



# World Journal of Gastroenterology®



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National Journal Award

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• BASIC RESEARCH •

## Fumagillin treatment of hepatocellular carcinoma in rats: An *in vivo* study of antiangiogenesis

I-Shyan Sheen, Kuo-Shyang Jeng, Wen-Juei Jeng, Chi-Juei Jeng, Yi-Ching Wang, Shu-Ling Gu, Shin-Yun Tseng, Chien-Ming Chu, Chia-Hui Lin, Kuo-Ming Chang

I-Shyan Sheen, Liver Reserch Unit, Chang Gung Memorial Hospital, Taipei, Taiwan, China

Kuo-Shyang Jeng, Department of Surgery, Mackay Memorial Hospital, Taipei, Taiwan, China

Wen-Juei Jeng, National Yang-Ming University Medical College, Taipei, Taiwan, China

Chi-Juei Jeng, National Taiwan University Medical College, Taipei, Taiwan, China

Yi-Ching Wang, Shu-Ling Gu, Shin-Yun Tseng, Chien-Ming Chu, Chia-Hui Lin, Department of Medical Research, Mackay Memorial Hospital, Taipei, Taiwan, China

Kuo-Ming Chang, Department of Pathology, Mackay Memorial Hospital, Taipei, Taiwan, China

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Correspondence to: Kuo-Shyang Jeng, M.D., F.A.C.S., Department of Surgery, Mackay Memorial Hospital, No.92, Sec2, Chung-san North Road, Taipei, Taiwan, China. issheen.jks@msa.hinet.net  
Telephone: +886-2-5433535 Fax: +886-2-7065704

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### Abstract

**AIM:** To investigate the effect and possible mechanisms of antiangiogenesis therapy for HCC in rats.

**METHODS:** Adult male LEW/SsN rats were divided into 3 groups, 25 animals each. Group A was the control group. Groups B and C were given diethylnitrosamine, 5 mg/kg/d. In addition, group C rats received an intraperitoneal injection of fumagillin, 30 mg/(kg·d). Five animals in each group were killed at 6<sup>th</sup>, 12<sup>th</sup>, 18<sup>th</sup>, 20<sup>th</sup> and 24<sup>th</sup> wk to evaluate the development of HCC and metastasis. Weight of the rats, liver tumors, and number of organs involved by HCC were measured at each stage. We compared methionine aminopeptidase-2 (MetAP-2) mRNA, Bcl-2 mRNA, telomerase mRNA, and telomerase activity at 24<sup>th</sup> wk in the liver tissue of group A rats and tumor tissue of HCC from group B and C rats.

**RESULTS:** No HCC developed in group A, but tumors were present in group B and C rats by the 18<sup>th</sup> wk. At wk 20 and 24, the median liver weight in group B was 0.64 g (range: 0.58-0.70 g) and 0.79 g (range: 0.70-0.90 g) ( $P = 0.04$ ), and that in group C was 0.37 g (range: 0.35-0.42 g) and 0.39 g (range: 0.35-0.47 g) ( $P = 0.67$ ). The liver weight in group C rats was significantly lower than that in group B rats ( $P = 0.009$ ). At the same time, the median metastasis score (number of organ systems involved) was 3 (range 2-3) in group B, and 1 (range 1-2) in group C, a significant difference between the groups ( $P = 0.007, 0.004$ ). The

levels of MetAP-2 mRNA were significantly higher in groups B and C than in group A ( $P = 0.025$ ), and significantly higher in group C than in group B ( $P = 0.047$ ). The level of Bcl-2 mRNA was significantly higher in group B than in group A ( $P = 0.024$ ), but lower in group C than in group B, although not significantly ( $P = 0.072$ ). Telomerase mRNA was significantly higher in group B than in group A ( $P = 0.025$ ), but significantly lower in group C than in group B ( $P = 0.016$ ). The same inter-group relationship was also true for telomerase activity ( $P = 0.025$  and  $0.046$ ).

**CONCLUSION:** Fumagillin effectively inhibits both liver tumor growth and metastasis in rats *in vivo*. A possible mechanism is fumagillin-induced inhibition of MetAP-2, which plays an essential role in endothelial cell proliferation. Inhibition of MetAP-2 also results in inhibition of Bcl-2 and telomerase activity.

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**Key words:** Hepatocellular carcinoma; Antiangiogenesis therapy; Fumagillin; MetAP-2

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### INTRODUCTION

Hepatocellular carcinoma (HCC), a leading cause of death in Taiwan and many Asian countries, is difficult to treat because of early progression and metastasis. It is well known that angiogenesis is essential for the survival, growth, and metastasis of tumor cells<sup>[1-5]</sup>. Angiogenesis, formation of new blood vessels from the existing vascular bed, is a complex multistep process. There is extracellular matrix remodeling and binding of angiogenic factors to specific endothelial cell (EC) receptors, which results in EC proliferation, invasion of basement membrane, migration, differentiation, and formation of new capillary tubes. Their anastomoses develop a vascular network. There is much interest in inhibiting angiogenesis as a treatment strategy<sup>[6-9]</sup>.

Fumagillin and its derivatives, such as TNP 470, are well-known antiangiogenic agents<sup>[9-18]</sup>. There are few published studies, however, of the *in vivo* effects of these agents on experimentally induced HCC in an animal model. The aim

of this study was to evaluate the therapeutic effect and possible mechanisms of antiangiogenesis in a rat model of HCC.

## MATERIALS AND METHODS

### Animals

Pathogen-free adult male LEW/SsN rats at the age of 8 wk were purchased from the National Science Council, Taiwan. They were fed standard diet chow pellets and water *ad libitum*. The study was begun when the rats were 12 wk old, and their median body weight (BW) was 372.5 g (range: 350-394 g). All experiments were performed according to standard guidelines for animal experiments and approved by the Animal Ethics Committees of Mackay Memorial Hospital.

### Treatment of rats

The 75 rats were divided into 3 groups, 25 each. Group A rats were used as controls, receiving food only and no medication. To induce hepatocarcinogenesis, groups B and C rats were given diethylnitrosamine (DEN) ( $C_4H_{10}N_2O$ ) (Sigma Chemical, St. Louis, MO, USA) in water at a dose of 5 mg/(kg·d). Group C rats received, in addition to DEN, intraperitoneal injections of fumagillin ( $C_{26}H_{34}O_7$ ) (Sigma Chemical, St. Louis, MO, USA) 0.3 mg/(kg·d) beginning at the 18th wk of DEN induction.

### Gross and histologic examination

Five rats in each group were sacrificed at 6<sup>th</sup>, 12<sup>th</sup>, 18<sup>th</sup>, 20<sup>th</sup> and 24<sup>th</sup> wk to evaluate the development of liver tumors and their changes. We measured the body weight, whole liver weight, and the number of involved organ systems of each rat. Liver and HCC specimens were examined by pathologists. The tumor weight was estimated by subtracting the liver weight of group A rats from that of group B or C rats. In examining for metastasis, we gave a score of 1 for HCC limited to the liver, 2 for extrahepatic extension or metastasis within the peritoneal cavity, and 3 if there were both intraperitoneal and lung metastases.

### Molecular mechanisms

To investigate the molecular mechanisms of HCC inhibition by fumagillin, we detected methionine aminopeptidase 2 (MetAP-2) mRNA, Bcl-2 mRNA, and telomerase mRNA and telomerase activity from samples of the resected livers of group A and the resected tumors of groups B and C, in the 24<sup>th</sup> wk of treatment. GAPDH mRNA was used as a control.

### Extraction of RNA

We homogenized resected tissue completely in 1 mL of RNA-Bee™ (Tel-Test, Protech Technology Enterprises Co., Ltd, Friendswood, TX), added 0.2 mL chloroform, and shook vigorously for 15-30 s. We stored the samples on ice for 5 min and then centrifuged at 12 000 g for 15 min. We transferred the supernatant to a new 1.5 mL Eppendorf tube and precipitated the solution with 0.5 mL of isopropanol for 5 min at 4 °C. We centrifuged the tube at 12 000 g for 5 min at 4 °C before removing the supernatant and washed

the RNA pellet with 1 mL of isopropanol, shook to dislodge the pellet from the side of the tube. We centrifuged the pellet again at 12 000 g for 5 min at 4 °C, removed the supernatant, and washed the RNA pellet once with 75% ethanol, shook to dislodge the pellet from the side of the tube. We suspended the pellet in at least 1 mL of 75% ethanol and centrifuged it at 7 500 g for 5 min at 4 °C before carefully removing the ethanol. The RNA was air dried and then dissolved in DEPC-H<sub>2</sub>O (50-100 µL) and stored at -80 °C.

### Reverse transcription

We heated the RNA sample at 55 °C for 10 min, chilled it on ice, and then added the following reagents: 4 µL 5 XRT buffer containing Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, and 10 mmol/L DTT (dithiothreitol); 3 µL 10 mmol/L dNTP (deoxyribonucleoside triphosphate); 1.6 µL Oligo-d (T)<sub>18</sub> and 0.4 µL random hexamers (N)<sub>6</sub> (1 µg/µL); 0.5 µL RNase inhibitor (40 units/µL); 3 µL 25 mmol/L MnCl<sub>2</sub>; 6 µL RNA in DEPC-H<sub>2</sub>O; and 0.5 µL DEPC-H<sub>2</sub>O. We incubated the mixture at 70 °C for 2 min and then chilled it to 23 °C to anneal the primer to the RNA. We added 1 µL of M-MLV RTase (Moloney murine leukemia virus reverse transcriptase, 200 units/µL, Promega) and incubated it for 10 min at 23 °C followed by 60 min at 40 °C. We then heated it at 94 °C for 5 min, chilled it on ice, and stored the cDNA at -20 °C.

### By PCR amplification of MetAP-2, Bcl-2, telomerase, and GAPDH cDNA

First-strand cDNA synthesis was carried out using 2 µg of total RNA purified from 50 mg tissue. Reverse transcription was performed in a 20 µL final volume containing 2 µg of random hexamer (Gene Tek Bioscience Inc., Taipei), and 1.5 mmol/L each of dATP, dCTP, dGTP, and dTTP. Each reaction mix was incubated for 8 min at 23 °C with 20 U of rRNasin (RNase inhibitor; Promega, Madison, WI) followed by incubation with 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Paisley, UK) for 60 min at 40 °C followed by 5 min at 94 °C. PCR was performed in a final volume of 50 µL, by using 2 µL of cDNA solution in a mix containing 0.4 mmol/L deoxynucleotide triphosphates, 40 pmol of both sense and antisense oligonucleotide primers according to the MetAP-2, Bcl-2 and telomerase type to be detected, 2.5 mmol/L MgCl<sub>2</sub>, 2.5 U of Taq DNA polymerase (Promega) and 5 µL of 10 min Taq DNA polymerase reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl [pH9.0], 1% Triton-X-100). PCR primer sequences of the sense and antisense oligonucleotides for MetAP-2, Bcl-2 and telomerase, as well as the direction, size and reaction conditions are shown in Table 1. For example, the MetAP2 phosphorothioate anti-sense oligonucleotide (5'-AGTATTT ACTTTCTCCCAAG-3') and its relative scrambled sequence (S'-CTTGG GAGAAAGTAAATACT-3') were synthesized by Sigma-Genosys Ltd, Woodlands, TX, USA. The anti-sense start position on the MetAP2 mRNA coding region was 1 284. This region corresponds to the large helical domain insertion on the surface of the type 2 isozyme. GAPDH was used as a control, with the quantities of the other mRNA products reported as a fraction of their intensity compared

**Table 1** Sequence of sense and antisense primers in reverse transcription-polymerase chain reaction (RT-PCR) analysis for MetAP-2, Bcl-2 and telomerase mRNA expression

Name	Sequence	Direction	Expected product size (bp)	PCR conditions for pair of primers
MetAP-2-S	TGG CGG GCG TGGAAG AGG	Sense	282	1 cycles: 94 °C, 7 min 50 cycles: 94 °C, 40 s; 54 °C, 40"; 72 °C, 1 min
MetAP-2-AS	GCA CCA TCA CCATCA CCA TCT CC	Antisense	282	1 cycle: 72 °C, 10 min; 4 °C overnight
Bcl-2-S	AGA TGA AGA CTCGCC GCC CCT CAG G	Sense	566	1 cycles: 94 °C, 7 min 50 cycles: 94 °C, 40 s; 54 °C, 40"; 72 °C, 1 min
Bcl-2-AS	CCA GGT ATG CACCCA GAG TGA TG	Antisense	566	1 cycles: 72 °C, 1 min; 4 °C overnight
Telomerase-S	GAC ATG GAG AACAAG CTG TTT GC	Sense	185	1 cycles: 94 °C, 7 min 50 cycles: 94 °C, 40 s; 54 °C, 40"; 72 °C, 1 min
Telomerase-AS	ACA GGG AAG TTCACC ACT GTC	Antisense	185	1 cycle: 72 °C, 10 min; 4 °C overnight
GAPDH-S	ACC ACA GTC CATGCC ATC AC	Sense	485	1 cycles: 94 °C, 7 min 50 cycles: 94 °C, 40 s; 54 °C, 40"; 72 °C, 1 min
GAPDH-AS	TCC ACC ACC CTGTG CTG TA	Antisense	485	1 cycle: 72 °C, 10 min; 4 °C overnight

to GAPDH mRNA. To eliminate any possibility of genomic DNA contamination, PCR amplification reaction was carried out on each sample for RNA extraction. As another internal contamination control, PCR amplification was also carried out on a sample of reaction mixture in the absence of cDNA.

#### Telomeric repeat amplification protocol (TRAP) assay

Either an unamplified conventional standard or a polymerase chain reaction-ELISA-based assay (Roche Molecular Biochemicals, Foster City, CA) was used to measure telomerase activity. Cell equivalents ( $1 \times 10^3$  to  $5 \times 10^3$ ) were used to visualize the DNA ladder according to the standard protocol. For polymerase chain reaction-ELISA,  $2 \times 10^3$  cell equivalents were used. The polymerase chain reaction-ELISA protocol was provided by the assay kit manufacturer (Roche Molecular Biochemicals). Each set of TRAP assays included control reaction tubes without any extract or with RNase A (200 µg/mL)-treated extracts. To quantify the levels of telomerase activity, the average densitometric optical density of the first six TRAP bands after a primer band was reported as a ratio of the internal TRAP assay standard band.

#### Quantification of telomerase activity

After the TRAP reaction, hybridization and ELISA, the level of telomerase activity in a given sample was determined by comparing the signal from the sample to the signal obtained using a control template (TS8; solutions 4 or 5). The control templates provided with the TeloTAGGG telomerase PCR ELISA<sup>plus</sup> are ready-to-use solutions containing TS8 at a concentration of 0.001 mol/mL and 0.1 mol/mL. The control templates used were identical to a telomerase elongation product with 8 telomeric repeats. However, because amplification of the TRAP products and the internal standard (IS) are competitive, the signal of the internal control might be near background level when analyzing samples with very high telomerase activity.

Relative telomerase activities (RTA) within different samples were obtained using the following formula:

$$RTA = \frac{(A_S - A_{S0}) / A_{S,IS}}{(A_{TS8} - A_{TS8,0}) / A_{TS8,IS}} \times 100$$

$A_S$ : absorbance of sample;  $A_{S,0}$ : absorbance of heat- or RNase-treated sample;  $A_{S,IS}$ : absorbance of internal standard (IS) of the sample;  $A_{TS8}$ : absorbance of control template (TS8),  $A_{TS8,0}$ : absorbance of lysis buffer;  $A_{TS8,IS}$ : absorbance of the internal standard of the control template.

#### Statistical analysis

A statistical software package (SPSS for Windows, version 8.0, Chicago, IL) was used, with Student's *t* test for continuous variables and  $\chi^2$  or Fisher's exact test for categorical variables. Non-parametric data were analyzed with Mann-Whitney test or Kruskal-Wallis test. Significance was accepted at  $P < 0.05$ .

## RESULTS

### Hepatocarcinogenesis

No tumor was found in the liver of group A rats at any time point in the study. All group B and C rats developed diffuse neoplasms in all lobes of the liver by the end of 18<sup>th</sup> wk. However, after fumagillin treatment, hepatic tumors in group C rats at wk 20 and 24 had necrosis and hemorrhage, no change was seen in group B. Both group B and C rats had a slight but insignificant increase in weight from wk 20 to 24; the difference in the changes between the two groups was also not significant ( $P > 0.05$ , Table 2).

**Table 2** Body weight of rats at wk 20 and 24

Rats ( <i>n</i> = 10)		Body weight (g)	
		20 <sup>th</sup> wk	24 <sup>th</sup> wk
Group B	DEN only	398 [386-409]	400 [384-411]
Group C	DEN + Fumagillin	397 [380-410]	398 [382-414]
<i>P</i> value		NS	NS

Weight: median [range]; *P* value: non-parametric test (Mann-Whitney *U* test)  
Group A (control): 372 [350-380] g at wk 20 and 380 [351-394] g at wk 24.

The median tumor weights in group B at wk 20 and 24 were 0.64 g (range 0.58-0.70 g) and 0.79 g (0.70-0.90 g) respectively, a significant increase. Those in group C were 0.37 g (range 0.35-0.42 g) and 0.39 g (0.35-0.47 g). The tumor weight in group C rats was significantly lower than that in group B at wk 20 and 24 ( $P = 0.009$ ,  $P = 0.009$ , Table 3), suggesting that fumagillin inhibited the tumors.

### Metastasis

Group B rats had a median metastasis score of 3 (range 2-3) at wk 20 and 24. Group C rats had a median score of 1 (range 1-2) at wk 20 and 24. The difference between the

two groups was statistically significant ( $P = 0.007$ ,  $P = 0.004$ , Table 3).

**Table 3** Inhibitory effect of fumagillin on hepatic tumor growth and metastasis in LEW rats at wk 20 and 24

Treatment groups	Liver tumor (B/C-A) (g, median)		No. of HCC-involved organs <sup>1</sup>	
	20 <sup>th</sup> wk	24 <sup>th</sup> wk	20 <sup>th</sup> wk	24 <sup>th</sup> wk
Group B DEN only ( <i>n</i> = 5)	0.64 <sup>1</sup> [0.58-0.70]	0.79 <sup>1</sup> [0.70-0.90]	3 [2-3]	3 [3-3]
Group C DEN + Fumagillin ( <i>n</i> = 5)	0.37 [0.35-0.42]	0.39 [0.35-0.47]	1 [1-2]	1 [1-2]
<i>P</i> value	0.009	0.009	0.007	0.004

Weight: median [range] of 5 rats in each step; *P* value: non-parametric test (Mann-Whitney U test); <sup>1</sup>organs involved: 1 (liver only), or 2 to 3 (lung or/and peritoneum, in addition to liver). Liver tumor (B/C-A): B rat liver tumor weight = liver weight of group B rat minus that of group A rat; C rat liver tumor weight = liver weight of group C rat minus that of group A rat.

### Microscopic findings

All rats had evidence of HCC after 18 wk of treatment with DEN. However, after administration of fumagillin for 2 wk, tumors in group C rats had dilated bile ducts and sinusoids, and karyorrhectic changes of endothelial cells lining the sinusoids. After 6 wk of fumagillin treatment, multiple areas with varying degrees of necrosis and hemorrhage were found in tumors of the group C rats. Some cancer cells had membrane blebbing, cytoplasmic vacuolization, and mitochondrial body formation, and there were neutrophil and histolytic infiltration. These changes were not present in HCC of group B rats at wk 20 and 24.

### Assays for MetAP-2, GAPDH, Bcl-2, and telomerase mRNA

The results of quantitative RT-PCR analysis are shown in Table 4. The median intensity of MetAP-2 mRNA (compared with GAPDH) in liver tissue of group A and HCC tissue of groups B and C at the 24<sup>th</sup> wk was 0.34 (range 0.32-0.36), 0.50 (0.33-0.70) and 0.58 (range 0.40-0.73) respectively. The groups all differed significantly from one another. Comparable results for Bcl-2 mRNA were 0 in group A (range 0-0.19), 0.45 in group B (range 0.22-0.63) and 0.38 in group C (range 0-0.32). Differences among the 3 groups and between groups A and C as well as between groups B and C were not significant. However, the difference between

groups A and B was significant ( $P = 0.024$ ). The median value of telomerase mRNA was 0.30 in group A (range 0.29-0.32), 0.43 in group B (range 0.35-0.47), and 0.34 in group C (range 0.29-0.37). The value for group B was significantly higher than that for group A or C ( $P = 0.025$ , 0.016), while the value did not differ significantly in groups A and C ( $P = 0.655$ ).

### Telomerase activity

The median telomerase activity of HCC tissue at wk 24 in the 3 groups was 47.6% (range 46-71%), 225.0% (187-310%), and 203.5% (94-292%) respectively. Telomerase activity in group A was significantly lower than that in group B ( $P = 0.025$ ) or C ( $P = 0.025$ ). The activity was significantly higher in group B than that in group C ( $P = 0.046$ , Table 4).

## DISCUSSION

Our study has confirmed the inhibitory effects of fumagillin on HCC and allowed us to develop a model describing its effects both at tissue and cellular level and at molecular level. The advantage of our investigation is that it was an *in vivo* rather than *in vitro* study or one, which used subcutaneously implanted tumors. Evaluating HCC progression and inhibition *in situ* in the liver can increase our understanding of the disease.

Tumor weight in the group B animals treated with DEN increased significantly from wk 20 to wk 24, confirming its effect on progression of HCC. However, in group C rats being given both DEN and fumagillin, the tumor weight was significantly lower than that in group B rats at wk 20 and 24. In addition, the fumagillin-treated rats had a lower metastasis score than those treated with DEN alone.

Fumagillin has been reported to cause weight loss<sup>[18,19]</sup>, although Kin *et al*<sup>[20]</sup> found that liver weight as a function of body weight is actually higher in rats treated with fumagillin derivative TNP-470<sup>[20]</sup>. In our fumagillin-treated rats, insignificant weight loss was only at wk 20 and 24. We think that this is most likely due to the low dose we used. In addition, the tumor weight was determined by comparing whole liver weight with that of controls. Thus, fumagillin-induced body weight loss cannot explain the lower tumor weight in the fumagillin-treated rats.

It is proposed that the mechanism by which fumagillin

**Table 4** Comparison of MetAP-2 mRNA, Bcl-2 mRNA, telomerase mRNA and telomerase activity among the 3 groups of rats at wk 24

Parameters	Group			<i>P</i> value		
	A ( <i>n</i> = 3)	B ( <i>n</i> = 5)	C ( <i>n</i> = 5)	A vs B	A vs C	B vs C
MetAP-2 mRNA (0.32-0.36)	0.34 (0.33-0.70)	0.50 (0.40-0.73)	0.58	0.025	0.025	0.047
Bcl-2 mRNA (0-0.19)	0 (0.22-0.63)	0.45 (0-0.42)	0.38	0.024	0.608	0.072
Telomerase mRNA	0.30 (0.29-0.32)	0.43 (0.35-0.47)	0.34 (0.29-0.37)	0.025	0.655	0.016
Telomerase Activity (%)	47.6 (46-71)	225.0 (187-310)	203.5 (94-292)	0.025	0.025	0.046

Non-parametric test: Mann-Whitney and Kruskal-Wallis tests.

inhibits HCC is by inhibiting angiogenesis, specifically by blocking EC proliferation. By inducing apoptosis of ECs, vascularization is disrupted, leading to infarction of HCC.

It is well documented that fumagillin agents directly inhibit proliferation and migration of ECs *in vitro* and *in vivo* in various tumor models, such as in tumors implanted subcutaneously in mice<sup>[21]</sup>. Folkman<sup>[22]</sup> has stated that the goal of antiangiogenic therapy is to maximize apoptosis of ECs in tumor vascular beds. Fox *et al.*<sup>[5]</sup> pointed out that this is a particularly attractive approach, as ECs are directly accessible through the blood and because they are 'normal' cells and therefore unlikely to become resistant to treatment. Similarly, according to Prox *et al.*<sup>[23]</sup>, fumagillin derivatives do not directly inhibit proliferation of pancreatic cancer cells, but they inhibit EC proliferation, increasing apoptosis of tumor cells by reducing microvessel density.

At 24 wk, our fumagillin-treated rats had massive hemorrhage and necrosis in the liver tumors, a finding not seen in the rats treated with DEN alone. Damage to the vessels supplying the tumor could certainly account for these changes, as ischemia can result in both apoptosis and necrosis of cancer cells. There is a critical difference between these two causes of cell death. Necrosis occurs when the cell suffers a major insult. Damage is generally so severe that the cell loses its ability to maintain membrane integrity, rapid swelling results in bursting of the membrane and release of its contents. This sets up a chain reaction, as toxic enzymes released from the dead cells attack surrounding cells. A wave of necrosis radiates out from the initial site of damage.

There is as yet no evidence that fumagillin can kill HCC cells. However, Catalano *et al.*<sup>[24]</sup> noted that fumagillin might induce apoptosis by early mitochondrial damage in malignant mesothelioma cells. Yoshida *et al.*<sup>[25]</sup> reported that fumagillin-like agents inhibit both the growth and migration of human hepatoma and vascular ECs *in vitro* and may suppress *in vivo* growth of hepatoma, associated with a reduction in the microvasculature and macrophage counts. The speculation that fumagillin inhibits not only ECs but also cancer cells to some degree warrants more studies.

Methionine aminopeptidases (MetAPs) are enzymes involved in the removal of N-terminal methionine from peptides and proteins. The molecular target of fumagillin is MetAP-2, which appears to be important in EC growth<sup>[26]</sup>. This enzyme's effects appear to include protein co-translational or posttranslational processing and myristoylation, as well as regulation of protein stability<sup>[27]</sup>. Sin *et al.*<sup>[28]</sup> demonstrated that fumagillin selectively inhibits MetAP-2 protein *in vivo* by covalently binding to it and blocking its aminopeptidase activity. This would disrupt post-translational modification with failure of myristoylation, contributing to EC cytostasis, with arrest in the late G1 phase.

If MetAP-2 plays a comparable role in tumor cells, that would further support the hypothesis that fumagillin directly inhibits tumor cells. Studies have shown that *in vitro* exposure of human microvascular endothelial cells (HMVECs) to 1 nmol/L fumagillin for 24 h results in a two- to six-fold increase in MetAP2 protein in all cell types<sup>[29]</sup>. Up-regulation of *MetAP-2* gene thus seems to be a common phenomenon in cells treated with fumagillin. It is hypothesized that the loss of MetAP-2 catalytic function in cells exposed to

fumagillin leads to up-regulation of the gene. This response might itself contribute to cytostatic inhibition of ECs, possibly via an excessive increase in the ribosomal regulatory (*p67*) function of increased MetAP-2 protein in its free form or bound to fumagillin.

DEN treatment significantly increased rat liver MetAP-2 mRNA over that in untreated rats in our study. We attribute this to rapid cell growth, and hence increased expression of MetAP-2 mRNA, during carcinogenesis. Fumagillin-treated rats had an even higher MetAP-2 mRNA level than those treated with DEN alone, a difference that achieved statistical significance ( $P = 0.047$ ). With fumagillin inhibition, MetAP-2 mRNA in HCC probably first decreased and then increased to compensate for loss of MetAP-2 catalytic activity. However, it did not quite achieve a two-fold increase over the level in the control rats. This might be due to the fact that we used a relatively low dose of fumagillin.

MetAP2 also affects two key regulators of proliferation and programmed cell death, namely Bcl-2 and telomerase. Inhibition of MetAP-2 in mesothelioma cells reduces both mRNA and protein expression of the anti-apoptosis gene *bcl-2* as well as telomerase activity. This suggests a major role for MetAP2 in proliferative and apoptosis pathways<sup>[24,30,31]</sup>. However, the mechanism by which MetAP2 regulates *bcl-2* expression remains unknown. MetAP2 is not a transcription factor; therefore, it is unlikely that it directly regulates *bcl-2* gene expression. Instead, by its posttranslational processing effects, MetAP2 may alter the function of *bcl-2* transcription factors.

The *bcl-2* gene encodes a 26-kDa protein that protects cells against apoptosis in a variety of experimental systems. Bcl-2 maintains mitochondrial integrity by regulating the opening of the transition pore, thus preventing release into the cytosol of caspase activators. Furthermore, Bcl-2 protein prevents apoptosis by inhibiting lipid peroxidation of the cell membrane. It may therefore be important in protecting ECs against apoptosis as they are engaged in forming new vessels in tumors. It may also potentiate their differentiation into functional blood vessels. Second, Bcl-2 might potentiate the ability of ECs to differentiate. Some authors have reported that MetAP2 inhibition causes a time-dependent down-regulation of the *bcl-2* gene, whereas it does not alter expression of the pro-apoptotic gene, *bax*. Another mechanism for downregulation of *bcl-2* expression, at least in selected systems, is by an increase in p53 expression induced by fumagillin. In our study, Bcl-2 mRNA was significantly higher in DEN-treated rats than in controls. It was somewhat lower in the fumagillin-treated rats than in group B, but the difference was not statistically significant. It is possible this was because of our small sample number. Or, it is possible that down-regulation of *bcl-2* does not play a significant role in this particular model of tumor inhibition.

In addition to deregulation of apoptosis, it is increasingly clear that oncogenesis is driven by the activation of telomerase, a ribonucleoprotein complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes. Telomerase is thought to be responsible for cell immortality, primarily by protecting chromosomes from rearrangement. Telomerase activity is not detected in normal liver, but it has been detected in the vast majority of human

cancer cells. This has raised the possibility that telomerase may be an important target for therapy aimed at controlling cell growth. Kishimoto *et al*<sup>[31]</sup> emphasized the critical step of telomerase activation in hepatocarcinogenesis and tumor progression. Takahashi *et al*<sup>[32]</sup> reported that telomerase reactivation during hepatocarcinogenesis might be regulated only by hTERT, whereas increased telomerase activity in tumor progression might be regulated by both hTERT (reverse transcript) and hTERT (RNA component). Shimada *et al*<sup>[33]</sup> maintained that the higher the telomerase activity in HCC, the higher the malignant potential.

The relationship between Bcl-2 and telomerase activity remains controversial. Recent studies have shown an association between telomerase activation and Bcl-2 deregulation in a wide range of human carcinoma cells. Elkak *et al*<sup>[34]</sup> found that telomerase activity is higher in the Bcl-2-expressing cases of colorectal cancer than in Bcl-2-non-expressing cases, suggesting that Bcl-2 expression may be related to telomerase activity in colorectal carcinoma. Iida *et al*<sup>[30]</sup> and Elkak *et al*<sup>[34]</sup> hypothesized that telomerase reactivation in human breast cancer is associated with increased immunohistochemical expression of Bcl-2. Mandal *et al*<sup>[35]</sup> reported that the stable overexpression of Bcl-2 in human cancer cells with low Bcl-2 expression is accompanied with increased levels of telomerase activity. In low-grade tumors, Bcl-2 is inversely correlated with telomerase activity. Ohmura *et al*<sup>[36]</sup> suggested that the biological role of the Bcl-2 protein is altered by the degree of tumor aggressiveness, so that it works with telomerase against genetic instability. HCC is an aggressive malignancy, and we propose Bcl-2 and telomerase work together in this tumor. It is possible that MetAP2 acts as upstream of Bcl-2, while the Bcl-2 site of action is likely to be upstream of that of telomerase and caspases.

We quantified both telomerase mRNA and telomerase activity to ensure the accuracy of our results. DEN-treated rats had significantly higher values for both at 24<sup>th</sup> wk compared to control and fumagillin-treated rats. Telomerase mRNA in fumagillin-treated rats did not differ significantly from that in controls, although the telomerase activity remained significantly higher in group C than in group A. There are three possible explanations for this. First, as suggested by Kenmochi *et al*<sup>[37]</sup> and Ohta *et al*<sup>[38]</sup>, differentiation may worsen as the tumor progresses; there may be localized spread of the tumor, including intrahepatic metastasis or portal vein thrombosis. Second, as the treated tumor necroses, regeneration of hepatocytes may increase telomerase activity. Third, it has been reported that telomerase may be expressed in lymphocytes. Lymphocytic infiltration may occur during tumor necrosis, so that the total telomerase activity we measured included a proportion generated by lymphocytes, thus overestimating that contributed by the tumor.

Our study demonstrated the ability of fumagillin to inhibit both progression of HCC in the liver itself and systemic metastasis *in vivo* in DEN-treated rats. We also examined three molecular targets of fumagillin in HCC. We found that HCC tissue in fumagillin-treated rats had a compensatory elevation of MetAP-2 mRNA after an initial decrease, with associated decreases in Bcl-2 mRNA, telomerase

mRNA, and telomerase activity. These results may be attributed mainly to inhibition of ECs by fumagillin. A possible mechanism is that fumagillin-induced inhibition of MetAP-2 plays an essential role in EC proliferation. Inhibition of MetAP-2 also results in inhibition of Bcl-2 and telomerase activity.

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• BASIC RESEARCH •

# Loss of heterozygosity: An independent prognostic factor of colorectal cancer

Shih-Ching Chang, Jen-Kou Lin, Tzu-Chen Lin, Wen-Yih Liang

Shih-Ching Chang, Jen-Kou Lin, Tzu-Chen Lin, Division of Colon and Rectal Surgery, Department of Surgery, Veterans General Hospital-Taipei, National Yang-Ming University, Taiwan, China  
Wen-Yih Liang, Department of Pathology, Veterans General Hospital-Taipei, National Yang-Ming University, Taiwan, China  
Shih-Ching Chang, Institute of Clinical Medicine, National Yang-Ming University, Taiwan, China

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Correspondence to: Jen-Kou Lin, M.D., Ph.D., Division of Colon and Rectal Surgery, Department of Surgery, Veterans General Hospital-Taipei, No. 201, Sec. 2, Shih-Pai Rd, 11217 Taipei, Taiwan, China. jklin@vghtpe.gov.tw

Telephone: +886-2-28757544-110 Fax: +886-2-28757639

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## Abstract

**AIM:** Colorectal cancers result from the accumulation of several distinct genetic alterations. This study was to investigate the frequency and prognostic value of loss of heterozygosity (LOH) and microsatellite instability (MSI) at 14 genetic loci located near or within regions containing important genes implicated in colorectal tumorigenesis.

**METHODS:** We studied colorectal cancers with corresponding normal mucosae in 207 patients (139 males and 68 females, mean age at the time of tumor resection 66.2±12.4 years, range 22-88 years). There were 37 right-sided colonic tumors, 85 left-sided colonic tumors and 85 rectal tumors. The distribution of tumor staging was stage I in 25, stage II in 73, stage III in 68, and stage IV in 41. We analyzed the LOH and MSI of *HPC1*, *hMSH2*, *hMLH1*, *APC*, *MET*, *P53*, *NH23-H1*, *DCC*, *BAT25*, *HMT26*, *D17S250*, *MYCL1* and *D8S254* with fluorescent polymerase chain reaction and denatured gel electrophoresis. High-frequency LOH was determined to be greater than three, or more than 50% of the informative marker with LOH. High-frequency MSI (MSI-H) was determined as more than four markers with instability (>30%). Correlations of LOH and MSI with clinical outcomes and pathological features were analyzed and compared.

**RESULTS:** The occurrence of MSI-H was 7.25%, located predominantly in the right colons (7/15) and had a higher frequency of poor differentiation (6/15) and mucin production (7/15). LOH in at least one genetic locus occurred in 78.7% of the tumors and was significantly associated with disease progression. Of the 166 potentially cured patients, 45 developed tumor recurrence within 36 mo of follow-up. Clinicopathological factors affecting 3-year

disease-free survival (DFS) were TNM staging, grade of differentiation, preoperative CEA level, and high LOH status. Patients with high LOH tumors had a significantly lower DFS (50%) compared with patients with low LOH tumors (84%). Of the patients developing subsequent tumor recurrence, the number and percentage of LOH were 2.97 and 46.8% respectively, similar to the stage IV disease patients. TNM staging had the most significant impact on DFS, followed by high LOH status.

**CONCLUSION:** Clinical manifestations of LOH and MSI are different in colorectal cancer patients. High-frequency LOH is associated with high metastatic potential of colorectal cancers.

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**Key words:** Colorectal Cancer; Loss of Heterozygosity; Prognosis

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## INTRODUCTION

Cumulated genetic alterations could affect proto-oncogenes such as *Ki-ras* and tumor suppressor genes, including *APC*, *DCC* and *TP53*<sup>[1,2]</sup>. The common event that inactivates tumor suppressor genes is the loss of one allele frequently associated with an inactivating point mutation in the remaining wild-type allele. This process is referred to as loss of heterozygosity (LOH)<sup>[3]</sup>.

In another mutational pathway, colorectal cancers display increased rates of intragenic mutation characterized by generalized instability of short, tandemly repeated DNA sequences known as microsatellites<sup>[4]</sup>. High frequency of microsatellite instability (MSI-H) has been found in more than 90% of tumors in patients with hereditary nonpolyposis colorectal cancer<sup>[5,6]</sup>. The presence of MSI could be the result of defective DNA mismatch repair genes that were misaligned in repetitive sequences (caused by slippage of polymerases) and left unrepaired. In sporadic cases of colorectal cancer, MSI-H occurred in approximately 15% of tumors<sup>[4,5,7]</sup>.

Previous studies have demonstrated that tumors with more alleles lost have a considerably worse prognosis<sup>[8,9]</sup>. In contrast, the presence of MSI-H carries significant prognostic implications for colorectal cancer patients<sup>[10,11]</sup>. In this study,

we evaluated 207 colorectal cancer patients. LOH and MSI at 14 markers were detected by microsatellite analysis to evaluate the clinical manifestations of LOH and MSI in sporadic colorectal cancers.

## MATERIALS AND METHODS

### *Patients and clinicopathological data*

A total of 207 patients with colorectal adenocarcinoma who underwent resection in Taipei Veterans General Hospital from January 1999 to December 1999 were enrolled in this study. Patients were excluded if they received preoperative chemotherapy and/or radiotherapy, and had evidence of hereditary non-polyposis colorectal cancer according to the criteria of Amsterdam or familial adenomatous polyposis, a malignant tumor outside the colon within the previous five years, or died from surgical complications. Clinical data were recorded prospectively and stored in computerized files. The database included (1) patients' names, gender, age, family history, and major medical problems, (2) location, size, gross appearance, tumor-node-metastasis (TNM) stage, differentiation, and relevant pathologic prognostic features, and (3) types of operations, complications, recurrence of disease, and follow-up conditions. After operation, patients were followed up every 3 mo in the first 2 years, every 6 mo between 2 and 5 years, and annually thereafter. All patients were followed up for at least 3 years after operation or until death.

### *Tumortissues*

The Institutional Review Board of the Taipei Veterans General Hospital approved the study program. Consents to tissue collection were obtained from all patients. Following removal, specimens were cleaned thoroughly. The tumors were dissected meticulously and samples were collected from 4 different quadrants of the tumor for consideration of intratumoral heterogeneity. The corresponding normal mucosae taken at least 10 cm away from the primary tumor were collected. The fragments of samples were immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Sections of cancer tissues and the corresponding normal mucosae were identified, reviewed, and analyzed by a senior gastrointestinal pathologist who did not know the clinical outcome of the patients. The stage of the disease was classified according to the TNM classification of the International Union Against Cancer<sup>[12]</sup>. There were 139 (67.1%) men and 68 (32.9%) women in the study group. The mean age at the time of tumor resection was  $66.2 \pm 12.4$  years (range 22–88 years). There were 37 (17.8%) right side colonic tumors (cecum to splenic flexure), 85 (41.1%) left side colonic tumors (splenic flexure to sigmoid colon) and 85 (41.1%) rectal tumors. The distribution of tumor staging was stage I in 25 (12.1%), stage II in 73 (35.3%), stage III in 68 (32.9%) and stage IV in 41 (19.8%).

### *LOH and MSI analysis*

High-molecular-weight genomic DNA was prepared from each tumor and the corresponding normal tissue using the QIAamp tissue kit (QIAGEN GmbH, Hilden, Germany). Normal and tumor genomic DNA sample pairs were amplified using the microsatellite instability MSI/LOH assay

starter kit (Applied Biosystems, Foster City, CA). According to the international criteria for the determination of MSI<sup>[13]</sup>, five reference markers including *D2S123*, *D5S345*, *Bat25*, *Bat26* and *D17S250* were enrolled in our microsatellite panel. The chromosomal location of the microsatellite markers and genes surrounding the markers was described in a previous report<sup>[14]</sup>. In brief, 25 ng template DNA was amplified with fluorescent-labeled primer. PCR was carried out in a GeneAmp PCR System 9, 600 thermal cycler (Applied Biosystems) as follows: a 10-min pre-PCR incubation step at  $95^{\circ}\text{C}$ , 30 cycles of amplification, each at  $96^{\circ}\text{C}$  for 10 s, at  $55^{\circ}\text{C}$  for 30 s, at  $70^{\circ}\text{C}$  for 3 min, and a final extension at  $70^{\circ}\text{C}$  for 30 min.

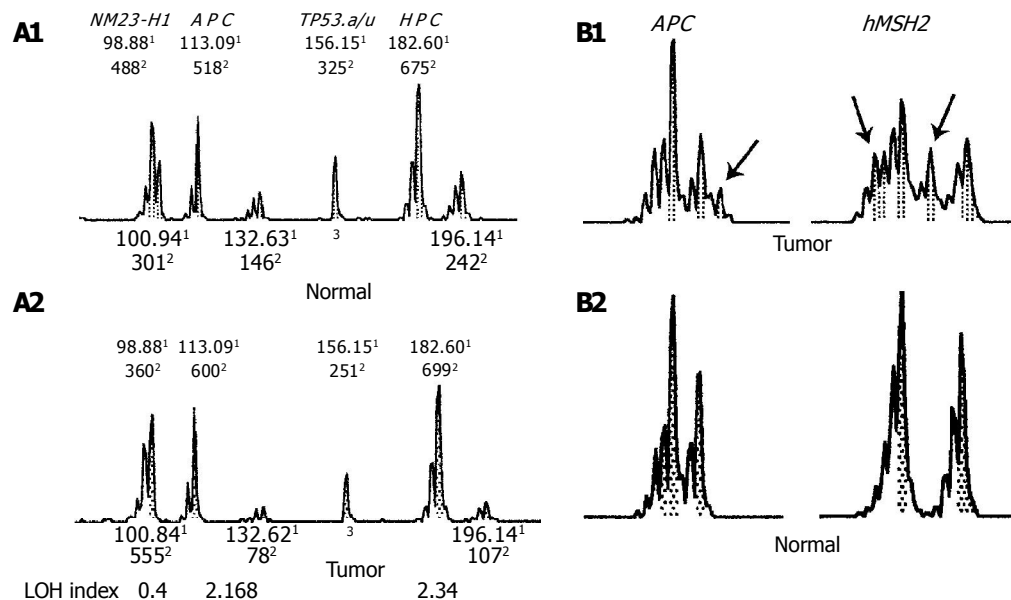
The amplification reactions were pooled and loaded onto a denaturing polyacrylamide gel, and the data were collected with an ABI 377 automated sequencer (Applied Biosystems). At the end of the run, each fluorescent peak was quantitated in terms of size (in base pairs), peak height, and area. Normal and tumor DNA pairs were compared for changes in peak height of each microsatellite marker *via* GeneScan analysis software (Applied Biosystems).

The LOH index was calculated using the modified method described by Cawkwell<sup>[15]</sup> for each paired normal and tumor samples. The peak height of two alleles in each tumor was divided by the peak height in normal samples:  $T1:T2/N1:N2$  where T1 and N1 are the peak heights of the tumor and normal samples, respectively for the corresponding allele one, and T2 and N2 for the corresponding allele two. Allele loss, according to the manufacturer's instructions, occurred with an LOH index of  $\leq 0.67$  or  $\geq 1.5$ . This allele loss also translated to a 33% decrease in peak height of one of the tumor alleles as compared with the normal alleles. The result of LOH analysis of a representative sample is shown in Figure 1. The tumors that exhibited LOH at more than 3 markers, or showed more than 50% of informative markers, were classified as the high-frequency LOH group (LOH-high). Others were classified as the low-frequency LOH group (LOH-low).

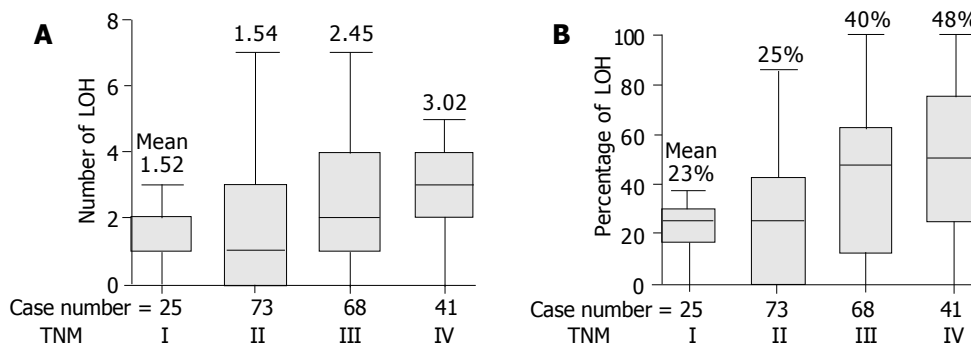
Tumor samples that exhibited novel allele peaks compared with the corresponding normal samples were classified as MSI at that marker. Such markers were considered uninformative for the LOH study. The pattern of MSI of a representative sample is shown in Figure 2. The analysis was performed twice if the data were controversial. According to the international criteria for the determination of MSI<sup>[13]</sup>, MSI in at least 4 or more loci was defined as high microsatellite instability (MSI-H); otherwise it was considered microsatellite stability (MSS).

### *Statistical analysis*

The statistical end point in our analysis was the occurrence of metastasis or death. The group distribution of each clinicopathological characteristic according to the presence or absence of LOH or MSI was compared using the  $\chi^2$  test. The numerical value was compared with the Student's *t* test. Statistical significance was defined as  $P < 0.05$  (SPSS for Windows version 10.0). The variables that resulted in *P* values  $< 0.1$  were entered into the Cox proportional hazards model for the determination of the independent prognostic factor for colorectal cancer.



**Figure 1** LOH at three genetic loci in a 52-yr-old male with stage IV disease (A) and MSI at two genetic loci in a 47-yr-old male with stage II disease (B) shown as representative results of GeneScan. A: LOH at three genetic loci in a 52-yr-old male with stage IV disease; B: MSI at two genetic loci in a 47-yr-old male with stage II disease. <sup>1</sup>Size of the PCR product (in bp). <sup>2</sup>Fluorescence intensity of peak. <sup>3</sup>Homozygosity. The arrowhead indicated novel alleles.



**Figure 2** The number and percentage of LOH. Panel A: The number of LOH; Panel B: The percentage of LOH.

## RESULTS

Markers with detectable heterozygous alleles were defined as informative. One hundred and sixty-three tumors (78.8%) exhibited LOH in at least one microsatellite marker. LOH in more than 3 markers (LOH-high) was noted in 93 tumors (44.9%). The highest frequency of LOH in the tumor suppressor gene was *TP53.alu* (65%), followed by *DCC* (64.3%), *D8S254* (51.7%) and *APC* (47.8%) (Table 1, Figure 1A). There was no difference in the distribution of gender, age, preoperative CEA level and location of tumors between the LOH-high and LOH-low groups. Considering the pathological variables, there was a linear trend between TNM staging and the LOH-high tumors ( $P < 0.001$ ). The mean number and percentage of LOH in stage I tumors were 1.52% and 22.8%, respectively, with an increase to 3.02% and 48%, respectively, in stage IV tumors (Figure 2). The other pathological variables such as lymph-vascular invasion, mucin component, invasive pattern of tumors, and grade of differentiation showed no significant association with the LOH-high tumors (Table 2).

**Table 1** Frequency of LOH and MSI of tumor suppressor genes in sporadic colorectal cancers

Marker	Informative / Total (%)	LOH/Informative (%)	MSI No./Total (%)
<i>NM23-H1</i>	125/207 (60.4)	43/125 (31.6)	4/164 (2.4)
<i>APC</i>	121/207 (58.5)	56/121 (47.8)	10/151 (6.6) <sup>1</sup>
<i>TP53.alu</i>	103/207 (49.8)	67/103 (65.0)	1/140 (0.7)
<i>HPC1</i>	127/207 (61.4)	15/127 (11.8)	6/192 (3.1)
<i>D8S254</i>	89/207 (43.0)	46/89 (51.7)	5/161 (3.1)
<i>DCC</i>	98/207 (47.3)	63/98 (64.3)	3/144 (2.1)
<i>hMSH2</i>	153/207 (73.9)	24/153 (15.7)	19/183 (10.4) <sup>1</sup>
<i>hMLH1</i>	99/207 (47.8)	26/99 (26.3)	11/181 (6.1)
<i>MET</i>	91/207 (44.0)	27/91 (29.7)	7/180 (3.9)
<i>TP53.pcr15</i>	80/207 (38.6)	31/80 (38.8)	5/174 (2.9)
<i>MYCL1</i>	110/207 (53.1)	38/110 (34.5)	5/169 (3.0)
<i>Bat-25</i>	30/207 (14.5)	3/30 (10)	22/204 (9.8) <sup>1</sup>
<i>Bat-26</i>	41/207 (19.8)	4/41 (4.4)	15/203 (7.4) <sup>1</sup>
<i>Mfd15</i>	33/207 (15.9)	2/33 (7.2)	9/205 (4.4) <sup>1</sup>

<sup>1</sup>Reference markers according to the international criteria for the determination of MSI<sup>[13]</sup>.

In a total of 2,451 MSI analyses, excluding LOH markers, 122 markers had MSI (4.97%, Figure 1B). Fifty-five tumors

**Table 2** Comparison between clinico-pathological features of loss of heterozygosity-high and loss of heterozygosity-low in colorectal cancer, *n* (%)

Features	Total	LOH-high	LOH-low	<i>P</i>
Number of cases	207	93 (44.9)	114 (55.1)	
Age (yr)	66.2±12.4	67.2±12.4	65.4±12.3	0.292 <sup>1</sup>
Gender (male/female)	139/68	61/32	78/36	0.778 <sup>2</sup>
Location of tumor				
Right colon	37 (17.9)	16 (17.2)	21 (18.4)	0.544 <sup>3</sup>
Left colon	85 (41.1)	42 (45.2)	43 (37.7)	
Rectum	85 (41.1)	35 (37.6)	50 (43.9)	
TNM stage				
I	25 (12.1)	7 (7.5)	18 (15.8)	<0.001 <sup>4</sup>
II	73 (35.3)	22 (23.7)	51 (44.7)	
III	68 (32.9)	37 (39.8)	31 (27.2)	
IV	41 (19.8)	27 (29.0)	14 (12.3)	
Invasive pattern				
Expanding	52 (25.1)	20 (21.5)	32 (28.1)	0.286 <sup>2</sup>
Infiltrative	155 (74.9)	73 (78.5)	82 (71.9)	
Grade				
Well-differentiated	5 (2.4)	2 (2.2)	3 (2.6)	0.263 <sup>3</sup>
Moderate differentiated	182 (87.9)	81 (87.0)	101 (88.6)	
Poorly differentiated	20 (9.7)	10 (10.8)	10 (8.8)	
Lymphovascular invasion				
Yes	48 (23.2)	27 (56.3)	21 (43.7)	0.102 <sup>2</sup>
No	159 (76.8)	66 (41.5)	93 (58.5)	
Mucin production				
Yes	29 (14.0)	9 (9.7)	20 (17.5)	0.155 <sup>2</sup>
No	178 (86.0)	84 (90.3)	94 (82.5)	

<sup>1</sup>Student *t* test, <sup>2</sup>Fisher exact test, <sup>3</sup>Pearson  $\chi^2$  test, <sup>4</sup> $\chi^2$  test with linear-by-linear association.**Table 3** Comparison between clinico-pathological features of MSI-H and MSS in colorectal cancer, *n* (%)

Features	Total	MSI-H	MSS	<i>P</i>
Number of cases (%)	207	15 (7.25)	192 (92.7)	
Age (yr)	66.2±12.4	69.7±14.8	65.9±12.2	0.248 <sup>1</sup>
Gender(male/female)	139/68	8/7	131/61	0.369 <sup>2</sup>
Location of tumor				
Right colon	37 (17.9)	7 (46.7)	30 (15.6)	0.005 <sup>3</sup>
Left colon	85 (41.1)	2 (13.3)	83 (43.2)	
Rectum	85 (41.1)	6 (40.0)	79 (41.2)	
TNM stage				
I	25 (14.2)	1 (6.7)	24 (12.5)	0.780 <sup>4</sup>
II	73 (36.8)	6 (40.0)	67 (34.9)	
III	68 (31.6)	6 (40.0)	62 (32.3)	
IV	41 (17.4)	2 (13.3)	39 (20.3)	
Invasive pattern				
Expanding	52 (25.1)	4 (26.7)	48 (25.0)	1.0 <sup>2</sup>
Infiltrative	155 (74.9)	11 (73.3)	144 (75.0)	
Grade of differentiation				
Well	5 (2.4)	(0)	5 (2.6)	<0.001 <sup>3</sup>
Moderate	182 (87.9)	9 (60.0)	173 (90.1)	
Poor	20 (9.7)	6 (40.0)	14 (7.3)	
Lymphovascular invasion				
Yes	48 (23.2)	1 (6.7)	47 (24.5)	0.209 <sup>2</sup>
No	159 (76.8)	14 (93.3)	145 (75.5)	
Mucin component				
Yes	29 (14.0)	7 (46.7)	22 (11.5)	0.001 <sup>2</sup>
No	178 (86.0)	8 (53.3)	170 (88.5)	

<sup>1</sup>Student *t* test, <sup>2</sup>Fisher exact test, <sup>3</sup>Pearson  $\chi^2$  test, <sup>4</sup> $\chi^2$  test with linear-by-linear association.

(26.6%) exhibited MSI in at least one genetic locus. MSI in at least 4 or more loci (MSI-H) was noted in 15 tumors (7.25%). The distribution of MSI at different markers is shown in Table 1. The highest frequency of MSI was *hMSH2* (10.4%), followed by *Bat25* (9.8%), and *Bat26* (7.4%). The information on the clinicopathological features of MSI-H and MSS colorectal cancers is shown in Table 3. Seven (46.7%) of the 15 MSI-H cancers were located in the right colon, a significantly greater number than that of the MSS

cancers (15.6%). Poor tumor differentiation was found in 40% of MSI-H cancers (6/15), significantly higher than that found in MSS cancers (7.3%, 14/192). High mucin component of tumors was found in 46.7% of MSI-H cancers (7/15), significantly higher than that found in MSS cancers (22/192). There was no difference in the distribution of gender, TNM stage, invasive pattern of tumors, and lymph-vascular invasion between the MSI-H and MSS cancers.

