the patients in the experimental group, smaller and falling of the body weight occurring earlier to prove that transfusion of hypertonic saline not only decreases volume of positive fluid balance after surgery but also makes negative fluid balance appear earlier. This has not been reported in foreign literature.

In 6 h after entering SICU, PaO_2/FiO_2 ratio of the patients in the experimental group was significantly higher than that of the control group ($P = 0.000111$), which shows that hypertonic saline can improve the patients' oxygenation conditions. Hypertonic saline may make intrapulmonary venous admixture less and improve alveolar gas exchange[6]. Someone also thinks it reduces systemic and pulmonary inflammatory responses as well as improves pulmonary perfusion and oxygenation[16].

The overall incidence of postoperative complication and the incidence of pulmonary infection of the experimental group were significantly lower than that of the control group ($P = 0.0175$, $P = 0.0374$; Table 6), which may relate to diuretic effect and improving effect of oxygen supply of hypertonic saline. The intestinal functional rehabilitation time (anus exsufflation) of the patients in the experimental group was significantly earlier than that in the control group ($P = 0.000006$), which may be the result of the fact that the hypertonic saline decreases fluid infusion volumes and volumes of the positive fluid balance to relieve postoperative intestinal edema.

After 6 h of transfusing hypertonic saline, five patients of the experimental group had hypernatremia, but serum sodium concentration was not above 153 mmol/L. After 6 h (after 12 h in ICU), serum sodium concentration returned to the normal value.

The foreign researchers concluded that the use of hypertonic saline might be of interest for use in surgical patients. The suggested explanations for the beneficial effects of hypertonic saline are (1) compartmental redistribution with a fluid shift to the vascular bed and consequent plasma expansion[17,18]; (2) a reduction in blood viscosity through hemodilution and reduced endothelial and red blood cell swelling improved microcirculation[19]; (3) an endothelial barrier mechanism decreased microvascular fluid loss during states of elevated microvascular leak[20,21]; (4) the effects of improving tissue oxygenation and perfusion, and reducing systemic and pulmonary inflammatory responses[22]; and (5) hormonal and immunologic effects[23] that are possible mechanisms of the long-lasting impact of hypertonic saline.

This study's findings through a prospective control study of 52 patients with gastrointestinal carcinoma undergoing radical surgery suggest that 7.5% hypertonic saline has an intense diuretic effect, which may decrease volumes of postoperative fluid infusion and positive fluid balance; and make negative fluid balance appear earlier. In addition, 7.5% hypertonic saline can also improve postoperative oxygenation conditions and reduce the incidence of overall postoperative complication and pulmonary infection.

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Nutritional status and quality of life of the gastric cancer patients in Changle County of China

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Abstract

AIM: To analyze the relation between nutrition and quality of life in the stomach cancer patients, evaluate the intake of daily nutrition of the patients, and study the feasibility of nutrition intervention in improving quality of life of the stomach cancer patients.

METHODS: A total of 285 surgical stomach cancer patients reported in the Changle Cancer Registry from 2002 to 2003 were investigated with respect to their diet and quality of life. Daily nutrition intakes of the patients were calculated according to the Food Composition Database, and these data were compared with the reference values proposed by the Chinese Nutrition Society. The partial correlation was used to analyze the relationship between nutrition and quality of life in the patients. Stepwise multiple regression analyses were conducted to analyze the factors influencing nutrition intake in stomach cancer patients.

RESULTS: Except vitamin C, there were statistical correlations between the nutrition and quality of life in stomach cancer patients, and differences of the daily nutrition intake among three groups (good, modest and bad quality of life) of the patients were significant. Most of the stomach cancer patients had a lower daily nutrition intake than the reference values. At the significance level $\alpha = 0.05$, the factors influencing the daily nutrition intake of the patients were number of meals a day, family income, way of operation, exercise and age.

CONCLUSION: The nutritional status of the operated patients with stomach cancer may impact on their quality of life. The stomach cancer patients in Changle County have a low level of daily nutrition intake, which suggests that they have a bad nutritional status. To improve the quality of life of the patients, the nutrition intervention should be conducted. Increasing times of meals a day and having a high-protein, high-calorie foods can improve the nutritional status of the stomach cancer patients. Moreover, exercise for rehabilitation can whet the appetite of the patients and recover their body function, which in turn may improve the quality of life of the stomach cancer patients.

Key words: Nutrition; Quality of life; Stomach cancer

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INTRODUCTION

Changle County is a high-incidence area of gastric cancer in Fujian Province. On an average, about 500 cases were operated a year in Changle County. Along with the increase in the patients with early stage and improvement in tumor treatment, the survival time of the patients with stomach cancer has been prolonged[1]. As there are more and more stomach cancer patients in the communities, how to make the stomach cancer patients rehabilitate and how to improve their quality of life, are important research contents. We had conducted the studies of the influencing factors on the quality of life of the stomach cancer patients in Fuzhou City and Changle County during 1998-2002. The results of these researches suggested that most of the gastric cancer patients had poor quality of life and the rural patients had lower quality of life than those of urban patients. Moreover, there were three aspects of factors, including the disease conditions, the social-economic status and rehabilitation status, influencing the quality of life of the stomach cancer patients[2], and their weights on quality of life were 0.35, 0.32, 0.33, respectively, and whether increasing nutritious food played an important role in rehabilitation status of the patients[2]. Increasing nutritious food in diet had effect not only on patients' physiological state, but also on their psychological state[3]. These results suggested that when seeking the countermeasure improving the quality of life of the stomach cancer patients, we should not neglect to study their nutritional status. Because the disease conditions and social-economic status of the patients cannot be changed, their quality of life may be improved by changing their nutritional status. Because our early studies only asked the patient whether to increase nutritious food or not (yes or no), there were some defect the information on the patient's nutrition intake. In order to quantitatively analyze

the association between nutrition intake and quality of life and to study the feasibility of nutrition intervention in improving the quality of life of the stomach cancer patients, we made an epidemiological survey during springtime in 2004. In this paper, we further quantitatively analyzed the relation between nutrition and quality of life among the stomach cancer patients in Changle County, evaluated the nutritional status of the patients and analyzed the influencing factors of the daily nutrition intake of the patients. The results of our research may be useful to doctors and nurses in the community health centers to help improve the quality of life of the gastric cancer patients.

MATERIALS AND METHODS

Materials

The stomach cancer patients who underwent operation in 2002-2003 and were still alive during the term of investigation in Changle County were the subjects of our research. The name and address list of the subjects were obtained from the tumor registration office in Changle City. The patients aged over 80 years or not finishing the questionnaires were rejected, so our study sample consisted of 285 patients. In the five age-groups, <40, 40-, 50-, 60- and 70-80 years, there were 8 (2.81%), 32 (11.23%), 84 (29.47%), 92 (32.28%) and 69 (24.21%) patients, respectively. There were 231 males with age (60.96±12.65) years and 54 females with age (58.67±15.39) years in our sample.

Methods

The epidemiological survey was conducted from April to May in 2004. The data were collected by means of the investigators going into the homes of the stomach cancer patients. The quality of life within two weeks was measured with a 21 items scale, with the Cronbach coefficient $\alpha = 0.9866$, and each of the 21 items was scored from 1 to 5. The higher score of the item is, the better the function corresponding to the item is. The total score of the quality of life was the sum of the scores of these 21 items.

The food frequency survey method[4] was used to obtain information about the diet for every patient within two weeks. Daily the ten kinds of nutrition intake for every patient were calculated according to the Food Composition Database[5]. On the basis of the reference values proposed by the Chinese Nutrition Society[5], the nutritional status of the patients was evaluated.

The partial correlation[6] and analysis of variance were used to analyze the relationship between nutrition and quality of life of the patients. The *u* test was used to compare the means of daily nutrition intake of the patients to the reference values. The stepwise multiple regression analyses were conducted to analyze the factors influencing nutrition intake of the stomach cancer patients. SAS software package was used for all analyses[7].

RESULTS

Status of quality of life of the stomach cancer patients

The distribution of the total score of the quality of life for 285 patients was shown in Table 1. Compared with that in 1999, the quality of life of the patients improved a little. However, the average of the total score of the quality of life was 65.69 (96%CI: 64.44-66.94), which was not statistically higher than that in 1999. The means of the total score of the quality of life for male and female were 66.9 (SD = 10.30) and 60.50 (SD = 11.22), respectively. This difference was significant ($P<0.05$).

Table 1 Distribution of the total score of the quality of life in the patients

Total score of quality of life	2002–2003		1999	
	Number of patients	%	Number of patients	%
<30	2	0.7	4	2.00
30-	1	0.35	6	3.00
40-	15	5.26	16	8.00
50-	55	19.30	54	27.00
60-	112	39.30	69	34.50
70-	71	24.91	41	20.50
80-	23	8.07	10	5.00
90–105	6	2.11	0	0.00

Relation between nutrition and quality of life

The Pearson partial correlation coefficients between daily nutrition intake and quality of life, adjusted for age, sex, way of operation and exercise, were shown in Table 2. Except vitamin C, each of the nutrition was positively correlated with the quality of life.

Table 2 Partial correlation analysis between nutrition intake and quality of life

Nutrition	*r*	*P*	Nutrition	*r*	*P*
Calorie	0.22	0.0002	Selenium	0.24	<0.0001
Protein	0.25	<0.0001	Thiamine	0.21	0.0004
Calcium	0.21	0.0004	Riboflavin	0.25	<0.0001
Iron	0.19	0.0012	Niacin	0.25	<0.0001
Zinc	0.24	<0.0001	Vitamin C	0.05	0.3891

A total number of 231 patients were grouped according to their total score of the quality of life. There were three groups: the quality of life was bad (the total score was under 60), modest (the total score was within 60-80) and good (the total score was over 80). The means of daily nutrition intake in each of the groups for male and female were calculated and shown in Table 3. For both male and female, the daily nutrition intake among three groups, except vitamin C, were statistically different, which suggested that the patients who had a better nutritional status had a higher quality of life.

Nutrition intake of the patients

There were 7 patients (2.5%) whose 10 kinds of nutrition all come to or more than the reference values, 51 patients (17.9%) whose 10 kinds of nutrition all lower than the reference values. The proportions of the patients whose

Table 3 Means of nutrition intake in three groups of the patients by sex

Nutrition mean	Total score of quality of life for male				Total score of quality of life for female			
	<60	60–80	>80	P^1	<60	60–80	>80	P^1
Calorie (kJ)	6 947.93	7 355.12	8 301.17	0.080	6 404.58	6 726.34	13 912.84	<0.001
Protein (g)	61.51	62.82	78.41	0.028	46.84	56.99	133.05	<0.001
Calcium (mg)	526.70	497.21	653.87	0.070	418.37	467.87	973.14	<0.001
Iron (mg)	21.22	20.00	27.98	0.034	15.01	18.60	46.67	0.002
Zinc (mg)	12.78	12.71	18.38	0.002	10.24	12.54	23.70	0.025
Selenium (μg)	24.33	24.98	30.74	0.028	20.38	23.89	40.58	0.122
Thiamine (mg)	1.48	1.57	1.82	0.045	1.35	1.42	2.76	0.007
Riboflavin (mg)	1.41	1.41	1.88	0.015	1.06	1.36	3.02	0.001
Niacin (mg)	11.72	13.36	16.39	0.036	10.89	12.45	27.35	0.007
Vitamin C (mg)	94.95	79.87	98.03	0.551	69.99	81.91	95.31	0.865

[1]*P* value derived from the analysis of variance among three groups.

daily nutrition intake were less than the reference values were 83.86% for calorie, 33.33% for protein, 66.67% for calcium, 35.79% for iron, 71.58% for zinc, 95.44% for selenium, 31.58% for thiamine, 45.26% for riboflavin, 51.93% for niacin and 53.33% for vitamin C, respectively, which suggested the bad diet and nutritional status of the stomach cancer patients. From Table 4, it could be seen that the intakes of calorie, protein, calcium and selenium were much lower than the reference values.

Table 4 Means of daily nutrition intake in the 285 patients

Nutrition	Male (n = 231)		Female (n = 54)	
	Reference value	mean±SD	Reference value	mean±SD
Calorie (kJ)	10 080	7 470.08 (2 722.52)[a]	8 820	6 900.39 (2 684.72)[a]
Protein (g)	70	65.37 (34.12)[a]	65	56.79 (30.21)[a]
Calcium (mg)	800	528.05 (385.61)[a]	800	509.73 (498.37)[a]
Iron (mg)	12	21.54 (17.44)[a]	18	18.57 (12.38)
Zinc (mg)	15	13.70 (9.42)[a]	15	12.27 (6.81)[a]
Selenium (μg)	50	25.90 (12.68)[a]	50	23.46 (13.30)[a]
Thiamine (mg)	1.2	1.60 (0.64)[a]	1.1	1.45 (0.62)[a]
Riboflavin (mg)	1.2	1.49 (0.93)[a]	1.1	1.34 (0.74)[a]
Niacin (mg)	12	13.69 (7.95)[a]	11	12.54 (7.14)
Vitamin C (mg)	60	84.86 (107.39)[a]	60	78.81 (83.93)

[a]P<0.05. *vs* reference value.

Factors influencing on nutrition intake of the patients

For each of the patient, his nutritional score was defined as the number of nutrition whose daily intakes were no less than the reference values. The stepwise regression analysis was conducted with the dependent variable as the patient's nutritional score and independent variables as sex (male 1, female 2), age, education (primary school 1, middle school 2, high school 3 and university 4), family income, way of operation (total gastrectomy 1, partial gastrectomy 2), times of meals a day and exercise (no 1, sometimes 2 and often 3).

At the significance level $\alpha = 0.05$, the factors influencing the nutritional status of the patients were age, family income, way of operation, times of meals a day and exercise (Table 5). According to the standard parameter estimate, times of meals a day had the most effect on the patients' nutritional status, and then in turn were family income, way of operation, exercise and age. The patients who had more times of meals a day, more family income and often taking part in exercise had a better nutritional status than those who had less times of meals a day, less family income and not taking part in exercise. Besides, total gastrectomy made the patients have worse nutritional status, and the older the patient was, the worse his nutritional status was.

Table 5 Result of stepwise regression at α = 0.05

Factor	Standard parameter estimate	Standard error	t	P
Age (yr)	-0.119	0.012	-2.12	0.035
Family income	0.206	0.142	5.71	<0.001
Way of operation	-0.151	0.366	-2.75	0.007
Times of meals a day	0.241	0.164	4.12	<0.001
Exercise	0.139	0.376	2.38	0.018

Table 6 Means of daily nutrition intake by way of operation

Nutrition	Total gastrectomy		Partial gastrectomy (n = 200)
	Times of meals (d) ⩽3 (n = 44)	Times of meals (d) ⩾4 (n = 41)	
Calorie (kJ)	5 553.66	7 394.73	7 759.08
Protein (g)	46.26	65.04	67.39
Calcium (mg)	406.13	536.43	548.50
Iron (mg)	15.13	22.03	22.06
Zinc (mg)	10.11	14.11	14.03
Selenium (μg)	20.36	26.76	26.10
Thiamine (mg)	1.17	1.63	1.72
Riboflavin (mg)	1.09	1.43	1.55
Niacin (mg)	9.71	14.16	14.17
Vitamin C (mg)	57.41	88.66	89.35

The patients who underwent total gastrectomy were grouped by the times of meals a day: one group had the times of meals a day less than or equal to three, and the other group had the times of meals a day more than or equal to four. The means of daily nutrition intake for both the groups of the patients and for the patients who underwent partial gastrectomy were shown in Table 6. From Table 6, we could see that means of daily nutrition intake in the total gastrectomy group which had the times of meals

a day more than four were close to those in the patients who underwent partial gastrectomy, which suggested that although the way of operation was the factor influencing the patient's nutritional status, the differences of daily nutrition intake between total gastrectomy and partial gastrectomy patients could be decreased by increasing the times of every day meals. Now that 62.11% of the patients had their meals no more than three times a day in our sample, increasing times of every day meals might be with great potential for improving the nutritional status of the stomach cancer patients in Changle County.

DISCUSSION

The results of our earlier research showed that the patients who often noticed to increase nutritious food in their diet had a better quality of life. However, the results of the researches on relationship between nutrition and quality of life were not consistent. Most of the researchers suggested that nutritional status of the cancer patients impacted on their quality of life[8,9,12,16-18]. Some researchers suggested that although cancer stage was the major determinant of patients' quality of life, nutritional deterioration combined with deficiencies in nutritional intake might be more important factors for the quality of life of the cancer patients[8]. Nutrient depletion adversely affects immune function, the patient's enjoyableness and social interactions with family and friends, which can further depress appetite[9]. Low hemoglobin levels were associated with fatigue, poor overall quality of life and decreased ability to work. Interventions that reverse fatigue and other anemia-related symptoms should have a positive effect on the quality of life[11]. However, some researchers have not found the statistical differences of the quality of life among the cancer patients with variant nutritional status, and there were little correlation between the quality of life and malnutrition[19-21]. In our present research, the results showed that the daily nutrition intake was different among the patients with variant quality of life, and it was positively correlated with the quality of life.

Our results showed that the stomach cancer patients in Changle County had a low nutrition intake. Most of them had a far lower daily nutrition intake than the reference values proposed by the Chinese Nutrition Society, especially for calorie and protein. Nutritional intake was associated with nutritional status of the cancer patients[22], so it could be inferred that the nutritional status of the stomach cancer patients in Changle County was bad. Some researchers have suggested that malnutrition has a significant impact on the survival of the cancer patients, and malnourished patients have depressed immune systems, which lead to unimpeded tumor growth[22]. Besides, under-nutrition or cachexia was the major cause of death in 1% of cancer patients[23,24]. Some researches on the survival of the head and neck cancer patients showed that patients supplemented with nutrition not only had a better quality of life[25], but tended to live longer also[22,26]. Protein-calorie malnutrition influenced functional status (eating, personal hygiene and toilet use) and psychosocial well being (initiative or involvement, unsettled relationships and past roles)[16,25-30]. The stomach cancer patients often have many difficulties in eating because of the operation, and disease and treatment make them lose appetite also, so the nutritional status of the stomach cancer patients is not good in general. Therefore, communal care for the stomach cancer patients is especially important. The health care services in the community should offer nutritional counseling to the patients and their nursing staff, encourage the patients to eat more than three times every day and have a balanced diet with emphasis on high-protein, high-calorie foods.

In summary, our data obtained by epidemiological survey have shown that the nutritional status of the operated patients with stomach cancer may impact on their quality of life. The stomach cancer patients in Changle County have a low level of daily nutrition intake, which suggests that they have a bad nutritional status. To improve the quality of life of the patients, the nutrition intervention should be conducted. Increasing times of meals a day and having high-protein, high-calorie foods can improve the nutritional status of the stomach cancer patients. Moreover, exercise for rehabilitation can whet the appetite of the patients and recover their body function, which in turn may improve the quality of life of the stomach cancer patients.

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• *COLORECTAL CANCER* •

Feasible economic strategies to improve screening compliance for colorectal cancer in Korea

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Abstract

AIM: While colorectal cancer (CRC) is an ideal target for population screening, physician and patient attitudes contribute to low levels of screening uptake. This study was carried out to find feasible economic strategies to improve the CRC screening compliance in Korea.

METHODS: The natural history of a simulated cohort of 50-year-old Korean in the general population was modeled with CRC screening until the age of 80 years. Cases of positive results were worked up with colonoscopy. After polypectomy, colonoscopy was repeated every 3 years. Baseline screening compliance without insurance coverage by the national health insurance (NHI) was assumed to be 30%. If NHI covered the CRC screening or the reimbursement of screening to physicians increased, the compliance was assumed to increase. We evaluated 16 different CRC screening strategies based on Markov model.

RESULTS: When the NHI did not cover the screening and compliance was 30%, non-dominated strategies were colonoscopy every 5 years (COL5) and colonoscopy every 3 years (COL3). In all scenarios of various compliance rates with raised coverage of the NHI and increased reimbursement of colonoscopy, COL10, COL5 and COL3 were non-dominated strategies, and COL10 had lower or minimal incremental medical cost and financial burden on the NHI than the strategy of no screening. These results were stable with sensitivity analyses.

CONCLUSION: Economic strategies for promoting screening compliance can be accompanied by expanding insurance coverage by the NHI and by increasing reimbursement for CRC screening to providers. COL10 was a cost-effective and cost saving screening strategy for CRC in Korea.

Key words: Cost-effectiveness analysis; Colorectal cancer; Screening; Compliance; National health insurance; Coverage; Reimbursement

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INTRODUCTION

Korea is known to be a low-risk area for colorectal cancer (CRC), but the incidence has been rapidly increasing during the last decade. From 1987 to 1996, the age-standardized mortality rate for CRC has roughly doubled from 8.7 to 16.5 per 100 000 for men and 6.3 to 14.3 per 100 000 for women[1]. Screening for CRC reduces mortality through detection of malignancy at an earlier, more treatable stage as well as by identification and removal of precursor lesion, the adenomatous polyp[2]. Recent panel in Korea recommends that an average-risk individual should begin CRC screening at the age of 50 with one of the two following guidelines[3]: 1. Colonoscopy (COL) every 5-10 years. 2. Flexible sigmoidoscopy (SIG) and double-contrast barium enema (DCBE) every 5 years.

However, these recommendations condoned by expert panels, were not based on economic evaluation. CRC screening tests vary considerably in terms of their performance characteristics, complication rates, acceptability and cost. Especially the cost structure for reimbursement of CRC screening and treatment in Korea is different from that in other countries. Colonoscopy, sigmoidoscopy and DCBE for CRC screening are not covered by national health insurance (NHI) scheme in Korea. Previous studies have demonstrated that out-of-pocket payment was a barrier to cancer screening and health insurance was an important determinant of the utilization of cancer screening[4,5]. In addition, physician's noncompliance with screening recommendation was known to be a major barrier to effective CRC control[6]. Perceived inadequacy of the reimbursement of colonoscopy or sigmoidoscopy was one of the factors affecting physician's compliance[7].

To improve the physician and patient compliance for CRC screening, some reports have demonstrated that the third-party payer should remove financial barriers by providing insurance coverage and raising reimbursement of CRC screening to physicians[8]. However, Korean NHI

has experienced an annual deficit since 1997 and fiscal stability is a major concern[9]. At the current status, new national policy on screening should not put financial burden on the Korean NHI system and needs to take into account economic consequences.

To suggest a feasible economic model to improve the compliance by raising insurance coverage and reimbursement without increasing financial burden on the NHI, we constructed a decision-analytic model to evaluate the cost-effectiveness of CRC screening for average-risk Korean individuals.

MATERIALS AND METHODS

Model

The natural history of a simulated cohort of 50-year-old Koreans in the general population was modeled with and without CRC screening until the age of 80 years (Figure 1). We evaluated 16 different screening strategies with Markov model. Persons representative of the 50-year-old Korean population were placed into health states defined by the presence or absence of a polyp or cancer (early or advanced). Cases of positive screening test results were worked up with a colonoscopy, and individuals diagnosed with polyp underwent polypectomy. Colonoscopy was repeated every 3 years for surveillance after polypectomy[10]. The probability of perforation was assigned to DCBE, SIG, COL and polypectomy[11-13]. Mortality caused by the risk of perforation was assumed to be 0.02%[13,14].

Our main outcome measures were discounted lifetime costs, life expectancy, lifetime NHI's financial burden and incremental cost-effectiveness ratio (ICER), which were compared for 16 different CRC screening strategies. Incremental cost-effectiveness analysis was performed by ranking the 16 strategies in the order of increasing effectiveness. After eliminating strategies, that were more or equally costly and less effective than a competing strategy (i.e., ruled out by simple dominance), we calculated the ICER for each strategy (additional cost divided by additional benefit) compared with the next least expensive strategy. If a strategy was less effective and had a higher ICER than another strategy, it was ruled out by extended dominance[15]. Strategies exhibiting extended dominance were eliminated from the rank-ordered list, and ICERs of the remaining strategies were recalculated. Future costs and life-years were discounted at an annual rate of 3%. The model was programmed in DATA Pro 4.0 software (TreeAge Software Inc., Williamstown, MA).

Clinical data

Natural history of colorectal polyps and cancer Table 1 showed selected parameter estimates. We estimated the age-specific prevalence of adenomatous polyps from previous studies in Korea[3,16]. The incidence of polyp is assumed to be constant calibrated with the two prevalence rates between age 50 and 65. The probability of transformation from polyp to cancer was estimated from the study of patients who refused the resection of polyp[17]. We assumed that the longer the duration of polyp, the greater the probability of transformation from polyp to cancer.

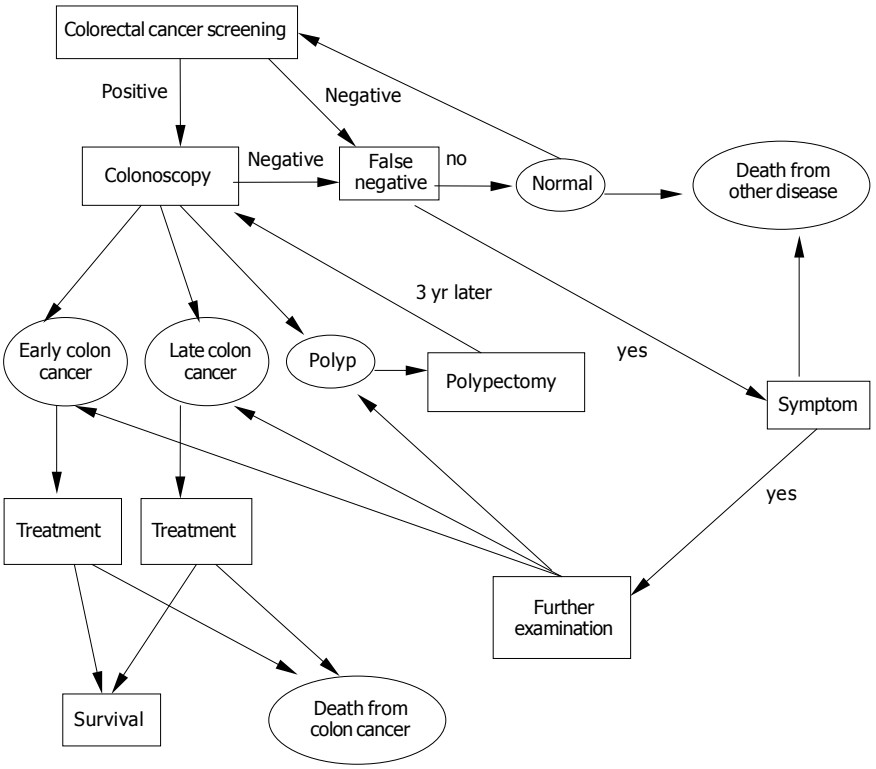

Figure 1 Markov model of colorectal cancer screening. The ovals represent Markov states in which patients remain for at least full 1-year cycle. The squares represent intermediate states of screening procedures, in which patients may enter and leave during one cycle. The arrows represent transitions between various states.

Table 1 Summary of assumption

	Parameter	Base case value	Reference
Sensitivity and specificity of screening and diagnosis	Sensitivity of FOBT for polyps/cancer	0.1/0.5	[15], [28], [29], [30]
	Sensitivity of colonoscopy for polyps/cancer	0.85/0.97	[2], [31], [32]
	Sensitivity of colon study for polyps/cancer	0.5/0.8	[2], [33], [34]
	Sensitivity of sigmoidoscopy for polyps/cancer	0.67	[1], [2], [31], [32]
	Specificity of FOBT	0.9	[15], [28], [29], [30]
	Specificity of colonoscopy	1	[2], [31], [32]
	Specificity of colon study	0.9	[2], [33], [34]
	Specificity of sigmoidoscopy	1	[2], [31], [32]
Natural history of polyp/cancer sequence	Prevalence of polyps at age 50	0.25	[13], [36]
	Annual polyp incidence rate	0.005	[13], [36]
	Percent of cancers originating as polyps	100%	[37], [38]
	Annual cancer incidence of polyp whose duration is below 5 yr	0.005	[17], [39], [40], [41]
	Annual cancer incidence of polyp whose duration is from 5 to 10 yr	0.01	[17], [39], [40], [41]
	Annual cancer incidence of polyp whose duration is above 10 yr	0.016	[17], [39], [40], [41]
	Dwelling time of cancer in early stages	2 yr	[29], [42]
	Percent of cancers detected in early stages with no screening	5%	[21]
	Five-year all cause survival for early cancer	90%	[21], [20]
	Five-year all cause survival for advanced cancer	54%	[20], [21], [23]
	Polyp recurrence rate after polypectomy in the first year	0.11	[2], [3]
	Polyp recurrence rate after polypectomy thereafter	0.03	[2], [3]
Complications and unintended consequences	Rate of perforation of colon in colonoscopy	0.002	[12], [13]
	Rate of perforation of colon in polypectomy	0.004	[12], [13]
	Rate of perforation from sigmoidoscopy	0.0001	[12], [13]
	Rate of perforation from colon study	0.00005	[7]
	Death rate from perforated colon	0.002	[16], [17]
Cost (won[1])	Sigmoidoscopy	26 620	[24]
	Colonoscopy	52 560	[24]
	Colon study	58 600	[24]
	FOBT	2 290	[24]
	Polypectomy	134 600	[24]
	Biopsy	24 160	[24]
	Treatment of early cancer for first year	5 150 000	[3], [17], [23]
	Treatment of advanced cancer for first year	10 300 000	[3], [17], [23]
	Treatment of cancer after first year	2 164 000	[3], [17], [23]
	Treatment of colonic perforation	3 000 000	[3]

[1]Exchange rate: 1200 Korean won for one US dollar.

We defined early stage cancer as modified Duke's stage A and advanced stage as modified Duke's stage B-D[18,19]. The latent period between early stage and advanced stage was assumed to be 2 years[14]. The stage-specific CRC mortality was applied uniformly to all malignancies, regardless of the means of detection (by symptoms or screen) or the state of detection (diagnose *vs* undiagnose cancer). Five-year survival rates from previous studies were used for the yearly probability of dying from CRC based on the stage and number of years with cancer[20,21]. Age-specific mortality from other causes was estimated, based on the above source combined with statistics published by the National Center for Health Statistics[22].

Cost

We obtained the data on the costs of CRC treatment by stage and time period from the National Health Insurance Corporation (social insurer of the NHI with a universal coverage of population)[23]. However, the co-payment that patients pay at the point of service amounts to about 50% of the total medical expenses of CRC treatment in Korea[3,9]. Therefore, the total medical cost of CRC treatment was assumed to be twice the expense that the NHI reimburses. Costs of screening test were obtained from the fee schedule of the National Health Insurance Corporation (the NHI of Korea has a fee schedule applied to all insured services)[24].

Compliance and screening cost

Compliance rates of 50-70% were obtained in the optimized setting of clinical trials of CRC screening[2]. However, colonoscopy, sigmoidoscopy and DCBE for CRC screening are not covered by the NHI in Korea. Therefore, the compliance is likely to be lower than that in other countries where CRC screening is covered by health insurance. At each particular screening event without NHI benefit coverage, we assumed that 30% of population underwent the initial screening test, independent of whether they were compliant with past tests. The compliance of follow-up or surveillance colonoscopy was assumed to be 20% higher than that of the initial screening.

If the NHI covered the CRC screening or the amount of reimbursement for screening to providers increased, the compliance was assumed to increase. If the NHI covered 50% and 100% of screening cost, the compliance was assumed to be 15% and 30% higher than that in case of non-coverage respectively, by reducing the financial barrier of patients. The Korean Medical Association had insisted that current reimbursement of colonoscopy to physicians was too low and the appropriate level should be 60% higher than the current level[25]. An increase in colonoscopy reimbursement, to 60% higher than the current level, was assumed to lead to 10% increase in the compliance due to financial incentives for physicians.

As there were no data available on the compliance changes resulting from the change in insurance coverage or reimbursement level, we performed sensitivity analysis to assess the stability of the results to plausible ranges of compliances. The compliance rate was set to vary from 10% lower to 10% higher than the baseline value.

RESULTS

In the base-case analysis at 30% screening compliance without NHI coverage, all screening strategies extended life expectancy. And the strategies which were not ruled out by simple dominance or extended dominance (non-dominated strategies) were colonoscopy every 5 years (COL5) and colonoscopy every 3 years (COL3). The screening strategies with colonoscopy or sigmoidoscopy showed lower total medical cost and lower financial burden on the NHI than the strategy of no screening (Table 2).

If the NHI covered 50% of the screening cost and the screening compliance was 45%, non-dominated strategies were colonoscopy every 10 years (COL10), COL5 and COL3. As the coverage of NHI increased, the financial burden on NHI increased. Nevertheless, the financial burden on the NHI associated with COL10 was smaller than that associated with no screening (Table 3). In the case that NHI covered 100% of screening cost, non-dominated strategies

Table 2 Cost-effectiveness of 16 strategies of colorectal screening among Korean adults without NHI coverage (NHI[a] coverage = 0%, screening compliance = 30%, follow-up compliance = 50%)

Strategy (abbreviation)	Lifetime cost per person, won[2]	Life expectancy, cost per day	Incremental person, won[2]	Incremental days of life gained	Lifetime financial burden of NHI[1], won[2]	Incremental C/E[3], won[2] per life-year gained
COL5	311 682	6 176.1			139 043	
COL3	313 877	6 181.1	2 195	5.0	128 757	160 965
COL10	321 407	6 171.7	7 530		151 394	(Dominated)[4]
COL at 55	336 367	6 167.9	22 490		164 547	(Dominated)
SIG3	346 903	6 172.4	33 026		155 607	(Dominated)
SIG5	352 290	6 167.9	38 413		164 996	(Dominated)
SIG10	356 222	6 165.8	42 345		171 689	(Dominated)
SIG at 55	359 939	6 164.4	46 062		177 231	(Dominated)
SIG5+DCBE5	368 560	6 168.1	54 683		165 557	(Dominated)
No screening	370 726	6 161.9	56 849		185 236	(Dominated)
FOBT2	375 772	6 165.5	61 894		187 015	(Dominated)
FOBT1+SIG5	384 709	6 169.7	70 832		187 443	(Dominated)
FOBT1	387 912	6 168.1	74 035		192 309	(Dominated)
DCBE10	390 767	6 164.1	76 890		177 826	(Dominated)
DCBE5	410 554	6 165.3	96 677		174 392	(Dominated)
DCBE3	435 775	6 169.3	121 898		169 661	(Dominated)

COL, colonoscopy; SIG, sigmoidoscopy; DCBE, double contrast barium enema; FOBT, fecal occult blood test. Ellipses indicate no data (incremental days or life gained and incremental CE ratio were not calculated for these strategies because they were dominated or extended dominated). [1]National Health Insurance of Korea. [2]Exchange rate: 1200 Korean won for one US dollar. [3]Incremental CE ratio (won/year) = Incremental cost per person/Incremental days of life gained×365 d. [4]Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy.

Table 3 Cost-effectiveness of 16 strategies of colorectal screening among Korean adults with changing NHI[1] coverage and compliance

Strategy (abbreviation)	Lifetime cost per person, won[2]	Lifetime financial burden of NHI[1], won[2]	Incremental C/E[3], won[2] per life-year gained	Strategy (abbreviation)	Lifetime cost per person, won[2]	Lifetime financial burden of NHI[1], won[2]	Incremental C/E[3], won[2] per life-year gained
	NHI[1] coverage = 50% Screening compliance = 45%, Follow-up compliance = 65%				NHI[1] coverage = 100% Screening compliance = 60%, Follow-up compliance = 80%		
COL10	310 354	178 233		COL10	307 395	226 848	
COL5	311 640	188 051	93 440	COL at 55	308 933	192 405	(Dominated)
COL at 55	321 624	172 824	(Dominated)[4]	SIG5	316 541	235 951	(Dominated)
SIG3	328 365	197 511	(Dominated)	SIG10	321 728	214 095	(Dominated)
SIG5	332 244	191 881	(Dominated)	SIG3	323 691	261 056	(Extended Dominated)[5]
COL3	336 101	207 072	2 113 350	COL5	325 435	267 054	1 371 670
SIG10	339 760	186 587	(Dominated)	SIG at 55	330 560	191 573	(Dominated)
SIG at 55	347 179	181 334	(Dominated)	FOBT2	370 827	216 521	(Dominated)
SIG5+DCBE5	369 225	213 205	(Dominated)	No screening	370 968	185 809	(Dominated)
No screening	370 847	185 499	(Dominated)	COL3	374 192	323 357	5 656 770
FOBT2	373 988	208 794	(Dominated)	FOBT1+SIG5	382 870	277 687	(Dominated)
FOBT1+SIG5	380 512	238 067	(Dominated)	SIG5+DCBE5	383 934	303 581	(Dominated)
FOBT1	388 456	234 316	(Dominated)	FOBT1	389 668	248 595	(Dominated)
DCBE10	394 938	209 420	(Dominated)	DCBE10	397 017	260 523	(Dominated)
DCBE5	420 550	229 974	(Dominated)	DCBE5	429 376	316 022	(Dominated)
DCBE3	455 758	255 914	(Dominated)	DCBE3	478 144	384 805	(Dominated)

COL, colonoscopy; SIG, sigmoidoscopy; DCBE, double contrast barium enema; FOBT, fecal occult blood test. [1]National Health Insurance of Korea. [2]Exchange rate: 1 200 Korean won for one US dollar. [3]Incremental CE ratio (won/year) = Incremental cost per person/ incremental days of life gained×365 d. [4]Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy. [5]Extended dominated: Extended dominated strategy is a strategy which is less effective and had a higher ICER than another strategy.

Table 4 Cost-effectiveness of 16 strategies of colorectal screening among Korean adults with raising reimbursement of colonoscopy to 60% higher than current level (Cost of colonoscopy = 85 000 won[1], NHI[2] coverage = 50% screening compliance = 55%, follow-up compliance = 75%)

Strategy (abbreviation)	Lifetime cost per person, won[1]	Life expectancy, day	Lifetime financial burden of NHI[2], won[1]	Incremental C/E[3], won[1] per life-year gained
COL at 55	339 486	6 173.1	184 815	
SIG at 55	353 851	6 169.0	182 912	(Dominated)[4]
COL10	362 230	6 179.1	208 801	1 401 600
SIG10	364 257	6 173.3	192 838	(Dominated)
No screening	371 238	6 161.9	185 704	(Dominated)
SIG5	377 039	6 178.0	201 390	(Dominated)
FOBT2	384 067	6 170.8	219 587	(Dominated)
SIG3	399 919	6 183.0	210 271	(Extended dominated)[5]
COL5	402 824	6 184.0	238 433	2 992 270
FOBT1	409 771	6 176.2	254 521	(Dominated)
DCBE10	410 690	6 169.1	221 430	(Dominated)
FOBT1+SIG5	426 305	6 180.8	262 952	(Dominated)
SIG5+DCBE5	435 850	6 178.8	235 034	(Dominated)
DCBE5	448 356	6 173.0	249 784	(Dominated)
COL3	474 893	6 187.5	281 257	7 487 245
DCBE3	499 560	6 178.5	284 941	(Dominated)

COL, colonoscopy; SIG, sigmoidoscopy; DCBE, double contrast barium enema; FOBT, fecal occult blood test. Current level of colonoscopy cost in Korea is about 53 000 won. [1]Exchange rate: 1 200 Korean won for one US dollar. [2]National Health Insurance of Korea. [3]Incremental CE ratio (won/year) = incremental cost per person/incremental days of life gained×365 d. [4]Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy. [5]Extended dominated: Extended dominated strategy is a strategy which is less effective and had a higher ICER than another strategy.

were COL10, COL5 and COL3, but theses strategies showed greater financial burden on the NHI than the strategy of no screening did (Table 3).

When the reimbursement of colonoscopy was 60% higher than the current level, along with 50% coverage of screening cost by the NHI and the compliance rate of 55%, non-dominated strategies were COL at age 55, COL10, COL5 and COL3. Total medical costs of COL at age 55 and COL10 were less than that associated with no screening. In addition, the NHI's financial burden in case of COL at 55 was lower than that of no screening, and COL10 had relatively low incremental burden on the financial status of the NHI (Table 4).

Results of sensitivity analyses consistently showed the dominance of colonoscopy. In all cases, COL10, COL5 and COL3 were non-dominated strategies. When the reimbursement of colonoscopy was 60% higher than the current level, along with NHI's 50% coverage of screening cost and the compliance rate of 65%, COL10 had slightly higher total medical cost than no screening. In other cases, total medical cost of COL10 was lower than that of no screening, and NHI's financial burden associated with COL10 was lower or slightly higher than that of no screening. In all scenarios of various compliance rates, COL10, COL5 and COL3 were non-dominated strategies, and COL10 had lower or minimal incremental total medical cost and NHI's financial burden than the strategy of no screening.

DISCUSSION

We compared 16 strategies for CRC screening, varying in the level of insurance coverage and reimbursement of colonoscopy by NHI to providers. In all scenarios, COL every 10 years, 5 years and 3 years were not ruled out by either simple or extended dominance, and COL every 10 years was associated with lower total medical cost than the strategy of no screening.

Public awareness of the importance of CRC screening is increasing although the rate of screening remains low[8]. Previous studies have shown that the cost was a barrier to cancer screening[4,26]. Removing the financial barrier by providing insurance coverage is one of the effective methods to raise the screening compliance, but the financial burden on the NHI can be increasing as well. In other countries, screening for CRC usually leads to greater life expectancy but is more costly than no screening. Interestingly, in our study of Korea, COL every 10 years has lower total medical cost than the case of no screening. This difference might be due to the difference in cost structure. In the US, published cost estimates for the medical care of patients with CRC range from $25 000 to $45 000 and the cost of COL is approximately $1 000[14]. In Korea, the cost estimate of CRC treatment in the first year ranges from $5 000 to $10 000 and the cost of COL was approximately $50[23,24]. The ratio of treatment cost to COL cost ranges from 25:1 to 45:1 in the US and 100:1 to 200:1 in Korea. Since the cost of COL is relatively low in Korea, the screening is more cost-effective than in the US.

In Korea, the government started the national cancer-screening program (NCSP) in 1999, which included CRC screening in 2004. The government covers 50% of the screening cost for the insured and 100% for the low-income people. The primary method for CRC screening in NCSP is FOBT. Our study shows that the strategy of 'FOBT annually' costs more and carries heavier burden on NHI than the strategy of no screening, while COL every 10 years is less costly than no screening. These results suggest that COL every 10 years can be recommended as a primary screening strategy for CRC in NCSP. However, if COL is to be promoted as a screening tool, there must be sufficient manpower to deliver colonoscopy to the public. Unfortunately,

there are only a few medical endoscopists available to undertake COL in Korea. Korean physicians insist that they are not willing to contribute to increase in CRC screening rate because of low reimbursement of COL[25]. Some surveys indicate that strong recommendation from the physician is highly correlated with patient participation in CRC screening[27]. Therefore, raising reimbursement rate for CRC screening to physicians can be effective in changing their behavior, which will eventually improve compliance rate. Our model shows that when the reimbursement for COL increases up to 85 000 (Korean) won, which is 60% higher than the current level, along with NHI's 50% coverage of screening cost, COL every 10 years or 5 years not only has lower total medical cost and lower financial burden on the NHI, but also improves lifetime expectancy than FOBT annually (Table 4). In addition, the total medical cost of COL every 10 years was lower than that of no screening in Korea. More investment in CRC screening is ideal because it reduces the cost of conventional treatment and extends life expectancy. Health policy makers should understand the need to train medical, and possibly even non-medical, personnel to perform endoscopy and to find an effective policy to lead physicians to perform colonoscopy[26].

Our analysis has several limitations. In the design of the model, we tried to reduce the complex natural history of CRC to a few essential states and to avoid assumptions on treatments for which little or no published data existed. For instance, we assumed that all cancers arose from polyps. And we used data from western countries if there were no published data available in Asia. There were possible differences between the races. Finally, we calculated only the direct costs and did not take into account the impact of CRC and screening on indirect costs.

In our conclusion, economic strategies for promoting screening compliance can be accompanied by expanding insurance coverage by the NHI and by increasing reimbursement for CRC screening to providers. And COL every 10 years is a cost-effective and cost saving screening strategy for CRC in Korea.

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Association of polymorphisms of interleukin-18 gene promoter region with chronic hepatitis B in Chinese Han population

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Abstract

AIM: To investigate the polymorphisms of interleukin-18 (IL-18) gene promoters, and to disclose whether such polymorphisms are associated with susceptibility to chronic hepatitis B in Chinese Han population.

METHODS: Using polymerase chain reaction with sequence specific primers (PCR-SSP) method, the single nucleotide polymorphisms (SNPs) of the promoter region of IL-18 gene at position -607 and -137 were detected in 231 patients with chronic hepatitis B and 300 normal controls.

RESULTS: Allele C at position -607 in the promoter of IL-18 gene was detected in 48.7% of normal controls and 51.9% of patients, while allele A at position -607 was detected in 51.3% of normal controls and 48.1% of patients. The frequencies of -607CC, -607 CA and -607AA genotypes in normal controls were 22.0%, 53.3% and 24.7% respectively and in chronic hepatitis B patients were 26.8%, 50.2% and 23.0% respectively. Allele G at position -137 in the promoter of IL-18 gene was detected in 82.3% of normal controls and 88.5% of chronic hepatitis B patients, while allele C at position -137 was detected in 17.7% of normal controls and 11.5% of patients. The frequencies of -137GG, GC and CC genotype were 67.3%, 30.0% and 2.7% in normal controls respectively, while in chronic hepatitis B patients were 78.8%, 19.5% and 1.7% respectively. The frequency of -137GG genotype in chronic hepatitis B groups was significantly higher than that in normal controls (χ^2 = 8.55, P = 0.003 <0.05), whereas the frequencies of -607C/-137C and -607A/-137C haplotypes in chronic hepatitis B groups were significantly lower than that in normal controls. The association between genotypes of IL-18 promoter region polymorphisms and HBV copies showed that the frequency of -607AA genotype in high HBV-DNA copies groups was lower than that in low HBV-DNA copies groups (χ^2 = 6.03, P = 0.014 <0.05).

CONCLUSION: The polymorphisms of the promoter region of IL-18 gene at position -607 and -137 are closely associated with susceptibility to chronic hepatitis B. The people with allele C at position -137 in the promoter of IL-18 gene may be protected against HBV infection; moreover AA genotype at position -607 may be closely linked to inhibit HBV-DNA replication. These findings give some new clues to the study of pathogenesis of chronic hepatitis B.

Key words: Interleukin-18 gene; Polymorphism; Chronic hepatitis B

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important chronic viral diseases in the world. An estimated 400 million people worldwide are carriers of HBV, and approximately 250 000 deaths occur each year as a consequence of fulminant hepatic failure, cirrhosis and hepatocellular carcinoma[1,2]. When HBV is acquired in adulthood, the majority of infections are cleared, with chronic infection occurring in 5-10% of cases. However, the dynamic interaction of the host inflammatory response with HBV and the subsequent impact of this interaction on the clinical outcome of HBV infection, are not yet fully understood, nor are the underlying mechanisms for the persistence of the virus. But it has been thought that genetic associations may also provide clues to the development of HBV infection. Some polymorphisms have been reported to be involved in susceptibility to chronic hepatitis B, in disease severity and progression, or in disease prognosis[3-5].

Several recent advances concerning the polymorphism of cytokines controlling the host response could play an important role in determining HBV infection outcome[6,7]. Being involved in the proinflammatory cytokine network, interleukin-18 (IL-18) is a novel cytokine that is mainly produced by activated macrophages and, like interleukin-12

(IL-12), is able to induce interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) induction, as well as enhancing the cytotoxicity of NK cells and FasL expression[8-10]. Clinical research showed that there was a correlation between the levels of serum IL-18 and disease severity in patients with viral hepatitis B[11]. Meanwhile, *in vitro* IL-18 can improve the peripheral blood monocytes from chronic hepatitis B patients to produce a great deal of IFN-gamma. These findings indicate the evidence of an association between susceptibility to chronic hepatitis B and IL-18 gene. Three single nucleotide polymorphisms (SNPs) in the promoter of IL-18 gene at the position -656G/T, -607C/A and -137G/C have been identified, and the two SNPs at position -607C/A and -137G/C in the promoter were predicted to be nuclear factor binding sites for cAMP-responsive element binding protein and H4TF-1 nuclear factor, respectively; moreover mutation of the two sites can influence the expression of IL-18 and also potentially of IFN-gamma[12]. In order to investigate the possible roles of the SNPs of IL-18 gene promoter region in the development and progression of chronic hepatitis B, we genotyped 231 patients with chronic hepatitis B and 300 control subjects for two IL-18 SNPs, using polymerase chain reaction with sequence specific primers (PCR-SSP) method.

MATERIALS AND METHODS

Subjects

A total of 231 unrelated Chinese subjects with chronic hepatitis B (156 males, 75 females) aged 5-82 years were recruited in Remin Hospital of Wuhan University. The diagnosis of all the patients was confirmed according to the criteria for chronic hepatitis B[13,14], and the patients did not have other viral hepatitis. Three hundred control subjects (183 males, 117 females) aged 18-81 years were randomly selected in Wuhan area, China during the same period, with definitely negative for HBsAg, anti-HBe and anti-HBc and with no history of HBV vaccination. They did not have any abnormalities based on physical examination, chest radiography, electrocardiogram, urinalysis and routine laboratory blood testing. Liver, renal, endocrine and cardiovascular disorders were excluded. All the subjects were Chinese Han people and they were recruited with their informed consent for genetic analysis.

Specimens preparation

Two microliters of peripheral venous blood were drawn from all the subjects after an overnight fasting and collected in an EDTA tube. Genomic DNA was extracted from peripheral blood leukocytes with standard techniques and frozen at -20 ℃.

Determination of the IL-18 genotypes

Polymorphisms were analyzed by using PCR-SSP, at the position -607 and -137 in the promoter of IL-18 gene[12]. For the position -607C/A-specific PCR, a common reverse primer 5'-TAACCTCATTCAGGACTTCC-3' and two sequence-specific forward primers 5'-GTTGCAGAAA GTGTAAAAATTATTAC-3' and 5'-GTTGCAGAAAG TGTAAAAATTATTAA-3' were used. An amplification product of 196 bp was detected. A control forward primer 5'-CTTTGCTATCATTCCAGGAA-3' was used to amplify a 301-bp fragment covering the polymorphic site as an internal positive amplification control. PCR reaction was performed in a final volume of 15 μL consisting of 1.5 μL 10× PCR buffer, 0.2 mmol/L dNTP, 30 ng genomic DNA and 0.5 U Taq polymerase. One sequence specific primer (for allele C or allele A) and the common reverse primer were included in every reaction mixture at a concentration of 0.6 μmol/L. In addition, the internal position control primer was added to the reaction mixture at a concentration of 0.15 μmol/L. Therefore, two PCR reactions were performed for every individual DNA.

Reactions were carried out in a GenAmp PCR system 2 700 thermal cycler. At the first step, denaturation for 2 min at 94 ℃ was performed, followed by seven cycles of 94 ℃ for 20 s, 64 ℃ for 40 s and 72 ℃ for 40 s and 25 cycles 94 ℃ for 20 s, 57 ℃ for 40 s, 72 ℃ for 40 s and 72 ℃ for 5 min. PCR products were visualized by 2% agarose gel electrophoresis stained by ethidium bromide.

For the -137 genotyping, a common reverse primer 5'-AGGAGGGCAAAATG CACTGG-3' and two sequence-specific forward primers 5'-CCCCAACTTTTACGGAA GAAAAG-3' and 5'-CCCCAACTTTTACGGAAGAAAAC-3' were used. An amplification product of 261 bp was detected. A control forward primer 5'-CCAATAGGAC TGATTAT TCCGCA-3' was used to amplify a 446-bp fragment covering the polymorphic site to serve as an internal positive amplification control. PCR reaction was performed in a final volume of 15 μL consisting of 1.5 μL 10× PCR buffer, 0.2 mmol/L dNTP, 30 ng genomic DNA and 0.5 U Taq polymerase. Concentrations were of 0.3 μmol/L of the control primer and 0.5 μmol/L of the reverse primer and sequence-specific primers used. At the first PCR step, denaturation for 2 min at 94 ℃ was performed, followed by five cycles of 94 ℃ for 20 s, 68 ℃ for 1 min and 25 cycles of 94 ℃ for 20 s, 62 ℃ for 40 s, 72 ℃ for 40 s and 72 ℃ for 5 min.

HBV-DNA measurement

Serum HBV-DNA levels in patients with chronic hepatitis B were detected with the real-time fluorescent quantitative PCR method (reagents supplied by Shanghai Fosun Co. Ltd) using a Lightcycler PCR system. Results were considered abnormal when HBV-DNA $>1\times10^3$ copies/mL.

Statistical analysis

The frequencies of genotypes and alleles in the promoter region of IL-18 gene at position -607 and -137 were calculated by counting. Data were analyzed with SPSS11.5 software and the Hardy-Weinberg equilibrium was determined by means of the χ^2 test. Comparison of allelic and genotypes between groups, and association of -607C/A, -137G/C polymorphisms with HBV-DNA replication were examined for statistical significance with χ^2 test. The odds ratio (OR) was calculated by means of logistic regression and the confidence interval (CI) was calculated at the 95% level. Statistical significance was assumed for *P* values less than 0.05.

RESULTS

Polymorphisms in promoter of the IL-18 gene

Polymorphisms at the position -607 and -137 in the promoter of IL-18 gene were analyzed by PCR-SSP. In every polymorphic site, a common reverse primer and two sequence-specific forward primers were used and two PCR reactions were performed for every individual DNA. The specific products of PCR from homozygous individuals were one DNA segment and from heterozygous individuals showed the expected two specific fragments. In total, 531 unrelated Chinese subjects were studied for IL-18 promoter polymorphisms. As shown in Figure 1, there were CC, CA and AA genotypes at position -607, and GG, GC and CC genotypes at position -137.

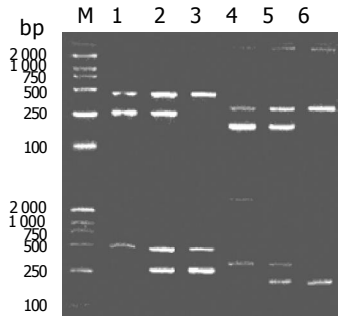

Figure 1 Genotyping for the IL-18 position -137 and -607 polymorphisms. M: DL2000 DNA Marker; lanes 1–3: -137 GG, GC and CC; lanes 4-6: -607 CC, CA and AA.

Frequencies of IL-18 promoter genotypes in both groups

Genotype and allele frequencies for IL-18 polymorphisms are summarized in Table 1. The genotype frequencies were in agreement with the Hardy-Weinberg ($P>0.1$ for all analyses). As the -607 genotypes, of 231 patients with chronic hepatitis B, 62 had the CC type (26.8%), 116 the CA type (50.2%) and 53 the AA (23.0%). Of the 300 control subjects, 66 had the CC type (22.0%), 160 the CA type (53.3%) and 74 the AA (24.7%). No significant difference in the genotype distribution or in the allele frequency between the patients with chronic hepatitis B and the control subjects was observed. As for the -137 genotypes, 182 of the 231 patients with chronic hepatitis B had the GG type (78.8%), 45 the GC type (19.5%) and 4 the AA type (1.7%). Two hundred and two of the 300 control subjects were type GG (67.3%), 90 were GC (30.0%) and 8 were CC (2.7%). There was a significant difference in the genotype distribution and in the allele frequency between the patients with chronic hepatitis B and the control subjects. In genotypes, the GG type at position -137 was present at a significantly higher frequency in the patients with chronic hepatitis B compared to those in the controls. OR of the GG genotype for the comparison with that of the GC and the CC genotype was 1.80 (95%CI 1.21-2.68, $P = 0.01 <0.05$). But in phenotypes, the allele C at -137 was of a significantly lower frequency in the patients with chronic hepatitis B than that in the controls ($\chi^2 = 7.87$, $P = 0.005 <0.05$).

Based on genotypes of IL-18 promoter polymorphisms, haplotype frequencies were estimated by the expectation-maximization method. Four haplotypes of the IL-18 promoter at position -607 and -137 were present in both patients and controls (haplotypes I, II, III and IV in Table 2). The frequencies of haplotype I, II, III and IV in the patients with chronic hepatitis B were 51.7%, 0.2%, 36.8% and 11.3%, respectively. The frequencies of haplotype I, II, III and IV in the controls were 47.4%, 1.3%, 35.0% and 16.3%, respectively. The frequencies of haplotype II and IV, which bear C at -137, in the patients were significantly lower than that in the health control subjects.

Table 1 Comparison of IL-18 gene promoter polymorphism between patients with chronic hepatitis B and controls

Polymorphism	Control $n = 300$ (%)	Patient $n = 231$ (%)	χ^2	P
Position -607				
Genotypes				
CC	66 (22.0)	62 (26.8)	1.67	0.196
CA	160 (53.3)	116 (50.2)	0.51	0.476
AA	74 (24.7)	53 (23.0)	0.21	0.645
Alleles				
C	292 (48.7)	240 (51.9)	1.12	0.842
A	308 (51.3)	222 (48.1)		
Position -137				
Genotypes				
GG	202 (67.3)	182 (78.8)	8.55	0.003
GC	90 (30.0)	45 (19.5)	7.62	0.006
CC	8 (2.7)	4 (1.7)	0.52	0.472
Alleles				
G	494 (82.3)	409 (88.5)	7.87	0.005
C	106 (17.7)	53 (11.5)		

Table 2 Haplotype frequencies of two interleukin-18 bi-allelic polymorphisms in chronic hepatitis B and health controls

Haplotype	-607C/A	-137G/C	Control n (%)	Patient n (%)	χ^2	P
I	C	G	284 (47.4)	239 (51.7)	2.02	0.155
II	C	C	8 (1.3)	1 (0.2)	3.87	0.049
III	A	G	210 (35.0)	170 (36.8)	0.37	0.545
IV	A	C	98 (16.3)	52 (11.3)	5.55	0.018
Total			600	462		

Association of IL-18 gene promoter polymorphism with HBV-DNA replication

For further analysis of the relationship between IL-18 gene promoter polymorphisms and HBV-DNA replication in patients with chronic hepatitis B, the patients were divided into two sub-groups (HBV-DNA $<1\times10^3$ copies/mL as sub-group I, HBV-DNA $\geqslant1\times10^3$ copies/mL as sub-group II). As shown in Table 3, the distribution of AA genotype in the IL-18 gene promoter at position -607 were significantly different between the two sub-groups ($\chi^2 = 6.03$, $P = 0.014 <0.05$).

DISCUSSION

Individuals, with an inadequate primary immune response to HBV, are at increased risk of developing chronic hepatitis

Table 3 The association between genotypes of IL-18 promoter region polymorphisms and HBV copies in the chronic hepatitis B patients

Polymorphism	Group I n = 97 (%)	Group II n = 134 (%)	χ^2	P
Position -607				
Genotypes				
CC	23 (23.7)	39 (29.1)	0.83	0.361
CA	44 (45.4)	72 (53.7)	1.58	0.209
AA	30 (30.9)	23 (17.2)	6.03	0.014
Alleles				
C	90 (46.4)	150 (56.0)	4.14	0.042
A	104 (53.6)	118 (44.0)		
Position -137				
Genotypes				
GG	76 (78.4)	106 (79.1)	0.02[1]	0.890
GC	20 (20.6)	25 (18.7)		
CC	1 (1.0)	3 (2.2)		
Alleles				
G	172 (88.7)	237 (88.4)	0.01	0.940
C	22 (11.3)	31 (11.6)		

Group I: HBV-DNA $<1\times10^3$ copies/mL; Group II: HBV-DNA $\geqslant1\times10^3$ copies/mL. GG compared with both GC and CC.

B. Age is the strongest host feature associated with chronic infection with 90% infants and 5-10% of adults developing chronic hepatitis B after exposure[15,16]. In addition, people belonging to the same age, sex and ethnical groups were exposed to the same HBV strain, which could cause a broad spectrum ranging from no infection to different clinical outcomes. These data suggest that host genetic factors are responsible for the clinical outcomes of HBV infection[17]. Clearance of HBV requires a coordinated innate and adaptive humoral and cell-mediated immune response. Cytokines are soluble polypeptide molecules that mediate cell-to-cell communication and regulate the intensity and duration of the immune response. Previous studies have shown that the maximal capacity of cytokine production varies among individuals and correlates with SNPs in the promoter region of various cytokine genes. Furthermore, cytokine gene polymorphisms were associated with liver disease severity in patients with viral hepatitis B[18-22], which may provide clues to understand the development of end-stage complications such as cirrhosis or hepatocellular carcinoma. In the present study, we compared the distributions of IL-18 gene promoter polymorphisms between patients with chronic B and control subjects.

IL-18 was first described as an IFN-gamma inducing factor, and has multiple functions including induction of the synthesis of IFN-gamma by T cells and NK cells, promotion of Th1-type immune responses, augmentation of proliferative response and cytokine production of activated T cells. Meanwhile, IL-18 leads to activities against pathogens, activate effector cells involved in the cellular interactions that occur during inflammation, and are part of the acute and chronic stages of viral hepatitis, induce target-cells apoptosis[23,24]. Recently, it was reported that injection of a single 10-μg dose of recombinant murine IL-18 rapidly, reversibly and non-cytopathically inhibited HBV replication in the livers of HBV transgenic mice. The anti-viral effect of IL-18 was mediated by its ability to activate resident intrahepatic NK cells and T cells to produce IFN-gamma and by its ability to induce IFN-alpha/beta production in the liver[25]. These results suggest that IL-18 has the potential to contribute to the control of HBV replication during self-limited infection and that it may have therapeutic value for the treatment of patients with chronic hepatitis. The human IL-18 gene is located on chromosome 11q22.2-q22.3, and is composed of six exons and five introns[26]. Giedraitis *et al*[12,27,28] described that there were three SNPs at position -656G/T, -607C/A and -137G/C in the promoter of IL-18 gene first exon. A change from C to A at position -607 disrupts a potential cAMP-responsive element-binding protein binding site and a change at position -137 from G to C changes the H4TF-1 nuclear factor binding site. Cloning and gene expression analysis showed that two SNPs of the promoter of IL-18 gene at position -607 and -137 were suggested to cause the differences in transcription factor binding and have an impact on IL-18 gene activity and potentially also to IFN-gamma. Potentially, the G/C polymorphisms at position -137 could play a main role in the expression of IL-18. Individuals with CC genotype at position -137 had higher levels of IL-18 mRNA compared to other genotypes, that had a clear correlation between IL-18 and IFN-gamma mRNA expression.

In the study, we identified two polymorphisms in the promoter regions of the IL-18 gene and demonstrated the association between these polymorphisms and chronic hepatitis B. The results showed that no significant differences were seen in the distribution of the genotypes or allelic frequencies for polymorphisms of IL-18 gene promoter at position -607 between patients with chronic hepatitis B and control subjects. However, the genotypes distributions and allelic frequencies at position -137 in both groups were statistically different. The genotype frequency of -137GG in chronic hepatitis B groups was significantly higher than that in normal controls ($\chi^2 = 8.55$, $P = 0.003 < 0.05$). The OR was 1.80 for chronic hepatitis B when the genotype at position -137 was GG. Meanwhile, haplotype frequencies' distributions suggested that the frequencies of -607C/-137C and -607A/-137C haplotypes in chronic hepatitis B groups were significantly lower than that in normal controls. These results indicated that the carriage of allele C at position -137 plays a protective role in the development of HBV infection. Further analysis of the relationship between IL-18 gene promoter polymorphism and HBV-DNA replication in patients with chronic hepatitis B showed that AA genotype at position -607 in the IL-18 gene promoter was associated with HBV-DNA replication ($\chi^2 = 6.03$, $P = 0.014 < 0.05$). To our knowledge, there is no published study, concerning the role of the promoter of IL-18 gene in other infection diseases, because of the complication of IL-18 regulation function. Taking into consideration our findings and those of Giedraitis *et al*, there may be a possible link between G→C polymorphism at position -137 of the promoter of IL-18 gene and the increased levels of IL-18, carriage of allele C at position -137 of each of these polymorphisms was related with high production of IL-18, which may augment the production of IFN-gamma, modulate activity of NK and CTL cells, and trigger the complex immunological processes to eliminate HBV and its complex. As for the

frequency of -607AA genotype in low HBV-DNA copies groups was higher than that in high copies groups, whether individualism with -607AA genotype could inhibit HBV-DNA replication, further investigation of the general association of the polymorphisms with cAMP-responsive element-binding protein binding site in an independent data set is needed.

In summary, the findings of this study and others may provide further evidence that genetic factors are important in the pathogenesis of HBV infection. Our results suggest that the carriage of allele C at position -137 in the promoter of IL-18 gene may play a protective role in the development of HBV infection and AA genotype at position -607 may be associated with HBV-DNA replication. However, the real roles of IL-18 gene promoter polymorphisms in the pathogenesis of developing chronic hepatitis B should be further investigated by large population-based studies.

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• *BASIC RESEARCH* •

Evaluation of diffusion in gel entrapment cell culture within hollow fibers

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Abstract

AIM: To investigate diffusion in mammalian cell culture by gel entrapment within hollow fibers.

METHODS: Freshly isolated rat hepatocytes or human oral epidermoid carcinoma (KB) cells were entrapped in type I collagen solutions and statically cultured inside microporous and ultrafiltration hollow fibers. During the culture time collagen gel contraction, cell viability and specific function were assessed. Effective diffusion coefficients of glucose in cell-matrix gels were determined by lag time analysis in a diffusion cell.

RESULTS: Significant gel contractions occurred in the collagen gels by entrapment of either viable hepatocytes or KB cells. And the gel contraction caused a significant reduction on effective diffusion coefficient of glucose. The cell viability assay of both hepatocytes and KB cells statically cultured in hollow fibers by collagen entrapment further confirmed the existence of the inhibited mass transfer by diffusion. Urea was secreted about 50% more by hepatocytes entrapped in hollow fibers with pore size of 0.1 μm than that in hollow fibers with MWCO of 100 ku.

CONCLUSION: Cell-matrix gel and membrane pore size are the two factors relevant to the limited mass transfer by diffusion in such gel entrapment of mammalian cell culture.

Key words: Hollow fiber; Mammalian cell culture; Collagen gel entrapment; Diffusion

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INTRODUCTION

Entrapment of mammalian cells inside semi permeable hollow fibers has been introduced as a commonly used technique[1-5]. Based on this technique, more tissue-like culture of cells by entrapped into gel within hollow fibers have been explored as novel applications in tissue engineering[6-9], toxicology[10] and pharmacology[11,12]. Such system is comprised of two major compartments: hollow fiber membrane and gel-cell matrix, which is typically featured by ultrafiltration hollow fiber membranes and collagen gels, respectively[13]. And mass transport across hollow fibers has been well studied by taking consideration of convection together with diffusion[14-18]. When hollow fiber bioreactor is operated at low flow rate, especially in medical-related field, the primary mass transfer mechanism should be the diffusion[14], where the solute effective diffusion coefficient, representative of diffusion ability, across membranes is proportional to the transmembrane concentration gradient and decreases with the solute to pore radius ratio[19]. However, even at a very slow flow rate, convection cannot be completely negligible. Also, the diffusion across cell-collagen gel is rarely known including the extent of gel contraction and its effect on the diffusion. These were drawbacks in evaluation of mass transfer and thus hinder its further exploration as a practical technology, for an example, the clinical application of bioartificial liver in the near future. Therefore, discrete evaluation of diffusion in either membrane side or gel side should be urgently required.

Our previous research found that the liver-specific functions were much higher in the hollow fiber bioreactor featured by microporous hollow fibers with membrane pore size of 0.1 μm than in that featured by ultrafiltration hollow fibers with MWCO of 100 ku, an equivalent pore size of 0.01 μm[20]. In those bioartificial livers, hepatocytes were entrapped into collagen gels in the lumen side of the hollow fiber bioreactors with each medium circulated in either lumen or shell side. But it is uncertain that the increase of liver-specific functions is related to the better diffusion caused by bigger membrane pore size in microporous hollow fibers, because uneven distribution of fluid flow and specific configuration of hollow fibers may cause additional convective flows[21]. It seems difficult to uncouple diffusion from convection in such device of hollow fiber

bioreactor. Also we never evaluated the diffusion within collagen gels.

In this paper, we evaluated the effect of diffusion by static cell culture, where solute mainly transfers by diffusion in two serial processes: membrane and cell-collagen gel. The effective diffusivities were measured, testing the diffusion ability across collagen gel with or without cells. And both the viability and specific function of collagen-entrapped cells within simple hollow fibers were examined for further evaluation of diffusion ability.