Of the 166 potentially cured patients, 45 developed tumor recurrence within a median of 36 mo of follow-up. The sites of tumor recurrence were: liver (20), lung (18), bone (5), peritoneum (5), remote nodes (4), and other sites (3). As shown in Table 4 and Figure 3, the clinicopathological factors affecting the 3-year DFS were TNM staging, grade of differentiation, preoperative CEA level, and high LOH status. Using multivariate analysis, we identified that TNM staging showed the most significant impact on DFS [hazard ratio (HR) = 3.31; 95% confidence interval (CI) = 1.82-6.01], followed by high LOH status (HR = 3.18; 95% CI = 1.68-5.98) and CEA level (HR = 2.30; 95% CI = 1.21-4.37). Of the respective markers, LOH of *Tp53.alu*, *D8S254*, *DCC*, and *Tp53.pcr15* had a significant impact on DFS, as shown in Table 5.

**Table 4** Correlation of the clinico-pathological factors with three-year disease-free survival in potentially cured colorectal cancer patients (stage I, II, III patients)

Variables	Number of cases	3-yr disease-free survival	P <sup>1</sup>
Gender			
Male	116	70	0.614
Female	50	75	
Preoperative CEA level			
<5 ng/mL	80	82	0.003
>5 ng/mL	86	63	
TNM stage			
I	25	95	<0.001
II	73	84	
III	68	47	
Lymphovascular permeation			
No	129	73	0.091
Yes	37	63	
Invasive pattern of tumor			
Expansive	45	73	0.517
Infiltrative	121	68	
Grade of differentiation			
Well	3	100	0.004
Moderate	146	75	
Poor	17	48	
Mucinous component			
<50%	143	72	0.642
>50%	23	68	
Microsatellite instability			
MSI-H	13	69	0.910
MSS	153	71	
LOH status			
Low	100	84	<0.001
High	66	50	

Multivariate analysis<sup>2</sup>

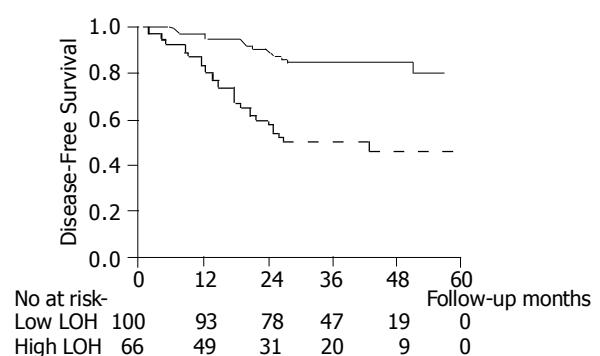
Factors	Hazard ratio	95% CI	P
LOH (High vs Low)	3.18	1.68-5.98	<0.001
TNM	3.31	1.82-6.01	<0.001
CEA level			
>5 ng/mL vs <5 ng/mL	2.30	1.21-4.37	0.011
Grade of differentiation	1.12	0.45-2.77	0.791
Lymphovascular invasion	1.0	0.49-2.01	0.981

<sup>1</sup>Log-rank test; <sup>2</sup>The P value results from the hypothesis that the relative risk as determined by a multivariate binary logistic regression analysis equaled 1.0.

**Table 5** Informative markers that influenced three-year DFS in potentially cured colorectal cancer patients (stage I, II, III patients)

Markers	Number of cases	3-yr disease-free survival	P <sup>1</sup>
<i>Nm23-H1</i>			
Non-LOH	68	75	0.266
LOH	28	62	
<i>APC</i>			
Non-LOH	54	75	0.881
LOH	45	72	
<i>Tp53.alu</i>			
Non-LOH	29	92	<0.001
LOH	55	51	
<i>HPC1</i>			
Non-LOH	87	75	0.743
LOH	12	70	
<i>D8S254</i>			
Non-LOH	39	75	0.030
LOH	31	52	
<i>DCC</i>			
Non-LOH	27	79	0.042
LOH	45	55	
<i>MSH2</i>			
Non-LOH	107	73	0.972
LOH	15	75	
<i>MLH1</i>			
Non-LOH	62	66	0.287
LOH	19	57	
<i>Met ONC</i>			
Non-LOH	50	72	0.350
LOH	16	64	
<i>Tp53.pcr15</i>			
Non-LOH	42	76	<0.001
LOH	20	33	
<i>MYCL1</i>			
Non-LOH	59	71	0.421
LOH	29	65	

<sup>1</sup>Log-rank test; The markers that had frequency of informative tumors lower than 30% were excluded in the survival analysis.



**Figure 3** Comparison of the clinicopathological factors with 3-yr survival in colorectal cancer patients. — : Low LOH group; ----: High LOH group.

## DISCUSSION

Our approach consisted of examining 14 microsatellite markers located near or within regions containing important genes implicated in the complex process of colorectal tumorigenesis. The use of automated assessment of loss of heterozygosity and microsatellite instability with fluorescent-PCR was described by Cawthell<sup>[15]</sup> and could be adapted to the analysis of both LOH and MSI, two important mechanisms of colorectal carcinogenesis. In our results, the frequency of MSI exhibited a trend to cluster

in *bMSH2*, *BAT25* and *BAT26* loci, but not in markers in regions displaying high levels of allelic loss (e.g., *p53* and *DCC*). The higher susceptibility of mononucleotide repeats to instability in MSI tumors has been reported by Ionov<sup>[4]</sup> and Dietmaier<sup>[16]</sup>. The microsatellite markers with a low frequency MSI might provide prognostic information about LOH. The present study demonstrated a significant inverse correlation between the two molecular events (LOH *vs* MSI). The tumors with MSI-H phenotypes had a lower frequency of LOH than those with MSS phenotype.

In the present series of patients with resected primary colorectal carcinomas, the frequency of MSI-H phenotypes was 7.25%, lower than in previous reports<sup>[7,17]</sup>. This may be attributed to several markers having a low-frequency MSI. If only considering five reference markers<sup>[13]</sup> as the current definition of MSI-H, the frequency of MSI-H could be as high as 11.1% of tumors. Previous reports indicated that MSI-H colorectal cancers were associated with distinct clinicopathological features, including poor differentiation, extracellular mucin accumulation, younger age at onset, and proximal tumor location<sup>[18-20]</sup>. Our MSI-H tumors displayed similar clinicopathological findings.

Loss of heterozygosity, i.e., loss of one allele at a constitutional heterozygous locus indicates the probability of loss of a tumor suppressor gene, which might promote neoplastic progression<sup>[21,22]</sup>. The frequency of an informative tumor for each microsatellite marker varied in the present study (range 14.5-73.9%). Analysis of a single marker may be biased by non-informative tumors, especially in the marker in which the frequency of an informative tumor was less than 50%. Therefore, we defined the LOH-high group as LOH at more than 3 markers, or more than 50% of informative markers for considering both the number and percentage of LOH. In our results, high-frequency LOH could be an independent prognostic factor affecting tumor recurrence. Of the 166 potentially cured patients, those with a high LOH tumor showed a DFS of 50 %, which was poorer than those with low LOH tumors (84%). Of the 45 patients who developed tumor recurrence, the mean number of LOH was 2.97 and the percentage of LOH in the primary tumor was 46.8%, which were similar to those tumors that already had distant metastasis at operation. These findings imply that high LOH tumors behave comparably to those stage IV diseases.

Metastasis is a complicated multistep process. The cells must detach and acquire motility, invade through the basement membrane and extracellular matrix, gain access to and survive in the vascular system, adhere to and immigrate into the target organ, and then proliferate in the new site<sup>[23]</sup>. It is reasonable to believe that multiple genes control the complicated process. Previous reports have hypothesized that accumulation of various genetic abnormalities is more important in tumorigenesis than the sequence of genetic alterations<sup>[2,24]</sup>.

Additionally, a previous report demonstrated that *p53* and *DCC* were the most common mutated tumor suppressor genes in colorectal cancer, and occurred mainly at the late phase of carcinogenesis<sup>[1]</sup>. In our study, the frequency of LOH was the highest at *p53* (65.0%) followed by *DCC* (64.3%), *D8S254* (51.7%) and *APC* (47.8%). There were

four markers associated with metastatic potential, including *p53*, *DCC*, and a tumor suppressor gene located in the 8p22. In our series, the incidence of LOH of *p53* was similar to a previous report<sup>[24]</sup>. The *p53* gene has been thought to modulate two discrete functions, both in response to stress (particularly DNA damage), cell cycle arrest, and apoptosis<sup>[25,26]</sup>. The loss of *p53* in the final stages of colon cancer not only allows the cells to divide at an unrestrained rate, but this rapid replication further promotes the development of even more genetic mutations because the cell cycle is not interrupted when errors occur. Several authors have found that mutations of *p53* or allelic loss of the *p53* was correlated with poor outcomes<sup>[27-29]</sup>. Our study yielded a similar finding. The incidence of allele loss of *DCC* in colorectal cancer has been reported to be from 33%<sup>[15]</sup> to 75%<sup>[29-31]</sup>. According to previous reports, LOH or decreased *DCC* expression was associated with liver and lymph node metastasis<sup>[29,31]</sup>. In our study, LOH of *DCC* was significantly associated with lymph node metastasis (data not shown). The *DCC* protein was a transmembrane glycoprotein similar to the neural cell adhesion molecules<sup>[32]</sup>. LOH of *DCC* could thus lead to impaired contacts between cells, thereby contributing to tumor growth and invasion. The prognostic value of LOH of *DCC* has been confirmed in several previous studies<sup>[31,33,34]</sup>. Our observations also showed that LOH of *DCC* was significantly associated with metastasis potential.

Another putative tumor suppressor gene located in the short arm of chromosome 8 had a high frequency of LOH in our study, as shown in a previous report<sup>[35]</sup>. Several studies showed that this novel tumor suppressor gene locus was related to the stage progression of colorectal cancer<sup>[36,37]</sup>, similar to our findings.

In the multivariate analysis, TNM stage was the most significantly independent prognostic factor for colorectal cancer, followed by high-frequency LOH and preoperative CEA level. The outcome of patients with colorectal cancer was determined by the presence or absence of metastasis at the time of operation. We further analyzed the prognostic impact of high LOH status on early state tumors (stage I or II). Of the 29 stage I or II tumor patients with high-frequency LOH, 9 patients (32.1%), developed distant metastasis within three years after surgery, which was significantly higher than those with low-frequency LOH (8.7%,  $P = 0.037$ ). This finding implies that early stage tumors with LOH-high have a stronger metastatic potential.

In conclusion, genetic change in both MSI and LOH phenotypes may occur in sporadic colorectal tumors, as established by microsatellite analysis. Variation of genetic phenotypes is related to different clinical manifestations, an important finding that may be useful in identifying subgroups of patients with different prognostic risks.

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• BASIC RESEARCH •

# Combination of telomerase antisense oligonucleotides simultaneously targeting hTR and hTERT produces synergism of inhibition of telomerase activity and growth in human colon cancer cell line

Xiao-Hua Fu, Jian-Song Zhang, Na Zhang, Yang-De Zhang

Xiao-Hua Fu, Jian-Song Zhang, Na Zhang, Medical College, Hunan Normal University, Changsha 410006, Hunan Province, China  
Yang-De Zhang, National Hepatobiliary and Enteric Surgery Research Center of Ministry of Health, China  
Supported by the Science and Research Foundation of Bureau of Health, Hunan Province, China, No. Y02-083  
Correspondence to: Dr. Yang-De Zhang, National Hepatobiliary and Enteric Surgery Research Center of Ministry of Health of China, Changsha 410006, Hunan Province, China. fuxh1@sohu.com  
Telephone: +86-731-8912446 Fax: +86-731-8912417  
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## Abstract

**AIM:** To investigate synergism of inhibition of telomerase activity and proliferation of human colon cancer cells by combination of telomerase antisense oligonucleotides (ASODNs) simultaneously targeting human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) *in vitro*.

**METHODS:** ASODN of hTR and ASODN of hTERT were transfected into human colon cancer SW480 cells by liposomal transfection reagents. Telomerase activity of SW480 cells was examined using telomeric repeat amplification protocol (TRAP)-enzyme-linked immunosorbent assay (PCR-ELISA). Proliferation activity of SW480 cells was tested by methyl thiazolyl tetrazolium assay. Apoptosis and cell cycle were analyzed by flow cytometry.

**RESULTS:** The telomerase activity and cell survival rate in SW480 cells transfected with 0.2  $\mu\text{mol/L}$  of ASODN of hTR or ASODN of hTERT for 24-72 h were significantly decreased in a time-dependent manner compared with those after treatment with sense oligonucleotides and untreated (telomerase activity: 24 h, 73%, 74% vs 99%, 98%; 48 h, 61%, 55% vs 98%, 99%; 72 h, 41%, 37% vs 99%, 97%;  $P < 0.01$ ; cell survival rate: 24 h, 88%, 86% vs 94%, 98%; 48 h, 49%, 47% vs 94%, 97%; 72 h, 44%, 42% vs 92%, 96%;  $P < 0.01$ ). Moreover, the telomerase activity and the cell survival rate in SW480 cells treated by the combination of telomerase anti-hTR and anti-hTERT were more significantly suppressed than single anti-hTR or anti-hTERT (telomerase activity: 24 h, 59% vs 73%, 74%; 48 h, 43% vs 61%, 55%; 72 h, 18% vs 41%, 37%;  $P < 0.01$ ; cell survival rate: 24 h, 64% vs 88%, 86%; 48 h, 37% vs 49%, 47%; 72 h, 25% vs 44%, 42%;  $P < 0.01$ ). Meanwhile, the apoptosis rates in the combination group

were markedly increased compared with those in the single group (24 h, 18.0% vs 7.2%, 7.4%; 48 h, 23.0% vs 13.0%, 14.0%; 72 h, 28.6% vs 13.2%, 13.75;  $P < 0.01$ ). Cells in combination group were arrested at  $G_0/G_1$  phase.

**CONCLUSION:** Telomerase anti-hTR and anti-hTERT suppress telomerase activity, and inhibit growth of human colon cancer cells probably via induction of apoptosis and retardation of cell cycle. Additionally, combined use of telomerase ASODNs targeting both hTR and hTERT yields synergistic action selective for human colon cancer.

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**Key words:** Telomerase reverse transcriptase; Telomerase RNA; Antisense oligonucleotides; Synergistic action; Colon cancer

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## INTRODUCTION

Telomerase is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric TTAGGG repeats at eukaryotic chromosome ends<sup>[1]</sup>. Telomeres are highly conserved in organisms ranging from unicellular eukaryotes to mammals, indicating a strong role of their protective mechanisms in preventing chromosomal ends from undergoing degradation and ligation with other chromosomes. Without telomeric caps human chromosomes would undergo end-to-end fusions, with the formation of dicentric and multicentric chromosomes<sup>[1]</sup>. These abnormal chromosomes would break during mitosis, resulting in severe damage to the genome and the activation of DNA damage checkpoints, leading to cell senescence or initiation of the apoptotic cell death pathway<sup>[2]</sup>. Indeed, it has been proposed that telomere length specifies the number of cell divisions a cell can undergo before senescence<sup>[3]</sup>. Telomerase activity is up-regulated in the vast majority of human tumors, as compared with normal somatic tissues. Expression of the catalytic subunit of telomerase, human

telomerase reverse transcriptase (hTERT), in cultured human primary cells reconstitutes telomerase activity and allows immortal growth<sup>[4-7]</sup>. The rate of telomere DNA shortening is regulated by telomerase expression and activity<sup>[8]</sup>. Therefore, telomerase inhibitors might be useful as anticancer agents, but there will be an expected lag phase between the time when the telomerase is inhibited and the time when the telomere of cancer cells is shortened sufficiently to produce detrimental effects on cell proliferation<sup>[9]</sup>.

Human telomerase mainly consists of three subunits: human telomerase RNA (hTR), human telomerase-associated protein 1 (TP1) and hTERT. Numerous studies have documented that antisense gene therapy directing against telomerase RNA or hTERT component could effectively inhibit telomerase activity and induce apoptosis in gastric cancer, malignant gliomas, colon cancer, and ovarian cancer<sup>[10-13]</sup>. We hypothesized that simultaneous use of antisense to the RNA portion of hTR, which blocks the template for telomere synthesis<sup>[8]</sup> and antisense to hTERT, which blocks the promoter of human telomerase catalytic subunit, may provide synergistic antitumor activity against tumor cells that depend on telomerase for telomere maintenance. In this study, we tested the hypothesis that telomerase antisense oligonucleotides (ASODNs) simultaneously targeting hTR and hTERT may provide synergistic antitumor activity against tumor cells that depend on telomerase. ASODNs combination targeting the hTR gene (anti-hTR) and the hTERT gene (anti-hTERT), modified by phosphorothiolation, was transfected into SW480 cells with Oligofectamine<sup>TM</sup>, which has been demonstrated to be telomerase-positive cells. The synergistic action of the combination of anti-hTR and anti-hTERT on telomerase activity, cell survival rates and apoptosis rate in SW480 cells were investigated respectively.

## MATERIALS AND METHODS

### Reagents

TRAPEZE<sup>®</sup> ELISA telomerase detection kit. Oligofectamine<sup>TM</sup> reagent was purchased from Invitrogen. RPMI-1640 was obtained from Gibco. Methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were provided by Sigma.

### Oligodeoxynucleotides synthesis

Antisense oligodeoxynucleotides against hTR (anti-hTR) with sequence 5'-CTCAGTTAGGGT'TAGAC-3', which blocks the template for telomere synthesis, and sense oligodeoxynucleotides (SODN) (s-hTR) with sequence 5'-CATTTCTTGCTCTCCACG-3', antisense oligodeoxynucleotides against hTERT (anti-hTERT) with sequence 5'-GGAGCGCGCGGCATCGCGGG-3', which blocks the promoter for telomere synthesis, and sense oligodeoxynucleotides (s-hTERT) with sequence 5'-CCCGCGATGCCGCGCGCTCC-3', were synthesized by Sangon Biotechnology Engineering Company of Shanghai. The synthesized oligodeoxynucleotides (ODNs) were modified by phosphorothiolation, purified by HPLC, stored at -20 °C.

### Cell culture

SW480 cells, a human colon cancer cell line, generously supplied by Department of Biology, Wuhan University,

Wuhan, China, were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum in a humidified 50 mL/L CO<sub>2</sub> atmosphere at 37 °C.

### Transfection of oligodeoxynucleotides

Cultured SW480 cells were divided into seven groups: ASODNs (anti-hTR, anti-hTERT, anti-hTR+anti-hTERT), SODNs (s-hTR, s-hTERT), Oligofectamine<sup>TM</sup> and blank control. The concentration of ASODNs was 0.05 μmol/L, 0.1 μmol/L, 0.2 μmol/L, respectively; the concentration of SODNs was 0.2 μmol/L; Oligofectamine<sup>TM</sup> concentration was varied depending on ODNs dose according to manufacturer's protocol.

The ODNs transfection was performed with Oligofectamine<sup>TM</sup> according to the manufacturer's protocol. Briefly, cells were plated into 96-well plates and incubated until the cells reached 30-50% confluency. Before the transfection, ODNs were diluted with serum-free medium. Then, the desired amount of ODNs was incubated for 15-20 min with diluted Oligofectamine<sup>TM</sup>. The ODNs/Oligofectamine<sup>TM</sup> mixture (20 μL) was added drop-wise in 80 μL serum-free RPMI-1640. After incubation for 4 h at 37 °C, 50 μL RPMI-1640 containing 30% fetal bovine serum was added into each well. Cells were analyzed after 24, 48 and 72 h, respectively.

### Telomerase assay

Polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) was performed according to the manufacturer's protocol with a minor modification. Cultured SW480 cells were harvested at a density of 1×10<sup>5</sup> per well and washed with PBS and then homogenized in 200 μL CHAPs lysis buffer and left on ice for 30 min. One hundred and sixty microliters of supernatant was collected after centrifugation (12 000 g, 20 min, 4 °C). PCR was performed in 50 μL supernatant containing 10 μL transfer reaction mixture, 2 μL cell extracts added to 48 μL nuclease-free water. The PCR condition is as follows: the telomerase reaction was carried out at 30 °C for 30 min, followed by a two-step PCR amplification (94 °C, 30 s and 55 °C, 30 s for 33 cycles). Five microliters of amplified product and 20 μL denatured reagent were incubated at room temperature, 25 μL hybridization buffer was then added and mixed, and 100 μL of them was distributed in the wells of a microtitering plate. After one hour of incubation (37 °C), 100 μL of anti-DIG-POD working solution was added and incubated for another 30 min followed by the addition of 100 μL TMB substrate solution, and 100 μL of stop reagent was added at last. The absorbance in each well was read at the wavelength of 450 and 690 nm by a microtiter plate reader. The results of  $A_{450} \text{ min } A_{690} > 0.80$  unit using TSR 8 were judged as a positive control. The negative control was considered as  $A_{450} \text{ min } A_{690} < 0.20$  unit using lysis buffer. Telomerase activity was considered positive when the value of  $A_{450} \text{ min } A_{690}$  of a sample was at least 0.15. Each sample was examined for more than twice.

At the same time, telomeric repeat amplification protocol (TRAP) products were analyzed on a 19% non-denaturing polyacrylamide gel. The gel was stained with silver and analyzed. The internal telomerase assay standard (ITAS)

was present in the lane, as described in the kit protocol.

### Cytotoxicity assay

The SW480 cells were seeded onto 96-well plates at the density of  $0.5 \times 10^4$  cells per well, and incubated in a humidified atmosphere of 50 mL/L CO<sub>2</sub>+95% air, until the cells reached 30-50% confluency, then they were transfected with ODNs/Oligofectamine™ mixture for 24, 48 and 72 h. After the treatment, 20  $\mu$ L MTT (5 g/L in PBS) was added. The plates were incubated for 4 h and the blue dye formed was dissolved in 100  $\mu$ L DMSO. The absorbance at 570 nm was recorded using an ELISA Multiskan reader. Survival rate was calculated as follows: survival rate (%) =  $(T-B)/(U-B) \times 100\%$ , where *T* is the absorbance determined when tumor cells were exposed to drugs; *U* is the absorbance of untreated cells; *B* is the absorbance when neither the drug nor MTT was added.

### Apoptosis assay

To analyze apoptosis and cell cycle distribution, SW480 cells were trypsinized, harvested with a density of  $1 \times 10^6$ /L, washed once in ice-cold PBS, fixed and stained with propidium iodide, and then analyzed by flow cytometry.

### Statistical analysis

The data were expressed as mean  $\pm$  SD. Results were analyzed with the software package SPSS 10.0, and one-way ANOVA. *P* value <0.05 was considered statistically significant.

## RESULTS

### Synergistic effect of combination of anti-hTR and anti-hTERT on inhibition of telomerase activity in SW480 cell line

The SW480 cells were transfected with ASODN, SODN and Oligofectamine™ and the untreated served as control; telomerase activity of the SW480 cells after transfection of anti-hTR and anti-hTERT was significantly decreased ( $P < 0.01$ ). In addition, after transfection with ASODN of combination of anti-hTR and anti-hTERT, the telomerase activity was significantly lower than that with anti-hTR or anti-hTERT alone ( $P < 0.01$ ). These findings suggested that the combination of anti-hTR and anti-hTERT had synergetic

effect on the inhibition of the telomerase activity in human colon cancer cell line (Figure 1A).

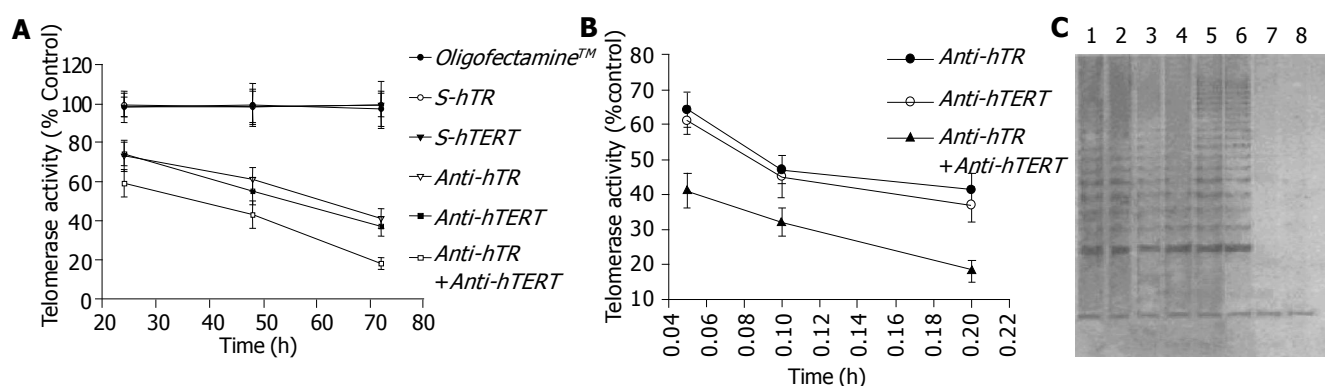
Meanwhile, inhibition of anti-hTR or anti-hTERT or both on telomerase activity was in a concentration-dependent manner. The telomerase activity (% control) of SW480 cells transfected with the combination of anti-hTR and anti-hTERT at various concentrations of 0.05, 0.1, 0.2  $\mu$ mol/L for 72 h decreased from 40.51 to 32.76%, finally to 19.55% (Figure 1B). Lanes shown in PAGE figures support the results (Figure 1C).

### Synergistic inhibitory effect of combination of anti-hTR and anti-hTERT on proliferation of SW480 cell line in vitro

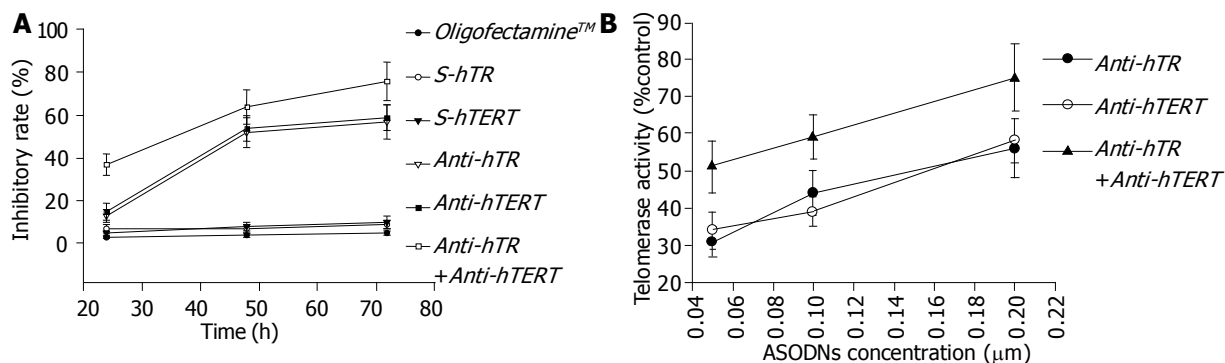
The inhibition on proliferation of SW480 cells was determined after transfection with ASODN, SODN and Oligofectamine™ for 24, 48, 72 h, respectively. At 24 h, the survival rates in anti-hTR or anti-hTERT or both groups were significantly decreased compared to those in SODN and Oligofectamine™ group ( $P < 0.01$ ). Similarly, combination of anti-hTR and anti-hTERT has synergistic inhibitory effect on cell growth ( $P < 0.01$ ); cell proliferation activity was inhibited in a concentration- and time-dependent manner. The rates of inhibition on proliferation increased from 33.58% at 24 h to 69.34% at 72 h (Figure 2A). Meanwhile, rates of inhibition increased depending on auto-concentration (Figure 2B).

### Synergistic effect of combination of anti-hTR and anti-hTERT on induction of apoptosis in SW480 cell line

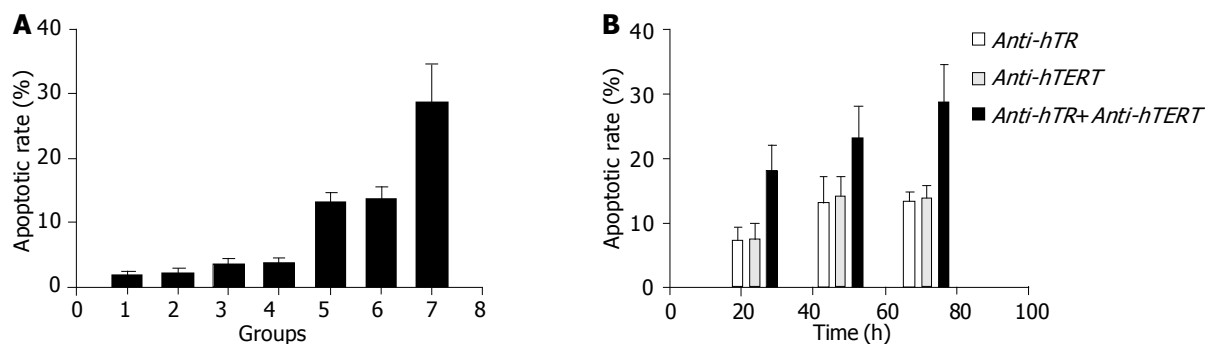
To explore the mechanism contributing to the inhibition of cell growth and the altered cellular morphology, we analyzed the apoptotic rate of SW480 cells treated with anti-hTR or anti-hTERT or both. Compared to cells treated with SODN, Oligofectamine™ and untreated control, there was a significant increase in the apoptotic rates of cells treated with anti-hTR and anti-hTERT at 0.2  $\mu$ mol/L for 72 h ( $P < 0.01$ ). Apoptotic rate of SW480 cells in combination group (28.61%) was markedly higher than that in anti-hTR (13.2%) or anti-hTERT (13.72%) alone ( $P < 0.01$ ) (Figure 3A). Furthermore, as shown in Figure 3B, apoptotic rates gradually increased with treatment time elongation, showing



**Figure 1** Inhibition of telomerase activity by telomerase ASODN. A: The relative telomerase activity (% control) of SW480 cells transfected with 0.2  $\mu$ mol/L ASODN for 24, 48, 72 h respectively. Extracts of SW480 cell were quantified with a TRAPEZE® ELISA telomerase detection kit. Sensitivity of telomerase PCR-ELISA (mean  $\pm$  SD of three experiments). B: Telomerase activity (% control) of SW480 cells transfected by ASODN with various concentrations for 72 h. The concentration of ASODN was 0.05, 0.1, 0.2  $\mu$ mol/L, respectively (mean  $\pm$  SD of three experiments). C: PAGE figure of TRAP-PCR: Lane 1, Oligofectamine; lane 2, S-hTR; lane 3, anti-hTR; lane 4, anti-hTERT; lane 5, S-hTERT; lane 6, control; lane 7, anti-hTR+anti-hTERT; lane 8, negative; ITAS was the 36-bp internal control. Negative was lysis buffer.



**Figure 2** The inhibition on proliferation of SW480 cells transfected with 0.2 μmol/L ASODN. A: Cells were exposed to various agents for 24, 48, 72 h, respectively; B: Cells were exposed to various concentrations of ASODN for 72 h (mean±SD of three experiments).



**Figure 3** Apoptosis induced by treatment with ASODN in SW480 cells. A: Cells were transfected with various agents (1: blank control, 2: s-hTR, 3: s-hTERT, 4: Oligofectamine™, 5: anti-hTR, 6: anti-hTERT, 7: anti-hTR+anti-hTERT) for 72 h; B: Cells were transfected with ASODN for 24, 48, 72 h, respectively (mean±SD of three experiments).

a time-dependent manner.

### Synergistic effect of combination with anti-hTR and anti-hTERT on cell cycle distribution in SW480 cell line

In SW480 cells treated with combination of anti-hTR and anti-hTERT for 72 h, the cells in G<sub>0</sub>/G<sub>1</sub> increased and in G<sub>2</sub>/M phases decreased. SW480 cells after treatment with anti-hTR or anti-hTERT alone were accumulated in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases, respectively (Table 1).

## DISCUSSION

Telomerase activity has been detected in 85-90% of various human tumor tissues but is stringently repressed in normal human somatic tissues making telomerase an attractive therapeutic target<sup>[14]</sup>. Telomerase-associated hTR functions as a template for telomerase elongation by telomerase<sup>[15]</sup>. hTERT contains reverse transcriptase motifs and functions as the catalytic subunit of telomerase<sup>[16]</sup>. hTR and hTERT are both attractive targets.

Bachand *et al*<sup>[17]</sup> have identified that two independent hTERT binding sites exist within hTR. hTR and hTERT react with each other. Our experiments showed that combination of anti-hTR and anti-hTERT possessed synergistic action on the inhibition of telomerase in the SW480 cell line. This is likely a result of the blocking of both hTR and hTERT components responsible for telomere elongation, thus enhancing the inhibition of telomerase

**Table 1** Cell cycle distribution of SW480 cells transfected with telomerase ASODNs, SODNs and Oligofectamine™ (mean±SD, n = 3)

	Cell cycle distribution (%)		
	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
Control	50.7±2.3	36.6±1.8	15.5±2.2
Oligofectamine™	53.8±2.7	34.1±3.6	12.3±2.6
S-hTR	53.1±2.8	34.4±13.2	12.5±1.3
S-hTERT	50.7±3.5	30.1±2.8	19.2±2.6
Anti-hTR	55.0±2.3	30.1±2.5	14.9±1.9
Anti-hTERT	49.2±3.4	28.8±3.1	21.9±2.8 <sup>b</sup>
Anti-hTR+anti-hTERT	63.8±2.3 <sup>b</sup>	24.9±1.6	12.3±2.9

<sup>b</sup>P<0.01 vs control group.

activity.

Telomerase is a ribonucleoprotein DNA polymerase that synthesizes telomeric repeats de novo and is involved in multiple cellular processes, including cell differentiation, proliferation, inhibition of apoptosis, tumorigenesis, and possibly DNA repair and drug resistance<sup>[18-21]</sup>. Telomerase activity has recently been implicated in the control of the proliferative capacity of normal and malignant cells<sup>[22]</sup>. In the present study, it has been shown that ASODNs against hTR component and hTERT significantly suppressed SW480 cell growth. In contrast, no significant proliferation inhibition in SW480 cells by sense hTR or sense hTERT was observed. In addition, combination of anti-hTR and anti-hTERT had synergistic inhibitory effects. We suppose that combination of anti-hTR and anti-hTERT augmented the effect on

telomerase repression, thus enhanced the inhibitory effect on cell growth.

Some experiments showed that telomerase inhibitors led to cell death probably because progressive telomerase shortening was responsible for decreased cell proliferation. However, a number of studies have documented that telomerase inhibitors resulted in proliferation inhibition likely via induction of apoptosis<sup>[23]</sup>. Kondo *et al.*<sup>[24]</sup> demonstrated that treatment with antisense telomerase inhibited telomerase activity and subsequently induced either apoptosis or differentiation. Regulation of these two distinct pathways may be dependent on the expression of interleukin-1 beta-converting enzyme (ICE) or cyclin-dependent kinase inhibitors (CDKIs). In the present study, it showed that anti-hTR and anti-hTERT induced apoptosis in SW480 cells. Apoptotic rates in combination group were markedly increased than that in the single group. It was established that anti-hTR and anti-hTERT inhibited the growth of SW480 cells through the induction of apoptosis. Combination of anti-hTR and anti-hTERT has synergistic induction of apoptosis.

Some human tumor cells without any telomerase activity are able to maintain the length of their telomeres, indicating the existence of one or more non-telomerase mechanisms for telomere maintenance which have been termed alternative lengthening of telomeres (ALT)<sup>[25,26]</sup>. ALT has been detected in a variety of human tumors, including sarcomas, glioblastomas, and cancers of the lung, kidney, adrenal, breast, and ovary<sup>[27]</sup>. An implication of the existence of ALT is that tumors using this telomere maintenance mechanism (including mixed telomerase-positive/ALT-positive tumors) will be resistant to telomerase inhibitors. Also, telomerase inhibitors will put tumors that are initially telomerase-positive under strong selection pressure for activation of ALT. Therefore, combination therapy using ALT and telomerase inhibitors may help prevent the emergence of drug resistance. A novel telomerase inhibitor, telomestatin, is a clinical candidate as a dual inhibitor of ALT and telomerase<sup>[28]</sup>. Lee *et al.*<sup>[29]</sup> reported that neoplastic cells from telomerase RNA null mice (mTERC<sup>-/-</sup>) showed enhanced chemosensitivity to doxorubicin or related double strand DNA break-inducing agents. Telomere dysfunction, rather than telomere inhibition, is proposed to be the principal determinant governing chemosensitivity specifically to double strand DNA break-inducing agents<sup>[29]</sup>. In this study, we detected apoptosis in tumor cells just 24 h after the combination of anti-hTR and anti-hTERT treatment in a dose-dependent manner. It is likely to raise the possibility that antitumor effect of the combination of anti-hTR and anti-hTERT occurs through the following two pathways: (1) A short-term effect on apoptosis was induced rapidly by the combination of anti-hTR and anti-hTERT. (2) A long-term inhibitory effect on telomerase activity was provoked, and cell death was caused when telomere length was critically shortened by telomeric DNA.

Zhu *et al.*<sup>[30]</sup> have reported that telomerase activity was likely to be regulated in a cell cycle-dependent manner. As the cell progresses through the cell cycle, telomerase activity gradually increased through the G<sub>1</sub>/S phase, reached its maximum level in S phase, and decreased through the G<sub>2</sub>/M

phase. These observations may be consistent with the present experimental results showing that inhibition of telomerase by the combination of anti-hTR and anti-hTERT led to increased G<sub>0</sub>/G<sub>1</sub> phase cells. Although SW480 cells treated with anti-hTERT were arrested in G<sub>2</sub>/M phase, we speculated that combination of anti-hTR and anti-hTERT augmented retardation, causing cells to accumulate in G<sub>0</sub>/G<sub>1</sub> phase.

In conclusion, in this study, it has been shown that anti-hTR and anti-hTERT have specific inhibition on SW480 cells. There is no significant difference between anti-hTR and anti-hTERT, but the combination of anti-hTR and anti-hTERT has significant synergistic action. Our data suggest that the strategy of telomerase ASODNs simultaneously targeting hTR and hTERT may offer a potential antitumor tool against human colon cancer and other telomerase-positive tumors.

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• BASIC RESEARCH •

## ***Helicobacter pylori* infection, glandular atrophy and intestinal metaplasia in superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer and early gastric cancer**

Chuan Zhang, Nobutaka Yamada, Yun-Lin Wu, Min Wen, Takeshi Matsuhisa, Norio Matsukura

Chuan Zhang, Department of Gastroenterology, Baogang Hospital, Shanghai Second Medical University, Shanghai 201900, China  
Nobutaka Yamada, Min Wen, Department of Pathology, Division of Surgical Pathology, Nippon Medical School, Tokyo 113-8602, Japan

Takeshi Matsuhisa, Department of Gastrointestinal Endoscopy Center, Tama-Nagayama Hospital, Tokyo 206-8512, Japan

Norio Matsukura, First Department of Surgery, Nippon Medical School, Tokyo 113-8602, Japan

Yun-Lin Wu, Department of Gastroenterology, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China

Correspondence to: Chuan Zhang, M.D., Department of Gastroenterology, Baogang Hospital, Shanghai Second Medical University, Shanghai 201900, China. zhangchuan71@hotmail.com  
Telephone: +86-21-56691101 Fax: +86-21-56691662

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### **Abstract**

**AIM:** To evaluate the histological features of gastric mucosa, including *Helicobacter pylori* infection in patients with early gastric cancer and endoscopically found superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer.

**METHODS:** The biopsy specimens were taken from the antrum, corpus and upper angulus of all the patients. Giemsa staining, improved toluidine-blue staining, and *H pylori*-specific antibody immune staining were performed as appropriate for the histological diagnosis of *H pylori* infection. Hematoxylin-eosin staining was used for the histological diagnosis of gastric mucosa inflammation, gastric glandular atrophy and intestinal metaplasia and scored into four grades according to the Updated Sydney System.

**RESULTS:** The overall prevalence of *H pylori* infection in superficial gastritis was 28.7%, in erosive gastritis 57.7%, in gastric erosion 63.3%, in gastric ulcer 80.8%, in early gastric cancer 52.4%. There was significant difference ( $P<0.05$ ), except for the difference between early gastric cancer and erosive gastritis. *H pylori* infection rate in antrum, corpus, angulus of patients with superficial gastritis was 25.9%, 26.2%, 25.2%, respectively; in patients with erosive gastritis 46.9%, 53.5%, 49.0%, respectively; in patients with gastric erosion 52.4%, 61.5%, 52.4%, respectively; in patients with gastric ulcer 52.4%, 61.5%, 52.4%, respectively; in patients with early gastric cancer 35.0%, 50.7%, 34.6%, respectively. No significant difference was found among the different site biopsies in superficial gastritis, but in the other diseases the detected

rates were higher in corpus biopsy ( $P<0.05$ ). The grades of mononuclear cell infiltration and polymorphonuclear cell infiltration, in early gastric cancer patients, were significantly higher than that in superficial gastritis patients, lower than that in gastric erosion and gastric ulcer patients ( $P<0.01$ ); however, there was no significant difference compared with erosive gastritis. The grades of mucosa glandular atrophy and intestinal metaplasia were significantly highest in early gastric cancer, lower in gastric ulcer, the next were erosive gastritis, gastric erosion, the lowest in superficial gastritis ( $P<0.01$ ). Furthermore, 53.3% and 51.4% showed glandular atrophy and intestinal metaplasia in angular biopsy specimens, respectively; but only 40.3% and 39.9% were identified in antral biopsy, and 14.1% and 13.6% in corpus biopsy; therefore, the angulus was more reliable for the diagnosis of glandular atrophy and intestinal metaplasia compared with antrum and corpus ( $P<0.01$ ). The positivity rate of glandular atrophy and intestinal metaplasia of superficial gastritis with *H pylori*-positivity was 50.7%, 34.1%; of erosive gastritis 76.1%, 63.0%; of gastric erosion 84.8%, 87.8%; of gastric ulcer 80.6%, 90.9%; and of early gastric cancer 85.5%, 85.3%, respectively. The positivity rate of glandular atrophy and intestinal metaplasia of superficial gastritis with *H pylori*-negativity was 9.9%, 6.9%; of erosive gastritis 42.5%, 42.1%; of gastric erosion 51.1%, 61.9%; of gastric ulcer 29.8%, 25.5%; and of early gastric cancer 84.0%, 86.0%, respectively. The positivity rate of glandular atrophy and intestinal metaplasia of superficial gastritis, erosive gastritis, gastric erosion, and gastric ulcer patients with *H pylori* positivity was significantly higher than those with *H pylori* negativity ( $P<0.01$ ); however, there was no significant difference in patients with early gastric cancer with or without *H pylori* infection.

**CONCLUSION:** The progression of the gastric pre-cancerous lesions, glandular atrophy and intestinal metaplasia in superficial gastritis, gastric erosion, erosive gastritis and gastric ulcer was strongly related to *H pylori* infection. In depth studies are needed to evaluate whether eradication of *H pylori* infection will really diminish the risk of gastric cancer.

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**Key words:** *Helicobacter pylori*; Glandular atrophy; Intestinal metaplasia; Early gastric cancer

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## INTRODUCTION

The discovery of *Helicobacter pylori* (*H. pylori*) offered the etiologic agent of the initiating event of the inflammatory cascade<sup>[1,2]</sup>. It has been confirmed that the development of gastric cancer spans over several decades sequentially starting with the acquisition of *H. pylori* infection and the development of chronic active gastritis<sup>[3,4]</sup>. Over time, the development of glandular atrophy and intestinal metaplasia takes place in a subset of patients. Finally, gastric cancer would eventually arise<sup>[5]</sup>. It was suggested that *H. pylori* infection leads to an increased risk, in the order of 4 to 9 folds, of developing precancerous gastric conditions especially when the infection occurs in childhood<sup>[6-9]</sup>. In 1994 the International Agency for Research on Cancer (IARC) monograph committee classified *H. pylori* as a class I carcinogen to humans<sup>[10]</sup>. On the other hand, *H. pylori* are also the cause of other gastric diseases, such as peptic ulcer, gastric mucosa-associated lymphoma<sup>[13-17]</sup>. Previous histological studies have reported the association between *H. pylori* infection and gastric cancer mainly using gastrectomy specimens from patients with advanced gastric cancer. However, the results were not always consistent; higher rates of serologically and histologically detected *H. pylori* positivity have been reported for early stage cancer than for advanced cancer<sup>[11,12]</sup>. Therefore, to evaluate the significance of *H. pylori* infection in gastric carcinogenesis, samples obtained from patients with an early stage cancer could be more informative than those from patients with advanced-stage cancer. Additionally, because both the incidence of gastric cancer and the frequency of *H. pylori* infection are much higher in Japanese than in most Western populations, it would be of particular interest to examine the association between endoscopically found superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer and early gastric cancer in Japanese patients with *H. pylori* infection. Further, it is more accurate to compare the presence of *H. pylori* infection, glandular atrophy and intestinal metaplasia in age- and gender-matched subjects<sup>[18]</sup>.

## MATERIALS AND METHODS

### Patients

All patients were prospectively selected from subjects who underwent upper gastrointestinal endoscopy screening with present or past abdominal complaints at Nippon Medical School hospitals from November 1994 to November 2003. To perform a case-controlled study, five age ( $\pm 2$  years) and sex-matched control subjects for each cancer patient was randomly selected from the same series of subjects with superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer or early gastric cancer. Subjects were considered to be eligible for inclusion into the present study when their endoscopic diagnosis was superficial gastritis, gastric erosion, erosive gastritis or gastric ulcer. Early gastric cancer was

pathologically diagnosed, as defined by the Japanese Gastroenterological Society, by the growth of malignant tumor confined to the mucosa and submucosa of the stomach regardless of the presence or absence of metastatic disease in the perigastric lymph nodes. Patients were excluded from the study if they had received anti-ulcer agents or antibiotics during the 2 mo before endoscopy or had previous histories of duodenal ulcers, or gastric surgery. The study comprised 286 patients with early gastric cancer aged from 38 to 90 years (mean age  $65.7 \pm 10.8$ ), which included 220 males and 66 females; 286 patients with superficial gastritis aged from 38 to 88 years (mean age  $65.3 \pm 9.9$ ); 286 patients with gastric erosion aged from 38 to 90 years (mean age  $65.3 \pm 10.4$ ); 286 patients with erosive gastritis aged from 38 to 90 years (mean age  $65.3 \pm 10.4$ ); 286 patients with gastric ulcer aged from 38 to 92 years (mean age  $65.7 \pm 10.8$ ). All patients gave informed consent before their endoscopies and the study was approved by the Ethics Committee of Nippon Medical School.

### Histological analysis

Biopsy specimens for histological diagnosis were obtained endoscopically from the greater curvature of the lower, the upper corpus and the lesser curvature of the lower corpus of the stomach, according to the triple-site gastric biopsy method, in all cases. Biopsy specimens were fixed overnight in buffered formalin, embedded in paraffin, cut to 3- $\mu$ m thickness, and stained with hematoxylin-eosin staining, improved toluidine-blue staining, Giemsa staining and *H. pylori*-specific antibody immune staining (Dako, Denmark). Identification of *H. pylori* was performed using the improved toluidine-blue staining, Giemsa staining and *H. pylori*-specific antibody immune staining. In accordance with the Updated Sydney System, the degree of gastric mucosal inflammation (mononuclear cell infiltration), polymorphonuclear cell infiltration, glandular atrophy, and intestinal metaplasia were classified into four grades as follows: 0 = none, 1 = mild, 2 = moderate and 3 = severe. Histologically, *H. pylori* infection was considered negative if *H. pylori* were absent from all biopsy sites stained with improved toluidine-blue staining, Giemsa staining and *H. pylori*-specific antibody immune staining. *H. pylori* infection was considered positive if at least one of the histology tests was positive<sup>[19,20]</sup>.

### Statistical analysis

The prevalence of *H. pylori* infection, rates of gastric mucosal inflammation, polymorphonuclear cell infiltration, glandular atrophy and intestinal metaplasia were compared using the  $\chi^2$  test for 4-fold table. The difference in grades of mononuclear cell infiltration, polymorphonuclear cell infiltration, glandular atrophy, and intestinal metaplasia between diseases was compared by Mann-Whitney *U*-test. *P* values  $< 0.05$  were considered statistically significant.

## RESULTS

### Prevalence of *H. pylori*

The positivity rates for *H. pylori* infection in studied patients are shown in Table 1. The overall prevalence of *H. pylori*

infection in superficial gastritis was 28.7%, erosive gastritis 57.7%, gastric erosion 63.3%, gastric ulcer 80.8%, and early gastric cancer 52.4%. The prevalence of *H. pylori* infection in early gastric cancer was significantly higher than that of superficial gastritis, lower than that of gastric erosion, gastric ulcer (all  $P < 0.05$ ); however, there was no significant difference in the prevalence of *H. pylori* infection between early gastric cancer and erosive gastritis. The prevalence was also higher in gastric ulcer and gastric erosion than in superficial gastritis and erosive gastritis (all  $P < 0.05$ ). No significant difference was found in the *H. pylori* infection rates among the different biopsy sites in superficial gastritis, but in the other diseases the rates were higher in corpus biopsy.

**Table 1** *H. pylori* infection identified in different biopsy sites in associated diseases

Endoscopic diagnosis	Cases	<i>H. pylori</i> infective rate (%)			
		Antrum	Corpus	Angle	Overall
Superficial gastritis	286	25.9	26.2	25.2	28.7
Erosive gastritis	286	46.9	53.5	49.0	57.7
Gastric erosion	286	52.4	61.5	52.4	63.3
Gastric ulcer	286	65.0	78.0	61.2	80.8
Early gastric cancer	286	35.0	50.7	34.6	52.4

#### Grades of mononuclear cell infiltration, polymorphonuclear cell infiltration, mucosal glandular atrophy and intestinal metaplasia

The grades of mononuclear cell infiltration and polymorphonuclear cell infiltration, mucosal glandular atrophy and intestinal metaplasia in patients are shown in Table 2. The grades of mononuclear cell infiltration and polymorphonuclear cell infiltration in early gastric cancer patients were significantly higher than that in superficial

gastritis patients and lower than that in gastric erosion and gastric ulcer patients (all  $P < 0.01$ ); however, there was no significant difference compared with erosive gastritis. The grades of mononuclear cell infiltration and polymorphonuclear cell infiltration in gastric ulcer, gastric erosion, and erosive gastritis patients were also significantly higher than that in superficial gastritis patients (all  $P < 0.01$ ). On the other hand, they were significantly lower than that in gastric ulcer patients; (both  $P < 0.01$ ).

The grades of mucosal glandular atrophy and intestinal metaplasia were significantly higher in early gastric cancer, lower in gastric ulcer, erosive gastritis, and gastric erosion and the lowest in superficial gastritis (all  $P < 0.01$ ). Furthermore, 53.3% and 51.4% showed glandular atrophy and intestinal metaplasia in angular biopsy specimens, respectively; however, only 40.3% and 39.9% were identified in antral biopsy, 14.1% and 13.6% in corpus biopsy. Therefore, the angulus was more reliable for the diagnosis of glandular atrophy and intestinal metaplasia compared to antrum and corpus (both  $P < 0.01$ ).

#### Rates of inflammation, activity, mucosal glandular atrophy and intestinal metaplasia in patients with and without *H. pylori* infection

Rates of mononuclear cell infiltration, polymorphonuclear cell infiltration, glandular atrophy and intestinal metaplasia in patients with and without *H. pylori* are shown in Table 3.

The positivity rate of chronic inflammation of superficial gastritis, erosive gastritis, gastric erosion, and gastric ulcer patients with *H. pylori*-positivity was significantly higher than those with *H. pylori*-negativity (all  $P < 0.01$ ); however, there was no significant difference in patients with early gastric cancer with or without *H. pylori* infection.

Mononuclear cell infiltration rate was significantly higher in superficial gastritis, erosive gastritis and gastric erosion, gastric ulcer or early gastric cancer patients with *H. pylori*-positivity than *H. pylori*-negative patients (all  $P < 0.01$ ).

**Table 2** The grade of mononuclear cell infiltration, polymorphonuclear cell infiltration, glandular atrophy and intestinal metaplasia in studied patients (%)

Diagnosis	Mononuclear cell infiltration				Polymorphonuclear cell infiltration				Glandular atrophy				Intestinal metaplasia			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Superficial gastritis	40.9	34.3	10.1	14.7	72.7	3.5	4.5	19.2	78.7	17.6	2.2	1.5	85.3	4.9	4.2	5.6
Erosive gastritis	11.2	32.9	19.2	36.7	40.2	6.6	12.9	40.2	38.8	30.6	17.3	13.3	45.8	16.4	14.7	23.1
Gastric erosion	3.8	29.0	21.0	46.2	33.9	3.8	10.1	52.1	28.3	23.2	24.5	24.1	21.7	18.5	23.4	36.4
Gastric ulcer	4.2	14.3	14.0	67.5	17.8	3.1	7.7	71.3	29.2	17.7	21.8	31.3	21.7	2.4	2.4	73.4
Early gastric cancer	0.7	42.3	29.4	27.6	40.2	10.1	1.9	37.8	20.3	20.7	18.1	40.9	14.3	12.2	18.9	54.5

**Table 3** Rates of inflammation, activity, glandular atrophy and intestinal metaplasia in patients with and without *H. pylori* infection (%)

Diagnosis	Mononuclear cell infiltration <i>H. pylori</i>		Polymorphonuclear cell infiltration <i>H. pylori</i>		Glandular atrophy <i>H. pylori</i>		Intestinal metaplasia <i>H. pylori</i>	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Superficial gastritis	93.9 <sup>b</sup>	45.1	90.2 <sup>b</sup>	2.0	50.7 <sup>b</sup>	9.9	34.1 <sup>b</sup>	6.9
Erosive gastritis	98.8 <sup>b</sup>	75.2	97.0 <sup>b</sup>	9.1	76.1 <sup>b</sup>	42.5	63.0 <sup>b</sup>	42.1
Gastric erosion	100.0 <sup>b</sup>	89.5	99.4 <sup>b</sup>	8.6	84.8 <sup>b</sup>	51.1	87.8 <sup>b</sup>	61.9
Gastric ulcer	99.1 <sup>b</sup>	81.8	99.1 <sup>b</sup>	10.9	80.6 <sup>b</sup>	29.8	90.9 <sup>b</sup>	25.5
Early gastric cancer	100.0	98.5	100.0 <sup>b</sup>	15.4	85.5	84.0	85.3	86.0

<sup>b</sup> $P < 0.01$  vs *H. pylori*-negative group.

The positivity rate of mucosa glandular atrophy and intestinal metaplasia of superficial gastritis, erosive gastritis, gastric erosion, and gastric ulcer patients with *H pylori*-positivity was significantly higher than those with *H pylori*-negativity (all  $P<0.01$ ); however, there was no significant difference in patients with early gastric cancer with or without *H pylori* infection ( $P<0.01$ ).

## DISCUSSION

Since the discovery of *H pylori*, many studies have implicated infection with this bacterium in the pathogenesis of gastric cancer. But prevalence of *H pylori* varies widely between and within populations. Risk factors for *H pylori* infection have been extensively studied. The prevalence of *H pylori* infection among males appears to be higher than that among females. It is also associated with age, lifestyle, ethnic, and economic factors. In order to decrease the effect of these risk factors on the study results as much as possible, we performed a case-controlled study. Five age- and gender-matched control subjects for each early gastric cancer patient was randomly selected from the same series of subjects with superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer<sup>[21-25,31]</sup>.

Our age- and gender-matched results suggest that from superficial gastritis, erosive gastritis, gastric erosion to gastric ulcer, as *H pylori* infection rates increased, the pre-malignant lesions of glandular atrophy and intestinal metaplasia also increased gradually. But in early gastric cancer patients the *H pylori* infection rate was not very high; the reason might be in early gastric cancer both mucosa atrophy and intestinal metaplasia were very serious, which was unfavorable for *H pylori* growth; therefore *H pylori* decreased gradually. As to the distribution of *H pylori* and inflammation in the stomach, Genta reported that *H pylori* were distributed evenly throughout the stomach<sup>[26]</sup>. In the present study, we found that the prevalence of *H pylori* infection was not significantly different among the different biopsy sites in superficial gastritis, but in the other diseases the detected rates were higher in corpus biopsy. This finding was a little different from Genta's observation. In comparison between *H pylori*-positive and *H pylori*-negative patients, mononuclear cell infiltration was more severe in *H pylori*-positive patients with superficial gastritis, erosive gastritis, gastric erosion, gastric ulcer or early gastric cancer than *H pylori*-negative patients, and it was related between the grade of mononuclear cell infiltration, polymorphonuclear cell infiltration and the grade of *H pylori* infection. More intense bacterial infection and more severe polymorphonuclear cell infiltration may contribute more to DNA damage and promote carcinogenesis in patients with gastric cancer. Furthermore, chronic *H pylori* infection is also associated with increased gastric cell turnover, probably of importance in malignant transformation<sup>[27,28]</sup>.

The finding of a high incidence of chronic gastritis in patients with gastric cancer and gastric ulcer supports previous studies. All of the gastric cancer and gastric ulcer were found in the setting of atrophic gastritis. Similarly, Sipponen has reported a study of 54 patients with gastric cancer, among whom 38 (70%) had *H pylori* infection. Only five patients (16%) had normal mucosa, but had no evidence

of *H pylori* infection by serology or histology<sup>[29]</sup>. Craanen showed that atrophic mucosal changes were present in 90.3% of patients with intestinal-type early gastric cancer. *H pylori* infection was found in 63.6% of patients with intestinal-type early gastric cancer and in 54.5% of patients with diffuse-type early gastric cancer<sup>[30]</sup>. In Western countries, the prevalence of *H pylori* infection is 70-80% in gastric cancer patients, but 10-20% of gastric cancer patients develop in an apparently *H pylori*-negative stomach. It is well known that the prevalence of *H pylori* infection and gastric cancer is higher in Japan than in Western countries. In our study, of 286 patients with early gastric cancer 150 patients had positive *H pylori* infection, 136 had negative *H pylori* infection, but most of them had moderate to severe atrophic gastritis, the atrophic rate was 85.5% and 84.0% respectively. The prevalence of *H pylori* infection and early gastric cancer in this study was 52.4%, also similar to the findings reported by Craanen<sup>[30]</sup>.

Glandular atrophy and intestinal metaplasia were found in more than half of *H pylori*-positive patients but were remarkably low in the *H pylori*-negative patients. These results confirm the tight link between *H pylori* infection, atrophic gastritis and intestinal metaplasia in the stomachs of Japanese. In early gastric cancer patients, both glandular atrophy and intestinal metaplasia were found to be higher; however, there was no significant difference between *H pylori*-positive and negative patients. Occasionally, it was found in glandular atrophy and intestinal metaplasia tissues *H pylori* negative, while in the tissues without glandular atrophy or intestinal metaplasia it might be found *H pylori* positive. These findings suggest that most patients with intestinal metaplasia and glandular atrophy have been infected with *H pylori* at some stage. *H pylori* infection may provide the proper environment for atrophic gastritis and intestinal metaplasia to occur. At the final stage of the disease, gastric atrophy with intestinal metaplasia is not a hospitable environment for *H pylori* and is associated with a dramatic reduction or even disappearance of the organism<sup>[31-35]</sup>. Furthermore, we found glandular atrophy and intestinal metaplasia were more frequent and severe in angulus and antrum, where gastric cancer occurs more frequently than in the corpus. In comparison with *H pylori* infection, the presence of intestinal metaplasia in the lesser curvature of the angulus was an increased risk for the development of gastric cancer.

In intestinal metaplasia in all patients with *H pylori* positivity, it was found that from superficial gastritis, erosive gastritis, gastric erosion to gastric ulcer, and early gastric cancer, both glandular atrophy and intestinal metaplasia significantly increased. This result is in accordance with the epidemiologic and pathologic studies of Correa, which revealed the temporal association of chronic superficial gastritis, atrophic gastritis, intestinal metaplasia, epithelial dysplasia, and finally gastric cancer evolution<sup>[36]</sup>.

There is increasing evidence that *H pylori* strains are highly diverse genomically. Several *H pylori* virulence-associated genes have been found in Western populations to be associated with an increased risk of gastric cancer and pre-cancerous lesions<sup>[37]</sup>. Studies from Japan have confirmed that IL-1 $\beta$  polymorphisms do contribute to the gastric acid secretory response to *H pylori* infection and

subsequently to clinical sequelae<sup>[38,39]</sup>. These outcomes range from, at one end of the spectrum, hypochlorhydria and atrophic gastritis with an increased risk of cancer, and on the other hand, high acid secretion and duodenal ulcer disease. In an important extension to this work, Figueriedo genotyped a large population with chronic gastritis and gastric cancer for polymorphisms of the genes for both IL-1 $\beta$  and its receptor, and for the *vacA* and *cagA* genotypes of the infecting *H. pylori* strain<sup>[40]</sup>. Combinatorial analysis of both bacterial and host genotypes demonstrated an enormous difference in the risk of gastric cancer, depending on particular mixtures of *H. pylori* virulence and host genetic factors, thus demonstrating the importance of considering both *H. pylori* and host genetics in gastric cancer risk assessment. Infection with the *vacA* s1a/m1 strain has also been shown to be associated with greater mucosa neutrophil and lymphocyte infiltration. However, previous studies have shown that most *H. pylori* strains in Japan are *cagA* positive and of the *vacA* s1a/m1 genotype<sup>[41-43]</sup>. Therefore, our results suggest that variabilities in host response to *H. pylori* infection play an important role in the occurrence of intense gastritis, glandular atrophy and intestinal metaplasia in patients with superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer or early gastric cancer. However, because we used forceps biopsies, there was also the possibility of sampling error in the case of focal atrophy or intestinal metaplasia.

On the other hand, in the present study, infection of *H. pylori*, glandular atrophy and intestinal metaplasia of gastric erosion were more serious than that of erosive gastritis by pathological diagnosis. These were not in accordance with our endoscopic findings in which erosive gastritis was more serious than gastric erosion. The possible reasons need to be evaluated further in the future.

In conclusion, the progression of gastric precancerous lesions, glandular atrophy and intestinal metaplasia in superficial gastritis, gastric erosion, erosive gastritis and gastric ulcer is strongly related to *H. pylori* infection. Prospective studies are needed to evaluate whether eradication of *H. pylori* infection will really diminish the risk of gastric cancer.

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• BASIC RESEARCH •

## Polymorphisms of uridine-diphosphoglucuronosyltransferase 1A7 gene in Taiwan Chinese

May-Jen Huang, Sien-Sing Yang, Min-Shung Lin, Ching-Shan Huang

May-Jen Huang, Department of Laboratory Medicine, Cathay General Hospital, Taipei, Taiwan, China

Sien-Sing Yang, Department of Liver Unit, Cathay General Hospital, Taipei, Taiwan, China

Min-Shung Lin, Department of Family Medicine, Cathay General Hospital, Taipei, Taiwan, China

Ching-Shan Huang, School of Medicine and Health Sciences, Foo-Yin University, Kaohsiung, Taiwan; Department of Laboratory Medicine, Cathay General Hospital, Taipei, Taiwan, China

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Correspondence to: Professor Ching-Shan Huang, Department of Laboratory Medicine, Cathay General Hospital, 280, Jen Ai-Road, Section 4, Taipei 106, Taiwan, China. chsh.huang@msa.hinet.net  
Telephone: +886-22-6360450 Fax: +886-22-6360462

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### Abstract

**AIM:** Single nucleotide polymorphisms (SNPs) of uridine-diphosphoglucuronosyltransferase 1A7 (UGT1A7) gene are associated with the development of orolaryngeal cancer, hepatocellular carcinoma, and colorectal cancer. We performed this research to establish the techniques for determining UGT1A7 gene and basic data of this gene for Taiwan Chinese.

**METHODS:** We collected blood samples from 112 healthy adults and 505 subjects carrying different genotypes of UGT1A1, and determined the promoter area and the entire sequence of UGT1A7 exon 1 by polymerase chain reaction. We designed appropriate primers and restriction enzymes to detect variant UGT1A7 genotypes found in the study subjects.

**RESULTS:** Six SNPs at nucleotides 33, 387, 391, 392, 622, and 756 within the coding region of UGT1A7 exon 1 were found. The incidence of UGT1A7 \*1/\*2 (N129R131W208/K129K131W208) was predominant (35.7%) while that of UGT1A7 \*3/\*3 (K129K131R208/K129K131R208) was the least (2.7%). The allele frequency of UGT1A7\*3, which exists in a considerable proportion of Caucasians (0.361) and Japanese (0.255), was identified only to be 0.152 in our study subjects. A novel variation at nucleotide -57 in the upstream was found, which was associated with SNPs at nucleotides 33, 387, 391, 392, and 622 in one of the variant haplotypes. The nucleotide changes at positions 387, 391, 392 and 756 were in linkage in another variant haplotype. The allele frequency of UGT1A7\*3 was 0.018, 0.158, 0.242, 0.433, and 0.920 in subjects carrying wild, A(TA)<sub>6</sub>TAA/A(TA)<sub>7</sub>TAA, A(TA)<sub>7</sub>TAA/A(TA)<sub>8</sub>TAA, 211G/211A, and 211A/211A variants of UGT1A1 gene, respectively.

By using natural or mutagenesis primers, we successfully detected the variations at nucleotides -57, 33, 387, and 622 with the restriction enzymes *Hpy*CH4 IV, *Taq* I, *Afl* II, and *Rsa* I, respectively.

**CONCLUSION:** The results indicate that the allele frequencies of UGT1A7 gene in Taiwan Chinese are different from those in Caucasians and Japanese. Carriage of the nucleotide 211- variant UGT1A gene is highly associated with UGT1A7\*3. The restriction-enzyme-digestion method for the determination of nucleotides -57 (or 33, or 622) and 387 can rapidly identify genotypes of UGT1A7 in an individual.

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**Key words:** UGT1A7 gene; Single nucleotide polymorphisms; Genotype; Taiwan Chinese

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### INTRODUCTION

Polymorphisms in genes encoding drug metabolism enzymes are known to play an important role in clinical response to drug therapy and disease susceptibility<sup>[1,2]</sup>. UDP-glucuronosyltransferase enzymes (UGTs) catalyze the reaction of glucuronidation, which is one of the most important conjugative pathways for the detoxification and elimination of endogenous and exogenous compounds<sup>[2]</sup>. Polymorphisms may decrease UGT activities and cause illness in affected individuals. For instance, variant UGT1A1 genes may result in serious and benign inheritable unconjugated hyperbilirubinemia, known as Crigler-Najjar syndrome and Gilbert's syndrome, respectively<sup>[2]</sup>. Two UGT gene subfamilies, UGT1 and UGT2, have been identified in humans up to now, based on evolutionary divergence<sup>[3]</sup>. Unlike the UGT2B family, which is encoded by several genes on chromosome 4q13-21, the 13 members of UGT1 family are all derived from a single gene on chromosome 2q37 and generated by alternatively splicing of exon 1 to the four common exons (exons 2-5)<sup>[4]</sup>. The genes are designated UGT1A1 through UGT1A13 with nine functional proteins (UGT1A1, UGT1A3-UGT1A10) and four pseudogenes (UGT1A2, UGT1A11-UGT1A13), which have either nucleotide deletions or flawed TATA boxes<sup>[4]</sup>.

Genetic polymorphisms of UGT1 have been described to date for only four enzymes in humans: UGT1A1<sup>[2]</sup>, 1A6<sup>[5]</sup>, 1A7<sup>[6]</sup>, and 1A8<sup>[7]</sup>.

The results of our previous study showed that the allele frequency of A (TA)<sub>7</sub>TAA in the UGT1A1 gene in Taiwan Chinese was 0.143<sup>[8]</sup>, comparable with that in Singaporean Chinese (0.162)<sup>[9]</sup>, Malaysians (0.188)<sup>[9]</sup>, and Japanese (0.1-0.168)<sup>[10-12]</sup>, but lower than that in Caucasians (0.357-0.415)<sup>[13-16]</sup> and Indians (0.351)<sup>[9]</sup>. In contrast, variation rate within the coding region of UGT1A1 gene was much higher in Taiwan Chinese than that in Caucasians (0.293<sup>[8]</sup> vs 0.001<sup>[13]</sup>). Moreover, the key UGT1A1-gene defect for the development of neonatal hyperbilirubinemia in Japanese and Taiwan Chinese is homozygous variation at nucleotide 211<sup>[11,17,18]</sup>, opposed to the homozygous variation in the promoter area, which has been reported in Caucasians<sup>[19,20]</sup>. Recently, we found a novel compound heterozygous variation of the UGT1A1 gene that caused Crigler-Najjar syndrome type 2 in a Taiwan Chinese<sup>[21]</sup>. A previous report indicated that there was a large difference in the number of UGT1A6 polymorphisms between Asians and Caucasians<sup>[5]</sup>. Those results reveal that the ethnic differences of UGT1 genes commonly occur and are worth studying.

Five and six single nucleotide polymorphisms (SNPs) have been discovered in the first exon of UGT1A7 gene in Caucasians and Japanese, respectively<sup>[6,22]</sup>. We hypothesized that the variations of UGT1A7 gene in Taiwan Chinese might be different from those in other ethnics and performed this research.

## MATERIALS AND METHODS

### Study subjects

Blood samples were collected from 112 healthy adult Taiwan Chinese and 505 subjects carrying different genotypes of UGT1A1 who gave their written consent to participate in this study. Among the 505 subjects, the number of different UGT1A1-genotypes carriage was 246 for wild type, 38 for A(TA)<sub>6</sub>TAA/A (TA)<sub>7</sub>TAA, 31 for A (TA)<sub>7</sub>TAA/A (TA)<sub>7</sub>TAA, 90 for 211G/211A, and 100 for 211G/211A.

### Determination of SNPs

Total genomic DNA was isolated from the blood cells using the blood DNA isolation kit (Maxim Biotech Inc., San Francisco, USA). The promoter area (beginning at -114

nucleotide in the upstream) and the entire sequence of UGT1A7 exon 1 were analyzed by polymerase chain reaction (PCR). The primers used for PCR are shown in Table 1. For sequencing promoter and second part of exon 1, the forward primers were used, while for the first part of exon 1 the reverse primer was utilized. The amplification reaction mixture (100 µL) contained 1 µg of DNA in 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 0.1% Triton X-100, 200 µmol/L of each dNTP, 100 ng of each primer, and 2 U of Dynazyme DNA polymerase (Finnzymes OY, Espoo, Finland). The reaction was performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) as follows: three cycles of denaturation at 94 °C for 80 s, annealing at 55 °C for 60 s, and primer extension at 72 °C for 110 s; seven cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 110 s; 30 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, and extension at 72 °C for 90 s; and a final extension step at 72 °C for 10 min. The PCR products were sequenced with an automated fluorescence sequencer (ABI Prism377, PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

### Restriction-enzyme-digestion method

We designed the natural or mutagenesis primers to detect the variant UGT1A7 genotypes found in the study subjects. PCR amplification was performed in a thermal cycler for 35 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 55 °C, primer extension for 60 s at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were digested with appropriate restriction enzymes, and analyzed on 3% agarose gel (NuSieve 3: 1, FMC Bioproduct, Rockland, ME, USA) containing ethidium bromide.

### Statistical analysis

The allele frequencies of UGT1A7 genotypes in our study subjects were compared with those found in Caucasians<sup>[6]</sup> and Japanese<sup>[22]</sup> by  $\chi^2$  test. For the analysis of association between UGT1A1 and UGT1A7 genes, the relative risk and its 95% confidence interval for carriage of UGT1A7\*3 in the subjects bearing variant UGT1A1 gene were calculated by comparing the allele frequency of UGT1A7\*3 with the subjects carrying wild UGT1A1-gene. A *P* value <0.05 was defined as statistically significant.

**Table 1** Primers used for PCR and sequencing of UGT1A7 gene

Region		Primer		Target size (bp)
		Name	Sequence	
Promoter	PCR:	U7F1	5'TGAATGAATAAGTACACGCC3'	439
		U7R1	5'ATAGAGAAAATGCACTTCGC3'	
Exon 1	Sequencing:	U7F1	5'TGAATGAATAAGTACACGCC3'	779
	PCR:	U7F1	5'TGAATGAATAAGTACACGCC3'	
		U7R2	5'TAGGGGCAAAATAAATGTTC3'	
	Sequencing:	U7R2	5'TAGGGGCAAAATAAATGTTC3'	
	PCR:	U7F2	5'ATACACTCTGGAGGATCAGG3'	774
		U7R3	5'GCTACCCAACAATTAAGTGA3'	
	Sequencing:	U7F3	5'TGTCCCCAGACTTCCTTAG3'	

bp: base pair.

## RESULTS

Six SNPs at nucleotides 33 (C to A), 387 (T to G), 391 (C to A), 392 (G to A), 622 (T to C), and 756 (G to A) within the coding region of UGT1A7 exon 1 were found in the study subjects. Among them, the variations at nucleotides 33 and 756 were wobbles. The functional polymorphisms at codons 129 (nucleotide 387), 131 (nucleotide 391 or 392) and 208 (nucleotide 622) are shown in Figure 1. Incidences of UGT1A7\*1/\*1 (N129R131W208/N129R131W208), \*1/\*2 (N129R131W208/K129K131W208), \*1/\*3 (N129R131W208/K129K131R208), \*2/\*2 (K129K131W208/K129K131W208), \*2/\*3 (K129K131W208/K129K131R208) and \*3/\*3 (K129K131R208/K129K131R208) are presented in Table 2. The incidence of UGT1A7 \*1/\*2 was predominant (35.7%), while that of UGT1A7 \*3/\*3 was the least (2.7%). As shown in Table 3, the allele frequency of UGT1A7\*1 (wild type) in Taiwan Chinese was higher and that of UGT1A7\*3 was lower when compared to that found in Caucasians. As compared with Japanese, the allele frequency of UGT1A7\*2 was higher and that of UGT1A7\*3 was lower in Taiwan Chinese.

**Table 2** Frequencies of UGT1A7 genotypes in 112 Taiwan Chinese

UGT1A7	Number (%)
*1/*1	36 (32.1)
*1/*2	40 (35.7)
*1/*3	17 (15.2)
*2/*2	5 (4.5)
*2/*3	11 (9.8)
*3/*3	3 (2.7)

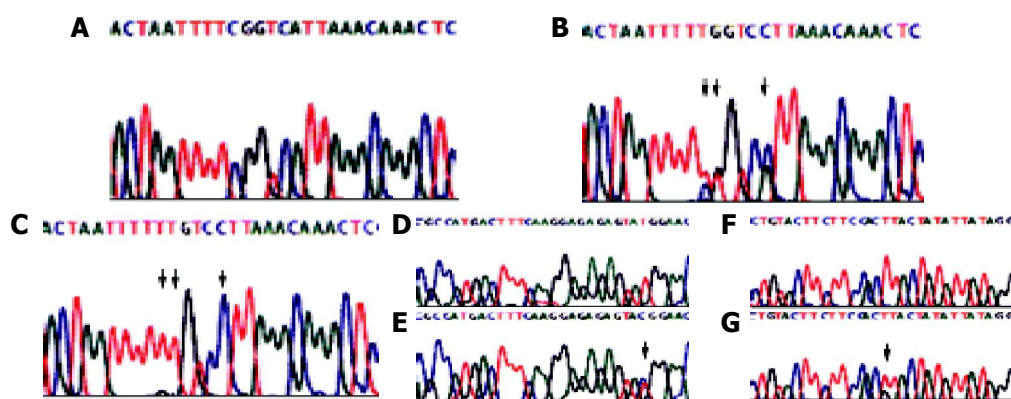
In addition to the six SNPs, a heterozygous or homozygous T to G conversion at nucleotide -57 in the upstream (Figure 1) was observed, which was associated with SNPs at nucleotides 33, 387, 391, 392, and 622 in one of the variant haplotypes. The nucleotide changes at positions 387, 391, 392 and 756 were in linkage in another variant haplotype. The functional variations in the 28 subjects with heterozygous T to G conversion at nucleotide -57 were UGT1A7\*1/\*3 in 17 and UGT1A7 \*2/\*3 in 11 subjects, respectively, while that in the three subjects with homozygous T to G conversion was UGT1A7\*3/\*3.

The distribution of the six UGT1A7 genotypes (\*1/\*1, \*1/\*2, \*1/\*3, \*2/\*2, \*2/\*3, and \*3/\*3) and the UGT1A7\*3 allele frequency in the subjects bearing the wild, A (TA)<sub>6</sub>TAA/A (TA)<sub>7</sub>TAA, A (TA)<sub>7</sub>TAA/A (TA)<sub>7</sub>TAA, 211G/211A, and 211A/211A variants of UGT1A1 gene are presented in Table 4. All the subjects carrying the wild UGT1A1 gene did not bear UGT1A7\*2/\*3 or UGT1A7\*3/\*3. All the individuals bearing the variant UGT1A1 gene were at a relative higher risk for carriage of UGT1A7\*3 in comparison with those with the wild type. The UGT1A7\*3 allele frequency in the subjects with A(TA)<sub>7</sub>AA/A (TA)<sub>7</sub>TAA was not significantly different from that in analogs with A (TA)<sub>6</sub>TAA/A (TA)<sub>7</sub>TAA. By contrast, the UGT1A7\*3 allele frequency in the 211A/211A subjects was approximately two-fold as many as the 211G/211A analogs. Of the individuals carrying 211A/211A in UGT1A1 gene, 84% had UGT1A7\*3/\*3 and 16% UGT1A7\*1/\*3 genes, respectively.

As shown in Table 5, by using the natural or mutagenesis primers, we successfully detected the variations at nucleotides

**Table 3** Allele frequencies of UGT1A7 gene among different ethnic groups

UGT1A7 alleles	Taiwan Chinese (224 chromosomes)	Caucasians (288 chromosomes)	Japanese (206 chromosomes)	P value (by $\chi^2$ test)
UGT1A7*1	0.576	0.358	0.593	<0.001
				0.73
UGT1A7*2	0.272	0.264	0.153	0.93
				0.004
UGT1A7*3	0.152	0.361	0.255	<0.001
				0.008



**Figure 1** Representative electropherograms of (A) homozygous N<sup>129</sup> R<sup>131</sup> (nucleotides 387-392: TGACCG), (B) heterozygous K<sup>129</sup> K<sup>131</sup> (nucleotides 387-392: T/GGACC/AG/A), (C) homozygous K<sup>129</sup> K<sup>131</sup> (nucleotides 387-392: GGACAA), (D) homozygous W<sup>208</sup> (nucleotide 622: T), (E) heterozygous W<sup>208</sup>/R<sup>208</sup> (nucleotide 622: T/C), (F) - 57 T, and (G) - 57 T/G. Sequences were read reversely and nucleotides were translated into complements for (A), (B), and (C). The arrows indicate the sites of single nucleotide polymorphism.

**Table 4** Allele frequency of UGT1A7\*3 in subjects carrying different UGT1A1 genotypes

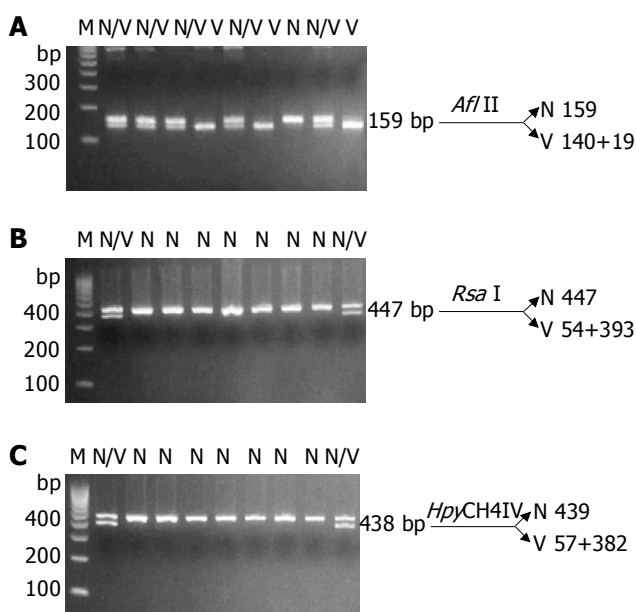
UGT1A1 gene	Number	UGT1A7 gene						Allele frequency of UGT1A7*3	Relative risk (95% CI)
		*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3		
Wild type	246	114	108	9	15	0	0	0.018	1.0
A(TA) <sub>6</sub> TAA/A(TA) <sub>7</sub> TAA	38	13	12	7	1	5	0	0.158	8.78 <sup>b</sup> (2.70-28.54)
A(TA) <sub>7</sub> TAA/A(TA) <sub>7</sub> TAA	31	13	5	9	1	0	3	0.242	13.44 <sup>b</sup> (4.41-40.99)
211G/211A	90	6	10	48	0	22	4	0.433	24.06 <sup>b</sup> (9.31-62.31)
211A/211A	100	0	0	16	0	0	84	0.920	51.11 <sup>b</sup> (20.24-128.80)

CI: confidence interval, <sup>b</sup>*P*<0.001 vs wild type.

**Table 5** Natural or mutagenesis primers, restriction enzymes and the results for UGT1A7 variations

Position (cDNA)	Primers	Sequence	Restriction enzyme	Result (bp)
-57 T→G	U7F1 U7R1	5'TGAATGAATAAGTACACGCC 5' ATAGAGAAAATGCACCTTCGC3'	HpyCH4IV	N <sup>2</sup> 439 V <sup>3</sup> 57+382
33 C→A	1 <sup>st</sup> PCR U7F1 U7R2	5'TGAATGAATAAGTACACGCC 5'TAGGGGCAAAAATGTTTC3'		
	2 <sup>nd</sup> PCR U7-33F1 U7-33R	5'GGGTGGACTGGCCTCCTG <sup>3'</sup> 5'ACTGCATGGTGAACCATGTCG	Taq I	N 115 V 19+96
387 T→G	1 <sup>st</sup> PCR U7F1 U7R2	5'TGAATGAATAAGTACACGCC 5'TAGGGGCAAAAATAAATGTTTC		
	2 <sup>nd</sup> PCR U7-387F U7-387R	5'AAATTGCAGGAGTTCTTA <sup>3'</sup> 5'TGGCAAAATATTCCTCCGC3'	Afl II	N 159 V 140+19
622 T→C	U7F3 U7R3	5'TGICCCAGACTTCTCTTAG 5'GCTACCCAACAATTAAGTGA	Rsa I	N 447 V 54+393

bp: base pair, <sup>1</sup>mutagenesis site, <sup>2</sup>N: digestion result of wild type, <sup>3</sup>V: digestion result of variant.



**Figure 2** Results of restriction fragments at nucleotides (A) 387, (B) 622, and (C) -57, digested by *Afl* II, *Rsa* I, and *Hpy*CH4 IV, respectively. The bands of 19, 54, and 57 bp were too small to be seen (bp = base pair, M: DNA size marker, N: wild type, V: variant).

-57, 33, 387, and 622 with restriction enzymes *Hpy*CH4 IV, *Taq* I, *Afl* II, and *Rsa* I, respectively. The example products are shown in Figure 2.

## DISCUSSION

In the studies of SNPs for UGT1 in general populations, most data were focused on UGT1A1 gene. Four common allelic variations in the TATA box of UGT1A1 promoter have been observed<sup>[13-16]</sup>. In addition, within the coding region of UGT1A1 gene, the variations at nucleotides 211, 686, 1 091, 1 099, and 1 456 have been found in Asians<sup>[8,10-12,17,18,23,24]</sup>. The other SNPs in UGT1 ever described are limited to UGT1A6, UGT1A7, and UGT1A8. For UGT1A6 gene, two close missense variations have been reported<sup>[5]</sup>. For UGT1A8 gene, four genotypes have been observed<sup>[7]</sup>. Recently, six SNPs in UGT1A7 have been identified<sup>[6,22]</sup>. There were missense variations in codons 129, 131, and 208, characterized by the substitution of N for K, R for K, and W for R, respectively. The allele containing all three missense variations, UGT1A7\*3, was found exhibiting a 5.8 fold lower relative activity compared to the wild type<sup>[6]</sup>.

UGT1A7 is a typical isoenzyme of the extrahepatic

UGT, with undetectable expression transcript in human liver<sup>[25]</sup>, while it is differentially expressed in human lung<sup>[6]</sup>, esophagus<sup>[26]</sup>, and stomach<sup>[27]</sup>. The main substrates conjugated by UGT1A7 are some phenolic compounds, carcinogens, and drugs<sup>[28,29]</sup>. In the studies of disease susceptibility for Caucasians, UGT1A7\*3 was found to be a risk gene for the development of orolaryngeal cancer<sup>[30]</sup>, hepatocellular carcinoma<sup>[31]</sup>, and colorectal cancer<sup>[27]</sup>. UGT1A7 might characterize a “trans-acting modifier gene” of cancer at the liver as well as at other sites of the body<sup>[27,31]</sup>. However, population study is the first step to set up the basic data for every ethnic. That is why we performed this research.

All the five and six SNPs of UGT1A7 observed in Caucasians and Japanese<sup>[6,22]</sup> were found in Taiwan Chinese. However, the allele frequencies of UGT1A7 were different among Taiwan Chinese, Caucasians, and Japanese. The low activity of UGT1A7\*3/\*3, which exists in a considerable proportion of population (15.3%) in Caucasians<sup>[6]</sup>, was identified only in 2.7% in our study subjects. The clinical significance of this difference warrants further investigation since hepatocellular carcinoma, one of UGT1A7\*3-related sicknesses, is still a life-threatening disease in Taiwan<sup>[32]</sup>. The UGT1A7\*4 allele (N129R131R208), with 0.017 in frequency in Caucasians<sup>[6]</sup>, was not found in our study subjects. The variation at nucleotide -57 in the upstream of UGT1A7 gene is a novel finding. The association between this variation and SNPs at nucleotides 33, 387, 391, 392, and 622, as well as the association of nucleotides 387, 391, 392, and 756 in another haplotype, might be a founder effect. Our results also indicate that the UGT1A1 gene variant at nucleotide 211 was highly associated with carriage of UGT1A7\*3. However, it remains possible that this phenomenon may be a founder effect. The results of our previous studies revealed that homozygous variation at nucleotide 211 of UGT1A1 gene was a risk factor for developing hyperbilirubinemia<sup>[17,18]</sup>. If UGT1A7\*3 is a risk SNP for the development of certain cancers, the interaction between UGT1A1 211A/211A and UGT1A7\*3, serum bilirubin level, and its effect on disease severity in patients are worthy of investigation. Interestingly, the 211 G to A variation has been found in Japanese, Koreans, Chinese<sup>[11]</sup>, and Taiwan Chinese<sup>[8,17,18]</sup>, but not in Caucasians<sup>[13]</sup>. This suggests that the clinical significance of the association between UGT1A1 211A/211A and UGT1A7\*3 is more important for the Orientals.

The rapid restriction-enzyme-digestion method for the detection of nucleotide -57 (or 33, or 622) can identify the genotypes of UGT1A7\*3/\*3 in an individual. The genotype is UGT1A7\*3/\*3 if the result is homozygous G at nucleotide -57 (or homozygous A at nucleotide 33, or homozygous C at nucleotide 622). The Afl II-digestion-method for nucleotide 387, following the detection of nucleotide -57 (or 33, or 622), can identify the genotypes in subjects carrying genes other than UGT1A7\*3/\*3. In the situation of wild type at nucleotide -57 (or 33, or 622), the genotypes are UGT1A7 \*1/\*1, \*1/\*2, and \*2/\*2 when the results of nucleotide 387 are wild, heterozygous variation, and homozygous variation, respectively. In the situation of heterozygous variation at nucleotide -57 (or 33, or 622), the genotypes are UGT1A7 \*1/\*3 and \*2/\*3 when the results of nucleotide 387 are heterozygous and homozygous

variations, respectively. Therefore, the restriction-enzyme-digestion method for the determination of nucleotides -57 (or 33, or 622) and 387 can rapidly identify the genotypes of UGT1A7 in an individual.

In conclusion, the features of UGT1A7 gene are found in Taiwan Chinese and a simple and rapid method to determine genotypes of UGT1A7 is established. The clinical studies for this important gene are on-going.

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• BASIC RESEARCH •

# Presence of CCK-A, B receptors and effect of gastrin and cholecystokinin on growth of pancreatobiliary cancer cell lines

Jin-Young Jang, Sun-Whe Kim, Ja-Lok Ku, Yong-Hyun Park, Jae-Gahb Park

Jin-Young Jang, Sun-Whe Kim, Ja-Lok Ku, Yong-Hyun Park, Jae-Gahb Park, Department of Surgery, Seoul National University College of Medicine, 28 Yongon-dong Chongno-gu, Seoul 110-744, Korea

Ja-Lok Ku, Jae-Gahb Park, Laboratory of Cell Biology, Korean Cell Line Bank, Cancer Research Institute, Seoul National University College of Medicine, 28 Yongon-dong Chongno-gu, Seoul 110-744, Korea

Jae-Gahb Park, National Cancer Center, Madu 1-dong, Goyang, Gyeonggi 411-764, Korea

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Correspondence to: Dr. Sun-Whe Kim, Department of Surgery, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul, 110-744, Korea. sunkim@plaza.snu.ac.kr

Telephone: +82-2-760-2315 Fax: +82-2-745-2282

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## INTRODUCTION

Carcinomas of the pancreas and biliary tract remain a challenge to clinicians. The anatomical complexity and late diagnosis of such carcinomas have led to a disappointingly low resectability rate of around 10-20% especially in pancreatic cancer<sup>[1]</sup>. Moreover, even if it is possible to resect the tumor with clear margin, early recurrence and metastasis are frequently observed. The overall 5-year survival rates are reported as below 15% in pancreatic cancer and 15-50% in other biliary and periampullary cancers<sup>[2,3]</sup>.

No adjuvant treatments have shown success in improving survival in periampullary cancers including pancreatic cancer until now. So with the progress of surgical treatment and early detection methods, new approaches including gene therapy and new chemotherapeutic drugs are definitely required to improve treatment results<sup>[4,5]</sup>. One such approach might be hormonal manipulation, which is currently accepted as the standard treatment modality for breast, prostate and thyroid cancers.

The gastrointestinal hormones gastrin and cholecystokinin (CCK) are structurally related. The former is known to be a stimulant of acid secretion by gastric mucosa, the latter a stimulant of enzyme secretion by the pancreas and contraction of the gallbladder. While exerting these classical actions, gastrin and CCK are also considered to act as growth stimulants for gastrointestinal malignancies such as gastric and colon cancer<sup>[6-9]</sup>.

However, studies on the trophic effect of these hormones on pancreatic cancer have provided conflicting results. Although some researchers have documented that gastrin and CCK stimulate the growth of pancreatic cancer cells<sup>[10,11]</sup> and are involved in the early carcinogenesis, others have refuted these effects<sup>[12,13]</sup>. Few investigations have been performed in the bile duct cancer due to the shortage of cancer cell lines and tumor models in the biliary tract.

In the present study, we investigated the effects of gastrin and CCK on the growth of pancreatic and biliary tract cancer cell lines established at the Cancer Research Institute of Seoul National University College of Medicine,

## Abstract

**AIM:** To investigate the effects of gastrin and cholecystokinin (CCK) and their specific antagonists on the growth of pancreatic and biliary tract cancer cell lines.

**METHODS:** Five pancreatic and 6 biliary cancer cell lines with 2 control cells were used in this study. Cell proliferation study was done using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test and direct cell count method. Reverse transcription-polymerase chain reaction (RT-PCR) and slot blot hybridization were performed to examine and quantify the expression of hormonal receptors in these cell lines.

**RESULTS:** SNU-308 showed a growth stimulating effect by gastrin-17, as did SNU-478 by both gastrin-17 and CCK-8. The trophic effect of these two hormones was completely blocked by specific antagonists (L-365, 260 for gastrin and L-364, 718 for CCK). Other cell lines did not respond to gastrin or CCK. In RT-PCR, the presence of CCK-A receptor and CCK-B/gastrin receptor mRNA was detected in all biliary and pancreatic cancer cell lines. In slot blot hybridization, compared to the cell lines which did not respond to hormones, those that responded to hormones showed high expression of receptor mRNA.

**CONCLUSION:** Gastrin and CCK exert a trophic action on some of the biliary tract cancers.

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**Key words:** Bile duct cancer; Gallbladder cancer; Pancreatic

and the expression of hormonal receptors.

## MATERIALS AND METHODS

### Cell lines

The human cancer cell lines in the biliary tract and pancreas used in this experiment were SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, SNU-213, SNU-324, SNU-410, PANC-1, Mia-PaCa. The SNU series were established at the Korean Cell Line Bank in the Cancer Research Institute of Seoul National University College of Medicine, Seoul, Korea<sup>[14,15]</sup>. Cell lines PANC-1, Mia-PaCa, LoVo and HT-1080 were obtained from the American Type Culture Collection (Rockville, MD).

SNU-245 was established from distal common bile duct cancer. SNU-478 and SNU-869 were obtained from ampulla of Vater cancer, SNU-1079 and SNU-1196 from upper bile duct cancer, and SNU-308 from gallbladder cancer. SNU-213, SNU-324 and SNU-410 were acquired from pancreatic head cancer. The characteristics of the cell lines are summarized in Table 1.

Cell lines were grown in RPMI 1640 (GIBCO-BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37 °C in an atmosphere of 50 mL/L CO<sub>2</sub>.

### Cell proliferation assay

**MTT assay** A colorimetric assay using tetrazolium salt, 3-[4,5 dimethyl thiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), was used to determine the hormonal stimulation effect on the cancer cell lines and the optimal stimulation concentration of the hormones. A single cell suspension was prepared and the cell number was calculated. MTT assay was performed as previously described<sup>[16]</sup>.

An equal number of cells were inoculated into each well in 100 µL of serum free culture medium to which gastrin-17 (Sigma, St. Louis, MO) or CCK-8 (Sigma, St. Louis, MO) was added in a range from 10<sup>-6</sup> mol/L to 10<sup>-12</sup> mol/L. Twenty µL of gastrin-17 or CCK-8 was added daily to each well in order to maintain the determined hormonal concentration. After five days of culture, 0.1 mg MTT was added to each well and incubated at 37 °C for 4 more hours. Plates were centrifuged at 450 g for 5 min at room temperature and the medium was then aspirated. Dimethyl sulfoxide (150 µL) was added to each well to dissolve the crystals. The plates

were read immediately at 540 nm on a scanning multi-well spectrophotometer. All experiments were performed 4 times.

We also performed another MTT assay to find the optimal inhibitory concentration of specific antagonists and to observe whether autocrine or paracrine effect of gastrin or CCK could affect the tumor cell growth by application of hormone receptor antagonists in the absence of exogenous ligand. L-364, 718 (antagonist for CCK-A receptor) and L-365, 260 (antagonist for CCK-B/gastrin receptor) were used in a range from 10<sup>-6</sup> mol/L to 10<sup>-12</sup> mol/L (10<sup>-9</sup> mol/L), which were kindly supplied by ML laboratory in UK. Test condition, except for antagonists, was same as above.

Test for optimal antagonist concentration was performed in cell lines, which showed hormone-dependent growth stimulation. Under the optimal growth stimulation concentration of hormones, various concentrations of specific antagonists (range: 10<sup>-6</sup>-10<sup>-12</sup> mol/L) were added (data not shown).

**Direct cell count** Cell lines showing a hormonal growth-stimulating effect in the MTT assay were selected for the direct cell count test to confirm the effect of hormones and whether the hormonal trophic effect on cancer cells could be blocked by specific antagonists with the concentration determined by MTT test.

Cells (30×10<sup>4</sup> for SNU-308; 5×10<sup>4</sup> for SNU-478) were plated onto 25 cm<sup>2</sup> flasks (Falcon, Franklin Lake, NJ) in serum free RPMI 1640 medium. After twenty-four hours, cells were treated with hormone (CCK-8 or gastrin-17), hormonal antagonists (L-364, 718 (10<sup>-9</sup> mol/L), antagonist for CCK-A receptors; L-365, 260 (10<sup>-9</sup> mol/L), antagonist for CCK-B/gastrin receptors), combinations of hormones with their antagonist, or medium alone (control). Medium and reagents were added daily. Every third day, we harvested the cells and counted the cell number using a hemocytometer. Each experiment was performed in triplicate.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted according to the manufacturer's instructions (TRIzol®, GIBCO-BRL, Rockville, MD). RNA concentration was measured spectrophotometrically at 260 nm and the integrity of mRNA was controlled by analyzing the ribosomal RNA content by electrophoresis

**Table 1** Characteristics of pancreatic cancer and periampullary cancer cell lines

Cell line	<i>In vivo</i>			<i>In vitro</i>			
	Origin	Degree of differentiation	Nodal status	Growth characteristics	Viability	Doubling time	Cell morphology
SNU-245	CBD	Well	0/9	Ad	85	54	P
SNU-308	GB	Moderately	-	Ad	88	48	P
SNU-478	AoV	Poorly	0/8	Ad	83	52	P/S
SNU-869	AoV	Well	5/10	Ad	91	48	P
SNU-1079	IHD	Moderately	-	Ad	89	72	Ple
SNU-1196	HDB	Moderately	0/1	Ad	94	48	Spindle to P
SNU-213	Pancreas	Moderately	1/8	Ad	89	48	P
SNU-324	Pancreas	Poorly	0/15	Ad+Fl	95	52	P/S
SNU-410	Pancreas	Poorly	-	Ad	92	72	P

CBD: common bile duct, GB: gallbladder, AoV: ampulla of Vater, Ad: adherent, Fl: floating, P: polygonal, S: spherical, Ple: pleomorphic.

on agarose gel.

After quantification of the extracted RNA, first strand complementary DNA (cDNA) of each cancer cell line was synthesized from 2 µg of total RNA using Molony murine leukemia virus reverse transcriptase (GIBCO-BRL, Rockville, MD). PCR reactions were performed in a total of 20 µg, composed of 2 µL cDNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.25 mmol/L dNTPs, 1 µmol/L of each oligonucleotide primer, and 1 U of Taq DNA polymerase (Takara, Shiga, Japan). We used the primers described by Mandair *et al.*<sup>[17]</sup> for CCK-A receptor, CCK-B/gastrin receptor, and β-actin synthesized in Bioneer Co. (Chungbuk, Korea). The forward primers were 5'-CCTAC GACACCGCCT-CCGC-3', 5'-ACCCCAACGACAGG AAAAGGT-3', and 5'-CACTGTGTTGGCGTACAGGT-3'; the reverse primers were 5'-TCCGTTCTTTCTT CTCTGCCTCCT-3', 5'-TTTGGGAAGGAAGGAG AGGGC-3', and 5'-TCATCACCATTGGCAA TGAG-3' for the CCK-A receptor, CCK-B/gastrin receptor, and β-actin respectively. The amplification reaction involved denaturation at 94 °C for 5 min followed by the following cycling: for CCK-A receptor, 40 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and extension for 1 min 30 s at 97 °C; for CCK-B/gastrin receptor, 40 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 58 °C and extension for 1 min 30 s at 97 °C; for β-actin, 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and extension for 1 min at 97 °C.

The reaction proceeded in a DNA thermal cycler (Hybaid, Middlesex, U.K.), and the products of amplification were submitted to electrophoresis on 1.5% agarose gel and visualized with ethidium bromide staining.

### Slot blot hybridization

Quantitative gene expression was determined by slot blot analysis. RNA samples were denatured at 68 °C for 15 min. Four different concentrations (10, 3, 1, and 0.3 µg) of RNA were loaded on per slot. Blots were prehybridized in buffer (5× SSPE, 30% formamide, 5× Denhardt's solution, 1% SDS and 100 µg/mL salmon sperm DNA) for 2 h at 42 °C. Overnight hybridization was performed at 42 °C with a

<sup>32</sup>P-radiolabeled probe. The complementary riboprobes were prepared by ligating PCR products for CCK-A receptor, CCK-B/gastrin receptor and β-actin gene cDNA into the pCR<sup>®</sup>II-TOPO<sup>®</sup> vector, using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The appropriate templates for each RNA probe were generated by linearization with a restriction endonuclease *Eco*RI. Radiolabeling of the probes was performed with [α-<sup>32</sup>P] deoxycytidine triphosphate using a Prime-It<sup>®</sup> II random primer labeling kit (Stratagene, La Jolla, CA, USA). After hybridization, the blots were washed twice in 2× SSPE plus 0.2% SDS at 42 °C for 20 min, then once with 0.1× SSPE at 42 °C for 15 min, and finally subjected to autoradiography with Fuji Super RX<sup>®</sup> film at -70 °C. Hybridization with a β-actin RNA probe was used to correct RNA loading. The autoradiograms were analyzed and quantitated by densitometry (BIPS<sup>®</sup>, Biomedlab, Seoul, Korea).

### Statistical analysis

Data were expressed as mean±SE. Comparison between groups was made using Mann-Whitney *U* test, *P*<0.05 was considered statistically significant.

## RESULTS

### MTT assay

The SNU-308 cell line originating from gallbladder cancer responded to the addition of gastrin-17, and in particular showed a maximum growth stimulating effect at the gastrin-17 concentration of 10<sup>-9</sup> mol/L. At this concentration 27% of the growth stimulating effect could be seen compared to the control (Figure 1A). SNU-478 from ampulla of Vater cancer exhibited similar stimulating effects to both gastrin-17 (21%) and CCK-8 (23%) (Figure 1B). However, no significant differences between control and hormone treated cultures were observed at any concentration in any of the other cell lines. MIA PaCa-2 and PANC-1 showed similar results to those of the other pancreatic cell lines (Tables 2, 3).

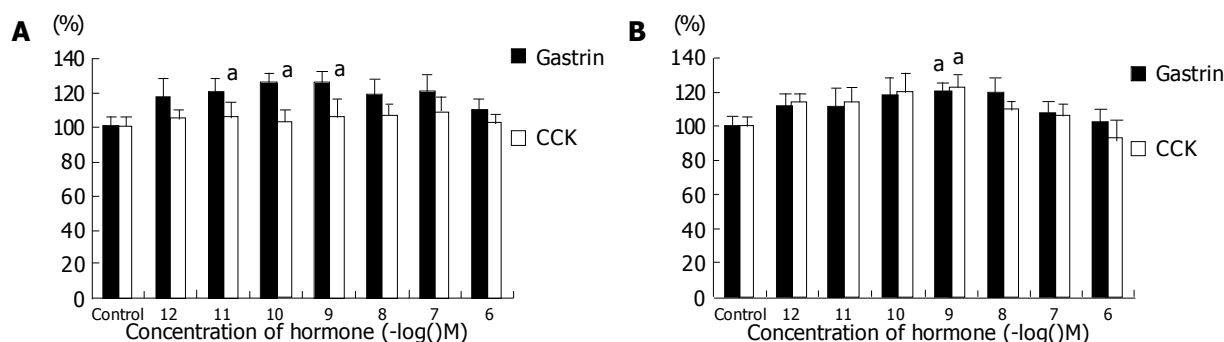
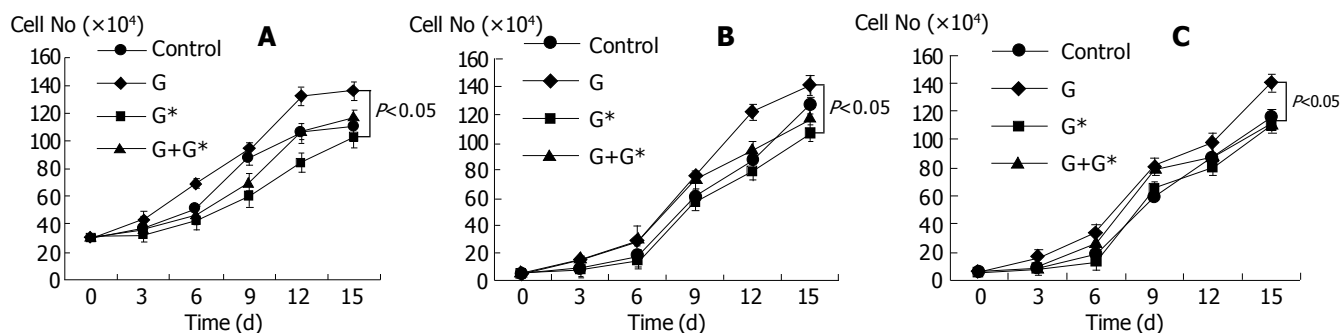
Suppression of cell growth by hormonal receptor antagonists (without exogenous hormone) was not definite

**Table 2** Effect of gastrin and L-365,260 on periampullary cancer cell lines

Cell line	Origin	Maximum response to hormones and hormone concentration					
		Gastrin			L-365, 260		
		Response (%)	Concentration (mol/L)	<i>P</i> -value	Response (%)	Concentration (mol/L)	<i>P</i> -value
SNU-245	CBD	108	10 <sup>-7</sup>	NS	98	10 <sup>-8</sup>	NS
SNU-308	GB	127	10 <sup>-9</sup>	0.038	96	10 <sup>-9</sup>	NS
SNU-478	AoV	121	10 <sup>-9</sup>	0.047	94	10 <sup>-9</sup>	NS
SNU-869	AoV	107	10 <sup>-6</sup>	NS	103	10 <sup>-7</sup>	NS
SNU-1079	IHD	107	10 <sup>-9</sup>	NS	104	10 <sup>-11</sup>	NS
SNU-1196	HDB	104	10 <sup>-8</sup>	NS	93	10 <sup>-7</sup>	NS
SNU-213	Pancreas	105	10 <sup>-10</sup>	NS	97	10 <sup>-8</sup>	NS
SNU-324	Pancreas	112	10 <sup>-11</sup>	NS	97	10 <sup>-9</sup>	NS
SNU-410	Pancreas	115	10 <sup>-12</sup>	NS	98	10 <sup>-8</sup>	NS
MIA PaCa-2	Pancreas	106	10 <sup>-10</sup>	NS	95	10 <sup>-9</sup>	NS
PANC-1	Pancreas	101	10 <sup>-7</sup>	NS	94	10 <sup>-8</sup>	NS

**Table 3** Effect of CCK and L-364,718 on periampullary cancer cell lines

Cell line	Origin	Maximum response to hormones and hormone concentration					
		CCK			L-364, 718		
		Response (%)	Concentration (mol/L)	P-value	Response (%)	Concentration (mol/L)	P-value
SNU-245	CBD	110	$10^{-12}$	NS	96	$10^{-10}$	NS
SNU-308	GB	109	$10^{-7}$	NS	94	$10^{-8}$	NS
SNU-478	AoV	123	$10^{-9}$	0.043	93	$10^{-9}$	NS
SNU-869	AoV	107	$10^{-10}$	NS	97	$10^{-10}$	NS
SNU-1079	IHD	113	$10^{-9}$	NS	101	$10^{-8}$	NS
SNU-1196	HDB	109	$10^{-8}$	NS	94	$10^{-8}$	NS
SNU-213	Pancreas	106	$10^{-7}$	NS	98	$10^{-6}$	NS
SNU-324	Pancreas	116	$10^{-12}$	NS	96	$10^{-10}$	NS
SNU-410	Pancreas	113	$10^{-9}$	NS	94	$10^{-8}$	NS
MIAPaCa-2	Pancreas	108	$10^{-10}$	NS	97	$10^{-9}$	NS
PANC-1	Pancreas	105	$10^{-8}$	NS	98	$10^{-8}$	NS

**Figure 1** Effects of gastrin and CCK on SNU-308 from gallbladder cancer (A), SNU-478 from ampulla of Vater cancer (B). <sup>a</sup>*P*<0.05 vs control.**Figure 2** Growth curves for SNU-308 (A), and SNU-478 (B, C) cells grown with either hormone or hormone combined with antagonist or antagonist, or medium only. G: gastrin-17, G\*: antagonist for CCK-B/gastrin receptor (L-365,260), C: CCK-8, C\*: antagonist for CCK-A receptor (L-364,718).

in all pancreatobiliary cancer cell lines (Tables 2, 3).

#### Effects of hormonal antagonists

The stimulatory effect of gastrin-17 exposure on the growth rate of SNU-308 cells was blocked by the specific antagonist L-365, 260. The growth stimulation effects of both the hormones on SNU-478 were blocked by their respective specific antagonists (Figure 2).