MATERIALS AND METHODS

Cultivation of rat hepatocytes

Hepatocytes from 4-6 wk old male Sprague-Dawley rats weighting 200-250 g were harvested by a two-step collagenase perfusion technique modified from that of Seglen[22]. Post-harvest viability was at least 85% by trypan blue exclusion. Hepatocytes were cultured in a basal medium of Williams' E (Gibco) complemented with 100 U/mL penicillin and 100 U/mL streptomycin, 5 g/L bovine serum albumin (Amresco, 0530S03) and 5% fetal calf serum (Hangzhou Sijiqing Biological Eng. Material Co., Ltd., China).

Freshly harvested hepatocytes were mixed with the collagen solution, a 3:1 (v/v) mixture of type I collagen (3 g/L, prepared from rat tails in our own lab) and 4 fold concentrated Williams' E medium with pH adjusted to 7.4 with 1 mol/L NaOH. The cell suspension with density of 1×10^6 cells/mL was loaded into the lumen of the long fibers with length of 80 cm. Hollow fibers were circuitously put into 12 cm dishes and maintained in a 50 mL/L CO_2 incubator for collagen gelation. And 20 min later, hollow fibers were cut into equal pieces of 4 cm and immersed into 5 mL prewarmed culture medium in 60 mm culture dishes. These dishes were put back into the incubator for cell culture.

Samples for analysis of urea secretion were taken at regular intervals from the medium after mild mixing. Urea assays were performed using a Urea Nitrogen Kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cultivation of KB cells

KB cells, kindly obtained from biomedical engineering institute in Zhejiang University, were cultivated in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 U/mL streptomycin and 10% new born calf serum (Hangzhou Sijiqing Biological Eng. Material Co., Ltd., China). KB Cells was subcultured in T-flasks at a density of 10^5 cells at 37 ℃ in a 50 mL/L CO_2 incubator with the medium changed every two days. Later, KB cells were detached from T-flasks by 0.5% trypin and entrapped into collagen gel within hollow fiber at the density of 4×10^5 cells/mL, following the same procedure as hepatocyte entrapment.

Measurement of collagen gel contraction

Cylindrical collagen gels were used to examine the gel contraction by both hepatocytes and KB cells in static culture. At different culture time, cylindrical collagen gels were carefully pushed out from the hollow fibers by injection with medium and suspended in the plates containing prewarmed medium. The gel diameter was then determined with a vernier eyepiece equipped on an inverted microscope.

Viability determination of entrapped cells with fluorescent microscopy

Hepatocytes cells or KB entrapped within collagen gels were stained twice with fluorescein diacetate (FDA) and ethidium bromide (EB)[23,24]. Cylindrical collagen gels drawn from hollow fibers were stained with 2 mL of a mixture of 10 g/mL FDA and 40 g/mL EB. After 2 min incubation at room temperature followed by three rinses with PBS, the sample was examined with an epifluorescence inverted microscope. The excitation wavelength was 488 nm and the emission wavelength was 600 nm for EB and 530 nm for FDA. Cell viability was assessed by counting the number of FDA- and EB-stained cells.

Diffusivity measurements

A modified diffusion cell described by Hannoun and Stephanopoulos[25] was used to measure the effective diffusion coefficient of glucose in collagen gel. A plexiglass ring, with outer diameter of 8.05 cm and inner diameter of 4.55 cm, was attached by a plastic mesh at bottom to hold the covered collagen gel. Solution of 2.25 g/L collagen at pH 7, a mixture of 3 g/L type I rat tail collagen and 4 fold concentrated PBS (3:1, v/v), was immediately poured on the plexiglass ring. After 30-40 min incubation at 37 ℃, collagen was gelatinized and its thickness was measured by micrometer. Then, the plexiglass covered with collagen gel was put into the diffusion cell where the upper chamber and the lower chamber were respectively 286 and 227.5 mL. The well mixing in two chambers was achieved by stirring at about 60 r/min with a magnetically driven bar in the lower cell and a stirrer in the upper cell at room temperature (21-22 ℃). By taking samples in the upper chamber, the transferred glucose concentrations were determined by means of 3,5-dinitrosalicylic (DNS) colorimetric determination[26].

The corresponding diffusion coefficients were calculated according to lag-time analysis[27-29]. Assuming that there was no film mass transfer resistance, the solute concentration in the lower chamber (c_1) and the solute concentration in the upper chamber (c_2) can be defined as follows:

$$c = c_1 \quad \text{at} \quad x = 0$$
$$c = c_2 \quad \text{at} \quad x = l$$
$$c = 0 \quad 0 \leqslant x \leqslant l \quad \text{at} \quad t = 0$$

Thus, the total amount Q of solute transferred through the membrane was given by the equation below:

$$Q|_{ts} = \frac{ADc_1}{l}(t_s - \frac{l^2}{6D})$$

where l was the membrane thickness, A the membrane area, D the effective diffusion coefficients and t_s the lag time. Figure 1 was the experimental graph of transferred glucose versus diffusion time for the diffusion of glucose. The intercept of the linear part of the curve was the so-called lag time.

To entrap hepatocytes into collagen gels, 2 mL of fresh hepatocyte slurry at density of 3×10^7 cells/mL was mixed with 10 mL solution of 2.7 g/L collagen to obtain a final

suspension with cell density of 5×10^6 cells/mL and collagen concentration of 2.25 g/L. In preparation of collagen gels entrapped with dead cells, gel-cell matrix formed above was immediately deactivated by exposure to ultraviolet for 10 min. While in preparing collagen gel entrapped with viable cells, the cell-collagen matrix was cultivated in a basal medium of Williams' E at a 50 mL/L CO_2 incubator for 24 h to develop gel contraction before it was deactivated by 10 min UV radiation for effective diffusion coefficient measurement. Membranes with cell density of 0.5×10^6 cells/mL were similarly prepared.

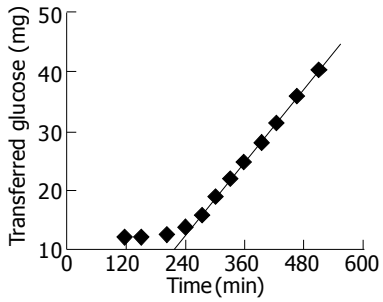

Figure 1 Transferred glucose through the collagen gel membrane *vs* time in a diffusion experiment (c_1 = 10 g/L, t_0 = 122 min).

Hollow fibers

Microporous polysulfone hollow fibers with average pore size of about 0.01 μm and ultrafiltration polysulfone hollow fibers with MWCO of 30 and 100 ku were purchased from Yuandong Pharmaceutical Machinery Corporation (Shanghai, China). All these hollow fibers with the same outer diameter of 1 mm, inner diameter of 0.7 mm and membrane porosity of about 90% had identical diffusion area.

RESULTS

Gel contraction measurements

Collagen contraction was frequently reported in hepatocyte culture by collagen gel entrapment, but its contraction extent was usually determined in disc collagen gel[7]. Also, the gel contraction was rarely reported in the continuous cell culture. Hereby, the contraction of collagen gel mediated by both primary hepatocyte and KB cells were first determined in this paper by measuring the diameter of cylindrical collagen gels over the culture period of five days. The entrapped cell density of hepatocytes was set at 1×10^6 cells/mL with the initial gel diameter of 0.68 mm. Figure 2 demonstrates the average percentage of diameter of 3 cylindrical gels entrapped with hepatocytes to the initial gel diameter. The gel diameter rapidly decreased almost to 60% of the initial diameter within three days cultivation, while the control gel containing no cells showed no signs of contraction. At a higher cell density of 5×10^6 cells/mL, cell-matrix gel contracted by 60% after one day cultivation but were stable since then (data is not shown here). This was possibly due to cell death because the rapidly decreased cell viability was suspected according to the prevalent morphological change since the culture time of one day with a microscope. To confirm that dead cells caused no gel contraction, we repeated the gel contraction experiment with dead cells killed by UV for 10 min. It was found that no sign of gel contraction was observed when dead cells were entrapped with collagen gels.

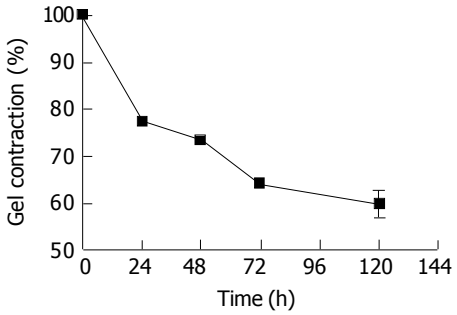

Figure 2 Contraction of cylindrical collagen gels entrapped with hepatocytes. Gel contraction was expressed as the average percentage of diameter of three cylindrical gels entrapped with hepatocytes to the initial gel diameter *vs* time.

Another anchorage-dependent KB cells were also randomly used to confirm gel contraction caused by cell entrapment. The initial entrapped KB cell density was set at 0.5×10^6 cells/mL. Compared with the initial value of 0.68 mm, the gel diameter decreased by about 14 % at 48 h and 30% at 120 h with a high cell density of around 1×10^7 cells/mL due to KB cell propagation. It seems primary cells are more likely to cause the gel contraction than continuous cells.

Effective diffusivity across collagen gels

Effective diffusivities of glucose were determined in collagen gels with or without cells. Glucose was commonly selected as a marker for chemical substances involved in cell metabolisms and hepatocytes were used here to represent for mammalian cells. The glucose concentration was set at 10 g/L and the collagen concentration was fixed at 2.25 g/L. The effects of entrapped cells on the effective diffusivities of collagen gels were listed in Figure 3. The statistical data were expressed as mean±SD. In cell-free collagen gel, this coefficient was determined as $1.441\pm0.017\times10^{-6}$ cm^2/s, which is much lower than the corresponding molecular diffusivity (6.7×10^{-6} cm^2/s). At a low cell density of 0.5×10^6 cells/mL, the effective diffusion coefficient was $1.512\pm0.042\times10^{-6}$ cm^2/s for collagen gels entrapped with dead cells, which was an equivalent of that for cell-free collagen gel. In contrast, this value decreased to $0.502\pm0.002\times10^{-6}$ cm^2/s in the comparatively contracted gel after 24 h interaction of cells with collagen gels. While at a high cell density of 5.0×10^6 cells/mL, this coefficient was $0.799\pm0.013\times10^{-6}$ cm^2/s for non-contraction gel and only $0.231\pm0.017\times10^{-6}$ cm^2/s for the contracted gel. It seems that collagen contraction resulted in a significant decrease of the effective diffusivity and should be the main reason for reduced diffusion across gels other than the obstruction of cell masses.

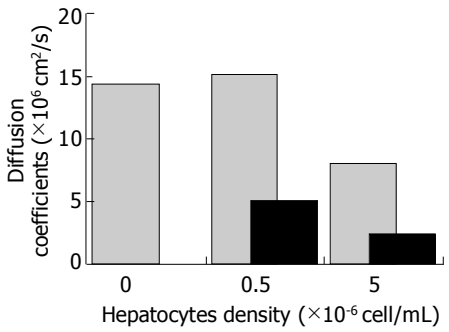

Figure 3 Effective diffusion coefficients of glucose in cell-free collagen gel and gel-cell matrices. Data from two independent experiments are shown.

Entrapment culture of hepatocytes and KB cells

By measuring the effective diffusivities, we know the diffusion situation inside collagen gels with or without the interaction of cells. And it is common knowledge that the diffusion across membranes was mainly determined by the transmembrane concentration gradient and the molecular sizes of both solutes and membrane pores. Thus, the integrated diffusion across both membrane and collagen gel can be reflected by cell cultivation itself within such bioreactor. Cell viability was first checked by cultivation of cells in hollow fibers with variant membrane pore size and different cell concentration. Cell function was later investigated.

To feature the cell surviving abilities in such configured system, both rat hepatocytes and KB cells were employed here. Hepatocyte cell culture at two different cell densities was employed in hollow fibers with three featured pore sizes, 0.1 μm, MWCO of 30 and 100 ku, respectively. Initial harvest viability of hepatocyte was about 85%. At the culture time of 78 h, the cell matrices were carefully taken out from the hollow fibers for FDA/EB staining. Hepatocytes at an inoculated cell density of 1×10^6 cells/mL almost maintain the initial viability by cultivated in hollow fibers with MWCO of 100 ku (Figure 4A) and in microporous hollow fibers with pore size of 0.1 μm (pictures are not shown here), but all appeared dead in the hollow fibers with MWCO of 30 ku (Figure 4B). No hepatocytes were survived when entrapped into the hollow fibers for 78 h at a higher cell density of 5×10^6 cells/mL, as expected from our early observation in gel contraction at this cell density. Considering their multiplication, KB cells was inoculated at a low cell density of 0.4×10^6 cells/mL in hollow fibers with MWCO of either 100 or 30 ku here. The viability of KB cells was rather high in hollow fibers with MWCO of 100 ku (Figure 4C) and it can also be noticed that KB cells propagate well according to the more concentrated cell density than the initial one (the similar density as that in Figure 4D). In contrast to the primary hepatocytes, KB cells are more robust to survive and can propagate quickly even with a basic mammalian cell culture medium, so its viability is usually above 98% on the condition without critical environmental problems such as nutrition shortage and physiological chaos. Even so, KB cells did not survive in hollow fibers with MWCO of 30 ku (Figure 4D) and its cell density was almost the same as the initial one. Viability measurement showed that cells grew well in hollow fibers with MWCO of 100 ku which provides a better diffusion property than hollow fibers with MWCO of 30 ku.

As the hepatocyte viabilities were similar in hollow fibers with either MWCO of 100 ku or pore size of 0.1 μm at the cell density of 1×10^6 cells/mL, cell functions were examined to reflect the diffusion ability in such system. Culture media were changed between 48 and 72 h and media samples were taken every 12 h. The urea production was used a marker for the liver-specific function of hepatocytes. The accumulative urea production throughout the culture period was calculated and shown in Figure 5. It can be seen that hepatocytes produced more urea in the microporous hollow fibers than in the ultrafiltration hollow fibers. By performing linear fit on data points, average urea production were determined and summarized in Table 1. Hepatocytes entrapped inside the microporous hollow fibers exhibited higher urea production (31.2 pg/cell per h) and one-third lower of urea production inside the ultrafiltration hollow fibers.

Table 1 Average urea production of hepatocytes cultivated by gel entrapment within either microporous or ultrafiltration hollow fibers

Hollow fiber feature	Microporous hollow fiber	Ultrafiltration hollow fiber
Average urea production (pg/cell per h)	31.2	20.7

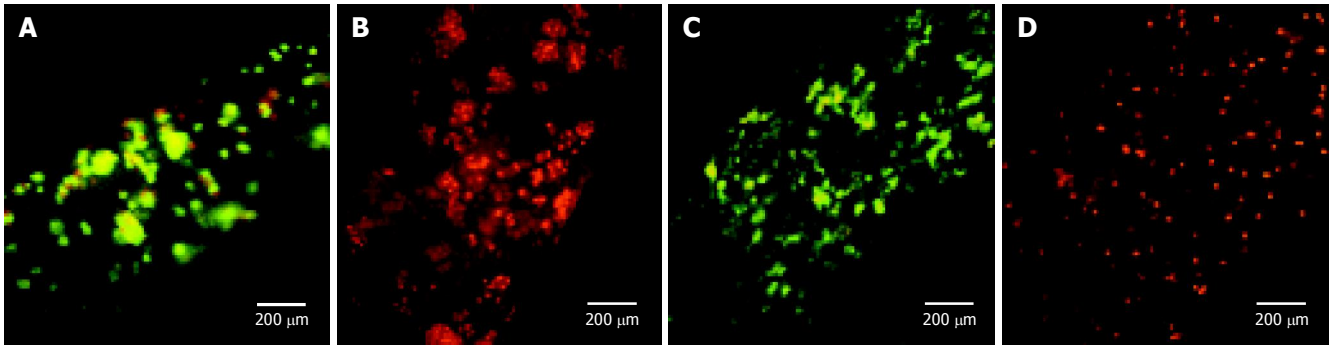

Figure 4 Fluorescent microscope images of rat hepatocytes entrapped for 78 h and KB cells entrapped for 72 h stained with FDA/EB. A: Hepatocytes entrapped in hollow fibers with MWCO of 100 ku; B: Hepatocytes entrapped in hollow fibers with MWCO of 30 ku; C: KB cells entrapped in hollow fibers with MWCO of 100 ku; D: KB cells entrapped in hollow fibers with MWCO of 30 ku.

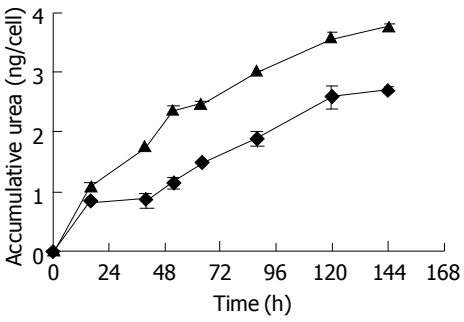

Figure 5 Accumulative urea production of hepatocytes by gel entrapment within hollow fibers featured by microporous membrane with pore size of 0.1 μm (triangle) and ultrafiltration membrane with MWCO of 100 ku (diamond). Average data from triplicate samples are shown. The error bars express standard deviation.

DISCUSSION

This paper discussed the possible diffusion barrier in the mammalian cell culture system featured by entrapment of cells into cell matrix in hollow fibers. We focused on two major mass transfers by diffusion in static culture. One was the diffusion across cell-matrix gel and the other was the diffusion across membranes. Because diffusion across the membranes has been well studied, the possible diffusion barrier caused by cell mediation was of great consideration in this paper. Also the integrated diffusion across both collagen gel and membranes was also evaluated through cell culture.

The cell-mediated collagen gel contraction and its effect on glucose diffusivities were first determined. Two types of mammalian cells, primary hepatocytes and continuous KB cells, were mixed with collagen gel and then incubated for a couple days to detect gel contraction. It was found that collagen contraction was caused not only by mediation of primary cells, as previously reported, but also by mediation of continuous cells, although the former was more significant. Such gel contraction was caused by the interaction between mammalian cells and cell matrix and could also serve as a crude estimate of cell viability. The measurement of the effective diffusion coefficients across cell-matrix gels reflected that the solute mobility by diffusion was inhibited more significantly by gel contraction than by the obstruction of entrapped cell mass. This is most possibly attributed to the densification of collagen gel. Thereby, it can be reached that the presence of cell masses together with cell-mediated gel contractions caused the remarkable diffusion resistance but the gel contraction was the major factor.

Cell viability measurement was then applied for evaluating diffusions in static culture of primary hepatocyte and continuous KB cells by collagen entrapment within hollow fibers. Hepatocytes maintained almost the initial viability in either microporous hollow fibers or ultrafiltration hollow fibers with higher MWCO of 100 ku but were all dead in ultrafiltration hollow fibers with lower MWCO of 30 ku. Similarly, KB cells presented quite high cell viability and even a well proliferation in hollow fibers with MWCO of 100 ku but did not survive in hollow fibers with MWCO of 30 ku. Based on the well studied mass transfer across membranes, the cell death in hollow fibers with lower MWCO is due to the exclusion of solutes with large molecular weight, for example, BSA with molecular weight of 67 ku. The shortage of such nutrients most possibly causes cell death.

Noticing that KB cells present much higher viability even at a high cell density due to propagation while hepatocytes cannot survive at the density of only 5×10^6 cells/mL, this could be contributed to the more roughly requirement of KB cells than primary hepatocyte, for example, primary cells usually need more nutrients for maintaining *in vitro* such as proteins or hormones. As the glucose effective diffusivity across gels decreases with cell density, it is understandable that hepatocyte viability also decreases with the inoculated cell density, or in another word, the limited mass transport by diffusion is prevalent inside collagen gels entrapped with high cell density of viable cells. Actually, in Nyberg's paper[30], around 50% of reduction on hepatocyte viability within one day culture was reported without further investigation where hepatocytes were statically cultivated in collagen noodles at a cell density of 4.4×10^6 cells/mL. This decrease in cell viability was quite unexpected in comparison with the high viability in his hollow fiber bioreactor running[31], indicating the possible limited diffusion of nutrients across gels in static culture. And it is worth mentioning that our lower viability in our hollow fiber culture at cell density of 5×10^6 cells/mL in comparison to their hepatocyte viability of 45%[30] could due to the extra membrane barrier and different medium composition and no daily medium changing.

As hepatocytes exhibit similar viabilities in hollow fibers featured respectively by large MWCO and microporous, liver-specific function on urea secretion was further examined to compare the effect of diffusion. The urea production was much higher in microporous hollow fibers than in the ultrafiltration hollow fibers with MWCO of 100 ku. This corresponded well with our previous results that higher liver-specific function was performed in bioartificial livers with microporous hollow membranes. Thus, by this experiment, it could be concluded that higher liver-specific functions were closely related to better diffusion. We did not discuss the possible diffusion resistance caused by a boundary layer or a stagnant film on surface of membranes or collagen gels, however, by considering the same materials (polysulfone and collagen gel) and the same configurations among all these hollow fiber systems, it is reasonable to exclude the effect of the diffusion in such boundary layer.

In summary, both cell-matrix gel and membrane compartment contribute to diffusion resistance in static culture of mammalian cells by gel entrapment within hollow fibers. Cell culture of continuous KB cell and primary hepatocyte confirm the cell-mediated gel contraction and the limited transportation of nutrients by diffusion.

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• *BASIC RESEARCH* •

Attenuation of graft ischemia-reperfusion injury by urinary trypsin inhibitor in mouse intestinal transplantation

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Abstract

AIM: Ischemia/reperfusion (I/R) injury is one of the major obstacles for intestinal transplantation (ITx). Urinary trypsin inhibitor (Ulinastatin, UTI) suppresses proteases and stabilizes lysosomal membranes. We supposed that Ulinastatin would diminish I/R injury of intestinal graft.

METHODS: UTI- treated group and untreated control group were investigated by histological assessment at 1.5, 4, 24, and 72 h after ITx. Myeloperoxidase (MPO) activity was used as the activity of neutrophils, and malondialdehyde (MDA) was used as an index of lipid peroxidation. TNFα and i-NOS mRNA expression in graft tissue were measured by semi-quantitative RT-PCR. $CD11b^+$ $Gr1^+$ cells in graft lamina propria were analyzed by flow cytometry.

RESULTS: Histological scores of the graft showed that the tissue injury was markedly attenuated by UTI treatment at different time points after ITx, with reduced MPO and MDA value in the grafts. The expression of TNFα and i-NOS mRNA was profoundly inhibited, while the infiltration of $CD11b^+$ $Gr1^+$ cells into the intestinal graft was decreased in UTI group.

CONCLUSION: Urinary trypsin inhibitor attenuates I/R injury in mouse intestinal transplantation by reducing monocytes infiltration and down-regulation of TNFα and i-NOS mRNA expression.

Key words: Ischemia/reperfusion injury; Ulinastatin; Intestinal transplantation

Yu JR, Yan S, Liu XS, Wu YJ, Fu PF, Wu LH, Zheng SS. Attenuation of graft ischemia-reperfusion injury by urinary trypsin inhibitor in mouse intestinal transplantation. *World J Gastroenterol* 2005; 11(11): 1605-1609
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INTRODUCTION

Intestinal transplantation (ITx) is the only definitive therapy for patients with irreversible intestinal failure who have developed life-threatening complications of total parenteral nutrition[1]. The outcome of clinical ITx, when compared to other solid organ transplants, is still far from acceptable[2]. Ischemia/reperfusion (I/R) injury has been one of the major obstacles, since the intestinal graft is exceedingly sensitive to ischemia caused by storage and implantation[3]. I/R injury subsequently leads to impairment of mucosal barrier and bacterial translocation, which provoke graft loss, fatal infection, and even multi-organ dysfunction in recipients. Therefore, more effective protocols for controlling graft I/R injury are demanded, in order to achieve a better outcome for this therapeutic approach.

Urinary trypsin inhibitor (Ulinastatin, UTI), a glycoprotein with a molecular weight of 67 000, is a protease inhibitor purified from human urine. It suppresses proteases such as trypsin, chymotrypsin and elastase, as well as stabilizes lysosomal membranes and thereby inhibits the release of lysosomal enzymes[4]. Ulinastatin has been found effective in relieving reperfusion injury in ischemic liver[5], intestine[6], and kidney[7]. In our knowledge, however, its effect on intestinal transplantation has not been reported. Distinct from other causes, the ischemic event in transplantation is inevitable, featured by a shorter warm ischemic and a longer cold ischemic period[3]. In our previous studies, we had found quick influx of monocytes, mainly neutrophils and macrophages, into intestinal graft even in syngeneic combination. I/R injury is relevant to the accumulation and eruption of these infiltrating monocytes. We supposed that the suppressive effects on neutrophil protease by Ulinastatin would diminish I/R injury of intestinal graft. In the present study, we examined the effects of Ulinastatin on I/R injury of intestinal grafts and the expression of TNFα and i-NOS mRNA in a mouse intestinal transplantation model.

MATERIALS AND METHODS

Animals

Balb/c ($H\text{-}2^d$) mice were purchased from *The Central Animal Facility of Medical School, Zhejiang University*, and were used at 8-12 wk of age. All animals were housed under specific

pathogen-free conditions with controlled light/dark cycles and free access to water and rodent chow. The investigations were performed in compliance with the policies of the animal care committee of the local government.

Heterotopic intestinal transplantation in mice

Mouse heterotopic intestinal transplantation was performed as described by Zhong *et al*[8] with minor modifications. Balb/c (H-2^d) mice were used as donors and recipients. Briefly, ITx was carried out under intraperitoneal anesthesia using ketamine (0.08 mg/g) and xylazine (0.012 mg/g). First, the donor jejunum and proximal part of the ileum were isolated with attached superior mesenteric artery and portal vein. After luminal irrigation and vascular perfusion, the graft was harvested and stored in 4 ℃ Ringer's solution until implantation. Graft portal vein and superior mesenteric artery were anastomosed to recipient's inferior vena cava and aorta in an end-to-side fashion. The distal end of the intestinal graft was connected to the host jejunum. The proximal graft lumen was exteriorized as a stoma. No antibiotics were administered perioperatively.

Experimental groups and peri-transplant therapy

Three experimental groups were established: Group 1, Sham operation (n = 6): Laparotomy and dissection of SMA and PV were performed without occlusion of these vessels in this group. Group 2, UTI-treated group (n = 24): UTI (50 000 U/kg, Techpool Bio-Pharma Co. Guangzhou, China) was given to the recipients via intravenous injection 5 min before revascularization of the intestinal graft. Thereafter, it was given at the same level of dose every 12 h after transplantation. Group 3, untreated controls (n = 24): Normal saline instead of UTI was given to the recipients. At 1.5, 4, 24, 72 h after transplantation, six mice of each group were killed and the grafts were harvested for histological grading, FACS analysis, and mRNA extraction.

Histological assessment

A 3-cm segment of the proximal portion of the graft was divided along its anti-mesenteric side and washed in PBS. The samples were fixed by formalin and embedded in paraffin by Swiss rolls with the luminal side facing outwards. Then, the rolled samples were cross-sectioned and stained with hemotoxylin-eosin. All microscopic slides were observed and assessed blindly with reference to the histological criteria. Briefly, specimens were scored as: (0) less than 50% of villous tips damaged, (1) more than 50% of villous tips damaged; (2) damage confined to distal 1/3 of villus; (3) damage confined to distal 2/3 of villus; (4) damage including all of villus, and (5) damage more proximal than villus.

Assay of myeloperoxidase (MPO) activity

MPO activity was assayed by spectrophotometrically measuring the H_2O_2 dependent oxidation of 3,3',5,5'-tetramethylbenzidine at 650 nm. All the reagents in the assay were afforded by a MPO kit, obtained from Jiancheng Bio-technology, Nanjing, PR China.

Assay of malondialdehyde (MDA)

The lipid peroxide products, malondialdehyde (MDA) was used as an index of lipid peroxidation and was expressed as nmol/g protein. It was determined by TBA assay, using a MDA kit obtained from Jiancheng Bio-technology, Nanjing, PR China.

Measurement of TNFα and i-NOS mRNA by semiquantitative RT-PCR

Graft RNA was extracted by Trizol kit from each 50-100 mg snap-frozen samples. The concentration and A_{260}/A_{280} (>1.8) was determined by ultraviolet photospectrometry. Total RNA (4 μg) was reverse-transcribed to c-DNA and expanded by PCR. The primers of TNFα, i-NOS and β-actin are as follows:

TNFα:	Primer 1:	5'-AGCCCACGTAGCAAACCACCAA-3'
	Primer 2:	5'-ACACCCATTCCCTTCACAGAGC AAT-3'
i-NOS:	Primer 1:	5'-ACCCCTGTCTTCCACCAGGAGTTGAA-3'
	Primer 2:	5'-TGCAGCCATGACCTTTCGCATTAGCATGG-3'
β-actin:	Primer 1:	5'-TGGAATCCTGTGGCATCCATGAAAC-3'
	Primer 2:	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

The expanded products were analyzed by 1.5% agarose gel electrophoresis and imaged by Kodak digital imaging system. The relative value of mRNA was determined by the ratio between the A of target gene and β-actin.

Flow cytometry

Three-color flow cytometry was conducted on isolated cells from the lamina propria of the intestinal grafts. Isolated cells from each preparation were centrifuged at 2 000 r/min for 3 min, then washed twice in FACS buffer (PBS+1% FCS+5% normal rat serum) and incubated with primary mAbs for 20 min at 4 ℃. The specimens incubated with biotinylated mAbs were subsequently developed with streptavidin PercP. After washing twice, all samples were analyzed on a FACScan® cytometer (Becton Dickinson). 10 000 to 20 000 events were collected from each sample. Data analysis was performed using WinMDI 2.8 software.

Statistical analysis

Histology score, and MPO, MDA, i-NOS/β-actin,TNFα/β-actin value, were calculated for each group. Student's t- test was used to determine the significance of differences in means. The non-parametric Mann-Whitney U-test was used to compare the medians of groups that did not follow a normal distribution according to the Shapiro-Witt test. A P-value of less than 0.05 was considered significant. SPSS 12.0 and Microsoft Excel software were used for the statistical analyses.

RESULTS

Histological finding of the intestinal grafts

The histological scores of the intestinal grafts in both control and UTI groups were shown in Figure 1 (A-D). At different time points after transplantation, the histological impairments in UTI group were apparently attenuated compared to the control group. At the early stage after reperfusion (1.5, 4, and 24 h after transplantation), the mucosal damage in the UTI group is confined to villous tips, obviously slighter than the control group, in which the damage developed in total villi. The histological changes nearly disappeared after 72 h

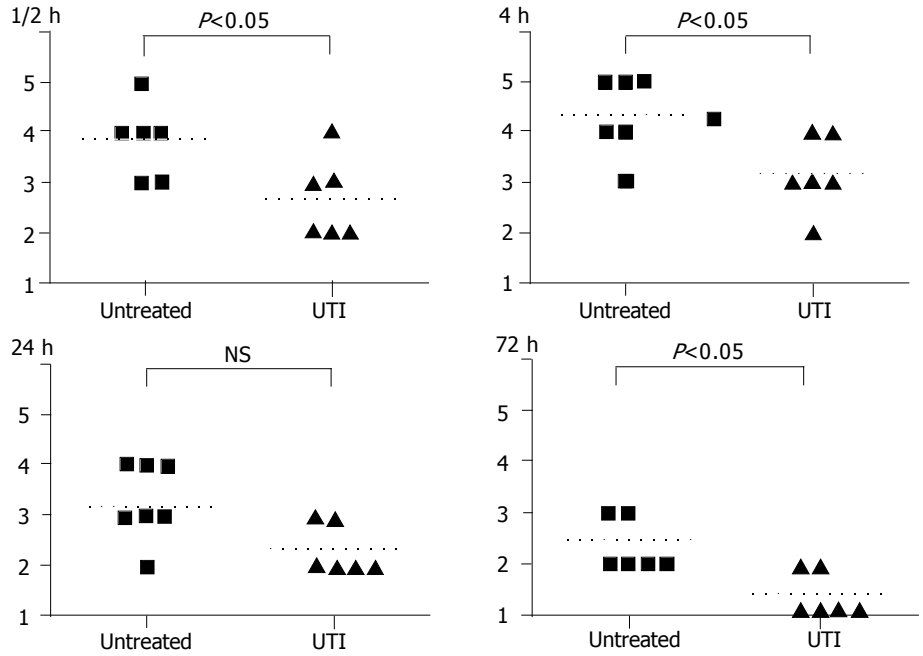

Figure 1 At 1.5, 4, 24, and 72 h after intestinal transplantation, the histological scores of tissue injury in the UTI-treated group were apparently less than the untreated group.

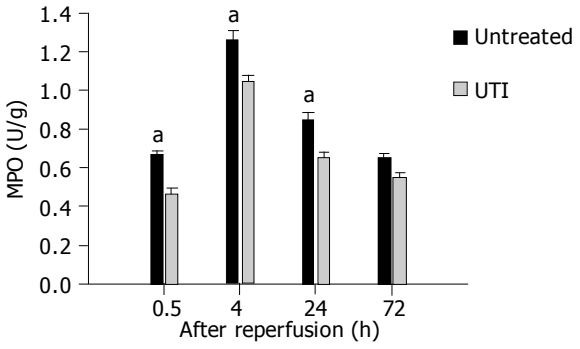

Figure 2 MPO activity in the intestinal grafts in the UTI-treated group remained at a lower level when compared with the control group in the earlier time points after IR (1.5, 4, and 24 h after transplantation respectively, [a]*P*<0.05 *vs* control group).

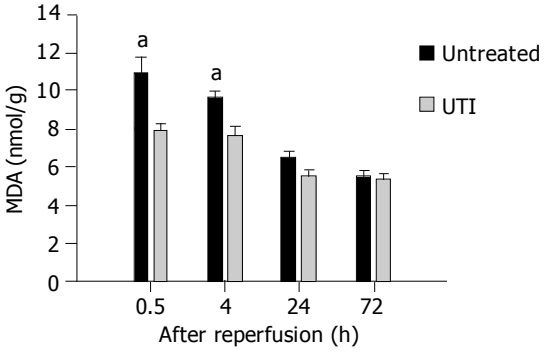

Figure 3 The MDA level at 1.5 and 4 h after transplantation was significantly suppressed in UTI-treated group, [a]*P*<0.05 *vs* the untreated control group.

in the UTI group, whereas villous impairments remained in the distal part of the villus in the control group. Moderate congestion and marked edema of the intestinal graft at early time point were found in control groups. In the UTI group, however, no congestion was found and the tissue edema was slighter. Comparing with the control group, the infiltration of monocytes was also reduced in UTI group at each time point after transplantation by light microscopic observation.

Myeloperoxidase (MPO) activity

As shown in Figure 2, the MPO activity of the intestinal graft tissue in the UTI group remained at a lower level when compared to the control group in the earlier time points after I/R (0.46±0.23 *vs* 0.63±0.17, 1.05±0.12 *vs* 1.25±0.15, 0.65±0.13 *vs* 0.85±0.18 at 1.5, 4, and 24 h after transplantation respectively, U/g, $n = 6$ for each group, $P<0.05$). At 24 h after transplantation, the MPO activity in both UTI and control groups were reverted to 0.69±0.14 U/g and 0.85±0.15 U/g, no statistical difference were found in this time point.

Malondialdehye (MDA) level

As shown in Figure 3, the lipid peroxidation derived MDA level at 1.5 and 4 h after transplantation was significantly suppressed by UTI treatment (7.93±0.32 *vs* 10.97±0.77, 7.70±0.44 *vs* 9.70±0.77 mmol/g, $n = 6$, $P<0.05$). At the following later time points (24 and 72 h after transplantation), however, the graft MDA in both groups reverted to a similarly lower level.

Graft infiltration by CD11b$^+$ Gr1$^+$ monocytes

As shown in Figure 4, when the monocyte gate of graft lamina propria was analyzed at the third post-transplant day, the sub-population of CD11b$^+$ Gr1$^+$ cells were found reduced in the UTI-treated group. The percentage of CD11b$^+$ Gr1$^+$ cells, mainly neutrophils and macrophages, in untreated allografts was 10.3±0.7%, and was decreased to 1.0±0.2% in the UTI-treated group ($P<0.01$). It indicated less inflammatory infiltrations, particularly neutrophils and macrophages, into the intestinal graft by UTI therapy.

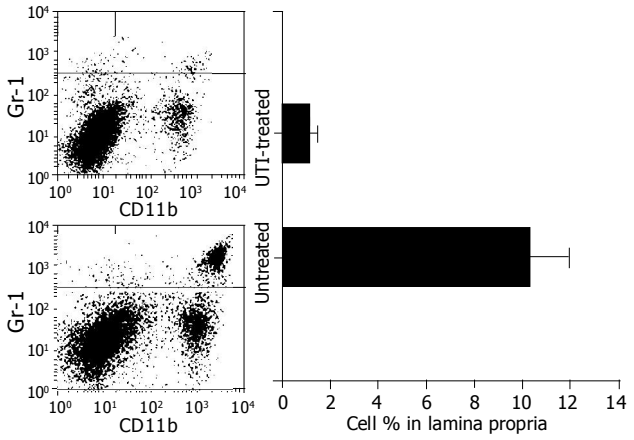

Figure 4 The sub-population of CD11b$^+$ Gr1$^+$ cells in the monocyte gate of graft lamina propria was found reduced in the UTI-treated group on the third post-transplant day.

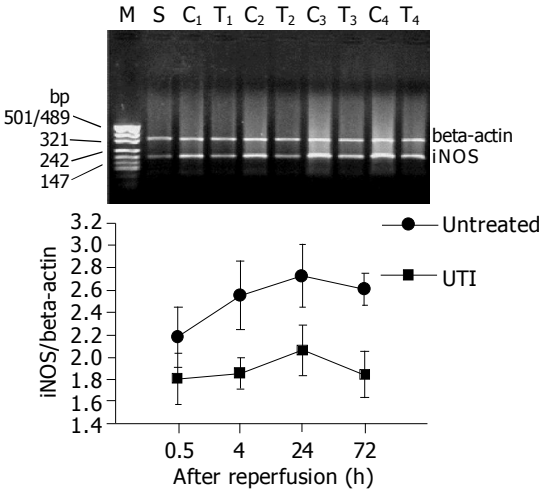

Figure 5 In UTI group, the expression of i-NOS mRNA was apparently inhibited at 1.5, 4, 24, and 72 h after intestinal transplantation. The upper bands show the representative images of the PT-PCR results for i-NOS. M: band for molecular marker; S: Sham operation group; C1-C4: bands for untreated group at 1.5, 4, 24 and 72 h after transplantation respectively; T1-T4: bands for UTI-treated group at 1.5, 4, 24 and 72 h after transplantation, respectively.

Expression of i-NOS, TNFα mRNA in intestinal grafts

In the sham-operated mice, the expression of i-NOS and TNFα mRNA in non-ischemic intestine is minor. However, the expression of the intestinal graft elevated dramatically due to I/R events after transplantation. The expression of i-NOS mRNA reached 2.18±0.28 to 2.73±0.28 (TNFα/β-actin, $n = 6$), and the TNFα mRNA were 1.71±0.24 to 2.67±0.71 (i-NOS/β-actin, $n = 6$) within the first 72 h after transplantation. However, in UTI group, the expression of i-NOS mRNA was apparently inhibited in each time point, and the level of TNFα mRNA was also lower than the control group at 1.5 and 4 h after transplantation. It indicated a profound suppression of i-NOS and TNFα expression by UTI treatment, when challenged by transplant-associated I/R. Figures 5, 6 displayed i-NOS and TNFα mRNA expression at different time points after transplantation and representative images.

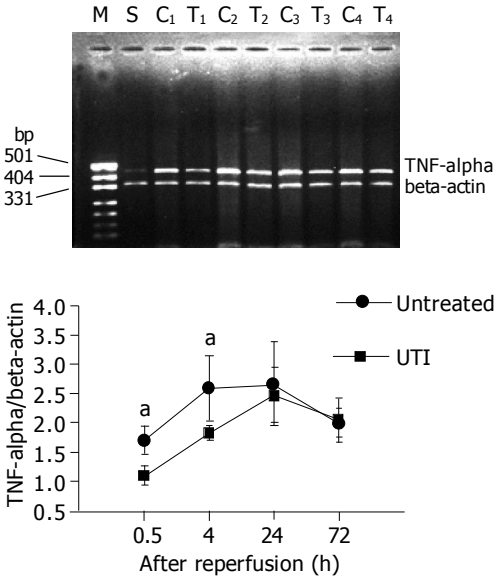

Figure 6 The expression of TNFα mRNA was inhibited at 1.5 and 4 h after intestinal transplantation in UTI-treated group, while no difference was found at 24 and 72 h after transplantation. The upper bands show the representative images of the PT-PCR results for TNFα. M: band for molecular marker; S: Sham operation group; C1-C4: bands for untreated group at 1.5, 4, 24 and 72 h after transplantation respectively; T1-T4: bands for UTI-treated group at 1.5, 4, 24 and 72 h after transplantation, respectively.

DISCUSSION

Intestine is one of the most vulnerable organs to I/R injury in solid organ transplantation[3]. The high morbidity and mortality after intestinal transplantation are unexceptionally associated to graft impairments due to I/R injury. Therefore, effective measures with minimal risk of evoking allogeneic rejection should be developed for mitigating the intestinal impairments caused by I/R response[9].

The most common model for investigations of intestinal I/R is performed by temporary occlusion of superior mesenteric artery (SMA)[10]. Easy as it is, however, it is unable to feature the I/R events occurring in intestinal transplantation. First, I/R injury, in transplantation, is mainly due to a longer cold ischemic preservation, while the SMA occlusion model can merely mimic a shorter warm ischemia. Second, collateral vascular supply cannot be excluded by SMA occlusion, which usually appears as sub-mucosal hemorrhage that is absent in transplantation. Other factors affecting the investigating outcomes, such as irrigation of the graft lumen during organ procurement, cannot be imitated by SMA occlusion either[3]. Thus, we took the more complicated mouse transplantation model to estimate the effect of UTI, in order to avoid possible errors.

Constitutive NO produced by c-NOS is a well-known mediator in several physiological processes. When insulted by inflammatory stimuli such as ischemia, i-NOS expression is promoted and a larger amount of NO is produced by i-NOS catalysis. Instead of being a signal transductor in lower levels, the highly concentrated NO acts as free radicals and eventually leads to local tissue damage[11]. Therefore, over expression of i-NOS is the key point for the NO mediated inflammatory reaction. TNFα is another crucial mediator in the onset and sustaining of the I/R response in the gut[12].

The present study reveals a profound inhibitive effect of UIT toward the expression of i-NOS mRNA in intestinal graft, which was challenged by long-term cold ischemia and followed reperfusion. The decreased levels of i-NOS mRNA and TNFα mRNA in the grafts are well consistent with the attenuated villous injury, as well as the reduced neutrophil index (MPO) and improved membrane stability (MDA). It suggested that the inhibition of TNFα and i-NOS, thereby, breaking down the cytokine cascade and the NO-induced tissue injury could be the underlying mechanism of the protective effects of UTI in intestinal transplantation.

Macrophages and other monocytes are the major source of TNFα and i-NOS mRNA,[13] therefore we isolated these infiltrated cells from the intestinal grafts and measured them by flow cytometry. Gr1 is usually expressed on granulocytes and macrophages, and CD11b has the similar tendency while it also appears on activated and mature lymphocytes[14]. The subpopulation, $CD11b^+$ $Gr1^+$ cells, including most neutrophils and macrophages, were found markedly reduced in UTI-treated grafts when compared to untreated grafts at the third post-transplant day. Hence, the lower level of TNFα and i-NOS mRNA in UIT-treated group might owe to reduced infiltration of macrophages and neutrophils. On the other hand, the suppressive effect of UTI on TNFα and i-NOS expression, if there is any, may lessen the severity of graft infiltration by abating the cytokine or chemotactic response. Shortly, prevention of macrophages and other monocytes into the graft by UTI is a possible mechanism for its alleviating effects on I/R injury.

As a safe and well-tolerated agent, UTI has not found any rejection-related adverse effects in clinical transplantation[15]. The revealed mechanism of UTI as well as our findings, such as inhibition of TNFα and i-NOS production, theoretically excludes the potential risk of provoking alloimmune response[16]. Consequently, UTI could be an ideal agent in protection of intestinal graft from IR injury. Further investigations for UTI in other solid organ transplants and clinical trials are required.

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• *BASIC RESEARCH* •

Analysis of the mechanisms of rabbit's brainstem hemorrhage complicated with irritable changes in the alvine mucous membrane

Xue-Long Jin, Yang Zheng, Hai-Ming Shen, Wen-Li Jing, Zhao-Qiang Zhang, Jian-Zhong Huang, Qing-Lin Tan

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Abstract

AIM: To explore the dynamic changes in the pressure of the lateral ventricle during acute brainstem hemorrhage and the changes of neural discharge of vagus nerve under the load of intracranial hypertension, so as to analyze their effects on the congestive degree of intestinal mucous membrane and the morphologic changes of intestinal mucous membrane.

METHODS: An operation was made to open the skull to obtain an acute brainstem hemorrhage animal model. Microcirculatory microscope photography device and video recording system were used to determine the changes continuously in the caliber of jejunal mesenteric artery during brainstem hemorrhage and the changes with time in the congestion of jejunal mucosal villi. We used HE stain morphology to analyze the changes of duodenal mucosal villi. A recording electrode was used to calculate and measure the electric discharge activities of cervical vagus nerve.

RESULTS: (1) We observed that the pressure of lateral cerebral ventricle increased transiently during acute brainstem hemorrhage; (2) The caliber of the jejunal mesenteric artery increased during brainstem hemorrhage. Analysis of red color coordinate values indicated transient increase in the congestion of jejunal mucous membrane during acute brainstem hemorrhage; (3) Through the analysis of the pathologic slice, we found enlarged blood vessels, stagnant blood, and transudatory red blood cells in the duodenal submucous layer; (4) Electric discharge of vagus nerve increased and sporadic hemorrhage spots occurred in duodenal mucous and submucous layer, when the lateral ventricle was under pressure.

CONCLUSION: Brainstem hemorrhage could cause intracranial hypertension, which would increase the neural discharge of vagus nerve and cause the transient congestion of jejunal mucous membrane. It could cause hyperemia and diffused hemorrhage in the duodenal submucous layer 48 h after brainstem hemorrhage.

Key words: Brainstem hemorrhage; Irritable changes; Alvine mucous membrane

Jin XL, Zheng Y, Shen HM, Jing WL, Zhang ZQ, Huang JZ, Tan QL. Analysis of the mechanisms of rabbit's brainstem hemorrhage complicated with irritable changes in the alvine mucous membrane. *World J Gastroenterol* 2005; 11(11): 1610-1615
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INTRODUCTION

The normal function of gastric juice is to digest food, but it can cause ulcer through digesting the gastric and duodenal mucosa. Gastric juice contains hydrochloric acid, which is a strong acid with pH1.0. Hydrochloric acid can prevent bacteria from breeding in stomach. The gastric mucosa is covered with the mucus of gastric juice, which can prevent the gastric mucosa from being digested. If the gastric mucosa was directly digested by gastric juice, the mucosa can be injured. Under stress, the discharge of sympathetic nerve and parasympathetic nerve will change, which will cause changes in gastrointestinal motility, the secretion of gastric juice, the mucosa blood stream, all of which are reasons that cause the stress ulcer[1,2]. Usually, the meaning of stress is emotional, such as agitation, anger, *etc.*, under the irritable environmental conditions breakthrough. They evoke the changes of the neural discharge of autonomic nerve[3]. The stress conditions in our experiment is brainstem hemorrhage[4-6]. The recovery of brainstem hemorrhage in different part and extent has significant variation, which is determined by the complex anatomic structure of brainstem[2,7-10]. The reticular formations of brainstem have many neurons, which are related to visceral activities. It was reported recently that the injury of the brainstem could cause a high incidence rate of stomach mucosa hemorrhage[11,12]. Some literatures also reported that the proportion of midbrain hemorrhage in brainstem hemorrhage was 28%, which is higher than 10 years ago[13]. The recovery of brainstem hemorrhage is

related not only with the hematoma, but also with the body conditions and complications of the patient[14]. Fatality rate is high in a man who has the complications of stress digestive tract ulcer with hemorrhages. The pathogenesis of digestive tract stress ulcer bleeding, induced by brainstem hemorrhage, was thought as the nerve and body fluid factors acting on the gastric mucosa[4]. The changes of vagus nerve discharge during acute brainstem hemorrhage are not clear. The aim of this research was to probe into the influence of brainstem hemorrhage on the intestinal mucous, the microcirculation of the mesentery, the regularity of the electric discharge of vagus nerve with the changes of intracranial pressure under brainstem hemorrhage at midbrain level, in order to guide the early diagnosis and treatment of brainstem hemorrhage complicated with digestive tract stress ulcer bleeding[12,15-19].

MATERIALS AND METHODS

Fifty male Japanese white rabbits with long ears (weighing 1.5-1.8 kg) were employed. Thirty rabbits of one group were used to observe the changes in the pressure of lateral cerebral ventricle and the blood stream of the duodenal mucosa and the jejunal mucosa, under the acute brainstem hemorrhage. The other 20 rabbits were used to analyze the changes of neural discharge of vagus nerves under the intracranial hypertension (36 mmHg, 1 min). Urethane 25% was used for intravenous anesthesia[20]. An operation was made to open the skull at interparietal bone and 0.1 mL blood of itself was injected into its brainstem to make the acute brainstem hemorrhage animal model at the level of inferior colliculus[21]. The hematoma was confirmed to compress the aqueduct of midbrain through the general dissection. Right lateral cerebral ventricle intubation was employed to calculate and measure the dynamic pressure, before and after the load of acute brainstem hemorrhage. We used the recording electrode to calculate and measure the changes of neural discharge of cervical vagus nerve under the intracranial hypertension (36 mmHg, 1 min). We used the software of the device, manufactured in Chengdu device factory, RM6204B organism signal acquisition processing system to measure the absolute value of an area of the nerve discharge activities wave[22]. Through the absolute value of an area of the wave, we could analyze the intensity of the nerve electric activities. By setting a single liminal value line to analyze the frequency of wave in each time segment, we could analyze the electric activities' intensity indirectly. Microcirculatory microscope photography device and video recording system were used to determine the changes continuously in the caliber of jejunal mesenteric mini-artery and the changes with time in the congestion of jejunal mucosal and duodenal mucosa during brainstem hemorrhage[11] (microcirculatory image process system, made in Optic Instrument General Factory of Xuzhou). The duodenal biopsies of normal and 12, 24, 48, 72 h after brainstem hemorrhage were taken, and fixed with 20% formalin, HE stained. We observed the morphologic changes of mucous layer and submucous layer under light microscope and oil immersion objective (magnification ×1 500).

We used paired *t*-test for statistical method-dependent samples. $P<0.05$ was considered significant.

RESULTS

(1) We observed a stationary baseline wave (about 9 mmHg) through lateral ventricle intubation, and intracranial pressure increased rapidly after acute brainstem hemorrhage animal model was made, which recovered subsequently, but the pressure level was still higher than it was loaded before (Figure 1); (2) The caliber of jejunal mesenteric artery increased after the load of brainstem hemorrhage. Statistical analysis showed a significant difference between pre-loaded and loaded brainstem hemorrhage (Figure 2); we also observed that the occurrence time sequence of mesenteric artery caliber dilation had individual difference. We analyzed the congestion of jejunal mucosa in acute brainstem hemorrhage by red color coordinate values, then we observed that the congestion increased transiently during 30 s after the load of hemorrhage (Figures 3A-D). The analysis of paired *t*-test for dependent samples showed that the increasing tendency was significant (Figure 4), and the time sequence of the phenomenon above had the individual difference; (3) We analyzed the intensity of the nerve electric activities through the absolute value of an area of the wave. We also analyzed the discharge activities' intensity indirectly by setting a single liminal value line to calculate the frequency of dischargeable wave in each time segment (Figures 5A-C). We observed that the neural discharge of vagus nerve increased after intracranial hypertension; (4) Comparing the pathologic slice of duodenal mucous membrane between

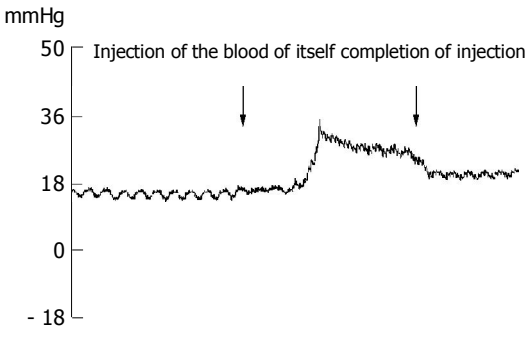

Figure 1 Changes in the pressure of the lateral ventricle during acute brainstem hemorrhage in rabbit.

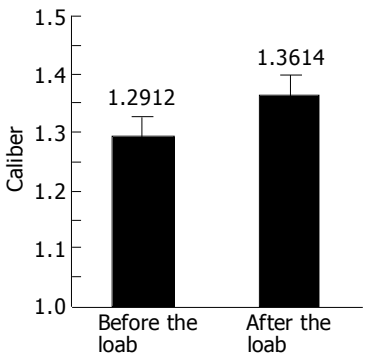

Figure 2 Changes in caliber of jejunal mesentery small vessels under the load of rabbit's brainstem hemorrhage.

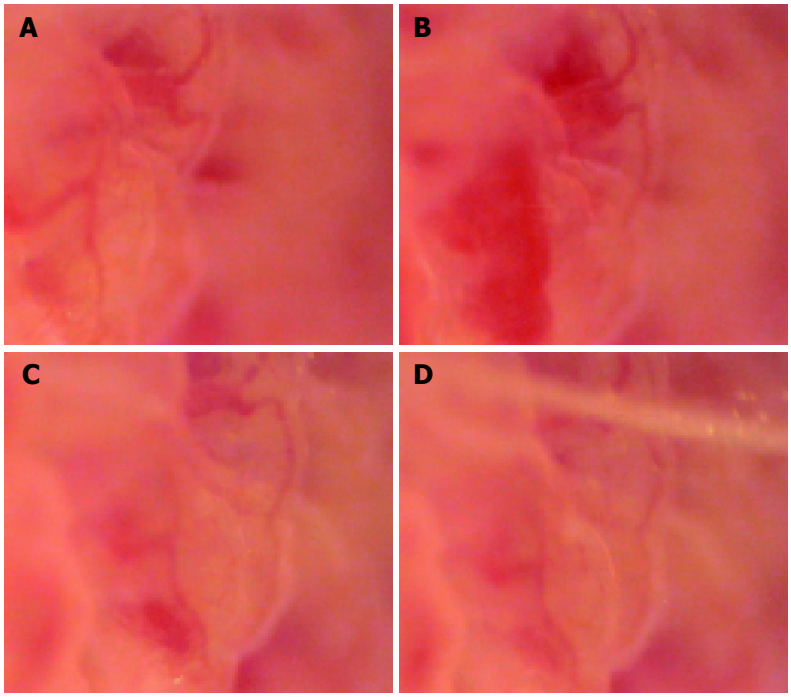

Figure 3 Changes of microcirculation of jejunal mucosa. A: Photograph of microcirculation of jejunal mucous membrane, 6 s before the load of rabbit's brainstem hemorrhage (×185); B: Photograph of jejunal mucous membrane, 10 min 26 s after the load of rabbit's brainstem hemorrhage; it showed the local congestion of villus mucosa (×185); C: Photograph of jejunal mucous membrane, 30 min 32 s after the load of rabbit's brainstem hemorrhage; it showed that the local congestion of villus mucosa had relieved (×185); D: Photograph of jejunal mucous membrane, 40 min 46 s after the load of rabbit's brainstem hemorrhage; it showed that the local congestion of villus mucosa had ameliorated obviously (×185).

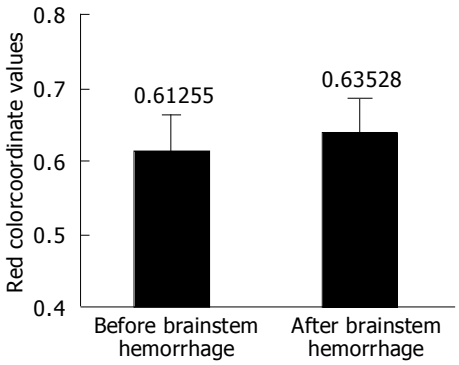

Figure 4 Paired *t*-test for red color coordinate mean values of jejunal mucous membrane before and after rabbit's brainstem hemorrhage.

normal and 48 h after brainstem hemorrhage, we observed that the duodenal submucous layer in normal rabbits did not have dilated minute blood vessel and red blood cells leaked out from capillary lumens to tissue space basically (Figure 6A, B). Analyzing rabbit's duodenum pathological slices that are 48 h after brainstem hemorrhage, we found that capillaries dilated in duodenal villi and duodenal glands, red blood cells increased in lumens and leaked out from capillary lumens to tissue space in local submucous layer (Figures 6C, D).

DISCUSSION

Duodenal wall is usually divided into four layers structurally, which are mucous layer, submucous layer, muscular layer, and adventitia. Mucous layer and submucous layer protrude into the intestinal lumens to form the plica. Many duodenal glands can be seen in the connective tissue of the submucous layer. Muscular layer consists of two layers of smooth muscles, inner-ring and outer-vertical. Adventitia is the plasma membrane. At present, it is considered that the digestive tract ulcer is caused by the imbalance between the attacking factors (gastric juice) that has the function of digestion and the defense factors (mucus, mucosa, blood stream of mucosa) that prevent the stomach and duodenum from being digested[15,23-32]. In recent years, some experiments emphasize that the bloodstream of duodenal mucosa and the buffer capacity of mucosa endothelial cell are important factors that protect mucosa cell from being injured; experimental study of the stress ulcer shows that the nutrition of gastrointestinal tract (such as glucose) can prevent the stress ulcer[19]. The excessive breeding of the bacterium and infection can influence the bloodstream of mucosa, and weaken the gastric mucosal protective barrier function[29]. The balance can be broken by the stimulation from brain through the action of the autonomic nerve[22,33]. This type of ulcer is not only a digestive disease, but also the result that was comprehensively effected by multiple factors. The occurrence is related to the disturbance of body neuroendocrine, the weakness of gastric mucosal protective barrier function and the augment of gastric mucosa injury factors[27,33,34]. It was reported that the integrity of midbrain structure can influence the hemodynamics during the hemorrhage, according to the result of empirical studying in recent years. In this experiment we investigated the upper gastrointestinal stress ulcer bleeding, with the animal model of hemorrhage in

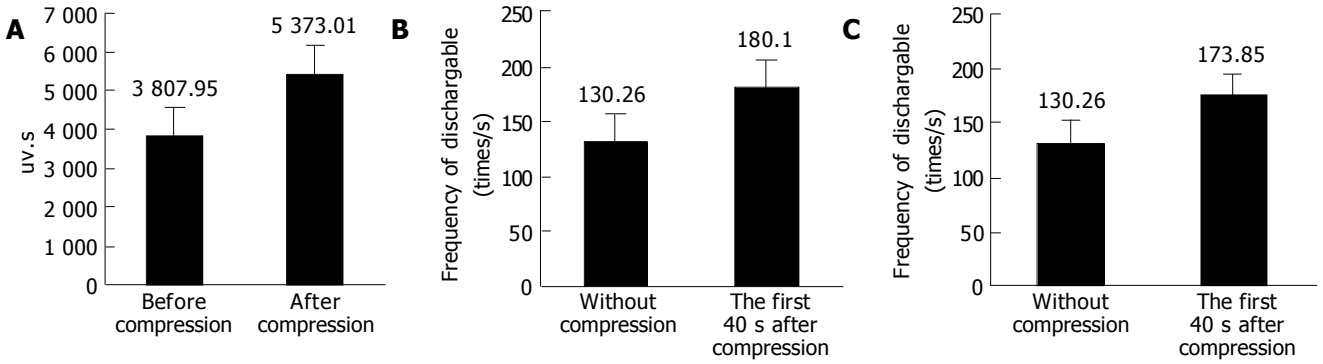

Figure 5 Comparison of electric discharge of vagus nerve. A: The comparison of the electric discharge of vagus nerve through analyzing the absolute value of an area of wave form before and after the compression (from 4 to 40 s under the intracranial hypertension), and the neural discharge of vagus nerve had increased after intracranial hypertension, $n = 20$, $P<0.05$; B: The comparison of the electric discharge of vagus nerve before and during the first 4 s after the rabbit's lateral ventricle compression. We analyzed the electric discharge frequency of the dischargeable wave in each time segment by setting a single liminal value line, and the neural discharge of vagus nerve had increased after intracranial hypertension; C: The comparison of the electric discharge of vagus nerve before the compression and during continuous compression (from 4 to 40 s). We analyzed the electric discharge frequency of the dischargeable wave in each time segment by setting a single liminal value line, and the neural discharge of vagus nerve had increased after intracranial hypertension.

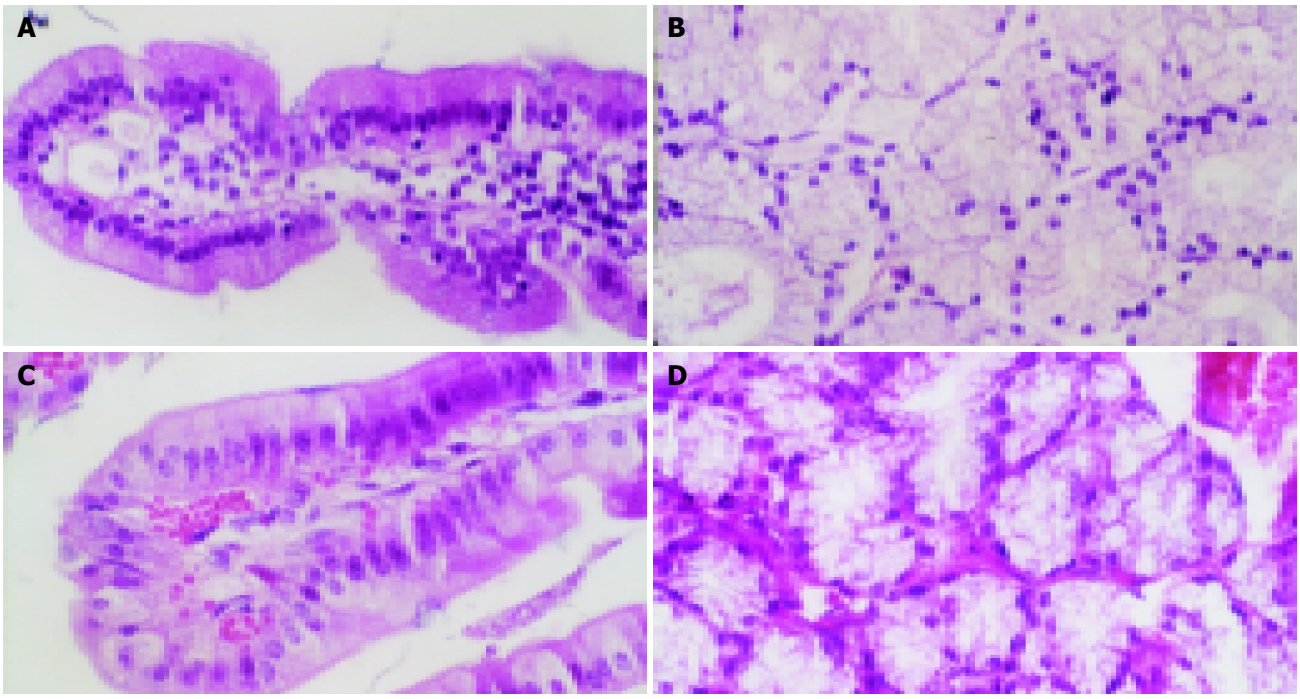

Figure 6 HE stain of duodenal structure of rabbits. A: HE stain of normal rabbit's duodenal villus. The minute blood vessel had not dilated and red blood cells had not leaked out from capillary lumens; B: HE stain of normal rabbit's mucous glands of duodenum. The minute blood vessel had not dilated; C: HE stain of the rabbit's duodenal villus, which were 48 h after brainstem hemorrhage. The capillaries had obviously dilated and red blood cells had leaked out from capillary lumens; D: HE stain of the rabbit's mucous glands of duodenum, which were 48 h after brainstem hemorrhage. The capillaries had obviously dilated and red blood cells had leaked out from capillary lumens.

inferior colliculus (midbrain), and found that it could influence the changes of bloodstream of the upper gastrointestinal mucosa[11]. After the primary and secondary lesion in brainstem, on one hand, the activities of sympathoadrenal system reinforced, the secretion of catecholamine *in vivo* had increased, the blood vessels in gastric mucosa contracted intensely, which caused the injury of the gastric mucosa; on the other hand, it stimulated the vagus nerve, which caused the increased excretion of gastric acid and pepsin, thus it aggravated the injury of gastric mucosal barrier. In the empirical study of observing and measuring the changes of bloodstream of gastroduodenal mucosa of shigellosis patients' biopsies by fluorescence microscopy, some researchers found that there were microcirculatory changes of leukocyte movement and platelet aggregation in mucosal blood vessels, and the injury of gastric mucosa was related to the movement of leukocyte and platelet activation[30,31]. The proportion of midbrain hemorrhage in the brainstem hemorrhage has increased in recent years. In our experiment, we observed that the baseline of intracranial pressure wave increased in the acute brainstem hemorrhage. We thought that it was caused by the local high intracranial pressure,

the occupation of hematoma in the brainstem at the level of inferior colliculus, and cerebrospinal fluid circulation disorder through hematoma compressing in aqueduct of midbrain. We also observed the reduction of the duodenal mucous membrane bloodstream, the dilatation of jejunal mesentery arterioles, and the transient increase in the congestion of jejunal mucous membrane in the early stage of acute brainstem hemorrhage model. The changes of bloodstream distribution were probably related to the changes of activities of sympathetic nerve and parasympathetic nerve[21]. It was indicated in a recent research that the decrease of gastroduodenal mucosal bloodstream, which is one of the important defense factors of mucosa, is related to the lack of NO synthetase and endothelium-derived relaxing factor in mucosal vascular endothelial cell[27]. The vagus nerve of rabbits initiates from the dorsal nucleus of vagus, its truncus goes into pectoral cavity and abdominal cavity through cervical part; it has branches which distribute to stomach and intestines. We calculated and measured the electric discharge activities of cervical vagus nerve continuously. It showed that the electric discharge of vagus nerve increased after intracranial hypertension. The average electric discharge intensity of vagus nerve increased rapidly during the first 4 s after the beginning of compression ($P<0.002$). Compared with the level before the compression of lateral ventricle, it also had significant increase during the 40 s followed ($P<0.002$). It implies that the factor of intracranial pressure should be considered in the mechanism of brainstem hemorrhage combined with the changes of digestive tract bloodstream. The intracranial hypertension could excite the vagus nerve to increase its electric discharge and influence the bloodstream of duodenum, jejunal mesentery and mucosa. Pathological slices, 48 h after acute brainstem hemorrhage, were analyzed and showed that there was a significant congestion of blood capillary in the duodenal villi and duodenal glands; some red blood cells leaked out into tissue space of submucous layer. These pathological changes are possibly the pathological basis of upper gastrointestinal hemorrhage caused by the stress ulcer in the condition of brainstem hemorrhage. It was reported that the weak stimulation of vagus nerve can cause the inhibition of intestinal movement; it was also reported that the vagotomy in the gastric mucosa can cause the reduction of gastric acid secretion. In this experiment, we found that the activity of vagus nerve has increased after the brainstem hemorrhage, and so we consider that the secretion of gastric acid should increase. We observed that the bloodstream of duodenal mucosa has decreased 20 s after the brainstem hemorrhage, and the minute blood vessel of duodenal mucosa, submucosa has obviously dilated and congested after the brainstem hemorrhage, for example, at 48 h after the brainstem hemorrhage. The stress ulcer with brainstem hemorrhage may relate to the activity of autonomic nerve and the blood stream of mucosa.

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• *CLINICAL RESEARCH* •

A community-based epidemiological study of elevated serum alanine aminotransferase levels in Kinmen, Taiwan

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Abstract

AIM: To explore any gender-related differences in prevalence of and condition-associated factors related to an elevated serum alanine aminotransferase (ALT) level amongst residents of Kinmen, Taiwan.

METHODS: A total of 11 898 of a potential 20 112 regional residents aged 30 years or more completed a related questionnaire that was carried out by the Yang-Ming Crusade between 1991 and 1994 inclusively, with blood samples being collected by public nurses. The overall questionnaire response rate was 59.3% (52.4% for males and 66.0% for females).