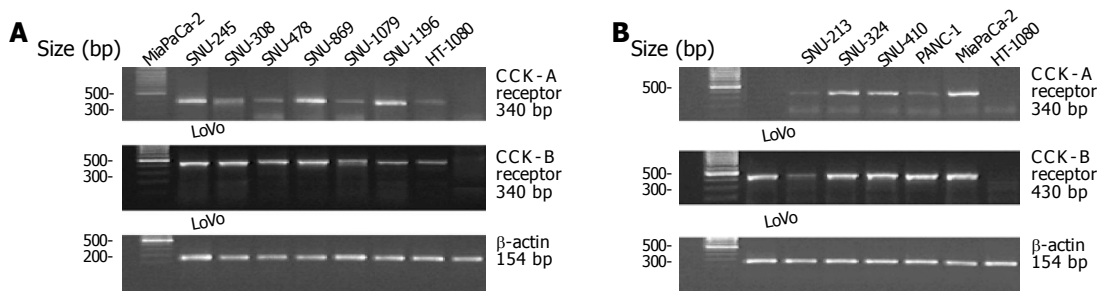
#### RT-PCR for CCK-A and CCK-B/gastrin receptors

Amplification of cDNA yielded an approximately 340 bp fragment from the CCK-A receptor and 430 bp fragment from the CCK-B/gastrin receptor (Figure 3). Sequence analysis confirmed that each kind of PCR products was identical to that of the expected sequence<sup>[18,19]</sup>. CCK-A

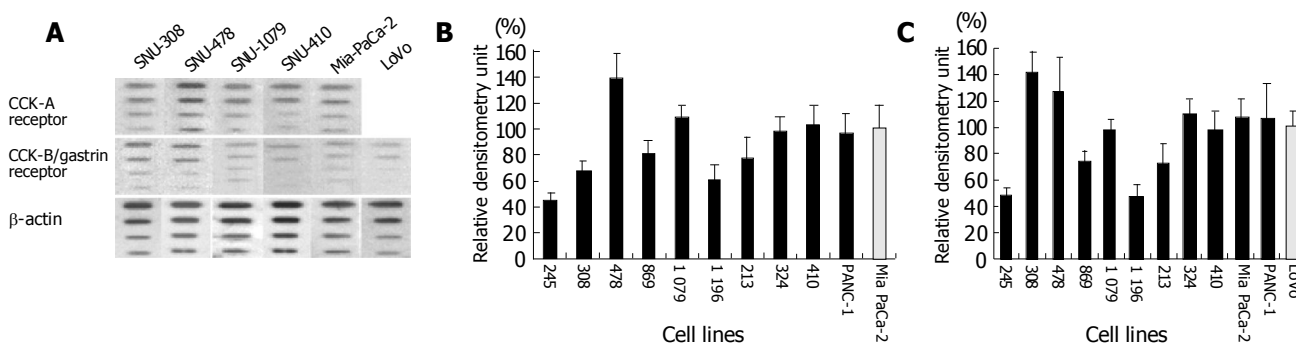
receptor and CCK-B/gastrin receptor mRNA were detected in all biliary and pancreatic cancer cell lines. We utilized the Mia PaCa-2 cell line as a positive control for the expression of CCK-A receptor<sup>[17]</sup> and the LoVo cell line for the CCK-B/gastrin receptor<sup>[20]</sup>.

#### Slot blot assay for CCK-A and CCK-B/gastrin receptors

CCK-A and CCK-B/gastrin receptor mRNA levels were measured by slot blot hybridization. The autoradiographic signals were compared with those of Mia PaCa-2 (for CCK-A receptor) and LoVo (for CCK-B/gastrin receptor). Figure 4 presents the slot blot assay results. SNU-308 and SNU-478 demonstrated a high expression of receptor mRNA compared with other cell lines that did not respond to



**Figure 3** Amplification of CCK-A and CCK-B receptor mRNA in human biliary tract cancer cell lines (A) and pancreatic cancer cell lines (B) by RT-PCR.



**Figure 4** Representative slot blot hybridization. (A) Data from SNU-308, 478, 1079, 410, Mia PaCa-2, and LoVo cell lines. (B, C) Densitometrically quantified slot blots.

hormones.

## DISCUSSION

Gastrin and CCK, which share a peptide structure including C-terminal pentapeptide amide (Gly-Trp-Met-Asp-PheNH<sub>2</sub>), exert a trophic effect on the normal gastrointestinal organs, including the stomach, gallbladder and duodenum, in addition to the stimulation of secretion in the stomach and pancreas<sup>[21-23]</sup>. Except for these classical actions, gastrin and CCK also appear to stimulate growth of gastrointestinal cancers. Chronic endogenous hypergastrinemia and exogenous pentagastrin administration are known to significantly increase tumor cell number and the concentration of DNA, RNA, and protein in colon and gastric cancers<sup>[6,24,25]</sup>.

Similar trophic effects of gastrin and CCK have been reported in pancreatic cancer. Smith *et al.*<sup>[10]</sup> reported that the growth responses of 6 human pancreatic cancer cell lines (SW-1990, PANC-1, MIA PaCa-2, BxPC-3, RWP-2 and CAPAN-2) are stimulated by CCK in serum-free medium, and its trophic effect can be blocked by a specific antagonist<sup>[26]</sup>. They also demonstrated that gastrin exerts a growth-stimulating effect on pancreatic cancer *in vivo* and *in vitro*<sup>[27]</sup>. Furthermore many reports about the trophic effect of CCK and gastrin on pancreatic cancer have been published<sup>[11,28-30]</sup> and some studies have demonstrated that these hormones play a role in gastrointestinal cancer carcinogenesis<sup>[31,32]</sup>. On the contrary, other studies have suggested that gastrin and CCK may have no trophic effect on pancreatic cancer, and even exert an inhibitory effect on pancreatic growth and carcinogenesis.

Liehr *et al.*<sup>[12]</sup> reported that CCK cannot affect the growth of PANC-1 and MIA PaCa-2 at the concentration of 10<sup>-12</sup>-

10<sup>-6</sup> mol/L. Robertson *et al.*<sup>[31]</sup> showed that gastrin also has no trophic effect on the same pancreatic cancer cell lines. Recently, Detjen *et al.*<sup>[33]</sup> demonstrated that CCK-A and CCK-B/gastrin receptors mediate growth inhibitory responses in pancreatic cancer in their experiment on the transfection of hormone receptors into the PANC-1 and MIA PaCa-2 lines.

In the experiment with cholangiocarcinoma cells (SLU 132), the growth of cancer xenografted in nude mice is significantly retarded by CCK<sup>[34]</sup>. Evers *et al.*<sup>[35]</sup> demonstrated that CR-1409, a CCK receptor antagonist, prevents caerulein-mediated inhibition of cancer growth when combined with caerulein administration in SLU 132. Though a few cancer cell lines have been established in the biliary tract, there are limitations in the inhibitory effect of CCK on bile duct cancer as described above. In our study, cancer cell lines originating from the biliary tract exhibited a growth stimulating effect by gastrin or CCK. Therefore, further study using well-established cancer cells is mandatory to determine the hormonal trophic effect on biliary tract cancer.

We found that the trophic action of hormones was influenced by the degree of expression of its receptors, suggesting that biological response to peptide hormones is modulated by the amount of receptors in target cells. Some investigators explained that the trophic response of cancer to gastrin and CCK is determined by the presence of specific hormonal receptors, so that the responsiveness of tumor to hormone treatment can be predicted by the presence or absence of the receptor<sup>[7]</sup>. However, this explanation does not account for the conflicting results of stimulatory or

inhibitory effect on the same cancer cells according to the investigators. In our study, most pancreatic and biliary cancer cell lines have CCK-A and CCK-B receptors. However, only 2 cell lines demonstrated tumor growth-stimulating effect by gastrin (SNU-308, SNU-478) and only one cell line exhibited tumor growth-stimulating effect by CCK (SNU-478). PANC-1 and Mia PaCa-2, the most prevalently studied cell lines in similar experiments, did not show any growth-stimulating effects by gastrin and CCK though both CCK-A and CCK-B/gastrin receptors presented. This non-response to exogenous hormone can be explained that endogenous hormonal stimulation is enough for the trophic action. However, we could not find sufficient endogenous growth stimulation, which is contrary to some reports<sup>[36]</sup>. We think endogenous stimulation effect is minimal even if it exists.

Another possibility is the diversity or mutation of specific hormonal receptors. Peptide hormones express their biological activity by binding to specific hormone receptors. Receptors for CCK have been pharmacologically classified on the basis of their affinity for both the peptide agonists CCK and gastrin, which share the same COOH-terminal pentapeptide amide sequence but differ in sulfation at the 6th (gastrin) and 7th (CCK) tyrosyl residue, and the recently developed subtype-specific antagonists.

Although two types of CCK receptors (CCK-A and CCK-B/gastrin) are well known, the possibility of new non-A non-B receptor types has recently been reported. Imdahl *et al*<sup>[37]</sup> reported that the expression of low affinity binding gastrin/CCK-C receptor is found in 75% of human colorectal carcinomas. Smith *et al*<sup>[38]</sup> recently showed that the new CCK receptor is expressed in pancreatic cancer specimens and they therefore designated it as CCK-C (cancer) receptor.

Recent advances in molecular biology reveal a broader distribution of CCK receptors in the gastrointestinal and central nervous systems than previously recognized, thereby suggesting additional physiological roles for these receptors. Although CCK receptors feature homology in structure among different species and even intra-species, slight differences in receptor structure and distribution result in significant pharmacological and physiological differences<sup>[39,40]</sup>. Minor peptide changes, even the difference of a single amino acid by mutation or polymorphism of the CCK receptor, can profoundly affect the binding affinities of hormones and antagonists, enabling antagonists to act as agonists and vice versa<sup>[41,42]</sup>. Actually, the functional effect and clinical significance of receptor mutations have been studied in some diseases<sup>[43]</sup>.

Based on the present study, it may be concluded that specific receptors for gastrin and CCK exert a trophic action on some of the biliary tract cancers. However, many cancer cell lines cannot be affected by hormones despite the presence of CCK-A and CCK-B/gastrin receptors, suggesting the possibility of hormonal manipulation in limited cases of pancreatic and biliary tract cancer. An accurate method for the identification of hormonally responsive cancers is therefore required before adjunctive hormonal or antihormonal therapy can be recommended. Therefore, further investigations are required to elucidate

the mechanism of the secondary signal pathway linked to the CCK receptor family, and to determine the functional and structural differences among the receptors. Furthermore, mutation or polymorphism studies of CCK receptors may be needed to ascertain the trophic or inhibitory effect of gut hormones.

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• BASIC RESEARCH •

# Comparison of intraperitoneal anti-adhesive polysaccharides derived from *Phellinus* mushrooms in a rat peritonitis model

Jae-Sung Bae, Kwang-Ho Jang, Hee-Kyung Jin

Jae-Sung Bae, Kwang-Ho Jang, Department of Surgery, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

Hee-Kyung Jin, Department of Laboratory Animal Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

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Co-correspondents: Kwang-Ho Jang and Hee Kyung Jin

Correspondence to: Dr. Hee-Kyung Jin, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea. hkjin@mail.knu.ac.kr

Telephone: +82-53-950-5966 Fax: +82-53-950-5955

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## Abstract

**AIM:** To assess the adhesion- and abscess-reducing capacities of various concentrations of polysaccharides derived from fungus, *Phellinus gilvus* (PG) or *Phellinus linteus* (PL) in a rat peritonitis model.

**METHODS:** In 96 SD rats, experimental peritonitis was induced using the cecal ligation and puncture model (CLP). Rats were randomly assigned to 8 groups; Ringer's lactate solution (RL group), hyaluronic acid (HA group), 0.025%, 0.25%, and 0.5% polysaccharides from PG (PG0.025, 0.25, and 0.5 groups), and PL (PL0.025, 0.25, and 0.5 groups). Adhesions and abscesses were noted at 7 d after CLP. RT-PCR assay was performed to assess the cecal tissue.

**RESULTS:** Adhesion formation was significantly reduced in PG0.25, 0.5, PL0.25, 0.5, and HA groups ( $2.5 \pm 0.7$ ,  $2.4 \pm 0.7$ ,  $3.8 \pm 1.0$ ,  $3.6 \pm 0.8$ , and  $2.7 \pm 1.1$ ,  $P < 0.05$ ). The incidence of abscesses was significantly reduced in all treated groups compared to RL group (58%,  $P < 0.05$ ). The urokinase-type plasminogen activator (uPA) gene expression was greatly up-regulated by increasing the concentration of polysaccharides. The urokinase-type plasminogen activator receptor (uPAR) and tumor necrosis factor (TNF)- $\alpha$  mRNA were highly expressed in PG0.25, 0.5, PL0.25, and 0.5 groups.

**CONCLUSION:** We concluded that 0.5% polysaccharide derived from PG and PL was the optimal concentration in preventing adhesion and abscess formation and may act by modulating activity of uPA and TNF- $\alpha$  in a rat peritonitis model.

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**Key words:** Adhesion; Abscess; *Phellinus gilvus*; *Phellinus*

*linteus*; Polysaccharides

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## INTRODUCTION

Intraperitoneal adhesions and abscesses are a major cause of morbidity and mortality in the adult intensive care unit. They are caused mainly by previous surgery and abdominal inflammation<sup>[1,2]</sup>. Abdominal infection is accompanied by peritoneal inflammation, including exudation of fibrinogen and fibrin formation into the abdominal cavity. In infectious conditions, these fibrin deposits may become a nidus for abscesses<sup>[3]</sup> and in turn become fibrous adhesions. Therefore peritoneal fibrinolytic system is crucial in preventing adhesion and abscess formation. Activation of fibrinolytic system results in the conversion of plasminogen into plasmin. The conversion is activated directly by tissue-type plasminogen activators (tPA) and uPA<sup>[4,5]</sup>. Various cells produce tPA, including endothelial cells, mesothelial cells, and macrophages. The uPA is also produced by the same cells and is equally effective in the degradation of fibrin<sup>[6]</sup>.

Numerous agents have been investigated in the prevention of adhesion and abscess formation. Recently, it was reported that beta-glucan in polysaccharides from chemical was capable of reducing the frequency of adhesion by preventing with beta-glucanase<sup>[7]</sup>. It is a potent macrophage stimulator that enhances macrophage cytotoxicity and phagocytic capacity and induces production of TNF- $\alpha$ <sup>[8,9]</sup>.

We have also previously demonstrated that 0.025% polysaccharides derived from PG and PL reduced both intraperitoneal adhesion and abscess formation by modulating activity of uPA and TNF- $\alpha$  produced from activated macrophages in a rat peritonitis model<sup>[10]</sup>. However, comparison of adhesion- and abscess-reducing capacity by varying concentrations of polysaccharides solutions derived from PG and PL has not been demonstrated to date.

Therefore, in this study, we hypothesize that intraperitoneal abscesses and adhesions could be reduced by increasing concentration of polysaccharides solutions derived from PG and PL, and differences of the effect could be related to uPA and TNF- $\alpha$  activity produced from activated macrophages. To test this hypothesis, we investigated differences by varying concentration of polysaccharides derived from PG and PL in preventing adhesion and abscess

formation in order to investigate influence of the *uPA* and *TNF- $\alpha$*  gene expression in a rat peritonitis model.

## MATERIALS AND METHODS

### Preparation of materials

The fruiting body of PG was kindly provided by Gyeongbuk Agricultural Technology Administration (Daegu, Korea). A seed culture was grown in a 250-mL flask containing 50 mL of PMP medium (2.4% potato/dextrose broth plus 1% malt extract, 0.1% peptone) at 28 °C on a rotary incubator at 150 r/min for 4 d. To obtain fruiting bodies of PG, a culture was grown in oak sawdust block for 90 d. The yield of fruiting bodies was 97 g dried weight per block. PL used in this study was developed for 3 years in routine artificial mulberry cultures and purchased from Sanwhang Mushroom Co. (Andong, Korea). The fruiting body of PG and PL was cut into small pieces, dried at 40–50 °C for 48 h. It was homogenized, extracted by optimal water extraction conditions, distilled water (1:25) at 100 °C for 10 h, and concentrated at 80 °C in a rotary evaporator. The recovery procedure of the polysaccharides from the fruiting body of PG and PL followed an established method in our previous study<sup>[10,11]</sup>. The concentration of 0.025%, 0.25%, and 0.5% polysaccharides solutions was determined by measuring total sugar using the anthrone method<sup>[12]</sup> using glucose as the standard material. The 0.2% HA was prepared by adding distilled water at HA (HYAL®, Shinpoong Pharm. Co., Korea). It was filtered through a 0.22  $\mu$ m membrane filter. All the materials were stored at 4 °C until used.

### Animals

Ninety-six male Sprague-Dawley (SD) rats (Charles River Korea Inc., Korea) weighing 237 to 261 g were acclimated under the controlled conditions for 1 wk before the experiments. The animals were fed with commercial rat feed from Orient Inc., Korea. Food and water were provided *ad libitum* to the animals. The *Guidelines for Animal Care and Use of Kyungpook National University* approved the housing, care and use of animals, as well as procedures to minimize discomfort.

### Surgical procedures

Bacterial peritonitis was induced by performing a CLP procedure according to Wichterman *et al.*<sup>[13]</sup>. Only water was provided in the 12 h preceding the experiments. The animals were weighed and anesthetized by intramuscular injection of a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg). They breathed spontaneously throughout the procedures. The abdominal skin was disinfected with 70% alcohol. All procedures were performed under sterile conditions. Routine midline celiotomy was performed with a 3-cm incision and the cecum was exposed. The cecum was ligated just distally to ileocecal valve with a 3-0 polyglactin 910 (VICRYL®, ETHICON, Inc., Johnson & Johnson Co., San Angelo, TX) suture to avoid intestinal obstruction, punctured once with a 19-G needle, squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity. After closing the abdomen in two layers with 3-0 polyglactin 910 sutures, the animals received

enrofloxacin (1 mg/kg) and 10 mL of isotonic sodium chloride solution subcutaneously for hydration. After 24 h, animals were weighed and the abdomen was reopened under the same anesthesia as the first celiotomy. Samples of peritoneal fluid were taken for microbiologic examination. The abdominal cavity was rinsed with 10 mL isotonic sodium chloride solution, and the cecum was resected. Before closure of the abdomen, the animals were randomly allocated to 8 groups of 12. One control group was treated with 8 mL ofringer lactate solution (RL group) and one other control group was treated with 8 mL of 0.2% HA solution (HA group) intraperitoneally through the urinary catheter. Six experimental groups were treated with 8 mL of 0.025%, 0.25%, and 0.5% polysaccharides derived from PG (PG0.025, 0.25, and 0.5 groups) and PL (PL0.025, 0.25, and 0.5 groups) intraperitoneally, respectively. All animals were given water only on the first postoperative day; standard rat chow and water *ad libitum* were provided on the second postoperative day. The animals were weighed again and killed with carbon dioxide asphyxiation a week after the first postoperative day. The abdomen was opened via a U-shaped incision for complete exploration. Adhesions and the incidence of abscesses were examined in a blind manner by one of us (HK Jin) according to the method of Zuhlke *et al.*<sup>[14]</sup>, whereby grade 0 means no adhesions and grade IV means firm extensive adhesions that are dissectible only with sharp instruments, with organ damage almost unavoidable. Sites of adhesions scored included the midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. The total score of these six locations was noted as the total adhesion score (0–24) (Table 1).

**Table 1** Grading of adhesions according to Zuhlke

Grade	Description
0	No adhesions
1	Filmy adhesions: gentle, blunt dissection required to free adhesions
2	Mild adhesions: aggressive blunt dissection required to free adhesions
3	Moderate adhesions: sharp dissection required to free adhesions
4	Severe adhesions: not dissectible without damaging organs

*Note.* Locations scored included midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. The sum of these locations formed the total adhesion score (0–24).

### Bacterial cultures

Samples of peritoneal fluid and abscesses were taken from all animals on the second postoperative day by swabs for verification of the induced peritonitis. The swabs were immediately introduced into medium and cultured semiquantitatively in aerobic and anaerobic conditions. Samples were incubated on blood and EMB agar for aerobic culture and layered on anaerobic blood agar and incubated in a Gas-Pak jar for anaerobic culture. After 24 and 48 h of incubation at 37 °C, growing colonies were identified with standard bacteriologic techniques.

### Tissue collection

The adhesion-carrying cecal site was resected carefully. The

cecal tissue was cut longitudinally to remove food contents, washed with sterile phosphate-buffered saline (PBS). Half the animals in each group were fixed in 10% formalin in PBS for histopathologic evaluation and the remaining animals in each group were stored at 80 °C for RT-PCR analysis until further processing.

### Histopathologic evaluation

After routine tissue processing, serial sections (5 µm) were stained with hematoxylin and eosin (HE). The inflammatory reaction was assessed for each group by light microscopy. The grade of inflammation was assessed using a semi-quantitative scoring system, the inflammation grading scale<sup>[15]</sup>. Grade 1 on this scale represents a mild inflammatory reaction with giant cells, occasionally scattered lymphocytes, and plasma cells. Grade 2 represents a moderate reaction with giant cells and increased admixed lymphocytes, plasma cells, eosinophils, and neutrophils. Grade 3 represents a severe inflammatory reaction with microabscesses present.

### RNA extraction

Total cellular RNA was extracted from rat cecum using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The purity and integrity of the RNA samples were assessed by  $A_{260}/A_{280}$  spectrophotometric measurements.

### RT

A 1 µg portion of total RNA was subjected to first-strand cDNA synthesis in a 20 µL reaction mixture containing Moloney murine leukemia virus reverse transcriptase (10 U), dNTP mixture (2.5 mmol/L concentrations of each dNTP), oligo (dT)<sub>12-18</sub> primers (10 µmol/L), and reaction buffer as supplied with the enzyme (50 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 10 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L spermidine, and 10 mmol/L dithiothreitol). The samples were incubated in a TOUCHgene DNA thermal cycler (Techne (Cambridge) Limited, U.K.) at 42 °C for 60 min followed by enzyme denaturation step at 94 °C for 2 min. The reverse transcription mixture was stored at -80 °C for use in PCR. All reagents were obtained from Promega (Madison, WI).

### PCR

PCR was performed on 2 µL of reverse transcriptase product using Gene Taq (Nippon Gene Co., Ltd., Toyama, Japan) containing Taq DNA polymerase, dNTPs, buffer, and 0.5 µmol/L concentrations of each gene-specific forward and reward primers (obtained from Bioneer, Daejeon, Korea) in a total volume of 50 µL. Gene-specific oligonucleotide primers were designed from published rat sequences. Primers used for amplification: TNF-α: sense, 5'-TACTGAACCTTCGGGGTGATTGGTCC-3', antisense, 5'-CAGCCTTGTCCTTGAAGAGAACC-3'; uPA: sense, 5'-TCGTGAATCAGCCAAAGAAGGAAGAGTACG-3', antisense, 5'-TTACAACTGACATTTTCAGGTCC-3'; uPAR: sense, 5'-CAGAACACTGTATTGAAGTG GTGACGCTCC-3', antisense, 5'-TCCAAGCACTGATTC ATTGGTCCCCG-3'; glyceraldehydes-3-phosphate

dehydrogenase (GAPDH): sense, 5'-TGAAGGTCGGTGT GAACGGATTGGC-3', antisense, 5'-CATGTAGGCCAT GAGGTCCACCAC-3'. The PCR was conducted in TOUCHgene DNA thermal cycler. After an initial denaturation at 95 °C for 5 min, amplification was conducted through 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (GAPDH) or 60 °C (for all the other transcripts) for 30 s and extension at 72 °C for 45 s. Final extension was at 72 °C for 10 min followed by a final hold at 4 °C. Negative controls (PCR mixture without cDNA) and positive controls (PCR mixture with a standard cDNA sample) were included in preliminary PCR runs. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers (data not shown). The PCR products were separated by electrophoresis using 2% agarose gels stained with ethidium bromide to visualize cDNA products.

### Statistical analysis

Values are expressed as mean±SD. Analysis of differences between treated groups and untreated groups was performed using analysis of variance followed by multiple comparisons and Fisher's LSD test using the SAS statistical package (release 8.1; SAS Institute Inc., Cary, NC). Differences at  $P<0.05$  were considered statistically significant.

## RESULTS

Following CLP, all rats showed symptoms of peritonitis-like apathetic behavior, ocular exudates, and piloerection. These symptoms resolved within 2 d following the re-celiotomy and removal of the necrotic, perforated cecum and peritoneal lavage. Survival rates in all groups were 100% by the end of the experiment.

### Body weights of the rats

The mean±SD body weight of the rats was 248.1±12.3 g at the time of the first operation. The rats lost weight during peritonitis (240.2±12.8 g) and recovered weight by the end of the experiment (255.3±10.7 g) in RL, PG0.025, PL0.025, and HA groups. The differences in weight gain were not statistically significant among the groups. But, the weight gain in the PG0.25, 0.5, PL 0.25, and 0.5 groups (270.2±12.8 g) was higher than that in RL, PG0.025, PL0.025, and HA groups.

### Microbiological examination

Culture results of the peritoneal fluid taken at the day of cecal resection revealed polymicrobial intraperitoneal infection. The most frequently isolated microorganisms were *Escherichia coli* (50.9%), *Proteus* species (57.5%), *Staphylococcus* (48.1%), *Streptococcus* (25%), Gram-positive *Bacillus* species (15%), and *Klebsiella* (4%). *E. coli* (84.7%) was the organism isolated most frequently from abdominal abscesses.

### Total adhesion score and site of adhesion

**The PG polysaccharide solutions** Rats treated with 0.25% and 0.5% of PG polysaccharide solutions had a significantly lower total adhesion score compared to that of the RL group ( $P<0.05$ ). The total adhesion score of rats treated

with the RL solution was  $12.3 \pm 5.2$ . Group treated with 0.25% ( $2.5 \pm 0.7$ ) and 0.5% ( $2.4 \pm 0.7$ ) of PG polysaccharide solutions was lower than the group treated with 0.025% ( $5.8 \pm 1.0$ ) solution in total adhesion score ( $P < 0.05$ ) (Figure 1). There was no significant difference in total adhesion score between PG0.25 and PG0.5 group. The prevention effect of adhesion of PG0.25 and 0.5 groups was slightly higher than that in PL0.25, PL0.5, and HA ( $2.7 \pm 1.1$ ) groups. There was no statistical difference in total adhesion score. Eight of 12 (67%) RL-treated rats had grade IV adhesions, in contrast to only 2 of 12 (17%) PG 0.025% and 0 of 12 (0%) PG0.25 and 0.5% treated rats. The site of the adhesions did not differ among the PG groups. Most of the adhesions were found between the bowel loops (81.1%), adnexa/epididymal fat bodies (62.5%), and the omentum (43.2%) in the PG groups ( $P < 0.05$ ) (Figure 2).

**The PL polysaccharide solution** Rat treated with 0.25 and 0.5% of PL polysaccharide solutions had a significantly lower total adhesion score compared to RL group ( $P < 0.05$ ) (Figure 1). The prevention effect of adhesion was slightly lower than the effect of PG0.25, PG0.5, and HA groups. Group treated with 0.25% ( $3.8 \pm 1.0$ ) and 0.5% ( $3.6 \pm 0.8$ ) of PL polysaccharide solutions was lower than the group treated with 0.025% PL ( $5.6 \pm 1.8$ ) solution in total adhesion

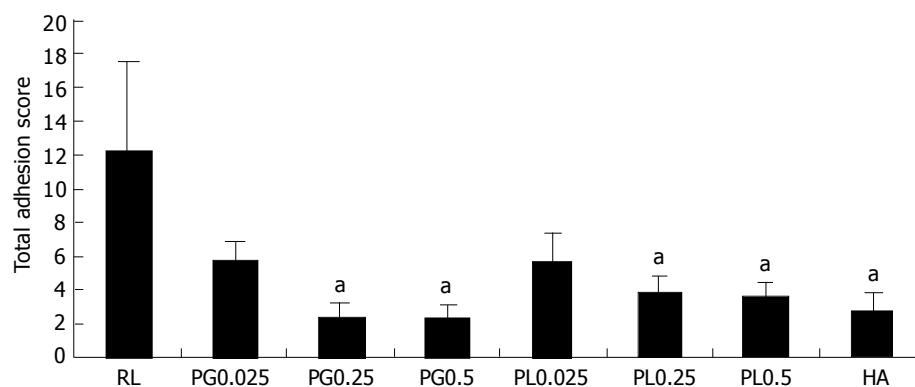
score ( $P < 0.05$ ). There was no significant difference in total adhesion score between PL0.25 and PL0.5 groups like groups treated with 0.25% and 0.5% of PG polysaccharide solutions. The prevention effect of adhesion of PL0.25 and 0.5 groups was slightly lower than that in HA ( $2.7 \pm 1.1$ ) groups. One of 12 (17%) PL-treated rats had grade IV adhesions. The site of the adhesions did not differ among the PL groups. Site of the adhesions was similar to the site of rats treated PG (Figure 2).

### Abscesses

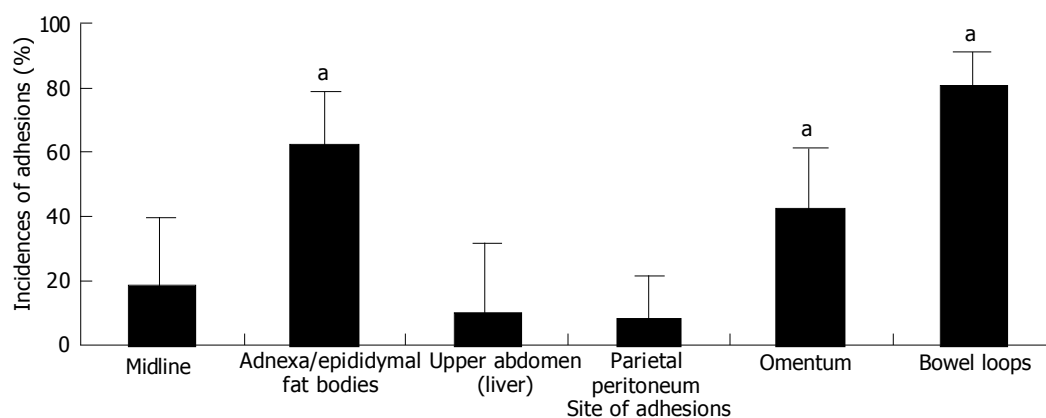
The incidence of intraperitoneal abscess significantly reduced in all treated groups ( $P < 0.05$ ) compared to that in RL group. No abscess occurred in rats treated with PG 0.25 and 0.5. Rats treated with HA (3 of 12, 25%) reduced the incidence of abscesses compared to RL (7 of 12, 58%).

### Histologic evaluation

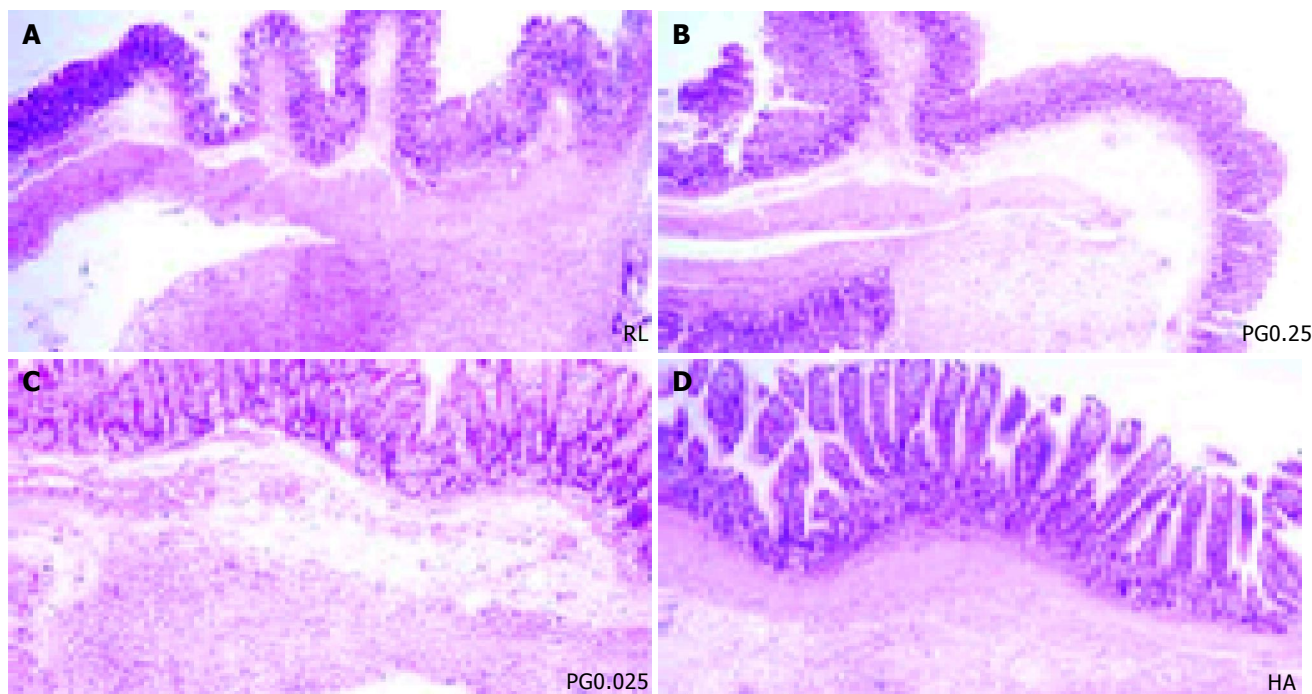
Mostly, the inflammatory reaction is dominant at mesenteric fat and serosal surface of cecum. Rats treated with PG0.25 showed markedly reduced inflammatory reaction compared to RL (Figure 3). The RL group showed increased admixed lymphocytes, plasma cells, eosinophils, and neutrophils (grade 3 on the inflammation grading scale). The grade of



**Figure 1** Total adhesion score of intraperitoneal adhesion in each group (in all rats). The animals were killed one week after the first postoperative day. The abdomen was opened for complete exploration. Rats treated with 0.25 or 0.5% polysaccharides solutions from both PG and PL showed a significantly lower total adhesion score compared with RL group ( $^aP < 0.05$  vs RL group).

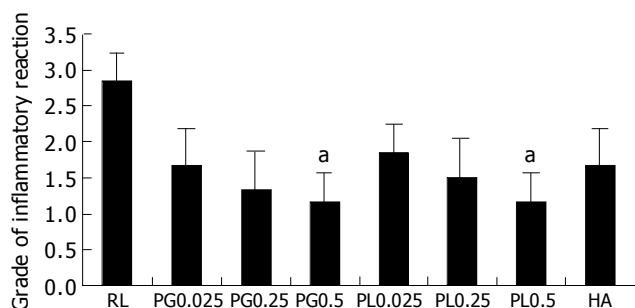


**Figure 2** The site of the intraperitoneal adhesions (in all rats). After the sacrifice, sites of adhesions were observed in the midline, adnexa/epididymal fat bodies, the upper abdomen (liver), the parietal peritoneum, the omentum, and between the bowel loops. Most of the adhesions were found between the bowel loops, adnexa/epididymal fat bodies, and the omentum compared with other sites ( $^aP < 0.05$ ).



**Figure 3** Evaluation of inflammatory reaction from formalin-fixed, paraffin-embedded section of cecal tissues. Compare the extent of mesenteric inflammatory reaction among (A). RL, (B). PG0.25, (C). PG0.025, and (D). HA ( $\times 40$ ). Note markedly reduced inflammatory reaction in PG0.25 treated groups ( $n = 6$  in each group).

inflammatory response for the PG0.5 and PL0.5 groups ( $1.1 \pm 0.4$  and  $1.2 \pm 0.4$ ) was significantly lower than the grade for the RL ( $2.9 \pm 0.4$ ) ( $P < 0.05$ ) and slightly lower than PG0.025 and 0.25 ( $1.7 \pm 0.5$  and  $1.3 \pm 0.5$ ), PL0.025 and 0.25 ( $1.8 \pm 0.4$  and  $1.5 \pm 0.5$ ), and HA group ( $1.6 \pm 0.5$ ) (Figure 4).

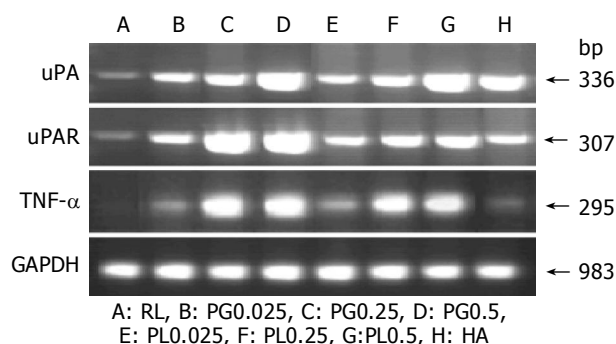


**Figure 4** The grade of inflammatory reaction in each group ( $n = 6$  per each group). The grade of inflammatory response for the PG0.5 and PL0.5 groups was significantly lower than the grade for the RL ( $^aP < 0.05$  vs RL group).

#### *TNF- $\alpha$ , uPA, and uPAR mRNA expression*

Experiments were carried out to demonstrate the effect of PG, PL, and HA on the gene transcription of  $TNF-\alpha$ , uPA and uPAR. In the PG and PL treatment groups,  $TNF-\alpha$  mRNA was highly expressed, as compared to treatment with RL in PG0.25, 0.5, PL0.25, and 0.5 groups. The level slightly increased compared to RL in PG0.025, PL0.025, and HA. The uPA gene expression was greatly up-regulated by increasing concentration of polysaccharides in the PG

and PL treatment groups. In HA group, the level was highly expressed, as compared to treatment with RL. The uPA receptor (uPAR) mRNA was expressed at the highest levels by the treatment with PG0.25 and 0.5. In PL and HA groups, the level was expressed compared to RL group. The GAPDH transcript levels among all groups were the same (Figure 5).



**Figure 5**  $TNF-\alpha$ , uPA and uPAR mRNA expression in each group ( $n = 6$  per each group). The  $TNF-\alpha$  mRNA was highly expressed, as compared to treatment with RL in PG0.25, 0.5, PL0.25, and 0.5 groups. The uPA gene expression was greatly upregulated by increasing concentration of polysaccharides in the PG and PL treatment groups. The uPAR mRNA was expressed at the highest levels by the treatment with PG0.25 and 0.5. The GAPDH transcript levels among all groups were the same.

#### **DISCUSSION**

Abdominal infections are associated with fibrin deposit, which may cause clinically significant adhesion and abscess

formation. Adhesions are the most common cause of intestinal obstruction in developed countries and important cause of chronic abdominal and pelvic pain<sup>[16-19]</sup>.

The CLP model has been used frequently for elucidating the pathophysiology of abdominal inflammation and developing new treatment modalities<sup>[20]</sup>, because it is caused by the intraperitoneal adhesions that are promoted by surgical trauma, bacterial contamination, allergic reactions to foreign bodies left in the abdomen, and tissue ischemia. Thus, in our study, we used the CLP model that underwent cecal resection to induce adhesion and abscess formation in infectious environment.

The number of different agents has been studied in the search to prevent intraperitoneal adhesions, with variable results. Many studies showed HA decreases inflammation<sup>[21]</sup>, interferes with fibrin formation<sup>[22]</sup>, prevents adhesion and abscess formation<sup>[23]</sup>, and stimulates fibrinolytic and TNF- $\alpha$  response<sup>[24,25]</sup>. In our study, we treated with 0.2% HA to prevent intraperitoneal adhesion in rats and this solution significantly reduced adhesion and abscess formation by modulating uPA and TNF- $\alpha$  production. This is in accordance with the results from earlier studies<sup>[23-25]</sup> for the prevention of intraperitoneal adhesions formation in rats using 0.2% HA. And, we have previously demonstrated that 0.025% polysaccharides derived from PG and PL reduced both intraperitoneal adhesion and abscess formation by modulating activity of uPA and TNF- $\alpha$  produced from activated macrophages in a rat peritonitis model<sup>[10]</sup>. In the present study, we treated with varying concentrations of 0.025%, 0.25%, and 0.5% of PG and PL in rats. It resulted that 0.25 and 0.5% solutions had higher adhesion- and abscess-reducing capacity than 0.025% solution in rats. The *uPA* gene expression was greatly up-regulated by increasing concentration of the solutions. Therefore, we think that PG and PL may act in a dose-dependent manner in the prevention of adhesion and abscess formation and the action may be determined by modulating fibrinolytic capacity of uPA produced from macrophages.

In short, PG and PL used in our study are fungi belonging to the species of Hymenochaetaceae (Basidiomycetes) and found mainly in tropical areas of America and Africa<sup>[26]</sup>. Polysaccharides isolated from them have received special attention due to their potent pharmacological activities. PL is well known as one of the most popular medicinal mushrooms due to its high anti-tumor<sup>[27]</sup> and immunostimulating activities<sup>[28]</sup>. It has been used medicinally in Korea and Japan. Recently, it was reported that polysaccharide solutions from PL and PG had anti-inflammatory activity related to arthritis, septic shock, and pulmonary inflammation<sup>[29-31]</sup>. Thus, we think that these anti-inflammatory activities of natural products like PG and PL may be beneficial in the treatment of intraperitoneal adhesion related to inflammation.

PG has other advantages over PL in that it has a very short growth period (3 mo) compared to PL (2-3 years) making it cheaper to produce and the safety of acute single orally-administered dose of PG has been demonstrated. In the present study, although it is not a statistically significant analysis, polysaccharides from PG had better capacity than PL in the prevention of adhesion and abscess formation. Therefore, this suggests an additional potential therapeutic

role for PG in the treatment of inflammation in future.

In an experimental study, Bedirli *et al.*<sup>[7]</sup> showed that beta-glucan in polysaccharides had a positive weight gain effect on the animals. In our study, although it is not a statistically significant analysis, weight gain in the 0.25% and 0.5% of PG and PL groups is higher than that in RL, HA, PG0.025, and PL0.025 groups. That is to say, it showed a positive weight gain in groups treated with high dose, 0.25% and 0.5% of polysaccharides. However, the precise mechanism of weight gain in these animals is uncertain. We postulate that this could be related to a difference in the mechanism by which adhesion formation is reduced.

Many studies showed that beta-glucan in polysaccharides is a potent stimulator of macrophage functions and it induces TNF- $\alpha$  production in wound tissue<sup>[8,9]</sup>. But the role of TNF- $\alpha$  in adhesion formation is not clear. More recently, Reijnen *et al.*<sup>[24]</sup> reported that HA counteracts the fibrinolytic decline induced by TNF- $\alpha$ . Boyce *et al.*<sup>[25]</sup> indicated that TNF- $\alpha$  down-regulates fibroblastic collagen synthesis within experimental wounds and HA stimulates TNF- $\alpha$  production by human macrophages. In our study, TNF- $\alpha$  mRNA was highly expressed in the PG0.25, 0.5, PL 0.25, and 0.5 groups compared to the RL group. This is in accordance with the results from Abel *et al.*<sup>[9]</sup>, which indicated that beta-glucan induced TNF- $\alpha$  production in wound tissue. In HA group, the level was slightly expressed compared to that of RL group. This is also in accordance with the results from Reijnen *et al.*<sup>[24]</sup> and Boyce *et al.*<sup>[25]</sup>. We infer that 0.25% and 0.5% of polysaccharides solutions derived from PG and PL stimulate macrophage activity and increase secretion of uPA, uPAR, and TNF- $\alpha$  by the stimulated macrophage activity. Thus, we conclude that high dose of polysaccharides solutions derived from PG and PL decrease adhesion formation by increasing macrophage activity and enhancing fibrinolytic activity.

In summary, high dose of polysaccharides solutions derived from the fungi, PG and PL are pharmacologic agents that rapidly enhance host resistance to a variety of biological insults through the fibrinolytic system and this involves macrophage activation. In the present study, 0.25 and 0.5% of polysaccharides significantly decreased intraperitoneal adhesion and abscess formation in a rat peritonitis model. It was as effective as HA in the prevention of intraperitoneal adhesion and abscess formation. Additional studies will help elicit whether the use of such polysaccharides and HA in combination may have wider clinical application.

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• BASIC RESEARCH •

## Effects of glutamine-containing total parenteral nutrition on phagocytic activity and anabolic hormone response in rats undergoing gastrectomy

Chen-Hsien Lee, Wan-Chun Chiu, Soul-Chin Chen, Chih-Hsiung Wu, Sung-Ling Yeh

Chen-Hsien Lee, Wan-Chun Chiu, Sung-Ling Yeh, Institute of Nutrition and Health Sciences, Taipei Medical University, Taipei, Taiwan, China

Soul-Chin Chen, Chih-Hsiung Wu, Department of Surgery, Taipei Medical University Hospital, Taipei, Taiwan, China

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Correspondence to: Sung-Ling Yeh, Ph.D., Institute of Nutrition and Health Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei, Taiwan 110, China. sangling@tmu.edu.tw

Telephone: +88-62-27361661-6551-115 Fax: +88-62-27373112  
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### Abstract

**AIM:** To investigate the effect of glutamine (Gln)-containing parenteral nutrition on phagocytic activity and to elucidate the possible roles of Gln in the secretion of anabolic hormones and nitrogen balance in rats undergoing a gastrectomy.

**METHODS:** Rats with an internal jugular catheter were divided into 2 experimental groups and received total parenteral nutrition (TPN). The TPN solutions were isonitrogenous and identical in nutrient compositions except for differences in amino acid content. One group received conventional TPN (control), and in the other group, 25% of the total amino acid nitrogen was replaced with Gln. After receiving TPN for 3 d, one-third of the rats in each experimental group were sacrificed as the baseline group. The remaining rats underwent a partial gastrectomy and were killed 1 and 3 d, respectively, after surgery. Plasma, peritoneal lavage fluid (PLF), and urine samples were collected for further analysis.

**RESULTS:** The Gln group had fewer nitrogen losses 1 and 2 d after surgery (d1,  $16.6 \pm 242.5$  vs  $-233.4 \pm 205.9$  mg/d, d2,  $31.8 \pm 238.8$  vs  $-253.4 \pm 184.6$  mg/d,  $P < 0.05$ ). There were no differences in plasma growth hormone (GH) and insulin-like growth factor-1 levels between the 2 groups before or after surgery. The phagocytic activity of peritoneal macrophages was higher in the Gln group than in the control group 1 d after surgery ( $41185 \pm 931$  vs  $323 \pm 201$ ,  $P < 0.05$ ). There were no differences in the phagocytic activities of blood polymorphonuclear neutrophils between the 2 groups at the baseline or on the postoperative days. No significant differences in interleukin-1 $\beta$  or interleukin-6 concentrations in PLF were observed between the 2 groups. However, tumor necrosis factor- $\alpha$  level in PLF was significantly lower in the Gln group than in the control group

on postoperative d 3.

**CONCLUSION:** TPN supplemented with Gln can improve the nitrogen balance, and enhance macrophage phagocytic activity at the site of injury. However, Gln supplementation has no effect on phagocytic cell activity in the systemic circulation, GH and insulin-like growth factor-1 might not be responsible for attenuating nitrogen losses in rats with a partial gastrectomy.

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**Key words:** Parenteral Nutrition; Glutamine; Phagocytosis; Gastrectomy

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### INTRODUCTION

Surgeries of the upper gastrointestinal tract usually produce a moderate degree of metabolic stress. Altered protein metabolism characterized by a negative nitrogen balance and changes in plasma-free amino acid pattern were often observed in surgical traumas<sup>[1,2]</sup>. For most gastrectomized patients with gastric diseases, preoperative protein-energy malnutrition was often present, and adequate oral intake after surgery was achieved late<sup>[3,4]</sup>. Artificial nutritional support is essential for these patients. Most surgeons use the parenteral route to administer nutrients before and after a gastrectomy. However, the optimal formulation of TPN for patients with gastrectomy is still unknown.

In recent years, glutamine (Gln) has elicited great attention for its therapeutic role in the treatment of diseases. Gln has traditionally been considered as a nonessential amino acid, but laboratory and clinical data suggest that it may become essential during certain catabolic conditions<sup>[5,6]</sup>, because studies have shown that hypercatabolic states are associated with significantly depressed plasma Gln levels<sup>[7-9]</sup>. A number of studies have demonstrated the beneficial effects of supplying exogenous Gln in the diet for metabolic-stressed conditions. These effects include increasing nitrogen retention, preserving the integrity of the intestinal mucosa and intestinal permeability, maintaining immunologic function,

and reducing infections<sup>[5,6,10,11]</sup>. Parry-Billings *et al*<sup>[6]</sup> demonstrated that depressed Gln concentrations were associated with depressed phagocytosis by peritoneal macrophages in normal mice. Ogle *et al*<sup>[12]</sup> also reported that Gln improved the bactericidal ability of abnormal neutrophils from pediatric patients after burns. Furukawa *et al*<sup>[13]</sup> revealed that supplemental Gln enhances phagocytosis by neutrophils from postoperative patients *in vitro*. Although Parry-Billings *et al*<sup>[6]</sup> and Ogle *et al*<sup>[12]</sup> suggested the efficacy of Gln supplementation, they did not supply Gln to their patients. The beneficial effect of Gln on phagocytosis in *in vitro* studies might not reflect *in vivo* situations. To our knowledge, no study has been carried out to date to investigate the effect of Gln supplementation on phagocytic activity after gastrectomy. Therefore, in this study, we infused Gln-containing parenteral nutrition before and after gastrectomy to investigate the effect of Gln on phagocytic activity at the site of injury and in systemic circulation. Growth hormone (GH) is an anabolic hormone that can reduce whole-body nitrogen loss after surgery<sup>[14,15]</sup>. A study showed that low-dose Gln supplementation was also capable of elevating plasma GH<sup>[16]</sup>. We analyzed plasma GH and insulin-like growth factor (IGF)-1 to elucidate whether Gln supplementation could enhance the secretion of anabolic hormones thus attenuating the nitrogen losses after gastrectomy.

## MATERIALS AND METHODS

### Animals

Male 7-wk-old Wistar rats weighing 170-210 g at the beginning of the experiment were used. All rats were housed in temperature- and humidity-controlled rooms, and allowed free access to a standard rat chow for 7 d prior to the experiment. The care of the animals followed the standard experimental animal care procedures. This study was approved by the Taipei Medical University Animal Care Committee.

### Study protocol and operation procedures

Rats were randomly assigned to 2 experimental groups, with 30 rats to each group. The average weight between the groups was adjusted as similar as possible. After an overnight fasting, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the right internal jugular vein was cannulated with a silastic catheter (Dow Corning, Midland, MI) under sterile conditions. The catheter was tunneled subcutaneously to the back of neck and exited through a coil spring that was attached to a swivel, allowing free mobility of animals inside individual metabolic cages. All animals were allowed to drink water during the experimental period. TPN provided 270 kcal/kg body weight, this level of energy was slightly higher than weight maintenance for normal TPN rats<sup>[17]</sup>. The kcal/nitrogen ratio in the TPN solution was 145:1. The calorie density was almost 1 kcal/mL. The TPN solutions were isonitrogenous (6.84 mg/mL) and identical in nutrient compositions except for the difference in amino acid content. One group received conventional TPN (control), the other group replaced 25% of the total amino acid nitrogen with Gln. Although the quantity of essential amino acids (EAA) was lower in the Gln group than that in the control

group, the EAA was adequate for maintenance according to the reported EAA requirements for rats<sup>[18]</sup>. The energy distribution of the TPN solutions in the experimental groups was 72% from glucose, 18% from protein, and 10% from fat (Table 1). Gln was dissolved and sterilized by passage through a 0.2- $\mu$ m Minisart NML filter (Sartorius, Goettingen, Germany) and stored at 4 °C until being used. Gln solution was stable at room temperature for at least 2 d as previously described<sup>[17]</sup>. The TPN solution was refilled daily and infused for 24 h at room temperature. Two milliliters per hour was administered on the first day, and then the rats received 48-57 kcal/d according to their body weight. The infusion rate was maintained with a Terufusion pump (model STC-503, Terumo, Tokyo, Japan). The TPN solution without fat was prepared every other day in a laminar flow hood, and the fat emulsion was added daily just before use. After receiving TPN for 3 d, one-third of the rats ( $n = 10$ ) in each experimental group were killed as the baseline group. The remaining rats underwent a partial gastrectomy on the 4<sup>th</sup> d of TPN, and were killed 1 or 3 d, respectively, after surgery. Partial gastrectomy was performed using the same method as in our previous study<sup>[19]</sup>. TPN was maintained for 3, 5, or 7 d according to the sacrifice schedule of the rats.

**Table 1** Formulation of the TPN solution

	Gln	Control
50% glucose	420	420
20% Lipofudin	50	50
<sup>1</sup> Moriamine 10%	345	450
NaCl <sub>3</sub> 3%	35	35
K <sub>3</sub> PO <sub>4</sub> 8.7%	10	10
KCl 7%	10	10
Calcium gluconate 10%	10	10
MgSO <sub>4</sub> 10%	4	4
ZnSO <sub>4</sub> 0.6%	2	2
<sup>2</sup> Infuvita	8	8
Choline chloride (g)	1	1
Gln (g)	8.4	---
H <sub>2</sub> O	105	---
Total volume	998	998
Total kcal	986	994

<sup>1</sup>From Chinese Pharmaceuticals, Taipei, Taiwan. Each deciliter contains: Leu 1 250 mg, Ile 560 mg, Lys acetate 1 240 mg, Met 350 mg, Phe 935 mg, Thr 650 mg, Trp 130 mg, Val 450 mg, Ala 620 mg, Arg 790 mg, Asp 380 mg, Cys 100 mg, Glu 650 mg, His 600 mg, Pro 330 mg, Ser 220 mg, Tyr 35 mg, and aminoacetic acid (Gly) 1 570 mg. <sup>2</sup>From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Each milliliter contains: vitamin A 660 IU, ascorbic acid 20 mg, vitamin A 660 IU, ergocalciferol 40 IU, thiamine HCl 0.6 mg, riboflavin 0.72 mg, niacinamide 8 mg, pyridoxine HCl 0.8 mg, d-panthenol 3 mg, and dl-alpha-tocopheryl acetate 2 mg.

### Measurements and analytical procedure

Rats in the respective groups were killed before or 1 or 3 d after surgery. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg BW). A middle abdominal incision was made, and 10 mL of phosphate-buffered saline (PBS) was intraperitoneally injected to elute the peritoneal cells. After the PLF was harvested, rats were exsanguinated by drawing arterial blood from the aorta. Blood samples were collected in tubes containing heparin and

immediately centrifuged. Plasma amino acids were analyzed by the standard ninhydrin technology (Beckman Instruments, model 6 300, Palo Alto, CA), after deproteinization of the plasma with 5% salicylic acid<sup>[20]</sup>. Plasma GH (Cayman Chemical, Ann Arbor, MI) and insulin-like growth factor (IGF)-1 (Diagnostic Systems, Webster, TX) were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  levels in plasma and PLF were measured using commercial ELISA microtiter plates, with antibodies specific for rat IL-1 $\beta$ , IL-6, and TNF- $\alpha$  coated onto wells of the microtiter strips provided (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Flow cytometric phagocytosis test was used to evaluate the phagocytic activity of blood polymorphonuclear neutrophils<sup>[21,22]</sup>. One hundred microliters of heparinized whole blood was aliquoted on the bottom of a 12 mm $\times$ 75 mm Falcon polystyrene tube (Becton Dickinson) and placed in an ice-water bath. Twenty microliters of precooled opsonized FITC-labeled *E. coli* (Molecular Probes, Eugene, OR) was added to each tube. Control tubes remained on ice, and assay samples were incubated for precisely 10 min at 37 °C in a shaking water bath. After incubation, samples were immediately placed in ice water, and 100  $\mu$ L of a precooled trypan blue (Sigma, St. Louis, MO) solution (0.25 mg/mL in citrate salt buffer pH 4.4) was added to quench the fluorescence of the bacteria merely adhering to the surface of phagocytosing cells. Cells were washed twice in Hank's buffered saline (HBSS), and erythrocytes were lysed by the addition of FACS lysing solution (Becton Dickinson). After an additional wash in HBSS, 100  $\mu$ L of propidium iodide (PI) solution (1  $\mu$ g/mL in HBSS) was added to stain the nuclear DNA 10 min before the flow cytometric analysis. Flow cytometry was performed on an FACS Calibur™ flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. A live gate was set on the red (PI) fluorescence histogram during acquisition to include only those cells with a DNA content at least equal to human diploid cells. The number of cells with phagocytic activity did not exceed 6% at 0 °C.

A Vybrant™ phagocytosis assay kit (molecular probes) was used to evaluate the phagocytic activity of peritoneal macrophages. After the peritoneal macrophages were washed 3 times with HBSS, the cell concentration was counted, and the cell number was adjusted to 10<sup>6</sup> cells/mL with RPMI-1640 supplemented with 5% fetal bovine serum and an adequate quantity of antibiotics. After 100  $\mu$ L of diluted solutions was distributed into each well on 96-well microplates, it was transferred to a 37 °C CO<sub>2</sub> incubator for 1 h to allow the cells to adhere to the microplate surface. The RPMI solution was removed from all microplate wells by vacuum aspiration, and then 100  $\mu$ L of the prepared FITC-labeled *E. coli* was added to each well for 2 h. Labeled bacteria were removed by vacuum aspiration, and 100  $\mu$ L of trypan blue suspension was added to all wells within 1 min. The excess trypan blue was immediately aspirated, and the experimental and control wells (without peritoneal macrophages) were read in the fluorescence plate reader using -480 nm for excitation and -520 nm for emission.

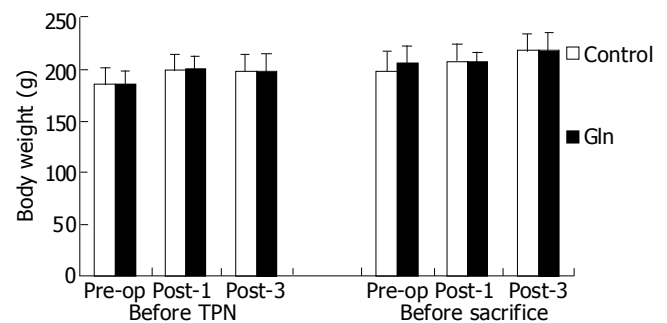
Twenty-four-hour urine specimens were collected during the 3 infusion days after surgery for determination of the nitrogen balance. Nonprotein nitrogen in urine was measured by a colorimetric method (Randox, Antrim, Ireland).

### Statistical analysis

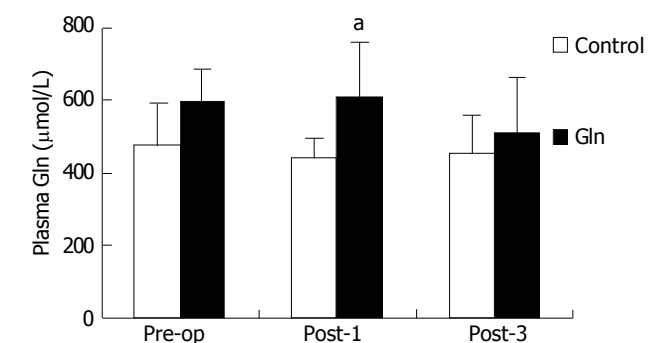
Data were expressed as mean $\pm$ SD. Differences among groups were analyzed by ANOVA using Duncan's test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

There were no differences in initial body weights between the 2 experimental groups at the beginning of TPN administration. All rats gained weight after TPN infusion, and weights were maintained postoperatively. No differences in body weights were seen between the 2 groups on postoperative d 1 and 3 (Figure 1). The Gln group had a higher plasma Gln level on postoperative d 1. No significant differences were observed before and 3 d after surgery (Figure 2).

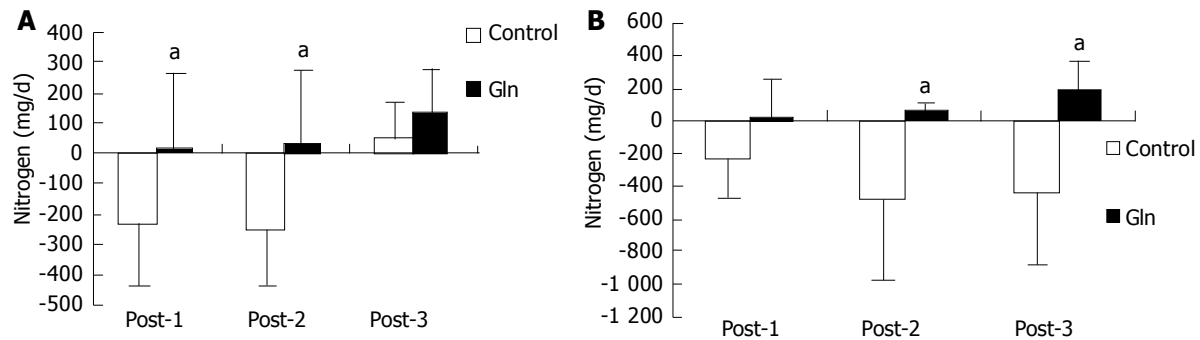


**Figure 1** Body weights of experimental groups at the beginning of TPN administration and before sacrifice.

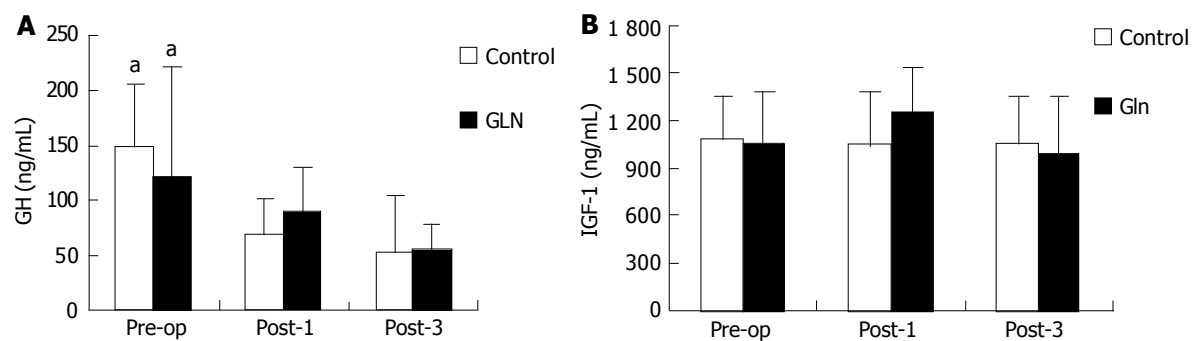


**Figure 2** Plasma glutamine (Gln) levels of the 2 groups before and after surgery. <sup>a</sup>*P*<0.05 vs control group on post-1d.

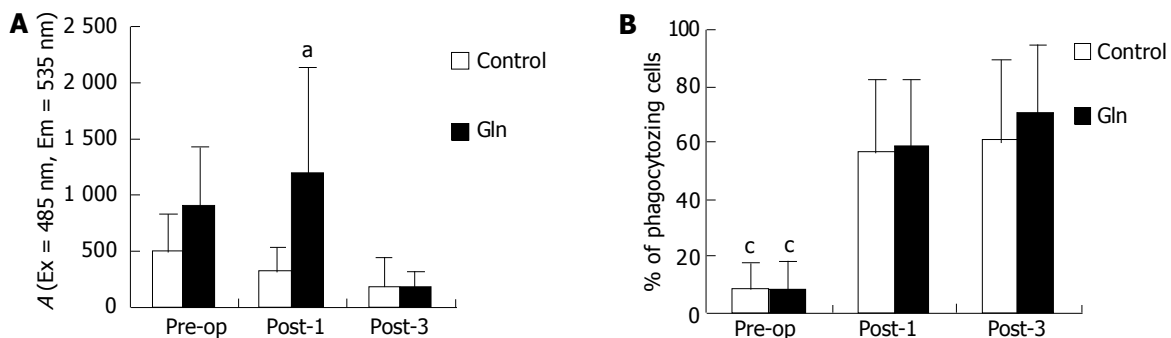
Compared with the control group, the Gln group had fewer nitrogen loss 1 and 2 d after surgery (Figure 3A). A significantly better cumulative nitrogen balance was observed in the Gln group on postoperative days (Figure 3B). Compared with the levels before surgery, plasma GH concentrations were significantly lower after surgery in the control group on both postoperative d 1 and 3, whereas there was only a difference on d 3 postoperatively in the Gln group. There



**Figure 3** Nitrogen balance and cumulative nitrogen balance between the 2 groups after operation. <sup>a</sup> $P < 0.05$  vs control group on post-operative days. A: Nitrogen balance between the 2 groups after operation; B: Cumulative nitrogen balance between the 2 groups after operation.



**Figure 4** Plasma growth hormone (GH) and insulin-like growth factor-I (IGF-I) concentrations between the 2 groups before and after operation. <sup>a</sup> $P < 0.05$  vs the corresponding group on post-operative days. A: Plasma growth hormone concentrations between the 2 groups before and after operation; B: Plasma insulin-like growth factor-1 concentrations between the 2 groups before and after operation.



**Figure 5** Phagocytic activity of peritoneal macrophages and peripheral blood neutrophils. A: Phagocytic activity of peritoneal macrophages measured by phagocytosis assay and read in the fluorescence plate reader using 480 nm for excitation and 520 nm for emission. <sup>a</sup> $P < 0.05$  vs control group on post-1d; B: Peripheral blood neutrophils measured by flow cytometry; <sup>c</sup> $P < 0.05$  vs the corresponding groups on post-operative days.

were no differences in GH and IGF-1 levels between the 2 groups before or after surgery (Figures 4A, B). The phagocytic activity of peritoneal macrophages was higher in the Gln group than in the control group on postoperative d 1 (Figure 5A). The phagocytic activities of blood PMNs were significantly higher after surgery than at the baseline, regardless of whether or not Gln was given. There were no significant differences in the phagocytic activities of blood PMNs between the 2 groups at various time points (Figure 5B). Plasma IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels were

undetectable. No significant differences in concentrations of IL-1 $\beta$  and IL-6 in PLF were observed between the 2 groups at the time we took the measurements. However, TNF- $\alpha$  levels in PLF were significantly lower in the Gln group than in the control group on postoperative d 3 (Table 2).

## DISCUSSION

In this study, 25% of total nitrogen in the TPN solution was supplied by Gln. This amount of Gln was previously

**Table 2** Interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  concentrations in PLF between the 2 groups before and after operation (mean $\pm$ SD)

	Pre-op (n = 10)	Post-1 (n = 10) pg/mL	Post-3 (n = 10)
IL-1 $\beta$			
Control	10.1 $\pm$ 6.8	13.6 $\pm$ 13.6	17.9 $\pm$ 22.6
Gln	8.7 $\pm$ 9.3	13.2 $\pm$ 5.6	5.9 $\pm$ 6.1
IL-6			
Control	88.9 $\pm$ 46.1	130.0 $\pm$ 21.7	131.5 $\pm$ 50.8
Gln	94.0 $\pm$ 10.4	144.5 $\pm$ 51.7	118.0 $\pm$ 64.3
TNF- $\alpha$			
Control	24.0 $\pm$ 16.6	10.2 $\pm$ 8.3	54.7 $\pm$ 28.5 <sup>a</sup>
Gln	12.7 $\pm$ 5.3	22.1 $\pm$ 24.9	27.1 $\pm$ 21.5 <sup>c</sup>

<sup>a</sup> $P < 0.05$  vs pre-op and post-1 groups in the same line, <sup>c</sup> $P < 0.05$  vs control group on post-3.

found to enhance the immune response in rodents<sup>[23,24]</sup>. We administered TPN before and after gastrectomy, to mimic the usual treatment for patients who were scheduled to undergo gastrectomy. These patients were frequently malnourished, and perioperative TPN was essential for adequate nutritional support. Since human studies may have wide variations owing to the age of patients, severity of disease, infected area of the stomach, and complications of other diseases, which may make interpretation difficult of the data, we used an animal model with a partial gastrectomy to investigate the effect of Gln on the catabolic and immune responses after abdominal surgery.

Injury to the body could result in a negative nitrogen balance together with a progressive loss of body protein<sup>[1,2]</sup>, possibly due to hormonal changes and cytokine secretion<sup>[25,26]</sup>. Many studies have shown that Gln supplementation could enhance skeletal muscle synthesis which might consequently improve nitrogen balance after elective surgery<sup>[5,27,28]</sup>. GH is known to exert many metabolic effects. Among them are nitrogen retention and preservation of muscle protein mass<sup>[14,15]</sup>. IGF-1 is one of the major effectors of GH action. The effects of GH are mediated in part by IGF-1 that is produced in the liver and locally in GH target tissues<sup>[29]</sup>. A study by Welbourne *et al*<sup>[16]</sup>, reported that oral Gln load was capable of elevating plasma GH in healthy adults. Hammarqvist *et al*<sup>[30]</sup>, demonstrated that GH together with Gln-containing TPN reduced nitrogen losses compared with Gln alone. The nitrogen retention data in the present study are in good agreement with those of previous reports<sup>[5,29,30]</sup>. However, we did not find an association between plasma GH, IGF-1 levels and Gln supplementation before or after operation. This finding suggests that GH and IGF-1 might not be responsible for attenuating nitrogen losses under the present experimental conditions.

Previous reports have shown that parenterally or enterally administered Gln lowered the incidence of infection in patients with bone marrow transplantation and multiple traumas<sup>[31,32]</sup>. Supplemental Gln improved the survival in experimentally *Escherichia coli*-induced peritonitis in rodents<sup>[33,34]</sup>. Nevertheless, the mechanisms underlying the enhancing effect of Gln on bactericidal capacity have not been fully elucidated. Gln is an important fuel for immune cells<sup>[6]</sup>. Macrophages could use Gln at a very high rate<sup>[35]</sup>. Some *in vitro* studies have shown

that Gln is required for macrophage phagocytosis<sup>[6,12,13,36]</sup>. In this study, we found that the phagocytic activity of peritoneal macrophages was much higher in the Gln group after surgery compared to the control group, whereas no differences were found in the phagocytic activities of blood PMNs between the 2 groups. These findings indicate that Gln supplementation can enhance the macrophage phagocytic activity at the site of injury. The effect of Gln on phagocytic cells in the systemic circulation was not obvious. In this study, we did not observe reduced plasma Gln levels after surgery. This result was consistent with the report by Parry-Billings *et al*<sup>[37]</sup> that plasma Gln levels did not change after a minor surgery. It is possible that partial gastrectomy performed in this study resulted in a minor metabolic stress. The rats were free of infection or other stresses that would cause a systemic response. Therefore, a tissue or an organ-specific nutrient like Gln exerted its effects locally but not systemically.

Cytokines are peptides produced by cells of the immune system that act as a mediator of the immune response and the response of tissues to injury. Studies have proposed that alterations in TNF- $\alpha$  and IL-6 can be used as biochemical markers of the stress response<sup>[27,28,38]</sup>. IL-6 has been considered as the most consistently identified cytokine mediator of postinjury infections<sup>[39]</sup>. High plasma concentrations of IL-1 and TNF- $\alpha$  were associated with increased severity of inflammatory diseases<sup>[39]</sup>. These cytokines in plasma were not detectable at the time we took measurements. However, cytokines in PLF were measurable. Compared with the baseline, IL-1 $\beta$ , and IL-6 levels did not change after surgery. This result may indicate that postinjury infection was not obvious in this study. We observed that TNF- $\alpha$  was lower in the Gln group than in the control group on postoperative d 3. This might mean that TPN with Gln could reduce the production of inflammatory mediators at the site of injury. An *in vitro* study by Rohde *et al*<sup>[40]</sup> showed that Gln had no effect on the production of IL-1 $\beta$ , IL-6, or TNF- $\alpha$ . Since it was an *in vitro* study, and samples used for evaluation were derived from healthy volunteers, responses to the stressful metabolic conditions observed in this study might differ and consequently lead to different immune responses.

In summary, parenterally infused Gln can significantly enhance peritoneal macrophage phagocytic activity, and the nitrogen balance can be improved. However, Gln supplementation has no effect on phagocytic cells in the systemic circulation, GH and IGF-1 might not be responsible for attenuating nitrogen losses in rats with a partial gastrectomy.

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• BASIC RESEARCH •

# Effect of Haimiding on the functioning of red cell membrane of FC and H<sub>22</sub> tumor-bearing mice

Yu-Bin Ji, Shi-Yong Gao, Wei-Ping Cheng

Yu-Bin Ji, Shi-Yong Gao, Wei-Ping Cheng, Postdoctoral Research Station, Institute of Materia Medica, Harbin Commercial University, Harbin 150076, Heilongjiang Province, China

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Correspondence to: Dr. Yu-Bin Ji, Postdoctoral Research Station, The Institute of Materia Medica, Harbin Commercial University, 138 Tongda Street, Daoli District, Harbin 150076, Heilongjiang Province, China. sygao2002@sina.com

Telephone: +86-451-84866001 Fax: +86-451-84866001

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## Abstract

**AIM:** To study the effect of Haimiding on the functioning of red cell membrane of FC and H<sub>22</sub> tumor-bearing mice.

**METHODS:** The membrane fluidity of red cells is measured with DPH fluorescence probe as a marker; the amount of red cell membrane proteins is measured using polyacrylamide gel electrophoresis; the amount of sialic acid (SA) on the surface of red cell membrane and the sealability of these cells are measured using colorimetric analysis.

**RESULTS:** Haimiding can lower the membrane fluidity of red cells in tumor-bearing mice and the amount of their membrane proteins, while increasing the amount of sialic acid in the membrane of red cells in these mice and enhancing the ability of the membrane of their red cells to reseal.

**CONCLUSION:** The anti-tumor effect of Haimiding on tumor-bearing mice is due to its ability to improve and restore the functions of the membrane of their red cell and to enhance the immune effect of the organisms.

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**Key words:** Membrane function; FC; H<sub>22</sub>

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## INTRODUCTION

Haimiding is a compound anticancer preparation combining

Chinese and Western medicines. It is prepared from the anticancer drug 5-Fu (5-fluorouracil) plus extracts from Chinese medicines including *Sargassum fusiforme*, *Ecklonia kurome*, *Astragalus chrysospermus*, and *Sophora flavescens*. Findings from our earlier studies show that Haimiding has some killing power on 8 kinds of human cancerous cells, namely, human oral epithelial KB, human esophageal carcinoma E<sub>ca</sub>-109, human proventriculus BGC-823, human pulmonary adenocarcinoma A<sub>549</sub>, human colon HCT-8, human breast MCF-7, human ovary A<sub>2780</sub>, and human liver Bel-7402. Its inhibitory effect is most prominent on BGC-823, E<sub>ca</sub>-109, and HCT-8 cells. It clearly can inhibit the formation of BGC-823, E<sub>ca</sub>-109, and HCT-8 colonies with the effect on E<sub>ca</sub>-109 colonies being the strongest. It has a clear inhibitory effect on the growth of FC tumors in mice, and can significantly prolong the life span of H<sub>22</sub> tumor-bearing mice<sup>[1]</sup>. The cell membrane, which constitutes a barrier between the cytoplasm and the tissue, contains many kinds of proteins<sup>[2-5]</sup> and receptors<sup>[6-8]</sup>, which are related to the functioning of the organism and are closely related to the genesis and development of tumors<sup>[10-14]</sup>. Based on these, we tried in the present study to observe the effect of Haimiding on the functioning of red cell membrane of tumor-bearing mice, aiming to explicate the mechanism of the anticancer effect of Haimiding.

## MATERIALS AND METHODS

### Materials

**Test animals** White mouse (of the Kunming strain), acquired from Laboratory Animal Center of Heilongjiang University of Chinese Medicine (certificate no.: Heilongjiang animal No. 00101003), half-male and half-female, weighing 20±2 g.

**Tumor strains** FC and H<sub>22</sub> tumor-bearing mice, acquired from the Institute of Tumor Research, Harbin Tumor Hospital.

**Major reagents** Haimiding is provided by the Institute of Materia Medica, Harbin Commercial University (lot no.: 20010517); 5-FU, acquired from Shanghai No. 12 Pharmaceutical Company (lot no.: 20010601); DPH (1,6-diphenyl-1, 3, 5-hexatriene), product of Fluke AG.

**Main equipment** RF-540 fluorescence spectrophotometer, produced in Shimadzu, Japan; thermostatic water bath, acquired from Xiamen Medical Electronic Instruments.

### Methods

**Development of the mouse tumor model** (1) Drawing abdominal dropsy. The mouse was killed by taking apart at the joint of its cervical vertebra 7 d after tumor cells were transplanted. The animal was then fixed on a wax plate. After sterilization, the abdominal skin was cut open and peeled off. A syringe sterilized under high temperature was

used to draw the abdominal dropsy, which was put in a germ-free container surrounded by ice cubes for storage. In addition, a small amount of abdominal dropsy was drawn and put in a test tube to be used for observing and counting the cells. A drop of the abdominal dropsy remaining in the syringe was put on a slide, which was smeared and dyed with Wright's staining, and the cells were assorted and counted. The sample was to be used only when 97% or more of the cells were cancerous. (2) Inoculation. The abdominal dropsy was diluted with normal saline to 1:4. The FC tumor-bearing mouse was created by injecting the dropsy at the animal's armpit, and the H<sub>22</sub> tumor-bearing mouse was created by injecting the dropsy at the animal's abdomen.

**Grouping and administration of drugs** (1) Grouping. The mice were randomly divided into 6 groups, with a normal group, a negative control group, a positive control group, and another 3 groups to be given high, medium, and low dosages of Haimiding. Each group consists of 10 mice. (2) Administration of drugs. Twenty-four h after tumor cells were transplanted into the mice, drugs were administered by abdominal injection once a d for 7 consecutive d. The positive control group was given 25 mg/kg of 5-FU, the treated groups were given 25, 50, and 100 mg/kg of Haimiding, and the negative control group was given normal saline of the same volume. On the day following the last day of drug administration, the animals were killed, and relevant measurements were taken.

#### **Effect of Haimiding on the immunological adhesiveness of red in tumor-bearing mice to tumor cells**

After the animal was killed, blood was drawn, centrifuged, and rinsed to make a red cell suspension ( $1 \times 10^8$  piece/mL) to be used later. When the experiment was conducted, H<sub>22</sub> tumor cells were drawn from a 7-d old abdominal cavity of the mouse and rinsed twice with Hanks' solution to make a tumor cell suspension ( $1 \times 10^6$ /mL). 0.1 mL of this suspension was taken and an equal volume of fresh guinea pig (or human) blood was added to the sample. The sample was then set in water bath at 37 °C for 1 h, centrifuged and rinsed twice, with the supernatant being discarded to yield serum-sensitized cancer cells. 0.05 mL of the red cell suspension was set in water bath at 37 °C for 30 min, and 0.25% glutaraldehyde was added to fix the solution, a slide was smeared with the preparation and dyed using Wright's staining. At this point, the tumor cells appeared to be blue, while red cells were red. One tumor cell joining the 3 or more red cells made up a tumor-red cell wreath. The percentage of tumor-red cell wreath was calculated.

#### **Effect of Haimiding on the fluidity of red cell membrane of tumor-bearing mice**

A 2 mL 0.1% heparin was added to each of the several 10-mL test tubes. Three drops of blood was drawn from the experiment animal by cutting its tail. The blood were put in those tubes, mixed, and centrifuged for 10 min at 3 000 r/min. After the supernatant was discarded, the residue was rinsed 3 times with isotonic phosphoric acid buffer solution, and the red cells were counted. With the number of red cells thus obtained, a red cell suspension with  $4 \times 10^7$  piece/mL was made. A 2 mL of the 4 mL red cell suspension was taken and added to a test tube as the blank tube, while the test tube containing the remaining 2 mL of the red cell suspension was used for

reference. A 2 mL of DPH probe solution was added to the reference tube, while the same amount of isotonic PBS buffer solution was added to the blank tube. Solution in the two tubes was mixed and incubated for 30 min under a temperature of 25 °C and then centrifuged for 10 min at 3 000 r/min. The remaining DPH probe solution was discarded, and the residue was rinsed twice with isotonic PBS buffer solution and then diluted into 4 mL of cell suspension with isotonic PBS buffer solution. Immediately after this, the fluorescence polarization of the residue was measured. RF-540 fluorescence spectrophotometer with xenon lamp as light source was used to measure the intensity P of fluorescence polarized light both when it was parallel to and when it was perpendicular to the direction of vibration of the excitation polarized light, at a fluorescence excitation wavelength of 362 nm, a radiation wavelength of 432 nm, and under a temperature of 25 °C. Then the micro viscosity was calculated.

$$\eta = \frac{2P}{0.46 - P}$$

#### **Effect of Haimiding on the membrane protein content in the red cells of tumor-bearing mice: Preparation of red cell membrane**

Whole blood

↓ centrifuged at 3 000 r/min for 10 min, rinsed twice with 1:3 saline, and suspended in 1:1 saline

Red cell suspension

↓ 5p 8.4 phosphoric buffer solution (1:30) was added at 4 °C and hemolysis was allowed to proceed for 1 h.

Hemolyzed solution of red cells

↓ centrifuged at 2 000 r/min at 4 °C for 40 min; the supernatant was discarded; the residue was rinsed 3 times and then suspended in 5p8.4 phosphoric acid buffer solution.

Membrane suspension

The concentration of red cell membrane protein was measured according to Lowry's rule. DSD-polyacrylamide gel electrophoresis was performed to separate the cell membrane protein and measure its content.

Gel, gel plate, gel repository solution, electrolytic tank buffer solution, and dyeing solutions I and II were prepared as described in literature<sup>[15]</sup>. SDS was used to solubilize membrane so as to separate the proteins from the membrane. The sample was loaded in the following manner: 50 μL of membrane sample was sucked with a microsyringe. After the sample was loaded, it was immediately covered with electrolytic tank buffer solution, and tap water and cooling water tube of the electrophoresis tank were turned on. Electrophoresis was conducted for 3.5 h with the upper tank as the cathode and the lower one as the anode, the voltage adjusted to 50 V and electric current to 30 mA. After electrophoresis, the gel was stripped off and the sample was dyed. The gel plate was placed on a CS-930 double-wavelength scanner and scanned at 560 nm to yield an electrophoretogram of the membrane protein, and then the percentages of the components of the membrane protein were calculated.

#### **Effect of Haimiding on the sialic acid content on the**

**Table 1** Steps for measuring the activity of the sialic acid content on the surface of the red cell membrane

Reagent	Blank	1	2	3	4	5	6	7
N-acetyl neuraminic acid Standard solution (mL)	0	0.05	0.1	0.2	0.3	0.4	0.5	0.8
N-acetyl neuraminic acid (μg)	0	5	10	20	30	40	50	80
Distilled water (mL)	1	0.95	0.9	0.8	0.7	0.6	0.5	0.2
Bialsche reagent (mL)	1	1	1	1	1	1	1	1
Boil for 12 min in water bath, then cool for 3 min with ice water								
Pentanol (mL)	5	5	5	5	5	5	5	5
Shaken thoroughly and then centrifuged for 10 min at 1 000 r/min; took the pentanol phase for colorimetric analysis at a wavelength 569 nm								

**Table 2** Steps for measuring the activity of the oxidoreductase NADH-cell pigment C

Reagent (mL)	No saponin added (-S)		Saponin added (+S)	
	Blank	Sample	Blank	Sample
0.6 mg protein/mL Membrane suspension	0.6	0.6	0.6	0.6
PBS (5p8.4)	2.4	2.2	2.2	2.0
0.1% saponin	/	/	0.2	0.2
5 mmol/L K <sub>3</sub> Fe (CN) <sub>6</sub>	/	0.1	/	0.1
Let the solution stand for 10 min, added NADH, and recorded the result after 1 min 6 mmol/L NADH	/	0.1	/	0.1

### surface of the red cell membrane of tumor-bearing mice

Red cell membrane was prepared as before.

The standard curve was plotted.

0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.8 mL of *N*-acetyl neuraminic acid was added to 8 test tubes each, which was to say the test tubes contained 0, 5, 10, 20, 30, 40, 50 and 80 μg respectively of *N*-acetyl neuraminic acid. Then the optic density (*A*) of each tube was measured according to the procedures in the following table, and a standard curve was plotted for the *N*-acetyl neuraminic acid content (Table 1).

### Measurement of sialic acid content in red cell membrane

Bialsche reagent was added to 1 mL of the membrane suspension prepared earlier (0.6 mg protein per millimeter membrane liquid) to measure directly the sialic acid (SA) content (by measuring the absorbance, or *A*). The procedure was the same as above.

### Effect of Haimiding on the sealability of the red cell membrane of tumor-bearing mice

**Preparation of closed membrane (isotonic closed membrane) from red cells** First, an unclosed membrane preparation was made in the same manner as before. The membrane was then rinsed once with isotonic phosphoric acid buffer solution, centrifuged for 40 min at 2 000 r/min, and then placed in isotonic solution, which was kept at 37 °C for 1 h to yield closed membrane. The activity of the oxidoreductase NADH-cell pigment C was measured by the method described in literature<sup>[16]</sup>: a 0.6 mL sample of membrane (membrane fluid) was put in a colorimetric cup, and reactants were added as shown in the Table 2. After the reactants were each mixed with the sample, their colors were compared at a wavelength of 420 nm. The time at which NADH was added was taken as time zero, and *A* was recorded once every min for 6 min after the reaction starts. The reaction was to take place under room temperature (25 °C). Colorimetric analysis was performed. The procedure was the same as above except that NADH was not added.

Specific steps were shown in Table 2.

**Calculation of sealability** The sealability of membrane (%) was calculated according to the formula below:

$$\text{Sealability (\%)} = \frac{\text{Activity when surfactant is added} - \text{Activity when no surfactant is added}}{\text{Activity when surfactant is added}} \times 100\%$$

### Statistical analysis

*t* test was performed on data obtained from the experiments, with the results expressed in the format mean±SD.

## RESULTS

### Effect of Haimiding on the immunological adhesiveness of red cells in tumor-bearing mice to tumor cells

Experimental results showed that the immunological adhesiveness of red cells in tumor-bearing mice, as measured by the rate of wreaths, was weaker than that in the control group, with the difference being more prominent with H<sub>22</sub> mice than with FC mice. And the rate of wreaths was significantly higher (*P*<0.01) in the treated group than in the group that was administered normal saline, with the level for FC group that was treated being close to the normal, while that for the 5-Fu group showing no clear increase, or even showing some trend toward decreasing (Tables 3, 4).

**Table 3** Effect of Haimiding on the immunological adhesiveness of red cells of FC mice to tumor cells (mean±SD)

Group	Number of animals ( <i>n</i> )	Route of administration	Dosage (mg/kg)	Rate of wreaths (%)
Control	10	Ip	Equal volume	36.36±5.42
Saline	10	Ip		10.46±3.55
5-Fu	10	Ip	25	8.41±2.56
Haimiding	10	Ip	50	28.96±4.41 <sup>b</sup>

<sup>b</sup>*P*<0.01 vs the saline group.

**Table 4** Effect of Haimiding on the immunological adhesiveness of red cells of H<sub>22</sub> mice to tumor cells mean±SD

Group	Dosage (mg/kg)	Route of administration	No. of animals (n)	Rate of wreaths (%)
Control		Ip	10	36.36±5.42
Saline	Equal volume	Ip	10	5.10±2.28
5-Fu	25	Ip	10	5.96±3.15
Haimiding	50	Ip	10	25.68±4.13 <sup>b</sup>

<sup>b</sup>P<0.01 vs the saline group.**Effect of Haimiding on the fluidity of intact red cell membrane of tumor-bearing mice**

From Tables 5, 6 it can be seen that, compared to the control, the micro viscosity of red cells in tumor-bearing mice had increased, and the membrane fluidity had decreased. But after treatment, the microviscosity of red cells in the two strains of tumor-bearing mice decreased to different degrees, which means membrane fluidity had increased. The effect was the most prominent with the group treated with medium dosage of Haimiding ( $P<0.01$ ).

**Effect of Haimiding on the membrane protein content in the red cells of tumor-bearing mice**

From Tables 7, 8 it can be seen that the percentage of polymers formed on the red cell membrane of the tumor-bearing mice of the saline group was higher than that in the control group, and that the level of polymers decreased significantly after the animals had been treated with medium dosage of Haimiding ( $P<0.01$ ). For different tumor-bearing organisms, the effect of high dosage of Haimiding differed to some extent. The results also showed that for the same dosage, the effect for H<sub>22</sub> mice was more prominent than that for FC mice.

**Table 7** Effect of Haimiding on the membrane protein content in the red cells of FC mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Membrane protein content %
Control	10	Ip	Equal volume	0.281±0.051
Saline	10	Ip	25	0.332±0.094
5-Fu	10	Ip	100	0.278±0.041
Haimiding	10	Ip	50	0.241±0.037 <sup>a</sup>
Haimiding	10	Ip	25	0.187±0.042 <sup>b</sup>
Haimiding	10	Ip		0.296±0.040

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs the saline group.**Table 8** Effect of Haimiding on the membrane protein content in the red cells of H<sub>22</sub> mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Membrane protein content %
Control	10		Equal volume	0.281±0.051
Saline	10	Ip	25	0.298±0.088
5-Fu	10	Ip	100	0.219±0.089
Haimiding	10	Ip	50	0.237±0.050
Haimiding	10	Ip	25	0.143±0.064 <sup>b</sup>
Haimiding	10	Ip		0.189±0.094 <sup>a</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs the saline group.**Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of tumor-bearing mice**

The results are shown in Tables 9, 10. From the study on the effect of Haimiding on the SA content in red cell membrane of tumor-bearing mice, it was found that the SA content for tumor-bearing mice especially for those of the H<sub>22</sub> strain was significantly lower than that for normal mice. After Haimiding was administered, the SA content in the red cell membrane of tumor-bearing mice increased to different degrees, with the medium- and high-dosage groups markedly different from the saline group

**Table 5** Effect of Haimiding on the fluidity of intact red cell membrane of FC mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Fluorescence polarization (P)	Micro viscosity, $\eta$ (P)	Membrane lipid fluidity (LFU)
Control	10		Equal volume	0.2006±0.0728	1.7557±0.9616	7.4472±2.3273
Saline	10	Ip	25	0.2438±0.0122	1.9458±0.2367	4.3134±1.4378
5-Fu	10	Ip	100	0.2169±0.0269	1.8183±0.3799	6.0234±1.9430 <sup>a</sup>
Haimiding	10	Ip	50	0.215±0.0242 <sup>b</sup>	1.796±0.1414 <sup>a</sup>	6.1913±1.9348 <sup>a</sup>
Haimiding	10	Ip	25	0.210±0.0137 <sup>b</sup>	1.701±0.2063 <sup>a</sup>	6.4966±1.9687 <sup>b</sup>
Haimiding	10	Ip		0.2203±0.0338	1.7330±0.4404	5.7670±1.6962 <sup>a</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs the saline group.**Table 6** Effect of Haimiding on the fluidity of intact red cell membrane of H<sub>22</sub> mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Fluorescence polarization (P)	Micro viscosity, $\eta$ (P)	Membrane lipid fluidity (LFU)
Control	10			0.2006±0.0728	1.7557±0.9616	7.4478±2.3273
Saline	10	Ip	Equal volume	0.2430±0.03017	2.1378±0.6186	4.3559±1.4051
5E-Fu	10	Ip	25	0.2275±0.0182	1.9760±0.6738	5.2606±1.5472
Haimiding	10	Ip	100	0.2331±0.0211	2.0819±0.1772	4.9153±1.5360
Haimiding	10	Ip	50	0.1770±0.0567 <sup>b</sup>	1.2775±0.4164 <sup>b</sup>	11.0370±3.4489 <sup>b</sup>
Haimiding	10	Ip	25	0.2111±0.0223 <sup>a</sup>	1.8176±0.2106	6.4776±2.0895 <sup>a</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs the saline group.

( $P < 0.01$ ), and the effect on the medium-dosage group being the greatest.

**Table 9** Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of FC mice

Group	Number of animals ( <i>n</i> )	Route of administration	Dosage (mg/kg)	A
Control	10	Ip		0.183±0.068
Saline	10	Ip	Equal volume	0.087±0.026
5-Fu	10	Ip	25	0.065±0.030
Haimiding	10	Ip	100	0.139±0.049 <sup>b</sup>
Haimiding	10	Ip	50	0.148±0.032 <sup>b</sup>
Haimiding	10	Ip	25	0.095±0.028

<sup>b</sup> $P < 0.01$  vs the saline group.

**Table 10** Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of H<sub>22</sub> mice

Group	Number of animals ( <i>n</i> )	Route of administration	Dosage (mg/kg)	A
Control	10	Ip		0.183±0.068
Saline	10	Ip	Equal volume	0.062±0.027
5-Fu	10	Ip	25	0.064±0.034
Haimiding	10	Ip	100	0.119±0.040 <sup>b</sup>
Haimiding	10	Ip	50	0.125±0.044 <sup>b</sup>
Haimiding	10	Ip	25	0.081±0.023

<sup>b</sup> $P < 0.01$  vs the saline group.

### Effect of Haimiding on the sealability of the red cell membrane of tumor-bearing mice

The results in Tables 11, 12 show that the sealability of the red cell membrane of tumor-bearing mice was lower than that for normal mice. The level of sealability remains constant even when positive control drug was administered, but the administration of Haimiding could raise the capacity of red cells to reseal. The improvement was significant ( $P < 0.01$ ) for all 3 groups administered with different dosages.

**Table 11** Effect of Haimiding on the sealability of the red cell membrane of FC mice

Group	Number of animals ( <i>n</i> )	Route of administration	Dosage (mg/kg)	Sealability (%)
Control	10	Ip		70.6±4.05
Saline	10	Ip	Equal volume	54.6±2.21
5-Fu	10	Ip	25	52.0±3.39
Haimiding	10	Ip	100	63.5±3.11 <sup>b</sup>
Haimiding	10	Ip	50	65.9±2.60 <sup>b</sup>
Haimiding	10	Ip	25	58.4±2.05 <sup>b</sup>

<sup>b</sup> $P < 0.01$  vs the saline group.

**Table 12** Effect of Haimiding on the sealability of the red cell membrane of H<sub>22</sub> mice

Group	Number of animals ( <i>n</i> )	Route of administration	Dosage (mg/kg)	Sealability (%)
Control	10	Ip		70.2±2.23
Saline	10	Ip	Equal volume	53.7±2.30
5-Fu	10	Ip	25	54.2±3.27
Haimiding	10	Ip	100	62.2±2.71 <sup>b</sup>
Haimiding	10	Ip	50	61.4±3.44 <sup>b</sup>
Haimiding	10	Ip	25	57.6±2.54 <sup>b</sup>

<sup>b</sup> $P < 0.01$  vs the saline group.

## DISCUSSION

The cell membrane is an important structure indispensable for all cells. It has extremely important biological functions. The immunity of the cell and tumor genesis have all to do with the cell membrane. The immune effect of red cells is realized through the CR1 receptors on the membrane of these cells<sup>[18,19]</sup>. The new concept “Red-Cell Immune System” was first proposed in 1981 by the American scholar Siegel. Through extensive research in the last two decades, scholars in China and abroad have discovered that red cells, which have their origin in hemopoietic stem cells, have the most complete immunizing functions in the human body. They contain many substances that have to do with immunity and are capable of self-adjustment and self-control as well as play a role in the organism’s immunological adjustment and control. Red cells are capable of recognizing, adhering to, and killing antigens, transmitting information about the antigens, and eliminating the immunity-inhibiting substances in circulation. Clinically, immunological defects of red cells play an important role in the pathogenesis of many diseases especially in the genesis of tumors.

In experiments we did in the past, we discovered that Haimiding can significantly improve the immunological functions of the red cells in tumor-bearing mice by significantly increasing the wreath rate of C3b receptors on red cells and the activity of promoters of immunological adhesiveness while significantly decreasing the wreath rate of immunological compounds in red cells and the activity of the inhibitors of immunological adhesiveness. In all 4 of these indices, Haimiding demonstrates an immune effect, which is not possessed by the chemotherapeutic drug 5-Fu. According to research results from recent years, red cells can immunologically adhere to various kinds of tumor cells, and under electron microscope it can be observed that tumor cells are damaged at points where they make contact with red cells, suggesting that red cells play the role of effector cells in the immune reactions against tumors. If there are autoantibodies for red cells in the serum of patients afflicted with tumors, this function of red cells is weakened<sup>[19,20]</sup>. In our experiments we also discovered that the capacity of red cells to adhere to H<sub>22</sub> tumor cells decreases significantly in tumor-bearing mice as compared to normal mice, but this index is significantly improved for the groups which are administered Haimiding, while the administration of 5-Fu does not yield this kind of effect.

Patients afflicted with tumor often are also anemic<sup>[21-23]</sup>. At the same time, both the number and the activity of the CR1 on their red cells decrease significantly, resulting in low immune efficacy of their red cells. The immune efficacy of red cells in metastatic cancer patients is even lower, but after operation on the digestive tract, the immunological adhesiveness of these cells increases significantly. Research in recent years shows that red cells can immunologically adhere to tumor cells and prevent cancer cells from undergoing deametastasis, which is the main route through which tumors spread. The number of red cells is 1 000 times that of white cells, and the chances for their getting into contact with tumor cells are much greater than that for white cells, so red cells play a more important role in preventing malignant tumor from metastasizing. Moreover, by

immunologically adhering to the CD2 on the membrane of T lymphocytes with the CD58 and CD59 on their own membrane, red cells can activate the immunological functions of T cells, which are expressed as an increase in the activity of IL-2R and in the secretion of  $\gamma$ -interferon. Red cells can release NK cell activators, thus increasing the activity of NK cells in killing cancer cells. Red cell membrane also contains receptors for chemotactic factors that can attract drifting chemotactic factors, thus reducing the concentration of chemotactic factors in blood circulation while enhancing the inflammation reaction of the tumorous tissue in question<sup>[24]</sup>. To sum up, red cells do not only perform antitumor immunological functions on their own, but play an important role in the overall control by the immune system of the human body that involves many kinds of cells and tissues and works through many routes, forming an antitumor immune control system.

Haimiding can enhance the capacity of red cells in tumor-bearing mice to adhere immunologically to tumor cells, which shows that it has an important effect on the spreading and metastasis of tumor. Furthermore, Haimiding can activate the immune system of the whole organism by enhancing the immunological effect of red cells, thus bringing about some overall therapeutic effects. The mechanism by which Haimiding enhances the immunological function of red cells may be as follows: The membranes of red cells contain some glycoprotein receptors, while *Astragalus chrysopterus*, a component of Haimiding, contains some glycoproteins. These glycoproteins can adhere to the glycoprotein receptors on the membrane of red cells, activating the CR1 on the membrane, thus enhancing the immunological effect of red cells.

The major types of membrane lipids of red cells include phospholipids (ovolecithin, cephalin, serine phosphatide, and sphingomyelin), cholesterol, and glycolipids. These lipids are arranged in a bi-layer molecular array, with the outer layer rich in ovecithin. Phospholipids, cholesterol, and glycolipids all contain asymmetric hydrophilic and hydrophobic groups. In the bi-layer structure, the hydrophilic parts point outward, while the hydrophobic parts point toward the center of the structure. The two layers of lipids are fluid. Fluidity is an important mechanical property of cell membrane. All the normal physiological functions of red cells depend on the integrity of their membranes and the fluidity of the bi-layer structure of membrane lipids. The fluidity of red cell membrane has implications for the permeability, deformability, fragility, translocation of solutes and enzyme activity. It is directly related to the constituents of the serum especially those of serum lipoproteins<sup>[25]</sup>. Therefore, the study of the effect of Haimiding on the fluidity of red cell membrane can help us explore at the cellular and molecular levels the mechanism by which Haimiding works. As is well known, part of the molecular basis of malignant proliferation of cells is abnormality in the transmembrane signal transmission chain. The malignant transformation and redifferentiation of tumor cells may be related to the damage and repair respectively of the transmembrane signal transmission chain. Modifying or multiplying certain molecules involved may induce abnormalities in the chain and disorganization of the molecular processes, resulting in

the loss of control of cell transformation and growth, which would lead to malignant tumors. Repairing or changing certain molecules involved can help restore the damaged transmembrane signal transmission chain, causing malignant tumor cells to redifferentiate and thus helping cancer cells to reverse their course of development. This procedure involves many kinds of molecules on the cell membrane, especially membrane proteins and membrane lipids. The canceration of cells and its reversion are both related to the fluidity of membrane<sup>[4,26]</sup>. Experiments in the present study show that Haimiding can lower the microviscosity of red cell membrane in tumor-bearing mice, resulting in the increase of membrane fluidity, and is thus helpful for adjusting the immunological functions of red cells in the organism and for restoring to normal the damaged transmembrane signal transmission chain, thus increasing the killing power of red cells on tumor cells.

After red cells are hemolyzed hypotonically and centrifuged at high speed, red cell membrane can be obtained. This is the so-called "ghost". Following treatment with detergent, SDS-polyacrylamide gel electrophoresis is performed on the sample. Red cell membranes are separated into different bands, which are treated with antibodies for shrinking protein, anchor protein, band 3 protein and band 4.1 proteins in immunoblot experiment. The results show that the polymers consist mainly of shrinking protein, anchor protein, and band 3 protein<sup>[14]</sup>. Thus far, we have discovered that the immune effect of red cells is mainly due to the protein molecules, such as CR<sub>1</sub> and CR<sub>3</sub>, on their membranes. These proteins on the membranes of red cells are specially structured. They are not fixed on the membrane with a transmembrane structure of more than 20 hydrophobic amino acids, but are anchored on the membrane by covalently binding with some glycosyl phosphoric acid myoinositol, which increases their movability on the membrane, enabling these protein molecules, which are of a limited number, to be in direct contact with a large number of ligands to bring out their biological functions. However, in certain circumstances, such as when the organism is experiencing abnormality in its physiological functions, or when the cell membrane is damaged, or when shrinking proteins (i.e., band 1 and band 2 proteins) and band 4.1 proteins are cross-linked into high polymers, the composition of membrane proteins would change greatly. The cross-linking of membrane proteins directly affects the degree of freedom with which their spatial configurations can be changed, leading to changes in the mechanical properties of the membrane<sup>[27]</sup>. But findings from experiments for the present study show that Haimiding can improve the spatial stability of membrane proteins that have already undergone changes. From comparison with the saline group, it can be shown that Haimiding can significantly lower the production of high polymers of red cell membrane proteins of tumor-bearing mice, with the effect being significantly better with the groups administered medium dosage of Haimiding (50 mg/kg) than with the other two groups ( $P < 0.01$ ). Decrease in the amount of high polymers of membrane proteins can increase the fluidity of the membrane, decrease its microviscosity, accelerate the speed of blood flow, and increase the capacity of red cells to adhere immunologically to tumor cells. These findings

show that Haimiding can enhance the immunological effect of red cells of the organism by lowering the production of high, protein polymers on the membrane of these cells, thus enabling them to adhere immunologically to tumor cells.

SA is a residue on the extremes of glycoproteins and the sugar chain of glycolipids on cell membrane that is widely distributed on the surface of the cell membrane (including that of red cells) of mammals and is the main source of negative cellular electric charges. It is also an important component of receptors on cell membrane, taking part in physiological processes such as cell differentiation, the metastasis of cancer cells, and the recognition, adhesion, and tactile inhibition of cells. It is a key to determining what important features the cell membrane has<sup>[28,29]</sup>. Therefore, changes in the SA content of cell membrane can lead to a series of biological changes inside the cell. SA is also an important component of the C3b receptor (CR1) of red cells<sup>[30]</sup>. As mentioned earlier, CR1 is an important material basis for red cells to perform their immunological functions, that is to say, the amount of SA determines the activity of CR1 receptors, thus also determining the immune effect of the organism's red cells. Experimental results show that the SA content of red cell membrane of tumor-bearing mice has clearly decreased, and that it further decreases after chemotherapy. The SA content of red cell membrane of groups to which Haimiding was administered, on the other hand, was clearly higher than that of the negative control and the positive groups, and the gap between them was very large. Based on the above analysis, it seems that Haimiding serves to enhance the immunity of the organism by affecting the SA content in red cell membrane and thus the activity of CR1.

Since the SA content of cell membrane is closely related to its activity, further research on the effect of Haimiding on the activity of red cell membrane is very important. In isotonic solution, the membrane of red cells can reseal, thus restoring the permeability barrier to macromolecules and cations, forming a sealed membrane. The index measuring the capacity of red cell membrane to reseal is sealability. Due to the growth of the tumor and the effect of anti-tumor drugs, the functioning of the membrane will inevitably change, and sealability can be used to indicate the extent of this change. Our experimental results show that the sealability of the red cell membrane of tumor-bearing mice is lower than that of normal mice, but Haimiding can increase sealability, suggesting that it can help restore the functions of red cell membrane. 5-Fu, on the other hand, does not have this effect. Increase in the activity of red cell membrane results in its various functions approaching the normal, which is the fundamental reason why the immune efficacy of red cells is enhanced and why the secretion of SA is increased. The membrane of tumor cells can also reseal, but its sealability is lower than that of normal red cells. Findings from the treated groups show that both Haimiding and 5-Fu can lower the sealability of tumor cells, suggesting that the activity of cell membrane has been lowered, which may lead to the disorganization and death of the tumor cells.

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• BASIC RESEARCH •

# Reversal of the phenotype by *K-ras<sup>val12</sup>* silencing mediated by adenovirus-delivered siRNA in human pancreatic cancer cell line Panc-1

Li-Mo Chen, Huang-Ying Le, Ren-Yi Qin, Manoj Kumar, Zhi-Yong Du, Rui-Juan Xia, Jing Deng

Li-Mo Chen, Ren-Yi Qin, Manoj Kumar, Zhi-Yong Du, Rui-Juan Xia, Department of Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Huang-Ying Le, Center for Biotechnology, College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian Province, China

Jing Deng, College of Life Sciences, Wuhan University, Wuhan 430072, Hubei Province, China

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Co-first-authors: Li-Mo Chen and Huang-Ying Le

Correspondence to: Ren-Yi Qin, Department of Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. ryqin@tjh.tjmu.edu.cn

Telephone: +86-27-83662389

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vectors can be used to mediate RNA interference (RNAi) to induce persistent loss of functional phenotypes. In gene therapy, the selective down-regulation of only the mutant version of a gene allows for highly specific effects on tumor cells, while leaving the normal cells untouched. In addition, the apoptosis of pancreatic cancer cell line Panc-1 can be induced after AdH1-*K-ras<sup>val12</sup>* infection. This kind of adenovirus based on RNAi might be a promising vector for cancer therapy.

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**Key words:** Pancreatic cancer; siRNA; Adenovirus; Phenotype

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## Abstract

**AIM:** To investigate the *in vitro* antitumor effect of adenovirus-mediated small interfering RNAs (siRNAs) on pancreatic cancer and the associated mechanism.

**METHODS:** A 63-nucleotide (nt) oligonucleotide encoding *K-ras<sup>val12</sup>* and specific siRNA were introduced into pSilencer 3.1-H1, then the H1-RNA promoter and siRNA coding insert were subcloned into pAdTrack to get plasmid pAdTrackH1-*K-ras<sup>val12</sup>*. After homologous recombination in bacteria and transfections of such plasmids into a mammalian packaging cell line 293, siRNA expressing adenovirus AdH1-*K-ras<sup>val12</sup>* was obtained. Stable suppression of *K-ras<sup>val12</sup>* was detected by Northern blot and Western blot. Apoptosis in Panc-1 cells was detected by flow cytometry.

**RESULTS:** We obtained adenovirus AdH1-*K-ras<sup>val12</sup>* carrying the pSilencer 3.1-H1 cassette, which could mediate gene silencing. Through siRNA targeted *K-ras<sup>val12</sup>*, the oncogenic phenotype of cancer cells was reversed. Flow cytometry showed that apoptotic index of Panc-1 cells was significantly higher in the AdH1-*K-ras<sup>val12</sup>*-treatment group (18.70% at 72 h post-infection, 49.55% at 96 h post-infection) compared to the control groups (3.47%, 3.98% at 72 and 96 h post-infection of AdH1-empty, respectively; 4.21%, 3.78% at 72 and 96 h post-infection of AdH1-p53, respectively) ( $P < 0.05$ ).

**CONCLUSION:** These results demonstrate that adenoviral

## INTRODUCTION

Pancreatic carcinoma is a very aggressive carcinoma and has the worst prognosis among common gastrointestinal cancers. Its carcinogenesis is a multistep process, often requiring 7 to 10 discrete (epi) genetic events that endow the cells with an ever-increasing proliferative advantage<sup>[1]</sup>. These multiple genetic alterations include dominant mutant oncogenes. It is often not clear which of these oncogenes is continuously required, and which may inhibit tumorigenesis when inactivated. In order to develop effective anti-cancer therapies, it is essential to know which of these events is still required late in the process of tumor progression to maintain an oncogenic phenotype. In 85% cases of pancreatic carcinoma, *Ras* genes were mutated<sup>[2,3]</sup>. The proteins encoded by the *Ras* genes (*K-ras*, *H-ras*, and *N-ras*) are guanine nucleotide binding proteins that associate with the inner plasma membrane and transduce external signals to the interior of cells. They regulate a broad spectrum of cellular activities, including cell proliferation, differentiation, and survival<sup>[4]</sup>. Mutant *Ras* oncogenes often contain point mutations that alter only a single amino acid, which locks the oncogenic *Ras* proteins in a persistently activated GTP-bound state<sup>[5,6]</sup>. Single amino acid substitutions at codons 12, 13 or 61 of *Ras* p21 protein result in aberrant *Ras* proteins that contribute to the formation of malignancy due to the disruption of normal GTPase activity of proteins<sup>[7]</sup>.

The codon 12 mutation has been identified as the most predominant mutation among the hot spots<sup>[8]</sup>. However, the variability of residue substitutions at this position seems limited. The substitution of the normal codon 12 Gly residue by Val residue has been documented<sup>[8]</sup>. In mouse models of cancer, somatic activation of oncogenic *K-ras* is necessary for early onset of tumors, and its continuous production for maintenance of tumor viability<sup>[9-12]</sup>. A difficulty in using *Ras* oncogenes as targets in anti-cancer therapy is that at present, it is not possible to specifically inhibit only the oncogenic *Ras* alleles<sup>[3,7,9]</sup>. This may be essential, since the wild-type *K-ras* gene appears to be required for viability, as evidenced by the embryonic lethal phenotype of mice nullizygous for *K-ras*<sup>[13]</sup>. Therefore, tools are required to effectively inhibit the activity of oncogenic *K-ras*, but not that of the wild-type *K-ras* protein in normal tissues. We report here that oncogenic alleles of *K-ras* could be specifically and stably inactivated in human pancreatic cancer cells using a viral RNA interference (RNAi) vector, leading to loss of tumorigenicity.