RESULTS: The prevalence of an elevated serum ALT level for this sub-population was found to be 7.2%, the prevalence revealing a statistically significant decrease with increasing population age ($P<0.0001$). Males exhibited a greater prevalence of elevated serum ALT level than did females (9.4% *vs* 5.3%, $P<0.0001$). Using multiple logistic regression analysis, in addition to male gender, a younger age, greater waist circumference, presence of type-2 diabetes and hyperuricemia were the significant factors associated with an elevated serum ALT level for both males and females. Gender-related differences as regards associated factors were also revealed. For males, obesity was significantly related to an elevated serum ALT level (OR = 1.28, 95%CI: 1.00-1.66) but this was not so for females (OR = 1.09, 95%CI: 0.84-1.42). Hypertriglyceridemia (OR = 1.80, 95%CI: 1.36-2.39) and hyperuricemia (OR = 1.61, 95%CI: 1.03-2.52) were significantly related to elevated serum ALT levels only for females.

CONCLUSION: Several gender-related differences were noted pertaining to the prevalence of and relationship between obesity, hypertriglyceridemia and hyperuricemia and elevated serum ALT level in the present study.

Key words: Alanine aminotransferase; Prevalence; Community-based study; Gender difference

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INTRODUCTION

Alanine aminotransferase (ALT) has, for some time, been viewed as a sensitive indicator of liver-cell injury[1]. Currently, the determination of serum ALT level constitutes the most frequently applied test for the identification of patients suffering from liver disease, this parameter also acting as a surrogate marker for disease severity and/or as an index of hepatic activity[2]. This cytosolic enzyme is able to be detected in many organs and is able to catalyze the transfer of the α-amino group from alanine to α-ketoglutaric acid[3]. The current understanding is that elevated serum ALT levels are associated with gender, age, obesity, waist-to-hip ratio, serum glucose concentration, serum triglyceride level, use of certain medication and history of viral hepatitis infection[4-6]. The early detection of this disorder by screening, followed by appropriate intervention, may offer a practical way for the prevention of condition-associated hepatocellular damage.

From the viewpoint of preventive medicine, it is not only important to be cognizant of the background prevalence of elevated serum ALT levels regionally, but also to explore the complete spectrum of demographic and biological markers which may be related to elevated serum ALT levels. Further, to the best of our knowledge, some uncertainty still exists as regards whether the prevalence of and the associated risk factors for an elevated serum ALT level reveal gender difference amongst a sub-population. Thus, in order to identify the prevalence of and associated risk factors for an elevated serum ALT level, a community-based screening program for the detection of elevated serum

ALT level was considered necessary. The present study was designed so as to attempt to explore the potential for condition-related gender difference, because it was considered that such difference might underscore important implications for the understanding of the overall pathogenesis of an elevated serum ALT level. The purpose of this study was to explore such gender difference in the context of prevalence of and associated risk factors for elevated serum ALT levels amongst the general population aged 30 years or more, as determined by the application of a community-based screening program to a well-organized, self-contained community-living on Kinmen Island, Taiwan.

MATERIALS AND METHODS

Study design and data resource

Kinmen is an island located around 90 km west of Taiwan in the Straits of Taiwan, close to the Chinese mainland. Based upon this island's population stability, geographical region and local community-support network, Kinmen was selected as a site to conduct various screening programs for chronic-related diseases, in this instance, elevated serum ALT levels. We conducted this community-based mass screening program targeting subjects aged 30 years or more during the period 1991-1994 inclusively. The details of the study design and execution, with respect to the mass-screening program, have been described in full elsewhere[7]. Briefly, according to resident household registration, a total of 20 112 subjects (10 136 males) were eligible to participate in such population screening. A total of 11 898 individuals (5 311 males) of the original 20 112 subjects underwent screening following their response to an invitation letter or telephone call, such individuals completing a questionnaire and providing a blood sample. The overall response rate was 59.2%, including 52.4% for males and 66.0% for females.

Data collection

Data pertaining to study participants was collected from them, the process including face-to-face interviews together with the provision of a structured questionnaire (questions pertained to demographic details, lifestyle information, and personal disease history), and the determination of participant blood pressure. All these investigations were carried out by the Yang-Ming Crusade, a group of well-trained and -organized medical students from the National Yang-Ming University, Taipei, Taiwan. During the study period, fasting blood samples were drawn via venipuncture from study participants by public-health nurses. Overnight-fasting serum and plasma (from whole blood preserved with EDTA and NaF) samples were kept frozen (-20 ℃) until ready for analysis. Subjects for whom the serum ALT level was ⩾40 U/L were classified as individuals who featured an elevated ALT level[8]. In addition, the study-used definitions of type-2 diabetes and hypertension derived from 1999 WHO criteria[9] and TNC VI (The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure)[10], respectively. Subjects featuring a personal disease history of type-2 diabetes or hypertension and who had received medication to treat such conditions were viewed as "known cases" of such disease. Definitions of the following diseases/conditions were obesity: a BMI ⩾25 kg/m^2, a large waist circumference ⩾90 cm for males and ⩾80 cm for females, hypercholesterolemia ⩾200 mg/dL, hypertriglyceridemia ⩾200 mg/dL, low HDL a level <35 mg/dL, high BUN a level ⩾20 mg/dL, high creatinine ⩾1.4 mg/dL and hyperuricemia ⩾7 mg/dL for males or ⩾6 mg/dL for females.

Statistical analysis

Statistical analysis was performed using SAS for Windows (SAS version 8.1; SAS Institute Inc., Cary, NC). A P value of <0.05 was considered to represent statistically significant difference between two test populations. For the univariate analysis, the two-sample independent t-test method was adopted to assess difference in the mean value of continuous variables between normal and elevated serum ALT levels. Crude and adjusted odds ratios (adjustment for sex and age) were estimated and 95% confidence intervals were used. Multiple logistic regressions were also performed in order to investigate the independence of risk factors associated with a high serum ALT level.

RESULTS

The overall mean of serum ALT level for the test population proved to be 20.85±44.82 U/L, males revealing a greater serum ALT level than was the case for females (respectively, 23.77±50.91 U/L *vs* 18.49±39.06 U/L, P<0.05). Table 1

Table 1 Gender- and age-specific prevalence of elevated serum alanine aminotransferase (ALT) level among subjects aged 30 and above in Kinmen

	Level of ALT (U/L)											
	Male (n = 5 311)				Female (n = 6 587)				Total (n = 11 898)			
Age (yr)	Screened No	⩾40 (U/L) No	Prevalence (%)	P-value for χ^2 test for trend	Screened No	⩾40 (U/L) No	Prevalence (%)	P-value for χ^2 test for trend	Screened No	⩾40 (U/L) No	Prevalence (%)	P-value for χ^2 test for trend
30–39	1 326	173	13.1		2 215	96	4.3		3 541	269	7.6	
40–49	1 255	144	11.5	<0.0001	1 581	94	6.0	0.192	2 836	238	8.4	<0.0001
50–59	1 456	112	7.7		1 224	75	6.1		2 680	187	7.0	
60–69	840	47	5.6		978	60	6.1		1 818	107	5.9	
⩾70	434	24	5.5		589	26	4.4		1 023	50	4.9	
Total	5 311	500	9.4		6 587	351	5.3		11 898	851	7.2	

Table 2 Comparison of characteristics of elevated serum ALT level among subjects aged 30 and over in Kinmen (mean±SD)

Variable	Level of ALT (U/L)			
	<40 (U/L) (n = 11 074)	≥40 (U/L) (n = 851)	Total (n = 11 898)	P-value for t test
Age (yr)	49.7±13.3	47.9±12.2	49.6±13.2	0.0001
BMI (kg/m^2)	23.3±3.4	24.6±3.4	23.4±3.5	<0.0001
Waist circumference (cm)	82.7±10.1	86.6±9.7	83.1±10.2	<0.0001
FPG (mg/dL)	100.3±28.2	105.4±28.8	100.9±28.2	<0.0001
SBP (mmHg)	129.8±21.0	132.7±19.7	129.9±20.9	<0.0001
DBP (mmHg)	80.0±12.4	83.5±12.0	80.3±12.4	<0.0001
Cholesterol (mg/dL)	201.4±39.5	211.3±44.2	202.2±40.0	<0.0001
Triglyceride (mg/dL)	91.8±57.7	120.9±79.2	93.9±60.0	<0.0001
HDL (mg/dL)	56.5±20.0	55.9±23.9	56.5±20.5	0.49
BUN (mg/dL)	16.6±5.5	16.8±5.1	16.6±5.5	0.39
Creatinine (mg/dL)	0.79±0.31	0.84±0.26	0.79±0.31	<0.0001
Uric acid (mg/dL)	5.5±1.6	6.3±1.7	5.6±1.6	<0.0001

presents the gender- and age-specific prevalence of an elevated serum ALT level (≥40 U/L) amongst study-participating subjects aged 30 years and over. The overall prevalence of an elevated serum ALT level for the test population was 7.2%, this parameter revealing a statistically significant decrease with increasing study-subject age by means of the χ^2 trend test (P<0.0001). The prevalence of an elevated serum ALT level proved to be substantially greater for males than for females (respectively, 9.4% *vs* 5.3%, P value for χ^2 test <0.0001). In addition, after stratifying data by age into one of five broad (age) groups, study-participating males exhibited a more-pronounced prevalence of elevated serum ALT level for all age groups apart from the 60-69-years-old age group than was the case for the female group. The age-specified prevalence of an elevated serum ALT level revealed a significant inverse relationship with age when applying the χ^2 trend test (P<0.0001) for male study subjects but not so for females (P = 0.192).

Table 2 illustrates the results of the comparison of a variety of test characteristics and their potential association with the specific (serum ALT) class value (either ≥40 U/L, or <40 U/L) for study-included subjects aged 30 years and over. Using the two-sample independent t-test, the associated factors that were significantly related to elevated serum ALT level included age [≥40 U/L (47.7±12.2 years) *vs* <40 U/L (49.7±13.3 years)], BMI [≥40 U/L (24.6±3.4 kg/m^2) *vs* <40 U/L (23.3±3.4 kg/m^2)], waist circumference [≥40 U/L (86.6±9.7 cm) *vs* <40 U/L (82.7±10.1 cm)], and serum level of FPG [≥40 U/L (105.4±28.8 mg/dL) *vs* <40 U/L (100.3±28.2 mg/dL)], SBP [≥40 U/L (132.7±19.7 mmHg) *vs* <40 U/L (129.8±21.0 mmHg)], DBP [≥40 U/L (83.5±12.0 mmHg) *vs* <40 U/L (80.0±12.4 mmHg)], cholesterol [≥40 U/L (211.3±44.2 mg/dL) *vs* <40 U/L (201.4±39.5 mg/dL)], triglyceride [≥40 U/L (120.9±79.2 mg/dL) *vs* <40 U/L (91.8±57.7 mg/dL)], creatinine [≥40 U/L (0.84±0.26 mg/dL) *vs* <40 U/L (0.79±0.31 mg/dL)], and uric acid [≥40 U/L (6.3±1.7 mg/dL) *vs* <40 U/L (5.5±1.6 mg/dL)].

Table 3 presents the crude and adjusted odds ratios for the association between certain relevant associated risk factors and elevated serum ALT level. Compared to individuals who exhibited a normal serum ALT level, subjects featuring an elevated serum ALT level revealed a more-

Table 3 Univariate analysis of associated factors for elevated serum alanine ALT level among subjects aged 30 and over in Kinmen

		Elevated ALT level (≥40U/L)		Crude OR (95%CI)	Adjusted OR[1] (95%CI)
		Yes (n = 851)	No (n = 11 074)		
Gender	Male	500	4 811	1.85	-
	Female	351	6 236	(1.60-2.13)	
Age (yr)	30-39	269	3 272	1.00	-
	40-49	238	2 598	1.11	-
				(0.93-1.34)	
	50-59	187	2 493	0.91	-
				(0.75-1.11)	
	60-69	107	1 711	0.76	-
				(0.60-0.96)	
	≥70	50	973	0.63	-
				(0.46-0.85)	
Smoking	Yes	267	2 941	1.26	0.87
	No	584	8 106	(1.08-1.47)	(0.72-1.04)
Alcohol drinking	Yes	266	2 362	1.67	1.24
	No	585	8 685	(1.44-1.95)	(1.04-1.48)
Obesity	Yes	360	3 032	1.94	2.05
	No	491	8 015	(1.68-2.23)	(1.77-2.37)
High waist - circumference	Yes	471	4 599	1.72	2.41
	No	380	6 388	(1.50-1.98)	(2.07-2.80)
Type-2 diabetes	Yes	134	938	2.01	2.39
	No	717	10 109	(1.66-2.45)	(1.94-2.93)
Hypertension	Yes	366	3 850	1.41	1.54
	No	485	7 197	(1.22-1.62)	(1.32-1.79)
Hypercholesterolemia	Yes	205	1 682	1.77	1.85
	No	646	9 362	(1.50-2.08)	(1.56-2.19)
Hypertriglyceridemia	Yes	99	518	2.66	2.65
	No	745	10 383	(2.12-3.34)	(2.10-3.33)
Low HDL	Yes	138	1 337	1.45	1.34
	No	663	9 324	(1.20-1.76)	(1.10-1.63)
High BUN	Yes	193	2 446	1.03	1.01
	No	658	8 598	(0.87-1.22)	(0.85-1.20)
High creatinine	Yes	7	132	0.69	0.66
	No	843	10 909	(0.32-1.47)	(0.31-1.42)
Hyperuricemia	Yes	342	2 680	2.10	2.08
	No	507	8 355	(1.82-2.43)	(1.79-2.40)

[1]Adjustment for gender and age.

pronounced prevalence of alcohol drinking (adjusted OR = 1.24, 95%CI: 1.04-1.48), in addition to obesity (adjusted OR = 2.05, 95%CI: 1.77-2.37), substantial waist circumference (adjusted OR = 2.41, 95%CI: 2.07-2.80), type-2 diabetes (adjusted OR = 2.39, 95%CI: 1.94-2.93), hypertension (adjusted OR = 1.54, 95%CI: 1.32-1.79), hypercholesterolemia (adjusted OR = 1.85, 95%CI: 1.56-2.19), hypertriglyceridemia (adjusted OR = 2.65, 95%CI: 2.10-3.33), a low HDL level (adjusted OR = 1.34, 95%CI: 1.10-1.63), and hyperuricemia (adjusted OR = 2.08, 95%CI: 1.79-2.40) subsequent to adjustment for gender and age.

The effect of independently associated risk factors upon elevated serum ALT level was examined using the multiple logistic regression models. As is depicted in Table 4, subsequent to adjustment for confounding factors, gender (female *vs* male, OR = 0.21, 95%CI: 0.11-0.39), age (OR = 0.95, 95%CI: 0.93-0.97), interaction between gender and age (OR = 1.02, 95%CI: 1.01-1.03), and the presence of obesity (yes *vs* no, OR = 1.20, 95%CI: 1.00-1.44), a large waist circumference (yes *vs* no, OR = 1.79, 95%CI: 1.48-2.17), type-2 diabetes (yes *vs* no, OR = 1.70, 95%CI: 1.35-2.13), hypercholesterolemia (yes *vs* no, OR = 1.46, 95%CI: 1.22-1.76), hypertriglyceridemia (yes *vs* no, OR = 1.40, 95%CI: 1.06-1.84), and hyperuricemia (yes *vs* no, OR = 1.63, 95%CI: 1.39-1.92) appeared to be statistically significantly related to an elevated serum ALT level. The data presented in Table 4 also show the dramatically different results of multiple logistic regressions of the data as stratified by gender. For males, the statistically significantly associated risk -factors related to an elevated serum ALT level included age (OR = 0.97, 95%CI: 0.96-0.98), and the presence of obesity (yes *vs* no, OR = 1.28, 95%CI: 1.00-1.66), a large waist circumference (yes *vs* no, OR = 1.88, 95%CI: 1.45-2.42), type-2 diabetes (yes *vs* no, OR = 1.74, 95%CI: 1.28-2.37), and hyperuricemia (yes *vs* no, OR = 1.47, 95%CI: 1.19-1.80). For female study participants, the statistically significant associated risk factors related to an elevated serum ALT level included age (OR = 0.98, 95%CI: 0.97-0.99), and presence of a large waist circumference (yes *vs* no, OR = 1.66, 95%CI: 1.24-2.21), type-2 diabetes (yes *vs* no, OR = 1.56, 95%CI: 1.11-2.21), hypercholesterolemia (yes *vs* no, OR = 1.80, 95%CI: 1.36-2.39), hypertriglyceridemia (yes *vs* no, OR = 1.61, 95%CI: 1.03-2.52), and hyperuricemia (yes *vs* no, OR = 1.92, 95%CI: 1.49-2.48).

DISCUSSION

Prevalence of an elevated serum ALT level

One of the important benefits of the ALT screening program was the chronic liver disease which was often identified by the detection of an (asymptomatic) elevated serum aminotransferase level and as such, a screening test was commonly included in the serum chemistry panels conducted on healthy individuals[11]. Further, the relative significance of such results was often ignored when the serum ALT level was deemed to be only just slightly abnormal[11]. However, it would appear that only few community-based studies have been published, attempting to determine the prevalence and possible etiology of an elevated serum ALT level for the general population of Taiwan[12], that also faced to the burden of liver disease. In the present study, an elevated serum ALT level appeared to be fairly common for the test population, the condition affecting an estimated 7.2% of the general population in Kinmen. The prevalence of an elevated serum ALT level amongst different test populations appears to vary according to the results of different studies conducted in different countries[6,11-13]. In addition to the differences in the specifics of diagnostic criteria for such an elevated serum ALT level, this disparity would likely be largely due to differences between different population stocks. The prevalence of an elevated serum ALT level for our study population (7.2%) was slightly lower than the corresponding figure presented in a previous population-based study conducted in A-Lein, Taiwan which was reported to be 7.5%[12]. The apparent slightly lower prevalence rate in our study may have been due to differences between the predominantly rural lifestyle of residents of Kinmen and the more urban lifestyle of

Table 4 Multiple logistic regression of associated factors for elevated serum ALT level among subjects aged 30 and above in Kinmen

Variable	ALT (≥40 (U/L) *vs* <40 (U/L))					
	Male		Female		Total	
	OR	95%CI	OR	95%CI	OR	95%CI
Gender (female *vs* male)	-	-	-	-	0.21	0.11-0.38
Age (yr)	0.97	0.96-0.98	0.98	0.97-0.99	0.95	0.93-0.97
Interaction between gender and age	-	-	-	-	1.02	1.01-1.03
Smoking (yes *vs* no)	0.93	0.76-1.14	0.68	0.33-1.38	0.90	0.74-1.10
Alcohol drinking (yes *vs* no)	1.14	0.93-1.40	1.48	0.85-2.59	1.17	0.96-1.41
Obesity (yes *vs* no)	1.28	1.00-1.66	1.09	0.84-1.42	1.20	1.00-1.44
High waist circumference (yes *vs* no)	1.88	1.45-2.42	1.66	1.24-2.21	1.79	1.48-2.17
Type-2 diabetes (yes *vs* no)	1.74	1.28-2.37	1.56	1.11-2.21	1.70	1.35-2.13
Hypertension (yes *vs* no)	1.12	0.91-1.38	1.18	0.90-1.55	1.14	0.97-1.34
Hypercholesterolemia (yes *vs* no)	1.26	0.98-1.61	1.80	1.36-2.39	1.46	1.22-1.76
Hypertriglyceridemia (yes *vs* no)	1.30	0.91-1.85	1.61	1.03-2.52	1.40	1.06-1.84
Low HDL (yes *vs* no)	0.96	0.74-1.25	1.12	0.79-1.60	1.02	0.82-1.25
High BUN (yes *vs* no)	0.92	0.73-1.16	1.10	0.82-1.48	0.99	0.82-1.19
High creatinine (yes *vs* no)	0.55	0.22-1.40	0.25	0.03-1.89	0.46	0.20-1.06
Hyperuricemia (yes *vs* no)	1.47	1.19-1.80	1.92	1.49-2.48	1.63	1.39-1.92

residents of A-Lein, Taiwan. The incidental finding in our study that approximately 47.6% of study-included males had not undergone any form of health screening previously might also partially explain the apparently low prevalence of elevated serum ALT level observed in our study. Further, another possible reason for such difference between the results of the A-Lein study and our results may simply have been related to the different age cut-off point for the two study populations.

Implications of gender difference as regards associated risk factors for elevated serum ALT level

Our results have revealed that male gender and a younger age both represented significant risk factors related to the likelihood of an elevated serum ALT level. Such a finding would appear to be consistent with the results of other hospital and community-based studies conducted elsewhere[6,11,14]. This apparent concentration of liver injury amongst predominantly younger adults remained unexplained at the time of publishing of these (overseas-based) studies, as is the case here, and clearly deserves further attention[6]. In addition, males exhibited a higher prevalence of elevated ALT level than did females (OR = 1.85, 95%CI: 1.60-2.13), such gender difference being consistent with a larger waist-to-hip circumference ratio for males, the latter possibly explaining the former[6].

A growing body of evidence appears to indicate that significant liver disease may accompany (seemingly) mild serum aminotransferase level elevations[15,16]. Consistent with the results of other studies[11,13], our results revealed that a larger BMI and a more-substantial waist circumference were both highly associated with an elevated serum ALT level. Previous studies have indicated that most obese individuals who featured an elevated serum ALT level did suffer from steatosis without any associated hepatic fibrotic reactions or sites of inflammation upon liver biopsy[17,18]. Further, approximately 30% of obese adults, who exhibited an elevated serum aminotransferase level, exhibited steatohepatitis associated with fibrosis or cirrhosis as demonstrated upon liver biopsy[17,18], approximately 40% of these cases revealed progressive liver disease[19]. In addition to such a finding, a larger waist circumference was also reported to be more strongly related to an elevated serum ALT level than was the case for an elevated BMI level for this last-mentioned study[19]. The possible mechanism for such a finding may relate to the observation that waist circumference is typically associated with visceral adipose tissue build-up, such a source of adipose tissue possibly providing a over-supply of potentially hepatotoxic fatty acids to the liver[20]. Further, it has been reported previously that visceral adipose tissue lipolysis was also less sensitive to insulin suppression than was the case for other fat deposits[21]. The present study has further demonstrated that a larger BMI is significantly related to an elevated serum ALT level for males but not so for females subsequent to adjusting for confounding factors. Further epidemiological and etiological investigations are clearly needed in order to clarify the pathophysiological mechanisms of gender difference between obesity and elevated serum ALT level.

In a previous study, type-2 diabetes has been reported to be associated with mild (asymptomatic) elevations in the serum levels of certain enzymes including serum ALT[22]. Elevated ALT levels have been reported as more frequently observed for diabetics than for the general population[23]. The Third National Health and Nutrition Examination Survey (NHANES III) reported that the likelihood of an individual featuring an elevated serum ALT level was greater amongst persons afflicted with type-2 diabetes than it was for non-diabetic individuals[11]. The Hispanic Health and Nutrition Examination Survey also reported that abnormal serum ALT levels were statistically significantly more common amongst Mexican Americans suffering from diabetes as compared to their non-diabetic counterparts[24]. Due to the liver playing an important role in the maintenance of glucoregulation, carbohydrate homeostasis and insulin degradation, it seems logical to conclude that the liver's normal functions might be significantly affected as a consequence of glucose intolerance and/or diabetes mellitus[6,24-26].

Hyperlipidemia is frequent amongst subjects who feature abnormal serum activities for certain liver enzymes[11,27]. Our findings have demonstrated that both hypercholesterolemia and hypertriglyceridemia are significantly related to the presence of an elevated serum ALT level. Recent studies have suggested that a considerable proportion of moderately obese individuals who feature hyperlipidemia might develop extensive fibrosis and cirrhosis, with a subsequent marked increase in mortality as a consequence of liver-related diseases[28]. Such observations imply that subjects suffering from obesity and hyperlipidemia should receive screening for liver function more regularly than normal individuals in order to avoid serious liver injury. Furthermore, previous study has shown that hypertriglyceridemia was related to serum ALT levels for both males and females, whilst an elevated serum cholesterol level only appeared to be related to an elevated serum ALT level for males[29]. A possible reason for such apparent discordant findings when compared to the results of the present study is that our study population was constituted by a slightly greater proportion of females who were post-menopausal (44%), such a post-menopausal subgroup likely featuring more-elevated serum total cholesterol and triglyceride levels than would be the case for pre-menopausal females[30]. At the time of study execution, we did not have any information pertaining to study participant hepatitis B surface antigen (HBsAg) and anti-HCV status, such that our failure to exclude positive such individuals from the study may also have confounded the results.

Previous clinical study has revealed that a fructose load might lead to a more substantial increase in serum uric-acid level amongst patients suffering from chronic hepatitis than would be the case for normal individuals[31]. In addition, serum uric-acid level has been reported to be elevated amongst subjects suffering from chronic liver lesions, especially those of a non-infectious origin. Further, the extent of such serum-level elevation appears to be dependent upon the specific severity of the hepatic lesions[32], although the previous results relating to the quite significant association between hyperuricemia and elevated serum ALT level appears to be similar to that proffered by other studies[32].

From the cross-sectional nature of our study design, we were not able to determine the degree to which could have occurred or to what extent the increase in serum uric acid level had arisen prior to liver disease having developed.

Although cigarette smoking and alcohol drinking might constitute important risk factors as regards liver-function abnormalities and/or specific liver diseases as inferred by the results of a number of previous studies[24,29], for the current study, habits such as smoking or alcohol consumption did not appear to be significantly related to elevated serum ALT level following multivariate adjustment. Such a result was not really surprising because we found it somewhat difficult to accurately identify the actual duration of such behavior as also the daily intake of alcohol and the number of cigarettes smoked daily in the context of such a large epidemiological study.

Perceived limitations

One of the major limitations to the present study was potential selection bias due to a relatively lower response rate. The potential impact on the prevalence and the study-observed elevated level-associated risk factors were, in our estimation, inevitable. Nevertheless, given the rather large sample size of this study, we still did retain sufficient statistical power to be able to effectively evaluate the presence of any gender differences between the various associated risk factors for an elevated serum ALT level subsequent to adjustment for confounding factors. Secondly, because it would appear that no standard cut-off level for serum ALT level elevation has yet been internationally accepted to constitute abnormality, different studies may elect to set slightly different normal *vs* abnormal cut-off levels, such that our estimation of what constituted an abnormal elevation of serum ALT level could have suffered from some level of misclassification-bias identification. Thirdly, since Kinmen is an offshore island from Taiwan and lacking in medical resources, the screening program involved a lot of difficulties such as mobilization of manpower and facility. For the cost consideration, we did not collect the information of hepatitis B and hepatitis C, tissue samples for histology and ultrasonography results as a part of our study, such that the "true" causes of observed elevated serum ALT levels for study-participating individuals were not able to be determined. Finally, our measurements were conducted at only a single point in time and, by clear inference, would not be able to be used to reflect long-term exposure to various demographic or biochemical aspects or factors, which might be important influencers of (an elevated) serum ALT level. The solution to such a quandary would best be accomplished by conducting a number of prospective longitudinal analogous studies, the results of which would be expected to complement the community-based (cross-sectional) findings of this study.

Conclusions

In conclusion, as a consequence of the conduct of this study, several gender-related dissimilarities were noted as regards the relationship between obesity, hypertriglyceridemia, and hyperuricemia and an elevated serum ALT level for study-included individuals, as also in regard to the actual prevalence of such level elevation. Further studies are not only needed in order to elucidate the temporal sequence of events that typically lead to elevated serum ALT levels and thus develop more satisfactory non-invasive indicators of liver pathology, but also to further explore the realm of gender-related differences that appear to be involved with elevated serum ALT levels.

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• *BRIEF REPORTS* •

Metabolic changes in the lower esophageal sphincter influencing the result of anti-reflux surgical interventions in chronic gastroesophageal reflux disease

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Abstract

AIM: With the availability of a minimally invasive approach, anti-reflux surgery has recently experienced a renaissance as a cost-effective alternative to life-long medical treatment in patients with gastroesophageal reflux disease (GERD). We are not aware of the fact whether reflux episodes causing complaints for a long time i.e., at least for one year are associated with metabolic changes in the lower esophageal sphincter, and if so, whether these may influence functional results achieved after anti-reflux surgery.

METHODS: Between 1 January 2001 and 31 December 2002 we performed anti-reflux surgery on 79 patients. Muscle samples were taken from the lower esophageal sphincter (LES) in 33 patients during anti-reflux surgery. Inclusion criteria were: LES resting pressure below 10 mmHg and a marked, pH proven acid exposure to the esophagus of at least one year's duration, causing subjective complaints and requiring continuous proton pump inhibitor treatment. Control samples were obtained from muscle tissue in the gastroesophageal junction that had been removed from 17 patients undergoing gastric or esophageal resection. Metabolic and lysosomal enzyme activities and special protein concentrations 16 parameters in total were evaluated in tissue taken from control specimens and tissue taken from patients with GERD. The biochemical parameters of these intra-operative biopsies were used to correlate the results of anti-reflux operations (Visick I and II-III).

RESULTS: In the reflux-type muscle, we found a significant increase of the energy-enzyme activities e.g., creatine kinase, lactate dehydrogenase, β-hydroxybutyrate dehydrogenase, and aspartate aminotransaminase-. The concentration of the structural protein S-100 and the myofibrillar protein troponin I were also significantly increased. Among lysosomal enzymes, we found that the activities of cathepsin B, tripeptidyl-peptidase I, dipeptidyl-peptidase II, β-hexosaminidase B, β-mannosidase and β-galactosidase were significantly decreased as compared to the control LES muscles. By analyzing the activity values of the 9 patients in Visick groups II and III at two months post-surgery, we found a significant increase in the activity of the so-called energy-enzyme values and in the concentration of structural and myofibrillar proteins as compared to the rest of the reflux patients.

CONCLUSION: Our results call attention to the metabolic changes that occurred in the LES muscles of reflux patients. The developing hypertrophy-like changes of LES muscles may be a reason for complaints after anti-reflux surgery, which consisted mainly of reports of persisting dysphagia.

Key words: LES muscle; Metabolic enzymes; Lysosomal enzymes; Anti-reflux surgery; Hypertrophy; Dysphagia; Gastroesophageal reflux

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INTRODUCTION

Clinical medicine has advanced by retrospective analysis, relating symptoms to anatomic or structural lesions and using this relationship prospectively to diagnose disease. In time, biochemical or histological abnormalities were identified as having a high probability of being caused by a disease process, such as metabolic alterations, neoplasia, inflammation, or ischemia. Consequently, biochemical and histological patterns are now used to recognize and identify specific diseases in symptomatic patients.

Functional disorders of the esophagus are abnormalities that can exist for a period of time without causing morphologic

changes even though considerable symptoms develop, such as heartburn, regurgitation, and dysphagia.

However, we are not aware of whether reflux episodes causing complaints for a long time at least for a year - induce any metabolic changes in the lower esophageal sphincter (LES), and if so, whether these may influence functional results achieved after anti-reflux operations.

It is true that with the availability of a minimally invasive approach, anti-reflux surgery has recently experienced a renaissance as a cost-effective alternative to lifelong medical treatment in patients with gastroesophageal reflux disease (GERD). The number of anti-reflux procedures performed has virtually exploded. The laparoscopic approach does not, however, reduce the prevalence of side effects usually associated with anti-reflux surgery even in experienced hands[1-3].

A review of the literature shows that between 5% and 20% of patients who had an anti-reflux operation would experience some form of recurrent or persistent symptoms, requiring continued or renewed medical attention. Today recurrent reflux is the most common reason for failure of anti-reflux surgery. This is followed by dysphagia and by a combination of dysphagia with reflux symptoms. The so-called "gas bloat syndrome" or gastric denervation symptoms are rare after anti-reflux surgery[4].

Although most patients with recurrent, persistent or new symptoms after an anti-reflux procedure can be managed medically, some will require revisional or salvage surgery and it is well-known that the rate of success exponentially decreases in proportion to the number of re-operations[5].

In the present study, we have analyzed the effect of persistent reflux on LES muscle metabolism based on the biochemical analysis of muscle samples taken from LES during 46 operations performed in the cardiac region.

The biochemical analyses used in this study included measurement of enzymes with functions in anabolic processes "energy enzymes": creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (HBDH), aspartate aminotransaminase (AST), lysosomal enzymes that play a role in catabolic processes, namely carbohydrate hydrolysis α-mannosidase (AMAN), β-mannosidase (BMAN), β-galactosidase (BGAL), β-glucuronidase (GCU), β-hexosaminidase B (HEX), and enzymes involved with protein degradation cathepsin B (CB), tripeptidyl-peptidase I (TPP I), dipeptidyl-peptidase II (DPP II). In addition, the myoglobin (MYO) and troponin I (TNI) proteins are important in skeletal muscle function and S-100 is a highly acidic calcium-binding protein found in various organs in the body.

MATERIALS AND METHODS

Between January 1 2001 and December 31 2002, we performed anti-reflux surgery on 79 patients. Out of the 75 cases we performed a laparoscopic operation, in 4 cases the intervention was conventional Nissen or Belsey-Mark IV fundoplication. In 33 patients the muscle sample was taken from the LES with scissors, without cauterization, during the operation from a standard site on the right side of the anterior vagus, on the intra-abdominal part of the esophagus, where the floppy-Nissen wrap was placed. First the mucosa was brought into the visual field similar to cardiomyotomy in order to ensure that the muscle samples, to be analyzed, represented both longitudinal and circular muscle fibers and the excision was made in this way. Inclusion criteria for patients in the reflux group were as follows: LES resting pressure below 1.33 kPa (10 mmHg), and a marked, pH proven acid exposure to the esophagus that has persisted for at least a year, causing subjective complaints and requiring continuous proton pump inhibitor (PPI) treatment. Those patients whose contraction amplitude was less than 2.66 kPa (20 mmHg) in one or more of the three lowest esophageal segments and/or for whom more than 20% simultaneous waves in these segments had been verified were excluded.

The control group contained muscle samples, obtained from the gastroesophageal junction, that was removed during 17 gastric or esophageal resections. Only patients with no reflux complaints in the history were included in the control group. Since in the majority of these patients the surgical intervention was necessary because of a tumor, we checked the muscle sample histology for tumor infiltrates.

The data of 46 patients, out of the 50 included (92%), were evaluated. Reasons for exclusion included histologically confirmed tumor infiltration (n = 2), progressive muscular dystrophy and congenital esophageal atresia (n = 1 each). Thus, GERD was represented with samples from 31 patients, while normal LES with samples from 15 patients. The anti-reflux operation was in each case a floppy-Nissen type reconstruction. The same surgeon performed the operations as well as muscle sampling.

Complaints of operated patients were evaluated according to the following Visick-classification: I: symptom-free. II: mild symptoms, requires no treatment. III: can be treated with medication or with dilation. IV: symptoms that cannot be controlled with conservative treatment, reoperation needed.

The muscle samples were frozen on dry ice immediately after dissection and stored at -70 ℃ prior to use.

Samples were thawed on ice, placed in 50 volumes (w/v) of 0.15 mol/L NaCl, 0.1% Triton X-100 and homogenized with a Brinkmann Polytron homogenizer. A soluble supernatant was prepared by centrifugation at 12 000 g at 4 ℃ for 25 min.

The activity of CK, LDH, HBDH, AST enzymes and CK-MB were measured with reagents produced by A.L. Instruments (Diachem Kft.; Budapest, Hungary), with kinetic UV photometry method, on an Olympus AU 600 chemical analyzer (Olympus Diagnostica GmbH; Hamburg, Germany).

Protein S-100 determination was performed with a two-step immunoluminometric sandwich assay (ILMA) technique, by applying LIAISON® Sangtec® 100 (AB Sangtec Medical; Bromma, Sweden) in a LIAISON® immunochemical automat (BYK SANGTEC Diagnostica; Dietzenbach, Germany).

The concentration of TNI and MYO were determined with the Microparticular Enzyme Immunoassay (MEIA) method (Lumi-Phos 530 is measured) in a Beckman Access immunochemical automat (Beckman Coulter Access

Immunoassay System; Fullerton, USA).

Glycosidase activities were measured using 4-methylumbelliferyl (4-MU) substrates described by Sleat *et al*[6] Protease assays using amino-4-methylcoumarin (AMC) substrates have been described by Sleat *et al*[7] and Sohar *et al*[8] Reactions were initiated by adding 40 μL substrate (various concentration 20 μmol/L), buffer (100 mmol/L) solution to 5 μL (CB) or 10 μL (other enzymes) of sample (after centrifugation, supernatants were diluted 2-, 4- and 8-fold in homogenization buffer in duplicate), incubated at 37 ℃, and terminated by the addition of 100 μL of 0.5 mol/L glycine, pH10.5 (at 4-MU substrates) or 0.1 mol/L monochloroacetic acid in 0.1 mol/L acetate, pH4.3 (AMC substrates). Buffers consisted of 0.1 mol/L citric acid or 0.1 mol/L sodium acetate adjusted to the indicated pH using sodium hydroxide, acetic acid, or HCl, respectively, and contained 0.15 mol/L NaCl with 0.1% Triton-X-100. Substrates were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were prepared as stocks in dimethyl sulfoxide that were added to the reaction buffer immediately prior to assay. Samples added to substrate solutions after addition of termination buffer were used as blanks. Fluorescent reaction products were measured using a CytoFluor II (PerSeptive Biosystems, Framingham, MA) fluorescence multiwell plate reader with excitation at 360 nm and emission at 460 nm.

Statistical analyses were performed using a one-factor variance-analysis and interval estimate method by applying MS Excel Analysis Toolpack program. Each subject enrolled into the study signed an informed consent form. Permission for the investigations was sought and obtained from the appropriate local Ethical Committee.

RESULTS

In 12 out of the 16 measured parameters, we found significant biochemical differences between reflux-type and control LES muscle. The activities of CK, LDH, HBDH, and AST enzymes were significantly elevated in the reflux-type LES muscle compared with the control group (Table 1). A significant increase was also found for the concentration of S-100 and TNI proteins in the reflux-type muscle. There were no statistically significant differences in myoglobin concentration or in CK-MB activity between the two examined groups, although seemingly marked individual differences were noted.

We found definite differences in relation to the lysosomal enzymes, since the activity of CB, TPP I, DPP II, HEX, BMAN and BGAL were significantly lower in the reflux-type LES as compared to the normal LES (Table 2).

Table 1 Metabolic enzyme activities and special protein concentrations in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
CK	73 027.3	32 312.4	398 220.7	210 587.2	*P*<0.05	111 904.3	29 131.0	1 098 105.3	514 604.5	*P*<0.001
LDH	23 805.7	9 561.9	127 786.9	62 210.7	*P*<0.05	44 229.0	7 850.8	332 039.5	54 804.5	*P*<0.001
HBDH	7 960.3	3 159.9	41 400.3	20 129.8	*P*<0.05	13 906.0	2 588.4	105 553.7	47 629.0	*P*<0.001
AST	2 869.5	1 453.0	13 397.2	6 825.7	*P*<0.05	3 697.0	923.0	36 030.9	14 938.4	*P*<0.001
CK-MB	269.1	103.9	1 580.3	1 332.6	NS	597.5	185.3	3 655.2	4 374.7	NS
S-100	1 252.5	844.0	10 569.1	5 038.1	*P*<0.05	3 986.5	2 123.0	26 018.5	11 485.7	*P*<0.001
MYO	750.3	538.3	11 339.8	14 029.3	NS	2 395.8	1 268.1	32 581.8	52 349.6	*P*<0.05
TNI	0.17	0.08	1.56	0.78	*P*<0.05	0.68	0.24	3.93	2.25	*P*<0.001

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II-III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; CK: creatine kinase; LDH: lactate dehydrogenase; HBDH: α-hydroxybutyrate dehydrogenase; AST: aspartate aminotransaminase; CK-MB: creatine kinase MB (U/g soluble protein); S-100; MYO: myoglobin; TNI: troponin I (μg/g wet weight).

Table 2 Lysosomal enzyme activities in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
CB	14 177.5	3 441.9	7 727.6	1 913.9	*P*<0.001	6 442.8	2 242.7	10 439.8	3 548.1	*P*<0.05
TPP I	74 318.0	10 742.6	47 509.9	5 339.3	*P*<0.001	47 541.6	6 760.4	47 438.5	1 1067.2	NS
DPP II	3 970.4	1 189.3	2 513.3	512.0	*P*<0.001	2 860.7	647.1	1 779.7	744.1	*P*<0.05
HEX	17 253.7	4 457.9	9 185.0	1 604.7	*P*<0.001	9 528.2	2 267.2	8 460.4	2 054.9	NS
GCU	17 345.0	5 145.4	13 123.5	2 307.5	NS	13 980.1	3 263.8	11 315.2	2 541.3	NS
BMAN	4 207.5	1 246.1	2 922.9	587.7	*P*<0.05	3 248.4	824.1	2 235.9	481.4	NS
AMAN	962.4	726.8	469.9	172.2	NS	567.4	232.8	238.5	108.9	NS
BGAL	17 714.3	3 561.0	11 396.7	1 567.3	*P*<0.001	11 733.9	2 241.9	10 684.9	1 821.4	NS

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II–III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; CB: cathepsin B; TPP I: tripeptidyl-peptidase I; DPP II: dipeptidyl-peptidase II; HEX: β-hexosaminidase B; GCU: β-glucuronidase; BMAN = β-mannosidase; AMAN: α-mannosidase; BGAL: β-galactosidase (pmoL/h·mg).

The HBDH/LDH ratio was significantly lower in the reflux-type LES muscle as compared to the control group, while the CK-MB/CK ratio showed no statistical differences (Table 3).

Patients were followed up in the 2nd and 12th month after the operation. Stratification of patients by Visick classification at two months post-surgery showed more patients with Visick I (n = 22) than with Visick II (n = 8) or III (n = 1). The biochemical parameters of Visick II-III patients were combined for comparison with Visick I patients. The leading symptom in the Visick II-III group was difficulty in swallowing (Table 4). In the Visick II-III group, the activities of CK, LDH, HBDH, AST, CB enzymes were significantly higher while DPP II lower than in the Visick I group. Similarly, the concentrations of S-100, TNI, and myoglobin were also significantly higher in Visick II-III patients as compared to Visick I. At the one-year control examination, four of these patients were dissatisfied with the results of the operation. We performed objective imaging and functional examinations for these four patients, and found that the complaints of only one patient could be explained by clear anatomical reasons, where recurring hiatal hernia developed as a consequence of latent brachy-esophagus.

DISCUSSION

With the renaissance of anti-reflux surgery, patients with persistent, recurrent, or newly developed symptoms following an anti-reflux procedure are likely to become a more common problem in the near future. Recurrent reflux is usually due to a breakdown of the repair and can frequently be treated medically or by repeating the procedure. In contrast, post-operative dysphagia with or without accompanying reflux symptoms may be due to a myriad of causes, which include a slipped wrap, a wrap that has been placed around the stomach rather than the esophagus, a too tight or too long wrap, the development of a stricture, the presence of a motor disorder of the esophageal body, hitherto unknown factors, or a combination of these.

So far, no adequately designed clinical trials have shown any benefit with a tailored approach to anti-reflux surgery, where motor function of the esophagus and in the gastroesophageal junction is assessed pre-operatively to determine the exact surgical procedure to be followed.

Lundell *et al*[9] supports this opinion, which found that pre-operative manometric observations had no predictive value regarding the outcome of either form of fundoplication i.e., Nissen-Rosetti total fundic wrap and the 180° partial wrap. An important question is, therefore, whether patients with chronic GERD benefit from anti-reflux surgery and if not, whether it is possible to define the patient profiles of these potential failures.

We looked for an answer by performing the biochemical analysis of LES muscles of those suffering from chronic gastroesophageal reflux disease. In the reflux-type muscle we found a significant increase in energy-enzyme activities CK, LDH, HBDH, AST, as well as, the concentrations of the S-100 protein and TNI. Among lysosomal enzymes, we found that the activities of CB, TPP I, DPP II, HEX, BMAN and BGAL were significantly decreased as compared to the control LES muscles. This is not in conflict with the observation that, in the early stage of hypertrophy, the protein synthesis and the activity of lysosomal glycosidases and proteases temporarily increase, since proteins and carbohydrates, that became unnecessary during muscle transformation, have to be eliminated. However, after the

Table 3 Metabolic enzyme ratios in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
HBDH/LDH	0.337	0.009	0.319	0.006	*P*<0.001	0.321	0.009	0.318	0.008	NS
CK-MB/CK	0.005	0.003	0.006	0.003	NS	0.006	0.003	0.005	0.007	NS

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II–III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; HBDH: α-hydroxybutyrate dehydrogenase; LDH: lactate dehydrogenase; CK-MB: creatine kinase MB; CK: creatine kinase.

Table 4 Patient satisfaction index according to Visick in the 2nd and 12th postoperative months of 31 patients who had laparoscopic floppy Nissen type operation due to gastroesophageal reflux disease

Visick	2 (mo, %)			12 (mo, %)		
I Symptoms-free	22 (71)			27 (87)		
II Mild symptoms	8 (26)	- Dysphagia	6/8	3 (10)	- Dysphagia	2/3
		- Bloating	3/8		- Bloating	2/3
		- Epigastric pain	2/8		- Heartburn	1/3
III Can be treated with medication or with dilation	1 (3)	- Dysphagia		1 (3)	- Dysphagia	
		- Heartburn			- Heartburn	
		- Belching			- Belching	
IV Reoperation needed		-			-	

alteration of muscle structure and metabolism, there is no need for increased protein degradation, as we found in a previous study of muscle hypertrophy with stimulation of muscle cells[10]. In our study the energy-enzyme activity and the specific protein content in the muscle are proportional to the protein synthesis processes, and the lysosomal enzyme activities are proportional to the protein degradation processes in the cardia muscle. The changes in lysosomal enzyme activities have been found to be similar to those found after treatment of rats with gamma irradiation; both irradiation and reflux disease produced oxygen free radicals[11,12].

The observation that increased muscular activity leads to muscle hypertrophy has been published for a long time. But only in the last decade, have newly developed models helped to identify the factors that play a role in the development of muscle hypertrophy. With continued mechanical stimulation in differentiated avian skeletal muscle cells, total protein degradation rate and several protease activities have been seen to increase in the first 2-3 h and return to control levels after several days, with total protein degradation rates falling to levels below those seen in static controls. Decreased protein degradation and the faster protein synthesis contributed to stretch-induced cell growth. Secretion and production of prostaglandin E2, F2 alpha[13] and insulin-like growth factor 1[14] were found to increase with the mechanical stimulation. Recent studies have also demonstrated that the calcium-activated transcription factor NFATC2 controls myoblast fusion by secretion of IL-4 and prostaglandin F2 alpha[15,16].

In reflux disease there is an increase in frequency of transient lower esophageal sphincter relaxations (TLESR) after a meal, which may be related to a greater acid reflux. Thus, an alteration in the triggering of TLESRs is now accepted as one of the key features in the development of gastroesophageal reflux disease[17].

This increased frequency of TLESR may be in the background of the statistically evaluable decrease of HBDH/LDH ratio found by us in chronic reflux-type LES muscles. The shift of oxidative metabolism into glycolytic i.e., anaerobic - direction can be explained by the greater overstrain.

Dysphagia following fundoplication is a common problem and generally occurs in all patients during the first week after surgery. In the great majority of patients the problem rapidly resolves, but in some patients it persists. In the review by Pope[18], dysphagia was reported in 2-44% in 6 different series. This wide variation is attributed to differing patient populations, differing techniques and differing methods of evaluation.

This brings the question of why dysphagia occurs in the first place. In the early post-operative days it is easy to imagine a certain degree of swelling associated with lower esophageal dysfunction, causing some difficulty in swallowing. In the longer term, the simplest explanation for dysphagia would be that the fundoplication is too tight. Several studies, however, suggest that the degree to which the LES region can be opened does not correlate with dysphagia[19-21].

By analyzing the activity values of the 9 patients in Visick groups II and III at two months after surgery, we found a significant increase in the activity of the so-called energy-enzyme values and in the concentration of structural and myofibrillar proteins as compared to the Visick group I patients. This fact also calls attention to the great individual differences of metabolic changes in the LES muscles of reflux patients. The developing hypertrophy of LES muscles may be a reason for complaints after anti-reflux surgery, persistent dysphagia in particular. It is not yet known when these metabolic changes begin to develop following reflux periods, and indeed whether these changes are reversible. It is readily evident, however, that interpatient variability in these metabolic/biochemical changes' rates of occurrence represents a prognostic barrier in the treatment of GERD, and that a "standard" mechanical wrap is not sufficient to recover the highly complex, neurohormonally controlled function of the LES in all patients.

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• *BRIEF REPORTS* •

Sofalcone, a mucoprotective agent, increases the cure rate of *Helicobacter pylori* infection when combined with rabeprazole, amoxicillin and clarithromycin

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CONCLUSION: The addition of sofalcone, but not polaprezinc, significantly increased the cure rate of *H pylori* infection when combined with the rabeprazole-amoxicillin-clarithromycin regimen.

Key words: *Helicobacter pylori* ; Sofalcone; Mucoprotective agents

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Abstract

AIM: The mucoprotective agents, sofalcone and polaprezinc have anti-*Helicobacter pylori* (*H pylori*) activities. We determined the therapeutic effects of sofalcone and polaprezinc when combined with rabeprazole, amoxicillin and clarithromycin for *Helicobacter pylori* infection.

METHODS: One hundred and sixty-five consecutive outpatients with peptic ulcer and *H pylori* infection were randomly assigned to one of the following three groups and medicated for 7 d. Group A: triple therapy with rabeprazole (10 mg twice daily), clarithromycin (200 mg twice daily) and amoxicillin (750 mg twice daily). Group B: sofalcone (100 mg thrice daily) plus the triple therapy. Group C: polaprezinc (150 mg twice daily) plus the triple therapy. Eradication was considered successful if ^{13}C-urea breath test was negative at least 4 wk after cessation of eradication regimens or successive famotidine in the cases of active peptic ulcer.

RESULTS: On intention-to-treat basis, *H pylori* cure was achieved in 43 of 55 (78.2%) patients, 47 of 54 (87.0%) and 45 of 56 (80.4%) for the groups A, B and C respectively. Using per protocol analysis, the eradication rates were 81.1% (43/53), 94.0% (47/50) and 84.9% (45/53) respectively. There was a significant difference in the cure rates between group A and B. Adverse events occurred in 10, 12 and 11 patients, from groups A, B and C respectively, but the events were generally mild.

INTRODUCTION

Helicobacter pylori (*H pylori*) causes type B chronic gastritis and plays a critical role in the pathogenesis of peptic ulceration[1,2]. Eradication of *H pylori* infection facilitates ulcer healing and prevents recurrence[3-5]. In the quest for optimal choice of drugs, dosage and duration, various therapeutic regimens have been studied extensively during the past few years. Among these, short-term low-dose triple therapy, comprising of one proton pump inhibitor and two antimicrobials from the choice of clarithromycin, amoxicillin and metronidazole, is currently considered the gold standard regimen[6,7]. The new proton pump inhibitor-based triple therapies provide high eradication rates, which are generally more than 80%. In fact, we reported that a 7-d course of rabeprazole, a novel potent proton pump inhibitor, 10 mg b.d. plus amoxicillin 750 mg b.d. and clarithromycin 200 mg b.d. showed satisfactory results[8]. Nevertheless, there are still some disadvantages to be addressed such as drug resistance when considering the PPI-based triple therapies[9-13]. With the rising prevalence of resistance of *H pylori* to metronidazole or clarithromycin, failure rates of the PPI-based regimens are expected to increase[12,13].

A variety of gastric mucoprotective agents have been used as anti-ulcer drugs, usually in combination with antacids, in the upper gastrointestinal tract[14]. Among these, rebamipide, ecabet sodium, sofalcone, polaprezinc, plaunotol and sucralfate are uncomplicated by drug resistance and have anti-*H pylori* activities[14-21]. Therefore, the theoretical rationale for adding such mucoprotective drugs to ordinary

eradication regimens is that these may meet demands of improved treatment outcome without the development of resistance.

There are several reports on the additive effects of mucoprotective drugs in eradication regimens, but most of the study designs were not randomized and the sample sizes were limited[14,22-27]. In addition, there is little information on the most effective mucoprotective drugs. This prospective, randomized study was designed to determine whether the inclusion of sofalcone or polaprezinc increases the cure rate of *H pylori* infection when combined with the rabeprazole-amoxicillin-clarithromycin therapy.

MATERIALS AND METHODS

Patients

The present study was designed as a prospective, open, randomized and controlled trial, which was performed between January 1999 and December 2002. The study was conducted according to Good Clinical Practice and the Declaration of Helsinki. All patients gave informed consent prior to their inclusion in the study.

The patient population comprised 165 consecutive outpatients with peptic ulcer and *H pylori* infection. Exclusion criteria were: age <18 years, pregnancy or lactation, severe concomitant diseases, previous medications effective against *H pylori* such as bismuth compounds, proton pump inhibitors, or antibiotics during the last 3 mo, alcohol abuse, drug addiction, chronic corticosteroid or nonsteroidal anti-inflammatory drug use, and previous gastroduodenal surgery. Information on alcohol intake and smoking habits was obtained at entry into the study. Ex-smokers and social drinkers were considered as nonsmokers and nondrinkers respectively.

Diagnosis of H pylori infection[28,29]

The presence of *H pylori* was confirmed by serology (anti-*H pylori* Immunoglobulin G antibody, HEL-p TEST, AMRAD Co., Melbourne, Australia), rapid urease test (Helicocheck, Otsuka Pharmaceutical Co., Tokushima, Japan) and histology (Giemsa staining) using two biopsy specimens obtained during endoscopy from each the antrum (within 2 cm of the pyloric ring) and the corpus (along the greater curvature). Patients were considered to be infected with *H pylori* when at least two of these examinations gave positive results. Patients were classified as *H pylori*-negative when all test results were negative. Patients who had only one positive result were not included.

Clinical trial

The enrolled patients were randomized by drawing a sealed envelope that contained pre-assigned treatment instructions. They were allocated to one of the following three groups and were medicated for 7 d: group A, which received rabeprazole 10 mg b.d., clarithromycin 200 mg b.d. and amoxicillin 750 mg b.d.; group B, which received sofalcone [2'-carboxymethyl 4, 4'-bis (3-methyl-2-butenyloxy) chalcone] 100 mg t.i.d., in combination with rabeprazole 10 mg b.d., clarithromycin 200 mg b.d. and amoxicillin 750 mg b.d.; group C, polaprezinc, zinc L-carnosine [N- -aminopropionyl-L-histidinato zinc (II) 150 mg b.d. in combination with rabeprazole 10 mg b.d., clarithromycin 200 mg b.d. and amoxicillin 750 mg b.d. Both the sofalcone and polaprezinc were prescribed as the standard daily dosage. If an active ulcer (defined as a circumscribed break in the mucosa measuring at least 5 mm in diameter with apparent depth and covered with an exudate[30]) was found at baseline endoscopy, it was treated with an H_2-receptor antagonist (famotidine 20 mg twice daily) for 4 wk after the eradication therapy. In cases of peptic ulcer scar, no other ulcer healing drugs were provided throughout the study. Participants returned at the conclusion of therapy for interview regarding adverse events. Compliance with medication was checked immediately after stopping treatment by counting the number of returned pills. Four weeks after cessation of eradication therapy, repeat endoscopy was performed to assess *H pylori* status by the rapid urease test and histology as before treatment. In patients with peptic ulcer in the active phase, the endoscopy-based tests were performed 4 wk after stopping famotidine (8 wk after the completion of eradication). Ulcer healing (defined as complete re-epithelialization) was assessed at the time of repeat endoscopy. Furthermore, we adopted the ^{13}C-urea breath test for the evaluation of *H pylori* cure 4 wk or longer after completion of treatment. In patients with active peptic ulcer, the ^{13}C-urea breath test was performed 4 wk or longer after completion of the ulcer treatment with famotidine (at least 8 wk after completion of the eradication therapy). The urea breath test was performed as described previously[31]. Briefly, ^{13}C-urea at 100 mg (Otsuka Pharmaceutical Co.) was dissolved in 100 mL of water. The test solution was ingested while in the sitting position, followed immediately by mouth rinsing. The patient was subsequently placed in the left decubitus position for 5 min and then in the sitting position for 15 min. Breath samples were collected, at baseline and 20 min after dosing and then analyzed using an isotope-selected nondispersive infrared spectrometer; UBiT-IR200 (Otsuka Electronics Co., Hirakata, Japan). The cut-off value was set at 2.5%; eradication of *H pylori* was considered successful if all test results were negative[31].

Statistical analysis

H pylori cure rate was evaluated by intention-to-treat (ITT) and per protocol (PP) analyses. ITT analysis included all enrolled patients and patients who dropped out were regarded as treatment failures. PP analysis included all patients who took at least 80% of each study medication as prescribed and returned for assessment of *H pylori* cure[24]. The cure rate was calculated together with 95% confidence intervals (CI). Statistical analyses were performed using the χ^2, Fisher's exact and Student's *t*-tests, as appropriate. A P value less than 0.05 was accepted as statistically significant.

RESULTS

The enrolled patients comprised 125 men and 40 women, with a mean age of 46 years (range, 21-73). They included 101 patients with gastric ulcer, 59 with duodenal ulcer and 5 with gastroduodenal ulcer. The peptic ulcer was in the

active phase in 87 patients. The baseline characteristics of the study population are listed in Table 1. The three treatment groups were well matched for gender, age, body weight, alcohol intake, smoking habits and baseline diagnosis. Of the 165 patients enrolled in this study, 4 patients (1 from group A, 1 from group B and 2 from group C) were lost to follow-up. Furthermore, 5 patients (1 from group A, 3 from group B and 1 from group C) were excluded from PP analysis as their compliance was less than 80%, leaving 156 patients for PP analysis.

Table 1 Patients' characteristics

	Group A[1]	Group B[1]	Group C[1]
Mean age, yr (range)	45.3 (21-73)	47.2 (27-71)	45.6 (21-71)
Male/female	41/14	42/12	42/14
Mean weight, kg (range)	60.7 (42.5-70.0)	59.8 (40.5-70.0)	58.7 (38.5-79.0)
Smokers (%)	23 (41.8)	28 (51.6)	24 (42.6)
Alcohol drinkers (%)	21 (38.1)	28 (51.6)	23 (41.1)
Gastric ulcer (%)	34 (61.8)	31 (57.4)	36 (64.3)
Duodenal ulcer (%)	19 (34.5)	21 (38.9)	19 (33.9)
Gastroduodenal ulcer (%)	2 (3.6)	2 (3.7)	1 (1.8)
Active ulcer (%)	27 (49.1)	32 (59.3)	28 (50.0)

[1]Group A: triple therapy with rabeprazole (10 mg twice daily), clarithromycin (200 mg twice daily) and amoxicillin (750 mg twice daily). Group B: sofalcone (100 mg thrice daily) plus the triple therapy. Group C: polaprezinc (150 mg twice daily) plus the triple therapy. Patients received the medications for 7 d in each group.

Table 2 shows ITT and PP *H pylori* eradication rates in each treatment group. In the ITT analysis, there were no significant differences in *H pylori* cure rates among the three groups, while the PP-based eradication rate in group B was significantly higher than that in group A ($P<0.05$). Background characteristics including age, gender, body weight, current tobacco use, alcohol intake and baseline diagnosis did not affect treatment outcome.

Table 2 Intention-to-treat and per protocol cure rates of *H pylori* infection

Group[1]	Intention-to-treat (%)	95% CI[2]	Per protocol (%)	95% CI
A	78.2 (43/55)	66.9-89.4	81.1 (43/53)	70.2-92.0
B	87.0 (47/54)	77.8-96.4	94.0 (47/50)	87.2-100.0
C	80.4 (45/56)	69.6-91.1	84.9 (45/53)	73.1-93.6

[1]See Table 1 for the definition of each group. Patients received the medications for 7 d in each group; [2]CI: Confidence intervals.

At the following endoscopy, ulcer healing was comparably observed in 24 out of 27 patients in group A (88.9%, 95%CI = 76.2-100%), 28 out of 32 in group B (87.5%, 95%CI = 75.4-99.6%) and 24 out of 28 (85.7%, 95%CI = 71.9-99.5%).

Adverse events were noted in 10 patients from group A, 12 from group B and 11 from group C. These included diarrhea or soft stools, heartburn, nausea and skin rash (Table 3), and resulted in discontinuation of treatment in one patient from group B due to skin rash. Overall, the adverse events were mild and self-limited. No significant differences in the incidence and proportion of adverse events were observed among the three groups.

Table 3 Adverse events in the three treatment groups (*n*, %)

	Group A[1]	Group B[1]	Group C[1]
Diarrhea or soft stool	8 (14.5)	7 (13.0)	9 (16.1)
Heartburn	1 (1.8)	4 (7.4)	1 (1.8)
Nausea	1 (1.8)	1 (1.9)	0 (0.0)
Skin rash	0 (0.0)	0 (0.0)	1 (1.8)
Total	10 (18.2)	12 (22.2)	11 (19.6)

[1]See Table 1 for the definition of each group. Patients received the medications for 7 d in each group.

DISCUSSION

The generally assumed mechanisms of action of mucoprotective agents involve up-regulation of gastric mucosal defense during the process of recovery from mucosal injury[14]. Some of these drugs are also reported to have anti-*H pylori* activities through different mechanisms[14-21]. Amidst this background, a series of trials have been carried out in which such mucoprotective agents were added to the original dual or triple therapy regimens in an attempt to improve the efficacy[22-27]. The meta-analysis study of Hojo *et al*[14], supported the view that the eradication rates can be enhanced by adding mucoprotective agents to dual therapies. However, such agents could not be employed as alternative drugs for antimicrobials such as metronidazole and clarithromycin as dual therapies plus any mucoprotective agents were still unacceptable for the treatment of *H pylori* infection[14,22,26]. On the other hand, Hojo *et al*[14], could not validate the positive effect of mucoprotective agents in triple therapies. While this lack of additive efficacy may be due to high eradication rates already provided by triple therapy regimens, most trials were not randomized and the sample sizes were limited. Thus, we sought to evaluate the efficacy of sofalcone or polaprezinc when combined with the new triple therapy. To our knowledge, this is the first prospective, randomized and controlled trial undertaken to determine which of the two mucoprotective agents produces better *H pylori* eradication.

Sofalcone is a type of flavonoid and a synthetic derivative of sophoradine isolated from the root of the Chinese medicinal plant *Sophora subprostrata*[32]. Besides its mucosal protective action, sofalcone has a direct bactericidal effect on *H pylori*, with a minimum inhibitory concentration of 55-222 μmol/L, anti-urease activity and reduces the adhesion of this organism to gastric epithelial cells[18,19]. Polaprezinc is an insoluble chelate compound consisting of a zinc ion and L-carnosine and exerts potent mucoprotective activities[33]. It also inhibits the growth of *H pylori*, in addition to its urease activity and adhesion to gastric mucin[17,25]. Thus, we expected additive effects of the two mucoprotective drugs in the original proton pump inhibitor-based triple therapy. This was the case in the inclusion of sofalcone, but not polaprezinc. A 7-d course of quadruple therapy consisting of sofalcone, rabeprazole, amoxicillin and

clarithromycin demonstrated satisfactory treatment outcome, *H pylori* eradication rate being not less than 94% on the PP basis. On the other hand, the co-prescription of polaprezinc did not improve the cure rate, which was comparable to the existing rabeprazole-amoxicillin-clarithromycin regimen.

Sakaki *et al*[24], reported that 7-d omeprazole-amoxicillin-clarithromycin combination therapy yielded satisfactory results by the addition of sofalcone. Moreover, adding this drug improved the eradication rate in dual therapy with lansoprazole and amoxicillin[34]. Kodama *et al*[23], reported that combination therapy of clarithromycin and sofalcone yield 69.2% of eradication rate without the incorporation of anti-secretory drug. Although the study designs were non-randomized and the sample sizes were not large enough to find any significant differences, it is of clinical importance that sofalcone may possess the ability to latently increase the efficacy in *H pylori* eradication.

In contrast to our study, the addition of polaprezinc to triple therapy with lansoprazole, amoxicillin and clarithromycin significantly improved the cure rate of *H pylori* in a randomized controlled trial[25]. Kuwayama *et al*[35] reported that this agent in combination with low dose metronidazole (750 mg/d) and amoxicillin (750 mg/d) produced a 100% *H pylori* eradication. The exact reason for this discrepancy remains unknown, but many factors may affect eradication efficacy such as physical structure of the patient, smoking habits, compliance in taking the therapeutic drugs, genetic predisposition of cytochrome p450 2C19, which metabolizes proton pump inhibitors, and frequency of strains resistant to antimicrobials[9-13,36,37]. In the present study, there were no significant differences in the baseline characteristics among the trial arms. The incidence of adverse events was comparable among the three treatment groups, affecting approximately 20% of patients, which is similar to previous data[28-30]. These adverse events were generally mild, causing discontinuation of therapy in only one patient. Thus, each treatment regimen was well tolerated, leading to excellent compliance in the current study. In addition, the action of rabeprazole is known to be less affected by genetic polymorphisms of the metabolic enzyme[38]. In this study, susceptibility of *H pylori* to antibiotics was not assessed, although we did endeavor to minimize the risk of antibiotic resistance by excluding patients who had taken medications, effective against the organism. However, Hoshiya *et al*[11], reported that based on the current wide use of clarithromycin, no less than 12.5% of their *H pylori* infected patients showed primary resistance to this agent. Clarithromycin resistance has been considered a key factor in determining the outcome of anti-*H pylori* therapies[10,12,13,28], and it might influence treatment results of the present eradication regimens incorporating this drug. Further studies should be conducted to determine the efficacy of mucoprotective agents in treating clarithromycin-resistant *H pylori*.

In conclusion, our results indicated that sofalcone provides a significant additive effect in the eradication of *H pylori* infection, when combined with rabeprazole, amoxicillin and clarithromycin. In contrast, polaprezinc inclusion did not improve treatment outcome of the original triple therapy. Each treatment arm yielded equally good acceptability and compliance. In clinical practice, further work should be conducted to identify the most effective mucoprotective agents with anti-*H pylori* activities when combined with existing eradication regimens.

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• *BRIEF REPORTS* •

A Thai family with hereditary pancreatitis and increased cancer risk due to a mutation in *PRSS1* gene

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Abstract

AIM: To investigate mutation of serine protease 1–cationic trypsinogen (CT, *PRSS1*) gene in members of a Thai family with hereditary pancreatitis and pancreatic cancer.

METHODS: Polymerase chain reaction and direct sequencing were performed to analyze the *PRSS1* gene in two members of the family affected by pancreatitis. Allele specific amplification (ASA) method was then developed to detect the mutation of the *PRSS1* gene in all available members of the family and normal control subjects.

RESULTS: A cytosine (C) to thymine (T) mutation at position 2441 (g.2441C>T) of the *PRSS1* gene, which results in a substitution of arginine by cysteine at position 116 (R116C) of CT, was identified by direct sequencing in both clinically affected members of the family but was not found in the unaffected member. This mutation, which might be arising from deamination of methylated cytosine in CpG dinucleotide of codon 116 (CGT>TGT), was also detected by the ASA method in the two affected members and a proband's brother but was not observed in unaffected members and 54 normal control subjects.

CONCLUSION: Autosomal dominant pancreatitis with increased cancer risk in the studied Thai family is most likely due to missense (R116C) mutation in the *PRSS1* gene.

Key words: *PRSS1*; Hereditary pancreatitis; Pancreatic cancer; Thai; R116C

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INTRODUCTION

Hereditary pancreatitis (HP; OMIM 167800) is an autosomal dominant disorder characterized by multiple episodes of acute pancreatitis, development of chronic pancreatitis and high incidence of pancreatic cancer (up to 40% of HP patients) with approximately 80% penetrance and variable expressivity[1-3]. Mutations in serine protease 1 or cationic trypsinogen (CT, *PRSS1*) gene have been initially identified as a causative mutation for HP[4,5]. *PRSS1* encodes CT protein, which plays a crucial role in food digestion at the duodenum. It is autoactivated to trypsin by cleavage of 8 amino acids, from alanine 16 to lysine 23 residues (APFDDDDK) next to the N-terminal signal peptide of 15 residues. The activated trypsin can then autolyze itself at the primary autolysis site, arginine 122[6,7].