RNAi is a process during which double-stranded RNA induces the homology-dependent degradation of cognate mRNA<sup>[14]</sup>. In several organisms, introduction of double-stranded RNA has been proven to be a powerful tool to suppress gene expression through a process known as RNAi<sup>[15]</sup>. However, in most mammalian cells this provokes a strong cytotoxic response<sup>[16]</sup>. This non-specific effect could be circumvented by use of synthetic siRNAs [21- to 22-nucleotides], which could mediate strong and specific suppression of gene expression<sup>[17]</sup>. Ever since synthetic 21-23 nucleotide siRNA has been shown to induce efficient RNAi in mammalian cells<sup>[17,18]</sup>, siRNA is routinely used in gene silencing by transfection of chemically synthesized siRNA<sup>[19]</sup>. However, this reduction in gene expression is transient, which would severely restrict its applications. To circumvent the high cost of synthetic siRNA and establish stable gene knock-down cell lines by siRNA, several plasmid vector systems have been designed to produce siRNA inside cells driven by RNA polymerase III-dependent promoters, such as U6- and H1-RNA promoters<sup>[20-24]</sup>. With these plasmid vectors, the phenotypes of gene silencing could be observed by stable transfection of cells<sup>[20]</sup>. Nevertheless, transient siRNA expression, low and variable transfection efficiency remain the problems for such plasmid vector derived siRNA. Recently, several virus vectors have been developed for efficient delivery of siRNA into mammalian cells<sup>[25-27]</sup>. Retroviral vectors have been designed to produce siRNA driven by either U6- or H1-RNA promoter for efficient, uniform delivery and immediate selection of stable knock-down cells<sup>[25,26]</sup>. But retroviruses can integrate into genome and have a narrower spectrum of cell types than adenoviruses<sup>[28-30]</sup>.

Decades of studies of adenovirus biology have resulted in a detailed picture of the viral life cycle and the functions of the majority of viral proteins. The genome of the most commonly used human adenovirus (serotype 5) consists of a linear, 36-kb, double-stranded DNA molecule. Both strands are transcribed and nearly all transcripts are heavily spliced. Additionally, high titers of viruses and high levels of transgene expression generally can be obtained. Recombinant

adenoviruses currently are used for a variety of purposes, including cancer gene therapy<sup>[28-30]</sup>. Two approaches are traditionally used to generate recombinant adenoviruses. The first involves direct ligation of DNA fragments of the adenoviral genome to restriction endonuclease fragments containing a transgene<sup>[31,32]</sup>. The low efficiency of large fragment ligations and the scarcity of unique restriction sites have made this approach technically challenging. The second and more widely used method involves homologous recombination in mammalian cells capable of complementing defective adenoviruses ("packaging lines")<sup>[33,34]</sup>. The desired recombinants are identified by screening individual plaques generated in a lawn of packaging cells<sup>[35]</sup>. Though this approach has been proven extremely useful, the low efficiency of homologous recombination, the need for repeated rounds of plaque purification, and the long time required for completion of the viral production process have hampered more widespread use of adenoviral vector technology. The problems noted above have stimulated novel methods for generating adenoviral vectors. Six years ago, a simplified system (AdEasy system) for generating recombinant adenoviruses was developed<sup>[36]</sup>. This system results in highly efficient viral production procedures that often obviate the need for plaque purification and significantly decrease the time required to generate usable viruses.

In this study, we developed a simple adenovirus system utilizing the well-defined polymerase III H1-RNA promoter to drive efficient expression of siRNA in human cancer cells. Our results demonstrate efficient and specific knock-down of *K-ras<sup>val12</sup>* in Panc-1 pancreatic carcinoma cell lines and indicate a promising application of this adenovirus system in cancer gene therapy.

## MATERIALS AND METHODS

### Plasmids, cell lines, medium, and reagents

Plasmids pSilencer3.1-H1 and pSP72 were purchased from Ambion Inc. (TX) and Promega Corp. (WI), respectively. Plasmid pAdTrack was kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, USA). Plasmid pSUPER-p53 was kindly provided by Dr. Reuven Agami (Center for Biomedical Genetics, Netherlands). All cell lines (Panc-1, HeLa) were purchased from the American Type Culture Collection. These lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, CA) supplemented with 100 mL/L fetal bovine serum (FBS, HyClone, UT), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in 50 mL/L CO<sub>2</sub>. Lipofectin was purchased from Invitrogen Corp. (CA). The antibodies used in this study were human K-ras (F234, mouse monoclonal IgG), p53 (DO-1, mouse monoclonal IgG) and actin (I-19, goat polyclonal IgG) from Santa Cruz Biotechnology Inc. (CA). <sup>32</sup>P-DNA probe was from Promega Corp. (WI).

### Plasmid constructs

The 63 nt oligonucleotides encoding human *K-ras<sup>val12</sup>* specific siRNA were 5'gatccgttgagctgttggcgtagttaagagactacgccacagctccaacttttggaaa3' and 5'agcttttccaaaaagtggagctgttggc

gtagtctcttgaactacgccaacagctccaacg3'. These oligonucleotides were annealed and ligated to the *Bam*HI and *Eco*RI sites of pSilencer3.1-H1 to get plasmid pSilencer3.1-H1-*K-ras*<sup>val12</sup>, subcloned into pSP72 (with *Hind*III and *Eco*RI) and then cloned into pAdTrack (with *Hind*III and *Bgl*II) to obtain plasmid pAdTrackH1-*K-ras*<sup>val12</sup>. pAdTrackH1-p53 (the pSUPER-p53 positive control contains the oligonucleotides encoding p53-specific siRNA) and pAdTrackH1-empty were constructed according to the procedures as described above. The inserted sequences were confirmed by dideoxy sequencing.

### Production of recombinant adenovirus and cell infection

We employed an efficient homologous recombination system as described<sup>[36]</sup>. The shuttle vectors pAdTrackH1-empty, pAdTrackH1-p53 and pAdTrackH1-*K-ras*<sup>val12</sup> were linearized with *Pme*I and transformed into BJ5183 cells (harbor pAdEasy-1) by electroporation. Positive clones were selected and confirmed by DNA miniprep and *Pac*I digestion. Plasmids from correct clones were amplified by transforming into DH10B cells. Miniprep plasmid DNA was prepared by a standard alkaline lysis procedure. The resulting adenoviral DNA (AdEasyH1-*K-ras*<sup>val12</sup>, AdEasyH1-empty and AdEasyH1-p53) was linearized with *Pac*I and purified by ethanol precipitation. The packaging cell line Ad-293 was grown in DMEM supplemented with 100 mL/L FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere containing 50 mL/L CO<sub>2</sub> in air at 37 °C. Twenty-four hours before transfection, 0.3×10<sup>6</sup> cells were plated in a 6-well plate to reach a 50-70% confluency. Cells were transfected according to the manufacturer's instructions (Invitrogen, CA). After 4-6 h, the medium containing the transfection mix was replaced with the growth medium. Transfected cells were incubated for an additional period of 7-10 d and medium was changed every 2-3 d. Viruses were harvested, amplified and titered according to the manufacturer's instructions (Stratagene, CA). The efficiency of packaging and amplification could be observed under the fluorescence microscope at 470 nm everyday.

Pancreatic carcinoma Panc-1 cells and cervical carcinoma HeLa cells were grown in DMEM supplemented with 100 mL/L FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in 50 mL/L CO<sub>2</sub>. On the day before virus infection, 0.3×10<sup>6</sup> Panc-1 or HeLa cells were plated in each well of 6-well plates. On the following day, the cells were incubated with recombinant viruses (AdH1-*K-ras*<sup>val12</sup>, AdH1-p53 or AdH1-empty) at a multiplicity of infection (MOI) of 10-20 at 37 °C. After adsorption for 1-2 h, 2 mL of fresh growth medium was added and cells were placed in the incubator for an additional period of 3-5 d.

### Western blot analysis

Panc-1 and HeLa cells were infected by adenoviruses and harvested after 60 h infection, washed once with cold phosphate buffered saline (PBS, pH 7.0) and lysed in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). Total protein (30 µg

per lane) was resolved on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane and incubated with anti-*K-ras*<sup>val12</sup>, anti-p53 and anti-actin antibodies, followed by incubation with corresponding secondary antibodies. The bands were visualized by using the enhanced chemiluminescence system (Pierce, Rockford, IL). As a control, Panc-1 and HeLa cells without adenovirus infection were used for detecting *K-ras*<sup>val12</sup>.

### Northern blot analysis

Panc-1 cells were infected as described above, and total RNA (30 µg) was extracted 60 h later. RNA was loaded on 11% denaturing polyacrylamide gels, and separated and blotted as described<sup>[37]</sup>. Anti-sense strand of *K-ras*<sup>val12</sup> was labeled by <sup>32</sup>P. The control 5S-rRNA band was detected with ethidium bromide (EB) staining.

### Flow cytometry analysis

At 72 and 96 h post-infection with viruses, Panc-1 cells were trypsinized, washed with PBS. The harvested cells were fixed in 750 mL/L ethanol overnight at 4 °C, washed with PBS, digested by DNase-free RNase A (to a final concentration of 100 µg/mL) for 30 min at 37 °C, stained with 50 mg/mL propidium iodide (PI) in PBS (1 h on ice in the dark), and then measured by flow cytometer (Beckman, IL) for relative PI fluorescence (FL-2). The apoptotic index was a mean of four independent experiments.

### Measurement of cell proliferation

In DMEM containing 100 mL/L serum, 2×10<sup>4</sup> Panc-1 and HeLa cells were infected with either AdH1-*K-ras*<sup>val12</sup>, control AdH1-p53 or AdH1-empty viruses. Then the cells were resuspended in 2 mL of 4 g/L low melting point agarose and seeded in duplicate into six-well plates coated with 10 g/L low melting point agarose in DMEM containing 100 mL/L FBS and adenoviruses. The number of foci was scored after 3 wk.

### Statistical analysis

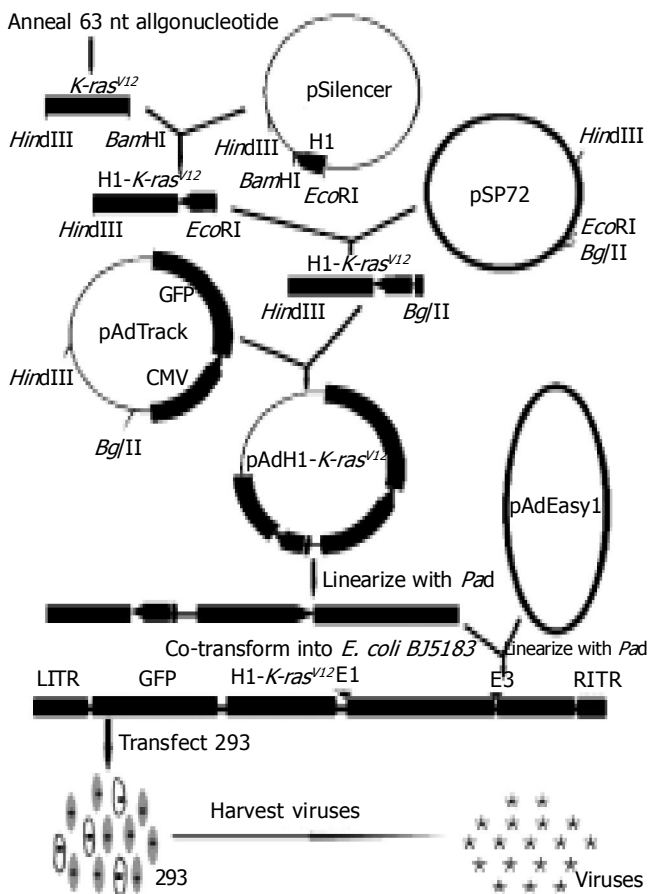
Results were expressed as mean±SD and the mean values were compared by using the ANOVA (SNK, Student-Newman-Keuls test) in the SAS 8.1 software. *P*<0.05 was considered statistically significant.

## RESULTS

### Generation of siRNA-expressing adenoviruses: AdH1-*K-ras*<sup>val12</sup>, AdH1-empty and AdH1-p53

We utilized an AdEasy-1 system by which adenoviruses were generated and a pSilencer™ Kit pSilencer 3.1-H1 containing the well-defined polymerase III H1-RNA promoter to deliver siRNA expressing cassettes into cells and to silence a specific gene in human cancer cells. After several rounds of cloning, the H1-RNA promoter and the 63 nt oligonucleotide were cloned into the shuttle vector pAdTrack to get pAdTrack-H1, which could drive the expression of siRNAs targeting different genes in recombinant adenoviruses (Figure 1). Here, the 63 nt oligonucleotide directed against *K-ras*<sup>val12</sup> was introduced into pAdTrack, then homologously recombined with pAdEasy-1 in *E. coli*

BJ5183 and packaged in 293 cells, after which adenovirus AdH1-*K-ras*<sup>val12</sup> was obtained (Figures 1, 2). In the same way, AdH1-empty and AdH1-p53 were obtained.



**Figure 1** Schematic outline of siRNA expressing adenovirus AdH1-*K-ras*<sup>val12</sup>, AdH1-p53 and AdH1-empty.

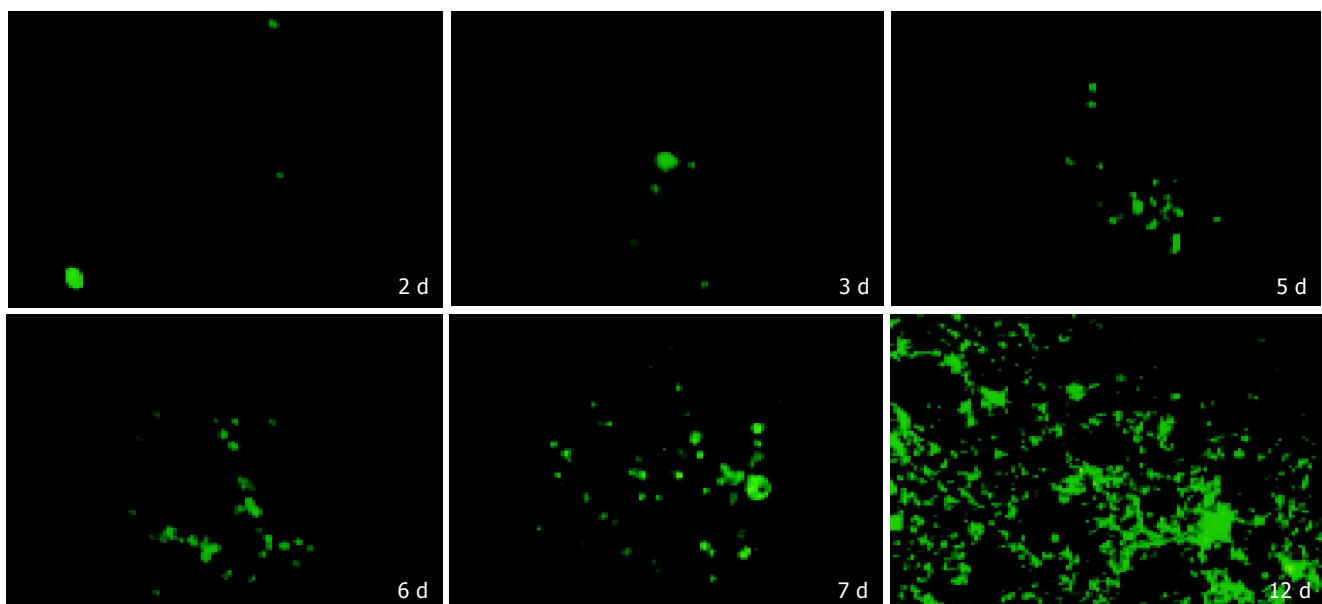
### Effect of siRNA-expressing adenoviruses at the molecular level

In this study, we tested our system by targeting *K-ras*<sup>val12</sup> gene as a model. To study the inhibitory effects of oncogenic *Ras* expression on the tumorigenic phenotypes of human cancer cells, we targeted the expression of endogenous mutant *K-ras*<sup>val12</sup> alleles with siRNA vector in the human pancreatic cell line Panc-1 (Figure 3). To target specifically the mutant *K-ras*<sup>val12</sup> alleles, the H1-RNA promoter was used in our strategy. We cloned a 19 nt targeting sequence spanning the region encoding valine 12 of mutant *K-ras* into the pSilencer 3.1-H1 vector, and the yielded pSilencer 3.1H1-*K-ras*<sup>val12</sup> (Figure 1) showed that human pancreatic carcinoma Panc-1 cells transiently transfected with pSilencer3.1H1-*K-ras*<sup>val12</sup> had significant suppression of endogenous *K-ras*<sup>val12</sup> expression, whereas control p53 protein levels were unaffected (data not shown). We then cloned the pSilencer3.1H1-*K-ras*<sup>val12</sup> cassettes into the pAdTrack to generate the adenovirus AdH1-*K-ras*<sup>val12</sup>. Viral stocks (AdH1-empty and AdH1-p53 viral stocks were used for control infections.) were used to infect Panc-1 cells that harbored *K-ras*<sup>val12</sup>. In AdH1-*K-ras*<sup>val12</sup> neither AdH1-empty nor AdH1-p53 infected Panc-1 cells, *K-ras*<sup>val12</sup> gene was efficiently silenced 60 h post-infection. After viral infection, Western blot analysis with anti-*K-ras*-specific antibodies revealed that the *K-ras*<sup>val12</sup> expression in AdH1-*K-ras*<sup>val12</sup>-infected Panc-1 cells was markedly suppressed compared to the control infections (Figure 4).

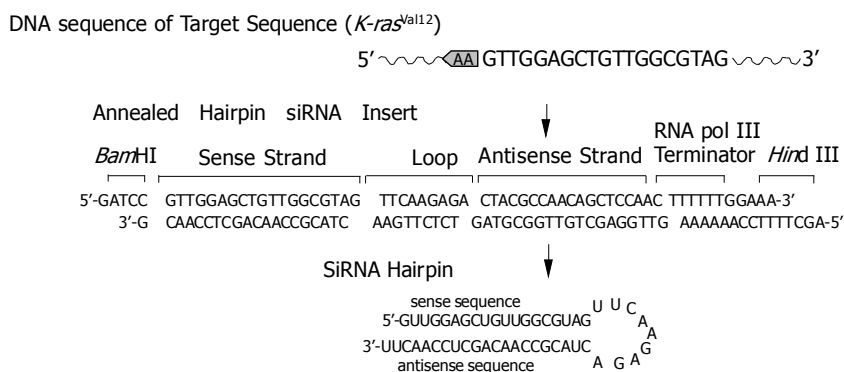
Consistent with this, Northern blot analysis with the anti-sense strand of *K-ras*<sup>val12</sup> as a probe detected *K-ras*<sup>val12</sup> mRNA generated in the control groups (AdH1-empty and AdH1-p53) but not in AdH1-*K-ras*<sup>val12</sup> group (Figure 5).

### Effect of siRNA-expressing adenoviruses at the cellular level

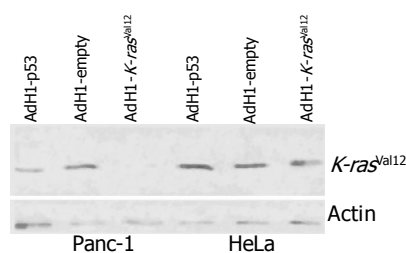
Flow cytometry analysis was performed to observe the apoptosis. As shown in Figure 6, knocking down *K-ras*<sup>val12</sup> Panc-1 displayed increased apoptotic cells compared to the



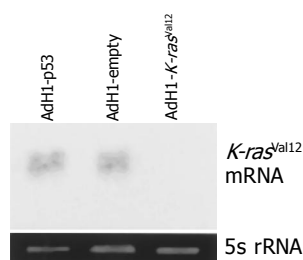
**Figure 2** Foci produced by adenoviruses on d 2-7 and 12 after transfection of 293 cells.



**Figure 3** Sequence (for detail, refer to<sup>[46]</sup>) of Val<sup>12</sup> mutant alleles of human *K-ras* and the predicted mutant-specific short hairpin transcripts encoded by AdH1-*K-ras*<sup>Val12</sup>.



**Figure 4** Representative Western blot analysis for *K-ras*<sup>Val12</sup> expression in Panc-1 and HeLa cells 60 h after infection with viral stocks (AdH1-*K-ras*<sup>Val12</sup>, AdH1-empty, AdH1-p53) of three separate experiments.



**Figure 5** Northern blot analysis with total RNA from same infected Panc-1 cells of single experiment.

control groups treated with AdH1-p53 or AdH1-empty. The percentage of apoptotic cells in the treatment group (18.70% 72 h post-infection, 49.55% 96 h post-infection) was significantly higher than that in the control groups (3.47%, 3.98% 72 and 96 h post-infection of AdH1-empty, respectively; 4.21%, 3.78% 72 and 96 h post-infection of AdH1-p53, respectively) ( $P < 0.05$ ).

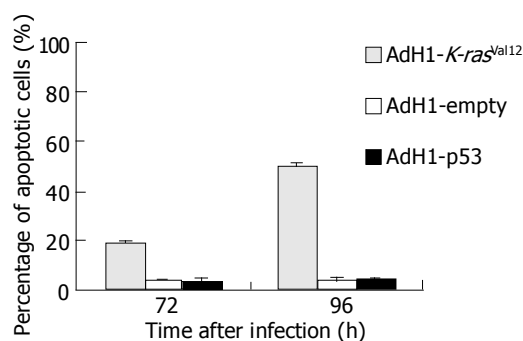
The presence of oncogenic *K-ras* alleles was frequent in human tumors, but almost invariably associated with other genetic events<sup>[3]</sup>. To address the question of whether the oncogenic phenotype of late stage human tumors still depended on the expression of oncogenic mutant *K-ras*, we again used Panc-1 cells. One phenotype associated with tumorigenicity had the ability to grow independent of anchorage when plated in a semisolid media (soft agar assay). We infected Panc-1 and HeLa cells with either AdH1-*K-*

*ras*<sup>Val12</sup>, control AdH1-p53, or AdH1-empty viruses. A total of  $2 \times 10^4$  cells were plated in soft agar and allowed to grow for three weeks. As expected from transformed human tumor cell lines, both Panc-1 and HeLa cell lines were able to grow and form colonies when infected with AdH1-empty and AdH1-p53 control viruses (Table 1). In contrast, infection of AdH1-*K-ras*<sup>Val12</sup> abolished almost completely the colony growth of Panc-1 cells in this assay. Importantly, the effect of AdH1-*K-ras*<sup>Val12</sup> was specific, as soft agar growth of HeLa cells (which contained *H-ras* oncogene) was unaffected (Table 1). Our results demonstrated a significant down-regulation of *K-ras*<sup>Val12</sup> expression in Panc-1 cells.

**Table 1** Cell proliferation assay in soft agar (mean $\pm$ SD)

Cell line	AdH1- <i>K-ras</i> <sup>Val12</sup>	AdH1-empty	AdH1-p53
Panc-1	4 $\pm$ 1.53	202 $\pm$ 7.77	201 $\pm$ 6.56
HeLa	300 $\pm$ 2	299 $\pm$ 12.06	301 $\pm$ 5.57

The number of soft agar colonies from three independent experiments is presented ( $P < 0.05$ ).

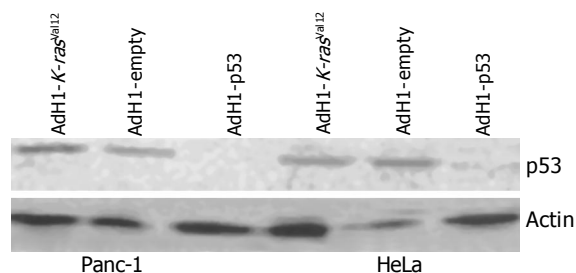


**Figure 6** Apoptotic indices shown as the mean of four separate experiments for each group ( $P < 0.05$ ).

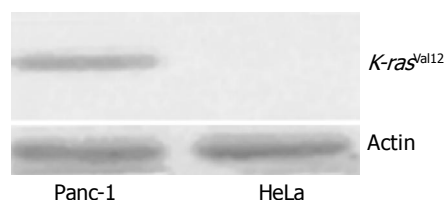
#### Test of the specificity of siRNA-expressing adenovirus AdH1-*K-ras*<sup>Val12</sup>

We tested the specificity of our targeting construct by examining the expression of wild type *K-ras*. We used HeLa cells, which could endogenously express wild type *H-ras* and

*K-ras* alleles. Western blot analysis revealed that comparable levels of wild type *H-ras* and *K-ras* proteins were expressed in HeLa cells, irrespective of whether they were infected with the same AdH1-*K-ras*<sup>val12</sup>, AdH1-p53, or AdH1-empty adenoviral stocks used for the Panc-1 cells (data not shown). In contrast, p53 expression was suppressed equally by AdH1-p53 in both HeLa and Panc-1 cell types, ruling out the possibilities that the HeLa cells were not infected or lacked components necessary for RNAi (Figure 7). In addition, *K-ras*<sup>val12</sup> was also measured at the protein level. It showed that *K-ras*<sup>val12</sup> expressed in Panc-1 but not in HeLa (Figure 8). Thus, the RNAi response provoked by AdH1-*K-ras*<sup>val12</sup> adenoviruses was powerful and sufficiently selective to distinguish the wild type *K-ras* alleles from the *K-ras*<sup>val12</sup> alleles, which differed in 1 bp only.



**Figure 7** Stable polyclonal pools of Panc-1 and HeLa cells harboring wild-type *H-ras* infected with the indicated viral stocks and immunoblotted for the detection of p53 and actin proteins in single experiment.



**Figure 8** Detection of *K-ras*<sup>val12</sup> at protein level in Panc-1 and HeLa cells without virus infection in single experiment.

## DISCUSSION

In cancer gene therapy, successful strategy depends on the cancer specificity and the efficient delivery into mammalian cells. In this study, RNAi technique and the human adenovirus serotype 5 were used to reach the goal. The availability of a high virus titer, infection of a broad spectrum of cell types and independence on active cell division make adenovirus the vector of choice for siRNA delivery. In the AdEasy-1 system, the backbone vector containing most of the adenoviral genomes, is used in supercoiled form, obviating the need to enzymatically manipulate it. The recombination is performed in *E. coli* rather than in mammalian cells. There are no ligation steps involved in generating the adenoviral recombinants, as the process takes advantage of the highly efficient homologous recombination machinery present in bacteria. The vector

contains a green fluorescent protein (GFP) gene incorporated into the adenoviral backbone, allowing direct observation of the efficiency of transfection and infection. In the system, the process of viral production can be directly and conveniently followed in the packaging cells by visualization of the GFP reporter. The packaging cell line 293 is highly transfectable by lipid-DNA complexes and constitutively expresses the *E1* gene products required for propagation of all recombinant adenoviruses. The system described herein has several advantages in terms of easy manipulation and speed. The ability to recover reasonable quantities of homogeneous viruses, without plaque purification, represents a major practical advantage. The GFP tracer makes it possible to follow all stages of the viral production process in a convenient fashion. In the case of cells that are inefficiently infected with adenoviruses, the GFP tracer additionally makes it possible to isolate expressing cells through fluorescence-activated cell sorting, thus facilitating several kinds of experiments. To generate viruses, we must attach importance to the following considerations. First, high efficient *E. coli* BJ5183 competent cells containing supercoiled circular pAdEasy-1 and constructs can completely digest with *PmeI*, guaranteeing the successful homologous recombination. Second, to produce viruses, recombinant plasmids can be digested with *PacI* to liberate linear adenoviral genomes, then transfected into 293 cells. It is critical to linearize the vectors at the *PacI* sites, as transfection of circular plasmids yields no viruses (data not shown). Third, homologously recombinant plasmids could be produced directly from *E. coli* BJ5183 cells with a relatively low yield. Therefore, miniprep DNA from *E. coli* BJ5183 cells should be used to transform DH10B cells, a *recA* strain in which high-quality and high yields of plasmid DNA could be obtained more easily. The *E. coli* strain BJ5183 is not *recA*, but it is deficient in other enzymes that mediate recombination in bacteria. It could be chosen from several strains mutated in *recA*, *recBCD*, *recJ*, or *recI*<sup>[38,39]</sup>, because of its higher efficiency of transformation and stable propagation of plasmid DNA in experiments. Once recombination is achieved and verified, the adenoviral recombinant DNA can be simply transferred to *recA* and *endA* strains (such as DH10B) for greater yields of DNA if desired. (Because of its *recA* status, DH10B can not be used to generate adenoviral recombinants by homologous recombination.) In contradiction to the previous literature<sup>[36]</sup>, we found that it was not important to place the insert in head-to-tail orientation when the siRNA expressing cassettes were cloned into pAdTrack. But we could not explain the phenomenon. If other kinds of expressing cassettes could be constructed like this, the system would be more convenient for the production of adenoviruses. Consistent with the observation of several groups<sup>[25-27]</sup>, these viral vectors could infect cells uniformly and rapidly. Given the simplicity of this system, which employs readily available reagents, most laboratories have the ability to silence their genes by recombinant adenoviruses.

RNAi has been widely used in identification and characterization of genes<sup>[40]</sup>. In functional genomics, a large number of genes controlling cell division and metabolism have been identified by screening with RNAi. Another

potential application is in the area of gene therapy<sup>[41]</sup>. It has been shown recently that RNA viruses are sensitive to RNAi<sup>[42-44]</sup>. Nevertheless, our results indicate that adenoviral vectors could also be used to mediate efficient integration of pSilencer3.1-H1 cassettes in human cells and direct the synthesis of siRNAs to suppress gene expression. It is suggested that the expression of siRNAs may depend on RNA polymerase III promoter but not on any kind of vectors.

To detect the effect of AdH1-*K-ras*<sup>val12</sup> at the cellular level, flow cytometry analysis and measurement of cell proliferation are employed. Determination of the relative DNA content of apoptotic nuclei (which are hypodiploid due to loss of fragments) by PI staining and cytofluorimetric analysis is a more sensitive way to demonstrate apoptotic cell death<sup>[45]</sup>. As shown in Figure 6, after AdH1-*K-ras*<sup>val12</sup> infection, the apoptosis of Panc-1 cells is detected by flow cytometry. But, in the study of Brummelkamp *et al.*<sup>[46]</sup>, flow cytometry analyses showed that knocking down *K-ras*<sup>val12</sup> had no significant effect on the ability of Capan-1 cells to proliferate adherently under standard tissue culture conditions. We think that it may be due to different viral vectors or different cell lines. Further researches are needed to explain the contradiction. Consistent with Brummelkamp *et al.*<sup>[46]</sup>, soft agar assay demonstrates that viral vectors could be used to mediate RNAi to induce persistent loss of functional phenotypes, suggesting that siRNAs-expressing adenoviruses can be used for cancer therapy at the late stage of human tumors. Nevertheless, adenoviruses must be employed repeatedly.

In mammalian cells, dsRNA larger than 30 bp was reported to induce generally non-specific suppression of gene expression by activating the antiviral interferon response<sup>[14]</sup>. This obstacle has been overcome by the discovery that the effector in RNAi is an siRNA which is 21-23 nt long with 2 nt 3'-overhang and 5'-phosphate. The successful gene silencing with chemically synthesized 21-22 nt siRNAs can rapidly trigger its wide application in mammalian cells<sup>[14,17,18]</sup>. It has been demonstrated that a vector derived from polymerase III-dependent H1-RNA gene promoter can produce siRNA and cause efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes<sup>[20]</sup>. Almost all the elements of H1-RNA promoter are located upstream of the transcribed region, and it is ideally suitable for the expression of about 19 nt siRNAs. It has been reported that stem-loop precursor transcripts are generated and processed to functional siRNA by cellular enzymes<sup>[20]</sup>. This small size of siRNA could prevent activation of the dsRNA inducible interferon system present in mammalian cells and avoid the non-specific phenotypes normally produced by dsRNA larger than 30 bp in somatic cells<sup>[20,46]</sup>. Similarly, our results showed the specificity. Adenovirus-delivered siRNAs may be an ideal method for tumor-specific gene therapy.

In conclusion, a simple siRNA delivery strategy can be developed by combination of well-defined H1-RNA promoters and pAdEasy-1 adenovirus system. Vectors like these have at least two potential applications. In gene therapy, the selective down-regulation of only the mutant version of a gene allows for highly specific effects on tumor cells,

while leaving the normal cells untouched. This feature greatly reduces the need to design viral vectors with tumor-specific infection and/or expression. By designing target sequences that span chromosomal translocation breakpoints found in cancer, these vectors may also be used to specifically inhibit the chimeric transcripts of these translocated chromosomes. The recent demonstration that siRNAs can inhibit gene expression *in vivo* provides further support for the notion that oncogene-specific RNAi may be a viable approach to the treatment of cancer<sup>[47,48]</sup>. This technology has a foreseeable wide application in experimental and clinical work for cancer therapy. In addition, these vectors can be used to efficiently identify the genetic events that are required for cancer cells to manifest a tumorigenic phenotype. Through the use of this technology, out of the many genetic alterations present in most human cancer cells, the most effective targets for drug development can be rapidly identified<sup>[46]</sup>. Furthermore, an apparent apoptosis in Panc-1 cells has been mediated by replication-incompetent adenoviruses. The mechanism needs to be further investigated. Since this kind of viruses transiently expresses exogenous genes, in order to get a persistent gene expression, we are developing a conditionally replicative virus that can express siRNA for cancer therapy.

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• BRIEF REPORTS •

## Correlation between severity of endoscopic findings and apnea-hypopnea index in patients with gastroesophageal reflux disease and obstructive sleep apnea

Pál Demeter, Katalin Várdi Visy, Pál Magyar

Pál Demeter, Department of Gastroenterology, St. Margaret's Hospital, Budapest, Hungary  
Katalin Várdi Visy, Pál Magyar, Department of Pulmonology, Semmelweis Medical University, Budapest, Hungary  
Correspondence to: Dr. Pál Demeter, Department of Gastroenterology, St. Margaret's Hospital, Bécsi út 132., Budapest 1032, Hungary. pauldemeter@axelero.hu  
Telephone: +36-30-9222985 Fax: +36-23-457656  
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### Abstract

**AIM:** To assess the relationship between severity of gastroesophageal reflux disease and apnea-hypopnea index (AHI) as an indicator of the severity of obstructive sleep apnea.

**METHODS:** Data of 57 patients with proven obstructive sleep apnea and gastroesophageal reflux disease were analyzed. Patients were divided into two groups according to severity of the sleep apnea: "mild-moderate" (A)-AHI  $\geq 5$ -30,  $n = 27$ , "severe" (B)-AHI  $> 30$ ,  $n = 30$ . All patients underwent apnea monitoring during the night, upper panendoscopy and were asked about typical reflux symptoms.

**RESULTS:** All examined patients in both groups showed a significant overweight and there was a positive correlation between body mass index and the degree of sleep apnea ( $P = 0.0002$ ). The occurrence of erosive reflux disease was significantly higher in "severe" group ( $P = 0.0001$ ). Using a logistic regression analysis a positive correlation was found between the endoscopic severity of reflux disease and the AHI ( $P = 0.016$ ). Forty-nine point five percent of the patients experienced the typical symptoms of reflux disease at least three times a week and there was no significant difference between the two groups.

**CONCLUSION:** A positive correlation can be found between the severity of gastroesophageal reflux disease and obstructive sleep apnea.

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**Key words:** Obstructive sleep apnea; Gastroesophageal reflux disease; Severity; Correlation

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### INTRODUCTION

There is an increasing mass of evidence for a link between the obstructive sleep apnea (OSA) and gastroesophageal reflux disease (GERD). The large negative intrapleural pressure swings during apnea should facilitate reflux events<sup>[1]</sup>. A further factor to be considered regarding the OSA-GERD relationship is that the diaphragm is connected to lower esophageal sphincter (LES) through the phreno-esophageal ligament (PEL). During apnea the respiratory work of the diaphragm increases extremely. This increased burden affects the cardia through the frequent change of the position of PEL. This leads partly to the loss of the cardia muscle tone<sup>[2]</sup>. The European Community Respiratory Health Survey published in 2002 noted that GERD development during sleep is an important determinant of the respiratory balance, since it may play a role as an aggravating or causal factor in relation to the nocturnal asthma, chronic cough, recurrent bronchitis and respiratory disorders during sleep<sup>[3]</sup>. A higher frequency of GER-related symptoms has been found in patients with OSA than in the control subjects<sup>[4,5]</sup>. More reflux events could be identified in OSA patients than in controls during one-channel esophageal pH-metry<sup>[6]</sup>. The number of reflux events could be reduced with nasal continuous positive airway pressure (nCPAP) treatment for both the patients with OSA and GERD<sup>[7-9]</sup>. These data suggest that OSA may be a significant cause of GERD.

The influence of the severity of OSA on endoscopic findings in patients with GERD and OSA has not been analyzed yet.

### MATERIALS AND METHODS

Fifty-five patients with proven OSA were referred for upper panendoscopy. Diagnosis of sleep apnea was based on a 12-channel polysomnography (2-channel EEG/electroencephalography/, EOG/electrooculography/, chin EMG/electromyography/, ECG/electrocardiography/, nasal and oral flow-metry, detection for O<sub>2</sub>-saturation, pulseoxymetry, detection for thoracic and abdominal movements, phonometry) performed using the Morpheus Medatech system in our sleep lab. All patients underwent upper panendoscopy and were asked about the frequency of typical reflux symptoms. Epworth sleepiness scale (ESS)<sup>[10]</sup> was completed by patients to measure their daytime sleepiness.

The classification of GERD was based on endoscopic findings. We used the conventional Savary-Miller classification of the disease<sup>[11]</sup>. The patients' data were collated on an Excel 9.0 worksheet, including the severity grades of GERD (0-4), the apnea-hypopnea index (AHI), the frequency of typical GERD symptoms (heartburn, regurgitation of gastric content, dysphagia, age, gender, the score of ESS and body mass index /BMI/. The patients were divided into two groups according to the severity of sleep apnea<sup>[12]</sup>: "mild-moderate" (A)-AHI  $\geq 5-30$ ,  $n = 27$ ; "severe" (B)-AHI  $>30$ ,  $n = 30$ .

### Statistical analysis

In case of continuous and category variables, a nonparametric  $t$  test (Mann-Whitney), one way ANOVA test and  $\chi^2$  test were used. In the event of dichotomous variables,  $\chi^2$  "for trend" test was performed. The relationship between the severity of reflux disease and AHI was analyzed with the help of logistic regression analysis.

We relied on the conventional  $P < 0.05$  critical values regarding the statistical tests of the results. We used the SPSS 9 for Windows software package for the statistical procedures.

## RESULTS

The total available population covered 57 patients. This population was characterized by an average age of 51.38 years ( $SD \pm 9.16$ ), a male/female ratio of 2.8:1 (73.7% *vs* 26.3%) and an average BMI of 34.20 kg/m<sup>2</sup> ( $SD \pm 8.79$ ). Using the Savary-Miller definitions, our patients displayed the following distribution alongside the endoscopic categorization of reflux disease: 11 (19.3 %) GERD 0 subjects, 13 (22.8%) GERD I subjects, 20 (35.1%) GERD II subjects, 7 (12.3%) GERD III subjects, and 6 (10.5%) GERD IV subjects (Table 1).

**Table 1 Distribution of GERD types**

Population ( $n = 57$ )	Occurrence	Percentage (%)
GERD 0	11	19.3
GERD 1	13	22.8
GERD 2	20	35.1
GERD 3	7	12.3
GERD 4	6	10.5

The population mean of AHI used as a direct measure of the severity of sleep apnea was 40.8 ( $SD \pm 35.18$ ). The patients were divided into two groups according to the severity of sleep apnea: "mild-moderate" (A)-AHI  $\geq 5-30$ ,  $n = 27$  and "severe" (B)-AHI  $>30$ ,  $n = 30$ . Comparison of clinical data of groups is summarized in Table 2.

The percentage of female patients was 37% in group A and 16% in B. The BMI was significantly higher in group B than in group A ( $P = 0.0002$ ). There was no significant difference between two groups in respect of typical reflux symptoms (52% *vs* 47%), but in 50.5% of the study population these symptoms were negative (poor) according to our determination. When the endoscopic findings of GERD were analyzed, a higher frequency of severe cases was found in group B ( $P = 0.0001$ ). In group A the incidence of GERD 0 was one-third of the cases, whereas in group B that was only 7%.

We compared the clinical (sleep) parameters and GER-related symptoms in each grade of GERD (Table 3). No significant difference was found between grades in respect of age, gender and GER-related symptoms. A positive correlation could be found between the severity of GERD and BMI. A very close connection was demonstrated between the severity of GERD and AHI values and the scores of Epworth scale.

**Table 2 Comparison of clinical data between A and B group**

Variable	"A" group (AHI $\leq 30$ )	"B" group (AHI $>30$ )	Total	$P^1$
Number of cases	27	30	57	-
Age (yr)	51 (41-54) <sup>1,2</sup>	53.5 (48-59.5)	52 (44.5-57.5)	0.07
Gender (women, %)	37	16	26	0.13
BMI	31.2 (27.1-34.3)	36.4 (32.3-41.9)	33.8 (28.6-38.0)	0.0002
AHI	10.1 (7-21)	56.7 (38.3-88.0)	35 (10.6-60)	$<0.0001$
GER-related symptoms + (%)	52	47	49.5	0.79
GERD endoscopic findings (%)	0:33	0:7	0:19	$<0.0001$
	1:33	1:13	1:23	
	2:34	2:37	2:35	
	3:0	3:23	3:12	
	4:0	4:20	4:11	

<sup>1</sup>Non-parametric  $t$ -test (Mann-Whitney),  $\chi^2$ -test, endoscopic findings:  $\chi^2$ -test for trend <sup>1,2</sup>median (interquartile range).

**Table 3 Relationship between the severity of endoscopic findings, clinical parameters and GER-related symptoms**

Variable	GERD "0"	GERD "1"	GERD "2"	GERD "3"	GERD "4"	$P^1$
Number of cases	11	13	20	7	6	
Age (yr)	52 (42-53) <sup>2</sup>	53 (45-55)	53 (43-59)	53 (48-60)	50 (48-50)	0.79
Gender (women, %)	18	31	35	29	0	0.197
BMI	29.5 (26.9-36.1)	33.9 (31.2-37.4)	32.3 (28.6-35.0)	37.8 (33.2-40.6)	40.8 (35.8-47.3)	0.04
Epworth	11 (8-14)	9 (7-12)	12.5 (10-16.5)	15 (14-19)	17 (16-18)	0.006
AHI	14 (8-26)	11.2 (10-35)	31.5 (16.4-66.1)	65 (56.7-99.3)	63.4 (45-83)	0.0001
GER-related symptoms + (%)	18.2	61.5	60	57.1	50	0.178

<sup>1</sup>In case of continuous variable one-way ANOVA and dichotomous variable  $\chi^2$ -test for trend ( $P < 0.05$  significant) <sup>2</sup>median (interquartile range).

We could reach essentially similar conclusions on the basis of logistic regressions investigating the severity of endoscopic findings and AHI values as indicator of the severity of OSA (Table 4).

**Table 4** Logistic regression analysis in terms of the severity of GERD<sup>1</sup> and the relationship between BMI and AHI

	Not-adjusted model	Adjusted at AHI <sup>2</sup>
BMI <sup>3</sup>	1.9 (0.64-5.88) 0.23 <sup>4</sup>	1.27 (0.38-4.15) 0.68
AHI <sup>3</sup>	4.09 (1.31-12.74) 0.016	3.79 (1.25-12.4) 0.028

<sup>1</sup>Severity of GERD: mild: 0-1, severe: 2-4 <sup>2</sup>Classifying the AHI values: low:  $\leq$  the median of all patients (35); high:  $>$  the median of all patients (35); <sup>3</sup>Classifying the BMI: low:  $\leq$  the median of all patients (33.8); high:  $>$  the median of all patients (33.8); <sup>4</sup>Confidence interval;  $P < 0.05$  significant.

This model showed that in case of a high AHI, the presence of severe GERD was 4.09-fold. The weak connection between BMI and the severity of GERD (Table 3) was not significant in this model.

## DISCUSSION

Characteristics of our OSA population were consistent with the patients who were overweight with males in the majority<sup>[13-15]</sup>.

In this study, the characteristics of GERD and their connection with the severity of OSA were demonstrated. We verified a positive and significant correlation between the severity of OSA and endoscopic findings of GERD with different statistical methods and established that more severe OSA was accompanied with more severe GERD. Indeed, a high frequency of reflux esophagitis (about 80%) in OSA patients was already found in contrast to the general population, however, 40-60% of patients with typical reflux symptoms had no esophageal erosions<sup>[16]</sup>. Ing *et al*<sup>[6]</sup> performed 24-h esophageal pH-metry in patients with OSA and found more severe reflux parameters in patients with more severe OSA. Even a higher frequency of GER-related symptoms was found in patients with OSA than in the control subjects<sup>[4,5]</sup>. In our study, typical and explicit complaints of GERD were also investigated. There was no difference between the two groups, but these symptoms were not significant in 50.5% of our patients. However, we classified the patients according to the endoscopic findings (Savary-Miller). A close connection was demonstrated between the severity of GERD and the scores of Epworth scale as an indicator of daytime somnolence. It is consistent with our previous results. We found a positive correlation between the body mass index and the degree of sleep apnea, which is also consistent with the reported data.

Even though the ultimate therapeutic solution was the nCPAP treatment in most cases, it appeared that the complementary treatment of GERD is important as well. This view seems to be shared by Foresman who noted that patients suffering from OSA and GERD simultaneously presented a therapeutic challenge. As an illustration of this

challenge, Wolf *et al* presented a case of a long-term severe GERD patient who was treated with a high dose of omeprazole and antacids. Eventually OSA was identified in the background of this condition, which came into light due to the complaint of daytime somnolence. The nCPAP therapy achieved a significant improvement regarding GERD and other symptoms as well. However, nCPAP could decrease gastroesophageal reflux in patients without OSA by increasing the intra-esophageal pressure<sup>[9]</sup>.

In conclusion, severity of OSA and GERD is probably parallel to each other. A high percentage of patients with fewer and indefinite complaints suggests the role of nocturnal GERD in OSA patients. Severity of OSA is correlated to the endoscopic findings in GERD.

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• BRIEF REPORTS •

## ***Helicobacter pylori* infection in hemodialysis patients: Susceptibility to amoxicillin and clarithromycin**

Selim Aydemir, Sedat Boyacioglu, Gurden Gur, Muge Demirbilek, Fusun Kamber Can, Murat Korkmaz, Ugur Yilmaz

Selim Aydemir, Department of Gastroenterology, Zonguldak Karaelmas University Faculty of Medicine, 67800, Zonguldak, Turkey

Sedat Boyacioglu, Gurden Gur, Murat Korkmaz, Ugur Yilmaz, Department of Gastroenterology, Baskent University Faculty of Medicine, 06000, Ankara, Turkey

Muge Demirbilek, Fusun Kamber Can, Department of Clinical Microbiology, Baskent University Faculty of Medicine, 06000, Ankara, Turkey

Correspondence to: Dr. Selim Aydemir, Department of Gastroenterology, Zonguldak Karaelmas University Faculty of Medicine, 67800, Zonguldak, Turkey. selimaydemir@hotmail.com  
Telephone: +90-372-2576169

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### **Abstract**

**AIM:** To evaluate susceptibility of *Helicobacter pylori* to amoxicillin and clarithromycin in end-stage renal disease (ESRD) patients and non-uremic controls.

**METHODS:** The subjects with dyspeptic complaints were 33 ESRD patients and 46 age- and sex-matched non-uremic controls who exhibited *H pylori* on antral biopsy specimens. The two groups were age and sex matched. The *H pylori* strains' pattern of susceptibility to amoxicillin and clarithromycin was investigated with the agar dilution technique.

**RESULTS:** None of the *H pylori* strains from either group showed resistance to amoxicillin with the agar dilution method. Twelve (36.4%) of the ESRD group strains and 7 (15.2%) of the control group strains showed resistance to clarithromycin, and this difference was statistically significant ( $P < 0.05$ ).

**CONCLUSION:** Resistance to amoxicillin does not appear to be an important problem in *H pylori*-infected ESRD and non-uremic patients in our region. In contrast, the rates of resistance to clarithromycin are high, particularly in the ESRD population.

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**Key words:** *Helicobacter pylori*; Chronic renal failure; Antibiotic resistance; Clarithromycin; Amoxicillin

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### **INTRODUCTION**

*Helicobacter pylori* (*H pylori*) infection is the most common chronic bacterial infection in humans. Estimates indicate that approximately 60% of the world population is colonized with this agent. In addition to the direct effects of *H pylori*, complications related to infection with this bacterium are serious public health problems<sup>[1]</sup>. *H pylori* is a gram-negative, spiral-shaped bacillus that colonizes the antrum of the stomach causing type B chronic-active antral gastritis. Research has established that a number of diseases of the gastrointestinal system are linked with *H pylori* infection. Specifically, this condition is known to play an important role in the pathogenesis of peptic ulcer disease, and is also associated with gastric cancer<sup>[2,3]</sup>.

The ideal treatment for *H pylori* infection is yet to be discovered. Triple-agent protocols involving a proton-pump inhibitor and two antibiotics are frequently used<sup>[4-6]</sup>. Most patients with *H pylori* infection respond well to these regimens; however, therapy may fail due to antibiotic resistance, poor compliance, or treatment-related factors (number/doses of combined medications, dosing frequency, and/or treatment duration). Antibiotic resistance is an important cause of *H pylori* treatment failure. In order to administer optimal treatment in a given region, it is critical to know the antibiotic susceptibility pattern for the strains that exist in the area<sup>[7-10]</sup>.

The epidemiological data concerning *H pylori* infection in end-stage renal disease (ESRD) patients are insufficient<sup>[11]</sup>. Most studies of the epidemiological features of *H pylori* infection have revealed similar findings in ESRD and non-uremic patients<sup>[11]</sup>. The prevalence rates of *H pylori* infection and dyspeptic complaints in ESRD patients are high. Most individuals with ESRD exhibit immune system dysfunction, and these patients also tend to require frequent invasive procedures. These factors result in increased rates of infection, and this explains the very high frequencies of antibiotic use in this population.

We designed a controlled study to investigate possible differences in antibiotic resistance pattern for *H pylori*-infected ESRD patients and non-uremic controls at a major center in Ankara, Turkey.

### **MATERIALS AND METHODS**

#### **Subjects**

Thirty-three ESRD patients who arrived at our gastroenterology clinic with dyspeptic complaints were enrolled in the study. Forty-six dyspeptic control patients with normal renal function and no other known health problems also participated. The ESRD group comprised of 17 (51.5%)

females and 16 (48.5%) males. The mean age of these patients was  $42 \pm 14$  years, and the mean hemodialysis duration  $4.1 \pm 2.8$  years. The control group comprised of 26 (56.5%) females and 20 (43.5%) males of mean age  $43 \pm 14$  years (Table 1). The two groups were age and sex matched ( $P > 0.05$ ). Each of the 79 subjects was investigated with upper gastrointestinal endoscopy. The exclusion criteria were as follows: prior treatment for *H. pylori* infection; use of any antibiotic, proton-pump inhibitor,  $H_2$ -receptor antagonist or bismuth-containing drugs in the 30 d before the study; negative *H. pylori* culture from an antral biopsy specimen; and positive *H. pylori* culture but insufficient growth for antibiotic susceptibility testing.

The study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Local Ethics Committee of Baskent University. Patients were enrolled in the study after getting their written informed consent.

### Endoscopic procedure

All the upper gastrointestinal system endoscopy procedures were performed under appropriate sedation (lidocaine 10 mg/puff for pharyngeal anesthesia and intravenous midazolam 2.5-7.5 mg for premedication) using the same videoendoscope (Olympus GIF Q240). Two biopsy specimens were collected in each case, one from the prepyloric region and one from the incisura angularis. Tissues were placed in selective medium (Brucella broth culture containing 20% glycerol) for transfer to the laboratory.

### Culture

Within 4 h of the endoscopy procedure, each biopsy specimen was placed in 0.5 mL sterile saline and homogenized in a tissue homogenizer. Two droplets of each homogenate were placed in 7% sheep blood Brucella agar, and the plates were incubated in microaerophilic conditions at  $37^\circ\text{C}$  for 3-7 d. Another sample was gram stained and examined under the microscope for gram-negative spiral bacilli. Isolates from the cultures were identified as *H. pylori* based on typical colony morphology on selective agar, and with gram staining and oxidase, catalase and urease tests.

The bacteria cultured from each case were kept in separate cryo vials with 0.75 mL of 20% glycerol containing Brucella broth medium at  $-80^\circ\text{C}$  until susceptibility testing was done.

### Minimum inhibitor concentration study

Each stock cultured specimen was warmed, re-plated on 7% sheep blood Brucella agar medium, and incubated in microaerophilic conditions at  $37^\circ\text{C}$  for 3-5 d. The pure cultured *H. pylori* strains were then tested for susceptibility to amoxicillin and clarithromycin using the agar dilution method, in accordance with the National Committee for Clinical Laboratory Standards (NCCLS)<sup>[12-14]</sup>. Using appropriate solvents, two-fold serial dilutions of each antibiotic were prepared resulting in final concentration ranges of 0.015-4.0  $\mu\text{g/mL}$  for amoxicillin (Sigma Chemical Co.) and 0.015-8.0  $\mu\text{g/mL}$  for clarithromycin (Abbott Laboratories). Five percent sheep blood containing Mueller-Hinton agar was used for the agar dilution method. Multiple

suspensions of each *H. pylori* strain to be tested were prepared in sterile saline, and were arranged in order of clarity according to the MacFarland 2 standard technique. Then a 3- $\mu\text{L}$  droplet of each suspension was placed in each of the above-described antibiotic dilutions and on control plates. All strains were studied in pairs, and the *H. pylori* strain NCTC 11637 was used as a control. The inoculated dilution tubes and plates were incubated in microaerophilic conditions at  $37^\circ\text{C}$  for 3 d. For each isolate, the dilution tube with the lowest antibiotic concentration that showed no visible growth was recorded as the minimum inhibitory concentration (MIC). To determine whether or not a strain was susceptible to each antibiotic, we compared the recorded MIC values for amoxicillin and clarithromycin to previously reported MIC cut-off values for these drugs (0.5 and 1  $\mu\text{g/mL}$ , respectively)<sup>[14-17]</sup>.

### Statistical analysis

Numeric values were expressed as mean  $\pm$  SD. The  $\chi^2$  test was used to compare mean values, and the Mann-Whitney *U* test was used to compare numeric values. *P* values  $< 0.05$  were considered to indicate statistical significance.

## RESULTS

A total of 79 *H. pylori* strains (one from each ESRD patient and control subject) were isolated from the antral biopsy specimens. The demographics for the two study groups are shown in Table 1. None of the *H. pylori* strains from either group showed resistance to amoxicillin. Twelve (36.4%) of the 33 ESRD group strains and 7 (15.2%) of the 46 control group strains showed resistance to clarithromycin, and this difference was statistically significant ( $P = 0.03$ ) (Table 1).

Table 2 shows the demographic characteristics of the ESRD patients and control subjects with clarithromycin-resistant and clarithromycin-susceptible *H. pylori* strains. Of

**Table 1** Demographic characteristics and frequencies of antibiotic resistance in the two study groups

	Non-uremic patients <i>n</i> = 46	ESRD patients <i>n</i> = 33	<i>P</i>
Mean age (yr)	$43 \pm 14$	$42 \pm 14$	$> 0.05$
Female/Male	26/20 (43.5%)	17/16 (48.5%)	$> 0.05$
Hemodialysis duration (yr)		$4.1 \pm 2.8$	
Clarithromycin resistance	15.2% (7/46)	36.4% (12/33)	$< 0.05$
Amoxicillin resistance	0% (0/46)	0% (0/33)	

**Table 2** Demographic characteristics of the ESRD and non-uremic patients with clarithromycin-resistant and susceptible *H. pylori* strains

	Resistant	Susceptible	<i>P</i>
ESRD patients			
<i>n</i>	12	21	
Mean age (yr)	$42.9 \pm 12.6$	$40.9 \pm 14.7$	$> 0.05$
Female/Male	7/5	10/11	
Hemodialysis duration (yr)	$5.6 \pm 3.8$	$3.3 \pm 1.8$	$> 0.05$
Non-uremic patients			
<i>n</i>	7	39	
Mean age (yr)	$49 \pm 17$	$42 \pm 14$	$> 0.05$
Female/Male	4/3	22/17	

the twelve ESRD patients with *H pylori* strains that were resistant to clarithromycin, five were males and seven were females. The mean age of these patients was  $42.9 \pm 12.6$  years, and the mean age in the susceptible cases was  $40.9 \pm 14.7$  years. This age difference was not statistically significant ( $P > 0.05$ ). The mean hemodialysis durations in the subgroups of ESRD patients with clarithromycin-resistant and susceptible *H pylori* strains were  $5.6 \pm 3.8$  years and  $3.3 \pm 1.8$  years, respectively ( $P > 0.05$ ).

Of the seven non-uremic control patients with *H pylori* strains that were resistant to clarithromycin, three were males and four were females. The mean age of these patients was  $49 \pm 17$  years, and the mean age in the susceptible cases was  $42 \pm 14$  years. This age difference was not statistically significant ( $P > 0.05$ ).

## DISCUSSION

*H pylori* infection is the most widespread bacterial infection in the world. Antibiotic resistance is important in the management of these patients, and knowledge of resistance pattern helps the clinician to establish the most appropriate treatment strategy<sup>[18-20]</sup>.

The therapy for *H pylori* infection in ESRD patients and non-uremic patients are approximately the same<sup>[21]</sup>. Drugs that contain bismuth may be toxic for individuals with ESRD, and are generally avoided in this population<sup>[11]</sup>. The combination of proton-pump inhibitors, clarithromycin and amoxicillin is frequently used to treat *H pylori* infection in both uremic and non-uremic patients<sup>[4,20,22]</sup>.

For some antibiotics, the *in vivo* and *in vitro* findings for *H pylori* susceptibility differ. Rates of antibiotic resistance in *H pylori* infection, particularly resistance to clarithromycin, are rising dramatically in Turkey and many other countries throughout the world, so it is more important than ever to be aware of this issue<sup>[23,24]</sup>.

One important barrier in the approach to this problem is the lack of a standard system for assessing *H pylori* microorganism susceptibility to antibiotics. The NCCLS subcommittee recommends the agar dilution method for this purpose, and this was our basis for using this technique in our study<sup>[14]</sup>. The NCCLS MIC value for *H pylori* resistance to clarithromycin is 1 µg/mL. There is no standard MIC value for *H pylori* resistance to amoxicillin, so we used the lowest value in the literature (0.5 µg/mL) as the cut-off in our study<sup>[14-17]</sup>.

The *in vitro* efficacy of amoxicillin for *H pylori* infection is high; however, when amoxicillin is used as a single therapeutic agent, this rate drops to approximately 20% due to low efficacy at acid pH<sup>[25]</sup>. This is the reason why amoxicillin is used in combination with anti-secretory drugs<sup>[23]</sup>. A recent report has identified a number of *H pylori* strains that are resistant to amoxicillin<sup>[26-29]</sup>, but none of the isolates in our study showed resistance to this agent.

Clarithromycin is the antibiotic to which *H pylori* is most sensitive *in vitro*, and this agent is widely used in combination regimens. Oral administration yields high serum and tissue concentrations, and the drug is stable in acid environments. Some authors have identified resistance to clarithromycin as the most important problem in *H pylori* treatment failure<sup>[30,31]</sup>.

It has been reported that clarithromycin resistance results in significantly lower *H pylori* eradication rates<sup>[7]</sup>, and studies from various nations have documented increasing rates of *H pylori* resistance to this agent in recent years<sup>[32-34]</sup>. Rates of *H pylori* resistance to clarithromycin are higher in countries where macrolide antibiotics are frequently used<sup>[32]</sup>. The estimated range of resistance to this antibiotic in *H pylori*-infected patients in various nations and regions of the world is 1-15%<sup>[32,35,36]</sup>.

In our *H pylori*-infected study subjects, the rates of *H pylori* resistance to clarithromycin were 15.2% in the otherwise healthy controls and 36.4% in the ESRD patients. These figures are both very high, and provide further evidence that *H pylori* resistance to clarithromycin is on the rise in our country. If this problem continues to escalate, there will be even more serious problems treating *H pylori* infection in future. In addition to the high frequencies we observed in both study groups, the significantly higher rate of clarithromycin resistance in our ESRD group compared to the controls is of particular concern. It is important to be aware that the risk of *H pylori* resistance to this agent is likely to be higher in a patient who takes antibiotics for a variety of reasons. It is essential to know the antibiotic susceptibility of *H pylori* strains in order to identify what drug combination will be effective and how to prevent antibiotic resistance, to avoid using inappropriate drugs, and to decrease costs. To our knowledge, this is the first study that has examined *H pylori* resistance to amoxicillin and clarithromycin in *H pylori*-infected ESRD patients.

In conclusion, it appears that *H pylori* resistance to amoxicillin is not a major problem in ESRD and non-uremic patients in Turkey. However, the *H pylori* isolates from the ESRD patients in this series showed high rates of clarithromycin resistance, and expansion of this problem in all patient groups is a concern worldwide. Patients with ESRD have a high probability of *H pylori* resistance to clarithromycin due to immune compromise and extensive use of antibiotics. In order to effectively treat *H pylori* in this patient group, it is important to know the antibiotic susceptibility pattern of the *H pylori* microorganism present in each individual.

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• BRIEF REPORTS •

# Disturbance of hepatic and intestinal microcirculation in experimental liver cirrhosis

Sasa-Marcel Maksan, Eduard Ryschich, Zilfi Ülger, Martha Maria Gebhard, Jan Schmidt

Sasa-Marcel Maksan, Department of Surgery, University of Mainz, Germany

Eduard Ryschich, Zilfi Ülger, Martha Maria Gebhard, Jan Schmidt, Department of Surgery, University of Heidelberg, Germany

Correspondence to: Sasa-Marcel Maksan, M.D., Department of Surgery, University of Mainz, Langenbeckstrasse 1, D-55131 Mainz, Germany. maksan@ach.klinik.uni-mainz.de

Telephone: +49-6131-171

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## Abstract

**AIM:** To analyze hepatic, mesenteric and mucosal microcirculation and leukocyte-endothelium interaction (LEI) in a rat model with liver cirrhosis.

**METHODS:** Hepatic cirrhosis was induced in Wistar rats by gavage with carbon tetrachloride, and intravital videomicroscopy was performed in liver, mesentery and small intestine mucosa. Special emphasis is given on microcirculatory and morphometric changes during cirrhotic portal hypertension.

**RESULTS:** LEI was influenced significantly in the cirrhotic liver but not in the gut. Blood flow measurement showed significant differences among liver, main mesenteric vessels and the mucosa. The results of our study indicate that liver cirrhosis leads to alterations in hepatic and mesenteric blood flow but not in mucosal blood flow.

**CONCLUSION:** The enhanced inflammatory response in hepatic microvessels may be caused by a decrease of antithrombin III levels and could be responsible for disturbances of organ pathology.

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**Key words:** Liver cirrhosis; Microcirculation; Small intestine; Liver

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## INTRODUCTION

Liver cirrhosis is recognized as an important risk factor for

the development of severe septic complications such as spontaneous bacterial peritonitis and bacteraemia<sup>[1]</sup>. The impairment of intestinal microcirculation and mucosal barrier may contribute to an increased intestinal permeability and bacterial translocation<sup>[2-4]</sup>. Hypoperfusion of the gut mucosa has been implicated as an important mechanism contributing to gut-derived endotoxemia and bacteremia in liver cirrhosis. Due to splanchnic arterial vasodilatation and portal hypertension, intestinal capillary pressure is altered and effective arterial blood volume decreases<sup>[5]</sup>.

It is generally recognized that there is a close relationship between the hepatic microcirculation and liver function and structure<sup>[6,7]</sup>. Recent studies established the important correlation between hepatic microcirculation and the development of liver pathology<sup>[8]</sup>. Furthermore, it has been described that leukocyte adherence in liver sinusoids is amplified after gut ischemia and reperfusion due to an up-regulation of adhesion molecules<sup>[9]</sup>.

We still lack quantitative data describing hepatic and mesenteric microvascular parameters such as blood flow velocity, volumetric blood flow, leukocyte-endothelial interaction and vessel size within one experimental setting. Currently, intravital videomicroscopy is the most pretentious technique to measure variables of microvascular perfusion *in vivo*. In addition to the measurement of mesenteric blood flow and leukocyte kinetics, we quantified mucosal blood flow in terminal arterioles directly, since the assessment of mucosal blood flow has been simplified<sup>[10]</sup>.

The present study was undertaken to quantitate basic microvascular parameters in normal rat liver and intestine and to investigate changes in these parameters associated with the development of cirrhosis.

## MATERIALS AND METHODS

Male Wistar rats weighing  $450 \pm 47$  g at the time of surgery (initial weight  $250 \pm 5$  g) were used in all experiments. They were housed in a controlled environment with a 12-h light: dark cycle and were fed standard rat diet with water *ad libitum*.

Chronic, progressive hepatic cirrhosis was induced according to the method of Proctor and Chatamra by gavage with carbon tetrachloride ( $\text{CCl}_4$ )<sup>[11]</sup>. Animals were given phenobarbital (35 mg/100 mL) in their drinking water to induce the enzyme cytochrome P450, which has been shown to increase rat liver sensitivity to  $\text{CCl}_4$ <sup>[12]</sup>. Two weeks later an initial dose of 0.04 mL  $\text{CCl}_4$  was given after orogastric intubation and enhanced weekly in steps of 0.04 mL to a maximum dose of 0.4 mL  $\text{CCl}_4$ . Animals were weighed weekly.

Six control animals without liver cirrhosis were observed for statistical analysis.

### Videomicroscopy

Videomicroscopy was performed under general anesthesia. Left carotid artery was cannulated with a polypropylene catheter (B. Braun, Melsungen, FRG) to monitor arterial blood pressure, heart rate and for blood-gas analysis (ABL Radiometer). The right jugular vein was cannulated for drug administration. The abdomen was opened via a median incision. The presence of ascites and macroscopic appearance of liver cirrhosis were noted.

For intravital microscopic investigations a Leitz microscope (Leitz GmbH, Wetzlar, Germany) was used. With different filter blocks in the epiillumination technique, selective visualization of FITC-labeled erythrocytes and rhodamine 6G-stained leukocytes was possible. For contrast enhancement, FITC-labeled albumin was administered intravenously.

### Hepatic microcirculation

The hepatic microvascular parameters were measured *in vivo* using the epiillumination microscopy setup described in detail elsewhere<sup>[13]</sup>. In brief, the left liver lobe was exteriorized onto a specially designed stage and prepared for intravital videomicroscopy. Continuous superfusion with buffered 37 °C Ringer's solution was provided. At the beginning of videomicroscopy, FITC-labeled erythrocytes (1 mL/kg BW) and rhodamine 6G-labeled leukocytes (0.05 nmol/kg BW) were injected intravenously. Using a FITC selective filter block, 5 fields of normal liver tissue during a period of 30 s were analyzed. Observations of leukocytes were done using a rhodamine-selective filter block. Vessels with a length of at least 100 µm were registered for a period of 60 s each.

### Intestinal microcirculation

In a second step, terminal ileum was exteriorized and placed on a glass slide. Videomicroscopy of mean mesenteric vessels was performed. Subsequently the bowel was opened along the antimesenteric border and fixed at the incision margins. The prepared bowel was bathed and superfused with buffered Ringer's solution. Mesenteric microcirculation was investigated in 10 fields of ileal arteries and corresponding veins following the scheme of hepatic videomicroscopy. Mucosal microcirculation was measured in the main arteriole of 5 single villus. Each vessel was observed for 30 s.

### Data interpretation

Measurements were videotaped and off-line analysis was performed by means of an image-processing system (Capimage®, Zeintl GmbH, Heidelberg, Germany)<sup>[14]</sup>. The red cell blood velocity and vessel diameter were measured using the frame-to-frame method. Leukocyte contact to endothelium was analyzed and described as leukocyte-endothelium interaction (LEI). The definition of the duration of this interaction results in leukocyte rolling and leukocyte sticking. Leukocyte rolling was based on the movement along the endothelial lining that was less than 66% of the red cell blood velocity. Temporary interactions with the endothelium of not more than 30 s duration were also considered as rollers. Leukocyte sticking was defined as the attachment

to the vessel endothelium for at least 30 s.

Microvascular blood flow ( $V_b$  in nL/min) was calculated using the following equation<sup>[15]</sup>:

$V_b = \pi \times v_c \times (D/2)^2$ . Determinants were erythrocyte velocity ( $v_c$ ) and vessel diameter ( $D$ ).

### Histology and blood tests

At the end of experiments, liver and small intestine specimens were taken and the sections were stained with hematoxylin and eosin. Blood samples were taken for measurement of serum concentration of GOT, GPT,  $\gamma$ -GT and AP. Prothrombin time and the antithrombin III level were taken for hemostatic analysis.

### Statistical analysis

Results were expressed as mean  $\pm$  SD. Statistical analysis was performed using Mann-Whitney *U* test. Results are considered significant at  $P < 0.05$ .

## RESULTS

Cardiorespiratory parameters did not differ between groups throughout the observation period (Table 1).

**Table 1** Cardiorespiratory parameters

	Cirrhosis	Controls	<i>P</i>
Mean arterial blood pressure (mmHg)	107 $\pm$ 8.4	112 $\pm$ 7.3	NS
Heart rate (bpm)	310 $\pm$ 9.2	304 $\pm$ 8.4	NS
paO <sub>2</sub> (mmHg)	89.1 $\pm$ 5.3	92.3 $\pm$ 4.8	NS

### Histological findings

Ascitic rats had microscopic evidence of cirrhosis in all cases. Liver histology revealed extensive deposits of fibrous tissue with regenerative nodules in at least some areas. Foci of necrotic cells were also observed. Sections of ileum showed a higher number of lymph vessels in cirrhotic rats than that in controls.

### Intravital observations

The quantitative measurements of microvascular parameters are summarized in Table 2. Blood flow in terminal arterioles of the villus was similar between both the groups (5.3 $\pm$ 0.3 *vs* 5.4 $\pm$ 0.2 nL/min in control animals, NS). Marked differences were observed in main mesenteric blood flow (135.1 $\pm$ 3.5 *vs* 156.5 $\pm$ 4.3 nL/min in controls,  $P < 0.01$ ). Liver blood flow remained comparable (32.1 $\pm$ 0.4 *vs* 31.2 $\pm$ 0.6 nL/min in controls, NS), although red blood cell velocity in cirrhosis was reduced significantly (0.93 $\pm$ 0.09 *vs* 1.22 $\pm$ 0.18 mm/s in controls,  $P < 0.05$ ).

Hepatic LEI was enhanced in cirrhosis. The number of rolling leukocytes and high-affinity leukocytes raised significantly (4.80 $\pm$ 0.90 *vs* 2.33 $\pm$ 0.75 roller/100 µm and 1.91 $\pm$ 0.28 *vs* 0.5 $\pm$ 0.5 sticker/100 µm in controls,  $P < 0.01$ ). Analysis of LEI in main mesenteric arterioles showed no differences between the groups (Table 3).

**Table 2 Microcirculatory parameters**

	Cirrhosis	Controls	P
Vessel diameter (μm)			
Liver	27.86±2.03	23.03±0.62	<0.01
Mesentery	32.06±8.91	33.46±11.66	NS
Mucosa	7.51±0.30	7.20±0.23	NS
Red blood cell velocity (mm/s)			
Liver	0.93±0.09	1.22±0.18	<0.05
Mesentery	2.81±0.49	3.11±0.35	NS
Mucosa	2.03±0.15	2.18±0.16	NS
Volumetric blood flow (nL/min)			
Liver	32.10±0.4	31.20±0.6	NS
Mesentery	135.10±3.5	156.50±4.3	<0.01
Mucosa	5.30±0.3	5.40±0.2	NS

**Table 3 Leukocyte-endothelium interaction**

	Cirrhosis	Controls	P
Adherent leukocytes (n/100 μm)			
Liver	1.91±0.28	0.50±0.5	<0.01
Mesentery	2.54±1.19	1.62±0.85	NS
Rolling leukocytes (n/100 μm)			
Liver	4.80±0.90	2.33±0.75	<0.01
Mesentery	7.68±3.18	6.88±1.94	NS

### Blood tests

Serum enzyme tests revealed significant disturbances of hepatocellular integrity and reduction of blood clotting capacity. Further antithrombin III levels were significantly reduced in cirrhotic animals (108.5±14.59 vs 125.0±8.96 IU in controls,  $P<0.05$ ) (Table 4).

**Table 4 Blood tests**

	Cirrhosis	Controls	P
GOT (U/L)	63.67±10.42	28.67±4.85	<0.01
GPT (U/L)	31.83±4.84	16.67±1.11	<0.01
GGT (U/L)	5.33±1.49	3.17±0.69	<0.05
AP (U/L)	130.50±35.75	56.83±2.67	<0.01
Prothrombin time (%)	75.30±5.4	102.4±2.7	<0.01
Antithrombin III (IU)	108.50±14.59	125.0±8.96	<0.05

## DISCUSSION

Liver cirrhosis has been detected to be an important risk factor for mortality in critically ill patients<sup>[16]</sup>. Its incidence in western countries is rising and represents the most common cause of mortality among the non-malignant digestive diseases<sup>[17]</sup>. The mechanisms for the increased morbidity and mortality may be immunological, mechanical or pharmacological. Special emphasize is given to hepatic and intestinal microcirculatory disorders. However, it is still not clear how alterations in hepatic microcirculation contribute to the progression of the disease and its sequelae.

In the present study, we have applied the technique of intravital videomicroscopy to quantitate intestinal and hepatic microcirculation and LEI in cirrhotic rats. We used a model of CCl<sub>4</sub>-induced cirrhosis. Liver damage is mainly

caused by hepatotoxic CCl<sub>4</sub> metabolites produced within the liver. The intensity of the hepatic damage is exaggerated when the microsomal enzyme oxidizing system is previously induced, for instance by the administration of phenobarbital<sup>[12]</sup>. Induced cirrhosis is associated with marked hemodynamic disturbances. These include changes in both the intrahepatic and splanchnic circulation resulting in portal hypertension<sup>[18,19]</sup>. Our quantitative studies of hepatic and mesenteric microvascular parameters indicate that cirrhosis leads to a marked arterial vasodilatation. This observation is in accordance with other experimental studies. The pathogenesis of vascular tone is explained by biochemical investigations and disorders of vascular heme oxygenase-1 expression and nitric oxide synthase expression in experimental cirrhosis<sup>[20,21]</sup>.

In this study, presence of liver cirrhosis had no effect on villus blood flow measured in the terminal, central villus arteriole. To our knowledge, there is no other study where the effects of cirrhosis and portal hypertension on villus blood flow were derived directly from data obtained on the level of single villus arterioles. The disturbance of mucosal blood flow may contribute to an impairment of the intestinal barrier function. It has been described that alterations of the mucosal permeability in cirrhosis are related to the degree of liver failure and to the progression of the liver disease<sup>[4]</sup>.

However, the impact of liver cirrhosis on hepatic microcirculation is more evident. LEI is enhanced significantly and may explain organ disturbances. LEI itself is modulated by a variety of adhesion molecules expressed on the surface of leukocytes and endothelial cells. These adhesion molecules mediate the decrease in leukocyte rolling and the increase in leukocyte adherence and migration.

In summary, this study demonstrates that in the gut mucosa, cirrhosis may not induce disturbances in the villus microcirculation, despite altered levels of mesenteric blood flow.

Experimental liver cirrhosis is associated with a marked increase of liver enzymes and a decrease of antithrombin III levels and prothrombin time. Antithrombin III is one of the most important physiological inhibitors of coagulation. It is synthesized in liver parenchymal cells. Moreover, it has been described that AT III has anti-inflammatory actions in experimental sepsis and hepatic ischemia-reperfusion<sup>[22,23]</sup>. It could be presumed that in cirrhosis a drop of the antithrombin III level leads to hemostatic disorders similar to disseminated intravascular coagulation (DIC) and inflammatory response in the microcirculatory units, even if clinical observations did not emphasize those findings<sup>[24,25]</sup>. In our study, hemostatic dysfunction could be measured and LEI in liver parenchyma and mesenteric vessels was enhanced significantly.

We conclude that experimental liver cirrhosis leads to disturbances in hepatic and mesenteric but not in mucosal microcirculation. Although not proven by our experiments, lower antithrombin III levels as found in our study could potentially maintain or aggravate hemostatic disorders and alterations in hepatic inflammatory response to portal hypertension associated with a deterioration of liver function.

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• BRIEF REPORTS •

# Cysteine peptidase and its inhibitor activity levels and vitamin E concentration in normal human serum and colorectal carcinomas

Robert Szwed, Zygmunt Grzebieniak, Yousif Saleh, Godwin Bwire Ekonjo, Maciej Siewinski

Robert Szwed, Zygmunt Grzebieniak, Clinic of Surgery and Oncology, Wrocław Medical University, 2 Skłodowskiej-curie Street, 50-368 Wrocław, Poland

Yousif Saleh, Godwin Bwire Ekonjo, Department of Obstetrics and Gynaecology, Wrocław Medical University, 3 Chalubenskiego Street, 50-368 Wrocław, Poland

Maciej Siewinski, Faculty of Public Health, Medical University of Wrocław; Bartla Street, 5, 50-618 Wrocław, Poland

Correspondence to: Maciej Siewinski, Ph.D., Faculty of Public Health, Medical University of Wrocław; Bartla Street, 5, 50-618 Wrocław, Poland. siewinski@op.pl

Telephone: +48-71-7842413 Fax: +48-71-7840111

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## Abstract

**AIM:** Cysteine peptidase (CP) and its inhibitor (CPI) are a matrix protease that may be associated with colorectal carcinoma invasion and progression, and vitamin E is also a stimulator of the immunological system. Our purpose was to determine the correlation between the expression of cysteine peptidases and their endogenous inhibitors, and the level of vitamin E in sera of patients with colorectal cancer in comparison with healthy individuals.

**METHODS:** The levels of cysteine peptidases and their inhibitors were determined in the sera of patients with primary and metastatic colorectal carcinoma and healthy individuals using fluorogenic substrate, and the level of vitamin E was determined by HPLC.

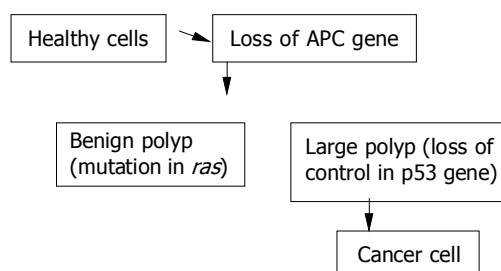
**RESULTS:** The levels of cysteine peptidases and their inhibitors were significantly higher in the metastatic colorectal cancer patients than that in the healthy controls ( $P < 0.05$ ). The activity of CP increased 2.2-fold, CPI 2.8-fold and vitamin E decreased 3.4-fold in sera of patients with metastasis in comparison with controls. The level of vitamin E in healthy individuals was higher, whereas the activity of cysteine peptidases and their inhibitors associated with complexes was lower than that in patients with cancer of the digestive tract.

**CONCLUSION:** These results suggest that the serum levels of CP and their inhibitors could be an indicator of the prognosis for patients with metastatic colorectal cancer. Vitamin E can be administered prophylactically to prevent digestive tract neoplasmas.

Szwed R, Grzebieniak Z, Saleh Y, Ekonjo GB, Siewinski M. Cysteine peptidase and its inhibitor activity levels and vitamin E concentration in normal human serum and colorectal carcinomas. *World J Gastroenterol* 2005; 11(6): 850-853  
<http://www.wjgnet.com/1007-9327/11/850.asp>

## INTRODUCTION

The first pathologic changes taking place in the organs of patients who presently are regarded as having neoplastic changes were recorded<sup>[1]</sup>. The results of diagnostic and statistical data have shown that in Poland, neoplastic diseases occur in about 75 000 people yearly and account for about 40% of these neoplastic changes in the digestive system. It is assumed that by the year 2010 the number of people with neoplastic changes will reach up to 80%<sup>[2]</sup>. It is also assumed that one of the basic causes of neoplastic changes is environmental pollution as a result of advances in industrialization<sup>[3]</sup>. Epidemiologic data were from the Centre of Oncology in Heidelberg, where examinations on the level of pollution in water, on land, and in the air by carcinogenic substances in Europe were carried out, and showed that the highest level of pollution caused by substances leading to cancer were observed in the region of Legnica and Głogów<sup>[4]</sup>. These results were supported by genetic test researches, showing that these substances could cause mutation of the digestive tract epithelial cells. It has also been shown that the mutated suppressor gene APC could take place in chromosome 5 (MCC - mutated in colorectal carcinoma), and the DCC gene (deleted in colorectal carcinoma) codes for proteins determining adhesion between mutated and unmutated cells. The information obtained from these examinations has made it possible to prepare a hypothetical scheme of neoplastic cell induction in the digestive tract (Figure 1)<sup>[5]</sup>.



**Figure 1** Hypothetic scheme of neoplastic cell induction in digestive tract.

For the further development of cancer, an important role is played by proteolytic enzymes like elastase, collagenase, metalloproteinases, cysteine peptidase, cathepsins B and L as well as cathepsin D. The researches carried out by many research groups have confirmed the fact that enzymes taking part in the process of carcinogenesis can autoactivate enzymatic transformation cascade in which cysteine peptidase plays a key role<sup>[6,7]</sup>. At the same time, it has also been shown that the relationship between cathepsins and caspases which are responsible for necrosis and apoptosis in cells helps the development of cancer<sup>[8]</sup>. A rise in the activity of cysteine peptidase was observed in the sera of patients<sup>[9]</sup> and in cell cultures and cancer tissues of the digestive tract<sup>[10]</sup>. We assumed that they could be used as components in the new generation of drugs referred to as inhibitherapy which could be used in cancer therapy as well as inhibiting the growth of pathogenic microorganisms<sup>[11,12]</sup>.

It was found that vitamin E could inhibit cysteine endopeptidase activities by increasing endogen inhibitor level. The level of vitamin E in healthy individuals was higher than that in patients who were exposed to the action of toxic substances as well as in patients with cancer<sup>[13]</sup>. In cell cultures, vitamin E could inhibit the expression of oncogenes *H-ras* and *c-myc* in cells, which are the factors determining the initiation of neoplastic processes<sup>[14]</sup>. Sakamoto *et al.*<sup>[15,16]</sup> suggested that they could activate macrophages leading to the production of interleukin-1, which acts on fibroblasts, lymphocytes catalyzing the secretion of interleukin 6, which increases the level of kininogen T, an autogenic inhibitor of cysteine peptidase.

The level of cysteine peptidases and their inhibitors and the level of vitamin E in the sera of patients with different degrees of digestive tract cancer were determined as compared to analogous markers in healthy individuals.

## MATERIALS AND METHODS

### Materials

All patients whose sera were tested were inhabitants of Legnica and Glogow, which is the most polluted region of Poland by carcinogenic substances, who had been living in this region for at least five years and the exposure level to the action of carcinogenic substances was the same (this concerns both healthy individuals and those with neoplastic changes in the digestive tract).

We purposely did not choose the people performing jobs which made them at a higher risk of exposure to toxic and carcinogenic elements. The aim was to eliminate the effect of toxic substances present in the working environment especially when it was a threat to the health of workers.

Blood samples were collected from the cubital veins of healthy males and females as well as those with neoplastic changes in the group aged 35-70 years. We determined the active cysteine peptidases and their inhibitors in each serum sample and the amount (free, total amount produced in the organism) bound to the enzyme-inhibitor complex. After routine diagnostic tests were carried out, the patients taking part in the research were divided into 5 groups, 25 in each group, so that the results obtained could be tested statistically. Group A: healthy individuals without neoplastic changes,

not genetically developing malignant cancer of the digestive tract; group B: patients with changes that did not show the qualities of malignancy (6 gastric adenomas, 13 colorectal adenomas, 5 ulcerative colitis, 1 patient with Crohn's disease); group C: patients with cancer before operation and chemotherapy; group D: patients operated once after chemotherapy; group E: patients with non-operative cancer and metastases.

### Cysteine peptidase activity (CP)

Sample probes of sera were filled up to 950 µL of 0.01 mol/L phosphate-buffered solution at pH 6.0 containing 2 nmol/L DL of cysteine and 2 nmol/L EDTA. Next, 50 µL 1.5 mmol/L of substrate benzoilo-arginylo β-naftylo amid (BANA) was added. The whole probe was incubated at 37 °C. After a 12-h reaction, a break was taken during which β-naftyloamine was released. As a unit of activity, we chose the amount of enzyme which hydrolyzed 1 nmol β-naftyloamine<sup>[17]</sup> within an hour.

### Cysteine peptidase inhibitor activity

Fifty microliters of 0.01% papain solution with activities of 3-4 units/mg protein was added to sera samples, and preincubated for 10 min at 37 °C, and then filled 950 µL 0.01 mol/L of phosphate buffered solution at pH of 6.0 containing 2 nmol/L DL cysteine and 2 nmol/L EDTA. Fifty microliters of 1.5 nmol/L of substrate solution was incubated at 37 °C, after 30 min the amount of released β-naftyloamine was determined. We assumed that a unit of inhibition was the amount of inhibitors in sera samples which inhibited one unit of papain<sup>[18]</sup>.

### Total amount of cysteine peptidase inhibitors in sera

Twenty to 200 µL sera samples each with the same amount of 0.02 mol/L HCl was preincubated for 20 min at 80 °C, cooled to room temperature and then filled up to 1.0 mL of 0.02 mol/L of phosphate-buffered solution at pH 6.0 containing 2 nmol/L DL cysteine and 2 nmol/L EDTA. One hundred microliters of each sample was collected and the inhibitory activity with respect to papain was determined as described in ICP<sup>37[18]</sup>.

### Cysteine peptidase inhibitors associated with complexes Δ ICP

The amount of inhibitors associated with enzyme-inhibitor complexes was calculated in each serum sample as a difference between the total amount of inhibitors and their active forms ( $\Delta\text{ICP} = \text{ICP}^{80} - \text{ICP}^{37}$ ).

### Determination of vitamin E level

Vitamine E was determined in sera samples by liquid chromatographic methods using HPLC apparatus from Philips and a detector Pye Unicam PU 4020 UV using a computer program, the peak simple chromatography data system<sup>[19]</sup>.

In all the samples tested, the enzymatic activity and the level of inhibitors and the amount of vitamin E were calculated and converted to 1.0 mL sera.

### Statistical analysis

Statistical determination was carried out with the help of

the program Statgraf. Distribution analysis of results was done using Colmogorov-Smirnoffs test. The examined parameters possessed a normal distribution in which parametric tests were used. The results obtained were compared using Student's *t*-test and Vilcox or Kruskal-Wallis.  $P < 0.05$  was considered statistically significant.

## RESULTS

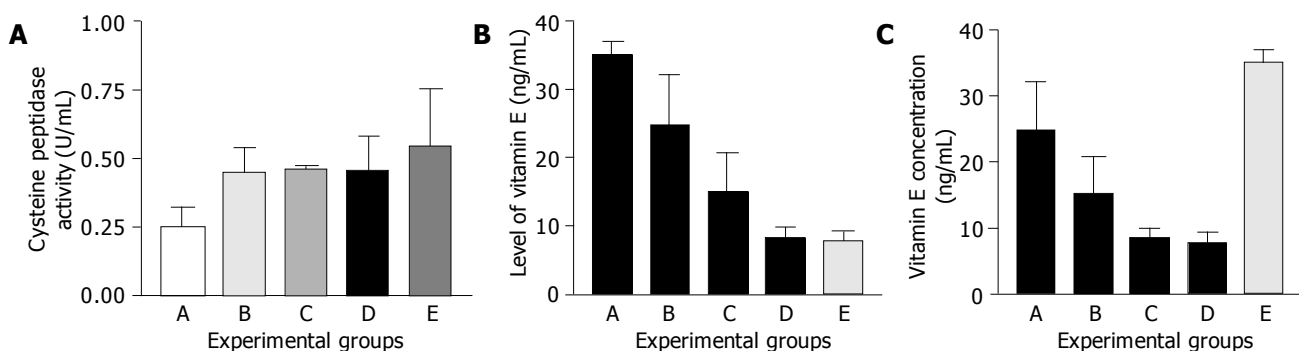
Figure 2A shows the activity of cysteine peptidases (cathepsins B and L) in sera of patients with different stages of colorectal cancer before and after chemotherapy and operation in comparison with control healthy blood. The mean activity of cysteine peptidases and their inhibitors was highly significant in comparison with controls ( $P < 0.05$ ). The activity increased 2.2-fold in sera of patients with metastasis in comparison with controls. The mean activity value of cysteine peptidases was  $0.250 \pm 0.077$  U/mL in group A,  $0.448 \pm 0.092$  U/mL in group B,  $0.461 \pm 0.0100$  U/mL in group C,  $0.452 \pm 0.132$  U/mL in group D, and  $0.547 \pm 0.212$  U/mL in group E. The mean value of inhibitor activity as a complex form ( $\Delta$  CPI) in each group was as follows:  $20.54 \pm 9.72$  U/mL in group A,  $32.92 \pm 11.17$  U/mL in group B,  $44.18 \pm 19.0$  U/mL in group C,  $27.64 \pm 8.23$  U/mL in group D, and  $58.22 \pm 20.12$  U/mL in group E. The differences were statistically significant (Figure 2B). The activity increased 2.8-fold in patients with metastasis in comparison with control group. By examining the level of free inhibitors of cysteine peptidase as well as the total amount, we were unable to show any statistically significant difference in the examined groups A, B, C, D and E. Compared with the level of vitamin E in the sera of examined patients, the highest value of vitamin E was observed in healthy individuals. It was  $35.0 \pm 0.03$  ng/mL in group A,  $24.71 \pm 7.41$  ng/mL in group B,  $15.08 \pm 5.67$  ng/mL in group C,  $8.42 \pm 1.54$  ng/mL in group D, and  $7.78 \pm 1.54$  ng/mL in group E. The difference was statistically significant in comparison with controls ( $P < 0.05$ ) and the results are presented in Figure 2C.

## DISCUSSION

In the research we wanted to find out if there was any

relationship between active cysteine peptidases, their autogenic inhibitors and the level of vitamin E. An examination of all these parameters was carried out on the same samples obtained from patients with neoplasmas of the digestive tract and healthy individuals.

In the organs of healthy people, equilibrium was observed between active enzymes from the cysteine peptidases and their autogenic inhibitors. In determining the activity of the inhibitors, we used papain which in laboratory examinations was used in stead of other cysteine peptidases such as cathepsins B and L<sup>[20]</sup>. The activity of cysteine peptidases (cathepsins B and L) in sera of patients with different stages of colorectal cancer before and after chemotherapy and operation was compared with controls. The mean activity of cysteine peptidases and their inhibitors was highly significant in comparison with controls ( $P < 0.05$ ). The activity of CP increased 2.2-fold and CPI 2.8-fold in sera of patients with metastasis in comparison with controls. During the development process of neoplastic diseases, the activity of these enzymes increased, and other activated proteolytic enzymes like elastase, kolagenase causing catalytic degradation of healthy tissues initiated the development of a disease and their autogenic inhibitors were not able to stop the developing changes<sup>[21,22]</sup>. The aim of many researchers was to know the cause of a shift in equilibrium between enzyme-inhibitors. It is assumed that the answer to this question may help gain a better knowledge of accompanying neoplastic changes. Information on this topic may support the conventional methods of treating malignant cancers<sup>[23,24]</sup>. Our research group dealt with the role of cysteine peptidases and their inhibitors in diagnosis and therapy of neoplastic changes<sup>[7,17,24,25]</sup>. The essence of information on enzymatic changes accompanying neoplastic processes was shown in the results obtained by a Japanese research group. They discovered that it was possible to increase the level of autogenic cysteine peptidase inhibitors after administration of large doses of vitamin E<sup>[15]</sup>. Our results showed that the level of vitamin E decreased 3.4-fold in patients with metastasis in comparison with healthy controls. Earlier researches showed that the level of vitamin E was lower in patients with cancer than in healthy people. This research was carried out in healthy individuals and patients with cancer<sup>[13]</sup>. Our research was based on this work and connected two



**Figure 2** Changes in cysteine peptidases and their inhibitor activities and vitamin E concentration in patients with colorectal cancer in comparison with controls. A: Healthy individuals (control); B: Patients with primary neoplasms; C: Patients with cancer before operation and chemotherapy; D: Patients operated once after chemotherapy; E: Patients with non operative cancer and metastases (A) Cysteine peptidase activities (U/mL) (B) level of vitamin E (ng/mL) (C) vitamin E concentration (ng/mL).

independent research areas. The first was associated with the role of enzymatic processes and their inhibitors as well as vitamin E in neoplastic processes. Our research was limited to know the role of substances in initiating neoplasmas of the digestive tract in the inhabitants of Legnica and Głogów, which is the most polluted region of Poland by carcinogenic substances. In our research, we presented the assay results of the level of active enzymes determining the autogenic intensity of neoplastic processes, and the autogenic inhibitors controlling these changes and vitamin E, which may affect the enzyme-inhibitor equilibrium<sup>[26,27]</sup>. The present research was to give an answer to the questions like the relationship between the level of neoplasm and the activity of enzymes belonging to the cysteine peptidase group, their autogenic inhibitors and vitamin E. The results obtained confirmed that in healthy people the level of vitamin E was the highest and the activity of cysteine peptidases and their inhibitors had the least values. We therefore believe that this idea has a close relationship with the research by Sakamoto *et al.*<sup>[15]</sup>. It is possible that vitamin E could induce an increase of the level of kininogen (an autogenic inhibitor of cysteine peptidase) in the organs, which play a key role in the transformation of neoplastic cells, invasion and metastases and in controlling the formation of apoptotic and necrotic changes in cells<sup>[28]</sup>.

In conclusion, the level of vitamin E in healthy individuals is higher, whereas the activity of cysteine peptidases and their inhibitors associated with complexes is lower than that in patients with cancer of the digestive tract. Vitamin E can be prophylactically administered to prevent digestive tract neoplasmas.

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• BRIEF REPORTS •

## Relationship between polymorphism of class II transactivator gene promoters and chronic hepatitis B

Ying-Ren Zhao, Ling Gong, Ying-Li He, Fang Liu, Chang Lu

Ying-Ren Zhao, Ling Gong, Ying-Li He, Fang Liu, Chang Lu,  
Department of Infectious Diseases, First Hospital of Xi'an Jiaotong  
University, Xi'an 710061, Shaanxi Province, China  
Supported by the National Natural Science Foundation of China,  
No. 30270597

Correspondence to: Dr. Ying-Ren Zhao, Department of Infectious  
Diseases, First Hospital of Xi'an Jiaotong University, Xi'an 710061,  
Shaanxi Province, China. zhaoyingren@sohu.com  
Telephone: +86-29-85323634 Fax: +86-29-85323647  
Received: 2004-04-06 Accepted: 2004-05-29

### Abstract

**AIM:** To investigate the relationship between the polymorphism of class II transactivator (CIITA) gene promoters and chronic hepatitis B (CHB).

**METHODS:** Genomic DNA was prepared from peripheral blood leukocytes. Promoters I, III and IV of gene were analyzed respectively with polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) in 65 patients with CHB, 26 patients with acute hepatitis B (AHB) and 85 normal controls.

**RESULTS:** No abnormal migration was found in PCR-SSCP analysis of the three promoters in the three groups. Also, no sequential difference was observed at the three promoters among the CHB patients, AHB patients and normal controls.

**CONCLUSION:** No polymorphism in promoters I, III and IV of CIITA gene exists in CHB patients, AHB patients and normal controls, suggesting that the promoter of CIITA gene might be a conserved domain.

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**Key words:** Class II transactivator gene promoter; Hepatitis B

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<http://www.wjgnet.com/1007-9327/11/854.asp>

### INTRODUCTION

Major histocompatibility complex (MHC) class II molecules play a central role in immune responses, and the regulation of this family of genes occurs primarily at the transcriptional

level. The class II transactivator (CIITA), a non-DNA-binding protein, is the master regulator of the MHC class II gene expression<sup>[1-5]</sup>. Human CIITA is encoded on chromosome 16, and the initial CIITA cDNA encodes an 1130-aminoacid protein. CIITA is the major rate-limiting factor for both constitutive and interferon- $\gamma$ -induced expression of MHC class II gene. Furthermore, the expression of MHC class II is quantitatively controlled at the CIITA level<sup>[4,6]</sup>. Human CIITA gene expression is controlled by 4 distinct promoters (PI to PIV), and they are cell-type-specific and have different activation profiles, leading to multiple CIITA transcripts with different first exons, and 3 of the forms predominating. Promoter I and promoter III direct dendritic cell and B-cell-specific expressions respectively, whereas promoter IV mediates IFN- $\gamma$ -inducible expression<sup>[7-10]</sup>. Recently, it has been shown that CIITA plays an important role in infectious diseases, cancer and autoimmune diseases<sup>[11-16]</sup>. Infection with hepatitis B virus (HBV) may result in different clinical outcomes<sup>[17]</sup>. There is strong evidence in HBV infection that host genetic factors play a major role in determining the outcome of the infection<sup>[18-23]</sup>. Therefore, to explore the relationship between polymorphism of CIITA gene promoters and chronic hepatitis B(CHB), we analyzed the sequence variability at promoters of CIITA transcription factor in CHB patients and compared with acute hepatitis B (AHB) patients and normal subjects by SSCP analysis of the PCR-amplified CIITA promoter region.

### MATERIALS AND METHODS

#### Subjects

Sixty-five patients (42 men and 23 women, mean age, 35.5 years) with CHB and 26 patients (18 men and 8 women, mean age, 32.3 years) with AHB, and 95 healthy blood donors (62 men and 33 women, mean age, 33.4 years) from the First Hospital of Xi'an Jiaotong University, Xi'an, China, were included in this study. The diagnosis of all the cases was made according to the criteria established on the Viral Hepatitis National Conference held in 2000 in Xi'an, China<sup>[24]</sup>. All the patients and controls were Chinese Han nationals without relatives from Xi'an.

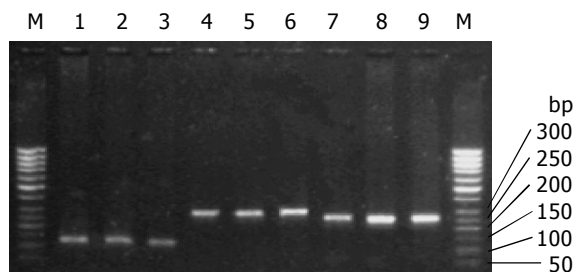
#### DNA preparation and PCR amplification

Genomic DNA was isolated from whole blood by the salt precipitation method, and dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) for polymerase chain reaction (PCR) analysis. The designed primers were synthesized by BioAsia Company, Shanghai, China. The specific primer sequences of promoters I, III and IV of C II TA gene are shown in Table 1. The PCR mixture (total

**Table 1** Primers used for CIITA promoters PCR amplification

Promoters	Primer sequence	Position	Region amplified	Annealing temperature (°C)
1 Sense	5'-TGGAGTCTGAATCAACCCAA-3'	-120/-101	120 bp	58
1 Antisense	5'-AGGGAACCTCTGCAATTTAT-3'	-20/-1		
3 Sense	5'-TGCAGAAGGTGGCAGATATT-3'	-321/-302	298 bp	56
3 Antisense	5'-CAAGCTAAGCCAACATGCAA-3'	-43/-24		
4 Sense	5'-GGTGGACTGAGTTGGAGAGA-3'	-354/-334	257 bp	60
4 Antisense	5'-GGAGCAACCAAGCACCTACT-3'	-117/-98		

volume 25  $\mu$ L) contained 50-100 ng of genomic DNA (0.5-1  $\mu$ L), 2.5  $\mu$ L of 10 mmol/L PCR buffer, 2.5  $\mu$ L of 25 mmol/L  $MgCl_2$ , 2  $\mu$ L of 2.5 mmol/L dNTPs, 1  $\mu$ L each of two fragment-specific primers, 15  $\mu$ L of triple-distilled water, and 1 unit of Taq DNA polymerase (Promega, USA). The PCR mixtures were pre-denatured at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s, and extension at 72 °C for 40 s, and the final extension at 72 °C for 5 min. The amplified fragments were run on a 15 g/L agarose gel, and confirmed to be existent (Figure 1).



**Figure 1** Identification of amplified CIITA gene promoters. Lane M: GeneRuler 50 bp DNA ladder; lanes 1-3: PCR product of promoter I of CIITA gene; lanes 4-6: PCR product of promoter III of CIITA gene; lanes 7-9: PCR product of promoter IV of CIITA gene.

### SSCP analysis

Two microliters of each PCR product was mixed with 8  $\mu$ L of denaturing dye (950 mL/L formamide, 10 mmol/L NaOH, 0.5 g/L bromophenol blue, 0.5 g/L xylene cyanol), denatured at 96 °C for 5 min, and transferred into an ice-cold water bath for 3 min. The PCR products were resolved on an 80 g/L polyacrylamide gel under non-denaturing

conditions in 1 g/L TBE buffer. Electrophoresis was performed at 250 V and at 4 °C for 2-3 h. Silver staining was done by fixing the gel in 100 g/L glacial acetic acid for 20 min, rinsed 3 times with ultra-pure water for 2 min each, immersed in 1 g/L  $AgNO_3$  solution for 30 min, and finally the gel was rinsed and developed with 30 g/L  $Na_2CO_3$  till the bands appeared (Figure 2 A-C). All the reagents were of analytical grade.

### DNA sequencing

The PCR products, which showed mobility shift in SSCP, were extracted from a 10 g/L agarose gel and column purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany), and direct sequencing was performed by BioAsia Company, Shanghai, China.

## RESULTS

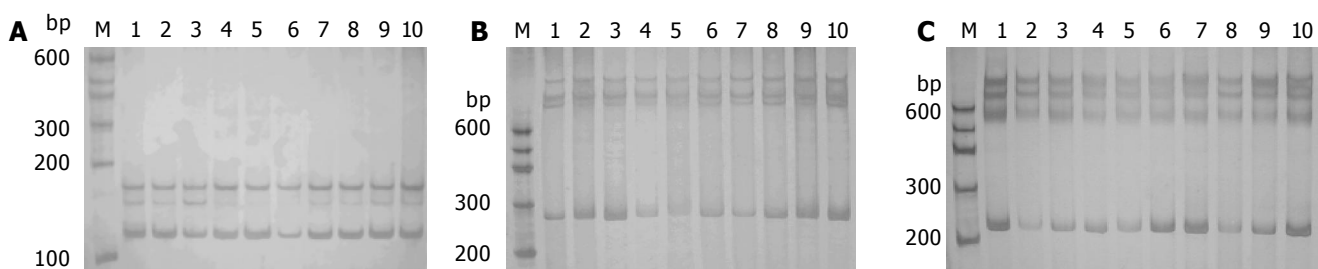
### Analysis of PCR products

The increment of all DNA samples from the CHB patients, AHB patients and normal controls was a single strand with 120, 298 and 257 bp in length respectively, which indicated that a large fragment insertion and deletion did not exist in the region of CIITA gene promoters I, III and IV among the subjects.

### SSCP analysis

For each promoter, identical SSCP patterns were detected in PCR products derived from all the subjects, whether CHB patients, AHB patients or healthy controls, with no evidence of polymorphism. Selective DNA bands detected by SSCP analysis randomly and direct DNA sequencing conformed to this result.

Thus, in the samples analyzed in the present study, no difference was found at CIITA gene promoters I, III and



**Figure 2** PCR-SSCP analysis of promoter I, III, IV of CIITA gene. A: Lane M: 100 bp DNA ladder; lanes 1-4: CHB patients; lanes 5-7: AHB patients; lanes 8-10: normal controls; B: Lane M: 100 bp DNA ladder; lanes 1-4: CHB patients; lanes 5-7: AHB patients; lanes 8-10: normal controls; C: Lane M: 100 bp DNA ladder; lanes 1-4: CHB patients; lanes 5-7: AHB patients; lanes 8-10: normal controls.

IV among CHB patients, AHB patients and healthy controls. The three CIITA promoters resulted in no polymorphism.

## DISCUSSION

It has been estimated that HBV is present in a reservoir of more than 130 million chronic carriers, accounting for more than 10% of Chinese population<sup>[25]</sup>. Infection with HBV can cause a broad spectrum of diseases, including asymptomatic HBV carriers, acute hepatitis, chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma. Virological factors may play a role in the outcome of the infection, while infection with the same HBV virus has been found to cause various clinical outcomes in different patients suggesting that there is a strong immunogenic component to affect the individual susceptibility to HBV. So far, histocompatibility leukocyte antigen (HLA)<sup>[26-29]</sup>, tumor necrosis factor alpha (TNF- $\alpha$ )<sup>[30]</sup>, interleukin-6 (IL-6)<sup>[31]</sup>, interleukin-10 (IL-10)<sup>[32,33]</sup>, mannose-binding lectin (MBL) gene<sup>[34]</sup>, and vitamin D receptor<sup>[35]</sup> have been investigated as potential candidate genes. However, no single allele has been clearly associated with HBV persistence or disease severity. Further study is needed to clarify these preliminary associations and to identify other potential candidate genes.

CIITA is encoded by the activator of immune response 1 (AIR-1) locus<sup>[36]</sup>. It was first discovered in a rare, severe immunodeficiency-bare lymphocyte syndrome (BLS), and the expression of CIITA was lacking in a BLS complementation of group<sup>[1]</sup>. It has been shown that CIITA can activate other genes involved in antigen presentation, such as the invariant chain, HLA-DM and MHC class I<sup>[37]</sup>, and can affect HIV long terminal repeat expression. Impaired induction of CIITA might decrease IFN- $\gamma$ -induced HLA-DR expression in cultured thymic epithelial cells (TEC) derived from thymoma and affect CD4<sup>+</sup> T cell development<sup>[38]</sup>. CIITA could compete with nuclear factor of activated T cells (NF-AT) to bind to the CREB binding protein (CBP/p300) and inhibit Th2-type cytokine production during Th1 cell differentiation to prevent interleukin-4 (IL-4) gene transcription<sup>[39]</sup>. Quan *et al*<sup>[40]</sup> investigated the underlying genetic defect in an immunodeficient patient who presented with recurrent bacterial infections in his late twenties and demonstrated that a single amino acid substitution, phenylalanine to serine, in the COOH-terminal portion of the CIITA sequence was correlated with reduced transcription of MHC class II genes. Marten *et al*<sup>[41]</sup> transfected dendritic cells (DCs) with the CIITA gene efficiently and found an increase in antitumoral immunostimulatory capacity of DCs. Sartoris *et al*<sup>[42]</sup> transfected an expressible CIITA cDNA in uninducible hepatocarcinoma cell lines, and found that there was a very high expression of HLA class II in the transfected cell surface and they had acquired antigen processing and presentation capacity.

The availability of CIITA appears generally essential for MHC class II gene expression, and hence its transcriptional regulatory mechanisms are of fundamental importance for a correct homeostasis of the immune response. Therefore, it is possible to hypothesize that variability at the CIITA-encoding locus, AIR-1, could constitute an additional source of susceptible traits to autoimmune diseases. Sartoris *et al*

analyzed the sequence variation at AIR-1/CIITA promoters by PCR-SSCP in 88 Caucasians from the Northeast of Italy, including 23 insulin-dependent diabetes mellitus (IDDM), 30 rheumatoid arthritis (RA) patients, compared with a sample of 19 unaffected normal controls and 16 unaffected IDDM family members, and they did not observe any sequence difference at the four AIR-1/CIITA promoters between autoimmune patients and normal controls. Rasmussen *et al* searched for genetic susceptibility to multiple sclerosis (MS). Polymorphism screening based upon detection of single strand conformational changes (SSCP analysis) followed by sequencing revealed six single nucleotide variations, i.e., one in the promoter-utilized by B cells and five in the 3' untranslated region (UTR) of the gene. The distribution of CIITA alleles did not differ between MS patients and control subjects. After sub-grouping the patients into relapsing-remitting MS and primary progressive MS, the distribution of promoter alleles at the *Bs/I* polymorphic site in the latter of the two patient groups differed from that of healthy control subjects. It is suggested that there is a weak association between one of the alleles at this site and primary progressive MS.

We studied the sequence variability at promoter I, III and IV of CIITA transcription factor in CHB patients, ABH patients and normal controls by SSCP analysis of the three PCR-amplified CIITA promoter regions to assess whether there was a relationship between polymorphism of CIITA gene promoters and the chronicity of hepatitis B. The similar report on the relationship between polymorphism of CIITA gene promoters and CHB has not been found abroad and in our country. We found no polymorphic variation at the three CIITA promoters significantly associated with CHB. Due to the importance of CIITA in the class II gene expression, the polymorphism in the coding region and 3'UTR of this gene needs to be studied further. We suppose that the promoter region of CIITA gene may be a conserved domain and the variability at CIITA promoters might not improve the overall potentiality of the system against pathogens. Furthermore, studies on a large number of cases will reveal significance of polymorphism in CIITA promoters and its relation with CHB.