Most of the 17 *PRSS1* mutations discovered, to date, occur within or near important enzymatic domains of CT and locate only in exons 2 and 3, and the promoter region of the *PRSS1* gene (http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/119620.html). The majority of mutation, accounting for approximately 70% of HP cases, affects CT at arginine 122 residue by changing either to histidine (R122H) in most cases or to cysteine (R122C) in rare cases. The second most often mutation, accounting for approximately 20%, is at asparagine 29, which is frequently changed to isoleucine (N29I) and rarely altered to threonine (N29T). The two most common *PRSS1* mutations (R122H and N29I) have been found in families with typical HP[4,8]. A16V and R116C are the mutations reported in several families with idiopathic chronic pancreatitis (ICP) but no history of HP with A16V having a low penetrance characteristic[9-12]. Other mutations identified in *PRSS1* include TCCdel-28, D22G, K23R, P36R, E79K, G83E, K92N, D100H, L104P, V123M and C139F. However, almost all of these mutations were individually reported in a single small family without history of HP, but with ICP[11-15].

The precise mechanism by which these *PRSS1* mutations cause HP disease remains unclear. However, it is believed to be a gain-of-function due to increased activity or stability of trypsin and/or decreased trypsin inactivation[7,16,17], leading to autolysis of the pancreatic tissue. Thus, the possible mechanisms may be: (1) enhanced autoactivation of trypsinogen by mutations at the autoactivation site (A16V,

D22G, K23R)[9,13,15], (2) stabilization of trypsin by N29I[16], and (3) disruption of the trypsin hydrolytic recognition site by R122H[16]. A recent report by Teich *et al*[18] has raised a novel mechanism of action for pancreatitis-associated trypsinogen mutations. Surprisingly, E79K markedly inhibited autoactivation of CT but, in contrast, could activate anionic trypsinogen encoded by *PRSS2*. These findings suggested that interactions between the two major trypsinogen isoforms might also play a role in the development of HP. It has been proposed that while gain-of-function mutations of *PRSS1* resulted in pancreatitis, loss-of-function mutations (Y37X and IVS2+1G>A) in the gene might provide protective effect against the disease[19].

Molecular study of HP patients has been carried out and reported mainly in Caucasian. There are limited data obtained from Eastern population, such as those obtained from Japanese, while studies of patients with chronic pancreatitis in Korea and Bangladesh could not show hot-spot mutations of *PRSS1*[20,21]. Here, we report a mutation of *PRSS1* in a Thai family with HP and pancreatic cancer.

MATERIALS AND METHODS

Patients

A 55-year-old Thai woman was admitted to Siriraj Hospital with 3-mo history of recurrent upper abdominal pain, jaundice and steatorrhea. Biochemical testing showed diabetes mellitus, whereas cholesterol, triglyceride and HDL-cholesterol were in normal ranges. Liver enzymes were elevated (SGOT 375 U/L and SGPT 634 U/L). Diagnostic imaging demonstrated a pancreatic pseudocyst and pancreatic duct stones at the head of pancreas. Endoscopic retrograde cholangiopancreaticography was performed and showed that the first part duodenum had generalized swelling and bulging papilla. Attempt to cannulate pancreatic duct was unsuccessful and patient underwent pancreato-jejunostomy. One year after being the operation, the patient developed adenocarcinoma of the head of pancreas from which she died shortly later without blood sample collection for genetic testing. Family history revealed two additional members with confirmed history of acute pancreatitis. Patient's son (III-2) and her younger sister (II-5) both suffered an episode of acute pancreatitis at the age of 15 and mid-30s, respectively. They are currently well without further episode, and after thoroughly examined by one of us (C.L.), no biochemical and radiographic evidence of pancreatitis could be demonstrated. Indeed, the son was the first member who became symptomatic. Moreover, the patient's mother died in her 60 s with unconfirmed diagnosis of "abdominal cancer". None of the symptomatic members consumed alcohol but there is a 44-year-old asymptomatic male (II-6) who had a drinking habit. There are 3 clinically symptomatic and 6 asymptomatic members of three generations in the pedigree of this family. The family consented for drawing blood in 2 affected and 6 asymptomatic members, while the proband did not give her consent before her death. Blood samples, from 54 persons who had no history of pancreatitis, were also collected as control subjects. This study was conducted in accordance with the Faculty of Medicine Siriraj Hospital Ethics Committee's guidelines according to the Helsinki Declaration.

DNA isolation and amplification by PCR

Genomic DNA samples from peripheral blood were prepared using standard phenol-chloroform extraction procedure. Nucleotide sequence of 390 bp encompassing exon 3 of the *PRSS1* gene was amplified by polymerase chain reaction (PCR) using a primer pair: PRSS1F (5'-TCCATGAGCAGAGAGCTTGAGGAA-3') and PRSS1R (5'-TGTGAGGATGGAGGGAAGTAGAAGGACT-3')[22]. PCR amplification was performed with 25 μL reaction volume containing 200 ng genomic DNA, 1× PCR buffer (QIAGEN, Germany), 0.2 mmol/L of each dNTP, 10 pmoL of each primer and 0.5 U *Taq* DNA polymerase (QIAGEN). The PCR product was generated by amplification under conditions as follows: the initial 5-min denaturation at 94 ℃, followed by 35 cycles at 94 ℃ for 30 s, 56 ℃ for 30 s, 72 ℃ for 30 s, and a final 7-min elongation step at 72 ℃. Aliquot (10 μL) of the PCR reaction was then loaded onto 25 g/L agarose gel to verify the size and quantity of the PCR product prior to sequencing.

DNA sequence analysis

The PCR products from all the samples were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Sequencing was accomplished by the use of the Applied Biosystems (ABI) Prism Big Dye Terminator Cycle Sequencing Reading Reaction Kit and ABI Prism model 310 DNA sequencer (PE Applied Biosystems, Foster City, CA). All the products were sequenced in both directions with the same forward (PRSS1F) and reverse (PRSS1R) primer pairs as used for the PCR.

Allele specific amplification

For subsequent determination of prevalence of R116C missense mutation in population, allele specific amplification (ASA) using two specifically designed primers, R116F-N (5'-ATCATGTTAATCAAGCCCTCCTGAC-3') for the wild-type allele and C116F-M (5'-ATCATGTTAATC-AAGCCCTCCTGAT-3') for the mutant allele, was performed in 54 normal control subjects (108 chromosomes). Each primer was included in a separate PCR mixture with a common primer, Ex3R (5'-AGCTCGTCTGGGTAGT-CGGCTGTGA-3') to yield an allele-specific 555-bp product. An internal control fragment of growth/differentiation factor 5 (*GDF5*) gene was amplified by a pair of primers, GDF-3F (5'-CTGAACCCAAGCCAGGACA-3') and GDF-3R (5'-GTACTCGTGGGGTGTGATG-3') producing a 206-bp product. Twenty-five microliters of PCR reaction mixture contained 200 ng genomic DNA, 1×PCR buffer (QIAGEN), 0.2 mmol/L of each dNTP, 20 pmoL of each allele-specific primer, 5 pmoL of both internal control primers and 0.5 U Hot Star *Taq* DNA polymerase (QIAGEN). The PCR cycling condition consisted of an initial denaturation step at 95 ℃ for 10 min, followed by 38 cycles of 94 ℃ for 20 s, 53 ℃ for 20 s and 72 ℃ for 45 s, with a final elongation step at 72 ℃ for 10 min. The PCR products were examined by agarose gel electrophoresis using a 25 g/L agarose gel run in 0.5× TBE and stained with ethidium bromide.

RESULTS

Exon 3 of *PRSS1* was amplified by PCR and directly

sequenced in two clinically affected members of the Thai HP family with HP. A heterozygous nucleotide substitution at position 2441 (g.2441 C>T) was identified (Figure 1), resulting in an amino acid substitution of arginine (CGT) by cysteine (TGT) at residue 116 (R116C) of the encoded protein. ASA was then developed to specifically detect the mutation in all available 8 family members as well as 54 normal control subjects. The results of ASA analysis in the HP family (Figure 2) showed the presence of R116C mutation in 3 individuals, while screening in normal control subjects did not identify the mutation (data not shown).

In the 3 mutation-positive individuals, 2 individuals (sister and son of the proband) had history of acute pancreatitis and 1 individual (younger brother of the proband) remained asymptomatic with an alcohol drinking habit.

DISCUSSION

CT is a trypsin precursor peptide that is synthesized within human pancreatic acinar cells and auto-activated to be trypsin by removing its signaling peptide portion of 23 amino acids. HP has been hypothesized to be caused by increased trypsin activity and/or decreased trypsin inactivation, leading to excessive trypsin in the pancreas and, ultimately, pancreatic autolysis. Mutations in *PRSS1* have been initially identified as the cause of this disease since 1996[4]. To date, a total of 17 *PRSS1* mutations that cause chronic pancreatitis have been described and reported (Figure 3). R122H is the first mutation identified by Whitcomb *et al*[4], and has been most frequently identified in more than 100 unrelated patients worldwide. N29I is the second most frequent mutation identified in more than 50 unrelated patients[8]. Comparison of patients with the two most common mutations has shown that the patients with R122H have younger age at onset and more requirements of surgical interventions than those with N29I[23,24].

This study was initially aimed to analyze the two most common mutations, R122H and N29I, of *PRSS1* gene in a Thai family with clear features of HP. The expected mutations were not found in the affected subjects. However, another missense mutation causing a replacement of arginine (CGT) with cysteine (TGT) at the 116th residue of CT, R116C, was identified. This mutation was initially reported as an allelic variant to GenBank as TRYP1-R116C allele (accession number AF315310). Screening of 54 normal control subjects (108 chromosomes) revealed no such change, supporting its pathogenicity. In addition, R116C is a known mutation previously reported on 3 occasions in a total of 8 patients: in two unrelated ICP patients from France[10]; in a Turkish family affected by atypical HP (only two of four members who carried this mutation in one allele developed ICP)[11]; and in a German family whose autosomal dominant inheritance of chronic pancreatitis was not clear (R116C was detected in unaffected mother and her affected young daughter)[12]. These data have demonstrated that R116C might lead to chronic pancreatitis with only low penetrance. In the present work, R116C was found in two symptomatic individuals (III-2 and II-5) and one asymptomatic member (II-6) of a Thai family. It may be possible that the disease in the latter member has not been developed or its penetrance is incomplete.

We believe that R116C occurs by deamination of cytosine in CpG dinucleotide, a well-known phenomenon that causes the C>T change. An argument can be made about the true pathogenicity of R116C since the arginine residue at position 116 is not conserved among species. However, as it has

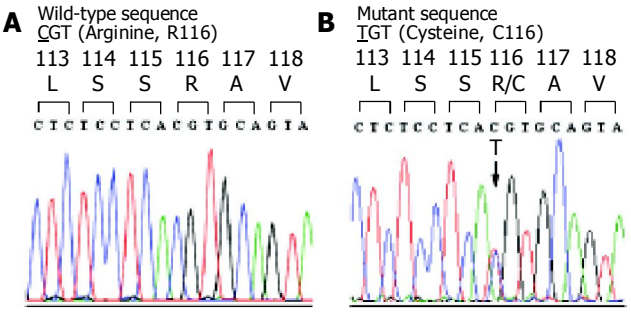

Figure 1 Chromatogram of DNA sequences in exon 3 of ***PRSS1*** gene in a normal control (A) and a patient with chronic pancreatitis (B). The tracing of the patient reveals a heterozygous missense mutation, g.2441C>T or c.346 C>T, which would result in a substitution of arginine (CGT) by cysteine (TGT) at amino acid residue 116 (R116C).

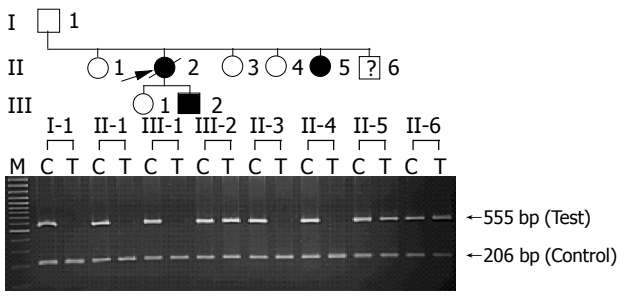

Figure 2 Allele specific amplification (ASA) analysis of R116C in the HP family. In two affected (II-5 and III-2) and one unaffected (II-6) individuals, two bands (555 bp) amplified from both wild-type (C) and mutant (T) alleles are shown as heterozygous genotype, whereas only wild-type alleles (C) are present in the rest of unaffected individuals (I-1, II-1, II-3, II-4 and III-1). PCR products of 206 bp are shown as internal control in all lanes.

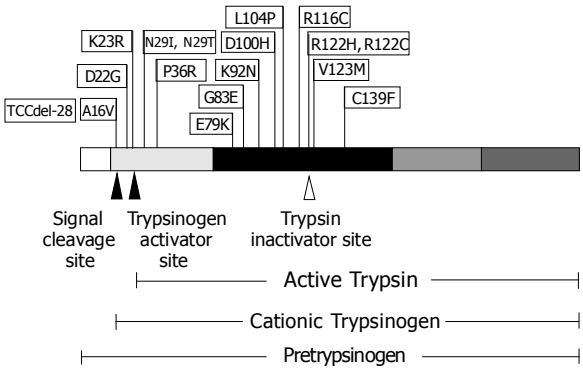

Figure 3 The schematic diagram of ***PRSS1*** mutations discovered, to date, and their positions on the encoded protein. ***PRSS1*** protein is shown with five regions, each of which encoded by a different exon, depicted with varying shaded blocks. The arrowheads represent the proteolysis cleavage sites of the protein resulting in various forms of trypsin biogenesis.

been shown from a recent computational analysis of mutational spectrum of 436 genes in the human genome, pathogenic mutations can indeed occur in non-conserved region of the gene[25]. Moreover, the nature of amino acid alteration in R116C (from non-sulfhydryl to sulfhydryl one) is considered to be far more radical than what usually observed among species[26]. Therefore, R116C is capable of being pathogenic from a functional point of view.

The mechanism of how R116C causes pancreatitis remains unclear. Computer modeling by Tautermann and colleagues[11] demonstrated the decreased chain flexibility of residues 112-118 of the CT molecule caused by the presence of cysteine at position 116. It is conceivable that the R116C mutation may have an effect on the motion of the semiflexible side chain containing R122 by interfering with the movement of oligopeptide chain into hydrolysis position and preventing an attack to the primary autolysis site at R122, resulting in an increased stability of trypsin[10]. Another potential mechanism is that the mutation results in disulfide-bridge formation with another proximal cysteine residue in the tertiary structure of trypsinogen polypeptide and, thus, plays a prominent role in increasing protein stability and decreasing its autolysis. However, none of these hypotheses has been critically studied.

The clinical manifestation of pancreatitis in this family merits some comments. Firstly, this is the first report of a family fulfilling the criteria for HP that has been shown to be due to R116C. The other previous three reports were mostly from sporadic symptomatic cases[10-12]. Our family confirms the dominant nature of this particular mutation. Secondly, this report exemplifies the intermittent nature of symptom shown in most family members rather than the chronic persistent symptom found in most HP. This phenomenon may not be common for all mutations and may be specific to this R116C mutation. This remains to be elucidated with further reports of similar and different mutations. Thirdly, the proband was later diagnosed with pancreatic cancer that was also suspected in her mother. This is contrary to the increased risk of pancreatic cancer found especially in the paternally inherited case[27]. If the diagnosis was certain, this might imply high cancer risk for this particular mutation that is different from the R122H mutation, which has negative contribution to the pathogenesis of a substantial fraction of pancreatic cancer[28]. It is well known that allelic heterogeneity of mutation may give rise to different clinical phenotypes. Cancer risks may therefore vary among different *PRSS1* mutations. This may eventually lead to different choices of surgery depending upon the type of mutation and its associated cancer risk. Lastly, this family demonstrates variable age of onset, as well as incomplete penetrance for HP. These characteristics make diagnosis of HP in the first case difficult. From our experience with this family, there may be a benefit in justifying molecular testing in proband with very young age even though the history is not consistent with typical chronic pancreatitis.

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• *BRIEF REPORTS* •

Serum IL-6, TNFα and CRP levels in Greek colorectal cancer patients: Prognostic implications

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Abstract

AIM: The significance of preoperative serum IL-6, TNFα and CRP levels in the progression of colorectal cancer (CRC) has not been fully elucidated. Our intention was to investigate their role and identify their prognostic significance.

METHODS: The IL-6, TNFα and CRP levels were measured in 74 CRC patients and the relationships between their elevations and both the clinicopathological factors and prognosis of patients were investigated. Serum concentrations of human IL-6 and TNFα were determined by enzyme-linked immunosorbent assay (ELISA). CRP was measured by an immunoturbinometric method.

RESULTS: Median IL-6, TNFα and CRP levels were significantly higher in CRC patients than in normal controls. High levels of serum IL-6, TNFα and CRP were correlated with larger tumor size. Furthermore, high IL-6 and high CRP levels were associated with reduced overall survival.

CONCLUSION: Serum IL-6, TNFα and CRP levels definitely increase in CRC patients. Pre-operative serum elevation of IL-6 and CRP was thus found to be predictor of the prognosis of CRC patients. The clinical value of TNFα in CRC needs to be further investigated.

Key words: IL-6; TNFα; CRP; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) represents a significant cause of morbidity and mortality worldwide. Currently available chemotherapy for the disease remains unsatisfactory, particularly in patients with advanced CRC. Previous attempts to use immune therapy to treat CRC did not seem to be very effective either[1]. The anti-tumor immune response is regulated by several factors, including cytokines produced by the tumor and other cells of tumor stroma[2]. Cytokines can modulate expression of tumor antigens, adhesion molecules and production of immunosuppressive factors by tumor cells. It is plausible that the local cytokine microenvironment, acting on the tumor cell or on the adjacent cells, can either block or facilitate tumor growth.

Cytokine regulation of human CRC is not clearly understood. However, it has been postulated that pro-inflammatory cytokines strongly influence the immunological status of CRC[3]. Among them, IL-6 and TNFα can initiate the innate immune response by inducing the acute phase of inflammation[4,5]. Additionally, IL-6 also appears to be involved in malignant transformation, tumor progression and tumor-associated cachexia, as reported in studies on Kaposi's sarcoma[6], multiple myeloma[7], renal cell carcinoma[8], prostate cancer[9], ovarian cancer[10] and breast cancer[11]. TNFα is also an important factor in the tumor microenvironment in human CRC. However, the role of TNFα in the local regulation of tumor growth is unclear. Barth *et al*[12] reported that better survival of CRC patients was associated with a larger number of TNFα expressing cells than in normal mucosa. However, TNFα may also play a negative role, favoring the growth of colorectal cancer by enhancing neo-vascularization and tumor metastases and down regulating the cell-mediated immune response by induction of soluble mediators such as IL-10[13-15].

Furthermore, C-reactive protein (CRP), a protein synthesized in the hepatocytes, has also been reported to be related both to the malignant potential of the neoplasms and to physical cachexia[16]. CRP belongs to the family of acute phase proteins, which are up regulated by cytokines, such as IL-6 and TNFα[17]. Studies in patients with colorectal cancer indicated that those with elevated serum CRP

concentrations had poorer prognoses than those whose CRP level were not increased. In addition, it has been suggested that increased CRP was associated with more frequent local tumor invasion[18], more advanced pathologic stage[19] and a higher rate of recurrence[20,21].

In light of the above, the purpose of our study was to measure the serum levels of IL-6, TNFα and CRP in Greek patients with colorectal cancer and to analyze these levels in relation to the clinicopathological findings.

MATERIALS AND METHODS

Patients

The study population consisted of 74 consecutive patients, who underwent resections for local colorectal cancer lesions, from September 2001 to September 2003 at D´Department of Surgery, Athens University Medical School. Patients with concomitant diseases (e.g., infectious diseases, inflammatory bowel disease, autoimmune conditions, allergy, asthma, *etc.*) capable of raising the serum levels of IL-6, TNFα, and/or CRP were excluded for the study. The patients ranged from 33 to 86 years of age (mean±SD: 66.83±10.45 years, mean±SE: 66.83±1.24 years) and consisted of 39 males and 35 females. All patients gave their informed consent and the hospital review board approved the study. The colorectal cancer was located in the cecum and ascending colon in 16 patients (21.62%), the transverse colon in 3 (4.05%), descending colon and sigmoid colon in 23 (31.1%), rectum in 24 (32.43%) and rectum and sigmoid colon in 8 (10.81%). The primary cancers of all patients were excised. Seventy-four patients had been followed up till April 2004 or death. The median time (±SD, ±SE) of follow-up and the follow-up range were 18.57 (±8.61, ±1.017) mo and 1-32 mo respectively.Twenty-six patients were lost to follow-up. The following parameters were recorded in all patients: age, sex, Dukes' staging[22], degree of histologic differentiation (good, moderate, or poor) and number of metastatic nodes. Serum samples from 25 sex- and age-matched normal healthy individuals were used as controls.

Serum samples

Blood samples were obtained before surgery to determine the serum concentration of IL-6, TNFα, and CRP. For all patients tumor marker serum levels [carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), cancer antigen 125 (CA 125), and tissue polypeptide antigen (TPA)] were also determined. The blood samples were centrifuged at 3 000 r/min for 5 min. Then the serum was removed and stored at -80 ℃ until biochemical analysis.

Biochemical determinations

Serum concentrations of human IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN). The limit of detection of the assay was 0.7 pg/mL. Because the cut-off point (ca. 8 pg/mL) was around the median value, we assigned patients with values less than 8 pg/mL to the low-level IL-6 group and those with equal or more than 8 pg/mL to the high-level IL-6 group. TNFα concentrations in sera were also determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN). The limit of detection of the assay was 0.12 pg/mL. Because the cut-off point (CA 4.71 pg/mL) was around the median value, we assigned patients with values less than 5 pg/mL to the low-level TNFα group and those with equal or more than 5 pg/mL to the high-level TNFα group. CRP was measured by an immunoturbinometric method, using a commercial kit (Dade Behring GmbH, Marburg, Germany). The serum levels ranging from 0 to 7 mg/L were normal in this assay. Because the median value was 6.79 mg/L, we defined values of less than 7 mg/L as low-level CRP and values equal to or above 7 mg/L as high-level CRP. Serum CEA, CA19-9, CA125 and TPA concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN).

Statistical analysis

Parametric two-factor ANOVA was used to test the differences among patient specific variables with the IL-6, TNFα and CRP dichotomies as factors. These factors were compared versus patient categories in contingency table analysis, based on the chi-square distribution. The survival curves were made using the Kaplan-Meier method and comparison was with the long rank test. Correlations between examined markers were evaluated by Spearman nonparametric rank test (r). The P values obtained were 2-tailed and significant differences were assumed below $P = 0.05$. The analysis was aided by GraphPad InStat (version 3.00, GraphPad Software Inc., San Diego, CA) and SPSS v. 11 (SPSS Inc., Chicago, IL).

RESULTS

The clinicopathological characteristics of the 74 CRC patients are summarized in Table 1. Serum IL-6 levels in CRC patients (median value: 8.11 pg/mL, range 1.09-188.42 pg/mL) were significantly higher than those in normal individuals (median value: 3.52 pg/mL, range 0.45-9.96 pg/mL, $P<0.01$). Taking 8 pg/mL as the threshold level, the serum IL-6 concentration was elevated in 35 of 74 patients (47.3%). The maximum size of the tumor in the high IL-6 group (4.8±1.9 cm) was significantly larger than that in low IL-6 group (3.8±1.3 cm, $P = 0.05$). The IL-6 levels in CRC patients with lymph node involvement was 15.2±24.3 pg/mL and the levels in patients without lymph node involvement was 26.9±29.1 pg/mL. The difference was not significant. No significant correlations were found between IL-6 levels and the serum levels of CEA, CA19-9, CA125, TPA, histological type, differentiation, and Dukes' stage (Table 1). Nevertheless, patients with increased serum IL-6 concentrations showed a decrease in overall survival ($P<0.05$, Figure 1).

The serum levels of TNFα in CRC patients (median value: 4.84 pg/mL, range 1.46-292.13 pg/mL) were found notably higher than in normal subjects (median value: 2.07 pg/mL, range 0-4.71 pg/mL, $P = 0.01$). Taking 5 pg/mL as the threshold level, the pre-operative serum TNFα concentration was increased in 33 of 74 patients (44.6%). Similar to the elevated IL-6 group, the maximal tumor size in CRC patients with high TNFα concentrations (4.7±1.6 cm) was significantly larger than that in the group with low TNFα

Table 1 Clinicopathological characteristics of colorectal cancer patients associated with high and low serum IL-6, TNFα and CRP levels

	Low IL-6 <8 pg/mL (*n* = 39)	High IL-6 ⩾ 8 pg/mL (*n* = 35)	Low TNFα <5 pg/mL (*n* = 41)	High TNFα ⩾5 pg/mL (*n* = 33)	Low CRP <7 mg/L (*n* = 36)	High CRP ⩾7 mg/L (*n* = 38)
Age (yr)	66[1]	70[1,a]	69[1]	67[1]	66[1]	69[1]
Gender ratio (M:F)	19:20	20:15	21:20	18:15	16:20	23:15
Tumor location						
Cecum/ascending	7	9	7	8	6	10
Transverse	1	2	0	2	1	2
Descending/sigmoid	15	8	15	7	10	13
Rectum	10	14	10	14	13	11
Rectum/sigmoid	6	2	9	2	6	2
Differentiation						
Good	1	2	2	2	2	1
Moderate	33	26	33	26	26	32
Poor	5	7	6	5	8	5
Preoperative CEA (ng/mL)	4.3[1]	4.4[1]	4.6[1]	4.3[1]	4.8[1]	3.6[1]
Preoperative CA19-9 (ng/mL)	12[1]	9[1]	12[1]	10[1]	10[1]	12[1]
Preoperative CA125 (ng/mL)	11.9[1]	13[1]	11.9[1]	14[1]	13[1]	11[1]
Preoperative TPA (ng/mL)	42[1]	71[1]	44[1]	70[1]	42[1]	60[1]
Maximum size of tumors (cm)	3.8±1.3[a]	4.8±1.9[a,2]	3.8±1.6[2]	4.7±1.6[a,2]	3.7±1.2[2]	4.8±1.9[2,b]
Growth characteristics						
Ulcerative	12	19	25	13	15	23
Protruding	27	16	16	20	21	15
Lymph node metastasis						
Positive	21	14	23	12	18	17
Negative	18	21	18	21	18	21
Dukes' stage (%)						
A	7 (54)	6 (46)	9 (69)	4 (31)[a]	10 (67)	5 (33)[a]
B	11 (46)	13 (54)	7 (29)	17 (71)	17 (59)	12 (41)
C	19 (63)	11 (37)	20 (67)	10 (33)	8 (35)	15 (65)
D	2 (29)	5 (71)	5 (71)	2 (29)	1 (14)	6 (86)

CRP, C-reactive protein; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; CA 125, cancer antigen 125; TPA, tissue polypeptide antigen. [1]Values are median, [a]P<0.05 *vs* controls, [2]Values are±SD, [b]P<0.01 *vs* low CRP group.

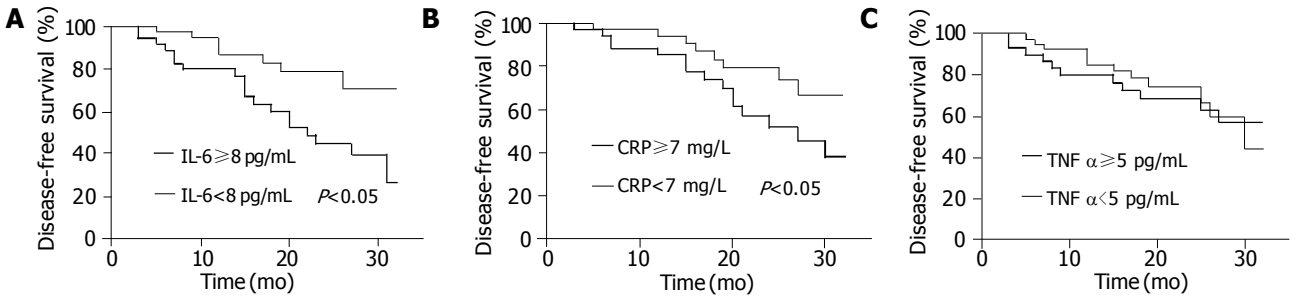

Figure 1 Overall disease-free survival of CRC patients stratified by IL-6, TNFα and CRP pre-operative serum concentrations. A: Disease-free survival in patients with IL-6<8 pg/mL; B: Disease-free survival in patients with CRP<7 mg/L; C: Disease-free survival in patients with TNFα<5 pg/mL.

levels (3.8±1.6 cm, P<0.05). Additionally, no significant difference in TNFα levels was observed between the CRC patients with lymph node involvement (6.6±8.7 pg/mL) and those without (16.7±46.2 pg/mL). The relationship of TNFα to serum level of CEA, CA19-9, CA125, TPA, histological type, differentiation, or overall survival, was not significant. Interestingly, a significant ratio of patients in stage B of Dukes' classification has showed elevated pre-operative levels of circulating TNFα (Table 1, Figure 1).

Serum CRP levels in CRC patients (median value: 6.79 mg/L, range 0.3-182 mg/L) were also elevated in CRC patients compared to the controls (median value: 3 mg/L, range 0.2-5.72 mg/L, P<0.05). Taking 7 mg/L as the threshold level, the pre-operative elevation of the serum CRP value was recognized in 38 patients (51.3%), whereas no such elevation was recognized in 36 (48.7%) patients. The maximal size of the tumors in the high CRP group (4.8±1.9 cm) was significantly larger than in the low CRP group (3.7±1.2 cm, P <0.01). As indicated in Table 1, no significant difference was observed between the proportion of histopathologically detected lymph node metastasis, differentiation, histological type, and serum levels of CEA, CA19-9 and CA125. However, TPA levels were significantly increased in the high CRP group *vs* the low CRP group (P<0.05). Notably, the ratio of stage C and D by Dukes' classification was significantly increased in the group with

elevated CRP (Table 1). The overall survival-rate in high CRP group was considerably more unfavorable than those in low CRP group ($P<0.05$, Figure 1).

Regarding quantitative associations between the examined markers, IL-6 levels were positively correlated with those of TNFα ($r = 0.55$, $P = 0.01$), and CRP ($r = 0.62$, $P = 0.01$).

DISCUSSION

Currently, there is ample literature regarding the role of various cytokines in CRC patients. IL-6 expression has been reported as secondary to a wide spectrum of malignancies and its clinical importance is being increasingly recognized[23]. In agreement with earlier studies, IL-6 levels in our CRC patients cohort were also found to be elevated[23-25].

The mechanisms leading to IL-6 induction and to IL-6 presence in high concentrations in the serum of those patients, include CEA-induced IL-6 production by Kupffer cells, malignancy-related chronic stress leading to increased IL-6 blood concentrations as well as direct IL-6 production and secretion by tumor-associated macrophages or the tumor cells themselves[23,24,26]. IL-6 appears to enhance tumorigenesis by a paracrine or autocrine mechanism and to have an inhibitory effect on the anti-tumor immune response[27]. Also the clinical significance of IL-6 pre-treatment levels has already been previously evaluated[24,25,27]. According to those authors, IL-6 concentrations reflected disease status, and were commonly associated with metastatic disease[24,25,27].

Our study, in a relatively limited number of CRC patients, failed to reveal a significant association with tumor stage. Moreover, we found that more patients with Dukes' stage C had low IL-6 levels, although the difference was not significant. However, our cohort contained only a small number of Duke's D patients. In the latter, 71% were found to have high IL-6 levels. Additionally, large tumor size correlated with elevated IL-6 levels. The aforementioned are partially consistent with previous observations that tumor burden and liver metastasis lead to higher IL-6 levels[27]. Liver metastasis may lead to hepatic dysfunction and subsequent decreased capacity for clearance of this cytokine[27]. One should not overlook the influence of different cut-off values used in the assessment of serum IL-6, as well as the ethnic origin of CRC patients in different studies.

Notwithstanding, in agreement with previous studies, serum IL-6 pre-operative levels were predictive of unfavorable prognosis with regard to CRC patients' survival[11,27,28]. Moreover, different from the prior studies, the fact that our cohort comprised more of non-metastatic stages strengthens the clinical relevance of this observation. In other words, IL-6 levels may be used as an adverse prognostic marker in the absence of advanced metastatic disease. Its independent prognostic significance, though, cannot be supported by our study since multivariant analysis was not performed due to the sample size. However, larger patients study-population and stage sub-groupings may aid in the more accurate evaluation of the cytokine's clinical significance.

TNFα has been detected in a number of different tumor types. Human epithelial tumor cells and infiltrated macrophages have been found to express TNFα protein. In certain malignancies increased production of TNFα correlated with worse prognosis[29]. On the other hand, high expression of this marker in Dukes' C CRC Japanese patients was a favorable indicator of prognosis[3]. Another study reported that, among other cytokines, TNFα was significantly elevated in stage B patients prior to surgery[30]. The latter is in keeping with our results, although a satisfactory explanation might not be easily offered. The relation to tumor- size but not other adverse histopathological variables could be related to the fact, that larger tumors may trigger a more potent immunological response manifested by the circulation of proinflammatory cytokines such as TNFα. It should be acknowledged though that the biological and clinical role of TNFα in CRC requires further elucidation.

The only parameter of the current study that demonstrated a clear association to the disease stage was CRP. It has also been suggested that an elevated pre-operative CRP may be an indicator of the malignant potential of the tumor and predictor of unfavorable prognosis in CRC patients. In accordance with the findings of Nozoe *et al*[19], our results have shown that the prognosis of patients without a pre-operative elevation of serum CRP level proved to be considerably better than that of patients with such an elevation. Tumor progression, among other pathophysiological sequelae, might lead to the development of an acute phase protein response, which is observed as an increase in CRP levels. Similar to the presently proved IL-6 prognostic significance as far as it concerns patients' overall survival, CRP elevation may be considered an unfavorable indicator. In spite of the usual clinical strategy to use tumor markers such as CEA and CA19-9 in determining disease progression, CRP may play an important role in signifying advanced disease.

Our study also shows that serum IL-6 levels are correlated with serum CRP levels. Apart from CRC, such a correlation has been observed in various malignancies[31]. IL-6 may play a critical role in controlling the production of acute phase proteins in liver cells and modulating host biological responses[27]. The observed inter-relation between IL-6 and TNFα could also be partially explained by the common denominator governing their production, i.e., the triggering of the malignancy-induced immunologic response.

The present study showed that IL-6, TNFα and CRP levels definitely increase in CRC patients. Although earlier observations of the IL-6 relation to disease stage were not fully replicated here, the current study in a Greek population adds to the growing evidence that IL-6 and CRP preoperative serum concentrations are correlated to decreased patients' overall survival. Such markers could be considered as appropriate predictors of CRC patients' prognosis and, to a certain extent could provide valuable information when determining treatment strategies.

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• *BRIEF REPORTS* •

Impact of *Helicobacter pylori* infection on ghrelin and various neuroendocrine hormones in plasma

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Abstract

AIM: Ghrelin, an endogenous ligand for growth hormone secretagogue receptor, influences appetite, energy balance, gastric motility and acid secretion. The stomach is the main source of circulating ghrelin. There are inconsistent reports on the influence of *Helicobacter pylori* (*H pylori*) infection on circulating ghrelin levels. We sought to elucidate the relationship between ghrelin and various peptides in plasma, with special reference to *H pylori*.

METHODS: Plasma ghrelin levels were measured by radioimmunoassay in 89 subjects who were referred for upper gastrointestinal endoscopy, consisting of 42 *H pylori* infected and 47 uninfected ones. Plasma gastrin, somatostatin, leptin, insulin-like growth hormone 1 (IGF-1) and chromogranin A concentrations were also measured. Twelve patients were treated with anti- *H pylori* regimen.

RESULTS: Ghrelin circulating levels were greatly decreased in *H pylori*-positive than negative individuals (194.2±90.2 fmol/mL and 250.4±84.1 respectively, $P<0.05$), but did not significantly alter following the cure of infection (176.5±79.5 *vs* 191.3±120.4). There was a significant negative correlation between circulating ghrelin and leptin levels, as well as body mass index, for the whole and uninfected population, but not in *H pylori*-infected patients. Plasma ghrelin concentrations correlated positively with IGF-1 in *H pylori*-negative group and negatively with chromogranin A in the infected group. There were no significant correlations among circulating levels of ghrelin, gastrin and somatostatin irrespective of *H pylori* status.

CONCLUSION: *H pylori* infection influences plasma ghrelin dynamics and its interaction with diverse bioactive peptides involved in energy balance, growth and neuroendocrine function.

Key words: *Helicobacter pylori*; Ghrelin; Leptin; Gastrin; Insulin-like growth factor

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INTRODUCTION

Helicobacter pylori (*H pylori*) is the major cause of chronic gastritis and peptic ulcer disease[1,2]. Chronic infection leads to atrophic gastritis, which increases the risk of gastric adenocarcinoma[2]. It is well- documented that the immune and inflammatory response against *H pylori* affects various cell types in gastric mucosa that are important in acid homeostasis, such as somatostatin-producing, gastrin-producing, chief and parietal cells[3-5]. *H pylori* gastritis causes a reduction of mucosal somatostatin levels and hypergastrinemia[3,4]. Leptin, a protein primarily secreted by the adipose tissue known to suppress appetite and modulate energy expenditure[6], is also produced by the chief and parietal cells[5]. Furthermore, gastric leptin levels are higher in *H pylori*-infected than in uninfected subjects, although serum levels may not be altered[5,7]

Ghrelin is a 28-amino acid peptide recently identified in the stomach as an endogenous ligand for growth hormone secretagogue receptor[8]. In addition to its potent growth hormone releasing activity, ghrelin influences appetite, energy balance, gastric motility and acid secretion[9,10]. This hormone is primarily produced by X/A-like neuroendocrine cells in the oxyntic glands[11] and the stomach is the main source of circulating ghrelin[8-11]. To date, conflicting results have been reported regarding the influence of *H pylori* status on ghrelin dynamics[12,13]. In addition, there is little information on the relationship between circulating ghrelin and other neuroendocrine hormones during the infection.

In the present study, we sought to determine the plasma concentrations of ghrelin, as well as leptin, gastrin and somatostatin, with special reference to *H pylori* infection. In addition, we assessed the relationship between circulating ghrelin and insulin-like growth factor 1 (IGF-1), the principal mediator of growth hormone axis[14], and chromogranin A, a reliable marker of gastric neuroendocrine proliferation[15].

MATERIALS AND METHODS

Patients

The study- subjects were 89 patients referred for upper gastrointestinal endoscopy between June 2003 and October 2003. The study was approved by Nagasaki University Ethics Committee. All samples were obtained with written informed consent of the patients prior to their inclusion, in accordance with the Helsinki Declaration. The exclusion criteria were: age <18 or >80 years, pregnancy, body mass index (BMI) >30 kg/m^2, diabetes mellitus, systemic infection, thyroid and liver diseases, renal impairment, use of medications effective against *H pylori* during the preceding 3 mo, alcohol abuse, drug addiction, and chronic corticosteroid or nonsteroidal anti-inflammatory drug use. None had undergone gastrointestinal surgery.

We treated 12 *H pylori*-positive patients with 7-d triple therapy consisting of lansoprazole, amoxicillin and clarithromycin[16]. Four weeks after cessation of the treatment, fasting plasma samples were also collected.

Plasma ghrelin concentrations

On the day of endoscopy, blood samples were taken between 8 and 10 a.m., after an overnight fast, transferred into chilled tubes containing ethylenediaminetetraacetic acid-2Na and aprotinin, stored on ice during collection, centrifuged, plasma separated and stored at -80 ℃ until assay. Plasma ghrelin concentrations were measured in-house in duplicate by radioimmunoassay (RIA), as described previously[11]. This RIA system employs a rabbit polyclonal antibody raised against the C-terminal fragment of human ghrelin, and can measure both the acylated and des-acyl forms. The intra-assay coefficient of variation was 2.8% and inter-assay coefficient of variation was 3.1%[11].

Circulating anti-H pylori antibody and other peptide concentrations

Plasma anti-*H pylori* immunoglobulin (Ig) G antibody was assessed by an enzyme-linked immunosorbent assay kit (HEL-p TEST, AMRAD Co., Melbourne, Australia). The cut-off value was determined according to the protocol provided by the manufacturer. Fasting plasma concentrations of gastrin, somatostatin, leptin, IGF-1 and chromogranin A were commercially determined by RIA (Mitsubishi Chemical Co., Tokyo, Japan).

Detection of H pylori Infection

H pylori status was assessed by anti-*H pylori* Immunoglobulin G antibody, ^{13}C-urea breath test (UBiT, Otsuka Pharmaceutical Co., Tokushima, Japan) or rapid urease test (Helicocheck, Otsuka Pharmaceutical Co.) using biopsy specimens endoscopically taken from the antrum within 2 cm of the pyloric ring and the corpus along the greater curvature. Patients were considered positive for *H pylori* infection when two of these examinations yielded positive results. On the other hand, patients were defined as *H pylori*-negative if all test results were negative[17]. Eradication of *H pylori* was considered successful when ^{13}C-urea breath test became negative[16].

Statistical analysis

Statistical analyses were performed using Fisher's exact, χ^2, Student's *t*, Mann-Whitney *U*, Kruskal-Wallis, Spearman rank and Wilcoxon signed ranks tests, as appropriate. A *P* value of less than 0.05 was accepted as statistically significant. Data were expressed as mean±SD.

RESULTS

Patient demographics

The study population consisted of 22 patients with chronic gastritis, 12 benign gastric polyps, 10 with gastric ulcer, 8 duodenal ulcer, 5 reflux esophagitis and 32 subjects with normal mucosa of the upper gastrointestinal tract at endoscopy. They included 44 men and 45 women, with a mean age of 53 years (range, 19-80). They consisted of 42 *H pylori*-infected and 47 uninfected subjects. The two groups were matched for age, sex, alcohol intake, smoking habit and BMI (Table 1).

Table 1 Baseline characteristics of the two groups (mean±SD)

Parameters	*H pylori* positive (*n* = 42)	*H pylori* negative (*n* = 47)
Age (yr)	52.7±16.9	53.4±14.3
Sex (Male:Female)	20:22	24:23:00
Alcohol intake	20	21
Smoking habit	18	21
Body mass index	23.1±3.3	22.8±3.2

Plasma concentrations of other peptides and H pylori status

As shown in Table 2, plasma ghrelin was significantly lower in *H pylori*-positive than negative subjects ($P<0.05$), whereas gastrin concentrations were significantly higher in *H pylori*-positive than negative subjects ($P<0.005$). There were no significant differences in plasma leptin, somatostatin, IGF-1 and chromogranin A levels with reference to *H pylori* status.

Table 2 Plasma concentrations of various peptides with respect to *H pylori* (mean±SD)

Peptide	*H pylori* positive (*n* = 42)	*H pylori* negative (*n* = 47)	*P*
Ghrelin (fmol/mL)	194.2±90.2	250.4±84.1	<0.05
Gastrin (pg/mL)	133.5±309.1	66.7±130.2	<0.005
Somatostatin (pg/mL)	11.3±5.7	9.7±6.2	Not significant
Leptin (ng/mL)	5.2±3.9	4.6±2.9	Not significant
Insulin-like growth factor 1 (ng/mL)	176.1±71.6	204.3±61.9	Not significant
Chromogranin A (ng/mL)	21.4±29.0	13.8±7.2	Not significant

Student's *t*-test was employed in statistical analyses.

Correlations between plasma ghrelin levels and plasma concentrations of other peptides

As shown in Table 3, there was a significantly negative correlation between plasma ghrelin and leptin levels for the

Table 3 Correlations of ghrelin with other peptides in plasma in terms of *H pylori* status

	Gastrin	Somatostatin	Leptin	IGF-1	Chromogranin A
H pylori positive	0.015 (NS)	0.076 (NS)	-0.239 (NS)	0.064 (NS)	0.285 (P<0.05)
H pylori negative	0.025 (NS)	0.100 (NS)	-0.353 (P<0.05)	0.272 (P<0.05)	0.073 (NS)
Total	0.018 (NS)	0.086 (NS)	-0.318 (P<0.05)	0.097 (NS)	0.200 (NS)

Data are correlation coefficients and (P values) of the correlations. IGF-1: insulin-like growth factor-1, NS: not significant.

whole and the uninfected cohort (P<0.05 for each). Within *H pylori* positive cohort, however, no such correlation was observed. In the whole series, there were no significant correlations between plasma ghrelin concentrations and gastrin, somatostatin, IGF-1 and chromogranin A levels. However, circulating levels of ghrelin correlated positively with those of IGF-1 within the uninfected population and negatively with chromogranin A within the *H pylori*-infected group (P<0.05 for each). There was a significant positive correlation between plasma gastrin and chromogranin A levels (r = 0.293, P<0.05), possibly reflecting gastrin-induced enterochromaffin-like cell proliferation[15].

Relationship between plasma ghrelin levels and baseline parameters

For the whole group, plasma ghrelin concentrations correlated negatively with BMI (r = -0.286, P<0.05). The same correlation was also noted in the uninfected (r = -0.562, P<0.005), but not infected group (r = 0.018). Plasma leptin levels correlated positively with BMI (r = 0.543, P<0.0001), irrespective of *H pylori* status (r = 0.473, P<0.01 for *H pylori*-infected group and r = 0.585, P<0.0001 for uninfected group). Other baseline characteristics including age, sex, alcohol intake and smoking habit were not associated with plasma ghrelin concentrations or circulating values of other peptides.

Changes in plasma levels of ghrelin and gastrin following eradication of H pylori

Successful eradication of the organism was confirmed in 10 of 12 patients treated with anti-*H pylori* regimen. Post-cure plasma gastrin levels tended to decrease although insignificantly (from 131.9±107.6 pg/mL to 100.8±78.0 pg/mL). There was no significant change in plasma concentrations of ghrelin after cure of the infection (from 176.5±79.5 fmol/mL to 191.3±120.4 fmol/mL).

DISCUSSION

Ghrelin is produced in a variety of human tissues[9,10], but its messenger ribonucleic acid (mRNA) is most highly expressed in the stomach[18]. Plasma ghrelin levels decrease by as much as 65% after gastrectomy[18], and this is consistent with the findings of decreased plasma levels after gastric bypass surgery[19]. Thus, the stomach is the major source of circulating ghrelin[9,10,18,19]. Our results showed that plasma ghrelin concentrations were significantly lower in *H pylori*-positive than negative group, in contrast to the Turkish study[12]. There are several possible explanations for this disparity, including differences in radioimmunoassay protocols for ghrelin, inadequate assessment of *H pylori* status in their series, i.e., only by histology, leading to underestimation of infection, differences in study populations with respect to baseline diseases, race, nutrient status and dietary habits and sample size. However, Nwokolo *et al*[20], reported that plasma ghrelin levels significantly increased following cure of the infection. In addition, ghrelin mRNA expression and peptide production are significantly decreased in *H pylori*-colonized gastric mucosa of chronically infected Mongolian gerbils[21], which is a good rodent model for various aspects of *H pylori*-associated pathogenesis[22]. These findings and our results indicate that *H pylori* status has a negative impact on gastric and plasma ghrelin dynamics.

However, we did not observe any significant post-cure rise in fasting plasma ghrelin levels following the same post-treatment period as the British study[20]. One possibility of the inconsistent results is that they assessed six-hour integrated plasma ghrelin (between 8:00 and 13:00) while we evaluated the value at only one point after an overnight fast. Another plausible explanation includes age-related differences in the severity or extent of gastritis, as the mean age of their population (36 years) was substantially lower than that of our population sample (53 years). Also, the likely possibility that there was a type 2 statistical error related to the small sample size might explain the negative results. Further larger study to compare the long-term effects of anti-*H pylori* therapy and placebo are warranted to elucidate the reversibility of ghrelin production.

Ghrelin has metabolic effects opposite to leptin. It stimulates food intake, enhances the use of carbohydrates and reduces fat utilization, contributing to weight gain[9,10]. Plasma ghrelin levels showed a diurnal rhythm over a 24-h period that was exactly in phase with that of leptin[9,10]. Consistent with these reports, we demonstrated here the significant negative associations between plasma ghrelin concentrations and leptin values and BMI within the *H pylori*-negative cohort. However, plasma ghrelin concentrations did not correlate with those of leptin, as well as BMI, within *H pylori*-positive group. Since ghrelin is predominantly secreted from the stomach[9,10,18,19], its products derived from other tissues are unlikely to be sufficient to compensate for the altered plasma ghrelin dynamics affected by inflammatory events associated with *H pylori* infection[13]. On the other hand, plasma leptin levels had strongly positive correlations with BMI, irrespective of *H pylori* status, as the primary contributor of circulating leptin is exclusively the adipose tissue[5,6].

Likewise, plasma ghrelin concentrations correlated positively with the circulating levels of IGF-1, which mediates most of the anabolic effects of growth hormone[14], within the uninfected population only. In line with this finding,

gastric ghrelin expression was found to depend on circulating IGF-1 levels in mouse models[23]. Muller *et al*[24], reported positive correlations among ghrelin, growth hormone and IGF-1 secretion in normal human volunteers, suggesting that ghrelin is potentially associated with linear growth, through the growth hormone-IGF-1 axis. Our results regarding the relationship between plasma ghrelin and IGF-1 levels, particularly in *H pylori*-positive cohort, may be relevant, since there have been conflicting data on the relationship between *H pylori*-infection and growth retardation in children[25-27].

In our study, there was no significant correlation between plasma ghrelin and gastrin levels, in agreement with the recent observation in human subjects of unaltered circulating gastrin concentrations following ghrelin administration[28]. In addition, increased levels of gastrin caused by omeprazole treatment failed to raise the level of ghrelin mRNA in oxyntic mucosa and circulating ghrelin concentrations in a rat model[29]. Based on these findings, we suggest that ghrelin production/release does not seem to operate under gastrin control.

Recent evidence has revealed that systemic infusion of somatostatin suppresses plasma ghrelin concentrations[30]. In the present study, however, there were no correlations between ghrelin and somatostatin levels both in *H pylori*-infected and uninfected settings. Although this hormone is released postprandially from somatostatin-containing cells into the blood stream[31,32], we assessed the values in fasting plasma, which might explain the lack of relationship between circulating levels of ghrelin and its potential regulator, somatostatin. In conclusion, we have demonstrated in the present study that circulating ghrelin levels were more decreased in *H pylori*-positive than in negative persons, albeit they did not alter after eradication of the organism. There were significant correlations of ghrelin with leptin, IGF-1 and chromogranin A in plasma, depending on *H pylori* status. Our results indicate *H pylori* infection may affect plasma ghrelin dynamics and be involved in energy homeostasis, growth and neuroendocrine function through the interaction with ghrelin.

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• *BRIEF REPORTS* •

A prospective cross-over study using a sphincterotome and a guidewire to increase the success rate of common bile duct cannulation

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Abstract

AIM: During endoscopic retrograde cholangiopancreatography (ERCP), selective cannulation of the common bile duct (CBD) is required in most of the cases.

METHODS: From June 2001 till December 2002, all patients referred to our unit for ERCP were considered for entry into the study. Selective CBD cannulation was first attempted with a standard catheter with or without the use of a guidewire. In cases, where CBD cannulation was considered unsuccessful, patients were crossed over to a double-lumen sphincterotome and a guidewire. All patients were hospitalized for 24 h after the procedure in order to assess the incidence of post-ERCP complications.

RESULTS: The study sample consisted of 158 patients. Selective CBD cannulation using a standard ERCP catheter with or without the assistance of a guidewire, was accomplished in 129 patients (success rate: 81.65%). From the 29 patients who were crossed over to a sphincterotome and a guidewire, selective CBD cannulation was achieved in 24; the overall success rate rising to 96.8%. Meanwhile, the use of this technique did not increase the incidence of post-ERCP complications.

CONCLUSION: The use of a sphincterotome and a guidewire increases the success rate of selective bile duct cannulation in cases that this has not been accomplished with a standard catheter.

Key words: Common bile duct; Cannulation; Sphincterotome; Guidewire

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INTRODUCTION

Selective cannulation of the common bile duct (CBD) is required, in the majority of cases when performing endoscopic retrograde cholangiopancreatography (ERCP). Experienced endoscopists, using a standard catheter, can achieve selective common bile duct cannulation, in most but not in all cases, with a success rate of approximately 65%[1-5]. Repeated unsuccessful attempts to cannulate, may result in trauma to the papilla; intramural injection and inadvertent pancreatic duct injections, increasing the risk of post-ERCP pancreatitis[2].

To increase the success rate of selective CBD cannulation several techniques have been described: additional use of guidewire precutting techniques and the use of sphincterotomes[1-3,6-11].

The aim of this prospective study was to determine whether the use of a sphincterotome with a guidewire increases the success rate of selective CBD cannulation and is a safe alternative in cases where cannulation has not been achieved with a standard catheter.

MATERIALS AND METHODS

From June 2001 to December 2002 all patients referred to our unit for ERCP were considered for entry into the study. We excluded patients who had a history of previous sphincterotomy or Billroth II gastrectomy, as well as patients who were found, during ERCP, to have an ampullary neoplasm.

All procedures were performed by the same experienced endoscopist using a side-viewing video endoscope (Fujinon ED-200XT; Tokyo, Japan). All patients received topical pharyngeal anesthesia (Xylocaine spray; Astra), as well as intravenous sedation with 3.0 to 6.0 mg of midazolam (Dormicum; Roche) and 150-500 mcg of fentanyl (Fentanyl; Janssen-Cilag). During ERCP, arterial oxygen saturation was continuously monitored by a pulse oximeter. In cases with an overactive duodenum, we also administered 20-40 mg

of hyoscine N-butylbromide (Buscopan; Boehringer Ingelheim).

In order to achieve CBD cannulation we first used a standard ERCP catheter (GLO-Tip, GT-1-T, Wilson Cook Medical Inc., Winston-Salem, NC, USA). Contrast medium was injected only once selective deep cannulation, in a direction strongly suggestive of the CBD, has been achieved. In cases that this was not accomplished, we used a straight tip Tracer-Hybrid (Wilson Cook Medical Inc., Winston Salem, NC, USA) guidewire to aid cannulation. To prevent excessive injury of the papilla, selective common bile duct cannulation was considered unsuccessful when five attempts using a standard catheter and ten more attempts using a guidewire through the catheter failed.

In those cases, where selective CBD cannulation was considered unsuccessful, patients were crossed over to a double-lumen 5 mm tip sphincterotome, with a 2.5 cm cutting-wire (CCPT-25-MONO Cotton cannulatome, Wilson-Cook Medical Inc., Winston Salem, NC, USA) and a guidewire (straight tip Tracer-Hybrid). No contrast medium was injected if we had not previously advanced the guidewire in a route strongly suggestive of the CBD. Once again, selective CBD cannulation was considered unsuccessful if 10 attempts to advance the guidewire failed.

Our technique consisted of the following steps:

(1) We brought the papilla as usual en face and inserted the tip of the sphincterotome, which has been preloaded with the guidewire, at the upper part of its orifice with or without slight flexion of the sphincterotome; (2) After complete flexion of the sphincterotome, we tried to push, very gently, the guidewire forward. We advanced the guidewire in several directions during slow progressive relaxation of the sphincterotome bow, under fluoroscopic control; (3) Whenever we managed to advance the guidewire in a direction strongly suggestive of the CBD, we pushed the sphincterotome over the guidewire a few centimeters further. After pulling back the guidewire, we injected contrast medium in order to ensure cannulation of the CBD; (4) In cases where we had difficulty in inserting the guidewire, we pulled the scope back with simultaneous step-by-step unflexing of the sphincterotome. This maneuver helped us to insert the tip of the sphincterotome slightly deeper into the papilla, onto the upper wall of the tortuous and folded ampullary channel. We then flexed the sphincterotome once again completely, trying to advance the guidewire during slow progressive relaxation of the sphincterotome.

Once selective CBD cannulation has been achieved, sphincterotomy, whenever needed, was performed using the same sphincterotome and applying 34 W of pure-cutting current from a commercially available electrosurgical generator (Plus II, Medical Systems, Teterboro, NJ, USA).

All patients were hospitalized for at least 24 h after the procedure in order to assess the incidence of post-ERCP complications. Pancreatitis was defined as serum amylase and lipase levels greater than two times the upper limit of normal, accompanied with abdominal pain. Serum amylase and lipase levels were determined 24 h after the procedure. Bleeding was defined as a drop in the hematocrit level greater than 5% or clinical signs of hemorrhage (e.g., melena)[12].

The study protocol accorded to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of our Hospital. All patients gave a written informed consent at the time of enrollment.

Statistical analysis

Values are expressed as mean±SD. A χ^2 test was used. A P value less than 0.05 was the accepted level of significance.

RESULTS

During the study period 202 patients were referred to our Unit for ERCP. Thirty-three of these patients have been subjected to sphincterotomy in the past, six had a history of Billroth II gastrectomy; 2 patients had evidence of an ampullary neoplasm; while in 3 patients ERCP was technically impossible due to inadequate sedation. Therefore, our study sample consisted of 158 patients. The characteristics of the study population as well as the reason they were referred for ERCP are presented in Table 1.

Table 1 Demographic characteristics of the study population and indications for ERCP

Mean age ± SD (yr)	63±11.9
Male / female	76/82
Indications for ERCP (number of patients, %)	
Documented or possible stones in CBD	132 (83.5)
Malignant obstruction	6 (3.8)
Miscellaneous	20 (12.7)

Selective common bile duct cannulation using a standard ERCP catheter, with or without the assistance of a guidewire, was accomplished in 129 patients (success rate: 81.65%). From the 29 patients who were crossed over to a sphincterotome and a guidewire, selective CBD cannulation was achieved in 24 (success rate: 82.75%). Therefore, with this technique, the overall success rate increased to 96.8%.

The endoscopic characteristics of the papilla in those 29 patients in whom cannulation with the use of a standard catheter, with or without the aid of a guidewire, was considered unsuccessful are presented in Table 2. A fleshy papilla was defined as a big, folded, oversized (>2 cm) papilla, protruding into the duodenum. A papilla was characterized as a floppy, if it was slippery when cannulation was attempted, due to loose tissue falling or protruding at the orifice site. Stenotic papilla was the one with a stenosis in the intraampullary distal CBD, evidenced before or after ERCP. An intra-diverticular papilla was the one lying inside the rims of a diverticulum. The success rate of selective CBD cannulation, among these patients, is also presented in Table 2.

Table 2 Endoscopic characteristics of the papilla in the 29 patients crossed over to a sphincterotome and a guidewire and success rate of selective CBD cannulation

Endoscopic characteristics of the papilla	Number of patients (n = 29)	Number of patients and success rate of CBD cannulation (n = 24, %)
Oversized fleshy papilla	19	18 (94.7)
Floppy papilla	5	4 (80.0)
Stenotic papilla	3	1 (33.3)
Intra-diverticular papilla	2	1 (50.0)

From those 5 patients, in whom selective CBD cannulation was unsuccessful even with the use of a sphincterotome and a guidewire, this was finally achieved with a precutting papillotomy, in 3. The remaining 2 patients were referred for percutaneous transhepatic cholangiography.

Post-ERCP pancreatitis developed in 8 out of 129 patients (6.2%) in whom cannulation was achieved with the use of a standard catheter with or without a guidewire and in 2 out of the 29 patients (6.9%) in whom CBD cannulation was achieved with the use of a sphincterotome and a guidewire. This difference was not statistically significant ($P>0.05$). None of our patients developed post-sphincterotomy bleeding and there was no incidence of perforation or evidence of a submucosal tract.

DISCUSSION

When performing ERCP, selective deep cannulation of the CBD is necessary in order to maximize the diagnostic and therapeutic benefits of the test, while diminishing the risk of post-procedure complications. The standard approach to selective CBD cannulation is the use of an ERCP catheter with or without the aid of a guidewire. According to the literature, even in experienced hands, this approach fails in approximately 10% of the cases[1-3,13].

Precutting may increase cannulation rates, however, it is said to carry a significant risk of complications. Therefore, it should be reserved for cases that all other methods have failed, while it should be performed only by experienced endoscopists[6-8].

Another alternative is the use of a sphincterotome[9]. Since a more acute angle is required in order to intubate the common bile duct, the bowed sphincterotome can lift the roof of the papilla facilitating entry into the bile duct. Indeed, studies performed in referral centers have demonstrated the superiority of sphincterotomes over standard catheters and have actually suggested that cannulation should be initially attempted with a sphincterotome[2,3].

The use of guidewires has, also, been described, since they have a hydrophilic coating, which when wet becomes slippery, facilitating cannulation[14].

When all endoscopic methods fail, the percutaneous or the combined approach could be an option, although this method is extremely difficult in the setting of a non-dilated biliary tree[14,15].

According to the results of our study, selective CBD cannulation, with the use of a standard catheter, with or without the aid of a guidewire, was accomplished in 81.65% of our patients. The relatively high failure rate could be attributed to the fact that we did not inject contrast material unless deep CBD cannulation has been achieved. When patients, in whom cannulation was unsuccessful, were crossed over to a sphincterotome and a guidewire, the overall success rate increased to 96.8%. As shown in Table 2 the success rate was particularly high in patients with an oversized, fleshy papilla and this can be attributed to the increased manoeuvrability of the wire tip into the long and tortuous intraampullary portion of the bile duct offered by the sphincterotome's flexing or unflexing.

Our study has been performed in a general hospital with an annual volume of no more than 150 ERCPs. All procedures were performed by a single endoscopist, since the aim of our study was to show if the technique described could enhance the cannulation rates of an endoscopist with certain abilities and skills. Although the endoscopist is experienced, he is not exposed to the number of ERCPs performed in a tertiary referral center. Therefore, we believe our study reflects the every day practice of a regular endoscopist, who works in a community Hospital, with an average load of cases. In such a setting, the use of a sphincterotome and a guidewire is helpful in increasing the success rate of selective CBD cannulation before employing more aggressive techniques.

We preferred a double-lumen sphincterotome instead of using a triple-lumen one although the latter permits injection of contrast medium through the additional channel without removing the guidewire. Our choice was based on the fact that double-lumen catheters, which are characterized by a wide wire channel that permits contrast injection without fierce push, which might result in submucosal injection.

We, also, chose to use a guidewire, to aid CBD cannulation, since modern wires are soft and not traumatic, while with their flexible tip they can enhance the ability to cannulate a CBD with a tortuous intrapapillary ending. Being aware that the use of a guidewire may lead to increased risk of perforation or to bile duct injury, we tried to advance it forward very gently and such an incidence was not encountered in our study. We chose to avoid contrast injection before deep cannulation, since it could potentially cause submucosal injection. In this way we also diminished the inadvertent pancreatic duct injections.

The results of our study show that the use of a sphincterotome and a guidewire is a safe alternative for CBD cannulation since this technique did not seem to be associated with a higher complication rate.

In conclusion, the use of a sphincterotome and a guidewire increases the success rate of selective bile duct cannulation in cases that this has not been accomplished with a standard catheter with or without the aid of a guidewire. This technique is non-invasive, safe and can be easily performed in routine practice.

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• *BRIEF REPORTS* •

Metabolic syndrome as a risk factor for gallstone disease

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Abstract

AIM: To establish an association between the presence of metabolic syndrome and the development of gallstone disease.

METHODS: We carried out a cross-sectional study in a check-up unit in a university hospital in Mexico City. We enrolled 245 subjects, comprising 65 subjects with gallstones (36 women, 29 men) and 180 controls (79 women and 101 men without gallstones). Body mass index, waist circumference, blood pressure, plasma insulin, and serum lipids and lipoproteins levels were measured. Insulin resistance was calculated by homeostasis model assessment. Unconditional logistic regression analysis (univariate and multivariate) was used to calculate the risk of gallstone disease associated with the presence of at least three of the criteria (Adult Treatment Panel III). Analyses were adjusted for age and sex.

RESULTS: Among 245 subjects, metabolic syndrome was present in 40% of gallstone disease subjects, compared with 17.2% of the controls, adjusted by age and gender (odds ratio (OR) = 2.79; 95%CI, 1.46-5.33; $P = 0.002$), a dose-dependent effect was observed with each component of metabolic syndrome (OR = 2.36, 95%CI, 0.72-7.71; $P = 0.16$ with one component and OR = 5.54, 95%CI, 1.35-22.74; $P = 0.02$ with four components of metabolic syndrome). Homeostasis model assessment was significantly associated with gallstone disease (adjusted OR = 2.25; 95%CI, 1.08-4.69; $P = 0.03$).

CONCLUSION: We conclude that as for cardiovascular disease and diabetes mellitus, gallstone disease appears to be strongly associated with metabolic syndrome.

Key words: Obesity; Metabolic syndrome; Gallstones; Insulin resistance

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INTRODUCTION

The phenotype characteristic of metabolic syndrome was first described by Archard and Thiers[1] in 1921, in association with polycystic ovary syndrome. In 1956, Vague[2] systematically described the features of the metabolic syndrome and in 1966, Welborn *et al*[3], studied 19 non-diabetic patients with essential hypertension and demonstrated that these individuals had significantly higher plasma insulin concentrations compared to a normotensive control group. These observations suggest that the prevalence of resistance to insulin-mediated glucose disposal would be increased in patients with essential hypertension, and it was the first time that the implications of insulin resistance to the development of metabolic syndrome were described. In 1988, Reaven[4] coined the term syndrome X.

The importance of metabolic syndrome is increasing, especially when associated co-morbidities are considered. The prevalence of metabolic syndrome varies according to the diagnostic criteria selected. The general prevalence is 23.7%, although the prevalence varies widely in population analyses[5] and is up to 58.3% in Mexican-American women between 40- and 74-year old[6]. Recently, the prevalence of metabolic syndrome in Mexican population was determined to be 26.6% according to NCEP-III criteria[7].

There are a cluster of metabolic syndromes, that include resistance to insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, increased very low-density lipoprotein cholesterol, triacylglycerol, diminished high-density lipoprotein cholesterol (HDL) and hypertension[4]. Furthermore, obesity, which has been progressively increasing worldwide, is closely associated with the increased morbidity and mortality caused by several of the most common diseases in the western world including diabetes, hypertension, cardiovascular diseases, cancer, and gallstone disease[4].

Gallstone disease is a major cause of morbidity in the United States[8,9], other western countries[10,11] and Latin American countries such as Chile[12] and Mexico[13]. In these countries, the economic impact of gallstone disease is high[8-11]. Epidemiological studies have identified risk -factors for cholesterol gallstones[10,14] and obesity and hyperinsulinemia

are the most related of these. In addition, hyperinsulinemia is considered to be a common factor linking cholesterol gallstone disease, diabetes mellitus and obesity[15,16]. Currently, it is thought that the pathogenesis of cholesterol gallstone disease is multifactorial and the disease probably develops from complex interactions between multiple genetic and environmental factors[17,18]. The aim of this study was to establish if there is an association between the presence of metabolic syndrome and the development of gallstone disease.

MATERIALS AND METHODS

Population and sample

We conducted a cross-sectional study in the check-up unit of the Diagnostic Clinic at the Medica Sur Clinic and Foundation. This hospital provides care for mainly middle- and high-income individuals from Mexico City and surrounding metropolitan areas. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the check-up unit by their companies as an annual requirement and not for symptomatic disease. The study included 245 subjects who agreed to participate (no one neglected our invitation to participate), 65 subjects found to have gallstones (36 women, 29 men) and 180 controls (79 women and 101 men without gallstones). Gallstones cases and controls were a series of consecutive asymptomatic subjects from the check-up unit. Real-time ultrasonographic studies were done while the subjects were fasting (as part of usual procedures in check-up unit). Gallstones were defined by the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing). At the completion of each patient's participation in the study, all ultrasonographic studies were evaluated again by the same radiologist. No discrepancies were found between the results of the first and second evaluations. In the second evaluation, all studies for each subject were viewed side-by-side in a masked fashion. The study was approved by the Medica Sur Clinic and Foundation Ethics Committee.

Questionnaire

Subjects were asked to complete a questionnaire that asked for information on demographic data, age, gender, alcohol consumption, smoking habits, diabetes mellitus, hypertension, chronic liver disease, hyperlipidemia, gastrointestinal surgery (vagotomy gastrectomy for peptic ulcer, ileal resection for inflammatory bowel disease, or any other disease or cause), gravidity, and the use of oral contraceptives.

Metabolic syndrome

Participants, having three or more of the following criteria, were defined as having the metabolic syndrome[19]. The waist to hip >0.85 and high body fat were defined according to previous definitions[20,21]. The other criteria were defined according to the Executive Summary of the Third Report of the National Cholesterol Education Program[19]. (1) Abdominal obesity: waist circumference >102 cm in men and >88 cm in women; (2) Hypertriglyceridemia: triacylglycerol $\geqslant$1.7 mmol/L; (3) Low high-density lipoprotein cholesterol: HDL <1.03 mmol/L in men and <1.3 mmol/L in women; (4) High blood pressure: $\geqslant$17.3/11.3 kPa; (5) High fasting glucose: $\geqslant$6.1 mmol/L.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. Body mass index was calculated as weight (kg) divided by height (m) squared. Waist circumference (at the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock. Body fat percentage was measured using bipolar electric impedance (OMRON model HBF-306INT).

Three blood pressure readings were obtained at 1-min intervals, and the second and third systolic and diastolic pressure readings were averaged and used in the analyses.

Analytical procedures

Insulin levels were measured using an immunoenzymometric assay (MEIA; Abbott Diagnostics), with inter- and intra-assay coefficients of variation less than 3%. Plasma glucose in the fasting state was measured in duplicate with an automated analyzer. The coefficient of variation for a single determination was 1.5%. Cholesterol, HDL-cholesterol, and triacylglycerol were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and TG assays (Roche Diagnostics Co., Indianapolis, IN). Low-density lipoprotein cholesterol (LDL) concentrations were calculated using the Friedewald formula[22].

Assessment of insulin resistance using the homeostasis model assessment (HOMA-IR)

HOMA-IR was calculated using the following formula: HOMA index = [fasting insulin (μU/mL) ·fasting glucose (mmol/L)] ·22.5^{-1}, high index of insulin resistance a value >2.5[23]. HOMA-IR has a close correlation with the insulin sensitivity index by the standard euglycemic hyperinsulinemic clamp, as shown by Matthews *et al*[23].

Statistical analysis

By means of cross-tabulations, the risks associated with the probability of developing gallstone disease were estimated. Odds ratios (OR) were calculated with the independent variables coded in a binary form. Statistical significance was determined by exact Fisher's test (two-tailed) and 95% confidence intervals and Mann-Whitney *U* test was used to determine non-normal distribution variables. To derive adjusted OR (by age and gender) associated with the probability of gallstone disease, multivariate unconditional logistic regression analyses were conducted. Multicollinearity in the adjusted models was tested by deriving the covariance matrix. All statistical analyses were carried out with the SPSS/PC v 10.0 program (SPSS Inc., Chicago, IL). The sample size for the study was chosen so that by assuming a prevalence of metabolic syndrome in cases (40%) and controls (17.2%), in a posthoc power calculation was analyzed to get statistically significant differences among cases and controls with >99% power, Table 2.

RESULTS

There were significant differences in the mean of clinical, laboratory and anthropometric data in relation to cases and controls. Cases were older, had greater waist and hip circumferences, body mass index, % body fat, and higher systolic and diastolic blood pressure, blood glucose and HOMA-IR (Table 1). Table 2 shows the crude (non-controlled) risks. In general terms, when anthropometric variables were tested, a higher obesity grade was associated with a higher probability of gallstone disease; however, non-statistical differences were showed related to gender. Waist circumference was also statistically associated with a higher risk of gallstone disease. Hypertension, which is a variable included within the definition of metabolic syndrome, was associated statistically with gallstone disease. An important fact is that none of the lipid variables (total cholesterol, LDL-cholesterol, low HDL-cholesterol and triacylglycerol) was statistically associated with the risk of gallstone disease (Table 2).

Table 1 Mean differences of continuous variables between cases and controls (mean±SD)

Variable	Cases *n*=65		Controls *n*=180		*P*
Age (yr)	51.6	13.7	45.0	11.2	<0.0001
Waist (cm)	97.0	15.6	89.9	13.2	<0.0001
Hip (cm)	105.0	12.9	99.7	11.6	0.001
Waist to hip ratio	0.92	0.07	0.91	0.14	0.08
Body mass index (kg/m²)	28.4	5.7	26.3	4.8	0.001
% body fat	33.1	7.8	28.8	7.5	<0.0001
Systolic blood pressure (kPa)	15.7	2.2	14.4	1.9	<0.0001
Diastolic blood pressure (kPa)	10.0	1.36	9.4	1.3	0.002
Glucose (mmol/L)	5.9	2.3	5.3	1.5	0.01
Insulin (μU/mL)	7.1	4.3	6.1	3.9	0.10
Total cholesterol (mmol/L)	5.3	1.2	5.3	1.0	0.80
HDL-cholesterol (mmol/L)	1.0	0.3	1.1	0.3	0.35
LDL-cholesterol (mmol/L)	3.3	0.9	3.42	0.9	0.53
Triacylglycerol (mmol/L)	1.9	0.9	1.8	1.2	0.18
Cholesterol/HDL ratio	5.3	1.4	5.1	1.6	0.34
HOMA index	1.9	1.2	1.5	1.5	0.02

Table 2 Clinical, demographic, anthropometric and biochemical variables associated with the probability of gallstone disease in univariate logistic regression analysis

Variable	Cases, *n* = 65		Controls, *n* = 180		OR	95%CI	*P*
	n	%	*n*	%			
Gender							
Men *vs* women	29	44.6	101	56.1	0.63	0.34–1.12	0.15
Age (yr)							
⩾45 men; ⩾55 women	38	58.5	69	38.3	2.26	1.27–4.03	0.006
Waist circumference (cm)							
>102 men; >88 women	34	52.3	40	22.2	3.84	2.11–7.00	<0.0001
Waist to hip ratio							
>0.85	55	84.6	139	77.2	1.62	0.76–3.46	0.29
Body mass index (kg/m²)							
⩾25	52	80.0	103	57.2	2.99	1.52–5.88	0.001
Body fat (%)							
⩾30	41	63.1	79	43.9	2.18	1.22–3.91	0.009
Glucose (mmol/L)							
⩾6.1	15	23.1	22	12.2	2.16	1.04–4.47	0.04
Triacylglycerol (mmol/L)							
⩾1.7	32	49.2	76	42.2	1.33	0.75–2.34	0.38
Triacylglycerol (mmol/L)							
⩾2.3	20	30.8	42	23.3	1.46	0.78–2.74	0.25
Total cholesterol (mmol/L)							
⩾6.2	13	20.0	31	17.2	1.20	0.59–2.47	0.71
LDL-cholesterol (mmol/L)							
⩾4.1	16	24.6	38	21.1	1.22	0.63–2.38	0.60
HDL-cholesterol (mmol/L)							
<1.03 male; <1.3 female	43	66.2	110	61.1	1.24	0.69–2.56	0.55
HDL-cholesterol (mmol/L)							
<0.9	19	29.2	51	28.3	1.05	0.56–1.95	0.87
HDL-cholesterol (mmol/L)							
<1.3	54	83.1	135	75.0	1.64	0.79–3.40	0.23
Total cholesterol/HDL-cholesterol							
>7	8	12.3	20	11.1	1.12	0.47–2.69	0.82
Systolic blood pressure (kPa)							
⩾17.3	21	32.3	18	10.0	4.30	2.11-8.76	<0.0001
Diastolic blood pressure (kPa)							
⩾11.3	12	18.5	14	7.8	2.69	1.17–6.16	0.03
Blood pressure (kPa)							
⩾17.3/⩾11.3	21	32.2	23	12.8	3.26	1.65–6.43	0.001
Metabolic syndrome (ATPIII)							
yes *vs* no	26	40.0	31	17.2	3.20	1.71–6.01	<0.0001
Metabolic syndrome (ATPIII)							
0 criterion	4	6.2	39	21.7	1		
1 criterion	16	24.6	58	32.2	2.69	0.84–8.65	0.10
2 criteria	19	29.2	52	28.9	3.56	1.12–11.31	0.03
3 criteria	16	24.6	17	9.4	9.18	2.67–31.55	<0.0001
4 criteria	7	10.8	12	6.7	5.69	1.42–22.80	0.01
5 criteria	3	4.6	2	1.1	14.63	1.86–115.2	0.01
HOMA index							
>2.5	17	26.2	24	13.3	2.30	1.14–4.64	0.03

Metabolic syndrome was associated with a more than three-fold risk of gallstone disease (OR = 3.20; 95%CI, 1.71-6.01; $P = 0.0001$), and the HOMA-IR with a risk of 2.30 (95%CI, 1.14-6.64; $P = 0.03$) (Table 2). Because age was identified as a potential confounder and there was a trend towards a greater proportion of women in the gallstone disease group, compared to the control group (Table 2), we adjusted the risks by both variables (age and gender) in multivariate models (Table 3). Metabolic syndrome increased its risk, with an OR of 2.79 (95%CI, 1.46-5.33; $P = 0.002$). Other anthropometric variables remained statistically associated. Low HDL-cholesterol had a borderline risk (OR = 2.32; 95%CI, 1.05-5.11; $P = 0.04$) in this adjusted analysis.

Table 3 Adjusted[1] risks associated with the probability of gallstone disease in multivariate logistic regression analysis

Model	OR	95%CI	*P*
Glucose (mmol/L)			
≥6.1	2.05	0.96–4.39	0.06
Metabolic syndrome (ATPIII)[2]			
Yes *vs* no	2.79	1.46–5.33	0.002
Metabolic syndrome (ATPIII)			
0 criterion	1		
1 criterion	2.36	0.72–7.71	0.16
2 criteria	3.89	1.20–12.59	0.02
3 criteria	7.89	2.25–27.73	0.001
4 criteria	5.54	1.35–22.74	0.02
5 criteria	7.46	0.89–62.85	0.07
HOMA index			
>2.5	2.25	1.08–4.69	0.03
Waist circumference (cm)			
>102 male; >88 women	3.61	1.95–6.71	<0.0001
Body mass index (kg/m^2)			
25–29.9 *vs* <25	2.35	1.09–5.09	0.03
≥30 *vs* <25	5.84	2.47–13.83	<0.0001
Body mass index (kg/m^2)			
≥25 *vs* <25	3.23	1.57–6.62	0.001
HDL-cholesterol (mmol/L)			
<1.3	2.32	1.05–5.11	0.04
Waist to hip ratio			
>0.85	2.20	0.95–5.12	0.07

[1]All of the models adjusted by: age (≥45 men; ≥55 women) and gender (men *vs* women). [2]Yes = 3 or more criteria of ATPIII. No = 0–2 ATPIII criteria.

DISCUSSION

This is the first study to show an association between gallstone disease and metabolic syndrome in a population with a high prevalence of both diseases[13,24]. The presence of metabolic syndrome was associated with an increased risk of gallstone disease (OR = 3.20; 95%CI, 1.71-6.01; $P = 0.0001$). Of all the characteristics of metabolic syndrome, the presence of high waist circumference was the most important factor associated with the risk of having gallstone disease (OR = 3.84; 95%CI, 2.11-7.00; $P<0.0001$), followed by body mass index (OR = 2.99; 95%CI, 1.52-5.88; $P = 0.001$). This observation coincides with the importance of obesity as a pathophysiological phenomenon in both the conditions[18,25,26]. Obesity is an important risk factor for gallstone disease, more so for women than for men, especially considering that women with a body mass index of 30 kg/m^2 or more have at least twice the risk of gallstone disease as women with a body mass index of less than 25 kg/m^2[27-33]. In our study, the presence of high waist circumference was common in patients with gallstone disease compared with the controls (52.3% *vs* 22.2%). The cornerstone of metabolic syndrome is the presence of obesity, especially considering its increasing prevalence, reaching 20.9% of the United States population[34]. In Mexican-Americans, the prevalence of high waist circumference in patients with metabolic syndrome is higher (62.7%)[35]. Furthermore, in the present study, the presence of insulin resistance in patients with gallstone disease is 26.2%, compared with 13.3% in controls ($P = 0.03$), which confers an increased risk of having gallstone disease (OR = 2.30; 95%CI, 1.14-4.66; $P = 0.03$). This increased risk could be attributed to the presence of obesity, which is a major risk factor for developing gallstone disease, mainly by the presence of lithogenic bile, a consequence of excessive synthesis of cholesterol[35,36]. In addition, increases in the plasma insulin levels seen in obesity could be a determinant in developing gallstone disease. Scragg *et al*[37], show that mean plasma insulin concentration was higher in patients with gallstone disease, independent of triglyceride levels, and the presence of a state of increased plasma insulin levels associated with obesity increases the bile cholesterol saturation index[38,39]. In fact, an increase in insulin concentration of 10 μU/mL was associated with an increased relative risk of developing gallstone disease in women (OR = 1.9; 95%CI, 1.1-4.2). The mechanism by which insulin may increase gallstone formation could be by increasing the activity of hydroxy-3-methylglutaryl-coenzyme A reductase[40], and insulin has been reported to stimulate the bile acid-independent flow of bile in whole animals[41], and in perfused liver[42]. In our multivariate analysis, the increased risk of having metabolic syndrome was maintained in all variables, and show the influence of low HDL cholesterol (OR = 2.32; 95%CI, 1.05-5.11; $P = 0.004$) on developing gallstone disease. One of the main metabolic characteristics of metabolic syndrome is diminished levels of HDL cholesterol[19], with this pattern having an increased risk of cardiovascular morbidity and mortality[43]. The importance of HDL cholesterol in developing gallstone disease has been shown in different analyses, which show that the bile cholesterol saturation index is negatively correlated with HDL cholesterol. Considering the high association between gallstone disease and metabolic syndrome in this study, the fact that blood pressure, especially systolic blood pressure, was associated with metabolic syndrome and gallstone disease appears logical. With more insulin resistance and higher levels of plasma insulin, this association could be explained by the action of insulin in hypertension. Finally, the presence of three criteria of metabolic syndrome confers a 7.89-fold increased risk of having gallstone disease. These data support the strong relationship between the entities that share the cornerstone phenomena of obesity and insulin resistance.

In conclusion, as in cardiovascular disease and diabetes mellitus, gallstone disease appears to be strongly associated with metabolic syndrome. These results are also consistent with the hypothesis that insulin resistance plays an important role in the pathogenesis of such diseases and that gallstone disease may be a part of metabolic syndrome.

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• *BRIEF REPORTS* •

"Defective" mutations of hepatitis D viruses in chronic hepatitis D patients

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Abstract

AIM: To verify whether "defective" mutations existed in hepatitis D virus (HDV).

METHODS: Hepatitis delta antigen (HDAg)-coding sequences were amplified using Pfu DNA polymerases with proof-reading activities from sera of five patients with chronic hepatitis D. Multiple colonies were sequenced for each patient. Pfu analyzed a total of 270 HDV clones. Three representative defective HDV clones were constructed in expression plasmids and transfected into a human hepatoma cell line. Cellular proteins were extracted and analyzed by Western blot.

RESULTS: Four of five cases (80%) showed defective HDV genomes in their sera. The percentage of defective genomes was 3.7% (10/270). The majority (90%) of the defective mutations were insertions or deletions that resulted in frameshift and abnormal stop translation of the HDAg. The predicted mutated HDAg ranged from 45 amino acids to >214 amino acids in length. Various domains of HDAg associated with viral replication or packaging were affected in different HDV isolates. Western blot analysis showed defected HDAg in predicted positions.

CONCLUSION: "Defective" viruses do exist in chronic HDV infected patients, but represented as minor strains. The clinical significance of the "defected" HDV needs further study to evaluate.

Key words: Defective virus; Hepatitis D virus; Hepatitis B virus; Polymerase chain reaction; Hepatitis delta antigen

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INTRODUCTION

The hepatitis D virus (HDV) is about 1.7 kb in genomic length and encodes a single protein, the hepatitis delta antigen (HDAg)[1,2]. The delta antigen exists in two forms: the large and the small HDAg. Both forms are identical in the 195 amino acids (AA) at the amino end, but the large form contains an additional 19 AA at the C-end due to RNA editing[1,2]. There are various domains in the HDAg. The coiled-coil structure (CCS; AA 31-52) is essential for small or large HDAg to form homo-dimers, hetero-dimers, or oligomers; the nuclear localization signal (NLS; AA 68-88) is essential for the nuclear translocalization of HDAg; the RNA-binding domain (RBD; AA 95-146) is essential for HDV RNA binding and subsequent replication. Lastly, the packing signal found in the large form of HDAg (PAS; AA 196-214) is crucial for the packaging of HDV virions. Changes or deletions of these domains usually result in impairment of HDV replication, packaging, or both[1,2].

HDV infection may induce fulminant hepatitis and exacerbations of chronic hepatitis B infection[3-8]. The great majority of patients with HDV superinfection progress to chronic disease[9]. The cause for the high chronicity rate is not completely clear. Recently, we found that selection of escape mutants from quasi-species may be an important mechanism for evasion of virus from immune-system attack[10]. In addition, recombination of HDV RNA sequences may play a role in the evolution and diversity of HDV sequences, which in turn, may contribute to evasion from immune selection and progression to chronic disease[10].

Defective viruses carry mutations in the wild type viral genome. They cannot replicate or package without help from coexisting wild type virus[11]. Defective viruses have been found in many viral infections, and they are associated with modulation of disease course and viral persistence[11]. There were several reports about the stability and heterogeneity of nucleotide sequence of HDV genome[12-15], but there have been no reports of the detection of defective viruses unable to replicate or assemble in human HDV infection or the interactions of wild type and defective HDV in cell

culture system. In the current study, we detected defective viruses from patients with chronic HDV infection.

MATERIALS AND METHODS

Patients

Serum samples were obtained from five patients with chronic hepatitis type D who had been under examination for several years[8-10,12]. Two patients were infected with genotype I HDV and the remaining three were infected with genotype IIa HDV[12,16]. They were positive for serum HBsAg and antibody to HDV antigen (anti-HDV), and were negative for immunoglobulin M antibody to hepatitis B core antigen (Ausria II-125, anti-Delta and CORAB-M; Abbott Laboratories, North Chicago, IL). Serum alanine aminotransferase (ALT) levels were measured by a sequential multiautoanalyzer (Technicon SMAC; Technicon Instruments, Tarry Town, NY).

Reverse transcription polymerase chain reaction

Viral RNA was extracted from 50 μL of serum. Reverse transcription polymerase chain reaction (RT-PCR) using primer #120 (homologous to a sequence from nt 889 to nt 912) and #88 (complementary to a sequence from nt 1663 to nt 1684) was performed as reported previously[10,17,18]. The cDNA was generated in the presence of reverse transcriptase (^{TM}GIBCO BRL, Life Technologies, Rockville, MD) according to the manufacturer's instructions. Each 100 μL of PCR reaction mixture contained 5 μL cDNA, 0.5 μL (5 units/μL) Pfu DNA polymerase (Promega, Madison, WI), 10 μL 10× PCR buffer, 8 μL dNTP mixture (2.5 mmol/L each), 4 μL primer (10 pmol/μL each) and 72.5 μL of water. The PCR was performed in a thermal cycler (Perkin Elmer Cetus Corp., Norwalk, CT). It was started at 95 ℃ for 2 min, followed by 35 cycles (each cycle: 95 ℃ for 20 s, 55 ℃ for 40 s, 72 ℃ for 1 min) of amplification and ended at 72 ℃ for 10 min. The RT-PCR products were analyzed in 2% agarose gel, followed by staining with ethidium bromide. Strict procedures were followed to avoid false positive results[19]. Negative control sera from normal control subjects without viral infection and chronic hepatitis B patients without HDV infection were included in experiments. And the results of the controls were negative.

PCR cloning and sequencing

The amplified PCR products were ligated into the plasmid pCR2 vector (Original TA Cloning Kit, Invitrogen Corporation, Carlsbad, CA) or pCR-Blunt II-TOPO (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. The ligation mixture was used to transform the competent *E.coli* strain DH5α (Gibco BRL, Life Technologies, Gaithersburg, MD)[10,17]. The colonies were lysed by heating at 95 ℃ for 5 min, followed by direct sequencing using primers #88 and #120[10,17]. Sequencing was performed with a dye terminator cycle sequencing kit (Dye terminator cycle sequencing core kit #402117, Perkin Elmer Cetus Corp., Norwalk, CT) according to the manufacturer's instructions, and sequencing products were analyzed in an ABI 373A sequencer (Perkin Elmer Cetus Corp., Norwalk, CT).

Plasmids for HDAg expression

The cDNA fragments encoding HDAg were obtained by RT-PCR of HDV genomes from one of the five patients. The PCR products were recovered from gel after electrophoresis and then cloned into a commercial TA cloning vector, pCRII (Invitrogen Corp., Carlsbad, CA). The inserted segments in the pCRII were completely sequenced and then cloned into *Xba*I/*Pst*I-digested pCMV-EBNA (Clontech, Laboratories, Palo Alto, CA). In order to be concise, the small and large forms of wild type HDAg derived from TW2479-12S and TW2697-51L are referred to in this paper as HDAg-S and HDAg-L, respectively. The letters TW indicate a Taiwanese origin and the Arabic numerical following TW is the serum sample number of the patient. The numbers 2 479 and 2 697 represent samples collected at different time points from the same patient with chronic hepatitis D. Similarly, HDAg-L-18d, HDAg-S-53d, and HDAg-S-13d represent the corresponding defective HDAgs derived from the TW2479-18, TW2697-53, and TW2479-13 isolates, respectively. HDAg-L-18d had a nucleotide guanine insertion in the HDAg-coding region that resulted in a frameshift and premature stop translation of HDAg due to the generation of a novel stop codon. HDAg-S-53d had a deleted segment (nt 1255 to 1329) that was substituted by a segment (nt 337 to 355) from a different region of the HDAg-coding sequence. This mutation also resulted in a frameshift and premature stop translation of HDAg. HDAg-S-13d had an insertion of two cytosines between the first and the second stop codons of the HDAg. This mutation resulted in the correct translation of a wild type small HDAg, but frameshift translation of the large HDAg. The predicted amino acid sequences of the wild type and defective HDAgs are shown in Figure 1. HDAg-S (24S) is a small HDAg isolate derived from sample TW2476, which came from another patient with genotype IIa HDV infection.

Transfection of cells

The human hepatoma cell line Huh-7 was used for DNA transfection[20,21]. Maintenance of cells and transfection of DNA by the calcium phosphate-DNA co-precipitation method were carried out as previously described[20,21]. In general, cells were seeded onto a 60 mm-diameter dish at 70% confluence one day prior to transfection. After transfection with a total of 10 μg DNA, the cells were incubated for an additional 20 h. The medium was then replaced at 3-d intervals thereafter. To produce virion-like particles (VLPs), the expression plasmid pS1X encoding the three forms of HBsAg was co-transfected with expressing plasmids of whole HDV genome[22], HDAg-S and HDAg-L. VLPs harvested from media 3 and 6 d after transfection were concentrated by centrifugation through a 20% sucrose cushion.

Western blot analysis of HDAg

To detect expression of HDAg, immunoblotting was performed as previously described[23-25]. Transfected cells were lysed in NET buffer containing 50 mmol/L Tris-HCl

Figure 1 Predicted amino acid sequences of the defective or wild type HDAg expressed by the plasmids. Dashes indicate conserved amino acids. The terminations of the wild type and defective HDAgs are indicated by asterisks (*). The coiled-coil domain is doubly underlined. The nuclear localization signal is marked by a thin line. The RNA-binding domain is marked by a thick line. The large HDAg package signal is marked by a hatched bar. The small and large forms of wild type HDAg are referred to as HDAg-S and HDAg-L, respectively. Similarly, HDAg-L-18d, HDAg-S-53d and HDAg-S-13d represent the defective HDAgs derived from the same patient. HDAg-L-18d had an insertion of a nucleotide G in the coding region of HDAg and resulted in frameshift and premature stop translation of HDAg due to the generation of a novel stop codon. HDAg-S-53d contained a segment (nt 1 255 to 1 329) of deletion and substituted by a segment (nt 337 to 355) from different regions of the HDAg-coding sequence. This mutation also resulted in frameshift and premature stop translation of HDAg. HDAg-S-13d had an insertion of two cytosines between the first and the second stop codons of HDAgs. This mutation resulted in translation of a wild type small HDAg, and a frameshift translation of large HDAg.

(pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. Human polyclonal antiserum with a high titer against HDAg was diluted 1:2 000 and used as primary antibody. The secondary antibody was HRP-conjugated goat anti-human antibody (Sigma, St. Louis, MO). To detect HDAg in released particles in either serum or culture medium, 1 mL culture medium from transfected cells was ultracentrifuged and particles were pelleted. Then pellets were treated and immunoblotted as cellular proteins. Membranes were finally developed with a Western blot chemiluminescence reagent (NEN Life Science, Boston, MA).

RESULTS

Detection of defective HDV genomes in human sera

In order to determine if PCR procedures will generate defective HDV genomes observed in this study, RT-PCR cloning followed by direct sequencing of serum HDV from the five patients were conducted using Pfu DNA polymerase with proofreading activity. In this study, defective mutations were focused on the HDAg-coding sequence. Variants with mutations that created novel stop codons or frameshift translation of HDAg were defined as defective HDV genomes. HDV genomes that encoded complete large or small HDAgs without frameshift or premature stop translation were defined as wild type. A total of 270 HDV clones from the five patients were analyzed. As shown in Table 1, all except one case showed defective HDV genomes in quasi-species of HDV in their sera. The percentage of defective genomes amplified was 3.7% (10/270). Four of the five patients had detectable defective HDV genomes in their sera. One of the wild type HDV plasmids had been amplified using Pfu, and the PCR products were transformed and cloned to serve as control. None of the 75 HDV clones showed defective mutations that resulted from PCR.

Of the 10 defective HDV mutations based on the amplification by Pfu DNA polymerase, only one (10%) was a transition that resulted in a novel stop codon and the majority (90%) were insertions or deletions that resulted in frameshift and abnormal stop translation of HDAg. The predicted mutated HDAg ranged from 45 amino acids to >214 amino acids in length. Various domains of HDAg including CCS, NLS, RBD and PAS were affected in different defective HDV isolates (Figure 1). Most defective mutations occurred randomly within the HDAg-coding region, but identical mutations could be found in two patients (patients D and E). In the patient (case no. E in Table 1) whose long-term follow-up sera were available for analysis, identical defective HDV clones were found at different time points. Because this patient had relatively higher percentage of defective HDV clones, the dominant defective HDV clones could be reproducibly obtained using Pfu. This patient cleared both HBV and HDV and went into biochemical remission after an increase of defective HDV clones up to 50% (2/4) of viral populations. This patient had not received anti-viral treatment before remission.

Table 1 Percentages of defective viral genomes in HDV genomic populations in the sera of patients with chronic hepatitis D

Patients	HDV genotype	Number of clones defective/analyzed (%)
A	IIa	0/36 (0)
B	I	1/52 (1.9)
C	I	2/60 (3.3)
D	IIa	3/58 (5.2)
E	IIa	4/64 (6.2)
Total		10/270 (3.7)

Expression of defective HDAg in cell culture

Of the viral genomic variants analyzed, three representative defective HDV clones and their wild type partners were cloned in expression plasmids and co-transfected into Huh-7 human hepatoma cells to observe viral interactions. To study if HDAg proteins were expressed by the defective HDV genomes, wild type and defective expression plasmids of HDAg were transfected into the Huh-7 hepatoma cell line. Cellular proteins were extracted, electrophoresed, and immunoblotted. As shown in Figure 2, wild type small and large HDAg proteins (abbreviated as HDAg-S and HDAg-L, respectively) were expressed with the expected electrophoretic sizes. Defective HDAg proteins (abbreviated as HDAg-L-18d and HDAg-S-53d, respectively) were also expressed, but in different molecular weights due to the underlying mutations. HDAg-S-13d had a mutation between the stop codons for the small and large HDAg-S. Therefore, wild type small HDAg was expressed and moved to an expected size by electrophoresis.

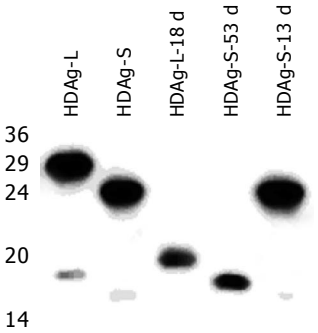

Figure 2 Western blot analysis of wild type and defective HDAgs. The wild type and defective expression plasmids of HDAg (see Methods and Figure 1) were transfected into the Huh-7 hepatoma cell line. Cellular proteins were analyzed by Western blotting. Wild type small and large HDAgs (HDAg-S and HDAg-L) were in proper positions and served as controls. Defective HDAgs (HDAg-L-18d and HDAg-S-53d) were of different molecular weights due to mutations. HDAg-S-13d had a mutation between the stop codons for the small and the large HDAg. Therefore, the small HDAg was expressed and was electrophoretically correct.

DISCUSSION

Defective viruses have been reported in many viral infections[11,26-29]. Defective hepatitis B and C viruses have also been reported[30,31]. Defective viral genomes may interfere with replication and suppress the direct cytotoxic effect of the wild type viruses, or they may induce immunity that protects hosts[11,26-30]. Defective genomes may also be a factor contributing to persistent viral infection[11]. However, there has been no report of defective viral genomes in human HDV infection. This study showed that defective viral genomes are very common (at least 80%) in HDV infections. If more HDV clones were analyzed, we suspect defective HDV genomes might be found in all cases with HDV infection. However, the amounts of defective viral genomes are rather low (usually <10% of viral population) compared to the amounts of wild type in HDV quasi-species. Therefore, defective viral genomes cannot be detected by screening only a few HDV clones. Direct sequencing of RT-PCR products of serum HDV genomes can only discover variants for more than 20% of viral populations[14].

One may argue that these mutations may result from fidelity problems in PCR due to the lack of proofreading activity of the polymerase[32,33]. To test this possibility, we conducted our experiment using Pfu DNA polymerase with proofreading activity. Pfu is currently reported to be the enzyme with the lowest error rate[32,33]. The average error rates (mutation frequency/bp/duplication) of various DNA polymerases have been reported as follows: Pfu (1.3×10^{-6}) <Deep Vent (2.7×10^{-6}) <Vent (2.8×10^{-6}) <Taq (8.0×10^{-6})[33]. Previous studies reported defective genome of HBV and HCV were using Taq DNA polymerases[30,31]. In the current study, defective viral genomes of HDV were detected using Pfu DNA polymerases. The percentages of defective HDV genomes are much higher than those that could be attributed to PCR error rates[32]. Moreover, the 75 HDV clones amplified and cloned from a wild type HDV plasmid did not show any defective mutation resulted form PCR. Therefore, the defective HDV genomes detected in this study were not likely due to PCR errors. In the study of HDV sequence stability by Netter *et al*[14], neither insertions nor deletions were detected in the finally cloned HDV sequences amplified by Taq polymerase from woodchuck serum after transfection of a single cDNA clone of HDV into a human hepatoma cell line followed by the innoculation of the assembled HDV particles in the medium into woodchucks for a total of six passages in different woodchucks. In previous studies evaluating the errors generated by PCR based on different DNA polymerases, most of the PCR errors were found to be transitions or transversion of nucleotides[32,33]. And the Taq polymerase has a higher single nucleotide substitution rate, especially a transition rate, compared to Pfu. While in the current study, the great majority (90%) of the defective mutations were insertions or deletions that resulted in frameshift and abnormal stop translation of the HDAg. This is also an evidence to support the existence of defective HDV genomes in human infection, which gradually accumulated after long history of evolution. Another finding to support the existence of defective HDV is the isolation of a unique defective clone, HDAg-S-53d, with two deleted segments (nt 1 255 to 1 329 and nt 1 627 to 1 635, respectively) and a replaced segment (nt 337 to 355) from a different region of the HDAg coding sequence. A frank mutation like this is unlikely to result from PCR error reported previously[32,33].

HDV is a RNA virus that replicates using a RNA polymerase lacking proofreading activity, a fact that contributes to a fast evolutionary rate. Mutations are expected to occur after several runs of replication in a large population of the HDV genome. In a recent report of transfection of HDV RNA into HuH7 hepatoma cells, they found that the polymerase was able to make an intramolecular template switch[34]. Furthermore, this switch produced small deletions of template sequences and in some cases even insertion of non-templated sequences. Although their experiments are not completely comparable to human infection, the results are at least supportive to the finding of naturally occurring defective viral genomes in human HDV infection. In the experiment of "serial passage of

hepatitis delta virus in chronic hepatitis B virus carrier chimpanzees", an effective infectious dose was estimated to be about 10 HDV genomes per mL. Although, defective HDV mutations were not described in that report, it is possible that some HDV genomes in the inoculum may be defective and a higher concentration of viral inoculum containing sufficient wild type HDV may be needed to induce effective infection in chimpanzees.

From another point of view, the detection rate of defective HDV may have been underestimated in the current work because some defective HDV genomes may not be secreted into serum. In addition, this study focused only on the HDAg-coding sequence. If more clones of whole HDV genome had been analyzed, the detection rate might have increased. The actual incidence and percentages of defective viral genomes in HDV quasi-species may vary depending on individual patients and the experimental conditions used. Nevertheless, the current study clearly indicates that defective HDV does exist in chronic HDV infection.

In summary, defective HDV genomes do exist in chronic HDV infection of humans. The clinical implication of the "defective" HDV needs further study.

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• *BRIEF REPORTS* •

Ribavirin monotherapy increases sustained response rate in relapsers of end treatment virologic responders

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Abstract

AIM: To assess the efficacy of ribavirin monotherapy in patients with biochemical relapse after combination therapy.

METHODS: Twenty-four weeks of ribavirin monotherapy was given to biochemical relapsers of end treatment biochemical responders within 6 mo after combination therapy, including non-responders with HCV-RNA level ≤0.2 Meq/mL and end treatment virologic responders (ETVRs) with or without reappearance of HCV-RNA.

RESULTS: Sixty-two chronic HCV-infected patients completed 24 wk of interferon-α plus ribavirin combination therapy. Fifty patients (80%) achieved end treatment biochemical response including 16 non-responders and 34 of 36 ETVRs. Twenty-six patients (41.9%) were non-responders. Ribavirin monotherapy was given to 20 biochemical relapsers including 12 non-responders with HCV-RNA levels ≤0.2 Meq/mL, four of eight HCV-RNA reappearing ETVRs, and four HCV-RNA negative ETVRs. After 24 wk of ribavirin monotherapy, one of 12 non-responders, two of four HCV-RNA reappearing ETVRs and all four RNA-negative biochemical relapsers of ETVRs showed sustained virologic response. Two of 12 monotherapy treated non-responders showed persistent normalization of liver function test. In total, 50% (31/62) of patients achieved sustained virologic response.

CONCLUSION: Resumption of ribavirin monotherapy in ETVRs at signs of viral rebound and recurrent biochemical abnormalities rather than continuation of monotherapy appears to be the key to success of ribavirin monotherapy after interferon-related combination therapy.

Key words: Ribavirin monotherapy; Interferon-α plus ribavirin combination therapy; Relapser; End treatment virologic responder

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INTRODUCTION

Interferon-α monotherapy for chronic hepatitis C virus (HCV) infection has been disappoint with a sustained response rate of only 20-25% after 24-48 wk of treatment[1,2]. Combination therapy with interferon-α and ribavirin has increased the sustained response rate to about 40%[3,4]. With the advent of pegylated interferon-α, the sustained response rate of pegylated interferon-α plus ribavirin combination therapy was further advanced to 54-56%[5,6].

In spite of these recent advances, patients with HCV genotype 1 infection have lower response rate to interferon-α related therapy than patients infected with HCV genotypes 2 and 3[7]. Patients with HCV genotype 1b are recommended to receive 48 wk of interferon-α-related combination treatments[5,8,9], which often include the pegylated interferon-α as a part of the treatment regimen. The side effect of pegylated interferon-α was reported to be similar[5] or milder than interferon-α[6], but the cost is much higher.

Ribavirin monotherapy has been unsuccessful in treating patients with chronic hepatitis C infection[10-13]. Ribavirin as an inhibitor of HCV viral replication, its mechanism of action in mono- or combination therapy of chronic hepatitis C remains unclear.

This study is designed to assess the efficacy of ribavirin monotherapy in treating chronic hepatitis C infected patients with low viral load after interferon-α plus ribavirin combination therapy, in the hope of reducing the medical cost and undesirable side effects due to resumption of interferon-related therapy.

MATERIALS AND METHODS

Materials

This study was conducted from March 1999 to July 2003. Sixty-five patients were recruited for the investigation. Eligible patients were previously untreated chronic C hepatitis patients with elevation of alanine transaminase (ALT) level 2-10 times over the normal upper limit for more than

six months. Patients were excluded if they had decompensated liver cirrhosis, alcoholism, hepatitis B virus superinfection, uncontrolled diabetes mellitus, autoimmune disorders, human immunodeficiency virus infection, uremia, or organ transplantation. Liver biopsies were done before and after the combination therapy and were read and given a Knodell histologic activity index (HAI) numeric score[14] by the same pathologist. Patients, who needed ribavirin dosage reduction, were excluded from the study.

Methods

Patients who fulfilled the above criteria were given combination therapy consisting of 3 million *U* alpha-2a interferon three times per week administered subcutaneously or intramuscularly plus ribavirin 1 000 or 1 200 mg/d based on the body weight (1 000 mg if body weight <70 kg, 1 200 mg if body weight ⩾70 kg). Liver function test and hemogram were performed monthly during the treatment. After the combination treatment, the liver function test was followed up monthly over the first 3 mo then bimonthly in all groups. The HCV-RNA level was measured again when the liver function test became abnormal during the follow-up period after the combination therapy.

Non-responder is defined as detection of HCV-RNA level at the end of 24 wk of combination therapy. End treatment biochemical response is defined as normalization of liver function test at the end of combination treatment with or without detectable HCV- RNA. End treatment virologic response is defined as normalization of liver function test and undetectable HCV-RNA (<100 copies/mL) at the cessation of combination treatment. Sustained virologic response is defined as normalization of ALT and undetectable HCV-RNA level (<100 copies/mL) at the cessation of the combination therapy and over the following 12 mo. Biochemical relapse is defined as elevation of ALT ⩾1.5× normal upper limit after combination therapy with or without detectable HCV-RNA within 6 mo after end treatment biochemical response.

Patients who experienced biochemical relapse within 6 mo after combination therapy, according to their intention and consent to be treated, received 24 wk of ribavirin monotherapy, and in dosages described above. Patients diagnosed as having diabetes mellitus, fatty liver, other viral infections, and other possible causes of abnormal liver function test were excluded. During ribavirin monotherapy, liver function test was followed up monthly over the first 3 mo, then bimonthly until the end of one year. HCV-RNA level was re-checked at the beginning of monotherapy, at 12 wk into the therapy and at the end of the monotherapy.

Virologic method

Quantification of HCV-RNA was first performed with a reverse transcription-polymerase chain reaction and a digoxigenin detection system[15]. The titer of HCV-RNA was expressed in million genome equivalent (Meq) per milliliter. The lower limit of detection of the test is 0.01 Meq/mL. For HCV-RNA titers less than 0.01 Meq, the specimens were reexamined using Amplicor qualitative HCV-RNA assay (Roche Diagnostics, Branchburg, NJ), which has a lower limit of sensitivity of 100 copies/mL. Bayer Versant 3.0 (bDNA) Quantitative Assay (Bayer Diagnostics, Emeryville, CA) was used to examine HCV-RNA levels between 100 and 10 000 copies/mL, the lowest level of detection is 3 200 copies/mL. The genotyping of HCV was carried out using reverse hybridization assay (Inno-LiPA HCV-II; Innogenetics, Gent, Belgium).

Statistical analysis

Baseline data of quantitative variables were expressed as mean±SD. Pair data of post-treatment response in groups were compared with *t* test, *F* test or χ^2 test. *P* values <0.05 were considered statistically significant.

RESULTS

Three of 65 patients failed to complete the study, including two patients who were unable to tolerate the side effect of interferon-α and one patient who experienced severe insomnia and dyspnea caused by ribavirin. Among 62 patients who completed the study, 26 (41.9%) were non-responders. The remaining 36 patients (58.1%) achieved end treatment virologic response. Fifty patients (80%) including 16 non-responders and 34 of 36 end treatment virologic responders (ETVRs) achieved end treatment biochemical response. The HCV-RNA levels in 12 of 16 non-responders who achieved end treatment bio-chemical response were ⩽0.2 Meq/mL as ALT relapsed. They were included in the monotherapy. Two among 36 ETVRs demonstrated persistent abnormal liver function test resulting from fatty liver as HCV-RNA levels were undetectable (<100 copies/mL) in four consecutive follow-up tests for one year and obvious fatty liver was demonstrated by ultrasonography. They were excluded from the monotherapy. Mild and transient elevations of ALT levels (<1.5 normal upper limit), which returned to normal one month later, were observed in four ETVRs at the fifth month after combination therapy. They were also excluded from the monotherapy. In total, 12 of 36 ETVRs demonstrated biochemical relapse within 5 mo after combination therapy including eight patients with HCV-RNA reappearance and four patients without detectable serum HCV-RNA levels (HCV-RN<100 copies/mL). Four of HCV-RNA reappearing ETVRs and all four RNA-negative ETVRs were included in the study. In total, ribavirin monotherapy was administered to 20 biochemical relapsers including four of eight HCV-RNA reappearing ETVRs, four HCV-RNA negative ETVRs, and 12 HCV-RNA levels ⩽0.2 Meq/mL non-responders. The baseline characteristics and virologic profiles of these 62 patients are divided into three groups according to treatment responses as presented in Table 1. No significant difference is seen in gender, age, HCV genotype, cirrhosis, patient number of HCV-RNA >2 Meq/mL, and HAI scores in pairwise comparisons among three treatment groups.

The initial HCV-RNA levels of 12 non-responders who were eligible for monotherapy ranged from 0.0046 to 0.2 Meq/mL at biochemical relapse. Three patients failed to normalize the liver function tests. Nine patients (75%) experienced normalization of ALT during the monotherapy. One of these nine patients achieved sustained virologic

Table 1 Base-line characteristics of patients who underwent combination therapy

	Total	Non-responder	SVR	Relapser
Sex (m/f)	25/37	10/16	10/14	5/7
Age (yr)	51.5±11.1	50.2±12.5	52.6±9.6	52.9±10.7
HCV genotype (%)				
1a	-	-	-	-
1b	49 (79)	22/26 (84.6)	18/24 (75)	9/12 (75)
2a	5 (8)	1/26 (3.8)	3/24 (12.5)	1/12 (8)
2b	8 (13)	3/26 (11.5)	3/24 (12.5)	2/12 (17)
Cirrhosis (%)	16/62 (26)	9/26 (35)	5/24 (21)	2/12 (17)
HCV-RNA (%) titer >2 Meq copies/mL	6/62 (10)	2/26 (8)	2/24 (8)	2/12 (17)
HAI score	9.5±3.8	10.6±4.3	8.6±2.4	8.7±31

No statistical difference in pair comparisons of sex, age, genotypes, cirrhosis, patient number of HCV-RNA titer >2Meq/mL and HAI scores in non-responders, sustained virologic responders and relapsers.

response when the monotherapy ended. The HCV-RNA level was 0.011 Meq/mL at the start of the monotherapy. This patient sustained the virologic response one year after monotherapy. Two of these nine patients, although HCV-RNA levels were 0.0046 and 0.08 Meq/mL, respectively at the beginning of the monotherapy, experienced 2 and 3 years of biochemical remission after the monotherapy. The HCV-RNA levels were 2.6 and 0.868 Meq/mL, respectively at the end of follow-up. The ALT levels of the remaining six patients became abnormal again and the HCV-RNA levels remained detectable at the end of the monotherapy.

In four HCV-RNA negative biochemical relapsers of ETVRs, the ALT levels normalized and the HCV-RNA levels remained undetectable for 2 years after monotherapy. In two of four HCV-RNA reappearing biochemical relapsers of ETVRs, ribavirin monotherapy was given at 1 and 3 mo after the detection of ALT elevation. The HCV-RNA levels in both patients became undetectable (<100 copies/mL) again at the end of the monotherapy and remained undetectable for over one year. The HCV-RNA levels were 3.8 and 0.061 Meq/mL, respectively at the start of monotherapy. The remaining two of four HCV-RNA reappearing biochemical relapsers of ETVRs received ribavirin monotherapy as late as 6 mo after the cessation of combination treatment and failed to respond to ribavirin monotherapy. The HCV-RNA levels were 1.06 and 2.7 Meq/mL, respectively at the beginning of monotherapy, and remained detectable at the end of monotherapy.

The demography of patients who had sustained virologic response after monotherapy is shown in Table 2. With seven additional sustained virologic responders after the monotherapy, the number of patients with sustained virologic response increased from 24 to 31 (50% of 62 enrolled patients) at the conclusion of the study.

DISCUSSION

In the present study, we have shown that adjuvant ribavirin monotherapy given early at the signs of recurrent liver function abnormalities following the completion of interferon-ribavirin combination therapy achieved sustained virologic response in seven of 20 patients. These results contrast with previous studies[10-12] wherein ribavirin monotherapy, used at the start of the anti-viral treatment or administered in non-responders when the viral loads were well established, showed little efficacy. Our results further contrast with a previous randomized controlled study by Shiffman *et al*[13], wherein no enhanced anti-viral response was observed in virologic responders of combination therapy who continued ribavirin monotherapy.

We think the different clinical outcome in our study may be attributable to the following. First, the patients studied here are treatment-naïve, unlike Shiffman's study in which the patients were relapsers after interferon monotherapy. Second, we employed ribavirin in a different treatment strategy-under the condition when the viral loads of the patients were low and soon after the combination therapy. The ribavirin monotherapy in our study was administered 1 to 3 mo after liver function abnormalities instead of continuing immediately after the combination or interferon therapy as carried out by Shiffman *et al*.

Mathematical analyses of viral load changes have provided critical insight into the pathogenesis of the chronic viral infection of HCV[16,17]. Dose-dependent exponential decline in viral load has been demonstrated by Lam *et al*[18] in interferon treatment as well as Buti *et al*[19] in pegylated interferon treatment. The decline is slower in patients infected by HCV genotype 1 and in African Americans[18,20]. After the rapid clearing of the viral RNA in the first phase, viral decline slows and becomes variable in the second phase [21]. The effect of the first phase is believed to be predominantly due to the inhibition of de novo infection of susceptible cells by interferon. The second phase of viral decline is thought to depend on the clearing of the virus from the infected cells with or without cell turnover[22]. To explain our results, we hypothesize that the significant reduction in viral load after the initial interferon-based mono- or combination-therapy may have created a genetic bottleneck wherein only attenuated HCV variants escaped and persisted. When reemerged, these attenuated HCV variants may have provided a boost to the immune system that was accompanied by liver function abnormalities. Indeed,

Table 2 Demography of patients with sustained virologic response after ribavirin monotherapy

Gender, age (yr)	Genotype (b)	HCV-RNA	ALT elevation (mo)	Monotherapy (mo)	Cirrhosis
F53 (Meq/mL)	1	3.81	2	3	+
F43 (Meq/mL)	2	0.061	1	1	–
F49[1] (Meq/mL)	1	0.011	2	1	–
F57 (copies/mL)	2	<100	1	2	–
F50 (copies/mL)	1	<100	2	1	–
M50 (copies/mL)	1	<100	1	2	–
M58 (copies/mL)	1	<100	5	2	–

Note: ALT elevation denotes the onset of ALT ⩾1.5× normal upper limit after combination therapy in months. Monotherapy denotes the onset of ribavirin monotherapy after biochemical relapse in months. [1] Non-responder at the cessation of combination therapy.

a previous report by Balladini *et al*[23] showed that serum ALT level is correlated with the number of lobular CD8+ cells and histologic manifestation. ALT elevation, in our cases, may coincide with a rebound of immune response to the reemergence of virus. The timely administration of ribavirin at this point may have reduced the viral load and/or impaired the virus further by introducing mutations into the viral genome. These conditions may have mimicked a booster effect in vaccination procedure and thus favor the induction of an effective host immune response against residual HCV, leading to the eventual viral clearance. This would explain why continuous ribavirin monotherapy is ineffective, while restarting ribavirin monotherapy at the signs of reemergence of viral RNA and/or liver function abnormalities as described here shows significant efficacy. Continuing ribavirin immediately after the interferon therapy may reduce HCV replication, but at the same time eliminate the immunological booster effect provided by the reemergence of low-level replication of "attenuated" HCV.

The mechanisms of action of ribavirin in the treatment of HCV infection remain incompletely understood. Ribavirin monotherapy has been shown previously to induce transient improvement of liver function test in chronic HCV-infected individuals, but had no sustained response[10-12]. While the added benefit of ribavirin to interferon treatment is obvious, the mechanism of its action is unclear, as it did not show significant inhibition of viral production[24]. Ribavirin has been shown to decrease HCV replicon RNA sequence moderately and can cause a significant reduction in viral infectivity in a single round of poliovirus infection by increasing the viral mutation rate[25,26]. Ribavirin has also been shown to enhance antiviral Th1 and suppress Th2 cytokines expression in human T cells[27]. Further studies to clarify the role of ribavirin in improving the efficacy of our treatment protocol are needed.

In agreement with the previous report where 5% of the patients with sustained virologic response had persistent rise of ALT[28], two of 36 ETVRs (5%) in this study were found to have fatty liver and persistently abnormal ALT. Critical to the significance of this study is the inclusion of those four biochemical relapsers who showed elevated ALT levels ($\geqslant$1.5-2.0× normal upper limit) but were serum HCV RNA-negative at the start of the ribavirin monotherapy. These four patients were able to normalize their ALT levels after the monotherapy. We think the conditions of these four patients would have worsenedif had they been left untreated. Their hepatic abnormalities are clear indications of viral reemergence despite negative serum HCV levels. Indeed, a recent report using quantitative hepatic HCV-RNA measurement found that intrahepatic HCV RNA was detectable in 2% of sustained virologic responders, among them, two of five patients had reappearance of HCV-RNA[29]. Two of four patients whose HCV-RNA recurred after end treatment virologic response responded to monotherapy is most intriguing in this study because monotherapy was minimally effective in non-responders. The observation requires a control and larger number of patients to clarify its role.

In conclusion, combination treatment with 24 wk of interferon and ribavirin achieved 40% of sustained virologic response in the previous study in this island[3]. In the present study, we show that the sustained virologic response rate can be increased up to 50% by adjuvant ribavirin monotherapy following the combination therapy upon recurrence of ALT abnormalities. The present result further indicates 24 wk of interferon and ribavirin combination treatment followed by adjuvant ribavirin monotherapy when signs of HCV-RNA and ALT abnormalities appear can achieve nearly the same treatment goal as 48 wk of combination treatment especially in biochemical relapsers of ETVRs before or on HCV-RNA reappearing, thus reducing the suffering associated with the latter treatment.

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• *BRIEF REPORTS* •

Detailed deletion mapping of loss of heterozygosity on 22q13 in sporadic colorectal cancer

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Abstract

AIM: Both development and progression of malignancies occur as a multistep process, requiring the activation of oncogenes and the inactivation of several tumor suppressor genes. The loss of heterozygosity (LOH) of tumor suppressor genes is believed to play a key role in carcinogenesis of colorectal cancer (CRC). In this study, we analyzed the LOH of seven loci on chromosome 22q13 in an effort to identify candidate tumor suppressor genes involved in colorectal carcinogenesis.

METHODS: Matched tumor and normal tissue DNA were analyzed by PCR using fluorescence-labeled polymorphic microsatellite markers in 83 CRC patients. PCR products were eletrophoresed and LOH was determined by calculating the peak height acquired through computer software. Comparisons between LOH frequency and clinicopathological features were performed by χ^2 test. $P<0.05$ was considered as statistical significance.

RESULTS: The average LOH frequency of chromosome 22q13 was 28.38%. The highest LOH frequency was 64.71% on D22S1160 locus, and the lowest was 21.43% on D22S1141 locus. We detected two obvious minimal deletion regions: one between markers D22S1171 and D22S274, the other flanked by markers D22S1160 and D22S1149, each about 2.7 and 1.8 cm, respectively. None had lost in all informative loci. LOH frequency on D22S1171 is 50% on distal colon, which was higher than that on proximal one ($P = 0.020$); on D22S114 locus, none LOH event occurred in patients with liver metastasis, whilst 46.94% occurred in patients without liver metastasis ($P = 0.008$); on D22S1160 locus, LOH frequency in lymph nodes metastasis patients was 83.33%, which was much higher than 43.75% without lymph nodes metastasis ones ($P = 0.016$). There was no statistical significance between clinicopathological features and other loci.

CONCLUSION: This study provides evidence of two minimal deletion regions, which may harbor putative tumor suppressor genes related to progression and metastasis in sporadic colorectal carcinoma on chromosome 22q13.

Key words: Heterozygosity; Chromosome 22; Sporadic colorectal cancer; Gene mapping

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INTRODUCTION

Colorectal cancer (CRC) is a predominant disease in the western world, after lung cancer in men and breast cancer in women, and CRC is the most common cause of cancer-related death. The peak incidence of CRC was in the seventh decade of the 20th century and it is fairly equally distributed between men and women[1]. Both development and progression of malignancies occur as a multistep process, requiring the activation of oncogenes and the inactivation of several tumor suppressor genes[2]. The loss of heterozygosity (LOH) of tumor suppressor genes is believed to be one of the key steps to carcinogenesis of CRC. LOH, the loss of one allele at a specific locus, is caused by a deletion mutation or loss of a chromosome from a chromosome pair. When this occurs at a tumor suppressor gene locus where one of the alleles is already abnormal, it can result in neoplastic transformation. In CRCs, frequent allelic loss has been identified in chromosome 5q (30%), 8p (40%), 17p (75-80%), 18q (80%) and 22q (20-30%)[3]. Indeed, much has been published on tumor suppressor genes APC, p53, and DCC, which have been localized to chromosome 5q, 17p and 18q, respectively. Recently, new tumor suppressor genes, such as PTEN (10q23), FHIT (3p14), Smad4 (18q), have been found. The LOH analysis became an effective way to find informative loci and then to find candidate tumor suppressor genes. In an attempt to integrally investigate the loss of tumor suppressor genes and search for putative suppressor loci associated with tumor occurrence and progression, we have conducted a genome-wide LOH study of 83 tumor samples obtained from Chinese patients with sporadic CRC. We found that LOH frequency was higher

than 35% in over 30 loci[4]. In this study, we analyzed the LOH of seven loci on chromosome 22q13 (encompassing D22S274 locus) of sporadic CRC in an effort to identify additional loci involved in colorectal tumorigenesis.

MATERIALS AND METHODS

Patient sample and DNA extraction

This study was based on 83 consecutively collected tumors, including 40 males and 43 females, from unrelated patients with CRC, treated at the surgical department in Shanghai No. 1 People's Hospital, China, between 1998 and 1999. The patients' ages ranged from 31 to 84 years with a median of 66 years. The cancerous tissue and adjacent normal tissue were frozen freshly. These tissues were cut into cubes of approximately 2 mm^3 and immediately frozen in liquid nitrogen. DNA was extracted using standard methods with proteinase K digestion and phenol/chloroform purification. All patients were confirmed by pathology, and were staged by Duke's criterion. Each patient was given his or her informed consent for the use of his or her tissues in this study.

Microsatellite markers and PCR

Seven fluorescence-labeled primers for polymorphic microsatellite markers (Shanghai Biological Technology Ltd, China), flanked on each side on D22S274 locus (Figure 1), were used to analyze matched pairs of normal and tumor DNA for LOH analysis. The sequence of markers was pter-D22S115-D22S1171-D22S114-D22S274-D22D1141-D22S1160-D22S1149-D22S1170-qter. The relative position was from the Genothon human genetic linkage map[5].

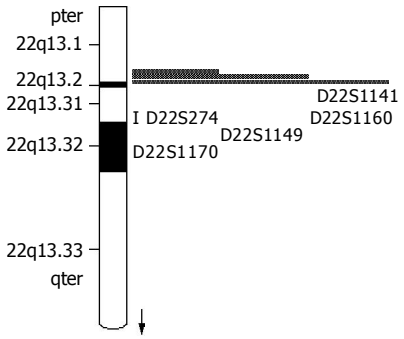

Figure 1 Microsatellite markers location on 22q13.

Polymorphic microsatellite markers were analyzed for each patient's tumor and normal DNA by PCR (GeneAmp PCR System 9 700, PE Applied Biosystems, Foster City, CA). PCR conditions were as follows: 5 μL total volume with approximately 1.4 ng of DNA as a template with 10 mmol/L standard buffer, 1.5 mmol/L Mg^{2+}, 80 mmol/L deoxynucleotide triphosphates, 0.3 unit of Hot-start Taq polymerase and 0.06 μmol/L of each oligonucleotide primer, with the forward primer fluorescence labeled with FAM. Cycling condition consists of three stages: an initial denaturation at 96 ℃ for 12 min in stage I; 14 cycles each at 94 ℃ for 20 s, 63-56 ℃ for 1 min (0.5 ℃ decreased per cycle), 72 ℃ for 1 min in stage II; 35 cycles each at 94 ℃ for 20 s, 56 ℃ for 1 min, 72 ℃ for 1 min in stage III.

LOH analysis

A portion of each PCR product (0.5 μL) was combined with 0.1 μL of Genescan 500 size standard (PE Applied Biosystems) and 0.9 μL of formamide loading buffer. After denaturation at 96 ℃ for 5 min, products were eletrophoresed on a 5% polyacrylamide gel on an ABI 377 DNA sequencer (PE Applied Biosystems) for 3 h. Genotyper 2.1 software displays individual gel lanes as electropherograms with a given size, height and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a LOH (Figure 2). Most amplification of normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygosity) and those PCR reactions, in which fragments were not clearly amplified, were scored as not informative. The LOH frequency of a locus is equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency of chromosome 22 long arm is the average value of each locus LOH frequency.

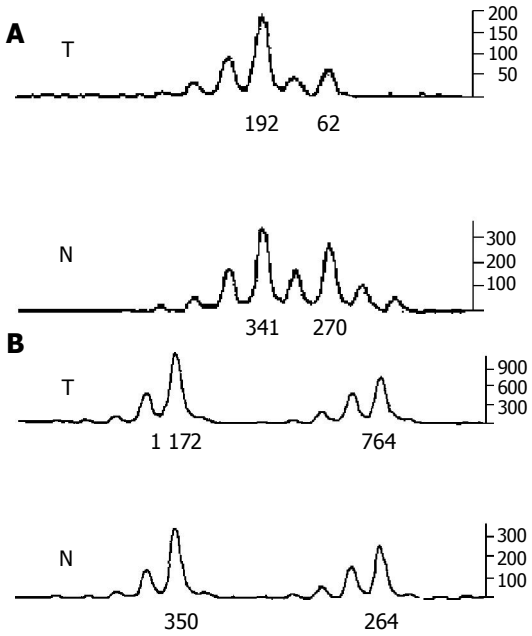

Figure 2 LOH demonstration. A: The typical peak of LOH: allele ratio = (T1/T2)/(N1/N2) = (190/62)/(341/270) = 2.43>1.5; B: The normal peak: allele ratio = (T1/T2)/(N1/N2) = (1172/764)/(350/264) = 1.15. T: tumor N: normal.

Statistical analysis

Comparisons between LOH and clinicopathological data were performed by χ^2 test. $P<0.05$ was considered as statistically significant.

RESULTS

LOH analysis on 22q13

The average LOH frequency of chromosome 22q13 is

28.38%. Fifty-five cases (66.27%) showed LOH on at least one marker on 22q13 (Table 1). LOH frequency on D22S1160 locus was the highest (64.71%), and on D22S1141 locus is the lowest (21.43%). We screened two obvious minimal deletion regions: one between markers D22S1171 and D22S274 (22q13.31), the other flanked by markers D22S1160 and D22S1149, each about 2.7 and 1.8 cm, respectively. None had lost in all informative loci. On D22S1149 locus, less information was got because of more homozygosity (Table 2).

Table 1 LOH result on every locus

No.	D22S115	D22S1171	D22S114	D22S1141	D22S1160	D22S1149	D22S1170
001T	△	△	L	△	N	△	N
019T	N	N	△	N	L	△	△
002T	N	△	N	N	△	△	L
006T	△	N	L	△	△	△	N
012T	L	N	L	△	L	△	△
016T	N	L	N	△	△	△	△
021T	N	N	△	N	L	△	△
008T	△	L	L	△	△	△	△
013T	N	△	N	N	L	△	△
017T	N	N	L	△	△	△	△
022T	N	△	L	△	△	△	△
010T	△	N	L	△	△	L	N
014T	△	△	△	N	L	△	L
018T	N	L	△	△	N	△	△
023T	△	L	△	N	N	N	△
029T	N	L	△	N	L	△	△
044T	△	L	L	L	L	△	△
101T	L	△	L	△	L	△	L
105T	N	L	△	△	L	△	△
030T	△	△	N	L	△	△	L
036T	N	△	N	N	N	△	L
041T	△	△	L	△	L	△	L
045T	L	L	△	L	L	△	N
102T	△	N	L	△	L	△	△
106T	L	N	△	△	△	△	△
032T	N	N	N	△	△	△	L
037T	N	L	△	L	△	△	L
042T	△	N	N	L	△	△	L
103T	N	△	△	N	L	△	△
033T	L	L	L	L	△	△	L
108T	N	N	N	△	△	△	L
116T	L	L	L	△	L	△	L
124T	L	N	L	△	△	△	△
128T	△	△	N	N	L	△	△
117T	L	△	△	△	△	△	△
121T	N	△	N	△	△	L	L
114T	L	N	L	△	N	△	L
122T	L	L	L	L	△	L	L
126T	△	△	△	L	L	△	△
130T	△	L	△	△	△	L	L
111T	L	L	△	N	N	△	L
127T	△	△	L	△	△	△	△
131T	L	N	N	△	L	△	△
132T	△	L	△	△	L	△	N
136T	△	N	L	△	N	L	△
140T	N	N	N	△	L	△	△
144T	N	N	N	△	△	△	L
133T	N	△	L	N	△	△	△
137T	△	L	L	N	△	△	△
141T	△	N	△	N	L	△	N
145T	△	N	L	N	△	△	△
134T	N	L	N	△	△	△	△
142T	L	△	L	L	L	N	△
139T	L	L	L	△	△	△	N
143T	△	L	N	N	L	△	△

L: loss of heterozygosity. N: retention of heterozygosity. △: non-informative (homozygosity/MSI/no production).

Table 2 LOH frequency statistics result on 22q13

Locus	Location	LOH cases	Normal cases	Informative rate	Distance (cm)	LOH rate (%)
D22S115	22q13.2	14	36	60.24	–	28
D22S1171	22q13.31	19	31	60.24	1.2	38
D22S114	22q13.31	23	35	69.88	0.7	39.65
D22S274	22q13.31	16	31	56.63	2.0	34.4
D22S1141	22q13.31	9	33	50.60	1.1	21.43
D22S1160	22q13.31	22	12	40.96	0.8	64.71
D22S1149	22q13.31	5	8	15.66	1.8	38.46
D22S1170	22q13.31	7	28	42.17	6.8	20

Relationship of clinicopathological features and LOH on 22q13

LOH frequency on D22S1171 is 50% on distal large intestinal cancers, which was higher than that on proximal ones ($P = 0.020$). On D22S114 locus, none exhibited LOH in patients with liver metastasis, whilst 46.94% without liver metastasis ($P = 0.008$). On D22S1160 locus, LOH frequency in lymph metastasis patients was 83.33%, much higher than that without lymph metastasis (43.75%, $P = 0.016$). There was no statistical significance between clinicopathological features and other loci (Table 3).

DISCUSSION

During tumorigenesis, loss of the wild-type allele is frequently observed at the appropriate locus. It was widely accepted that LOH on tumor suppressor genes played a key role in CRC transformation. LOH analysis of sporadic CRC can promote the discovery of unknown tumor suppressor genes[6,7]. Allelic loss on chromosome 22q is present not only in CRC but also in oral (40%)[6], brain (40%)[7], ovarian (55%)[8], breast (40%)[9], pancreatic endocrine tumor (30%)[10], gastrointestinal stromal tumor (77%)[11], and even hepatocellular carcinoma[12]. After microsatellite DNA analysis, several attempts were made to identify a region of deletion and eventually the tumor suppressor gene(s) responsible for these neoplasms. Allelic deletions were restricted to D22S274 (22q13) marker in oral squamous cell carcinoma[6].

We have made the LOH analysis on the long arm of chromosome 22 in the previous report[13] in CRC research, and found that there was a relatively high LOH frequency (34.04%) on D22S274 locus. In order to detect unknown tumor suppressor genes on this region, in this study, LOH scanning was carried out in 83 sporadic CRC samples with eight high-density polymorphic markers lying on each side on D22S274 locus (the average hereditary distance, 1.9 cm). By Genotyper software, i.e., by the ratio of the fluorescence intensity of allele, we hope to identify additional high-deletion loci involved in colorectal tumorigenesis and progression.

Through refined mapping, we detected two obvious minimal deletion regions: one between markers D22S1171 and D22S274 (22q13.31), LOH frequency was 34.4-39.65%, about 2.7 cm; the other flanked by marker D22S1160 and D22S1149 locus, LOH frequency was 38.46-64.71%, about 1.8 cm. Castells *et al*[14,15], report a allelic loss interval to a

Table 3 Relationship between clinicopathological features and LOH cases on 22q13

		D22S1157		D22S1171		D22S114		D22S1141		D22S1160		D22S1149		D22S1170	
		L	N	L	N	L	N	L	N	L	N	L	N	L	N
Gender	Male	4	18	10	10	10	16	6	19	7	7	2	7	4	14
	Female	10	18	9	21	13	19	3	14	15	5	3	1	3	14
Age (yr)	>60	11	26	14	26	20	26	8	22	14	10	4	7	6	25
	≤60	3	10	5	5	3	9	1	11	8	2	1	1	1	3
Location	Proximal colon	8	12	3	15	11	12	2	8	6	6	3	3	3	15
	Distal colon	5	9	9	7	7	7	3	8	8	4	1	2	2	5
	Rectum	1	15	7	9	5	16	4	17	8	2	1	3	2	8
Gross pattern	Massive	7	14	7	13	11	14	4	14	5	7	4	4	4	11
	Ulcerative	5	19	11	13	8	17	4	16	15	4	0	2	3	15
	Encroaching	2	3	1	5	4	4	1	3	2	1	1	2	0	2
Size (cm)	≥5	7	14	8	13	9	13	4	16	5	6	2	5	4	17
	<5	7	22	11	18	14	22	5	17	17	6	3	3	3	11
LN metastasis	LN(+)	7	20	10	17	12	17	4	16	15	3	2	3	3	13
	LN(-)	7	16	9	14	11	18	5	17	7	9	3	5	4	15
Liver metastasis	LM(+)	2	5	3	4	0	9	1	6	2	2	0	0	2	4
	LM(-)	12	31	16	27	23	26	8	27	20	10	5	8	5	24
Differentiation	Well	5	7	3	5	6	7	2	9	4	3	1	2	1	3
	Moderately	6	23	13	15	10	21	6	15	10	6	2	3	6	16
	Poorly	1	1	1	3	2	1	0	1	0	1	0	0	0	2
	Mucinous	2	5	2	8	5	6	1	8	8	2	2	3	0	7
Dukes stage	A	1	3	2	0	3	3	0	4	1	3	0	1	0	3
	B	4	9	4	10	6	9	4	6	6	3	3	4	2	7
	C	6	19	10	15	13	13	5	15	12	4	2	3	3	14
	D	3	5	3	5	1	10	0	8	3	2	0	0	2	4

0.5 cm on 22q13 in CRC and breast cancer. The region was flanked by D22S1171 and D22S298, which just included our first detected deletion region. Huang *et al*[16], found that allelic loss on 22q was 36.4% (12/33), and identified two common regions of deletion. One candidate region between D22S274 and D22S1149, about 2.4 cm, was just overlapping with our second deletion region.

LOH on D22S1171 locus was associated with tumor location, i.e., distal large intestinal cancers are prone to cause LOH than proximal cancers ($P = 0.020$). Zhou *et al*[10], found the same phenomenon on D22S274 locus. By the same result, it may be inferred that distal CRC reveals a different mechanism on tumorigenesis. Now, it is admitted that the mechanism of carcinogenesis in distal colon was different from that in proximal one[17-19]. And the mechanism in rectal cancer was also different from that in the proximal colon[20]. Distal colon cancer displayed a higher frequency of 17p and 18q allelic loss, p53 accumulation[21], *c-myc* expression and aneuploidy[22]. Right-sided tumors are more often diploid[23] and of the microsatellite instability (MSI) phenotype. We can conclude that LOH (17p, 18q) plays an important role in the formation of distal CRC.

Furthermore, we found that D22S1160 locus is associated with lymph nodes metastasis; 83.33% (15/18) LOH cases show lymph nodes metastasis, while only 43.75% (7/16) LOH cases without lymph nodes metastasis. This region may harbor tumor suppressor gene which associates with metastasis and results in lymph nodes invasion. But no significant difference was seen with liver metastasis. On D22S114 locus, LOH frequency is negatively associated with liver metastasis, none exhibited LOH in nine cases with liver metastasis (0/9), but 46.94% (23/49) exhibited LOH in patients without liver metastasis ($P = 0.008$). No significant difference was seen with lymph metastasis on this locus. These results indicate that LOH on the two loci are late events in tumorigenesis; lymph nodes metastasis and liver metastasis may reveal a different molecular mechanism.

We scanned GeneMap' 99 database, and found that no known gene exists in these regions. Castells *et al*[14], also have not found known gene between D22S1171 and D22S298 locus in colorectal and breast cancer. The completion of the chromosome 22q sequencing project permitted the prediction of unknown genes using computer-based approaches. Following this strategy, the Sanger Center predicted the existence of eight genes and four pseudogenes between D22S1171 and D22S298 locus[23]. Castells and his colleague made further study and identified several DNA variants that are not compatible with pathogenic mutation. Accordingly, PARVG genes were excluded as tumor suppressor gene on 22q13 involved in CRC and breast cancer development and progression[24]. With the cloning of new genes and further function recognizing of known genes, new foundation will be achieved on 22q13.

In summary, by detailed deletion mapping, we detected two obvious LOH deletion regions, one between markers D22S1171 and D22S274 (22q13.31), the other flanked by markers D22S1160 and D22S1149, about 2.7 and 1.8 cm, respectively. These regions may harbor candidate tumor suppressor gene related to tumorigenesis and progression in CRC. Our study provided the significant data to reveal the mechanism of colorectal carcinogenesis. And further LOH scanning with high-density microsatellite markers and selected genes mutant and methylation analysis in the region

may provide much more genetic and epigenetic information and find the potential tumor suppressor genes.

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• *BRIEF REPORTS* •

Clinicopathological and molecular genetic analysis of HNPCC in China

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Abstract

AIM: To explore the clinicopathological and molecular genetic features of hereditary nonpolyposis colorectal cancer (HNPCC) in Chinese population.

METHODS: We collected 16 Chinese HNPCC families from Wenzhou, Zhejiang Province, China. Tumor tissues and peripheral white blood cells were studied using microdissection, microsatellite analysis, immunostaining of hMSH2 and hMLH1 proteins and direct DNA sequencing of hMSH2 and hMLH1 genes.

RESULTS: (1) A total of 50 patients had CRC. Average age at diagnosis of the first CRC was 45.7 years; 40.9% and 28.7% of the CRCs were located proximal to the splenic flexure and in the rectum, respectively. Thirty-eight percent of the colorectal cancer patients had synchronous and metachronous CRC. 34.4% and 25% of the CRCs were poor differentiation cancer and mucinous adenocarcinoma, respectively. Fourteen extracoloni tumors were found, and the hepatic cancer was the most common tumor type. Twenty-one patients whose median survival time was 5.7 years died during 1-23 years. Twenty-nine patients have survived for 1-28 years, 58.6%, 41.4% and 24.1% patients have survived for more than 5, 10 and 15 years, respectively; (2) All nine tumor-tissues showed microsatellite instability (MSI) at more than two loci. Four tumor-tissues lost hMSH2 protein expression and one lost hMLH1 protein expression. Three pathological germline mutations were identified from five genetically analyzed families; two of three mutations had not been reported previously as they were a transition from C to A in exon 14 (codon 743) of hMSH2 and a TTC deletion in exon 14 (codon 530) of hMLH1.

CONCLUSION: Chinese HNPCC have specific clinicopathological features, such as early onset, propensity to involve the proximal colon, and high frequency of multiple CRCs, liver cancer more frequent than endometrial cancer. Chinese HNPCC showed relatively frequent germline mutation of mismatch repair (MMR) genes that correlated closely with high-level MSI and loss of expression of MMR genes protein.

Key words: Colorectal neoplasms; Hereditary nonpolyposis; Sequence analysis; Microsatellite instability; Mutation; Immunohistochemistry

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INTRODUCTION

In 1895, Warthin first described some families with an excess of colorectal, uterine and gastric cancers. In 1960s, Lynch accurately described these cancer-prone families. This condition was first termed the "cancer family syndrome" and was later renamed hereditary nonpolyposis colorectal cancer (HNPCC). According to the absence or presence of extracolonic malignancies, these families were divided into Lynch syndrome I (hereditary site-specific colorectal cancer) and Lynch II syndrome (colorectal cancer in association with extracolonic cancer)[1]; which accounts for 1-10% of the total colorectal cancer population[1-5]. Clinically, it is diagnosed by Amsterdam criteria[6]: (1) three or more relatives with histologically verified colorectal cancer, one of whom is the first-degree to the other two; (2) colorectal cancer affecting at least two generations; and (3) one or more colorectal cancer cases diagnosed before the age of 50. In addition, familial adenomatous polyposis (FAP) must be ruled out. As the criteria are too rigid for small families, they exclude extra-colonic cancers associated with HNPCC. In Asia, on the other hand, the Japan Research Society for Cancer of the Colon and Rectum developed the clinical criteria (Japanese criteria) for HNPCC in 1991[7]. The criteria

include A: a case with three or more colorectal cancers within the first-degree relatives; B: a case with two or more colorectal cancers within the first-degree relatives meeting the following criteria: (1) age onset of colorectal cancers being earlier than 50 years old; (2) with right colon involvement; (3) with synchronous or metachronous multiple colorectal cancers; (4) associated with synchronous or metachronous extracolonic malignancies.

Germline mutations of six genes involved in DNA mismatch repair (MMR), i.e., hMSH2, hMLH1, PMS1, PMS2, MSH6 (also known as GTBP) and MLH3, have been identified in patients with the disease, and the former two genes account for the large majority of mutations found in families with HNPCC. Totally, these genes are now believed to account for about 50-70% of all families with HNPCC and over 90% of the identified mutations focused on the two genes, hMSH2 and hMLH1[8-14]. There are many studies about the procedures of genetic testing of HNPCC, such as microsatellite instability (MSI), immunohistochemistry (IHC) and direct DNA sequencing[15-18].

It is of no doubt that there is a large population of HNPCC in China[19]. In 1996, Mo *et al*[20] first reported the clinical features of HNPCC cases. Until now there have been only some case reports of HNPCC in China and no systemic study of molecular genetic aspects of HNPCC has been presented. In the present study, 16 Chinese HNPCC families are included of which nine families fulfilling the Amsterdam criteria and seven families fulfilling the Japanese criteria B. We conducted clinicopathological and molecular genetic analyses of Chinese HNPCC families.

MATERIALS AND METHODS

Materials

From 1999 to 2004, 16 Chinese HNPCC families that were registered at the Department of Surgical Oncology in the Second People's Hospital of Wenzhou and at the Department of Surgery in the First Affiliated Hospital of Wenzhou Medical College were collected, of which nine HNPCC families fulfilled the Amsterdam criteria and seven HNPCC families fulfilled the Japanese criteria B. When the probands were verified, we investigated the more detailed family history of patients in the hospital or through inquiry by telephone, mail or visit. The tree of each family pedigree was drawn. And these HNPCC patients are being followed up.

Tumor tissues of nine identified patients, peripheral white blood cells of probands and members (over the age of 18) in the five genetic analyzed kindreds were collected for the study.

Methods

Microdissection and DNA extraction The 7-μm paraffin-embedded sections were deparaffinized. They were lightly stained with hematoxylin for microdissection. The microdissection was performed under the dissection microscope with a scalpel. The microdissected tissues were transferred directly into a centrifugation tube with 150 μL cell lysis buffer (0.5 mol/L Tris, 20 mmol/L EDTA, 10 mmol/L NaCl, 10 g/L SDS, 0.5 g/L proteinase K). The subsequent DNA extraction was performed according to the protocol of the DNA extraction kit (Daxia Biotech Ltd, Shanghai). So was Genomic DNA from peripheral white blood cells.

Microsatellite instability analysis Matched normal and tumor DNA were investigated with a panel of five microsatellite markers (including BAT26 and BAT25, D2S123, D5S346 and D17S250) that were recommended by the International Collaborative Group on Hereditary Non-Polyposis Cancer and the National Cancer Institute[21,22]. The primer sequences have been published elsewhere[22]. The primer pairs were synthesized by Shenyou Biotech Ltd. Each forward primer was labeled with a fluorescent dye at 5' end. The mixture was denatured, snap cooled and electrophoresed on ABI 310 automated DNA sequencer according to the manufacturer's instructions. The electrophoresis results were analyzed by GeneScan software. MSI was determined according to Gebert *et al*[23]. Additional peaks (bands) at a microsatellite locus in the tumor compared with the normal tissue from the same patient were interpreted as MSI. Cases with MSI in more than two of the five loci were interpreted as exhibiting high microsatellite instability (MSI-H).

Immunostaining for hMSH2 and hMLH1 Immunostaining was performed using a monoclonal antibody against the hMSH2 (Oncogene Ltd) and a monoclonal antibody against the hMLH1 (Pharmingen Ltd) at 1:40 dilutions. The antibodies were detected by the Envison two-step method. The slides were counter stained with hematoxylin. Infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor area served as internal positive controls. Diminished expression of hMSH2 or hMLH1 in cancer tissues were demonstrated when there was complete absence of detectable nuclear staining of neoplastic cells.

Sequencing analysis All 19 exons of hMLH1 gene and all 16 exons of hMSH2 gene (including all intron-exon borders) from proband's genomic DNA were individually amplified. PCR reaction was set in 25 μL volume containing 100 ng template DNA, dNTPs 0.2 mmol/L, $MgCl_2$ 1.5 mmol/L, Taq polymerase 1 U, and then denatured for 5 min at 94 ℃, cycled (45 s at 94 ℃, 45 s at 55 ℃ and 45 s at 72 ℃) 35 times and extended for 7 min at 72 ℃. The PCR products were purified using the QIAquick-spin PCR purification kits (Qiagen Inc.) and then performed on an ABI 310 automated sequencer (ABS Inc.).

RESULTS

Clinicopathological characteristics

From 1999 to 2004, we investigated 16 Chinese HNPCC families of which nine families fulfilled the Amsterdam criteria and seven families fulfilled the Japanese criteria B. A total of 56 patients developed malignant tumors, 50 of whom had CRC. There were 37 male and 13 female, age ranging from 19 to 73 years (average 45.7), 36 patients (72%) developed CRC below 50 years, including 17 patients (34%) under 40 years of age, four patients (8%) under 30 years of age. 40.9% and 28.7% of the colorectal cancers' loci were located in the colon proximal to spleen flexure and in rectum, respectively. The remaining loci were not

clear. Synchronous and metachronous colorectal cancer occurred in 19 (38%) patients. We reviewed the HE of 32 cases available. From Table 1 we found 11 (34.4%) cases of poor differentiation, eight (25%) cases of mucinous adenocarcinoma. Fourteen extracoloni tumors of 13 patients were found in nine Lynch syndrome II families, including four hepatic cancers, three endometrial carcinomas, three breast cancers, two stomach cancers, one bladder cancer, and one neck cancer. Hepatic cancer is the most frequent extracolonic cancer in our series, accounting for 30.7%. Twenty-nine patients have survived for 1-28 years, 17 (58.6%) of 29 patients have survived for more than 5 years, 12 (41.4%) of 29 patients have survived for more than 10 years, 7 (24.1%) of 29 patients have survived for more than 15 years. Twenty-one patients whose median survival time was 5.7 years died during 1-23 years.

Microsatellite instability

All nine tumors in the five HNPCC families showed MSI at more than two loci (MSI-H). Four tumors showed MSI in 5/5 loci, two tumors displayed MSI in 4/5 loci, two tumors presented MSI in 3/5 loci, the other had MSI in 2/5 loci (Table 1, Figure 1).

Expression of hMSH2 and hMLH1 protein

Lack of hMLH1 immunostaining was observed in tumors from H2 proband. Tumors from probands of H1 and H7 were negative for hMSH2 immunostaining. Normal expression of hMSH2 and hMLH1 protein was observed in tumors from H5 and H6 proband (Table 1, Figure 2).

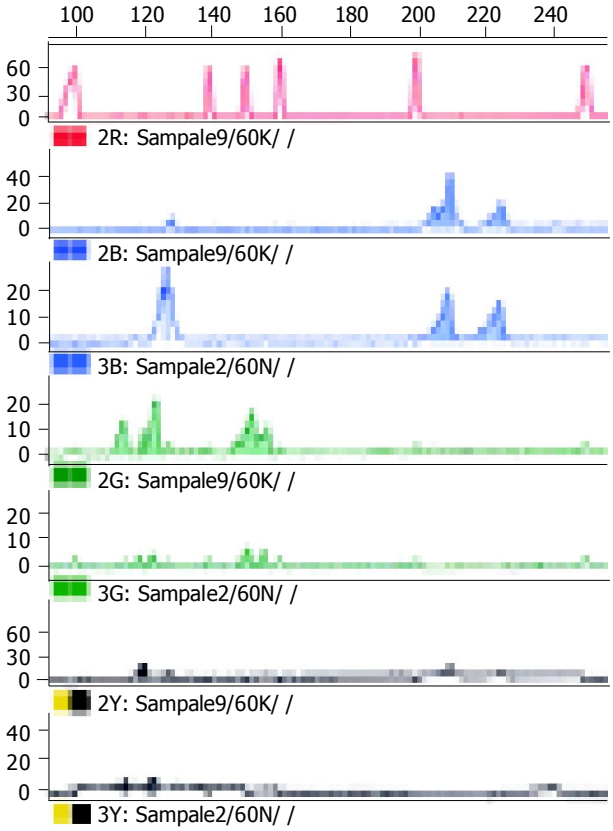

Figure 1 MSI status of H1 proband. Microsatellite analysis of H1 proband with five microsatellite markers, MSI in 5/5 loci.

Table 1 MSI status, immunostaining of nine tumors in five HNPCC kindreds

Family	Criteria	Tumor	BAT26	D2S123	BAT25	D3S346	D17S250	MSI status	Determination	hMSH2 protein	hMLH1 protein
H1	AC	CRC	+	+	+	+	+	5/5	MSI-H	Negative	N
H2	AC	CRC	+	+	+	+	+	5/5	MSI-H	N	Negative
H5-1	AC	CRC	+	+	+	+	+	5/5	MSI-H	N	N
H5-2	AC	CRC	+	–	+	+	+	4/5	MSI-H	N	N
H5-3	AC	CRC	+	NR	+	NR	NR	2/2	MSI-H	N	N
H6	JC	CRC	+	+	+	–	–	3/5	MSI-H	N	N
H7-1	AC	CRC	+	+	+	+	+	5/5	MSI-H	Negative	N
H7-2	AC	CRC	+	NR	+	+	+	4/4	MSI-H	Negative	N
H7-3	AC	CRC	–	+	+	–	+	3/5	MSI-H	Negative	N

AC: Amsterdam criteria; JC: Japanese criteria; NR: no result; MSI-H: high-level microsatellite instability; N: normal expression protein.

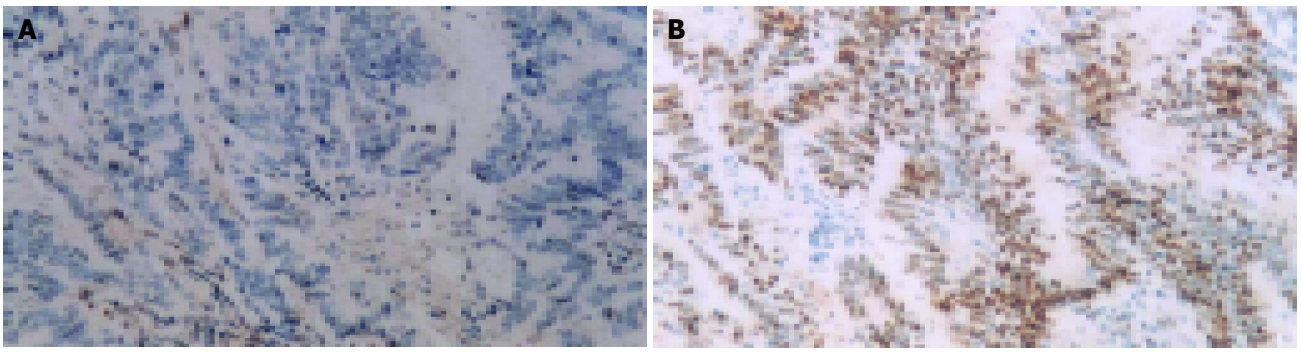

Figure 2 A: Immunohistochemical staining. Lack of hMSH2 protein expression in carcinoma area of H1 proband tumor section. ×400; B: Immunohistochemical staining. Normal expression of hMLH1 protein in tumor from H5 proband. ×200.

Germline mutation of hMSH2 and hMLH1 gene

Germline mutations were found in three of the five HNPCC families (60%). The first pathological mutation was a transition from C to A in exon 14 (codon 743) of hMSH2 (family H7). The second mutation was a TTC deletion in exon 14 (codon 530) of hMLH1 (family H5). The third mutation was a transition from G to A in intron 15-exon 15 borders of hMLH1 (family H6)(Figure 3). All the three were definitely pathological mutations, of which the former two had not been reported previously. All three mutations give rise to protein truncation or protein structure alteration. In addition, the affected sister and father of H7 proband also carried the same mutation in exon 14 of hMSH2. Two sons of H5 proband also suffered from colorectal cancer at young age, both carrying the same germline mutation as their father did.

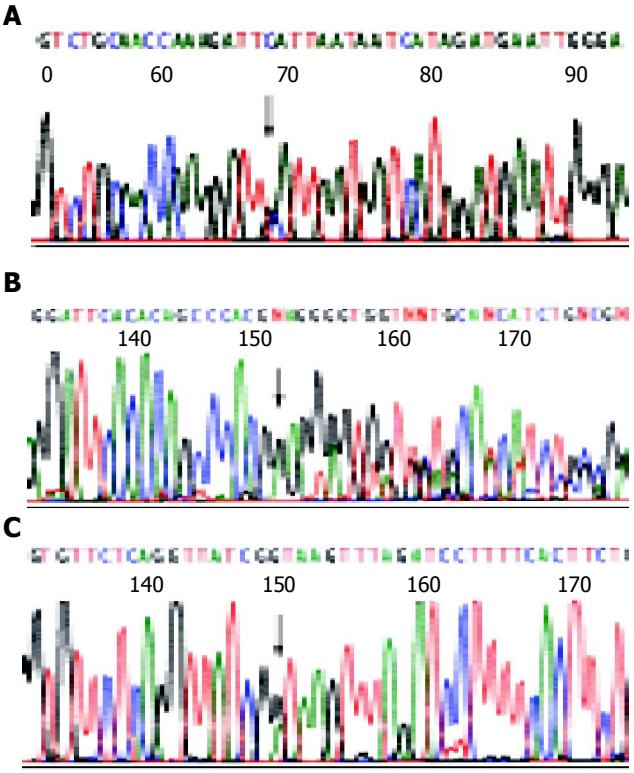

Figure 3 A: C-A transition (TCA-TAA) at codon 743 in exon 14 of hMSH2 gene in H7 proband; B: TTC deletion at codon 530 in exon 14 of hMLH1 gene in H5 proband; C: G-A transition (TCGgta →TCGata) at intron 15-exon 15 borders of hMLH1 gene in H6 proband.

DISCUSSION

HNPCC is an autosomal, dominantly inherited disease characterized by the development of cancer at an early age[5,24-27], predominance of proximal colonic cancer[26,28-30], excess of multiple cancers[31-33], poorly differentiated cancer[34], an increased risk for selected extracolonic adenocarcinomas[35] and better prognosis[36,37].

Early age of cancer onset is one of the most striking features about HNPCC. The average age to develop colorectal cancer was 45 years, 20 years earlier than the sporadic colorectal cancer. The study involving 43 HNPCC kindreds and 140 HNPCC patients by Bertario *et al*[26], showed that the average age of onset was 49 years. Cai *et al* [19], reported the mean age of cancer onset in 30 Chinese HNPCC families, which was 44.1 years in 140 patients, with 74.6% under 50 years of age and 8.5% under 30. In the present study, the median age of onset of the first colorectal cancer was 45.7 years, with 72% under 50 years of age and 8% under 30. Moreover, we found that the age of onset of the first colorectal cancer decreased generation after generation. So to a relatively young colorectal cancer patient, especially younger than 50 years, special attention must be paid while inquiring about family history.

HNPCC is inclined to be located in the proximal colon. Colon cancers are more often right-sided, constituting 47.8% of total cancer and 74.6% of colorectal cancers[5]. A Swedish national investigation in 2001 showed that the proportion of cancers located in the proximal colon was 51% of the total[38]. In our study, the colorectal cancer is found proximal to the splenic flexure constituting 40.9% of the total cancer. Because of the early age of the onset of cancer and as most of the tumor would develop in the proximal colon, we recommend that colonoscopy be performed for the family members with HNPCC and repeated annually or biannually thereafter from the age of 25. In this series, we found three colon cancers and five adenomas by colonoscopy in the family members with HNPCC.

HNPCC showed tendencies of multiple synchronous and metachronous colon cancer[27,32,33]. Fitzgibbons reported that the percentage of synchronous and metachronous colorectal cancers were 18.1% and 24.2%, respectively. It was higher than sporadic colon cancer, being 4.8% and 7.2%, respectively. And the difference was statistically evident[5,27]. Cai *et al*, reported that 23 (19.5%) of 118 patients presented with multiple cancers in the colorectal cancer[19]. Zhao *et al*, reported that 39.5% of colorectal cancer patients developed metachronous colorectal cancer within 10 years after their initial colorectal cancer resection. In our series, 19 of 50 (38%) patients presented with multiple cancers in the colorectal cancer. The high incidence of multiple cancers implies that subtotal colectomy is an appropriate management when colon cancers are found in affected patients. It can reduce the chance of developing synchronous colorectal cancer and simplify the endoscopic examination[27]. But considering the effect on the quality of life after subtotal colectomy and the psychological attack on the patients, we usually chose segmental resection for colorectal carcinoma and gave intensive follow-up.

Extracolonic tumors were often seen in HNPCC kindred[27,35,39,40], such as carcinomas of endometrium, ovary, stomach, small bowel, urologic system, hepatobiliary system, breast, brain, larynx, pancreas and as well as leukemia, lymphoma, soft tissue sarcoma, and cutaneous tumors. According to the reports of Western countries, the endometrial and stomach cancers are the first and the second most common tumor in HNPCC, respectively[33,41,42]. In our series, 14 extracolonic tumors of the 13 patients in the nine Lynch syndrome II families were found, of which four were hepatic cancers, three endometrial carcinomas, three breast cancers, two stomach cancers, one bladder cancer,

one neck cancer. Hepatic cancer is the most frequent extracolonic cancer in our series, accounting for 30.7%. Zhao *et al*, in China reported 34 cases of extracolonic cancer in 16 HNPCC families. He also found that stomach cancer was the most common extracolonic tumor in HNPCC (11 cases of stomach cancers) and endometrial cancer was less common (seven of 34) than gastric cancer. The difference in extracolonic tumor spectrum between China and Western countries may lie in many aspects. The small size of Chinese sample may be one of the reasons. Besides, life style, ethnicity and genotype may also contribute to the observed variation.

They were recognized by histopathological criteria in HNPCC: (1) mucinous histotype; (2) poorly differentiated tumors; (3) presence of peritumoral lymphocytic infiltrate, with Crohn's-like lymphoid reaction. Jass *et al*[34], reported that there is an excess of poorly differentiated and mucinous tumors in HNPCC. Similarly, in our series, poor differentiation cancers and mucinous adenocarcinoma accounted for 34.4% and 25%, respectively. But, there are rarely mucinous histotypic presence of peritumoral lymphocytic infiltrate, with Crohn's-like lymphoid reaction in our study, perhaps it was due to the small sample of our study or the Chinese racial difference.

Patients with HNPCC have been suggested to have a better prognosis than patients with common sporadic colorectal cancer. Sankila *et al*, compared the survival rates of 175 patients with HNPCC with those of 14000 patients with sporadic colorectal cancer diagnosed at <65 years of age in Finland from 1953 to 1993. They showed that the overall five-year cumulative relative survival rate was 65% for patients with HNPCC and 44% for patients with sporadic colorectal cancer[36]. In our study, 21 patients whose median survival time was 5.7 years died during 1-23 years. Twenty-nine patients have survived for 1-28 years, 58.6%, 41.4% and 24.1% patients have survived for more than 5, 10 and 15 years, respectively. The better survival rates may be caused by the heavy mutation burden affecting MMR-deficient tumor cells[36]. Takemoto *et al*[37], suggest that there might be a possibility of ITCIL having a role for a better prognosis after colorectal cancer surgery, which is closely related to MSI. But there is a conflicting data existing on the prognosis of hereditary colorectal cancer. Bertario *et al*[26], found no substantial survival advantage for HNPCC patients compared with the sporadic group, after adjustment for age, gender, stage and tumor location.

Microsatellite has been widely considered as an ideal genetic marker. MSI reveals loss of the function of MMR genes. It can serve as a reliable preliminary screening strategy of HNPCC family as several studies have shown that MSI occurs in about 80-90% of HNPCC tumors[43-47]. In the current study, we adopted a panel of five sensitive microsatellite markers accepted by the International Collaborative Group for HNPCC and the National Cancer Institute to detect the MSI status[48]. One hundred percent (9/9) of the nine tumors displayed high-level MSI, which showed high-level defection of MMR function in the affected patients from HNPCC families fulfilling Amsterdam or Japanese criteria B. Sixty percent of the families that have affected patients with high-level MSI were found with germline mutation of hMLH1 or hMSH2 gene, which showed that MSI status and germline mutation of hMLH1 and hMSH2 gene correlated closely with each other.

Ninety percent of HNPCC cases are associated with the mutation of hMSH2 and hMLH1 genes[49]. Recent studies showed that the immunostaining of proteins produced by these two genes could serve as a convenient, rapid and cheap approach in screening HNPCC families[50-52]. In our study, three tumors showed the lost expression of hMSH2 protein and a germline mutation of hMSH2 gene was identified, four tumors showed hMLH1 protein expression and a germline mutation of hMLH1 gene was identified. Two tumors showed that no pathological germline mutation had been detected, but one tumor displaying no expression of hMSH2 protein, one tumor showed the lost expression of hMLH1 protein. In general, immunohistochemical alteration of hMSH2 and hMLH1 proteins and germline mutation of hMLH1 and hMSH2 gene correlated closely with each other. IHC is also very useful in screening HNPCC families.

Direct gene sequencing remains the most reliable method for HNPCC diagnosis. Till now about 400 different predisposing mutations have been reported, mainly affecting the MMR genes hMLH1 (about half), hMSH2 (about 40%) and MSH6 (about 10%)[49]. There appeared no hot spot mutations among those found in these mutations. The three mutations (one in hMSH2 and two in hMLH1) found in five collective families are all pathological mutations. The rate of mutation is 60% (3/5). The first pathological mutation in H7 proband was a transition from C to A in exon 14 (codon 743) of hMSH2. The second mutation in H5 proband was a TTC deletion in exon 14 (codon 530) of hMLH1. The third mutation in H6 proband was a transition from G to A in intron 15-exon 15 borders of hMLH1. The former two pathological mutations had not been reported before (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). All three mutations gave rise to protein truncation or protein structure alteration. The mutations existed also in genomic DNA from other affected family members. For example, the affected sister of H5 proband also carried the same mutation in exon 14 of hMSH2. Two sons of H7 proband also suffered from colorectal cancer at the age of 37 and 38, both carrying the same germline mutation as their proband did, the other son was not found to have any tumor till the age of 43 and also his daughter till the age of 46, both the same germline mutation had not been identified. All new mutations appearing in our study demonstrates the wide spectrum of the mutation responsible for HNPCC. MMR gene mutation analysis will give both HNPCC proband and his family members better management and surveillance, and it will also support genetic counseling as well as gene therapy in the future. For the proband himself, it is helpful for us to conduct positive and effective therapy to reduce the occurrence of possible metachronous multiple colorectal cancer. To the mutation carriers in a family who have not yet suffered from colorectal cancer, close follow-up and early diagnosis are more likely to be performed. To the non-mutation carriers, we should free them from unnecessary psychological and economical burden[50]. But the mutation may be different in a variety

of races and geographical regions. Mutations of hMSH2 and hMLH1 accounted for 25-86% of the total cases[40,53,54]. A deeper study of germline mutation remains a heavy assignment for us.

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• *BRIEF REPORTS* •

One-year follow-up study of *Helicobacter pylori* eradication rate with ^{13}C-urea breath test after 3-d and 7-d rabeprazole-based triple therapy

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Abstract

AIM: To investigate the long-term role of a 3-d rabeprazole-based triple therapy in patients with *Helicobacter pylori* (*H pylori*)-infected active peptic ulcers.

METHODS: We prospectively studied 115 consecutive patients with *H pylori*-infected active peptic ulcers. *H pylori* infection was confirmed if any two of *H pylori* DNA, histology, and rapid urease test were positive. Patients were assigned to either an open-labeled 3-d course of oral amoxicillin 1 000 mg b.i.d., clarithromycin 500 mg b.i.d., and rabeprazole 20 mg b.i.d., or 7-d course of oral amoxicillin 1 000 mg b.i.d., clarithromycin 500 mg b.i.d., and rabeprazole 20 mg b.i.d. Subsequently, all patients received oral rabeprazole 20 mg once daily until the 8th wk. Three months after therapy, all patients were followed-up endoscopically for the peptic ulcer, *H pylori* DNA, histology, and rapid urease test. One year after therapy, *H pylori* infection was tested using the ^{13}C-urea breath test.

RESULTS: The ulcer healing rates 3 mo after therapy were 81.0% *vs* 75.4% for the 3-d and 7-d groups [intention-to-treat (ITT) analysis, $P = 0.47$] respectively, and 90.4% *vs* 89.6% for the 3-d and 7-d groups [per-protocol (PP) analysis, $P = 0.89$] respectively. The eradication rates 3 mo after therapy were 75.9% *vs* 73.7% for the 3-d and 7-d groups (ITT, $P = 0.79$) respectively, and 84.6% *vs* 87.5% for the 3-d and 7-d groups (PP, $P = 0.68$) respectively. One year after therapy, seventy-five patients returned to receive the ^{13}C-urea breath test, and the eradication rates were 78.4% *vs* 81.6% in 3-d and 7-d groups (PP, $P = 0.73$) respectively.

CONCLUSION: Our study showed the eradication rates against *H pylori* infection 3 and 12 mo after triple therapy were not different between the 3-d and 7-d rabeprazole-based groups. Therefore, the 3-d rabeprazole-based triple therapy may be an alternative treatment for peptic ulcers with *H pylori* infection.

Key words: Eradication rate; Short-term triple therapy

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INTRODUCTION

Helicobacter pylori (*H pylori*), a gram-negative spiral organism, was isolated from the mucosal biopsies of patients with chronic active gastritis by Marshall and Warren in 1983[1]. *H pylori* infection plays an important role in the pathogenesis of peptic ulcer disease, type B gastritis, gastric carcinoma, gastroesophageal reflux disease and lymphoma of mucosa-associated lymphoid tissue[2-6].

At present, the eradication of *H pylori* infection is considered to be a cost-effective method in the treatment of peptic ulcer disease[7,8]. Furthermore, in a recent randomized controlled trial, Wong *et al*[9] found that in *H pylori* carriers without precancerous lesions, eradication of *H pylori* significantly decreased the development of gastric cancer in a high-risk region of China. One-week proton pump inhibitor (PPI)-based triple therapy has been widely accepted as the standard treatment for *H pylori* eradication[10-12]. The *H pylori* eradication rate is higher than 90.0% with the combination of two antimicrobials and a PPI.

There has been recent studies in the eradication of *H pylori* infection[13,14] using short-term (<7-d duration) PPI-based therapy. This is because if a short-term eradication regimen is proven to be effective, it would provide advantages in cost-saving and better patient compliance. Rabeprazole is a newly developed PPI with potent and rapid acid-suppression[15]. It has been demonstrated in one study to have a higher eradication rate than omeprazole[16]. The long-term role of a 3-d rabeprazole-based triple therapy against *H pylori* infection is not known at present. We, therefore,

conducted a randomized, prospective study in patients with active peptic ulcer disease and *H pylori* infection to compare the efficacy and one-year eradication rates between 3-d and 7-d rabeprazole-based triple therapy.

MATERIALS AND METHODS

Selection of patients

From February 2002 to February 2003, we prospectively studied 115 consecutive outpatients (73 male and 42 female, Table 1) documented with *H pylori* infection as well as active gastric ulcers or duodenal ulcers or both at the China Medical University Hospital. All peptic ulcers were confirmed by the first upper gastrointestinal endoscopy.

Table 1 Demographic data of patients who underwent triple therapy

	3-d group	7-d group
Patients (M/F)	36/22	37/20[1]
Age (yr)	48.3 ±12.4	48.7±15.4[2]
Peptic ulcers[3]		
Gastric ulcers	9	12
Duodenal ulcers	40	38
Both	9	7

[1]P = 0.75 (χ^2 test); [2]P = 0.99 (two-sample t-test); [3]P = 0.70 (χ^2 test).

Patients were not allowed to receive aspirin, H_2-receptor blockers, antibiotics or any non-steroidal anti-inflammatory drugs (NSAIDs) in the 4 wk prior to the study. All females with active gastrointestinal bleeding, pregnancy, lactation, delayed last menstrual period or previous curative therapy for *H pylori* infection were excluded. Patients with a history of hypersensitivity to PPIs, penicillin groups, amoxicillin, or clarithromycin, concurrent serious systemic disease including malignancy, renal, hepatic or cardiac insufficiency, or previous esophageal or gastric surgery were also excluded.

The present study was approved by the Ethics and Science Committee of the hospital. Written informed consent was obtained from all patients before the study.

Diagnosis of H pylori infection

During the first endoscopic examination, two biopsy specimens were obtained from the gastric antrum within 2 cm proximal to the pyloric ring. One antral biopsy specimen was assessed for *H pylori* DNA. The *H pylori* DNA was detected using PCR. Another antral biopsy specimen was sent for rapid urease test (RUT) (CLO test, Ballard Medical Products, Draper, UT, USA). In addition, one biopsy specimen was taken from the gastric corpus and sent for histological examination for *H pylori* using hematoxylin-eosin staining or Giemsa staining if necessary. For those patients with gastric ulcers, additional biopsies were made from four sites of the margin of gastric ulcers to rule out possible malignancy. Patients were considered to have *H pylori* infection if they were positive for any two of the three tests.

Clinical treatment

All patients were randomly assigned into two groups. Group I patients received a 3-d course of oral amoxicillin 1 000 mg b.i.d., clarithromycin 500 mg b.i.d., and 7-d course of oral rabeprazole 20 mg b.i.d., Group II patients received a 7-d course of oral amoxicillin 1 000 mg b.i.d., clarithromycin 500 mg b.i.d., and rabeprazole 20 mg b.i.d. All patients further received oral rabeprazole 20 mg once daily until the end of the 8th wk. All the medications were open-labeled. Both groups of the patients were questioned about the occurrence and intensity of adverse effects one week later after triple therapy.

Three months follow-up

Three months after the completion of triple therapy, a repeat endoscopy was performed for examination of the peptic ulcers, *H pylori* DNA, RUT, and histology. *H pylori* infection was considered to be eradicated if all the three tests were negative.

Long-term follow-up

One year after the completion of triple therapy, *H pylori* infection was diagnosed using the ^{13}C-urea breath test (^{13}C-UBT) (Pei Li Pharmaceutical Industrial Ltd, Taichung, Taiwan).Patients were not permitted to receive antacids within 4 wk prior to the ^{13}C-UBT. A negative ^{13}C-UBT indicated no *H pylori* infection.

Statistical analysis

The eradication and healing rates of ulcers were evaluated by intention-to-treat (ITT) analysis and per protocol (PP) analysis. The ITT analysis included all enrolled patients including those cases dropped from the study. The PP analysis included all patients who took at least 80.0% of each study medication and returned for assessment of eradication of *H pylori* infection after three months and one year. Two-sample test or χ^2 test was used to assess significant differences between values in various groups of patients. The eradication rate and ulcer healing rate were calculated for 95.0% confidence intervals. A P-value of less than 0.05 was regarded as statistically significant. Statistical analysis was performed using commercial software (SAS v.8.0, SAS Institute Inc., Cary, NC, USA).

Results were expressed in the form of mean±SD.

RESULTS

Of the 115 patients, 21 patients had gastric ulcers, 78 had duodenal ulcers and 16 had both ulcers. Fifty-eight patients received the 3-d course and 57 received the 7-d course of triple therapy. Fifteen patients, who did not complete the 3-mo follow-up after treatment, were dropped from the study; six of the dropped patients were from the 3-d group and nine patients from the 7-d group.

The ulcer healing rate 3 mo after triple therapy showed no difference with 81.0% in 3-d group and 75.4% in 7-d group (ITT, P = 0.47), and 90.4% in 3-d group and 89.6% in 7-d group (PP, P = 0.89, Table 2).

The eradication rate of *H pylori* infection three months after triple therapy showed no difference with 75.9% in 3-d group and 73.7% in 7-d group (ITT, P = 0.79), and 84.6% in 3-d group and 87.5% in 7-d group (PP, P = 0.68).

Table 2 Eradication rate of patients who underwent triple therapy

	3-d group	7-d group	*P*	OR (interval)
Ulcer healing rate				
Patients number	58	57		
ITT	47 (81.0%)	43 (75.4%)	0.47	0.72 (0.30-1.75)
PP	47 (90.4%)	43 (89.6%)	0.89	0.92 (0.25-3.38)
Eradication rate				
3 mo after therapy				
Patients number	58	57		
ITT	44 (75.9%)	42 (73.7%)	0.79	0.89 (0.3-2.07)
PP	44 (84.6%)	42 (87.5%)	0.68	1.27 (0.41-3.98)
One year after therapy				
Patients (M/F)	37 (23/14)	38 (24/14)	0.93[1]	1.22 (0.39-3.80)
PP	29 (78.4%)	31 (81.6%)	0.73	0.82 (0.26-2.54)

ITT, Intention-to-treat analysis; PP, Per-protocol analysis, [1]$P = 0.93$ (χ^2test).

One year after triple therapy, seventy-five patients had ^{13}C-UBT for the assessment of *H pylori* infection; the eradication rate showed no difference between 3-d and 7-d groups (78.4% *vs* 81.6%; $P = 0.73$, PP). Eight patients in the 3-d group were positive for ^{13}C-UBT. There were 4 cases with treatment failure at 3 mo after treatment, including 1 case of gastric ulcer and 3 cases of duodenal ulcer. Seven patients in the 7-d group were positive for ^{13}C-UBT. There were 4 cases with treatment failure at 3 mo after treatment, including 2 cases of gastric ulcer and 2 cases of duodenal ulcer.

The eradication rate (PP) decreased from 84.6% to 78.4% in the 3-d group and from 87.5% to 81.6% in the 7-d group from 3 mo to 12 mo after rabeprazole-based triple therapy.

During the period of therapy, severity of symptoms such as epigastric pain, acid regurgitation, anorexia, nausea, vomiting, belching and flatulence showed rapid decline on the first 2 d after triple therapy in both groups. Drug compliance during this study was excellent - 100% in the 3-d RAC group and 99.4% in the 7-d RAC group. Both regimens were well tolerated. Adverse events in both groups were mild, which included taste disturbance, diarrhea, oral discomfort and chill sensation. Taste disturbance (bitter taste) was the most common event, comprising about 50% of cases in 3-d and 7-d triple therapies groups. The adverse events were mild and self-limiting and disappeared after one week.

DISCUSSION

Even though many different therapeutic regimens studied for *H pylori* eradication in acid-related disorders[7,8], 1-wk PPI-based triple therapy has been widely accepted for the standard treatment of peptic ulcer with *H pylori* infection[10-12,17]. However, the optimal duration of triple therapy remains to be established. More recently, many newer PPIs have been developed, and they possess potent and rapid inhibition of gastric acid secretion[18,19]. Therefore, short-term regimens (<7-d) have been suggested for the eradication of *H pylori* infection[13,14]. Lara *et al*[20] performed a randomized and prospective study for the eradication of *H pylori* infection in patients with dyspepsia. They found 1-d quadruple therapy (bismuth, metronidazole, amoxicillin and lansoprazole) was statistically similar to 7-d triple therapy (clarithromycin, amoxicillin and lansoprazole) (95% *vs* 90%, ITT). However, Wermeille *et al*[21] reported that the eradication rate of 1-d high-dose quadruple therapy (lansoprazole 30 mg t.d.s., amoxicillin 2 000 mg q.d.s., clarithromycin 500 mg q.d.s., and bismuth 240 mg q.d.s.) was significantly less (20% *vs* 80%) than 7-d triple therapy (lansoprazole 30 mg b.d., amoxicillin 1 000 mg b.d., and clarithromycin 500 mg b.d.).

More recently, a new PPI, rabeprazole, has been demonstrated to be effective in the treatment of acid-related disorders including peptic ulcer disease[22-24]. Rabeprazole has rapid and potent inhibition of gastric acid secretion[18]. In addition, this PPI can induce earlier stabilization of antibiotics and has higher eradication rate compared to other PPIs[16,22,25].

Several studies have compared the eradication rate of short-term course with standard course rabeprazole-based triple therapy for *H pylori* infection. In a study by Gambaro *et al*[26], non-ulcer dyspepsia patients were randomized to receive rabeprazole, clarithromycin and metronidazole for 4 or 7 d, and similar *H pylori* eradication rates were achieved with both regimens (81% *vs* 78%, ITT; 88% *vs* 85%, PP). Yang *et al*[27] compared the efficacy of a 4 and a 7-d rabeprazole-based regimen (rabeprazole, clarithromycin and amoxicillin) with a 7-d omeprazole-based regimen (omeprazole, clarithromycin and amoxicillin) in the eradication of *H pylori* in peptic ulcer patients. This study showed equal efficacy among the three groups (87% *vs* 83% *vs* 88%, ITT; 91% *vs* 95% *vs* 100%, PP). Isomoto *et al*[28] randomly compared a rabeprazole-clarithromycin-amoxicillin regimen for 5 and 7 d in *H pylori*-infected patients. Their results showed 5-d regimen had lower eradication rate than the 7-d regimen (66% *vs* 84%, ITT; 70% *vs* 91%, PP [$P<0.05$]). Recently, a multicenter, double blind, randomized, parallel-group clinical study was performed by Vakil *et al*[17], in the USA. The trial results showed that 7-d therapy with rabeprazole-clarithromycin-amoxicillin is similar in efficacy to 10-d therapies and had similar efficacy in patients with and without ulcer disease. Wong *et al*[29] performed a randomized study to compare rabeprazole-amoxicillin-clarithromycin administration for 3 and 7 d. They found 3-d regimen had a lower *H pylori* eradication rate than 7-d regimen (72% *vs* 88%, ITT; 72% *vs* 91%, PP). Hence, they concluded 7-d rabeprazole-based triple therapy is superior to 3-d regimen ($P = 0.04$).

Interestingly, our study showed that a 3-d rabeprazole-based triple therapy regimen had similar eradication rates (84.6% *vs* 87.5%, PP, $P = 0.68$) against *H pylori* infection and similar ulcer healing rates (90.4% *vs* 89.6%, PP, $P = 0.89$) compared to a 7-d rabeprazole-based triple therapy regimen in 3 mo follow-up. The major differences between our study and Wong's previous study were the following. First, we gave the drugs of amoxicillin 1 000 mg, clarithromycin 500 mg twice daily for 3 d and rabeprazole 20 mg twice daily for 7-d, then tapered to once daily for 7 wk. In Wong *et al*'s study, however, they only gave additional 4-wk course of famotidine 20 mg b.i.d., for all gastric ulcer patients after *H pylori* eradication. Second, all the patients in our study suffered from active peptic ulcer with *H pylori* infection,

but not all the patients were with peptic ulcer disease in Wong *et al*'s study. Actually, we considered that the large dose of PPI combined with antibiotics in the first week of treatment may be an important factor for *H pylori* eradication.

There is no doubt that an ideal *H pylori* treatment must be safe, cheap, easy and tolerable with more than 80% eradication rate and must have a low rate of antibiotic resistance[30,31]. Although treatment failures of *H pylori* eradication are influenced by several factors[32,33], many of the currently used *H pylori* eradication regimens fail to cure the infection due to either antimicrobial resistance or poor patient compliance[34-36]. In the past, there have been concerns about antimicrobial resistance of *H pylori* eradication in many studies[37,38]. A high prevalence of metronidazole resistance has been reported in different regions in Asia[39]. However, clarithromycin resistance is still low in the United States and most communities[40,41]. In standard 1-wk PPI-based triple therapy, the large number of pills needed to be taken daily, the duration of the therapy and the presence of adverse events can limit patient compliance. A recent article has concluded that patient noncompliance is a major cause of failure of *H pylori* eradication therapy[36]. A short-term PPI-based triple regimen will have a smaller overall amount of pills and possibly fewer adverse effects, and thereby improve patient compliance compared with standard 7-d regimens. In addition, there will be health economic and cost saving advantages for patients.

Our study also showed drug compliance in the 3-d regimen was also excellent compared to the 7-d regimen (100% *vs* 99.4%). Adverse events were mild, few and self-limiting in both study groups. Taste disturbance (oral bitter taste) was the most common adverse event in both groups, but this disappeared a few d later.

To our knowledge, however, the long-term follow-up results of *H pylori* eradication after short-term triple therapy have not been reported in published scientific English literature until now. The ^{13}C-UBT is a simple, noninvasive and rapid method for the initial diagnosis of *H pylori* infection and for confirmation of *H pylori* eradication after treatment. Its sensitivity and specificity rates are more than 90%[42,43]. We therefore performed the ^{13}C-UBT to detect *H pylori* infection one year after triple therapy in our study. The eradication rates of *H pylori* infection were similar (74.8% *vs* 81.6%, PP [P = NS]) in the 3-d and 7-d rabeprazole-based triple therapy regimens after 1-year follow-up.

The study demonstrates that 3-d rabeprazole-based triple therapy has the similar efficacy and safety as standard 7-d triple therapy in *H pylori* eradication and long-term follow-up. Hence, 3-d rabeprazole-based triple therapy may be an alternative treatment for peptic ulcer disease with *H pylori* infection.

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• *BRIEF REPORTS* •

Gallbladder motor function, plasma cholecystokinin and cholecystokinin receptor of gallbladder in cholesterol stone patients

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Abstract

AIM: To study the interactive relationship of gallbladder motor function, plasma cholecystokinin (CCK) and cholecystokinin A receptor (CCK-R) of gallbladder in patients with cholesterol stone disease.

METHODS: Gallbladder motility was studied by ultrasonography in 33 patients with gallbladder stone and 10 health subjects as controls. Plasma CCK concentration was measured by radioimmunoassay in fasting status (CCK-f) and in 30 min after lipid test meal (CCK-30). Radioligand method was employed to analyze the amount and activity of CCK-R from 33 gallstone patients having cholecystectomy and 8 persons without gallstone died of severe trauma as controls.

RESULTS: The percentage of cholesterol in the gallstone composition was more than 70%. The cholesterol stone type was indicated for the patients with gallbladder stone in this study. Based on the criterion of gallbladder residual fraction of the control group, 33 gallstone patients were divided into two subgroups, contractor group (14 cases) and non-contractor group (19 cases). The concentration of CCK-30 was significantly higher in non-contractor group than that in both contractor group and control group (55.86±3.86 pmol/L *vs* 37.85±0.88 pmol/L and 37.95±0.74 pmol/L, $P<0.01$), but there was no difference between contractor group and control group. Meanwhile no significant difference of the concentration of CCK-f could be observed among three groups. The amount of CCK-R was lower in non-contractor group than those in both control group and contractor group (10.27±0.94 fmol/mg *vs* 24.59±2.39 fmol/mg and 22.66±0.55 fmol/mg, $P<0.01$). The activity of CCK-R shown as KD in non-contractor group decreased compared to that in control group and contractor group. Only was the activity of CCK-R lower in contractor group than that in control group. The ejection fraction correlated closely with the amount of CCK-R ($r = 0.9683$, $P<0.01$), and the concentration of CCK-30 correlated negatively with the amount of CCK-R closely ($r = -0.9627$, $P<0.01$).

CONCLUSION: The distinctive interactive relationship of gallbladder emptying, plasma CCK and CCK-R in gallbladder from this study suggested that the defect of CCK-R may be a key point leading to the impairment of gallbladder motor function and the pathogenesis of cholesterol gallstone formation may differ in two subgroups of gallstone patient, gallbladder non-contractor group or contractor group.

Key words: Cholesterol stone disease; Gallbladder motility; Cholecystokinin; Cholecystokinin receptor

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INTRODUCTION

Gallbladder motility is regulated by cholinergic system and gastrointestinal hormone[1,2]. Postprandial gallbladder emptying is triggered mainly by plasma cholecystokinin (CCK) from small intestine. CCK interacts with CCK receptor-A (CCK-R) in gallbladder, which in turn elicits the contraction of gallbladder by the activation of post-membrane signaling passage in smooth muscle[3].

Abnormal gallbladder emptying may play an important role in cholesterol gallstone formation[2,4]. Recent studies have revealed that patients having gallbladder stone can be divided into two subgroups with regard to gallbladder emptying: normal contractor and pathological contractor. The two subgroups may differ in the pathogenesis of cholesterol stone formation[5,6]. The purpose of this study was to examine if there is a difference in gallbladder motor function, plasma CCK concentration, the amount and activity of CCK-R in the two subgroups, and to identify the role in pathogenesis of cholesterol gallstone formation.

MATERIALS AND METHODS

Subjects

Thirty-three patients with gallbladder stone (16 male and

17 female, median age 48.3±10.8 years) and 10 controls (4 male and 6 female, age 38.7±9.0 years) were enrolled into this study. Thirteen of gallstone patients suffered from episodes of biliary colic one month ago, and the others had vague right upper quadrant pain. Gallstone volume was no more than one-third of gallbladder volume as assessed by ultrasonography. None of subjects had diabetes mellitus, history of diseases or operations to affect gallbladder motility. None received any medication such as cholic acid and somatostatin to influence gallbladder motor function in recent times. All of subjects had normal gallbladder wall (no more than 2 mm), common bile duct by ultrasound study, and normal liver function by blood biochemistry test. With the analysis of gallstone composition, more than 70% cholesterol was shown in the gallstones from cholecystectomy in 33 patients. Control group for CCK-R measurement was composed of 8 persons (7 males and 1 female) without gallstone died of severe trauma. All samples were maintained in a highly controlled manner, insuring patient confidentiality.

Methods

Gallbladder motor function Gallbladder volumes were measured sonographically by Aloka 650 B-type ultrasonograph equipped with a transducer 3.5 mHz. The calculation was performed according to the sum of cylinders method[7,8]. The subjects in two groups were studied after an overnight fast. Fast volume (FV) was measured before the liquid test meal (500 mL, fat 25 g, protein 40 g and carbohydrates 65 g). The postprandial gallbladder volume was recorded 30, 60 and 90 min after the liquid test meal. Residual volume (RV) was defined as the smallest postprandial volume. Residual fraction (RF) was calculated with the formula (RV/FV×100%) and ejection fraction (EF) with the formula ((FV-RV)/FV×100%)[9].

Plasma CCK concentration measurements Venous blood samples were collected in iced tubes containing heparin for plasma CCK determination before the test meal and 30 min afterwards. Aprotinin (Sigma, Co.) was added immediately. After centrifugation (3 000 r/min, 15 min), plasma was frozen at -20 ℃ until a specific radioimmunoassay for plasma CCK[10]. We measured postprandial CCK concentration at 30 min after test meal in view of the fact that plasma CCK concentration reaches peak and keeps constant for a short period of time[10]. Both fasting plasma CCK concentration (CCK-f) and CCK concentration at 30 min after test meal (CCK-30) were taken into account in the study.

CCK-R concentration measurements Gallbladder samples were collected from the 33 gallbladder stone patients having cholecystectomy and 8 control subjects without gallstone. Tissue samples were snap-frozen in liquid nitrogen immediately. CCK-R was determined by radioligand blinding assay as described previously[11,12]. ^{125}I-BH-CCK$_8$ (Amersham, Co., radioactivity 2 000 Ci/moL) was used as the specific radioligand and CCK$_8$ (Sigma, Co.) as the non-radioactive ligand for competition. The activity of CCK-R was defined as KD (Equilibrium Association Constant), which meant the affinity between CCK and CCK-R. The lower KD of CCK-R suggested the higher activity[12,13].

Statistical analysis

Results were expressed as the mean±SE. The mean and slope of regression were evaluated by the Student's *t*-test. The relationship between two groups of variants was examined by linear regression analysis (Pearson's correlation coefficient), and a *P* value less than 0.05 was considered statistically significant.

RESULTS

According to Pomeranz and Shaffer, pathological contractor patients (non-contractor) were defined by a RF exceeding the mean of controls added 2 SD[5]. In this study, patients having gallbladder stone were divided into two subgroups: gallbladder contractor (14 subjects) and non-contractor (19 subjects).

Gallbladder emptying

Not only the patients in non-contractor group but also those in contractor group exhibited a significantly enlarged RV than in control group and the patients in non-contractor group had a markedly enlarged RV than in contractor group ($P<0.01$). FV in patients of contractor group was distinctly large than that in control ($P<0.05$); however, it did not show any significant difference between contractor group and non-contractor group, as well as between control group and non-contractor group (Table 1).

Table 1 Gallbladder volume and contraction in gallbladder stone patients and controls (mean±SE)

	Normal control	Gallbladder stone patient	
		Contractor	Non-contractor
Number	10	14	19
FV (mL)	21.15±1.67	32.03±3.01[a]	27.01±2.16
RV (mL)	5.27±0.48	7.19±0.60[a]	17.43±2.30[b,d]
RF (%)	24.87±1.22	24.60±1.13	61.32±4.16[b,d]
EF (%)	75.13±1.22	75.40±1.13	38.68±4.16[b,d]

[a]$P<0.05$ *vs* control; [b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* contractor.

Plasma CCK concentrations

There was no difference of CCK-f in plasma among three groups. Plasma CCK-30 of patients in non-contractor group was much higher than in contractor group and control group ($P<0.01$). But no difference could be identified in CCK-30 between contractor group and control group (Table 2).

Table 2 CCK and CCK-R in gallbladder stone patients and controls (mean±SE)

	Control	Gallstone patient	
		Contractor	Non-contractor
Number	10	14	19
CCK-f (pmol/L)	2.62±0.25	2.99±0.30	2.79±0.29
CCK-30 (pmol/L)	37.95±0.74	37.85±0.88	55.86±3.86 [b,d]
Number	8	14	19
CCK-R (fmol/mg)	24.59±2.39	22.66±0.55	10.27±0.94 [b,d]
KD (nmol/L)	0.022 (0.003)	0.036 (0.001) [b]	0.051 (0.002) [b,d]

[b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* contractor.

CCK-R measurements

The amount and activity of gallbladder CCK-R from three groups was summarized in Table 2. The amount of CCK-R in non-contractor group was significantly lower than that in contractor group and control group ($P<0.01$). The amount of CCK-R in contractor group tended to decrease slightly compared to that in control group despite of no significant difference. The activity of CCK-R (shown as KD of CCK-R) in gallstone patients of two groups was much lower than that in control group *(*$P<0.01$). In addition, the activity of CCK-R in non-contractor group decreased markedly than that in contractor group ($P<0.01$).

Correlation analysis

The relationship between amount of CCK-R of gallbladder and EF of gallbladder in gallstone patients was shown in Figure 1. Figure 2 showed the amount of CCK-R of gallbladder and CCK-30 concentration. EF of gallbladder correlated with the amount of CCK-R of gallbladder closely in 33 gallstone patients with positive coefficient of correlation of 0.9683, $P<0.01$. The concentration of CCK-30 in plasma also showed a close correlation with the amount of CCK-R of gallbladder in gallstone patients but with negative coefficient of correlation of -0.9627, $P<0.01$. We did not include 8 control subjects in the correlation analysis for CCK-R measurements because we could not measure their gallbladder motor function and CCK concentrations in plasma.

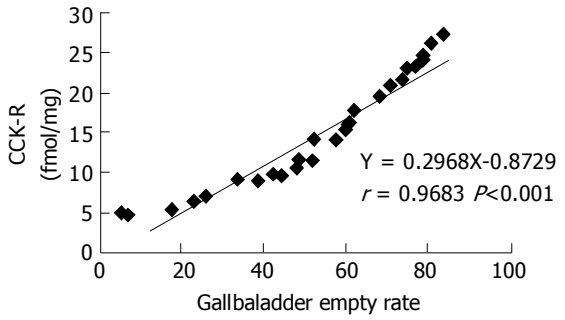

Figure 1 Correlation between EF and amount of CCK-R in gallbladder stone patients.

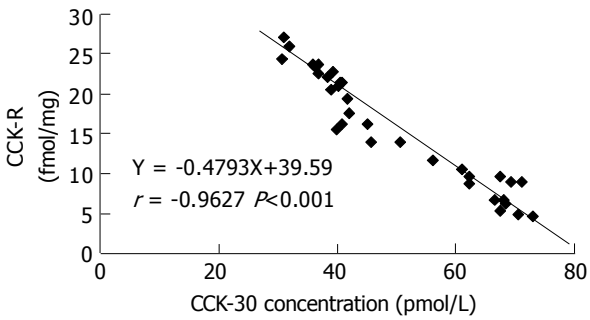

Figure 2 Relationship between CCK-30 concentration and the amount of CCK-R in gallstone patients.

DISCUSSION

The purpose of this study was to explore the possible role of gallbladder in cholesterol stone formation. The analyses of gallstone composition confirmed the patients in this study suffering from the cholesterol gallstone disease. Impaired gallbladder motility might be one of the key determinants of cholesterol stone formation[2,4]. It has been reported that the process of plasma CCK binding its receptor in gallbladder plays an important role in the regulation of gallbladder emptying, but the results from recent studies were still controversial[14]. The reason may result from the evidence that two modes of gallbladder motility exist in gallstone patients, and the change in CCK and CCK-R may differ from each other. Our study confirmed that there were two groups of cholesterol gallstone patients, gallbladder contractor group and non-contractor group. Gallbladder motor function of the patients in the two groups will be analyzed. The association of gallbladder motility with CCK in plasma and CCK-R of gallbladder will be focused as following.

Gallbladder motor function

Patients in both contractor group and non-contractor group had an enlarged RV than control subjects. The enlarged RV induces the retention of bile in gallbladder, which may facilitate the nucleation of cholesterol crystals and stone growth in gallbladder. The enlarged RV might be the final consequence of gallbladder motility[6,9]. The mechanism of the enlarged RV may be different in two gallstone subgroups. A markedly increased FV was found in patients of contractor group compared to that in control group. There was an increased RV in patients of contractor group as a result of EF unchanged. A lower EF in patients of non-contractor group would result in RV increased, which induced bile stasis in spite of FV of gallbladder similar to that in control. So the reason of enlarged RV was an increased FV for the patients of contractor group and a lower EF for the patients of non-contractor group. It can be speculated from this study that the difference in gallbladder motility between two gallstone subgroups might result from the alteration of plasma CCK and CCK-R of gallbladder.

Plasma CCK concentration and CCK-R

As shown in Table 2, the activity of gallbladder CCK-R in patients of contractor group decreased with stable amount of CCK-R and the postprandial concentration of CCK-30. It was inferred that the postprandial EF would enhance to compensate for increment in FV following the increase of the CCK-R activity. The RV of gallbladder in patients of contractor group would be close to the RV of control group, which leads to excreting the surplus of bile. Meanwhile, the decreased activity of CCK-R could be responsible for an abnormally increased FV in patients of contractor group. Gallbladder tension at rest controls the FV of gallbladder and FV may increase abnormally based on the gallbladder hypotonus. The gallbladder tension is maintained by myogenic tonus depending on some humoral and neural transmitters such as CCK and acetylcholine[1,4]. The decrease of CCK-R activity of gallbladder may induce its low affinity to plasma CCK, which contributes to gallbladder hypotonus[9,13].

Abnormally increased FV could be a risk factor of gallstone formation and an early indicator of prognosis of gallstone disease[9,15,16].

Not only the activity but also the amount of CCK-R decreased to a great extent in patients of non-contractor subgroup (Table 2). It was evident that gallbladder hypomotility in non-contractor group results from the defect of CCK-R of gallbladder[11,17,18]. On the contrary, postprandial CCK concentration (shown as CCK-30 in Table 2) of non-contractor group increased significantly compared to that in contractor group and control group, which is considered to be receptor resistance. This phenomenon could be also related with the defect of CCK-R of gallbladder[1,6]. Plasma CCK concentration may reach the peak at a specific postprandial time and keep constant for a period of time, which is important in the maintenance of continuous gallbladder contraction[10]. We speculated that there may be a dynamic equilibrium between the measurable plasma CCK and the CCK combined with CCK-R of gallbladder at the specific postprandial time and they would transform each other. A decreased amount of CCK-R in gallbladder led to the decreased combination of CCK, which could contribute to the measurable plasma CCK increasing. Therefore, a decreased amount of CCK-R of gallbladder should be responsible for an abnormally increased concentration of postprandial plasma CCK in patients of non-contractor group. This result was similar to that in a recent research, which showed postprandial plasma CCK concentration increased significantly in patients with cholecystectomy[19].

Analysis of correlation between CCK-R and gallbladder motor function along with CCK-30

Our result confirmed that the EF of postprandial gallbladder correlated closely with the amount of CCK-R in gallbladder. The result was similar to that in recent studies[11,13,17], which also showed that the impaired gallbladder motility could result from the defect of CCK-R of gallbladder. The cytobiological studies on gallbladder smooth muscle cells also supported the fact that gallbladder hypomotility results from the decreased amount and activity of CCK-R in gallbladder. The conclusion may be traced back to the post-membrane message passage, which induces the contraction of gallbladder smooth muscle cells, since the intact number and function of the second messages (inositol trisphosphate and diacylglycerol), Ca^{++} associated proteins and G proteins[20-22].

A negative linear correlation between postprandial plasma CCK (CCK-30) and CCK-R of gallbladder was found and shown in Figure 2. This result could be due to the mechanism of receptor resistance[1,6]. The other possible reasons of negative correlation between CCK-30 and CCK-R might include the physiologic or physiochemical property of postprandial CCK concentration in plasma and the combination of CCK with CCK-R, such as saturability, reversibility and specificity[1,4]. Based on the negative correlation between CCK-30 and CCK-R it may be feasible to estimate the amount of CCK-R in gallbladder by measuring postprandial CCK concentration in plasma, which would be helpful to evaluate gallbladder motor function.

In conclusion, the impaired gallbladder emptying was identified in patients having cholesterol stone, which might result from the defect of CCK-R of gallbladder. The different mode of gallbladder emptying in cholesterol stone patients was associated with the alterations of CCK-R of gallbladder. In patients of contractor group, the activity of CCK-R decreased predominantly accompanied by an abnormally increased FV. As to the patients of non-contractor group, the activity and amount of CCK-R decreased simultaneously, with higher postprandial plasma CCK concentration. The results of our study demonstrated that the pathogenesis of gallstone formation could be quite different in the two subgroups of cholesterol stone patients. As to the clinical treatment for these two different groups of patients without symptoms, conservative therapy is suitable only for patients of contractor group. If gallstone patients with gallbladder non-contractor have conservative therapies, they are prone to have gallstone recurrence. Symptomatic patients of gallstones need cholecystectomy, whether their gallbladders are contractive or non-contractive.

Our study confirmed that there were two groups of cholesterol gallstone patients, gallbladder contractor group and non-contractor group. Gallbladder motor function of the patients in two groups will be analyzed. The association of gallbladder motility with CCK in plasma and CCK-R of gallbladder will be focused as following.

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• *BRIEF REPORTS* •

HBVPathDB: A database of HBV infection-related molecular interaction network

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Abstract

AIM: To describe molecules or genes interaction between hepatitis B viruses (HBV) and host, for understanding how virus' and host's genes and molecules are networked to form a biological system and for perceiving mechanism of HBV infection.

METHODS: The knowledge of HBV infection-related reactions was organized into various kinds of pathways with carefully drawn graphs in HBVPathDB. Pathway information is stored with relational database management system (DBMS), which is currently the most efficient way to manage large amounts of data and query is implemented with powerful Structured Query Language (SQL). The search engine is written using Personal Home Page (PHP) with SQL embedded and web retrieval interface is developed for searching with Hypertext Markup Language (HTML).

RESULTS: We present the first version of HBVPathDB, which is a HBV infection-related molecular interaction network database composed of 306 pathways with 1 050 molecules involved. With carefully drawn graphs, pathway information stored in HBVPathDB can be browsed in an intuitive way. We develop an easy-to-use interface for flexible accesses to the details of database. Convenient software is implemented to query and browse the pathway information of HBVPathDB. Four search page layout options-category search, gene search, description search, unitized search-are supported by the search engine of the database. The database is freely available at http://www.bio-inf.net/HBVPathDB/HBV/.

CONCLUSION: The conventional perspective HBVPathDB have already contained a considerable amount of pathway information with HBV infection related, which is suitable for in-depth analysis of molecular interaction network of virus and host. HBVPathDB integrates pathway data-sets with convenient software for query, browsing, visualization, that provides users more opportunity to identify regulatory key molecules as potential drug targets and to explore the possible mechanism of HBV infection based on gene expression datasets.

Key words: Hepatitis B viruses; Pathway database; Molecular interactions

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INTRODUCTION

Worldwide spread of hepatitis B viruses (HBV) involves up to 350 million people infected, and areas of endemic infection such as China has about 120 million carriers[1-3]. Approximately 80% of the carriers have different levels of hepatocyte destruction, and most of them, transfer to serious consequences progressively: liver cirrhosis and hepatocellular carcinoma[4]. Information about HBV infection-related molecular interaction network is not only helpful to comprehend the mechanism of transient and chronic infections of the liver caused by HBV, but also instructive to find targets for developing new anti-HBV drugs.

Database has proved to be an efficient tool for storage and retrieval of information on molecular interaction network[5]. However, much of the available pathways' databases at present time such as CSNDB (Cell Signaling Networks database) focus on general information of signal transduction and metabolic pathways, but not specific disease. Although some commercial pathways' databases such as transpath[6] offer analysis, based on pathways, to user, they are not available freely and their data sets are limited. We develop a database about HBV infection-related molecular interaction network named HBVPathDB. The pathway information stored in HBVPathDB is composed of numerous reactions involved in the interaction between HBV and its host. To be convenient for integration in the web severs or lab severs, a web retrieval interface for HBVPathDB is also developed. A web retrieval interface with four-search mode is also developed to make the database convenient for the integration in the web server or lab server. HBVPathDB is freely available at http://www.bio-inf.net/HBVPathDB/HBV/.

MATERIALS AND METHODS

Data resources

Since a lot of physiological and biochemical processes have been involved in the complicated interaction network between HBV and host cells, it is naturally difficult to organize a series of pathways into the complex network. A pathway that refers to local response of network under interior regulating factor or exterior stimulating signal is an essential element of the network in which plenty of molecules interact with others directly or indirectly. In HBVPathDB, pathway is the essential record type of the database. Public databases are rich resources of original pictures of signal transduction and metabolic pathways. The reference databases on the web are shown in Table 1.

Table 1 Reference databases

	Database	URL (uniform resource locator)
HemoPDB		www.bioinformatics.med/ ohio-state.edu/HemoPDB
Cytokines online	Pathfinder encyclopedia	www.copewithcytokines.de
Cell signaling	Networks database	www.geo.nihs.go.jp/csndb
PathDB		www.ncgr.org/pathdb/ architcture.htm
Signal	Pathway database	www.grt.kyushu-u.ac.jp/ eny-doc/spad.html
Biocarta		www.biocarta.com
Gene	Ontology	www.geneontology.org
Cell signaling	Technology	www.cellsignal.com
Database of interacting	Protein	www.dip.doe-mbi.ucla.edu

Pathway graphs drawing

To enable users to browse the pathway information in an intuitive way, Freehand™ 10.0 is chosen as the tracer of pathway for HBVPathDB. Molecular mode criterion provided by Biocarta™ (download freely at http://www.biocarta.com/genes/index.asp) is also used to achieve standardization of graphical representation.

Database implementation

HBVPathDB is a relational database implemented using MYSQL DBMS. This allows the HBVPathDB to grow without concerns for performance issues and, more importantly, to discover relations between the data. The data are organized in the format appropriate for quick search in the database.

Web retrieval interface

A web retrieval interface for HBVPathDB is developed to make the database easier to use. The search engine consists of a suite of programs written in Personal Home Page and Structured Query Language (SQL). This choice was made partly to ensure that the software would work on as many hardware configurations as possible. The front-end data input part of the interface is coded in Hypertext Markup Language. The web retrieval interface has been tested on Windows 98/NT/2000/XP and Linux via the most popular web browsers Netscape and Internet Explorer.

RESULTS

Content of the database

As of August 2004, HBVPathDB contains 306 pathways with 1 050 molecules involved. These pathways can be classified into adhesion, apoptosis, cell activation, cell cycle regulation, cell migration, cell signal transduction, development, hematopoiesis, immunology, metabolism and neuroscience.

Search modes

HBVPathDB allows users four search modes: (1) Category search, which allows users to query pathway by category names. The search result is a list of pathways, which belong to one category or multi-category; (2) Gene search, which allows users to query pathway by gene names (five genes at most). The search result is a list of pathways with the genes involved; (3) Description search: allows users to query pathway by imprecise description; (4) Unitized search: allows users to query pathway by category name, gene name and imprecise description. The search result is a list of pathways, which belong to the category, and include the gene, and possess the description.

Results are returned as a list of pathway term with a brief account of description. And user can explore the details of specific pathway through hyperlinks.

Case study for interpreting differential gene expression based on pathway information

A pathway database is an extremely useful tool to identify regulatory key molecules as potential drug targets and discover mechanism of HBV-related diseases based on gene expression datasets. The query server of pathway information is available through HBVPathDB's web retrieval interface, which provides users with a searchable index of pathway data and gives users opportunity to analyze differential gene expression.

The expression of *Fibronectin* of hepatocyte infected with HBV is much more higher than that of normal hepatocyte in our experiment (details will be reported elsewhere). After searching for *Fibronectin* in HBVPathDB, we get a form with a list of five pathways associated with *Fibronectin* and these pathways are automatically classified into four categories: adhesion, apoptosis, cell migration and development. Click on "*Integrin Signaling Pathway*" in the list, then access a pathway information for which the picture is illustrated in Figure 1.

Figure 1 shows that there are a series of physiological and biochemical reactions generated after *HBsAg* interacted with *fibronectins*[7], 20 types of molecules are involved in these progresses. As a kind of cell surface receptors, *integrins* mediate intracellular signals in response to *fibronectins*, with the result that a series of pathways are activated. One of the important ultimate events shown in Figure 1 is the high expression of *c-Jun* gene coding a kind of DNA binding protein, which can regulate the expression of some specific genes. Furthermore, the gene of *c-Jun* is a type of proto-oncogene whose high expression implies carcinomatous change.

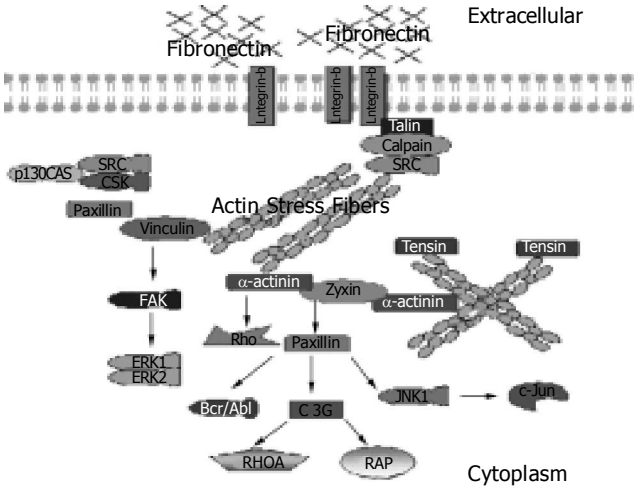

Figure 1 *Fibronectin*-related signaling pathway graph stored in HBVPathDB.

DISCUSSION

The HBVPathDB Database presented by us contains abundant information about HBV infection, ranging from HBV duplicate to cell response in host and provides user with intuitive interfaces to explore the related molecular interaction network in HBV infection process.

HBVPathDB is more than an electronic encyclopedia of HBV infection. With powerful pathway information query, navigation and visualization tools, it enables users to interpret differential gene expression generated by high-throughput technologies such as gene microarray from the point of view of pathways, and not just to find the co-express gene using clustering. The database would help to find the factors and drug targets of HBV-related diseases hidden in the complex molecular interaction network.

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• *BRIEF REPORTS* •

Effect of indomethacin on cell cycle proteins in colon cancer cell lines

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Abstract

AIM: To study the effect of indomethacin (IN) on human colon cancer cell line SW480 with p53 mutant and SW480 transfected wild-type p53 (wtp53/SW480) *in vitro* and investigate molecular mechanism of anti-tumor effect of IN on colon cancer.

METHODS: SW480 cells and wtp53/SW480 cells were treated with different concentrations of IN respectively, the expressions of CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein were detected by Western blotting.

RESULTS: IN gradually down-regulated the expression of CDK_2, CDK_4 protein of wtp53/SW480 cells in a dose-dependent manner, and inhibitory effect reached the maximum level at 600 μmol/L; IN up-regulated the expression of $p21^{WAF1/CIP1}$ protein in a dose-dependent manner at a certain concentration range, and the expression reached the maximum level at 400 μmol/L, and returned to the base level at 600 μmol/L. The expression of CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein of SW480 cells did not change.

CONCLUSION: IN exerts antitumor effect partly through down regulation of the expression of CDK_2, CDK_4 protein and up regulation of the expression of $p21^{WAF1/PIC1}$.

Key words: CDK_2; CDK_4; $p21^{WAF1/CIP1}$; SW480; Colon cancer

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the incidence and mortality from colorectal cancer[1,2]. Indomethacin (IN), a NSAID, is related with the decrease of incidence rate and mortality rate of colon cancer when administered on a long-term basis. Previous studies demonstrated that IN inhibit growth and metastasis of tumor by non-selectively inhibiting cyclooxygenase (COX)[3-5]. It is found in recent years that inhibiting cell proliferation and inducing cell apoptosis is another mechanism by which IN inhibit tumor[6-8]. In this study, Western blot analysis was employed to examine the effect of IN on the expression of cyclin-dependent kinase (CDK) CDK_2, CDK_4 and cyclin-dependent kinase inhibitor (CKI) $p21^{WAF1/CIP1}$ in wild-type p53 transfected colon cancer cell line SW480 (wtp53/SW480), so as to provide experimental evidence to elucidate the anti-tumor mechanism of IN.

MATERIALS AND METHODS

Reagents

p53-Mutated human colon cancer SW480 cell line was purchased from ATCC. wtp53/SW480 was SW480 transfected with wild-type p53 allele according to Bampoe and Shetty ascribed previously[9,10], wtp53/PLXSN recombinant plasmid was kindly provided by Dr. Zhou Xiao, wtp53/PLXSN plasmid was transfected into SW480 cells. Eight-hundred milligrams per liter of G418 was added 48 h after transfection and kept at 200 mg/L concentration for G418 resistance screening. G418-resistant clones of wtp53/SW480 cells were selected randomly. The cells were amplified in media with 200 mg/L G418. The blank plasmid PLXSN was transfected into SW480 cells for the control. Total RNA was extracted from wtp53/SW480 cells, SW480 cells and blank plasmid transfected SW480 cells using Trizol isolation kit according to the manufacturer's instructions. cDNA was synthesized from total RNA in 20 μL reaction mixture using a RT-PCR kit (GIBICO-BRL), including 25 mmol/L MgCl 24 μL, 10× RT buffer 2 μL, 10 mmol/L dNTP 2 μL, Rnasin 40 *U*, AMV 200 *U*, Oligo(dT) 151 μL. The mixture was incubated at 42 ℃ for 60 min, 95 ℃ for 5 min. Fifty microliters of PCR reaction mixture contained 5 μL product of the reverse transcription as a template, 10×PCR buffer 5 μL, sense and anti-sense primers 50 pmol, 10 mmol dNTP 2 μL, Taq DNA polymerase 2 *U*. The PCR condition was 94 ℃ for 3 min pre-denaturing, then 30 cycles at 94 ℃ for 50 s, 53 ℃ for 30 s, and 72 ℃ for 40 s, and further extension at 72 ℃ for 7 min, PCR products were resolved on 1% agarose gels and visualized by ethidium

bromide staining. RPMI-1640 was purchased from GIBICO-BRL, IN was dissolved in DMSO (Sigma) to make 200 mmol/L stock solution and kept at -20 ℃. Rabbit anti-CDK_2, CDK_4, and $p21^{WAF1/CIP}$ polyclonal antibodies were purchased from Santa Cruz; anti-β-actin antibody was kindly provided by Dr. Zhi-Ming He from the Cancer Research Institute, Xiangya Medical College, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse second antibodies, NC membranes were from Huamei Biotech Company (Luo Yan, China); BCA™ Protein Assay Reagent Kit was from Pierce Chemical Co. (Rockford, IL), ECL chemiluminescence kit was from NEN™ life science.

Cell culture

SW480 and wtp53/SW480 were maintained in RPMI-1640 medium containing 10% inactive fetal bovine serum, 50 IU/L penicillin and 50 μg/L streptomycin at 37 ℃ under an atmosphere of 5 mL/L CO_2.

Drug treatment

In experimental group, the cells were treated with IN at final concentration of 100, 200, 400 and 600 μmol/L; in the control group, the cells were treated only with medium or DMSO. The cells were harvested in 24 h treatment.

Protein extraction

The cells were washed twice with PBS, followed by 5 000 r/min centrifugation at 4 ℃ for 10 min, lysed with lysis buffer (50 mmol/L Tris-Cl pH 6.8, 1 mmol/L EDTA, 2%SDS, 5 mmol/L DTT, 10 mmol/L PMSF) on ice, denaturation at boiling water, renaturation on ice, disruption with ultrasound for 30 s, 12 000 *g* centrifugation again for 30 min and discard supernatant. The protein concentration was analyzed with Protein Assay Reagent Kit, and aliquots of cell lysates were stocked at -70 ℃.

Western blotting

Aliquots of cell lysates containing 80 μg of total proteins were separated by SDS-polyacrylamide gel for 2-3 h, and transferred to nitrocellulose filters. The filters were blocked with PBS-T buffer containing 5% skimmed milk for 4-6 h, incubated with rabbit anti-CDK_2 (1:200), anti-CDK_4 (1:200), anti-$p21^{WAF1/CIP1}$ (1:500) polyclonal antibodies respectively for 1 h, then the filters washed with PBS-T for 4 min×15 min and followed by the addition of horseradish peroxidase-linked goat anti-rabbit IgG and ECL visualization of the bands.

For internal control, the same filters were washed with PBS-T for 30 min×3 min, followed by incubation with mouse anti-β-actin in 5% skimmed milk (1:10 000 dilution) for 1 h, incubation with horseradish peroxidase-linked goat anti-mouse IgG for 1 h, and ECL visualization of the bands.

RESULTS

Effect of IN on CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein expression in SW480 cells

After 24 h of action of IN at different concentration, it was found by Western blot analysis that the protein expression of not only CDK_2 and CDK_4 but also $p21^{WAF1/CIP1}$ were not obviously changed in a certain range of IN concentration (Figure 1).

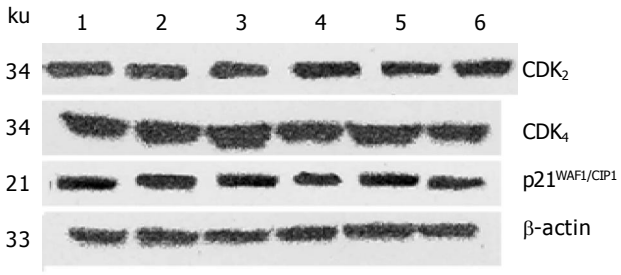

Figure 1 The effect of IN on CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein expression in SW480 cells. Lane 1: Control group; lane 2: DMSO group; lane 3: IN 100 μmol/L; lane 4: IN 200 μmol/L; lane 5: IN 400 μmol/L; lane 6: IN 600 μmol/L.

Effect of IN on CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein expression in wtp53/SW480 cells

After 24 h of action of IN at different concentration, it was found by Western blot analysis that the expression of CDK_2 and CDK_4 was decreased with the increase in IN concentration in certain range. Figure 2 shows that IN can significantly down-regulate the protein expression of CDK_2 and CDK_4 in a dose-dependent manner in a certain range, and the inhibitory effect was most evident at a IN concentration of 400 and 600 μmol/L; CDK_4 seems to be more effective. Meanwhile, the protein expression of $p21^{WAF1/CIP1}$ was increased with the augment of IN concentration in a dose-dependent manner in certain range, most evidently at a IN concentration of 400 μmol/L, and reduced to basic level at a IN concentration of 600 μmol/L.

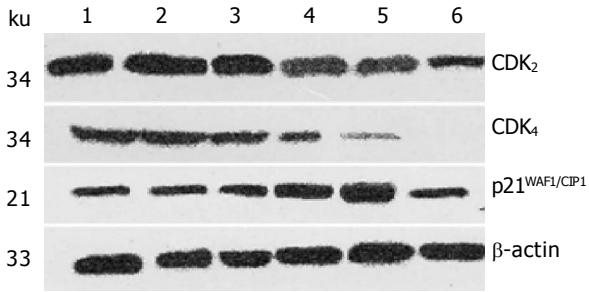

Figure 2 The effect of IN on CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein expression in wtp53/SW480 cells. Lane 1: Control group; lane 2: DMSO group; lane 3: IN 100 μmol/L; lane 4: IN 200 μmol/L; lane 5 IN 400 μmol/L; lane 6: IN 600 μmol/L.

DISCUSSION

Inhibition of tumor cells proliferation and induction of tumor cells apoptosis are associated with the blocking of the programmed progression of cell cycle[11-13]. NSAIDs inhibit proliferation and induce apoptosis in human colorectal cancer cells *in vitro*[14]. Our previous studies have

shown that IN inhibit proliferation of colon cancer cells by increasing the ratio of cells in G_0/G_1 phase in tumor cells, reducing the ratio of cells in S phase and G_2/M phase and thus lead to cell apoptosis, western blot analysis was employed to investigate the effect of IN on colon cancer cell line HCT116 contained with wild-type p53 and found it could down-regulate the expression of CDK_2 and CDK_4, and up-regulate $p21^{WAF1/CIP1}$ expression.

For its instability in solution and a short half-life of only few minutes, p53 protein is difficult to be detected by western blot. In this study, Western blot analysis was employed to detect the expression of CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein. To eliminate the error caused by the volume of protein, β-actin protein was used as control, for its stable expression and abundant content in the same class of cells which could ensure the reliability of objective protein and β-actin protein.

The results of the study showed that: IN down-regulated the expression of CDK_2 and CDK_4 protein, especially caused dramatic decreases in Cdk_4 activities, which leads to a G_1 phase cell cycle arrest, in the meantime, up-regulated $p21^{WAF1/CIP1}$ protein in wtp53/SW480 cells, in a dose-dependent manner within a certain range of concentration; while it had no effect on the expression of CDK_2, CDK_4 and $p21^{WAF1/CIP1}$ protein in SW480 cells, suggesting that the existence of wild-type p53 was required for the down-regulation of CDK_2 and CDK_4 expression by IN[15]. Therefore, we can speculate that up-regulation of $p21^{WAF1/CIP1}$ protein expression inhibits CDK_2 and CDK_4 expression as well as their activity, $p21^{WAF1/CIp1}$ being an important physiological regulator of CdK_4 complexes[16], it increased with the augment of IN concentration, most evidently at 400 μmol/L, and reduced to a basic level at 600 μmol/L, the most effective being 400 μmol/L; the drop of CDK_2 and CDK_4 activity in turn decreases the activity of CDK_2 or CDK_4 complex and induces cell cycle arrest in G_2 phase, indicating the effect of IN on colon cancer cells transfected with wild-type p53, namely wtp53/SW480 cells, being to induce cell apoptosis depending on p53 in a dose-dependent manner. Based on the results of previous studies and current research, we conclude IN induces cell cycle arrest in G_2 phase in a p53-dependent way and inhibits cell proliferation in a p53-$p21^{WAF1/CIP1}$ dependent way.

As it is well known, cell cycle is dominated by a regulatory network. A cell proliferation-regulating system composed of cyclins, CDKs and CKIs, can accelerate the progression of cell cycle and thus promote cell proliferation. The main regulatory point to start cell cycle is in G_1 phase, the core in cell cycle regulation are cyclin and CDK[17], and the formation of CyclinD/CDK_4 and CyclinE/CDK_2 is the rate-limiting step for progression of G_1 phase. Activation of CDK_2 and CDK_4 can yield many cell cycle related proteins, thus prompting progression from G_1 phase to S phase[18,19]; $p21^{WAE1/CIP1}$, a major member of CIKs family, which is closely related with growth and differentiation of tumor cells, is an extensive inhibitor for CDK, it can bind and block Cyclin/CDK complex and thus inhibit the activity of CDK_2 and CDK_4, stop the progression of cell cycle and induce G_2 phase arrest[11]. Wild-type p53 and $p21^{WAF1/CIP1}$, which are functionally related, are the inhibitors for CDKs-cyclin complex[20,21], so abnormality in the p53 or $p21^{WAF1/CIP1}$ gene will lead to abnormal cell proliferation[22-24]. There is a high frequency of p53 mutation in colon cancer, and mutant p53 can neither stop cell growth nor induce cell apoptosis, therefore, colon cancer is resistant to conventional chemotherapy drugs and not sensitive to assistant treatment of DNA damage. Transfection of wild-type p53 together with chemotherapy and radiotherapy can evidently induce apoptosis, reduce the dosage of chemotherapy and radiotherapy and decrease side effects[25,26]. Thus, it is held by some that for many tumors with p53 mutation or deletion which has a very poor sensitivity to radiotherapy and chemotherapy, gene transfection should be considered to enhance the sensitivity[27-29]. So the tumor-suppressing protein p53 mediate chemotherapy drugs induced apoptosis through the regulatory effect of p21 protein on cell cycle progression[30,31]. *In vitro* experiments have proved that IN inhibits cell proliferation, alters cell cycle and arrests G_1 phase possibly through down-regulating CDK_2 and CDK_4 protein expression and up-regulating $p21^{WAF1/CIP1}$ protein, giving elementary experimental evidence to elucidate the mechanism by which IN inhibits colon cancer, but much needs to be done to elucidate the anti-tumor mechanism of IN in detail[32] and on a network basis.

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• *BRIEF REPORTS* •

Post-radiation survival time in hepatocellular carcinoma based on predictors for CT-determined, transarterial embolization and various other parameters

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Abstract

AIM: In this retrospective study of unresectable hepatocellular carcinoma (HCC), we have investigated the efficacy of CT-derived parameters, laboratory measurements, clinical assessment and associated transarterial embolization (TAE) as predictors of post-radiotherapy survival time.

METHODS: Sixty-six patients diagnosed with unresectable HCC that had undergone radiotherapy at two medical university hospitals in Taipei were enrolled in the study. Using multivariant analysis, pre-treatment parameters including tumor number and CT confirmation of PVT and ascites were compared. Multivariant analysis was also used for comparison of the mean pretreatment values for laboratory measurements, including alpha-fetoprotein, direct/total bilirubin and GOT/GPT levels, and clinical history of chronic hepatitis across the three survival-time categories. The χ^2 was used to test the significance of the relationship between survival time and TAE procedure. The *P* values for the above tests were deemed statistically significant where $P<0.05$.

RESULTS: Portal vein thrombosis ($P = 0.032$) and ascites ($P<0.05$) were negative predictors of post-radiation survival time. Low-grade liver cirrhosis (A or B), lower tumor volume and low levels of AFT, GOT/GPT, and total bilirubin were predictors of longer post-radiation survival time ($P<0.05$).

CONCLUSION: The CT and clinical and laboratory assessment provide a reference for, and enable estimation of, probable survival times in HCC patients after radiotherapy. Tumor volume, severity of liver cirrhosis, status with respect to portal vein thrombosis and ascites and AFT, GOT/GPT and total bilirubin values were significant predictors of survival in this study.

INTRODUCTION

Radiotherapy treatment for hepatocellular carcinoma (HCC)[1] is still considered unorthodox in Taiwan. However, there has been a trend towards increased utilization in unresectable and TAE-nonsusceptible cases.

In this study, we share our experience over the past six years with HCC irradiation to provide a reference for prediction of survival time based on clinical, laboratory, and image-derived information[2], and particularly to discuss the value of pre-TAE treatment.

We also compared our results with those of other analogous research[1].

MATERIALS AND METHODS

Patients

From June 1998 to June 2004, radiotherapy for a total of 66 patients diagnosed with unresectable HCC was performed at Taipei Medical University Hospital (TMUH) and Taipei Wan-Fang Hospital (TWH).

The mean age for the 35 male (53%) and 31 female (47%) patients was 58 years. Diagnosis was based on cytology, high serum alpha-fetoprotein and image findings characteristic of malignancy (including angiography and CT follow-up).

Various factors were considered, including: laboratory data for alpha-fetoprotein, total bilirubin, and GOT/GPT; tumor number and volume and existence of portal vein thrombosis (PVT) and ascites, based on pre-treatment CT; clinical grade of liver cirrhosis (Child's A, B, C); history of chronic hepatitis; and status for the associated therapeutic factor, transarterial embolization (TAE).

Pre-treatment spiral CT examination (5-mm slices) was

performed, and the following parameters recorded: tumor number and volume (as described below), and presence of PVT or ascites.

VARIAN Eclipse 7.1.35.7 software (Integrated Treatment Planning System) was used for CT-based measurement of tumor volume, with the images made available on a large screen monitor. The distance-reference line on the CT images was used to calculate the pixel size. The tumor outlines were traced manually on the screen using a mouse-controlled cursor. Tumor volume was calculated by multiplying the sum of the traced areas by the image reconstruction interval (summation-of-areas technique). All three-dimensional reformation volumes were calculated in milliliters.

The patients were treated using limited-field radiotherapy mostly irradiated with daily fractions of 180/250 cGy to a total dose of 5 000 cGy.

Survival time after radiation was divided into three categories: (1) 6 mo; (2) 6-24 mo; (3) >24 mo; with these considered dependent variables. Other variables included PVT, ascites, hepatitis, liver cirrhosis (A, B or C) and tumor number (n = 1, 2, or >2) with multivariant analysis used to determine statistical significance as independent predictors where $P<0.05$. Mean AFP, tumor size, and GOT/GPT and total bilirubin [Bili(T)] levels were compared for each of the three survival categories, with multivariant analysis used to verify statistical significance as independent predictors where $P<0.05$.

Statistical significance was also determined for the associated therapeutic factor, TAE, for the three survival-time categories. The significance of the relationship between TAE and survival time was evaluated using the χ^2 ($P<0.05$).

RESULTS

The overall survival rates for the 66 irradiation patients[3] for <6, 6-24 and >24 mo were 18%, 32% and 50% respectively. Of these 66 cases: 46 (70%) involved single lesions detected by pre-treatment spiral CT; 12 patients (18%) had two visible tumors; and, 8 individuals (12%) had multiple or diffuse lesions. Tumor volumes ranged from 4-1 948 mL. Pre-treatment CT scan was used for 16 (24%) and 29 instances (44%) of portal vein thrombosis and ascites respectively (Table 1).

The clinical assessments and grading for the liver cirrhoses are presented in Table 2. A total of 40 cases (61%) were associated with TAE treatment[4] before or during radiation therapy[5].

The pre-treatment laboratory data for alpha-fetoprotein, direct/total bilirubin, GOT/GPT and clinical history of chronic hepatitis are presented in Tables 2, 3.

We found that, for our sample, PVT[6] ($P = 0.032$) and ascites ($P = 0.025$) were predictors[7] of shorter survival time[8] after radiation therapy (Table 1). By contrast, the early stages of liver cirrhosis (A or B; $P = 0.03$ and 0.02 respectively) were predictors of longer survival time. The variant factors of chronic hepatitis and tumor numbers were, however, not significant predictors. Mean values for the study parameters were compared across the three survival times using multiple analysis (Tables 1, 2). It was demonstrated that AFP <91.8 ng/mL, tumor volume <178 mL; GOT <59 IU/L; GPT <43.5 IU/L, and total bilirubin <0.799 mg/dL were predictors of longer survival time post radiation therapy.

We found that the associated procedure for TAE did not predict survival time after radiation therapy (Table 4).

DISCUSSION

Our results may provide a reference for prediction of outcome after radiotherapy for HCC, based on CT and clinical and laboratory assessment.

In this retrospective study, the number of eligible cases from the radiation oncology departments at TMUH and TWH was scarcely sufficient. In comparison to the reference study of Guo and colleagues[1], high grade criteria were not set to exclude advanced or terminal-stage disease from our sample.

Table 1 Parameters of CT findings

Parameters	Survival time	<0.5 yr	0.5-2 yr	>2 yr	P
Tumor number					
	1	2	4	9	0.31
	2	2	6	4	0.38
	Multiple or diffuse	1	4	3	0.24
PVT		7	8	1	0.032
Ascites		7	16	6	0.025

Table 2 Parameters of clinical assessment

Parameters	Survival time	<0.5 yr	0.5-2 yr	>2 yr	P
Hepatitis		8	11	14	0.37
Cirrhosis					
	A	3	5	22	0.03
	B	5	13	6	0.02
	C	1	0	0	0.28

Table 3 Parameters of laboratory data

AFP	<0.5 yr	0.5-2 yr	>2 yr	P
Range	2.7-146 700	10-265 891	2.3-40 248	<0.0001
Mean	23 844	47 594.8	91.8	
Tumor size	<0.5 yr	0.5-2 yr	>2 yr	P
Range	126-916	4-1 948	19-1 405	<0.0001
Mean	365	178	204	
GOT	<0.5 yr	0.5-2 yr	>2 yr	P
Range	20-330	2.1-270	20-153	<0.0001
Mean	99	59	59.8	
GPT	<0.5 yr	0.5-2 yr	>2 yr	P
Range	31-92	0.8-138	22-442	<0.0001
Mean	50	43.5	94.2	
Bili (T)	<0.5 yr	0.5-2 yr	>2 yr	P
Range	0.4-2	0.5-21	0.19-1.84	<0.0001
Mean	1.39	1.12	0.799	

Table 4 Parameters of TAE treatment

Parameters	Survival time	<0.5 yr	0.5-2 yr	>2 yr	Total	P
TAE						
	Performance	9	13	18	40	0.47
	Not performance	3	8	15	26	

The two studies differed in terms of overall survival rate, with markedly shorter post-RT survival time demonstrated for our sample population. Basically, this is due to fundamental differences in the two studies. In the above-mentioned study, the cases were first treated in the same hospital. By contrast, the source of our cases could be divided into two categories. The first consisted of referrals from other hospitals or medical units without initial treatment at our two institutions, such that, in most cases, we were not able to take advantage of the 'golden period' in terms of successful TAE treatment. Further, many patients had already undergone multiple sequential TAE procedures and as such, poor response to further TAE could be anticipated. Further, we also suggest that our cases were more severe and more advanced in terms of HCC stage as determined from CT and clinical and laboratory parameters. Our second category of patients were initially treated in the same hospital after HCC was proven, as in the study of Guo and colleagues[1], with some of these referred to diagnostic radiation departments for TAE. Unfortunately, the first group outnumbered the second by more than nine-fold in our study, resulting in a significant decrease in prediction of success rate for our TAE cases, as well as, shorter median survival time post-RT treatment, compared to the reference study. Therefore, it appears reasonable to assume that the difference in patient-selection criteria explains the disparity between these two otherwise similar studies.

In conclusion, we found that the presence of PVT ($P = 0.032$)[9] and ascites ($P = 0.025$) after radiation therapy were predictors of shorter survival time. By contrast, the early stages of liver cirrhosis (A and B; $P = 0.03$ and 0.02 respectively) were predictors of longer survival time[10].

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• *BRIEF REPORTS* •

Correlation of CD95 and soluble CD95 expression with acute rejection status of liver transplantation

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Abstract

AIM: To analyze the expression levels of soluble form of CD95, CD95 ligand (sCD95 and sCD95L, respectively) in plasma and CD95 expression on $CD3^+$ cells in liver-transplanted recipients with acute rejection (AR).

METHODS: Peripheral blood mononuclear cells (PBMCs) were isolated from 30 clinically liver transplanted recipients. CD95 expression on $CD3^+$ cells was quantitatively measured by two-color fluorescence activated cell sorter (FACS) analysis. Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry. Plasma levels of sCD95 and sCD95L were detected by Enzyme Linked-Immuno-Sorbent Assay (ELISA). The results were compared with that from normal healthy volunteers (n = 15 individuals).

RESULTS: FACS analysis showed that CD95 expression on $CD3^+$ T cells was significantly increased in liver transplanted recipients with AR compared to that in stable recipients without rejection and infection or healthy individuals who did not undergo transplantation (18 676.93±11 588.34/molecule, 6 848.20±1 712.96/molecule, 6 418.01±2 001.95/molecule, respectively, P<0.01). Whereas no significant difference was seen between liver-transplanted stable recipients and healthy individuals. Furthermore, no significant differences were detected between each group with CD4/CD8 ratio or the percentage of $CD16^+56^+$ cells. Plasma levels of sCD95 were significantly higher in transplanted recipients with AR compared to that in stable recipients or healthy individuals (391.88±196.00, 201.37±30.30, 148.83±58.25 pg/mL, respectively, P<0.01). In contrast, the plasma levels of sCD95L in liver- transplanted recipients were not significantly different from that in healthy individuals.

CONCLUSION: The present results indicate that the increased CD95 expression on $CD3^+$ cells and the increased levels of sCD95 in plasma may modify the immunological situation of the recipients after transplantation or represent the ongoing graft rejection.

Key words: Liver transplantation; Acute rejection; CD95

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INTRODUCTION

Numerous studies have proposed several mechanisms of graft rejection in liver transplantation such as the manipulation of intragraft cytokines, apoptosis of graft infiltrating lymphocyte and so on. Cytokines play an important role in regulating immunological responses of the host against transplanted organs. They control the activation and differentiation of immune effector cells and mediate cytotoxic activity of the effector cells. It has been suggested that Type I $CD4^+$ and $CD8^+$ T cells (Th1, Tc1) cytokines (interleukin-2, interferon-γ and tumor necrosis factor (TNF)-α) might promote cellular rejection[1-3], whereas type II $CD4^+$ and $CD8^+$ T cells (Th2, Tc2) cytokines (interleukin-4 and interleukin-10) might suppress graft rejection[4].

T cells stimulated via the T-cell receptors (TCRs) not only proliferate, but also undergo subsequent apoptosis by activation-induced cell death (AICD)[5,6]. Much evidence indicates the implication of AICD in the immune responses against alloantigens. Recent studies have been reporting that some pathways involved AICD of T cells[7,8]. CD95/CD95 ligand (CD95L) pathway has been shown to be a major AICD mediator of T cells. CD95 (Fas or APO-1) and its ligand are cell surface proteins. CD95 is a 48-KD type I transmembrane protein member belonging to the TNF receptor family, and CD95L is a 40-KD type II integral membrane protein belonging to the TNF family[9]. The interaction between the CD95 and CD95L is recognized as a major pathway for the induction of apoptosis in cells and tissues[10]. It has been suggested that the CD95 and CD95L

play an important role in the regulation of immune responses to foreign antigens and in the induction of peripheral tolerance[11]. sCD95 has recently been detected in the serum of the patients with liver diseases, including injury, hepatitis, cirrhosis, hepatocellular carcinoma (HCC) and in the systemic lupus erythematosus patients[12,13]. It has been shown that human CD95L was secreted from activated T cells[14] and the sCD95L may act as a cytotoxic cytokine, although Tanaka and colleagues recently suggested its inhibitory function[15]. CD95/CD95L pathway is regulated by a number of implicating factors. Till now, the role of CD95 and CD95 ligand system in graft rejection is not fully understood and changes of their expression during liver allograft rejection have not been elucidated.

Given the above considerations, we aimed to examine CD95 expression by $CD3^+$ T cells and the plasma levels of sCD95 and sCD95L in human recipients after liver transplantation, and estimated their relation to the liver rejection. The significance of these results on liver transplantation will be discussed below.

MATERIALS AND METHODS

Patient

Thirty liver -transplanted recipients (24 men, 6 women) who were treated at Organ Transplantation Center of Tianjin First Central Hospital were divided into post-transplanted AR (n = 15) and post-transplanted stable (n = 15) groups. The patients had been transplanted within 3 mo of immunosuppressed treatment with FK506, zenapax, MMF, and steroids. AR was diagnosed by means of clinical, laboratory, and histologic evidence. Methylprednisolone was used for treatment. Blood samples were collected from AR group and stable group after liver- transplantation. The control group consisted of 15 healthy individuals. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation from ethylenediaminetetraacetic acid (EDTA) blood 5 mL. Plasma was collected from healthy donors and patients by centrifugation of heparinized peripheral blood (PB) at 3 000 r/min for 10 min. Plasma samples were divided into aliquots and stored at -70 ℃ until measured.

Methods

Plasma cytokines The concentration of sCD95 in plasma was determined by a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) (DIACLONE France). Briefly, a monoclonal antibody (mAb) specific for sCD95 has been coated onto the wells of the microliter strips provided. Plasma and standards of known sCD95 concentrations are pipetted into these wells. During the first incubation, the antigen and a biotinylated mAb specific for sCD95 are simultaneously incubated. After five washes with 0.05% Tween 20-phosphated buffered saline (PBS), pH7.4, the enzyme (streptavidin-peroxydase) is added. After incubation and washing, to remove all unbound enzyme, a substrate solution, which acts with the bound enzyme, is added to induce a colored product. The intensity of this colored product is directly proportional to the concentration of sCD95 present in the plasma. The same ELISA system for sCD95L was used for the *in vitro* quantitative determination of sCD95L in plasma.

Determination of lymphocyte subpopulations Staining with mAb was performed in 100 μL aliquots of heparinized PB, followed by lysis of red blood cells and fixation with 1% paraformaldehyde (PAF). Phycoerythrin (PE) conjugated mouse anti-human CD4, CD8, CD16, fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD56 and isotype-matched control mAbs (All from Becton Dickinson) were used. 10 000 events were acquired using fluorescence activated cell sorter (FACS) Calibur and analysis was performed using CELLQUEST software (Becton Dickinson).

Quantitative measurement of cell surface expression of CD95 (Fas/Apo-1) expression on $CD3^+$ lymphocytes by dual-color flow cytometry Fifty microliter of cell suspension was added to each of 2-5 mL polystyrene snap cap tubes. To the first tube (T1), 25 μL negative isotypic control (BIOCYTEX, France) was added. To the second tube (T2), 25 μL FITC conjugated mouse anti-human CD95 (Fas/Apo-1) mAb (BIOCYTEX) was added. To the third tube (T3), 50 μL of QuantiBRITE beads (BDB) suspension (BIOCYTEX), which were coated with increasing and accurately known quantities of mouse immunoglobulins G was added. Samples were incubated for 10 min at room temperature. Subsequently, in each of the tube, 10 μL of PE conjugated mouse anti-human CD3 mAb (Becton Dickinson) was added. Samples were incubated at room temperature for 10 min, followed by washing in PBS with 2% fetal calf serum and fixation in 1% PAF and then analyzed by flow cytometry. The standard curve (Figure 1C) was made by plot the MFI (mean fluorescence index) calibration values obtained from T3 on the X-axis and their corresponding number of mAb molecules on the Y-axis (Figure 1B). Note the MFI values of T1 and T2 obtained on the corresponding histogram after gating $CD3^+$ cells (Figure 1A). Interoplate the MFI values of T1 and T2, then read the corresponding number of mAbs directly off the curve. The number of specific sites was obtained by subtracting T1 value to T2 value.

Statistical analysis

All data are presented as the mean±SD. Statistic analyses were performed with the t-tests. P-values of less than 0.05 were regarded as significant throughout the study.

RESULTS

We analyzed and compared freshly isolated PBMCs from liver-transplanted patients and healthy individuals as regards CD95 expression on $CD3^+$ T cell by dual-color flow cytometry (Figure 1). We did not find any significant difference between post-transplanted stable recipients and healthy individuals. However, CD95 expression on $CD3^+$ T cells was significantly increased in liver transplanted recipients with AR compared with that in stable recipients without rejection and infection or healthy individuals who did not undergo transplantation (P<0.01, Table 1).

We also investigated the sCD95 and sCD95L in plasma by ELISA. Plasma levels of sCD95 were significantly higher

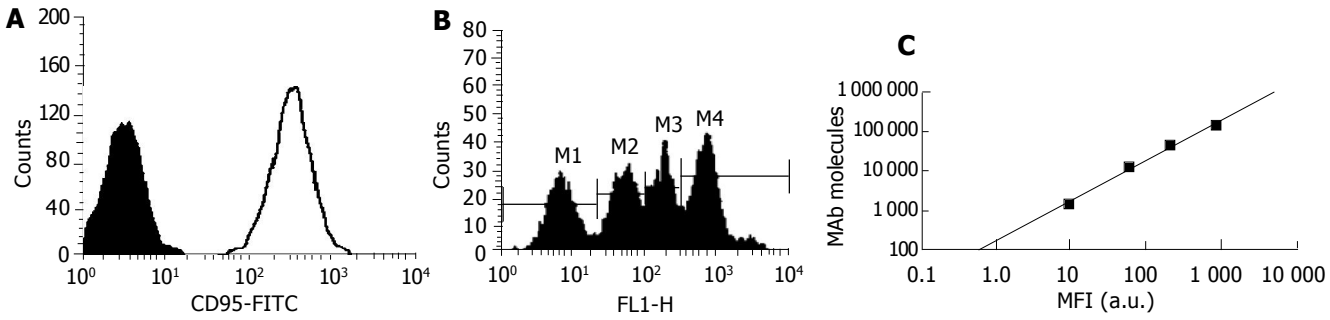

Figure 1 Quantitative measurement of cell surface expression of CD95 expression on $CD3^+$ lymphocytes PBMCs were labeled with QuantiBRITE beads suspension (T3), CD95-FITC (T2) or isotype control IgG1-FITC (T1) and CD3-PE as described in materials and methods. A: Histogram showed the expression of CD95 on $CD3^+$ cells. Solid histogram is isotype control. Bold line: CD95; B: Histogram showed the fluorescence intensity of the mouse immunoglobulins G in T3; C: The standard curve was made by plot the MFI calibration values obtained from Figure 1B on the X-axis and their corresponding number of mAb molecules on the Y-axis.

in transplanted recipients with AR than that in stable recipients or healthy individuals ($P<0.01$). In contrast, the levels of sCD95L in liver transplanted recipients were not significantly different from that expressed in healthy individuals (Table 1).

As concerns T lymphocyte subpopulations, no significant differences were seen among each group with CD4/CD8 ratio and the percentage of $CD16^+56^+$ cells (Table 2).

Table 1 Levels of CD95 expression on $CD3^+$cells (/molecule) and levels of sCD95, sCD95L (pg/mL) in plasma from post-transplanted AR group, post-transplanted stable group and healthy group

	Post-transplanted stable group ($n=15$)	Post-transplanted AR group ($n=15$)	Healthy group ($n=15$)
CD95 (/molecule)	6 848.20±1 712.96[b]	18 676.93±11 588.34	6 418.01±2 001.95[d]
sCD95 (pg/mL)	201.37±30.30[b]	391.88±196.00	148.83±58.25[d]
sCD95L (pg/mL)	279.42±79.72	226.82±132.63	254.38±96.29

[b]$P<0.01$ *vs* post-transplanted stable group; [d]$P<0.01$ *vs* health group.

Table 2 CD4/CD8 ratio, the percentage of $CD16^+56^+$ cells in post-transplanted AR group, post-transplanted stable group and healthy group

	Post-transplanted stable group ($n=15$)	Post-transplanted AR group ($n=15$)	Healthy group ($n=15$)
CD4/CD8	1.5±0.5	1.8±0.4	1.5±0.2
$CD16^+56^+$ (%)	15.4±9.5	20.4±9.4	17.0±7.5

DISCUSSION

Acute rejection (AR) is a well-known complication of allograft transplantation. T lymphocytes appear to be absolutely required in this rejection process[16,17]. Fas (CD95) is expressed in resting peripheral blood T cells and the Fas antigen as well as the ligand (FasL, CD95L) are up-regulated following T cell activation. Increased expression of FasL may activate the cytotoxic pathway, leading to the graft damage by Fas system activation and result in apoptosis.

The studies of Rivero *et al*[18], showed that up-regulated expression of Fas antigen in liver tissue with liver rejection but not in liver tissue without rejection, suggesting the importance of the Fas system in the rejection of liver grafts. Our studies, which focused on the peripheral blood lymphocytes, showed that CD95 expression on $CD3^+$ cells did not have any significant difference between liver-transplanted stable recipients without rejection and infection and healthy individuals who did not undergo transplantation. This result was similar to that of Renzo, who demonstrated that Fas on $CD3^+$ T cell and FasL mRNA expression in PBMC have no significant difference between cardiac-transplanted subjects and normal controls[19]. However, when compared to the recipients with AR to the stable recipients or healthy individuals, we found that the CD95 expression on $CD3^+$ cells was significantly increased. This increasing may have been caused by antigen stimulation. Stimulation through the CD3-TCR complex up-regulates CD95 expression and induces CD95L expression[20,21]. Through these cell surface molecules, activated T cells can commit suicide through formation of CD95-CD95L complexes[22,23]. Therefore, we suppose that because of the higher CD95 expression, $CD3^+$ T lymphocyte in AR patients may undergo more apoptosis than that from stable recipients, so that the spontaneous tolerance to the allograft may develop. On the other hand, previous animal studies of cardiac, renal and liver transplantation demonstrated that FasL up-regulation in allografts in rejection condition[24,25]. Therefore, we cannot exclude that in AR patients, the CD95 higher expression T lymphocyte may infiltrate in the allograft and induce apoptosis through formation of CD95-CD95L complex, then accelerate the rejection process.

In the meantime, we determined the plasma levels of sCD95 in liver- transplantation recipients. The sCD95 results from the deletion of the transmembrane domain of CD95. The levels of sCD95 were significantly increased in liver transplanted recipients with AR compared to that in stable recipients or healthy individuals. It has been speculated that sCD95 may reflect the expression levels of Fas antigen on tissue and the severity of inflammatory activity[26-30]. Therefore, the sCD95 detected in the present samples might be shedding from injured organs that express CD95. The significant increase of sCD95 in AR recipients were strong associated with the serum levels of total bilirubin, AST and ALT (unpublished data), implying that the levels of sCD95 may reflect the graft damage, which could be useful when

evaluating response to treatment. A previous study demonstrated the expression of CD95L mRNA in kidney-transplanted grafts at AR[31]. However, in this study, plasma levels of sCD95L in liver- transplanted recipients were not significantly different from that in healthy individuals, indicating that sCD95L might not contribute to AR. Concomitant immunosuppression, which is routinely administered in human transplantation cases, must also be a main cause for no increasing the FasL expression in rejection condition. Further, studies will be needed to elucidate the role of sCD95L in chronic rejection.

T lymphocytes can be separated into two sub-sets based on their expression of the CD4 and CD8 molecules on the cell surface. There are contradictory opinions on the relative contribution of $CD4^+$ and $CD8^+$ T cells to allograft rejection. Some animal models indicate that there is an absolute requirement for $CD4^+$ T cells in allogeneic rejection, whereas in others CD4-depleted mice reject certain types of allografts[32]. Haskova *et al* used CD4- or CD8-deficient knockout mice to investigate the role of T cell subsets in allograft rejection. The results showed that $CD4^+$ T cells play a critical role in the rejection of corneal allografts, whereas $CD8^+$ T cells appear to be involved in the rejection of skin allografts[33]. In our study, the CD4/CD8 ratio had showed no significant difference among AR group, stable group and healthy group. This finding is not compatible with that of Sadeghi *et al*[34] who showed that T lymphocyte sub-populations were lower in rejecting than in non-rejecting patients after renal transplantation. This difference may result from different organ allograft. Further studies will be needed to better understand the role of CD4 and CD8 T lymphocyte in the allograft rejection.

Human natural killer (NK) cells are defined by their ability to lyse target cells without prior sensitization and without restriction by major histocompatibility (MHC) antigens. It has been shown that these cells play an important role in immune defenses, especially after hematopoietic transplantation[35]. In our study, we demonstrated that the percentage of $CD16^+CD56^+$ cells did not change in AR patients, implying that NK cells may not play an important role in AR in liver transplantation or the immunosuppressive therapy interfere with the generation of NK cells.

In conclusion, monitoring of CD95 on $CD3^+$ T cells and in plasma may provide an important clue to a better understanding of the pathogenesis of liver graft rejection and would be a helpful tool to develop new therapeutic approaches for the prevention of AR by controlling the CD95/CD95L signaling.

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• *BRIEF REPORTS* •

Expression of hypoxia-inducible factor 1α and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival

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Abstract

AIM: To study the expression of hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) in hepatocellular carcinoma (HCC) and the impact on neovascularization and survival.

METHODS: Expressions of HIF-1α, VEGF and microvessel density (MVD) are studied through immunohistochemistry in 36 cases of HCC and the corresponding paraneoplastic tissue and 6 cases of normal liver tissue. The relationship of the expressions of HIF-1α and VEGF with the clinicopathological data and survival are analyzed.

RESULTS: The positive rate of VEGF in HCC was 32/36, which is significantly higher than that in paraneoplastic tissue and normal liver tissue ($P<0.05$). The expression of HIF-1α in HCC tissue is 24/36, also higher than that in paraneoplastic tissue and normal liver tissue ($P<0.05$). The expression of VEGF and HIF-1α in HCC with microscopic venous invasion is significantly higher than that in HCC without microscopic venous invasion ($P<0.05$). Spearman correlation analysis does not only show the expression of HIF-1α as correlated with the expression of VEGF ($r_s = 0.459$, $P<0.01$), but it also shows the expression of HIF-1α and VEGF as correlated with MVD ($r_s = 0.412$ and 0.336, respectively, $P<0.05$). The differences of the survival rates among VEGF positive group and VEGF negative group are significant ($P<0.05$), whereas the differences of the survival rates among the HIF-1α negative group and positive group are not significant ($P>0.05$).

CONCLUSION: HIF-1α plays important roles in neovascularization in HCC possibly through regulation of VEGF transcription.

Key words: Hypoxia-inducible factor 1 alpha; Vascular endothelial growth factor; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China. Owing to the improvement of surgical technique and early diagnostic methods, the resection rate of HCC has increased[1,2]. However, the postoperative relapse and metastatic rate remains high[3]. Neovascularization may play important roles in the relapse and metastasis of HCC[4-6].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional factor composed of the two basic-helix-loop-helix (bHLH) -PAS α and β subunits[7,8]. HIF-1α is the unique, O_2-regulated subunit that determines HIF-1 activity. A series of genes and proteins that may increase the survival of tumor cells under hypoxia conditions, including vascular endothelial growth factor (VEGF), insulin-like growth factor, inducible nitric oxide synthase, platelet-derived endothelial growth factor, glucose transporter 1, lactate dehydrogenase, erythropoietin and nitric oxide synthase gene, are regulated by HIF-1α[9-12]. Thus, HIF-1α may play important roles in tumor progression.

VEGF is a potent proangiogenic agents. There is a specific binding site with HIF-1α in the initiating area of VEGF genes[13-15]. Thus, HIF-1α may also be important in the regulation of neovascularization of malignant tumors.

The objective of this clinical study is to investigate the expression of HIF-1α and VEGF in HCC and the impacts of the expressions of the two biomarkers on neovascularization and survival in HCC.

MATERIALS AND METHODS

Patients

Thirty-six patients (32 men, 4 women) with HCC who underwent hepatic resection between March 2000 and October 2001 are included in this retrospective study. The hepatitis B surface antigens are positive in all HCC cases. None of the patients received other therapy before the operation. Median patient age, at time of surgery, is 45.9 (19-77) years.

Paraneoplastic liver tissue is taken from non-cancerous tissue 1 cm away from the tumor margin. Six samples of normal liver tissue are taken from the liver tissue around the hepatic hemangioma. All the sections are re-evaluated and the diagnosis are confirmed.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens are obtained. Serial 4 μmol/L sections are prepared, and one is stained with H&E. Sections are stained for HIF-1α, VEGF and CD34 based on streptavidin-biotin-horseradish peroxidase complex formation as mentioned before[16]. In brief, after deparaffinization and rehydration, endogenous peroxidase is blocked with methanol containing 0.3% hydrogen peroxide for 30 min. Slides are treated with target retrieval solution. Primary antibodies include monoclonal anti-HIF-1α antibody (clone H1alpha67; NEOMARKERS), monoclonal anti-VEGF antibody (clone JH121; NEOMARKERS) and monoclonal anti-CD34 antibody (clone QBEnd/10; NEOMARKERS). The peroxidase reaction is developed, using diaminobenzidine and the slides used are washed and mounted in mountant. Nuclei are lightly counterstained with hematoxylin. Negative controls are performed using PBS instead of the MAb. Two investigators (Bao-An Liu and Seng-Lin Chen) independently evaluate the immunohistochemistry. The immunohistochemical results for HIF-1α and VEGF are classified as follows: -, no staining; +, weak staining; ++, strong staining. Microvessel density, assessed by immunostaining for CD34, is determined according to Weidner[17]: the immunostained sections are scanned at low magnification (×40), and the tumor area with the highest density of distinctly highlighted microvessels ('hot spot') is selected. Microvessel density then is determined in the hot spot by counting all vessels at a total magnification of ×200. Each stained lumen is regarded as a single CD34 positive cell is visible, this cell is also interpreted as representing a microvessel.

Follow-up

Survival are calculated from the date of operation. Follow-up is performed through letter and telephone. Median duration of follow-up is 300 d (60-960 d). Follow-up rate is 91.6% (33/36).

Statistical analysis

Mann-Whitney test and Spearman coefficient of correlation are used as appropriate. Survival curves are calculated using Kaplan-Meier estimates and differences among groups are tested by log rank test. For all tests, a P value of less than or equal to 0.05 is considered as significant. All statistics are calculated through SPSS 10.0 software.

RESULTS

Expression of HIF-1α

There are no positive staining in negative control. The positive staining is located in the cytoplasma and/or the nucleus (Figure 1). There are 24 sections with positive staining of HIF-1α, including 3 strongly positive sections and 21 weakly positive, among the 36 sections of HCC. However, among the 36 sections of paraneoplastic tissue, all but 2 sections with weakly positive stain of HIF-1α are negative and all the 6 sections of normal liver tissue are negative. The expressions of HIF-1α in the bile duct and the vessels are negative. The expression of HIF-1α in the HCC tissue is statistically significantly higher than that in the paraneoplastic tissue and normal liver tissue (P<0.01).

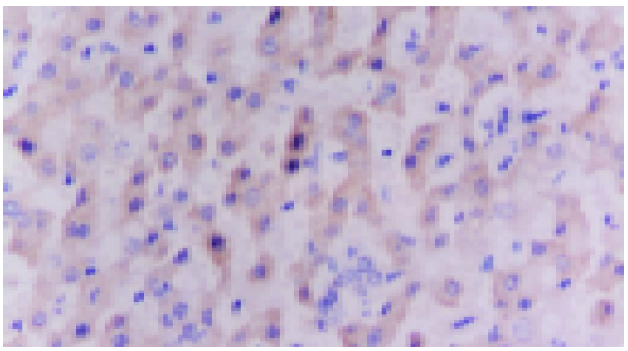

Figure 1 A specimen of HCC with strong expression of HIF-1α. Immunohistochemistry, original magnification, ×400.

Expression of VEGF

The positive staining of VEGF is located in the cytoplasma (Figure 2). The positive rate of VEGF in HCC is 32/36, including 16 strongly positive and 16 weakly positive. Among 36 sections of paraneoplastic tissue, all but 6 sections with weakly positive staining of VEGF are negative and all the 6 sections of normal liver tissue are negative. The expression of VEGF in the HCC tissue is statistically significantly higher than that in the paraneoplastic tissue and normal liver tissue (P<0.05).

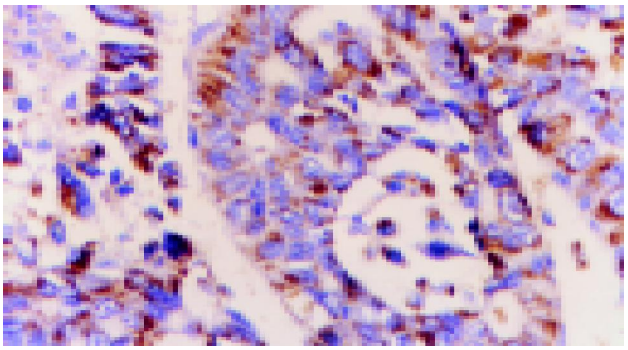

Figure 2 A specimen of HCC with strong expression of VEGF. Immunohistochemistry, original magnification, ×400.

Association of HIF-1α and VEGF expression with clinicopathological data

Significant association is only found between HIF-1α and VEGF expression and microscopic venous invasion (P<0.05), among the 5 clinicopathological data including serum alpha fetoprotein concentration, cirrhosis, Edmondson grading, tumor size and microscopic venous invasion

(Table 1). The expression of HIF-1α or VEGF in HCC with microscopic venous invasion are significantly higher than those in HCC without microscopic venous invasion (P<0.05).

Table 1 Association between expression of HIF-1α and VEGF with clinicopathological data in HCC

	n	HIF-1α		VEGF		P
		-	+	-	+	
AFP concentration (ng/mL)						
≤50	14	2	12	2	12	
>50	22	10	12	2	20	>0.05
Cirrhosis						
With	25	10	15	4	21	
Without	11	2	9	0	11	>0.05
Edmondson grading						
I–II	12	2	10	1	11	
III–IV	24	10	14	3	21	>0.05
Tumor size (mm)						
≤50	9	3	6	1	8	
>50	27	9	18	3	24	>0.05
Microscopic venous invasion						
With	24	3	21	1	23	
Without	12	9	3	3	9	<0.05

Association of HIF-1α and VEGF expression with neovascularization

CD34 expression is positive in 33 cases of HCC (Figure 3). Spearman correlation analysis does not only show the expression of HIF-1α as correlated with the expression of VEGF (r_s = 0.459, P<0.01), but also shows the expressions of HIF-1α and VEGF as correlated with that of CD34 (r_s = 0.412 and 0.336, respectively, P<0.05).

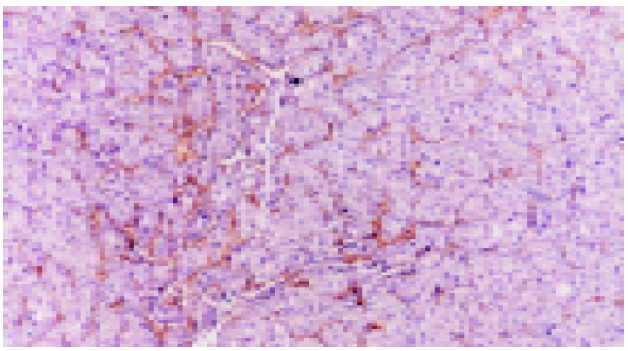

Figure 3 A specimen of HCC with strong expression of CD34. Immunohistochemistry, original magnification, ×100.

Survival analysis

The overall 1, 2-year survival rates of the 36 patients are 52.5% and 28.9%. The 1, 2-year survival rates are 70.7% and 48.0% in HIF-1α negative HCC group, 43.4% and 14.9% in HIF-1α positive group. Log rank test shows the differences among the two groups are not significant (P = 0.10, Figure 4).

The 1, 2-year survival rates are both 75.0% in VEGF negative HCC group, 49.2% and 14.5% in VEGF positive group. Log rank test shows the differences among the three group are significant (P<0.05, Figure 5).

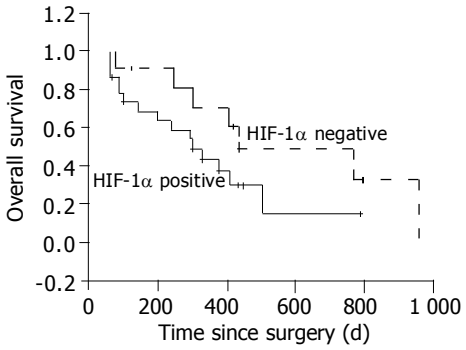

Figure 4 Overall survival curve of patients with different expression of HIF-1α.

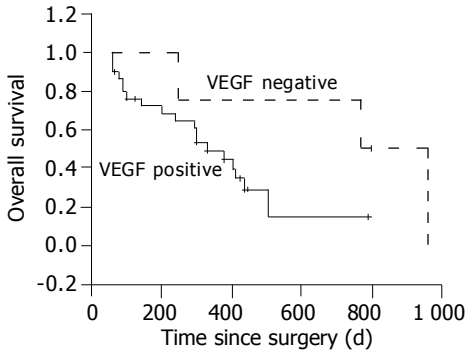

Figure 5 Overall survival curve of patients with different expression of VEGF.

DISCUSSION

The rate of respectability of HCC has risen and the 5-year survival rate has improved[1-3]. However, the postoperative relapse rate of HCC remains higher, which becomes one of the main obstacles to improve the survival rate. The molecular mechanisms of the relapse and metastasis of HCC are thus of interest. Neovascularization is one of the key steps[4-6]. HIF-1α is thought to be involved in the regulation of neovascularization in malignancies. The purpose of this study is to determine whether the expression of HIF-1α increases in HCC tissue and the impacts of HIF-1α expression on neovascularization and survival.

Most studies about the effects of HIF-1α on the neovascularization in malignancies have used *in vitro* or animal models. It has been confirmed that in many cancer cell lines, hypoxia can induce the expression of some proangiogenic factors, including VEGF, insulin-like growth factor, platelet-derived endothelial growth factor, through HIF-1α-dependent manner. It has also been proven through the method of gene knockout that the loss of HIF-1α may significantly suppress the growth of tumors including glioblastoma and malignant teratoma and most importantly decrease the neovascularization of those malignancies[18,19].

HIF-1α is also proven to highly express in many kinds of malignancies, such as stomach, colon, breast, thyroid, pancreas, ovary, lung and otopharyngeal cancer[11,20-23]. Zhong *et al*[11] and Talks *et al*[20] have studied the expression of HIF-1α in 8 cases and 5 cases of HCC tissue using immunohistochemistry. The results show that 2 cases out

of 13 have positive HIF-1α expression. However, it is evident that the amount of the samples in the two studies is too small to conclude. In the current study, the expression of HIF-1α in 36 cases of HCC are studied through immunohistochemistry and the positive rate is 24/36, higher than that in paraneoplastic liver tissue and normal liver tissue. Also, we demonstrate for the first time to our knowledge that the expression of HIF-1α in HCC does not only have relationship with that of VEGF, but also with that of MVD and microvenous invasion. Thus, this clinical study has supported that HIF-1α may play pivotal role in the neovascularization in HCC through regulation of VEGF expression. Our findings are consistent with some previous reports in other cancers, such as ovarian cancer[21], ductal carcinomas *in situ*[22], non-small cell lung cancer[23] and supratentorial pure oligodendrogliomas[24].

In the present study, we find no significant difference in survival among HIF-1α negative group and positive group. However, overexpression of HIF-1α is demonstrated to be an independent prognostic factor by univariate and multivariate analysis in some malignancies, such as early-stage invasive cervical cancer[12], non-small cell lung cancer[23], oligodendroglioma[24] and oropharyngeal cancer[25]. Thus to definitely clarify possible differences in survival in HCC, larger scale studies will be needed.

We conclude that overexpression of HIF-1α protein is associated with neovascularization in HCC, possibly through regulation of VEGF transcription.

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• *BRIEF REPORTS* •

Application of atomic force microscopy in blood research

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Abstract

AIM: To find suitable solutions having lesser granules and keeping erythrocytes in normal shapes under atomic force microscopy (AFM).

METHODS: Eight kinds of solutions, 1% formaldehyde, PBS buffer (pH7.2), citrate buffer (pH6.0), 0.9% NaCl, 5% dextrose, TAE, 1640 medium and 5% EDTA-K_2, were selected from commonly used laboratory solutions, and venous blood from a healthy human volunteer was drawn and anticoagulated with EDTA-K_2. Before scanned by AFM (NanoScopeIIIa SPM, Digital Instruments, Santa Barbara, CA), a kind of intermixture was deposited on freshly cleaved mica and then dried in the constant temperature cabinet (37 ℃).

RESULTS: One percent formaldehyde, citrate buffer, 5% dextrose, TAE, were found to keep human erythrocytes in normal shape with few particles. Processed by these solutions, fine structures of human erythrocyte membrane were obtained.

CONCLUSION: One percent formaldehyde, citrate buffer, 5% dextrose and TAE may be applied to dispose erythrocytes in AFM. The results may offer meaningful data for clinical diagnosis of blood by AFM.

Key words: Atomic force microscopy; Solution; Erythrocyte; Fine structures

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INTRODUCTION

Human beings have experienced three revolutions in the observation of cells. Since 1665, when the first light microscopy was invented, the revolution of this kind of instruments has seen great improvements. But with the limitation of the wavelength of visible light, smaller structures of cells cannot be observed.

In the 20th century, with the development of electronics, electron microscope emerged. Though organelles could be detected under electron microscope, the samples often required special treatment, such as coating, staining and drying in vacuum. Due to these complicated preparation steps, surface information ran a risk of artifacts.

In 1986 the invention of atomic force microscopy (AFM)[1] provided a great source of information the detection of cell structures and this was a turning point. In principle, the AFM images are generated by moving a tip attached to a soft cantilever over the sample surface while recording its deflections as change of position. With atomic resolution and simple sample preparation, since the invention of atomic force microscopy (AFM) in 1986, AFM has been applied to many fields, such as physics, chemistry, biology and mechanics[2-4]. However, the application of AFM in medicine especially in clinical diagnosis is far from being developed. To dispose human cells with suitable solutions AFM is necessary in clinical diagnosis. The aim of this experiment was to find suitable solutions, which could easily be obtained, having lesser granules and keeping erythrocyte in normal shapes, under AFM. Here, eight kinds of laboratory's common solutions were selected to dilute human erythrocytes from which we found four of them were suitable for the observation of erythrocytes under AFM. These four kinds of solutions kept human erythrocytes in normal shape and had few particles. Processed by these solutions, fine structures of membranes of human erythrocytes were also scanned.

MATERIALS AND METHODS

Sample preparation

The experiment had two parts. In the first part, eight kinds of solutions were selected from commonly used laboratory solutions. They were 1% formaldehyde, PBS buffer (pH7.2), citrate buffer (pH6.0), 0.9% NaCl, 5% dextrose, TAE, 1640 medium and 5% EDTA-K_2. These solutions were separately deposited on freshly cleaved mica and then dried in a constant temperature cabinet (37 ℃) for AFM observation. In the second part, venous blood from a healthy human volunteer was drawn and anticoagulated with EDTA-K_2 Then normal human blood was mixed with

the solutions separately and stored in a refrigerator (4 ℃). Before scanning by AFM, a kind of intermixture was deposited on freshly cleaved mica, then dried in the constant temperature cabinet (37 ℃).

Atomic force microscope

AFM (NanoScope IIIa SPM, Digital Instruments, Santa Barbara, CA) was placed on an air-supported anti-vibration table. In the first part, commercially available contact mode cantilevers were used (Digital Instruments). Ten areas of each kind of solutions were obtained with scan area 50 μm ×50 μm and scan rate 1 Hz. In the second part, commercially available tapping mode cantilevers were used (Digital Instruments). Five areas of each sample were scanned. Each area was observed with a scan size of 30 μm, erythrocyte and 1 μm as well as 0.5 μm (0.5 μm area was the magnification of 1 μm area) in both periphery and middle of membranes of erythrocytes. Scan rate was 0.5-1.5 Hz.

RESULTS

Visualization of eight kinds of solutions

In daily clinical pathological diagnosis with light microscope and electron microscope, it is very important to observe human cells. We tried to apply AFM for pathological diagnosis. Thus the observation of human cells was necessary. But in most cases human cells should be disposed by solutions. Therefore, the solutions, which have less granules[5] and could keep cells in normal shapes and diameters, were required. We selected eight kinds of solutions, which were commonly used in laboratory to be observed by AFM.

A few of irregular granules were scanned in 1% formaldehyde with sizes of 0.5-2 μm. Several irregular granules were scanned in citrate buffer with sizes of 0.5-3 μm. Some irregular granules were scanned in 5% dextrose with sizes of 0.5-4 μm (Figure 1). A few of irregular granules were scanned in TAE with sizes of 0.6-7 μm. Several irregular granules were scanned in 5% EDTA-K_2 with sizes of 0.5-4 μm. A lot of granules were observed in PBS buffer with four kinds of shapes; finely branched granules with a size of 6 μm (Figure 2), bulkily branched ones with a size of 10 μm, cross ones with sizes of 3-6 μm and irregular ones with sizes of 0.3-1.5 μm. Many granules were also found in 0.9% NaCl with four kinds of shapes; butterfly granules with sizes of 0.3-7 μm, branched ones with sizes of 8-20 μm, diamond-shaped ones with a size of 24 μm and irregular ones with sizes of 0.4-10 μm. A lot of snow-shaped granules were observed in 1 640 medium.

From the above data, 1% formaldehyde, citrate buffer, 5% dextrose, TAE and 5% EDTA-K_2 had a few granules. These five solutions may be applied to further observations.

Visualization of intermixtures of eight kinds of solutions and human blood

To further select the suitable solutions, which could be applied to dispose human cells for AFM observation, these eight kinds of solutions were mixed with human blood. In samples of 1% formaldehyde with blood, citrate buffer with blood, 5% dextrose with blood, and TAE with blood, the following were found; firstly, few granules existed in images; secondly, human erythrocytes were bi-concave with diameters from 7 to 8 μm (normal diameters of human erythrocytes is 7 to 8.5 μm); thirdly, fine structures of human erythrocyte membranes featured with the shape of pores (15-100 nm) and protrusions (15-100 nm), the protrusions were around the pores, and the protrusions and the pores were in a waffle-like pattern (Figure 3). In terms of sizes and arrangements of pores and protrusions, there were no differences between the periphery and the middle of erythrocyte membranes. However, in samples of PBS buffer with blood (Figure 4), 0.9% NaCl with blood, 1 640 medium with blood, and 5% EDTA-K_2 with blood, a lot of

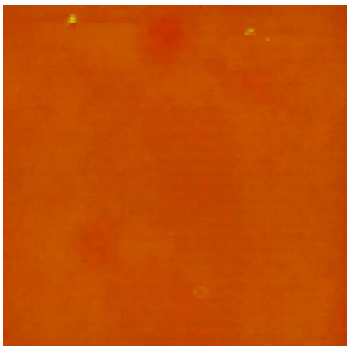

Figure 1 Some irregular granules were scanned in 5% dextrose. Scan size was 50 μm.

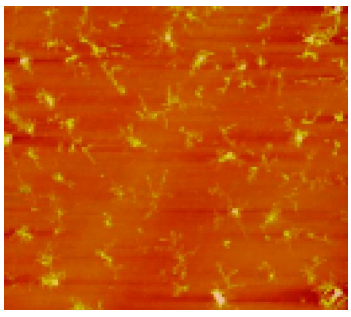

Figure 2 Finely branched granules were observed in PBS buffer. Scan size was 50 μm.

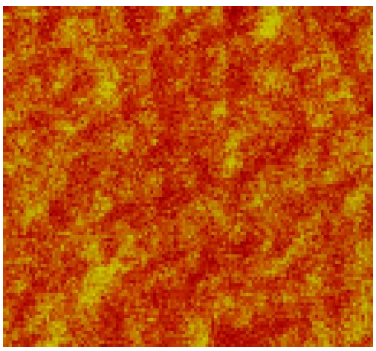

Figure 3 Fine structures of human erythrocyte membranes featured with the shape of pores and protrusions. The erythrocyte was mixed with 5% dextrose. Scan size was 500 nm.

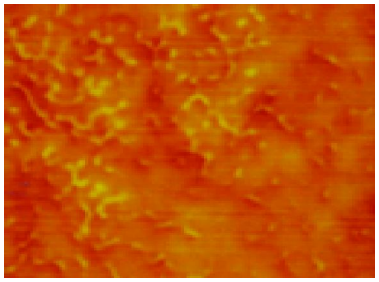

Figure 4 Fine structures of human erythrocyte membranes is shown. The erythrocyte was mixed with PBS buffer. A lot of granules existed in the image. Scan size was 500 nm.

granules existed in images, which were interferences in the observation. So these four solutions were abandoned. Human blood was also mixed separately with 5% formaldehyde, 3% glutaraldehyde and Tris buffer (0.05 mol/L, pH7.2). In 5% formaldehyde, the diameters of human erythrocytes were about 6 μm, which were smaller than normal ones. Three percent glutaraldehyde did not make the erythrocytes scatter evenly, and at the same time this kind of solution is poisonous. Tris buffer was mixed with human blood with the phenomena of hemolysis. Thus 5% formaldehyde, 3% glutaraldehyde and Tris buffer were also abandoned. From the above, 1% formaldehyde, citrate buffer, 5% dextrose and TAE are suitable for observation of human erythrocytes under AFM.

DISCUSSION

The lipid-globular protein mosaic model (LGPM) was developed by Singer and Nicolson in 1972[6]. They introduced that integral proteins and peripheral proteins are associated with lipid bilayer membranes. The pores and the protrusions observed in fine structures of human erythrocyte membranes might reflect the LGPM model.

As is known, there are several forms of transmembrane transport. In facilitated diffusion, substances with little solubility are transferred by special proteins. There are two types of facilitated diffusion. The first one is conducted by carriers which take dextroses, amino acids and mesostates through. The second one is carried out by channels, which let Na^+, K^+, Ca^{2+}, Cl^- through. The pores and the protrusions observed in fine structures of human erythrocyte membranes might be these special proteins of facilitated diffusion especially the pores were very similar with the channels. In active transport, the most famous is Na^+-K^+ pumps, which are special proteins embedded in membrane lipid bilayers. This kind of pumps transfers Na^+ and K^+ with consumption of ATP. With the methods of molecular biology, it is found that Na^+-K^+ pump is made up of α subunit and β subunit. The pores and the protrusions observed in fine structures of human erythrocyte membranes might be Na^+-K^+ pumps.

Receptors are very important in membranes, which are associated with many kinds of functions. In the surface of erythrocytes, there exist blood group antigens. The fine structures of human erythrocyte membranes probably contained receptors, for example, transferrin, and blood group antigens.

In terms of transmembrane signaling, voltage-gated channel especially Na^+ channels have been well studied. Na^+ channels consist of three subunits - α peptide chain, β_1 peptide chain and β_2 peptide chain, while their functions are really done by α peptide chain. α subunit contains four similar domains, and each domain has six transmembrane α-spirals, which are made up of hydrophobic amino acids. These four domains and hydrophobic α-spirals form a channel-like structure. When transmembrane potential is changed, these α-spirals move their sites because of their own electron charges; therefore, the "gates" of channels are opened. Other studies suggest that the second and the third α-spirals form the "inner wall" of the channel. The channel features are consistent with what had been observed in fine structures of human erythrocyte membranes.

Many kinds of diseases are pathological changes of proteins, which are difficult to be detected by light microscopy and electron microscopy. However, with the advantage of high resolution, the emergence of AFM makes it easy. By AFM the lesions of proteins could be distinctly observed. Therefore, AFM will be a very important instrument in daily diagnosis. Notwithstanding some methods of disposing erythrocytes[7-9], which are complicated and many of them follow the ways of electron microscope, in this experiment, a new way of disposing human cells was found for pathological diagnosis with AFM. Four kinds of suitable solutions were selected and the fine structures of human erythrocyte membranes observed might form the basis of further studies and diagnosis.

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• *BRIEF REPORTS* •

^{14}C-urea breath test in patients undergoing anti-tuberculosis therapy

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Abstract

AIM: Urea breath test (UBT) is a non-invasive diagnostic test for detecting the presence of *Helicobacter pylori* (*H pylori*). In this study we evaluated the effect of anti-tuberculosis therapy on the results of ^{14}C-UBT.

METHODS: Patients, with the diagnosis of tuberculosis (TB) who had a positive UBT at the point of starting anti-TB therapy, were included. None had a history of peptic ulcer disease or had taken antibiotics, bismuth compounds and/or PPI in the previous month. ^{14}C-UBT was repeated at the end of the second month and the end of treatment period and one month after completion of treatment course.

RESULTS: Thirty-five patients (23 males) were enrolled. 14C-UBT was negative in all 35 patients (100%) at the end of the second month and remained negative in 30 cases (85.7%) at the end of the treatment course. One month after completion of treatment course, UBT remained negative in 13 patients (37.1%).

CONCLUSION: Our report underscores the need for caution while interpreting urea breath test results in patients undergoing anti-TB therapy. Furthermore, the combination of drugs used in this study resulted in *H pylori* eradication in a minority of patients.

Key words: Urea breath test (UBT); *Helicobacter pylori*; Tuberculosis

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INTRODUCTION

Helicobacter pylori (*H pylori*) is the most renowned factor among peptic ulcer risk factors[1]. Eradication of this germ has contributed to a significant reduction in the peptic ulcer prevalence[2-5]. Several drug regimens have been introduced for *H pylori* eradication[6,7]. Urea Breath Test (UBT) is currently the standard means of determining *H pylori* eradication. Some drugs, including antibiotics are known to lower the accuracy of this test. In the present study, we evaluated specifically the effect of a four-agent anti-tuberculosis therapy on the results of ^{14}C-UBT in a group of patients with tuberculosis and positive baseline UBT.

MATERIALS AND METHODS

All patients referred to Amir-Alam General Hospital from January 2002 to December 2003 with a diagnosis of tuberculosis (TB) were evaluated. TB had been documented based on clinical and laboratory findings and anti-tuberculosis treatment was ordered for all of them. Patients with a history of documented peptic ulcer before treatment or using Bismuth, proton pump inhibitors (PPIs), H_2 blocker agents or antibiotics in the month before were excluded from the study. None of the enrolled patients had ever been treated for *H pylori* eradication or undergone gastric resection. UBT test was done for all patients at the time of starting anti-TB therapy and patients with positive tests were enrolled. The anti-TB regimen in all patients consisted of Isoniazid, Rifampicin, Ethambutol and Pyrazinamide for two months, after which the latter two drugs were stopped and the treatment was carried on with Isoniazid/Rifampicin until the end of the treatment course. Cases of spinal tuberculosis were planned for a 12-mo course of therapy, whereas a 6-mo course was considered for other types of tuberculous organ involvement.

^{14}C-UBT was repeated three times for every enrolled patient: (1) at 2 mo (time of stopping Ethambutol/Pyrazinamide); (2) end of treatment course (mo 12 for spinal TB cases); (3) one month after completion of the anti-TB treatment course. The tests were all performed in the Nuclear Medicine Laboratory, Shariati Hospital, Tehran University of Medical Sciences, by a single team of specialized staff. Each overnight fasting patient was given 1 μCi (37 kBq) of ^{14}C-urea

dissolved in 250 mL water, after thorough brushing. Breath samples were collected once before ingestion of the tracer and subsequently at 15 min after ingestion. The breath samples were trapped in 1 mmoL ethanolic hyamine hydroxide in 10 mL toluene-based scintillation fluid. Carbon-14 content was measured in disintegration per minute (DPM) mode using a liquid beta-scintillation counter. A cut-off value of 200 was set for the positive test result. Intermediate test result was defined as 50-200 DPM, and test results of <50 DPM were considered negative.

RESULTS

During the study period, 44 patients with a definite diagnosis of tuberculous infection were planned for anti-TB therapy. Three patients revealed a history of antibiotic therapy during the month before and were therefore excluded. Six more patients had negative or intermediate UBT results and were also excluded. Thirty-five patients including 23 males (age 17-55 years; mean age: 38.5) and 12 females (age 16-39 years; mean age: 24) were eligible for the study. Among the enrolled patients there were 12 pulmonary and 23 extra-pulmonary cases of TB including 5 patients with a diagnosis of vertebral tuberculous osteomyelitis (Table 1). None were critically ill or under treatment with immunosuppressive drugs.

At the end of the second month of therapy, UBT became negative in all 35 patients (100%). The test results at the end of the treatment course were still negative in 30 cases (85.7%). One month after completion of anti-tuberculosis therapy, UBT turned positive in 17 of 30 patients, so 22 patients (62.9%) had positive results at this point, and the test remained negative in 13 patients (37.1%, Table 2).

DISCUSSION

H pylori is a slow-growing, microaerophilic, gram-negative bacterium, whose most striking biochemical characteristic is the abundant production of urease. This bacterium colonizes gastric mucosa and elicits both inflammatory and immune lifelong responses, with release of various bacterial and host-dependent cytotoxic substances[8]. *H pylori* eradication can be established reliably by histology, rapid urease testing and the urea breath test (UBT). The UBT uses labeled urea (^{13}C or ^{14}C) that, in the presence of *H pylori*, is metabolized by urease to yield CO_2. The labeled gas is absorbed across the gastric mucosa and subsequently measured in the patient's expired breath.

Analysis of the results reported in studies in which urea breath-tests were evaluated against an accepted gold standard, confirms the great accuracy (sensitivity 97%; specificity 95%) of this technique[9].

There is general consensus[10-13] regarding the adverse effect of proton pump inhibitors (PPIs) on the UBT (false negative results range from 17% to 61%). Moreover, antibiotics and bismuth compounds reduce *H pylori* load such that infection may be undetectable. Thus, urea breath-tests should not be performed within 4 wk of receiving such drugs, whether given specifically to treat the infection or not[14].

In 1992, Mitchell found that a history of pulmonary TB might be associated with an increased prevalence of *H pylori* infection[15]. More recently, Woeltje assessed the prevalence of tuberculin skin test (TST) positivity in a cohort of 346 newly hospitalized patients. A history of peptic ulcer disease was one of the identified risk factors for a positive TST test (odds ratio: 4.53, $P = 0.017$)[16]. Increased risk of TB for persons with a history of peptic ulcer disease has also been reported[17]. *H pylori* is seen in high prevalence in some populations around the world[18] especially in regions having lower socioeconomic status[19-21]. The same is true for the distribution of tuberculosis which is, to a great extent, clustered in some developing countries[22]. Rationally, there seems to exist a population of considerable size, potentially exposed to both microorganisms.

In-vitro studies of Rifampicin and Streptomycin, two drugs commonly used in anti-tuberculosis regimens have suggested the efficacy of these agents against *H pylori*[23-25] and a decrease in *H pylori* seroprevalence during anti-tuberculosis therapy has been reported[26]. There is no report of using Rifampicin in *H pylori* eradication regimens but recently Rifabutin from the same family of agents has been implemented as rescue therapy against resident species in combination with Pantoprazole and Amoxicillin[27]. Isoniazid is used in treating mycobacterial species and acts via inhibiting mycolic acid synthesis. There is no report so far of the efficacy of this agent on non-mycobacterial microorganisms[28].

To our knowledge, there has been no specific report of the effect of anti-tuberculosis therapy on the accuracy of UBT. Our report shows that anti-TB therapy causes negative UBT results in a considerable fraction of patients, and so underscores the need for caution while interpreting urea breath test results in patients undergoing anti-TB therapy.

Table 1 Patient characteristics

Gender	Male	23 (65.7%)
	Female	12 (34.3%)
	Total	35
Age (yr)	Male	17-55 (38.5±11.2)
	Female	16-39 (24.0±8.6)
Type of infection	Pulmonary TB	12
	TB adenitis	9
	TB enteritis	5
	TB osteomyelitis (vertebra)	5
	Meningeal TB	2
	Peritoneal TB	2
	Total	35

Table 2 ^{14}C-urea breath test results among 35 patients during the course of anti-tuberculosis therapy

	Baseline	End of 2nd mo of therapy	End of treatment course	One month after completion on therapy
Positive (%)	35 (100)	0 (0)	4 (11.4)	22 (62.9)
Negative (%)	0 (0)	35 (100)	30 (85.7)	13 (37.1)

Furthermore, the combination of drugs used in this study resulted in *H pylori* eradication in a minority of patients.

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• *BRIEF REPORTS* •

Sacral anterior root stimulated defecation in spinal cord injuries: An experimental study in canine model

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Abstract

AIM: To investigate whether there was a dominant sacral root for the motive function of rectum and anal sphincter, and to provide an experimental basis for sacral root electrically stimulated defecation in spinal cord injuries.

METHODS: Eleven spinal cord injured mongrel dogs were included in the study. After L4-L7 laminectomy, the bilateral L7-S3 roots were electrostimulated separately and rectal and sphincter pressure were recorded synchronously. Four animals were implanted electrodes on bilateral S2 roots.

RESULTS: For rectal motorial innervation, S2 was the most dominant (mean 15.2 kPa, 37.7% of total pressure), S1 (11.3 kPa, 27.6%) and S3 (10.9 kPa, 26.7%) contributed to a smaller part. For external anal sphincter, S3 (mean 17.2 kPa, 33.7%) was the most dominant, S2 (16.2 kPa, 31.6%) and S1 (14.3 kPa, 27.9%) contributed to a lesser but still a significant part. Above 85% L7 roots provided some functional contribution to rectum and anal sphincter. For both rectum and sphincter, the right sacral roots provided more contribution than the left roots. Postoperatively, the 4 dogs had electrically stimulated defecation and micturition under the control of the neuroprosthetic device.

CONCLUSION: S2 root is the most dominant contributor to rectal pressure in dogs. Stimulation of bilateral S2 with implanted electrodes contributes to good micturition and defecation in dogs.

Key words: Spinal cord injury; Defecation; Sacral root; Electrical Stimulation

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INTRODUCTION

Spinal cord injuries often occur in fairly young people, who have the prospect of an almost normal life expectancy but a considerably impaired quality of life. These patients not only experience severe dysfunction of voluntary movement of limbs but also have impaired pelvic organ functions such as bladder, bowel, and sex[1].

Urinary problems in patients with spinal cord injuries have been extensively studied, and with the advent of intermittent catheterization (CIC), electrical stimulation of the bladder, and advances in diagnostic techniques, considerable improvements have been made in strengthening the lower urinary tract and renal function. In contrast, the management of bowel disorders has remained essentially unchanged in the past three decades[2-4].

One of the most distressing aspects of spinal cord injury is not able to regulate bowel function. Patients with complete supraconal lesions lose conscious control of defecation. Although they may be able to defecate reflexly by anorectal stimulation, evacuation is often inefficient and incomplete, resulting in a high incidence of constipation[5-8].

The first Finetech-Brindley's sacral anterior root stimulator (SARS)[9,10] was implanted in a patient with spinal cord injury in 1976. Since then, the device has been implanted in about 2 000 patients suffering from complete supraconal spinal cord injury (SCI) with intact bladder innervation to induce urine evacuation[11]. The stimulator was initially developed to improve bladder emptying, but as the parasympathetic and somatic nerves that supply the distal colon, anorectum, and anal sphincter are all derived from the same sacral spinal roots that are used for electrical micturition, it seems likely that the device can also be used to induce defecation in paraplegic patients.

Several authors[12-15] have reported the clinical and manometric results of the implanted neuroprosthesis. However, experimental studies concerning electrically stimulated defecation are fen[15]. In the present study, we reported a procedure that could allow selective sacral anterior root stimulation by application of electrodes to achieve controlled rectal evacuation.

MATERIALS AND METHODS

Animals

Eleven adult male mongrel dogs, weighing 12±2.5 kg

(range, 10-15 kg), were used in this study. The animals were kept in cages for 1 wk prior to the study.

The dogs were with diazepam at a dose of 0.4 mg/kg body weight (bw). They were anesthetized with intravenous sodium pentobarbital at a dose of 25 mg/kg bw with a bolus injection of 15-20 mg/h to maintain adequate anesthesia with spontaneous respiration. Intravenous infusion of normal saline solution was given at a dose of 15-20 mL/kg bw per hour. All animals were administered antibiotics (penicillin) peri-operatively.

Surgical techniques

With the animal lying prone, the back was shaved and prepped with betadine solution. A supraconal spinal cord transection was made at the T10 vertebral level. Then L5-S2 laminectomy was performed. L7 and S1-3 nerve roots were exposed extradurally. They were identified anatomically and by specific motoric responses on stimulation using a bipolar hook electrode. Nerve stimulation was given using 5 to 10 trains of constant current square pulses (200 s', 2.0 mA) delivered at a rate of 50 Hz using electromyography (Cantata-2 000, Dantec, Denmark). Then the dura mater was opened in posterior midline and retracted with 3-0 silks to allow access to the conus medullaris and the cauda equina. The intradural L7-S3 roots were confirmed by their corresponding extradural roots that passed through the dura cuff[17]. By meticulous microsurgical dissection, each anterior and posterior root of L7-S3 on each side was identified and separated. Then the posterior components of sacral roots were cut and a segment of 5 mm was removed to get sacral deafferentation.

Manometric studies

A 10F catheter was introduced into the rectum and anal canal. One balloon-ended catheter was introduced into the rectum up to 5-7 cm from the anal orifice. The balloon-catheter was filled with 50 mL of water and connected to a strain gauge pressure transducer (D3-manometrics, Medic Instrument Con., Hefei, China). Another catheter was introduced into the rectal neck (anal canal) up to 2-3 cm from the anal orifice and was also connected to another pressure transducer of the manometer. With individual root stimulated, rectal pressure and anal pressure were recorded simultaneously by the D3-manometer.

Balloon expulsion test

The test was performed with S2 root stimulation. By manually flow-out of the filled water, the volume of rectum balloon was diminished to 10 mL, which represented simulated stools[16].

Implantation of sacral anterior root stimulator

The stimulator (Tc-2 000 electrodes of bladder controller) was provided by Shanghai Tongji University[18]. The device consists of three components: the implantation part, the external control part and the testing block. The implantation part is composed of two electrodes, a connecting cable and a magnetic receiver-stimulator. The external control part is composed of a control box, a cable and a magnetic transmitter. The testing block was used to monitor whether the transmitter worked well or not.

In four animals, Tc-2 000 electrodes of bladder controller were implanted and trapped bilaterally to S2 nerve roots in sacral canal. The magnetic receiver-stimulator was implanted beside the sacral incision via a subcutaneous tunnel. The implanted components were fixed by suturing to sacral periosteum and subcutaneous tissue.

After operation, these four dogs were stimulated 4 times daily by the external transmitter, with an interval of 5 burst-on and 10 burst-off, at a frequency of 36 Hz and an intensity of 12 V.

RESULTS

Animal survival

The animals survived 17-41 d (average 23 d) postoperatively. No complications were encountered during the test. Electrodes did not migrate or break. All 11 dogs were evaluate during the operation and all 4 dogs with implanted electrodes were evaluate the operation.

Efficacy of differential sacral root innervation

With differential nerve root stimulation during operation, rectum and anal pressure elevated significantly as compared with the silent rest values (mean, 3.3 kPa in this group). For rectal innervation, S2 was the most dominant (mean 15.2 kPa, 37.7% of total pressure), S1 (11.3 kPa, 27.6%) and S3 (10.9 kPa, 26.7%) contributed to a smaller part (S2>S1>S3). For sphincter, S3 (mean 17.2 kPa, 33.7%) was the most dominant, S2 (16.2 kPa, 31.6%) and S1 (14.3 kPa, 27.9%) contributed to a lesser but still a significant part (S3>S2>S1) (Figure 1). Although the efficacy of L7 root contribution to pressure elevation in rectum (3.3 kPa, 8%) and anus (3.5 kPa, 7.1%) was minimal, 85% of L7 roots (frequency) provided some function to rectum and anal sphincter. For both rectum and sphincter, the right sacral roots seemed to contribute more than the left ones.

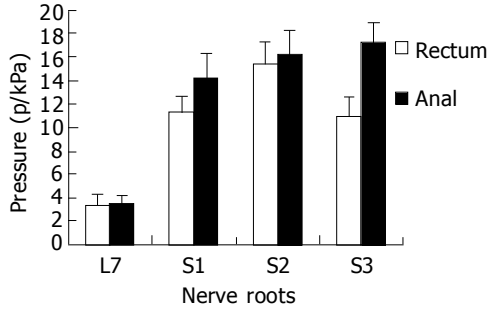

Figure 1 Efficacy of differential sacral root innervation to rectum and anus determined by intra-operative nerve root stimulation and pressure measurement. For rectum, the contribution was S2>S1>S3, and for anus, the contribution was S3>S2>S1.

Balloon expulsion test

During electrical stimulation of S2 root, the rectal neck (anal canal) pressure exceeded the rectal pressure, thus rectal balloon expulsion did not occur. However, as the pressure

of anal canal reduced more rapidly than that of the rectum, rectal balloon expulsion occurred in all 11 dogs as a post-stimulus defecation, which was similar to post-stimulus voiding.

Neuroprosthetic controlled defecation

After paraplegia and electrode implantation and sacral deafferentation, the bladder of animals was electrically evacuated four times per day. During electrically stimulated micturition, defecation occurred 2-3 times per day, providing there were stools in the rectum. With electrical stimulation of S2 nerve root, defecation was successful in all 4 dogs (Figure 2). No lubrication or other bowel management was needed for the dogs during their paraplegic life (mean, 23 years) after operation.

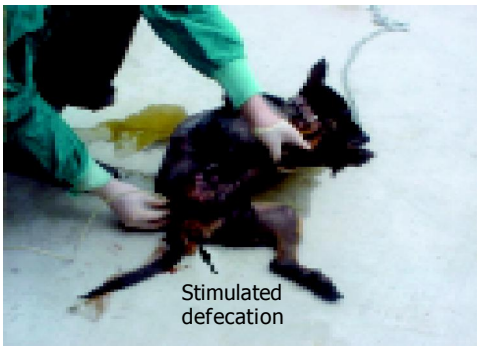

Figure 2 Electrically stimulated defecation in dogs controlled by neuroprosthetic device.

DISCUSSION

Concomitant neuropathic bladder and bowel dysfunction are common in patients with spinal cord injury[1-8]. Bladder dysfunction in these patients is commonly attributable to hyperreflexia and detrusor-sphincter dyssynergia. However, constipation in these patients is commonly caused by fecal stasis with colonic dilation[3-7]. Constipation causes substantial morbidity, and is usually managed empirically with aids, such as laxatives, suppositories, enemas or even digital evacuation[3,4]. Indeed, fecal impaction is the most common gastrointestinal complication sustained by the patients with spinal cord injury[8]. The pathophysiology of this problem is now better understood[19,20]. Menardo *et al*[21], in 1987 demonstrated that the main site of stasis was in the left colon and rectum following spinal cord injury.

Anatomically, dogs have seven pairs of lumbar roots and three pairs of sacral roots. The innervations of rectal detrusor and external anal sphincter (EAS) in dogs are provided by the ventral roots of L7, S1, S2, and S3, which originated from the sacral spinal cord parasympathetic and somatic centers, respectively[17]. Our experimental study demonstrated that only a pair of the most efficacious roots (S2) was needed to produce electrically stimulated micturition and defecation.

Since large diameter fibers (somatic motor) need a smaller stimulus threshold for their excitation than small ones (parasympathetic motor), activation of the small parasympathetic fibers (detrusor) is always accompanied with the activation of the larger somatic ones (anal sphincter). Attempts to empty the rectum by sacral ventral root stimulation have always been hampered by concurrent contraction of the anal sphincter, which far exceeds that of rectum. All dogs in our study were unable to expel the rectum balloon at the time of stimulation. However, the sacral anterior root stimulator took the advantage of the differences in biomechanical characteristics of the smooth (detrusor) and striated muscles (EAS). The contraction and relaxation speed of the striated muscles was faster than those of the smooth ones. When bilateral S2 anterior roots were stimulated (the posterior component was cut as deafferentation), the rectal and anal pressures increased at the same time, and the increasing amplitude of the anal pressure was remarkably higher than that of the rectal pressure. Therefore, defecation did not occur at this time. After stimulation was stopped, the EAS relaxed instantaneously and the pressure decreased rapidly to the baseline, but the rectal detrusor relaxed slowly, this slow decay resulted in a period of rectum pressure higher than the relaxed anal pressure. Thus, a positive recto-anal pressure difference developed, and defecation might occur. The evacuation mode is called post-stimulus defecation, similar to post-stimulus voiding in urinary system[10].

For human beings, the parasympathetic outflow to left colon, rectum, and internal anal sphincter is supplied by anterior sacral roots of S2, S3, and S4. These nerve roots also innervate the external anal sphincter (EAS) via its somatic contribution. Similar sacral nerve roots also innervate bladder and its sphincter. Bladder control can be successfully achieved by using Brindley's anterior root stimulator implant. Because most of these patients had concomitant bowel dysfunction, its effect on bowel function as defecation has been documented by Varma *et al*[12], in 1986, MacDonagh *et al*[13], in 1990, Binnie *et al*[14], in 1991, Chia *et al*[15], in 1996 and Creasey *et al*[11], in 2001. The mechanism of improvement in bowel function is attributed to the activation of contraction of the terminal colon and rectum, resulting in the movement of feces caudally into the anal canal. And this further initiates reflex relaxation of the pelvic floor muscles[22].

A recent intraoperative electrical study in patients with spinal cord injury demonstrated that, S3 root was the most efficacious contributor (52.2% of total pressure), S4 was the second but still significant efficacious one (44.9%), and S2 was the last and the least contributor (2.9%)[23]. The S2 root is mainly related to sexual function, and probably serves as a vital pathway for sensory feedback that is necessary for proper function of the viscera, especially for penile sensation, erection and ejaculation[24]. Based on this experimental study, the authors would like to propose a cheaper modification of Brindley's sacral anterior root stimulated micturition and defecation in Asians, i.e., using one cable with two electrodes to trap on the bilateral S3-4 roots[25].

In conclusion, S2 root is the most dominant contributor to rectal pressure in dogs. Stimulation of bilateral S2 with implanted electrodes contributes to good micturition and defecation in dogs.

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• *CASE REPORT* •

Endoscopic ultrasonographic appearance of gastric emphysema

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Abstract

Emphysematous gastritis (or phlegmonous gastritis) and gastric emphysema (or gastric pneumatosis) are variations of conditions associated with the presence of intramural air in the stomach. The presence of air in the gastric wall is a very rare clinical condition, associated with bacterial infection, increased intragastric pressure from gastric outlet obstruction, gastric mucosal disruption or air dissection from the mediastinum. In adults, this can occur in the setting of instrumentation-related injury, gastric outlet obstruction by gastric, duodenal or pancreatic malignancies or bowel ischemia. Here we describe a case of gastric emphysema related to repeated biliary stenting and partial duodenal obstruction in a patient with inoperable periampullary cancer, and provide the first description of the endoscopic ultrasonographic findings of gastric emphysema in the literature. In our case, endoscopic ultrasound showed a band of bright echogenicity arising from the submucosa layer, representing air in the gastric wall.

Key words: Endoscopic ultrasound; Gastric emphysema; Gastric pneumatosis

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INTRODUCTION

Gastric emphysema or pneumatosis is a rare finding. It was first described by Brouardel in 1895 and only 42 cases have been described in the literature[1]. Although uncommon, the radiologic[2], endoscopic[3], histologic[3], and percutaneous ultrasound[4,5] findings of this condition are well described. Since its development in the 1980s, endoscopic ultrasound[6-8] has been used for evaluating various esophageal, gastric and pancreatic-biliary diseases[9]. Here, we present the first description of the endoscopic ultrasonographic appearance of gastric emphysema.

CASE REPORT

An 87-year-old man presented to our gastroenterology ward with jaundice, fever, and abdominal pain in January 2002. He had been diagnosed with periampullary adenocarcinoma in June 2001. The tumor was not resected due to his advanced age and comorbid conditions; obstructive jaundice was treated with repeated sessions of palliative endoscopic biliary stenting. On admission, the patient appeared cachectic and complained of some abdominal pain. Physical examination was remarkable for scleral icterus, abdominal distension and epigastric tenderness. His hemogram showed mild anemia with a mildly elevated white cell count of 11 300/mm^3 (84% neutrophils). Total serum bilirubin level was 1.54 mg/dL.

Abdominal ultrasonography revealed multiple- liver metastases, pneumobilia and persistent air in the gastric wall despite changing the patient's position (Figure 1). Gastric emphysema was suspected. Because of this, endoscopic ultrasonography was performed using an Olympus GFUM200 echoendoscope with Olympus EU-M20 system (Olympus America Inc., Melville, NY). This showed a band of bright echogenicity arising from the submucosa layer, representing a large amount of gas in the gastric wall (Figure 2). Abdominal computerized tomography confirmed the presence of air in the gastric wall, pneumobilia and multiple-liver metastases (Figure 3). The mesenteric artery and vein were both patent.

Nasogastric aspirate yielded coffee-ground material on the third hospital day and prompting upper endoscopy revealed diffuse erythema, edema, erosions and sloughing of the mucosa throughout the stomach, without active bleeding (Figure 4). There was also partial duodenal obstruction by the periampullary cancer.

The patient was treated with acid suppression therapy using intravenous omeprazole and the bleeding resolved. The patient died 1 mo later from an episode of nosocomial pneumonia and progressive liver failure. Gastric emphysema was persistently detected throughout this period by serial abdominal ultrasonography.

DISCUSSION

The presence of air in the gastric wall is a very rare condition. Emphysematous gastritis (or phlegmonous gastritis) and gastric emphysema (or gastric pneumatosis) are variations

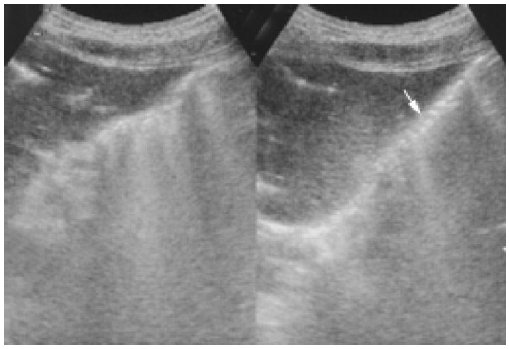

Figure 1 Abdominal sonography showing air in the gastric wall (arrow).

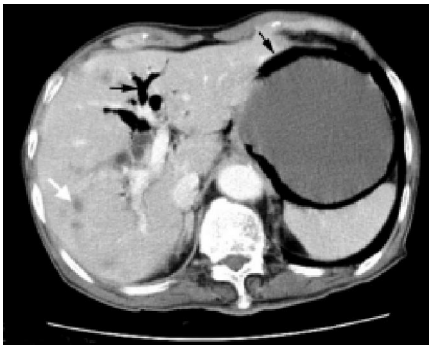

Figure 3 Abdominal computerized tomography revealing multiple liver metastases (white arrow), pneumobilia (thin arrow) and persistent air in the gastric wall (thick arrow).

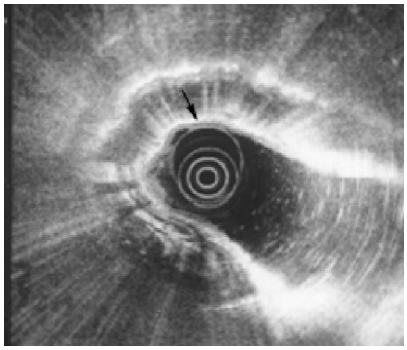

Figure 2 Endoscopic ultrasonography showing a band of bright echogenicity arising from the submucosal layer with shadowing (arrow), representing air in the gastric wall.

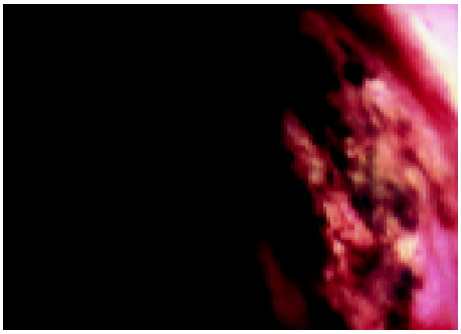

Figure 4 Upper endoscopy showing diffuse erythema, edema, erosions and sloughing of the gastric mucosa.

of conditions associated with the presence of intramural air in the stomach[1,10]. In children, this is associated with pyloric stenosis, gastric malrotation, annular pancreas, cardiac surgery and incorrect positioning of feeding catheters[11-15]. In adults, instrumentation-related injury[16], gastric outlet obstruction by gastric, duodenal or pancreatic malignancies, and bowel ischemia or infarction account for the majority of cases[1].

Four theories have been proposed to explain the development of gas within the gastric wall[17]. In the bacterial theory, *Clostridium welchii* and other gas-forming aerobic colonic bacilli, including *Escherichia coli, Streptococcus, Bacillus subtili* and *Bacillus proteus* generate the intramucosal gas seen in emphysematous gastritis[10]. The outcome in these situations is almost always fatal despite surgical intervention and broad spectrum antibiotic therapy. In the mechanical theory, air is thought to enter the gastric wall due to increased mural pressure[17], possibly caused by air insufflation during endoscopy[18] or intestinal obstruction[19,20]. In the mucosal damage theory, air enters the gastric wall through disrupted mucosa. This may account for gastric wall air associated with a penetrating gastric ulcer[5]. In the pulmonary disease theory, alveolar air dissecting down the mediastinum into the gastric wall in patients with severe asthma or emphysema results in the presence of gastric wall air. In our present case, we believe that repeated biliary stenting led to dissection of air into the gastric wall. In addition, increased intragastric air pressure due to partial duodenal obstruction may have contributed to the development of gastric emphysema as well.

The diagnosis of gastric wall air is usually made radiographically. Two radiological patterns of gastric intramural air have been described. The linear lucency pattern is usually associated with gastric emphysema and the cystic, mottled pattern is usually associated with emphysematous gastritis, a much more serious condition[1]. However, these patterns are not specific enough to distinguish between these two clinical entities[1,2]. Gastric emphysema can also mimic pneumoperitoneum[16]. Therefore, computerized tomography is the test of choice because it evaluates the entire abdominal cavity[2,21,22].

Endoscopic findings in patients with gastric emphysema or emphysematous gastritis have been described. These include submucosal gas bubbles, necroinflammatory changes, and erosions; in some cases, the mucosa appears normal[3]. Endoscopic biopsy may reveal numerous empty spaces in the lamina propria[3]. In our patient, endoscopy revealed only non-specific inflammatory changes and did not help in the diagnosis or management of gastric emphysema.

Unlike the nonspecific endoscopic findings, endoscopic ultrasonography clearly demonstrates the presence of a linear band of air in the submucosal layer. To our knowledge, this is the first report of endoscopic ultrasonographic findings in gastric emphysema or emphysematous gastritis. Although the presence of air in the gastric wall can be easily diagnosed by non-invasive tests such as abdominal ultrasonography or computerized tomography, endoscopic ultrasonography allows better visualization of the gastric

wall and be useful in differentiating between gastric emphysema and emphysematous gastritis. More data from additional patients are needed to establish the role of endoscopic ultrasonography in this rare condition.

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• *CASE REPORT* •

Actinomycosis mimicking recurrent carcinoma after Whipple's operation

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Abstract

Actinomycosis is a rare, chronic, spreading, suppurative, granulomatous and fibrosing infection. Actinomyces are normal inhabitants of the oral cavity and gastrointestinal tract. They rarely cause disease and are seldom reported as pathogens. Herein, we reported on a 69-year-old male patient who had undergone Whipple's operation due to ampulla Vater carcinoma, and became infected with actinomycosis at the pancreaticojejunostomy, which mimicked a recurrent malignancy. He was treated with radical resection of the mass at the pancreaticojejunostomy and had an uneventful postoperative course.

Key words: Actinomycosis; Carcinoma; Pancreaticojejunostomy; Recurrent; Whipple

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INTRODUCTION

Actinomycosis is a rare, chronic, spreading, suppurative, granulomatous and fibrosing infection characterized by the formation of multiple abscess, draining sinuses and the release of characteristic "sulfur granules"[1]. This infection is normally caused by *Actinomyces israelii*, which are anaerobic gram-positive filamentous rods. These organisms are not regarded as virulent human pathogens and are best considered as opportunistic pathogens, as they are normally present in healthy individuals, especially in the oral cavity and gastrointestinal tract[2,3]. This disease usually follows perforation of an abdominal viscus because of inflammatory or neoplastic disease, surgery, or trauma[4-7] and has also commonly associated with the long-term use of an intrauterine device[8-10]. Herein, we reported a patient who had undergone Whipple's operation due to ampulla Vater carcinoma, and succumbed to actinomycosis at the pancreaticojejunostomy, which mimicked local recurrence of cancer. He was treated with radical resection of the mass and had an uneventful postoperative course. He regained body weight steadily and had no recurrence of actinomycosis at the postoperative 6 mo follow-up.

CASE REPORT

A 69-year old male patient was referred to our hospital because of common bile duct (CBD) stone with biliary tract infection with an initial presentation of abdominal fullness, general malaise, nausea, vomiting, poor appetite and body weight loss for 2 wk. A panendoscopy revealed an enlarged, easy touch-bleeding papilla with an infiltrating mass. Endoscopic retrograde cholangiopancreatography (ERCP) showed a dilated biliary tree and pancreatic duct with stricture of the distal CBD and pancreatic duct (double duct sign). The serum level of the carbohydrate antigen 19-9 (CA19-9) was 126.26 u/mL. Biopsy of the ampulla Vater demonstrated moderately differentiated adenocarcinoma. He then received a radical pancreaticoduodenectomy with Child's reconstruction. A right subhepatic abscess was noted 10 d after the operation and was managed by computed tomography (CT) guidance percutaneous drainage. The patient was discharged 3 wk after surgery and received regular follow-ups at our outpatient department.

Unfortunately, an abdominal CT scan showed abnormal soft tissue enlargement at the pancreaticojejunostomy (Figure 1) 2 years after surgery. Meanwhile, the CA19-9 level had also elevated comparing to the immediate postoperative value (from 4.43 to 50.63 u/mL). Magnetic resonance cholangiopancreatography (MRCP) revealed tapering of distal bile duct with upstream dilation and a dilated pancreatic duct with sudden obliteration at the pancreaticojejunostomy (Figure 2). The patient had no fever, leukocytosis or jaundice (white blood cells, 5 900/mm^3; total bilirubin, 0.6 mg/dL). Only body weight loss of about 10 kg and fatigue had been noted over the last 2 mo. Therefore, he received surgery again under the strong impression of a suspected local recurrence of ampulla Vater carcinoma.

During the operation, a hard mass at the pancreaticojejunostomy with extension to para-aortic area and stenosis of previous pancreaticojejunostomy was identified. A frozen section revealed no evidence of malignancy. Nevertheless, the mass was resected and the pancreaticojejunostomy was re-constructed. The final pathology showed transmural

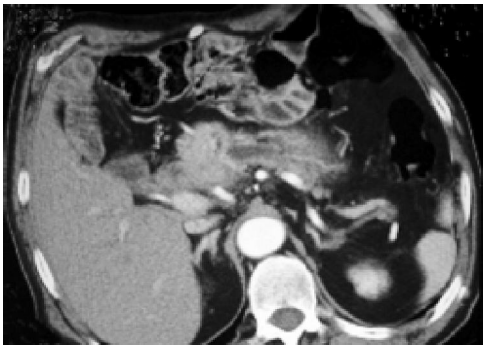

Figure 1 Abdominal CT scan reveals abnormal soft tissue enlargement at the pancreaticojejunostomy.

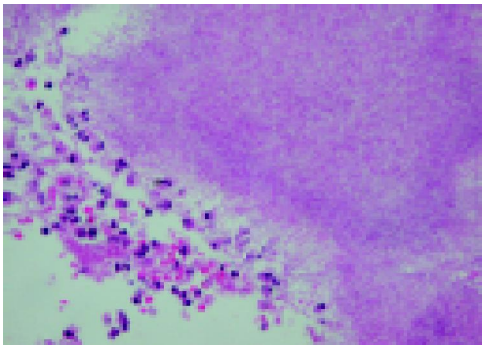

Figure 3 Histopathological examination demonstrates many sulfur granules, which are positive for Periodic acid and Giemsa stains, which is consistent with actinomycosis.

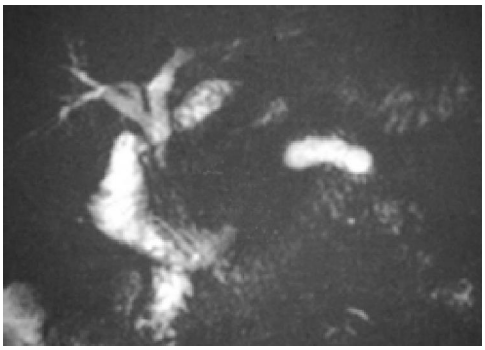

Figure 2 MRCP depicts tapering of the distal bile duct with upstream dilatation and a dilated pancreatic duct with sudden obliteration at the pancreaticojejunostomy.

inflammation of the segment of resected jejunum. There were many sulfur granules, which were positive for Periodic acid and Giemsa stains (Figure 3), consistent with actinomycosis. The patient had an uneventful postoperative course without administration of penicillin and was discharged home 2 wk after operation with steady regaining of body weight.

DISCUSSION

Actinomycosis, which was first described by Israel in 1878[11], is a rare, chronic, spreading, suppurative, granulomatous and fibrosing infection characterized by the formation of multiple abscesses, draining sinuses and the release of characteristic "sulfur granules"[1]. It is found worldwide and occurs at any age, but is rare at ages younger than ten. The peak incidence is between 15 and 30 years, and males are more frequently infected than females[2].

Actinomyces are anaerobic, gram-positive bacteria that form filaments[12]. They are normally present in healthy individuals, especially in the oral cavity and gastrointestinal tract[2,3]. All tissues and organs can be infected[3] when the mucosal barrier is broken, leading to multiple abscess formation, fistula, or a mass lesion[2,4]. Actinomycosis commonly occurs in three distinct forms. The majority of examples of the clinical disease are cervicofacial (55%), with only 20% occurring in an abdominopelvic form and 15% as a thoracopulmonic form[7,13]. Abdominopelvic actinomycosis has been associated with abdominal surgery, such as appendectomy, or bowel perforation, diverticulitis, trauma, foreign bodies and neoplasia[5-7,14-16]. Establishment of human infection may also require the presence of companion co-infection bacteria, which releases a toxin or enzyme inhibiting the host defenses. This enhances the relatively low invasive power of actinomycetes. Immune suppression and (surgical) trauma may also play an important role. Various abdominal organs may be involved in abdominopelvic actinomycosis including the gastrointestinal tract, ovaries, liver, gallbladder, and pancreas[2,17]. To the best of our knowledge, actinomycosis at a pancreaticojejunostomy has not been reported in the literature. In our case, the immune suppression due to ampulla Vater carcinoma along with the surgery and postoperative subhepatic abscess formation might have contributed to the development of the actinomyces infection.

In most cases, patients present with an abdominal mass were frequently mistaken for a neoplasm, as was the case here. At a more advanced stage, the abdominal mass is accompanied with extensive sinus, fistula, and abscess formation, usually draining to the skin. Although the clinical features depend on which organs are involved, common symptoms and signs include fever and leukocytosis, fatigue, anorexia, weight loss, and night sweats[2,14,15,18]. Our case presented with only body weight loss and fatigue without fever or leukocytosis, which led to the reasonable preoperative diagnosis of a cancerous recurrence rather than actinomycosis. Most adjunctive diagnostics are very not very specific. In only 10% of the cases is the diagnosis made preoperatively[19] and differentiation from a malignancy is very difficult[14,20]. A CT scan can be helpful in locating and determining the extent of the lesion. Additionally, CT-guided fine needle aspiration of the mass may help diagnosis by cytological examination[15].

High-dose intravenous penicillin injection is the treatment of choice[7,21] and the response is usually favorable[13,22,23]. Therefore, early diagnosis is important to minimize morbidity due to this disease and avoid unnecessary surgery. However, the diagnosis is often obtained postoperatively from a pathology report. Surgery is reasonable and usually necessary because of the difficulty of diagnosis. Debridement of necrosis and relieving the related symptoms such as obstruction and cramping pain can be quickly

achieved by surgery. In our case, an intra-operative frozen section of the mass at pancreatojejunostomy revealed only chronic inflammation without any evidence of malignancy. Resection of the mass was justified to rule out the recurrence of ampulla Vater carcinoma and to relieve the pancreatic duct obstruction. Postoperative penicillin was not administered to this patient because a radical resection of the actinomycosis was carried out and no sign of clinical infection was found.

Because of its rarity, intramural actinomycosis is an entity that is often overlooked by most surgeons. A high index of suspicion may help increase awareness of this important and curable disease. Actinomycosis should be taken into account as a differential diagnosis in patients having an intra-abdominal mass with unusual fever or leukocytosis after gastrointestinal surgery.

In summary, actinomyces rarely cause disease and are seldom reported as human pathogens. The symptoms of actinomycosis are non-specific, which leads to great diagnostic difficulty. Actinomycosis should be considered when a cancerous patient, after gastrointestinal surgery, experiences an intra-abdominal mass along with unexplained fever or leukocytosis. Penicillin G is still the medical treatment of choice. However, surgical intervention can still play a role in facilitating the recovery in selected patients and is useful to rule out malignancy in some instances. For this particular patient, re-operation was justified to rule out the recurrence of ampulla Vater carcinoma and to relieve the pancreatic duct obstruction.

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• *CASE REPORT* •

Candidal liver abscesses and cholecystitis in a 37-year-old patient without underlying malignancy

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Abstract

We report a case of candidal liver abscesses and concomitant candidal cholecystitis in a diabetic patient, in whom differences were noted relative to those found in patients with hematologic malignancies. In our case, the proposed entry route of infection is ascending retrograde from the biliary tract. Bile and aspirated pus culture repeatedly tested positive, and blood negative, for *Candida albicans and Candida glabrata*. Cholecystitis was cured by percutaneous gallbladder drainage and amphotericin B therapy. The liver abscesses were successfully treated by a cumulative dosage of 750 mg amphotericin B. We conclude that in cases involving less immunocompromised patients and those without candidemia, a lower dosage of amphotericin B may be adequate in treating candidal liver abscesses.

Key words: Candida; Liver abscess; Cholecystitis; Amphotericin B; Endoscopic papillotomy; Biliary prosthesis

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INTRODUCTION

Candidal liver abscess is a rare disease, and most of the reported cases have been diagnosed in patients with hematologic malignancies during periods of neutropenia resolution[1,2]. In a review by Thaler *et al*, only 8 of 73 patients had an underlying disease other than a malignancy. Clinical features and management of this disease may be different in those with a malignancy versus without a malignancy. We reported the case of a patient with diabetes mellitus in order to add further experience to the limited data regarding patients without malignancy.

CASE REPORT

A 37-year-old male, who was an alcoholic and heavy smoker, was admitted for right upper-quadrant abdominal colic, with pain radiating to the back, which had been present for 2 wk. Two years before this admission, he had suffered an accident that resulted in pancreatic rupture. Partial pancreatectomy was performed and insulin-dependent diabetes mellitus was noted soon. Since then, he had suffered from several episodes of pancreatitis. He visited our emergency department 1 year ago for treatment of biliary tract obstruction caused by pancreatic pseudocyst. Meanwhile, diabetic ketoacidosis and pulmonary tuberculosis were also found, which were appropriately treated.

On admission, poor nutritional status was evident (body weight 49 kg and body mass index 17.16 kg/m^2). His temperature was 37.6 ℃, blood pressure was 101/63 mmHg, pulse rate was 126 beats/min, and respiratory rate was 17/min. Remarkable findings on physical examination included rales over the left upper-lung field, tenderness over the right upper-abdominal quadrant with a positive Murphy's sign, but without rebounding pain.

Laboratory examinations revealed a white blood cell count of 16.5×10^9/L, with 88% of neutrophils, a hemoglobin level of 110 g/L, and a platelet count of 331×10^9/L. Serum chemistry results revealed the following values: glucose level, 324 mg/dL; sodium, 130 mmol/L; potassium, 3.7 mmol/L; blood urea nitrogen, 5 mg/dL; and creatinine, 0.8 mg/dL. Results of a liver function test performed on admission were as follow: aspartate aminotransferase, 26 IU/L; alanine aminotransferase, 16 U/L; total bilirubin 2 mg/dL (normal range, 0.2-1.6 mg/dL); direct bilirubin, 1.9 mg/dL (normal range, 0-0.3 mg/dL); alkaline phosphatase, 1645 IU/L (normal range, 10-100 IU/L); and gamma glutamyl transferase, 1214 IU/L (normal range, 8-61 IU/L). The initial C-reactive protein concentration was 12.06 mg/dL (normal value, <0.5 mg/dL). The amylase and blood gas analyses were within normal limits. Computed tomography (CT) scanning of the abdomen revealed multiple hypoechoic lesions over bilateral liver lobes, with the largest one located over segment 6, which had ruptured but was localized in

the subhepatic area (Figure 1). Other findings included features suggestive of cholecystitis with gallbladder empyema (Figure 2), chronic pancreatitis, intrahepatic and common bile duct dilatation, splenomegaly, and splenic vein occlusion with collateral circulation. Percutaneous transhepatic gallbladder drainage was performed and a pigtail was inserted into the ruptured abscess, but was removed 3 d after no drainage whatsoever occurred. The bile culture, aspirated pus from the abscess and blood, was negative for bacteria (aerobic and anaerobic) and *Mycobacterium tuberculosis*. However, cultures of bile and aspirated pus yielded yeast that was subsequently identified as *Candida glabrata* and *Candida albicans*. Initially, enteral fluconazole was given (6 mg/kg per d). Endoscopic retrograde cholangiography revealed stricture over the distal common bile duct but without evidence of malignancy. Endoscopic papillotomy was performed, and a biliary prosthesis was placed. Both *C. glabrata* and *C. albicans* persisted in the bile and liver abscess after 15 d of fluconazole therapy. Susceptibility testing was done using the ATB Fungus kit (bioMérieux, France). Both species were susceptible to amphotericin B (AmB) (minimal inhibitory concentration <1.00 μg/mL) and flucytosine (<0.25 μg/mL). Therefore, antifungal therapy was switched to intravenous AmB (0.5 mg/kg per d). The bile and liver aspirates were sterile 2 wk after this treatment. After a cumulative dosage of 750 mg AmB, the patient's symptoms had subsided progressively, laboratory data were normalized, and the CT scan showed no evidence of cholecystitis and only a small residual hypoechoic lesion without enhancement over segment 6. Enteral fluconazole (6 mg/kg per d) was prescribed for an additional month. Diabetes mellitus was well controlled by insulin administration during hospitalization. By his 2-year follow-up, he had suffered from several episodes of pancreatitis and bacterial cholangitis caused by biliary prosthesis occlusion with sludge, which was treated with percutaneous drainage and antibiotics, however, no recurrence of candidal liver abscess and cholecystitis was found.

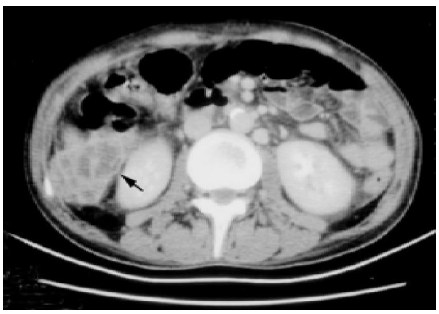

Figure 1 Computed tomography scanning with contrast showed a hypoechoic lesion with irregular and enhanced margin located over segment 6 of liver (arrow), which had ruptured but localized in subhepatic region.

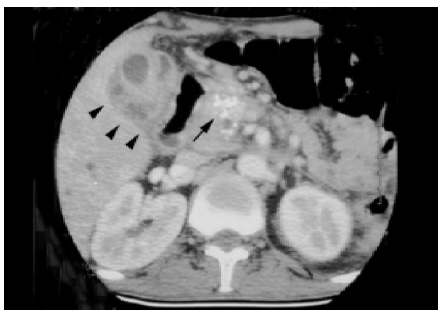

Figure 2 Presence of coarse calcification in the pancreatic head (arrow) indicating presence of chronic pancreatitis. There is inhomogenous enhancement of the gallbladder wall with pericholecystic fluid, suggesting presence of cholecystitis with empyema (arrowhead).

DISCUSSION

Most of the candidal liver abscesses in patients with hematologic malignancies are a manifestation of disseminated candidiasis and have a high mortality rate[2,3]. However, this disease can also be acquired by fungemia from the portal vein[1,4] or an ascending retrograde infection from the biliary tree[5,6], which is the proposed route of infection, as the patient had a biliary stricture, and the infection was localized in the liver and gallbladder, but negative in other parts and without candidemia. Biliary stricture may lead to cholestasis, which in turn can allow micro-organisms from the gut to ascend into the biliary tract. In patients with hematologic malignancies, the yield of positive culture is often less than 50%, with the diagnosis usually based on microscopic examination or histopathological finding from deep tissues[1,3,4,7]. Nevertheless, fungal cultures had repeatedly been positive in our patient. Taking into consideration the negative cultures for other pathogens, pure culture for *Candida,* and good clinical response after AmB therapy, *Candida* was considered a causative pathogen in our case. Interestingly, the infection was caused by 2 species of *Candida* concomitantly. In fact, Domagk *et al*[8] also reported a case of common bile duct candidiasis caused simultaneously by *C. albicans* and *C. glabrata*. For patients with hematologic malignancies who have suffered from candidal liver abscesses, higher cumulative dosages of AmB (2-9 g) are recommended by most experts, because some evidence reveals that a cumulative dose of less than 2 g AmB correlated with residual lesions at autopsy[1,4,9]. Cases of hepatosplenic candidiasis that were successfully treated with fluconazole have been reported by Kauffman *et al*. The symptoms improved within 3-8 wk, but resolution of lesions on CT scan was noted after at least 1 mo of fluconazole therapy[10]. In addition, fluconazole concentrations in the bile were equal to or slightly higher than serum concentrations reported in the study conducted by Bozzette *et al*[11]. However, culture from the liver abscess and bile remained positive despite 2 wk of fluconazole therapy in our case, but became sterile 2 wk after AmB therapy. It is possible that longer course of therapy with fluconazole might have led to resolution of infection in our patient. Notably, the disease improved remarkably with only a cumulative dose of 750 mg AmB. However, he received fluconazole for an additional month, and the role of this therapy is difficult to assess.

The management for candidal cholecystitis is not well established. Surgical drainage or cholecystectomy is

considered adequate treatment for isolated candidal cholecystitis in nonneutropenic patients, but the addition of antifungal therapy is required in critically ill or immunocompromised patients, or patients with extrabiliary tract candidiasis[12,13]. Our patient received cholecystotomy, endoscopic biliary drainage, and fluconazole as initial therapy. Again, the bile culture became sterile only after initiation of AmB. In a study by Adamson *et al*[14], AmB was concentrated in the bile and the outcome was favorable in their case of candidal cholecystitis treated with this antifungal agent.

In conclusion, candidal liver abscesses and cholecystitis are rare, especially in patients without malignancies. Mortality from candidal liver abscesses in patients with hematologic malignancy is high even with high cumulative dosage of AmB. However, in less immunocompromised patients and those without candidemia, this disease may be cured with a lower dosage of AmB.

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• *BRIEF REPORTS* •

Fatal thrombotic complications of hepatic cystic compression of the inferior vena: A case report

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Abstract

Of 5% of patients who develop liver cysts, only 10-15% of them come for medical attention, typically because of dull right upper quadrant pain, abdominal bloating or early satiety. We treated a 77-year-old female with a rare complication of inferior vena cava thrombosis. The patient expired due to septic shock and multiple organ failure.

Key words: Liver cyst; Inferior vena cava thrombosis; Non-parasitic cyst; Cellulitis; IVC compression; Sepsis

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CASE REPORT

A 77-year-old female patient presented at a local emergency room with complaint of epigastric and abdominal fullness of 1-week's duration. The patient had a history of endometrial carcinoma 10 years prior, treated with total hysterectomy. She had completed a course of chemotherapy and radiation therapy at that time. Bilateral ureteral strictures were noted. Subsequently, she did well except for episodic upper urinary tract infection.

One week prior to admission, the patient noted dull abdominal pain and nausea, aggravated by food intake, as well as, dyspnea. Temperature was 38 ℃. She was referred to our hospital for further evaluation and management.

An ultrasound study revealed a large cystic lesion occupying most of the right lobe and segment IV of the liver, with an echogenic shadow layer[1-4] measuring 15 cm×12 cm. The patient was treated with antibiotics for presumed sepsis due to a hepatic abscess.

Progressive, bilateral swelling of the lower extremities was followed by cyanosis of the right foot. The foot was cool and pulseless. Abdominal spiral CT with and without contrast medium showed a 15-cm hepatic cyst with non-enhancing homogeneous low density (about 15 HU). There was a mild bilateral dilatation of the intrahepatic ducts. The cyst was associated with a mass compression effect on the inferior vena cava, with a 20-cm segment of thrombus extending from the inferior aspect of the porta hepatis through the right renal vein to the right femoral vein. Minimal ascites, bilateral basal pleural effusions and atelectasis were also noted. A sonographically guided drainage tube was inserted into the hepatic cyst[3,4]. The aspirate was grossly purulent and culture yielded *E. coli* (Figures 1, 2).

Cellulitis of the right lower extremity persisted in spite of ongoing heparin therapy. Despite the risks of conservative treatment[5-7], the patient's general condition precluded surgical intervention. Though follow-up CT scan done a few days later, showed decrease in size of the cyst to approximately 9×4 cm^2, with relief of mass effect, segmental thrombi continued to occupy the venous trunk and bilateral renal, right common iliac, right femoral and right popliteal veins. Cellulitis progressed with involvement of the buttocks and bilateral lower extremities. There was no evidence of pulmonary embolization. However, the patient expired 10 d later from multiple organ failure (Figure 3).

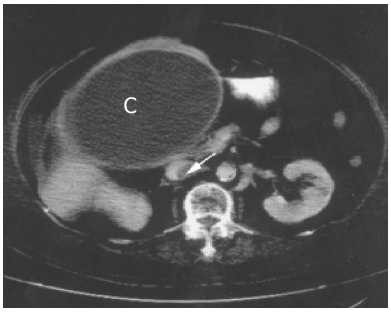

Figure 1 First CT scan shows that huge hepatic cyst (C) exhibits mass compression effect on IVC with thrombosis (arrow).

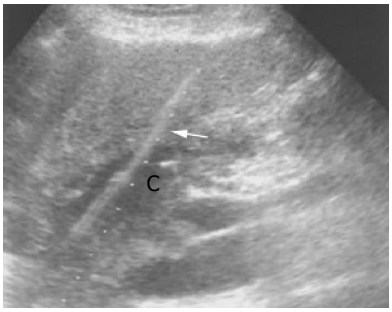

Figure 2 Under sono-guiding, drainage tube (arrow) put into adequate position for hepatic cyst (C).

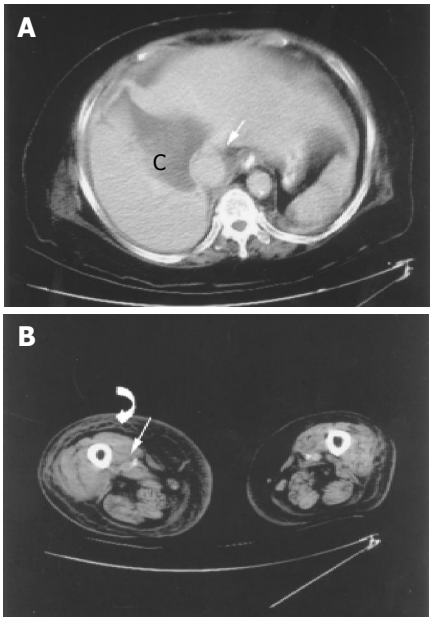

Figure 3 A: The post-drainage follow-up CT scan shows remarkable shrinkage of the hepatic cyst (C), no thrombi of IVC at this level (straight arrow). B: However, there is still long segmental thrombi occupation of lower trunk of IVC and right lower leg, including right popliteal vein (straight arrow) and subcutaneous tissue swelling of lower extremity due to cellulitis (curve arrow).

DISCUSSION

In general, inferior vena cava thrombosis (IVCT) results from deep vein thrombosis. However, isolated IVCT may occur. Examples include complication of malignancy (most often renal cell carcinoma, other genitourinary tumors, or hepatoma), extrinsic compression (most often due to retroperitoneal tumors and aneurysms of the abdominal aorta), post-traumatic hematoma (most often psoas muscle or common iliac artery injury), coagulopathy, iatrogenic causes, pregnancy or use of oral contraceptives and miscellaneous causes (including developmental anomalies and retroperitoneal fibrosis). Compression of the inferior vena cava by an intra-abdominal or pelvic mass has been associated with leiomyoma[8,9], adult polycystic kidney[10,11] and hepatic abscess[12].

A solitary liver cyst formation IVCT is very rare[13]. There are only three cases reported in the literature[1,2,13]. Reports of successful therapies for IVCT have included anticoagulation[1,2], placement of an inferior vena cava filter[2,13], or surgical intervention (resection)[5-7]. Such cysts, reported previously, have been even larger than this patient's[1,2,13], whose cyst was not recognized prior to the onset of acute epigastric pain and nausea. Unfortunately, anti-coagulation was not successful and her condition precluded surgical intervention.

Because of her past history of endometrial cancer, the possibility of malignant recurrence was excluded by clinical, laboratory and imaging studies. The extent of thrombosis led us to suspect an underlying coagulopathy. In addition, normal venous return and lymphatic drainage of the pelvic cavity were possibly already distorted or damaged, perhaps by prior irradiation, resulting in venous stasis and turbulent flow. This hypothesis is supported by the evidence of post-irradiation sequelae of bilateral ureteral strictures and repeated urinary infection.

Medical management of IVCT includes anti-coagulation and thrombolytic agents[1,2]. Surgical therapy[1,2] involves vena cava interruption and thrombectomy. Both of these modalities are gradually being replaced by use of the relatively non-invasive venous filters. In this case, although shrinkage of the hepatic cyst occurred after sonographically guided drainage, the patient's condition deteriorated due to septic shock and multiple organ failure, limiting surgical intervention.

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Meetings

Major meetings coming up

Digestive Disease Week
106th Annual Meeting of AGA, The American Gastroenterology Association
May 14-19, 2005
www.ddw.org/
Chicago, Illinois

13th World Congress of Gastroenterology
September 10-14, 2005
www.wcog2005.org/
Montreal, Canada

13th United European Gastroenterology Week, UEGW
October 15-20, 2005
www.uegf.org/
Copenhagen, Denmark

American College of Gastroenterology Annual Scientific Meeting
October 28-November 2, 2005
www.acg.gi.org/
Honolulu Convention Center, Honolulu, Hawaii

Events and Meetings in the upcoming 6 months

EASL 2005 the 40th annual meeting
April 13–17, 2005
www.easl.ch/easl2005/
Paris, France

21st annual international congress *of Pakistan society of Gastroenterology & GI Endoscopy*
March 25–27, 2005
www.psgc2005.com
Peshawar

World Congress on Gastrointestinal Cancer
June 15–18, 2005
Barcelona

British Society of Gastroenterology Conference (BSG)
March 14–17, 2005
www.bsg.org.uk
Birmingham

Digestive Disease Week *DDW 106th Annual Meeting*
May 15–18, 2005
www.ddw.org
Chicago, Illinois

Events and meetings in 2005

Canadian Digestive Disease Week Conference
February 26-March 6, 2005
www.cag-acg.org
Banff, AB

2005 World Congress of Gastroenterology
September 12–14, 2005
Montreal, Canada

International Colorectal Disease Symposium 2005
February 3–5, 2005
Hong Kong

13th UEGW meeting *United European Gastroenterology Week*
October 15–20, 2005
www.webasistent.cz/guarant/uegw2005/
Copenhagen-Malmoe

7th International Workshop on Therapeutic Endoscopy
September 10–12, 2005
www.alfamedical.com
Theodor Bilharz Research Institute

EASL 2005 the 40th annual meeting
April 13–17, 2005
www.easl.ch/easl2005/
Paris, France

Pediatric Gastroenterology, Hepatology and Nutrition
March 13, 2005
Jakarta, Indonesia

21st annual international congress *of Pakistan society of Gastroenterology & GI Endoscopy*
March 25–27, 2005
www.psgc2005.com
Peshawar

8th Congress of the Asian Society of HepatoBiliary Pancreatic Surgery
February 10–13, 2005
Mandaluyong, Philippines

APDW 2005 - Asia Pacific Digestive Week 2005
September 25–28, 2005
www.apdw2005.org
Seoul, Korea

World Congress on Gastrointestinal Cancer
June 15–18, 2005
Barcelona

British Society of Gastroenterology Conference (BSG)
March 14–17, 2005
www.bsg.org.uk
Birmingham

Digestive Disease Week *DDW 106th Annual Meeting*
May 15–18, 2005
www.ddw.org
Chicago, Illinois

70th ACG Annual Scientific Meeting and Postgraduate Course
October 28-November 2, 2005
Honolulu Convention Center, Honolulu, Hawaii

Events and Meetings in 2006

EASL 2006 - THE 41ST ANNUAL MEETING
April 26–30, 2006
Vienna, Austria

Canadian Digestive Disease Week Conference
March 4–12, 2006
www.cag-acg.org
Quebec City

XXX pan-american congress of digestive diseases *XXX congreso panamericano de anfermedades digestivas*
November 25-December 1, 2006
www.gastro.org.mx
Cancun

World Congress on Gastrointestinal Cancer
June 14–17, 2006
Barcelona, Spain

7th World Congress of the International Hepato-Pancreato-Biliary Association
September 3–7, 2006
www.edinburgh.org/conference
Edinburgh

71st ACG Annual Scientific Meeting and Postgraduate Course
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World J Gastroenterol 2005;11(11):Appendix 2-3
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*WJG,* ISSN 1007-9327 CN 14-1219/R) is a weekly journal of more than 48 000 circulation, published on the 7th, 14th, 21st and 28th of every month.

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Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was accomplished, disclosure of any financial support for the research, and the name, full address, telephone and fax numbers and email address of the corresponding author should be involved. Titles should be concise and informative (removing all unnecessary words), emphasize what is NEW, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initials and/or first name, middle name or initial(s) and full family name.

Abstract
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The format of structured abstracts is at: http://www.wjgnet.com/wjg/help/11.doc

Key words
Please list 3-10 key words that could reflect content of the study.

Text
For most article types, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or Figures and Tables, not both.

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Figures should be numbered as 1, 2, 3 and so on, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. No detailed legend should be involved under the figures. This part should add into the text where the figures are applicable. Digital images: black and white photographs should be scanned and saved in TIFF format at a resolution of 300 dpi; color images should be saved as CMYK (print files) and not RGB (screen-viewing files). Place each photograph in a separate file. Print images: supply images of size no smaller than 126×76 mm printed on smooth surface paper; label the image by writing the Figure number and orientation using an arrow. Photomicrographs: indicate the original magnification and stain in the legend. Digital Drawings: supply files in EPS if created by Freehand and Illustrator, or TIFF from Photoshop. EPS files must be accompanied by a version in native file format for editing purposes. Scans of existing line drawings should be scanned at a resolution of 1200 dpi and as close as possible to the size at which they will appear when printed, not smaller. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes of atrophic gastritis after treatment. A: ...; B: ...;C: ...; D: ...; E: ...; F: ...; G: ...

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Three-line tables should be numbered as 1, 2, 3 and so on, and mentioned clearly in the main text. Provide a brief title for each table. No detailed legend should be involved under the tables. This part should add into the text where the tables are applicable. The information should complement but not duplicate that contained in the text. Use one horizontal line under the title, a second under the column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

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illustrations should be expressed as ^1F, ^2F, ^3F; or some other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc*. in a certain sequence.

Acknowledgments

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Standard jounal article (list all authors and include the PubMed ID [PMID] where applicable)

1 **Das KM,** Farag SA. Current medical therapy of inflammatory bowel disease. *World J Gastroenterol* 2000; 6: 483-489 [PMID: 11819634]

2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]

3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287 [CMFAID:1082371101835979]

Books and other monographs (list all authors)

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

Chapter in a book (list all authors)

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

Electronic journal (list all authors)

6 **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

PMID requirement

From the full reference list, please submit a separate list of those references embodied in PubMed, keeping the same order as in the full reference list, with the following information only: (1) abbreviated journal name and citation (e.g. *World J Gastroenterol* 2003;9(11): 2400-2403; (2) article title (e.g. Epidemiology of gastroenterologic cancer in Henan Province, China; (3) full author list (e.g. Lu JB, Sun XB, Dai DX, Zhu SK, Chang QL, Liu SZ, Duan WJ; (4) PMID (e.g. 14606064). Provide the full abstracts of these references, as quoted from PubMed on a 3.5 inch disk or CD-ROM in Microsoft Word format and send by post to The *WJG* Press. For those references taken from journals not indexed by *Index Medicus*, a printed copy of the first page of the full reference should be submitted. Attach these references to the end of the manuscript in their order of appearance in the text.

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Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those that are linked with a hyphen and the difference between the two numbers at two sides of the hyphen is more than 5. For example, [1-6], [2-14] and [1,3,4-10,22] are all considered as inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Present as mean±SD and mean±SE.

Statistical expression

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

Units

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

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Italicization

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

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World Journal of Gastroenterology ISSN 1007-9327

World Journal of Gastroenterology standard of quantities and units

Number	Nonstandard	Standard	Notice
1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku,18 500u or M_r18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μmol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD_{260}	A_{260}	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260}nm	*A* should be in italic.
	^bP<0.05	^aP<0.05	In Table, no note is needed if there is no significance in statistics: ^aP<0.05, ^bP<0.01 (no note if P>0.05). If there is a second set of P value in the same table, ^cP<0.05 and ^dP<0.01 are used for a third set: ^eP<0.05, ^fP<0.01.
45	**F*=9.87, §*F*=25.9, #*F*=67.4	^1F=9.87,^2F=25.9, ^3F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	10×10×10cm^3	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$\bar{x}$±s	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	