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• BRIEF REPORTS •

## Interleukin-10 -1082 promoter polymorphism is not associated with susceptibility to esophageal squamous cell carcinoma and gastric cardiac adenocarcinoma in a population of high-incidence region of north China

Wei Guo, Na Wang, Yi-Min Wang, Yan Li, Deng-Gui Wen, Zhi-Feng Chen, Yu-Tong He, Jian-Hui Zhang

Wei Guo, Na Wang, Yi-Min Wang, Yan Li, Deng-Gui Wen, Zhi-Feng Chen, Yu-Tong He, Jian-Hui Zhang, Hebei Cancer Institute, Hebei Medical University, Shijiazhuang 050011, Hebei Province, China

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Correspondence to: Professor Jian-Hui Zhang, Hebei Cancer Institute, Hebei Medical University, Jiankanglu 12, Shijiazhuang 050011, Hebei Province, China. jianhui@hotmail.com

Telephone: +86-311-6093338 Fax: +86-311-6077634

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### Abstract

**AIM:** To investigate the possible association of G→A single nucleotide polymorphism (SNP) at the -1082 position of interleukin (IL)-10 promoter with susceptibility to esophageal squamous cell carcinoma (ESCC) and gastric cardiac adenocarcinoma (GCA) in a population of a high incidence region of North China.

**METHODS:** IL-10-G1082A promoter SNP was genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) in 355 cancer patients (203 ESCC and 152 GCA) and 443 healthy controls.

**RESULTS:** Smoking significantly increased the risk of ESCC and GCA development (the age and sex adjusted OR = 1.42 and 2.64, 95%CI = 1.11-1.81 and 1.46-4.76, respectively). Similarly, family history of upper gastrointestinal cancer (UGIC) significantly increased the risk of developing ESCC and GCA (the age and sex adjusted OR = 1.44 and 3.10, 95%CI = 1.18-1.75 and 1.94-4.97, respectively). The A/A, A/G and G/G genotype frequencies of IL-10-G1082A were 60.3%, 37.0% and 2.7% in healthy controls, 57.6%, 39.9% and 2.5% in ESCC and 61.2%, 36.8% and 2.0% in GCA patients, respectively. The frequencies of A and G alleles were 78.8% and 21.2% in healthy controls, 77.6% and 22.4% in ESCC patients and 79.6%, 20.4% in GCA patients. The distribution of genotype and allelotype in ESCC and GCA patients was not significantly different from that in healthy controls ( $P>0.05$ ). Compared to the A/A genotype, the combination of A/G and G/G genotypes did not show a significant effect on the risk of developing ESCC and GCA; the adjusted odds ratio was 0.92 (95% CI = 0.76-1.11) in ESCC and 0.95 (95% CI = 0.61-1.46) in GCA, respectively. When stratified for smoking status

and family history of UGIC, the combination of A/G and G/G genotypes also did not show any significant influence on the risk of ESCC and GCA development compared to A/A genotypes.

**CONCLUSION:** IL-10-G1082A polymorphism might not be used as a stratification marker to predicate the risk of ESCC and GCA development in North China.

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**Key words:** Polymorphism; Esophageal squamous cell carcinoma; Gastric cardiac adenocarcinoma

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### INTRODUCTION

China is a country with high-incidence regions of esophageal squamous cell carcinoma (ESCC) and gastric cardiac adenocarcinoma (GCA). Exogenous factors including nutrition deficiency<sup>[1]</sup>, unhealthy living habits<sup>[2]</sup>, consumption of alcohol and tobacco<sup>[2,3]</sup>, pathogenic infections<sup>[4,5]</sup> are generally considered as the risk factors for developing these two cancers in China. However, only a subset of individuals exposed to the above listed exogenous risk factors would develop ESCC and GCA, suggesting a role of host susceptibility factors in cancer development. The role of a genetic background in developing these cancers has also been strongly suggested by familial clustering of upper gastrointestinal cancer (UGIC) patients in high-incidence regions<sup>[6,7]</sup>. Some candidate genes have been identified to modify the susceptibility to ESCC and GCA in Chinese or other populations<sup>[8]</sup>.

Cytokines play an important role in regulating both humor and cell-mediated immune responses. Promoter regions of some cytokine genes contain polymorphisms that may directly influence the cytokine transcription or expression<sup>[9]</sup>. These promoter polymorphisms may lead to either high- or low-level production of the given cytokines,

cause inter-individual differences in antitumor immune response and subsequently influence the susceptibility to cancers. For example, vascular endothelial growth factor (VEGF) polymorphism has been suggested to affect the susceptibility to prostate cancer by promoting angiogenesis<sup>[10]</sup>. Interleukin-1 polymorphism has been demonstrated to be a risk factor for gastric cancer in subjects with *H pylori* infection<sup>[11]</sup>.

Interleukin (IL)-10, which is produced by T-lymphocytes, is an important anti-inflammatory and immuno-suppressive cytokine and may regulate angiogenesis in various cancers. The IL-10 gene is located on chromosome 1q31-32. Polymorphisms at positions -1082, -819 and -592 of the IL-10 promoter region are correlated with IL-10 production<sup>[12]</sup>. The expression of IL-10 is correlated to angiogenic factor expression in ESCC that may influence the development and progression of this tumor<sup>[13]</sup>. It has been reported that IL-10-G1082A polymorphism is correlated with the expression of IL-10 and accordingly affects the susceptibility to some types of tumors, such as cervical cancer<sup>[14]</sup> and prostate cancer<sup>[10]</sup>. Although the expression of IL-10 gene might be associated with the susceptibility to ESCC<sup>[13]</sup>, the correlation of IL-10 promoter polymorphism with the susceptibility to ESCC and GCA has not been reported so far. Therefore, in this study, we conducted a population-based case control study to investigate the association of IL-10 -G1082A polymorphisms with the risk of ESCC and GCA development in Cixian County and Shexian County, a high-incidence region of ESCC and GCA in Taihang Mountain, North China.

## MATERIALS AND METHODS

### Subjects

This study included 355 patients (203 with ESCC and 152 with GAC) and 443 healthy individuals without overt cancer.

The cases were outpatients for endoscopic biopsy or inpatients for tumor resection in the Fourth Affiliated Hospital of Hebei Medical University or local tumor hospitals in Cixian County and Shexian County between 2001 and 2003. Histological tumor typing was carried out on the basis of biopsy or resection specimens. Esophageal carcinomas were all squamous cell carcinomas. Gastric cardiac carcinomas were all adenocarcinomas with their epicenters at the gastroesophageal junction, i.e., from 1 cm above until 2 cm below the junction between the end of the tubular esophagus and the beginning of the saccular stomach<sup>[15]</sup>. Healthy subjects were recruited from Cixian County and Shexian County during the endoscopic screening campaign between 2001 and 2003. All the cancer patients and control subjects were unrelated Han nationals. Information of sex, age, smoking habit and family history was obtained from cancer patients and healthy controls by an interview following sampling. Smokers were defined as former or current individuals smoking 5 cigarettes per day for at least two years. Individuals with at least one first-degree relative or at least two second-degree relatives having esophageal/cardiac/gastric cancer were defined as having a family history of upper gastrointestinal cancers (UGIC). Smoking status and family history were only available from a sub-set of cancer patients and healthy controls (Table 1). The study was approved by the Ethics Committee of Hebei Cancer Institute and informed consent was obtained from all recruited subjects.

### DNA extraction

Five milliliters of venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted within one week after sampling by using proteinase K (Merck, Darmstadt, Germany) digestion followed by a salting out procedure according to the method published by Miller<sup>[16]</sup>.

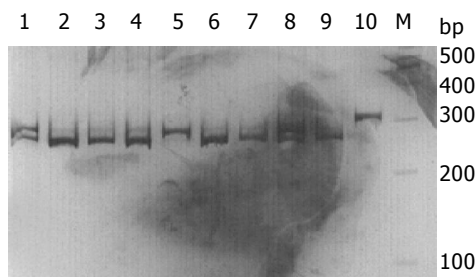
**Table 1** Selected characteristics of ESCC, GCA patients and healthy individuals

Groups	Control, n (%)	ESCC, n (%)	<i>P</i> <sup>a</sup>	GCA, n (%)	<i>P</i> <sup>1</sup>
Sex					
Male	269 (60.7)	135 (66.5)	0.16	102 (67.1)	0.16
Female	174 (39.3)	68 (33.5)		50 (32.9)	
Mean age, in years (SD)	57.1 (10.27)	59.2 (9.58)	0.06 <sup>2</sup>	60.7 (8.07)	0.06 <sup>2</sup>
Smoking status <sup>3</sup>					
Ex- or current smoker	144 (33.2)	106 (55.5)	<0.001 <sup>5</sup>	88 (66.7)	<0.001 <sup>5</sup>
Non-smoker	290 (66.8)	85 (44.5)		44 (33.3)	
Family history of UGIC <sup>4</sup>					
Positive	164 (37.0)	93 (52.2)	<0.001 <sup>6</sup>	74 (61.2)	<0.001 <sup>6</sup>
Negative	279 (63.0)	85 (47.8)		47 (38.8)	
IL-10 -G1082A SNP genotype					
A/A	267 (60.3)	117 (57.6)	0.78	93 (61.2)	0.88
A/G	164 (37.0)	81 (39.9)		56 (36.8)	
G/G	12 (2.7)	5 (2.5)		30 (2.0)	
IL-10 -G1082A SNP allelotype					
A	698 (78.8)	315 (77.6)	0.63	242 (79.6)	0.76
G	188 (21.2)	91 (22.4)		62 (20.4)	

ESCC: esophageal squamous cell carcinoma, GCA: gastric cardiac adenocarcinoma, UGIC: upper gastrointestinal cancer. <sup>1</sup>*P* value for  $\chi^2$  test; <sup>2</sup>*P* value for *t* test; <sup>3,4</sup>information of smoking status and family history was available from a subset of subjects; <sup>5</sup>smoking significantly increased the risk to the ESCC development (the age and sex adjusted OR = 1.42, 95% CI = 1.11–1.81); and GCA development (the age and sex adjusted OR = 2.64, 95% CI = 1.46–4.76); <sup>6</sup>positive family history of UGIC significantly increased the risk to develop ESCC (the age and sex adjusted OR = 1.44, 95% CI = 1.18–1.75) and GCA (the age and sex adjusted OR = 3.10, 95% CI = 1.94–4.97).

### IL-10-G1082A genotyping by PCR and restriction fragment analysis

IL-10-G1082A genotyping was determined by polymerase-chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. PCR primers used for amplifying IL-10-G1082A fragments were 5'-CACTACTAAGGC TTCCTTGGA-3' (sense) and 5'-GTGAGCAAAGTGA GGCACAGACAT-3' (antisense). The sense primer was specially designed to introduce a recognition site of restriction enzyme *Eco*NI (CCTNN\NNNAGG, MBI Fermentas, Lithuania) by replacing a T with a C at the seventh position close to the 3' end of the primer. PCR was performed in a 20  $\mu$ L volume containing 100 ng of DNA template, 2.0  $\mu$ L of 10 $\times$  PCR buffer, 1.5 mmol of  $MgCl_2$ , 1 U of *Taq*-DNA-polymerase (BioDev-Tech., Beijing, China), 200  $\mu$ mol of dNTPs and 200 nmol of sense and antisense primers. The PCR cycling conditions were at 94  $^{\circ}C$  for 5 min followed by 35 cycles at 94  $^{\circ}C$  for 30 s, at 60  $^{\circ}C$  for 50 s, and at 72  $^{\circ}C$  for 40 s, and with a final step at 72  $^{\circ}C$  for 5 min to allow the complete extension of all PCR fragments. An 8  $\mu$ L aliquot of PCR products was digested overnight at 37  $^{\circ}C$  in a 10  $\mu$ L reaction containing 10 units of *Eco*NI and 1 $\times$  reaction buffer. After an overnight digestion, the products were resolved and separated on a 10% polyacrylamide gel followed by staining with 0.25% silver nitrate. After electrophoresis, the AA homozygote was represented by three DNA bands being 252, 39 and 19 bp in length. The GG homozygote was represented by two DNA bands being 271 and 39 bp in length, whereas the heterozygote displayed four DNA bands being 271, 252, 39 and 19 bp in length (Figure 1). For a negative control, each PCR reaction used distilled water instead of DNA in the reaction system. For 10% of the samples, the reaction was repeated once for IL-10 genotyping and all of the genotypes matched with the original results.



**Figure 1** Polyacrylamide gel electrophoresis of IL-10-G1082A digested by *Eco*NI. Lanes 1 and 8: AG genotype; lanes 2-4 and 6, 7, 9: AA genotype; lane 5: GG genotype; lane 10: PCR product; M: Marker.

### Statistical analysis

Statistical analysis was performed using SPSS10.0 software package (SPSS Company, Chicago, IL). Hardy-Weinberg analysis was performed by comparing the observed and expected genotype frequencies using  $\chi^2$  test. Comparison of the IL-10-G1082A genotype distribution in ESCC and healthy controls was performed by means of two-sided contingency tables using  $\chi^2$  test. A probability level of 5%

was considered statistically significant. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model and adjusted by age and sex accordingly.

### RESULTS

The mean age of ESCC cases, GCA cases and controls was 59.2 $\pm$ 9.58 years (range 34-85), 60.7 $\pm$ 8.07 years (range 37-86) and 57.1 $\pm$ 10.27 years (range 31-78), respectively. The gender distribution in ESCC and GCA patients (66.5% and 67.1% men) was comparable to that in healthy controls (60.7% men) ( $P = 0.16$  and  $0.16$ , respectively). The proportion of smokers in ESCC and GCA patients (55.5% and 66.7%) was significantly higher than that in healthy controls (33.2%) ( $\chi^2 = 27.53$  and  $46.92$ , respectively,  $P < 0.001$ ). Therefore, smoking significantly increased the risk of ESCC and GCA development (the age and sex adjusted OR = 1.42 and 2.64, 95% CI = 1.11-1.81 and 1.46-4.76, respectively). In addition, the frequency of positive family history of UGIC in ESCC (52.2%) and GCA (61.2%) patients was significantly higher than that in healthy controls (37.0%) ( $\chi^2 = 12.14$  and  $22.70$ , respectively,  $P < 0.001$ ). Thus, family history of UGIC significantly increased the risk of developing ESCC (adjusted OR = 1.44, 95%CI = 1.18-1.75) and GCA (adjusted OR = 3.10, 95%CI = 1.94-4.97). The demographic distribution of ESCC and GCA patients as well as healthy controls was shown in Table 1.

IL-10 genotyping was successfully performed in all study subjects. The genotype distribution in ESCC and GCA patients and healthy controls was consistent with Hardy-Weinberg equilibrium ( $P = 0.30$ ,  $0.47$  and  $0.23$ , respectively). The genotype distribution was not correlated with gender and age both in healthy controls and in ESCC and GCA patients (data not shown). In healthy controls, the frequencies of the A/A, A/G and G/G genotypes were 60.3%, 37.0% and 2.7%, while the distribution of A and G alleles was 78.8% and 21.2%, respectively. As shown in Table 1, there was no statistical difference in allele distribution between ESCC, GCA patients and healthy controls ( $\chi^2 = 0.24$  and  $0.09$ ,  $P = 0.63$  and  $0.76$ , respectively). The overall IL-10 genotype distribution in ESCC and GCA patients was also not significantly different from that in healthy controls ( $\chi^2 = 0.50$  and  $0.26$ ,  $P = 0.78$  and  $0.88$ , respectively). Compared to the A/A genotype, the combination of A/G and G/G genotypes did not show a significant effect on modifying the risk of ESCC and GCA development; the adjusted OR for ESCC and GCA was 0.92 (95%CI = 0.76-1.11) and 0.95 (95% CI = 0.61-1.46), respectively.

When stratified for smoking status and family history of UGIC, the frequency of IL-10 genotypes in ESCC and GCA patients was also not significantly different from that in healthy controls. Compared to the A/A genotype, the combination of A/G and G/G genotypes did not show any significant influence on the risk of ESCC and GCA in each stratification group (Table 2).

### DISCUSSION

Cixian County and Shexian County lie in Taihang Mountain

**Table 2** Association analysis of the IL-10 -1082 promoter polymorphism with the risk of ESCC and GCA development

	A/A	A/G+G/G	aOR (95%CI) <sup>3</sup>
Overall			
Normal	267 (60.3)	176 (39.7)	
ESCC	117 (57.6)	86 (42.4)	0.92 (0.76–1.11)
GCA	93 (61.2)	59 (38.8)	0.95 (0.61–1.46)
Nonsmoker <sup>1</sup>			
Normal	181 (62.4)	109 (37.6)	
ESCC	52 (61.2)	33 (38.8)	0.89 (0.67–1.18)
GCA	26 (59.1)	18 (40.9)	0.79 (0.39–1.63)
Smoker			
Normal	80 (55.6)	64 (44.4)	
ESCC	61 (57.5)	45 (42.5)	1.01 (0.77–1.33)
GCA	54 (61.4)	34 (38.6)	1.11 (0.61–2.02)
Negative family history <sup>2</sup>			
Normal	168 (60.2)	111 (39.8)	
ESCC	48 (56.5)	37 (43.5)	0.92 (0.70–1.20)
GCA	30 (63.8)	17 (36.2)	1.10 (0.54–2.22)
Positive family history			
Normal	99 (60.4)	65 (39.6)	
ESCC	56 (60.2)	37 (39.8)	0.96 (0.71–1.29)
GCA	40 (54.0)	34 (46.0)	0.71 (0.37–1.36)

ESCC: esophageal squamous cell carcinoma. GCA: gastric cardiac adenocarcinoma. <sup>1,2</sup> information of smoking status and family history was available from a subset of subjects; <sup>3</sup> The age and sex adjusted odds ratio of the A/G+G/G genotype against the A/A genotype.

located at the southern part of Hebei Province. These two counties are famous in the world for their high-incidence and mortality of esophageal cancer. GCA, which was formerly registered as esophageal cancer or gastric cancer, has been diagnosed independently in very recent years, due to the improvement in early endoscopic screening and pathologic diagnosis. It has been suggested by our epidemiological data that GCA is also a prevalent tumor type in Cixian County and Shexian County; its incidence is about one-third of ESCC in these counties. Therefore, we selected ESCC and GCA patients as well as demographically matched healthy controls as the resources of this population-based case control study.

In the present study, smoking, which was considered as one of the important risk factors in Western countries, was also suggested to significantly increase the risk of ESCC and GCA development. In addition, the study strongly supported that the genetic background might play an additional role in the development of these two tumor types. Therefore, smoking control may play an important role in the prevention of ESCC and GCA in this region. In addition, individuals with a family history of UGIC should pay more attention to their living habits and undergo a regular endoscopic inspection to prevent and early detect ESCC or GCA.

In recent years, host-dependent susceptibility to ESCC and GCA has been widely exploited especially in China<sup>[8]</sup>. Some polymorphic genes encoding metabolic enzymes, cell cycle regulators and mismatch repair enzymes, such as aldehyde dehydrogenase-2 (ALDH2)<sup>[17]</sup>, cytochrome P450 (CYP)<sup>[18]</sup>, glutathione S-transferase (GST), methylenetetrahydrofolate reductase (MTHFR)<sup>[8]</sup>, NAD (P) H: quinone oxidoreductase 1 (NQO1)<sup>[19]</sup> have been found

to be able to modify the susceptibility to chemically induced cancers including esophageal and gastric cardiac cancer. Therefore, these polymorphic genes may be used as predictive parameters for screening individuals at a high risk of ESCC and GCA.

Polymorphisms in cytokine genes might be associated with functional differences in cytokine transcription and could alter clinical performance in a variety of diseases<sup>[10,14]</sup>. IL-10 is a cytokine with anti-inflammatory and B-cell-stimulating activity. It may also influence tumor development via its action on pathways of tumor angiogenesis. It has been reported that the IL-10-1082 A/A genotype (low producer of IL-10) significantly increased in prostate cancer<sup>[14]</sup> and cutaneous malignant melanoma<sup>[20]</sup>. In contrast, high levels of IL-10 were tumor promoting and elevated serum IL-10 levels were observed in patients with various solid tumors<sup>[21]</sup>. However, in this population-based case-control study, difference in IL-10-G1082A genotype distribution was not found between ESCC or GCA patients and healthy controls, as well as in the stratification analyses according to smoking status (never smoking or current and ever smoking) and family history of UGIC, suggesting that although IL-10-1082 polymorphism has been correlated with some cancer types, this genetic alteration may not be associated with the susceptibility to ESCC and GCA.

In summary, the findings in this study indicate that IL-10-1082 promoter polymorphism might not be used as a stratification marker to predicate the susceptibility to ESCC and GCA in North China.

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• BRIEF REPORTS •

## Effect of rhubarb on contractile response of gallbladder smooth muscle strips isolated from guinea pigs

Ya-Li Luo, Jun-Wei Zeng, Mei Yu, Yu-Ling Wei, Song-Yi Qu, Wei Li, Tian-Zhen Zheng

Ya-Li Luo, Jun-Wei Zeng, Mei Yu, Song-Yi Qu, Wei Li, Tian-Zhen Zheng, Department of Physiology, Lanzhou Medical College, Lanzhou 730000, Gansu Province, China

Yu-Ling Wei, Drug Control Institute of Gansu Province, Lanzhou 730000, Gansu Province, China

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Correspondence to: Tian-Zhen Zheng, Department of Physiology, Lanzhou Medical College, Lanzhou 730000, Gansu Province, China. wjztzl@126.com

Telephone: +86-931-8617647

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isolated gallbladder muscle strips from guinea pigs. The stimulation of rhubarb might be relevant with M receptor,  $Ca^{2+}$  channel and  $\alpha$  receptor partly.

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**Key words:** Rhubarb; Gallbladder smooth muscle strips; M receptor;  $Ca^{2+}$  channel;  $\alpha$  receptor

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### Abstract

**AIM:** To investigate the effect of rhubarb on contractile response of isolated gallbladder muscle strips from guinea pigs and its mechanism.

**METHODS:** Guinea pigs were killed to remove the whole gallbladder. Two or three smooth muscle strips (8 mm×3 mm) were cut along the longitudinal direction. The mucosa on each strip was carefully removed. Each longitudinal muscle strip was suspended in a tissue chamber containing 5 mL Krebs solution (37 °C), bubbled continuously with 950 mL/L  $O_2$  and 50 mL/L  $CO_2$ . The resting tension (g), mean contractile amplitude (mm), and contractile frequency (waves/min) were simultaneously recorded on recorders. After 2-h equilibration, rhubarb (10, 20, 70, 200, 700, 1 000 g/L) was added cumulatively to the tissue chamber in turns every 2 min to observe their effects on gallbladder. Antagonists were given 3 min before administration of rhubarb to investigate the possible mechanism.

**RESULTS:** Rhubarb increased the resting tension (from 0 to  $0.40 \pm 0.02$ ,  $P < 0.001$ ), and decreased the mean contractile amplitude (from  $5.22 \pm 0.71$  to  $2.73 \pm 0.41$ ,  $P < 0.001$ ). It also increased the contractile frequency of the gallbladder muscle strips in guinea pigs (from  $4.09 \pm 0.46$  to  $6.08 \pm 0.35$ ,  $P < 0.001$ ). The stimulation of rhubarb on the resting tension decreased from  $3.98 \pm 0.22$  to  $1.58 \pm 0.12$  by atropine ( $P < 0.001$ ), from  $3.98 \pm 0.22$  to  $2.09 \pm 0.19$  by verapamil ( $P < 0.001$ ) and from  $3.98 \pm 0.22$  to  $2.67 \pm 0.43$  by phentolamine ( $P < 0.005$ ). But the effect was not inhibited by hexamethonium ( $P > 0.05$ ). In addition, the action of mean amplitude and frequency was not inhibited by the above antagonists.

**CONCLUSION:** Rhubarb can stimulate the motility of

### INTRODUCTION

Muscular contraction of the gallbladder is the primary determinant factor of bile delivery into the duodenum. Rhubarb has been reported to promote bile secretion and dredge liver fine bile ducts. Moreover, rhubarb relaxes Oddi sphincter. It has been used to treat cholecystitis and bile duct infection, which was caused by manifold bacteria. But the action and mechanisms of rhubarb on the gallbladder smooth muscle strips *in vitro* are not reported. In this study, we observed the effects of rhubarb on the gallbladder muscle strips isolated from guinea pigs and investigated the possible mechanism concerned.

### MATERIALS AND METHODS

#### Animal preparation

Guinea pigs of either sex (grade I, purchased from Animal Center of Lanzhou Biology Institute), weighing 350-450 g, were fasted with free access to water for 24 h, and killed to remove the whole gallbladder. Two or three smooth muscle strips (8 mm×3 mm) were cut along the longitudinal direction. The mucosa on each strip was carefully removed.

#### Experiment

The muscle strips were suspended in a tissue chamber containing 5 mL Krebs solution, constantly warmed by a circulating water jacket at 37 °C, bubbled continuously with 950 mL/L  $O_2$  and 50 mL/L  $CO_2$ . One end of the strip was fixed to a hook on the bottom of the chamber. The other end was connected to an external isometric force transducer (JZ-BK, BK). The preparation was subjected to 1-g-load tension and washed with 5 mL Krebs solution every 20 min. The motility of gallbladder strips in tissue chambers was

simultaneously recorded on ink writing two channel recorders (LMS\_ZB, Chengdu). After 2-h equilibration, 10, 20, 70, 200, 700, 1 000 g/L of rhubarb were added cumulatively in turns every 2 min to observe their effects on gallbladder. Cumulating final concentration of rhubarb was 0.05, 0.15, 5, 15, 50, 100 g/L in the tissue chamber. Atropine (1  $\mu$ mol/L), hexamethonium (10  $\mu$ mol/L), phentolamine (1  $\mu$ mol/L) and verapamil (0.05  $\mu$ mol/L) were added 3 min before the administration of rhubarb to investigate whether the actions of rhubarb were relevant with M receptor,  $\alpha$  receptor,  $Ca^{2+}$  channel and N receptor. The concentrations of antagonists were the final concentrations<sup>[1]</sup>.

### Drug preparation

Rhubarb was broken into pieces, boiled, filtrated, and diluted to 1 000 g/L, and then diluted to 10, 20, 70, 200, 700, 1 000 g/L (the drug was appraised and prepared by Drug Control Institute of Gansu Province). The following antagonists were used: atropine (Pharmaceutical Factory in Yancheng, Jiangsu Province), hexamethonium (Sigma Chemical Company), phentolamine (Beijing Thirteen Pharmaceutical Factory), and verapamil (Lanzhou Pharmaceutical Factory).

### Data analysis

The results were presented as mean $\pm$ SE, and statistically analyzed by ANOVA,  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of rhubarb on spontaneous contraction of gallbladder muscle strips

Rhubarb (0.05, 0.15, 5, 15, 50, 100 g/L) increased the resting

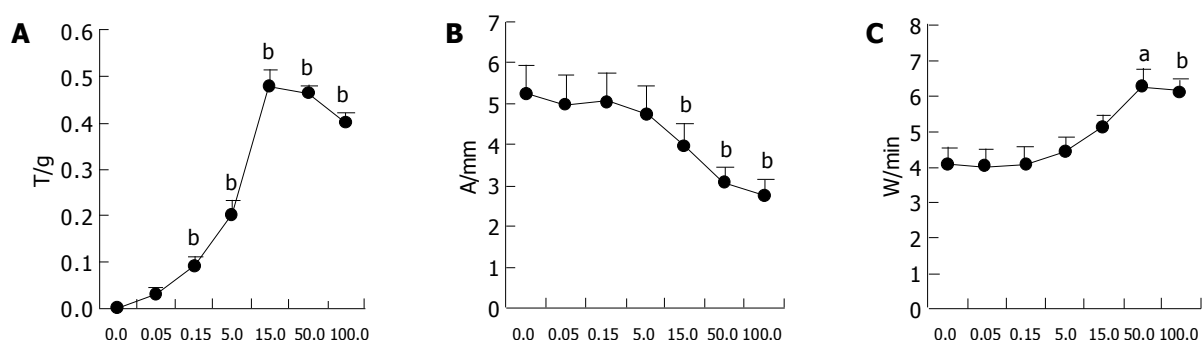
tension, and decreased the mean contractile amplitude. It also increased the contractile frequency of gallbladder muscle strips isolated from guinea pigs (Figure 1).

### Effect of atropine, verapamil, phentolamine, and hexamethonium on responses caused by rhubarb

Atropine (1  $\mu$ mol/L), hexamethonium (10  $\mu$ mol/L), phentolamine (1  $\mu$ mol/L) and verapamil (0.05  $\mu$ mol/L) had no significant effect on gallbladder muscle strips isolated from guinea pigs. But when given 3 min before the administration of rhubarb (0.05, 0.15, 5, 15, 50, 100 g/L), atropine, verapamil, and phentolamine reduced the increasing action of rhubarb on the resting tension of gallbladder muscle strips at different degrees (Table 1). They had no significant effects on the other actions of rhubarb (Tables 2, 3). Hexamethonium given 3 min before the administration of rhubarb had no significant effects on the action of rhubarb.

## DISCUSSION

The effects of Chinese herbs on the gallbladder motility have been reported<sup>[2-5]</sup>. It was reported that rhubarb could be used to treat cholecystitis<sup>[6-9]</sup> based on the fact that some substances extracted from rhubarb have significant effects on promoting gallbladder contraction. Also, rhubarb can relax Oddi sphincter and restrain the activity of pancreas succus amylase. These effects may be the basis of treating acute pancreas adenitis. But investigation of the effects of rhubarb on gallbladder smooth muscle *in vitro* is rare. In this experiment, we found that rhubarb significantly increased the resting tension and contractile frequency of isolated guinea pig gallbladder strips, and the mean amplitude



**Figure 1** Effect of rhubarb on resting tension (g), mean contractile amplitude (mm) and frequency (waves/min) of isolated guinea pig gallbladder muscle strips ( $n = 12$ ). A: resting tension; B: the mean contractile amplitude; C: frequency. <sup>a</sup> $P < 0.005$ , <sup>b</sup> $P < 0.001$  vs control (the gallbladder spontaneous contraction under 1 g initial load when rhubarb was 0 g/L). The resting tension of each strip in control was 0.

**Table 1** Effect of rhubarb on resting tension (g) of isolated guinea pig gallbladder muscle strips after pretreatment with antagonists (mean $\pm$ SE)

Resting tension (g)	Rhubarb (g/L)						
	0	0.05	0.15	5	15	50	100
Rhubarb	0	0.03±0.01	0.09±0.02 <sup>c</sup>	0.20±0.03 <sup>c</sup>	0.48±0.03 <sup>c</sup>	0.46±0.02 <sup>c</sup>	0.40±0.02 <sup>c</sup>
Phe+Rhu	0	0.04±0.01	0.06±0.01 <sup>e</sup>	0.11±0.01 <sup>c,g</sup>	0.33±0.05 <sup>c,g</sup>	0.30±0.05 <sup>c,g</sup>	0.27±0.04 <sup>c,g</sup>
Hex+Rhu	0	0.04±0.01	0.08±0.01 <sup>e</sup>	0.17±0.02 <sup>g</sup>	0.39±0.02 <sup>g</sup>	0.40±0.02 <sup>g</sup>	0.35±0.02 <sup>g</sup>
Atr+Rhu	0	0 <sup>g</sup>	0.03±0.01 <sup>g</sup>	0.07±0.01 <sup>a,g</sup>	0.22±0.04 <sup>c,g</sup>	0.19±0.03 <sup>c,g</sup>	0.16±0.01 <sup>c,g</sup>
Iso+Rhu	0	0.03±0.01	0.07±0.01 <sup>c</sup>	0.10±0.01 <sup>c,e</sup>	0.30±0.01 <sup>c,g</sup>	0.28±0.02 <sup>c,g</sup>	0.21±0.02 <sup>c,g</sup>

<sup>a</sup> $P < 0.05$ , <sup>c</sup> $P < 0.005$  vs control (the gallbladder spontaneous contraction under 1-g initial load when rhubarb was 0 g/L)  $n = 12$ . The resting tension of each strip in control was 0. <sup>e</sup> $P < 0.05$ , <sup>g</sup> $P < 0.005$  vs rhubarb (the resting tension of adding each concentration of rhubarb)  $n = 12$ .

**Table 2** Effect of rhubarb on the contractile amplitude (mm) of isolated guinea pig gallbladder muscle strips after pretreatment with antagonists (mean±SE)

Amplitude (mm)	Rhubarb (g/L)						
	0	0.05	0.15	5	15	50	100
Rhubarb	5.2±0.7	4.9±0.7	5.0±0.7	4.8±0.7	4.0±0.5	3.0±0.4 <sup>a</sup>	2.7±0.4 <sup>c</sup>
Phe+Rhu	3.3±0.4	3.2±0.4	3.1±0.4	3.0±0.4	2.9±0.4	2.3±0.4	1.7±0.4 <sup>c</sup>
Hex+Rhu	4.7±0.6	4.5±0.6	4.3±0.6	4.2±0.6	3.8±0.8	3.1±0.5	2.7±0.5 <sup>a</sup>
Atr+Rhu	5.4±0.9	5.3±0.9	5.1±0.8	4.8±0.7	4.5±0.7	3.8±0.6	3.1±0.5 <sup>a</sup>
Iso+Rhu	5.2±0.9	5.1±1.0	4.9±1.0	4.7±0.8	4.4±0.9	3.4±0.6	3.0±0.5

<sup>a</sup>P<0.05, <sup>c</sup>P<0.005 *vs* control (the gallbladder spontaneous mean contraction amplitude under 1-g initial load when rhubarb was 0 g/L) *n* = 12.

**Table 3** Effect of rhubarb on the contractile frequency (waves/min) of isolated guinea pig gallbladder muscle strip after pretreatment with antagonists (mean±SE)

Frequency (w/min)	Rhubarb (g/L)						
	0	0.05	0.15	5	15	50	100
Rhubarb	4.1±0.5	4.0±0.4	4.1±0.5	4.4±0.4	5.0±0.3	6.3±0.5 <sup>c</sup>	6.1±0.4 <sup>c</sup>
Phe+rhu	4.2±0.5	4.5±0.5	4.6±0.6	4.6±0.5	4.8±0.6	5.5±0.6	6.0±0.5 <sup>c</sup>
Hex+rhu	3.7±0.3	3.8±0.4	3.8±0.4	4.1±0.5	4.9±0.6	5.3±0.7 <sup>c</sup>	5.5±0.5 <sup>c</sup>
Atr+rhu	3.5±0.4	3.4±0.4	3.5±0.4	3.7±0.4	4.0±0.4	4.8±0.5 <sup>c</sup>	5.0±0.5 <sup>c</sup>
Iso+rhu	4.1±0.4	4.1±0.4	4.1±0.4	4.2±0.4	4.5±0.4	5.0±0.5	4.9±0.4

<sup>a</sup>P<0.05, <sup>c</sup>P<0.005 *vs* control (the gallbladder spontaneous contraction frequency under 1-g initial load when rhubarb was 0 g/L) *n* = 12.

decreased at the same time. Atropine, phentolamine and verapamil could block this exciting action partly, whereas hexamethonium had no inhibitory effects. Our results suggested that the stimulating action of rhubarb on gallbladder smooth muscle strips was relevant with M receptor, Ca<sup>2+</sup> channel and  $\alpha$  receptor, but irrelevant with N receptors.

The presence of M receptors in guinea pig gallbladder smooth muscle cells has been reported recently<sup>[10-12]</sup>. The majority of these receptors are said to be M<sub>2</sub> subtype. However, there are controversial reports about the functional muscarinic receptors that mediate contraction in this tissue. Kurtel *et al.*<sup>[13]</sup> presumed that M<sub>4</sub> receptors and M<sub>5</sub> receptors played a major role in gallbladder contraction. But von Schrenck *et al.*<sup>[11]</sup> reported that M<sub>3</sub> receptors mediated the movement. The study of Akici *et al.*<sup>[14]</sup> supported the conclusion that the majority of muscarinic receptors of M<sub>2</sub> did not mediate the contractile responses. When M receptors were stimulated, the potential sensitive Ca<sup>2+</sup> channels were opened, which would cause the influx of extracellular Ca<sup>2+</sup> and induce the contraction of smooth muscles. The key determinant of smooth muscle contractility is the concentration of intracellular free calcium Ca<sup>2+</sup>, which could trigger a sequence of events leading to the generation of forces<sup>[15-17]</sup>. When smooth muscle cells were stimulated, extracellular Ca<sup>2+</sup> entered into cells. Meanwhile sarcoplasmic reticuli could bring into play the function of Ca<sup>2+</sup>. When some exciting transmitters, hormones and drugs combined with muscular receptors, the secondary message was generated via G protein, then Ca<sup>2+</sup> was released. The result was a rise of [Ca<sup>2+</sup>]. This increased calcium sequentially bound to the four binding sites on the regulatory protein, calmodulin (CAM). The activated calmodulin bound to myosin light chain kinase (MLCK) to form an active complex (Ca<sup>++</sup><sub>4</sub>CaM.MLCK). Phosphorylation of myosin produced

a conformational change in the myosin head group that could activate ATPase. The interaction between the phosphorylated myosin heads and actin filaments generated forces, filament movements and cell shorting, with sequential attachment and detachment of the cross-bridges, leading to contraction<sup>[18]</sup>. In this experiment, verapamil (an inhibitor of Ca<sup>2+</sup> channel) significantly blocked the contraction response of rhubarb. This result is consistent with the above results.  $\alpha$  receptor has been found in gallbladder smooth muscles that could mediate the exciting action. After phentolamine (an inhibitor of  $\alpha$  receptor) was added, the contraction response of rhubarb significantly decreased. This mechanism will allow us to gain more information about the effects of rhubarb on gallbladder.

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• BRIEF REPORTS •

## YMDD mutations in patients with chronic hepatitis B untreated with antiviral medicines

Zhong-Min Huang, Qi-Wen Huang, Ya-Qin Qin, Yan-Zhuan He, Hou-Ji Qin, Yao-Nan Zhou, Xiang Xu, Mei-Jin Huang

Zhong-Min Huang, Qi-Wen Huang, Ya-Qin Qin, Yan-Zhuan He, Hou-Ji Qin, Yao-Nan Zhou, Xiang Xu, Mei-Jin Huang, Department of Infectious Diseases, The Affiliated Hospital of YouJiang Medical College for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

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Co-first-authors: Ya-Qin Qin and Zhong-Min Huang

Correspondence to: Dr. Ya-Qin Qin, Department of Infectious Diseases, The Affiliated Hospital of YouJiang Medical College for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China. zhongminhuang@msn.com

Telephone: +86-776-2836942 Fax: +86-776-2825603

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### Abstract

**AIM:** To polymerase P region (YMDD) mutations of hepatitis B virus gene (HBV DNA) in patients with chronic hepatitis B (CHB) untreated with antiviral medicines and to explore its correlation with pre-c-zone mutations, HBV genotypes and HBV DNA level, and to observe its curative effect.

**METHODS:** A total of 104 cases (38 cases in group of familial aggregation and 66 cases in group of non-familial aggregation) were randomly chosen from 226 patients with CHB who did not receive the treatment of lamivudine (LAM) and any other antiviral drugs within the last one year. Their serum YMDD mutations were detected by microcosmic nucleic acid and cross-nucleic acid quantitative determination, HBV genotypes by PCR-microcosmic nucleic acid cross-ELISA, HBV DNA quantitative determination and fluorescence PCR analysis, hepatitis B virus markers (HBVM) by ELISA. LAM was taken by 10 patients with YMDD mutations and its curative effect was observed.

**RESULTS:** Twenty-eight cases (26.9%) had YMDD mutations, of them 11 cases (28.9%) were in familial aggregation group (38 cases) and 17 cases (25.8%) in non-familial aggregation group (66 cases) with no significant difference between the two groups. Twenty-seven point one percent (16/59) cases were positive for HBeAg YMDD mutations, and 26.7% (12/45) cases were negative for HBeAg and positive for anti-HBe. There was also no significant difference between the two groups. Different YMDD incidence rate existed in different HBV genotypes. HBV DNA level did not have a positive correlation with the incidence of YMDD mutations. LAM was effective for all patients with mutations.

**CONCLUSION:** Wild mutant strains in HBV and their

incidence rate have no significant difference between familial aggregation and non-familial aggregation. It may have no significant relationship between YMDD mutations and pre-c-zone mutations. HBV DNA level may not have a positive correlation with YMDD mutations. LAM is clinically effective for CHB patients with YMDD mutations.

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**Key words:** Hepatitis B virus; Chronic hepatitis; Genotypes; YMDD mutation; Lamivudine

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### INTRODUCTION

To investigate the polymerase P region (YMDD) mutations of hepatitis B virus gene (HBV DNA) in patients with CHB who had not received antiviral treatment, 104 cases were randomly chosen from 226 such cases to detect their serum YMDD mutations. To explore its correlation with HBV genotypes, HBV DNA level and HBV e antigen system, and to investigate its incidence rate between familial aggregation and non-familial aggregation. LAM treatment was given to some patients with YMDD mutations. The therapeutic results were analyzed.

### MATERIALS AND METHODS

#### Patients and materials

A total of 104 patients were from out-patient and in-patient departments of our hospital. Among them 38 cases were in familial aggregation group (2 or more close-contacted family members were infected with HBV but had no direct evidence of blood transmission), including 26 males and 12 females aged 17-69 years (average, 35 years). Sixty-six cases were in non-familial aggregation group, including 45 males and 21 females aged 16-56 years (average, 34.6 years). All the cases met the diagnosis standards made at the Tenth Viral Hepatitis Conference<sup>[1]</sup> and had not received LAM treatment or any other antiviral treatment within one year before detection of serum YMDD.

#### Detection of YMDD mutations

YMDD mutations of HBV DNA were detected by micro-

osmic nucleic acid cross-nucleic acid quantitative determination. The detection of 25 cases was completed at the Biomedicine Diagnosis and Research Center of Basic Medicine Department of the First Military Medical University, and the detection of the other 79 cases was completed under the same conditions at our hospital's central laboratory. For specimen processing, 50  $\mu$ L serum to be tested and 50  $\mu$ L processed liquid were fully mixed, boiled at 100 °C for 5 min, spun by centrifugation at 12 000 r/min for 5 min, and 8  $\mu$ L supernatant was put into a tube containing reaction liquid, spun by centrifugation for 10 s at 10 000 r/min, then put into the processing machine and pre-denatured at 92 °C for 2 min, followed by 35 cycles (denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, extension at 72 °C for 65 s) and a final extension at 72 °C for 3 min. For HBV DNA primer design, HBV W1 (TGGA CGTCGCATG GGAGAACCACCGTGAA) and HBV W2 (GAAAGCTTCTGCGACGCCGTGATTGAG) were taken as primers of YMDD. For hybridization, amplified products were denatured at 98 °C for 10 min and then cooled in an ice-bath for 10 min. Two hundred micro liters of hybridization liquid was added and kept at 55 °C for 6 min, and the step was repeated twice. One hundred micro liters of enzyme anti-body liquid was added and kept at 37 °C for 30 min, discarded gently and completely, then 200  $\mu$ L enzyme lotions was added, kept at 37 °C for 5 min, and the step was repeated twice. Color reagents A and B (30  $\mu$ L) were added, kept at room temperature for 10 min, then 60  $\mu$ L termination liquid was added and the results were observed. The spatial absorption value (A) was detected with an enzyme labeling machine. YMDD was considered to be positive if  $P/N \leq 3.5$ .

### Detection of HBV genotypes

HBV genotypes were detected by PCR-microcosmic nucleic acid cross-ELISA<sup>[2]</sup>. Twenty-five cases were completed in Biomedicine Diagnosis and Research Center, Basic Medicine Department of the First Military Medical University, and the other 79 cases were detected in our hospital according to the introduction manual.

### Quantitative determination of HBV DNA

Shanghai FX990 micro-fluorometer and HBV-PCR fluoroscopy reagent kits (termination value,  $10^3$  copy/mL), provided by the Shanghai Fu Xing Company, were used. The procedure was strictly manipulated according to the introduction manual and estimated by experts.

### Detection of HBVM

For the detection of HBVM, ELISA was adopted. Reagent kits were provided by Zhongshan Bioengineering Co. Ltd, Guangdong. The manipulations were strictly completed according to the introduction manual.

### Treatment

LAM (10 mg/d p.o.) was given to patients with YMDD mutations and their liver functions, HBVM and HBV DNA were detected every 4 wk and observed continuously for 42 wk.

### Statistical analysis

The incidence rate of YMDD mutation was compared by

$\chi^2$  test between different genotypes, HBV DNA level, HBV e system, familial aggregation and non-familial aggregation case. The statistical analysis was processed with SAS statistical software.  $P < 0.05$  was considered significant.

## RESULTS

### YMDD mutation data of 104 CHB cases

Twenty-eight cases (26.9%) out of 104 had YMDD mutations. The incidence rate of YMDD mutations was 25.8% (17/66) in non-familial aggregation cases, and 28.9% (11/38) in familial aggregation cases. No significant difference could be found between the two groups. ( $\chi^2 = 0.124$ ,  $P > 0.05$ ).

### Relationship between HBeAg system and YMDD mutations

Of the 59 cases who were positive for HBeAg, 16 had YMDD mutations and the incidence rate was 27.1%. Of the 45 cases who were negative for HBeAg and positive for anti-HBe, 12 cases had YMDD mutations and the incidence rate was 26.7%. There was no significant difference between the two groups ( $\chi^2 = 0.0027$ ,  $P > 0.05$ ).

### YMDD mutations in different HBV genotypes

Genotypes of all these cases included D, C, B, non-classified types and mixed forms of CD, CB and DB. YMDD mutations of different genotypes are shown in Table 1. It demonstrated that the mutations mostly occurred in genotype C and its mixed genotypes, accounting for 71.4% (20/28).

**Table 1** YMDD mutations in different HBV genotypes

Patterns of genotypes	<i>n</i>	Case number of YMDD mutations	Incidence rate (%)
CD	25	8	32.0
CB	16	6	37.5
C	15	6	40.0
D	9	2	22.2
B	9	-	-
DB	12	-	-
Non-classified types	18	6	33.3
Total count	104	28	26.9

Comparison of mutations in different groups ( $\chi^2 = 0.9334$ ,  $P > 0.05$ ).

### Relationship between HBV DNA level and YMDD mutations

There was no significant difference between high and low HBV DNA level groups, suggesting that HBV DNA level might not have a positive correlation with YMDD mutations (Table 2).

**Table 2** Comparison of YMDD mutations in different HBV DNA levels

HBV DNA (copy/mL)	<i>n</i>	YMDD mutations	
		Positive	Positive rate (%)
$\leq 10^5$	56	16	28.6
$> 10^5$	48	12	25.0

Comparison between the two groups ( $\chi^2 = 0.1676$ ,  $P > 0.05$ ).

### Observation of curative effect

Of the 28 patients with YMDD mutations, 18 gave up treatments because of financial difficulty, the other 10 had abnormal ALT level and were positive for HBeAg at the same time. In the 10 cases, HBV DNA level decreased by different degrees 4 wk after LAM treatment. At the end of 42 wk, HBV DNA level in 8 cases (80%) was lower than  $10^3$ /mL, and ALT was returned to normal and baseline level of ALT did not rebound. HBeAg converted to negative in 4 (40%) cases and 1 (10%) case had HBeAg/anti-HBe serum conversion. It was suggested that LAM had a good curative effect in the patients with wild YMDD mutations.

## DISCUSSION

HBV belongs to DNA viral species and its duplication course is similar to that of reversed transcription viruses. HBV DNA polymerase has an activity of reversed transcription and a highly conservative YMDD order locating in the polymerase structural region C area, which is the combining and functioning site of LAM (nucleoside antiviral medicine). A great number of studies in recent years showed that long-term (longer than 6 mo) LAM treatment could contribute to YMDD mutations<sup>[3-12]</sup> and disease recurrence. Some patients even went worse, and eventually died<sup>[13-17]</sup>. But it has been seldom reported whether YMDD mutations have natural existence. Some scholars<sup>[18,19]</sup> found that YMDD mutational strains were positive in the serum of the cases infected with CHB who did not receive LAM treatment. Yan *et al.*<sup>[20]</sup> showed that 19 cases had YMDD mutations out of 110 CHB cases untreated with LAM. Fontaine *et al.*<sup>[21]</sup> reported that 5 had YMDD mutations out of 18 cases of asymptomatic HBV carriers, and so they considered that the natural existence of YMDD mutational strains was associated with a great amount of HBVs existing in CHB patients and its mutations. Fontaine *et al.*<sup>[21]</sup> revealed that YMDD mutations also occurred in HBV-infected cases who underwent kidney transplantation and dialysis therapy. In addition, Zhang *et al.*<sup>[22]</sup> found that 32 (26.2%) cases (including wild YMDD genotypes and mutant genotypes) had YMDD mutations in 122 CHB cases by genetic chip determination. Matsuda *et al.*<sup>[23]</sup> reported that a few CHB cases not treated with LAM had YMDD mutations. Of the 104 CHB cases in this study who had not received LAM and any other Chinese traditional or Western antiviral treatment, 28 (26.9%) had YMDD mutations and the detection rate of mutational strains was in accordance with that reported by Zhang *et al.*<sup>[22]</sup>, showing that wild YMDD mutational strains were in existence in HBV DNA. However, the spontaneous incidence rate of YMDD mutations had no correlation with familial aggregation.

Kobayashi *et al.*<sup>[19]</sup> found that anti-HBe was positive in all patients with YMDD mutations, and Ye *et al.*<sup>[24]</sup> found that anti-HBe was positive in most patients with YMDD mutations and considered that YMDD mutations might occur more easily if mutations took place in pre-c-zone. In this study, we found that the incidence rate of YMDD mutations was 27.1% in patients with negative HBeAg while 26.7% in patients with negative HBeAg and positive anti-HBe. There was no significant difference between the two groups. These results do not accord with those of Kobayashi

*et al.*<sup>[19]</sup> and Ye *et al.*<sup>[24]</sup> but accord with the studies of Da Silva *et al.*<sup>[25]</sup> and Yan *et al.*<sup>[20]</sup>. Therefore, YMDD mutations might not have a relationship with pre-c-zone mutations.

We found that there was a difference in the incidence rate of HBV YMDD wild mutational strains between HBV genotypes C, D, non-classified types and mixed genotypes of CD and CB. Most of the mutations (71.4%) occurred in genotype C and its mixed forms partly because genotype C occupied a great proportion in CHB cases. Though there was no significant difference among these genotypes, the relation between YMDD mutations and HBV genotypes could not be excluded as the cases were comparatively small in our study.

Some scholars<sup>[26, 27]</sup> proposed that a high HBV DNA level of serum had a positive correlation with the incidence of mutations. After HBV DNA was detected in 104 CHB patients, we found 12 YMDD mutations (25.0%) in 48 cases with a higher level of HBV DNA ( $>10^5$  copies/mL), while 16 (28.6%) mutations were found in 26 cases with a lower HBV DNA level ( $\leq 10^5$  copies/mL) and there was no significant difference between the two groups, suggesting that HBV DNA level might not be positively correlated with YMDD mutations. Further studies with more samples should be conducted.

LAM has been acknowledged and identified as one of the first-line medicines for CHB at World Gastroenterology Conference, and by Asian-Pacific Region Hepatology Association and European-American Hepatology Association<sup>[28,29]</sup>. In our study, 10 patients with YMDD mutations were treated with LAM. We found that HBV DNA level decreased by different degrees 4 wk after treatment, and at the end of 42 wk HBV DNA level in 8 cases was lower than  $10^3$  copies/mL, and their ALT recovered to normal and baseline level of ALT did not rebound, HBeAg became negative in 4 cases and 1 case had HBeAg/anti-HBe conversion. These indicated that LAM had a short-term curative effect on CHB with YMDD mutations. The results might be associated with the fact that wild viral strains were dominant while YMDD mutational strains were minor and had a lower duplication activity and weaker pathogenicity. The samples were small and the course of treatment was not long enough in our study to assess. To know whether and how the curative effect of LAM was affected by YMDD mutational strains, a contoured study with a non-YMDD mutation group and larger sample size is needed. Some scholars<sup>[30-34]</sup> considered that LAM combined with other antiviral medicines in the treatment of CHB patients would partly improve the curative effect and could delay or decrease the occurrence of drug-tolerant YMDD mutation strains. Thus, it is worthwhile to explore antiviral medicines for the treatment of patients with natural YMDD mutations.

In conclusion, YMDD wild mutational strains exist in HBV and its incidence rate has no relation to familial aggregation. LAM has a good short-term curative effect in CHB patients with YMDD mutations. But some issues still need further research, such as the factors that contribute to mutations, the relationship between mutations and the development of hepatic diseases, the correlation of pre-c-zone mutations with genotypes, the relationship between HBV DNA level and the incidence of mutations.

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• BRIEF REPORTS •

# Clinical significance of matrix metalloproteinase-9 expression in esophageal squamous cell carcinoma

Zhen-Dong Gu, Ke-Neng Chen, Ming Li, Jin Gu, Ji-You Li

Zhen-Dong Gu, Ke-Neng Chen, Department of Thoracic Surgery, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

Ming Li, Jin Gu, Department of General Surgery, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

Ji-You Li, Department of Pathology, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

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Correspondence to: Ji-You Li, Professor of Department of Pathology, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China. [lijyou@263.net](mailto:lijyou@263.net)

Telephone: +86-10-88121122 Fax: +86-10-88122437

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## Abstract

**AIM:** To evaluate the expression of matrix metalloproteinase-9 (MMP-9) and its clinical significance in esophageal squamous cell carcinoma (ESCC).

**METHODS:** The expression of MMP-9 in 208 cases of ESCC was detected by immunohistochemistry (IHC) and its clinical significance in ESCC especially the relationship with the clinicopathological parameters was analyzed.

**RESULTS:** The percentage of positive cases for MMP-9 detected by IHC was 49.0%. MMP-9 was mainly expressed in the cytoplasm of cancer cells especially in the invasive front. Only weak expression was detected in the stromal cells and no expression in non-cancerous mucosa. The expression of MMP-9 was positively correlated with poorer differentiation ( $P = 0.001 < 0.01$ ), existence of vessel permeation ( $P = 0.027 < 0.05$ ) and lymph node metastasis ( $P = 0.027 < 0.05$ ).

**CONCLUSION:** The expression of MMP-9 correlates with the cancer cell differentiation, vessel permeation and lymph node metastasis. It may be a novel biomarker for the diagnosis and treatment of ESCC.

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**Key words:** Matrix metalloproteinase-9; Esophageal squamous cell carcinoma; Immunohistochemistry; Clinicopathological parameters; Biomarker

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## INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignant tumors, and China is one of the leading ESCC high-incidence areas in the world. In general, patients with ESCC have a worse prognosis than those with other digestive tract cancers even after curative surgical resection, due to the extensive local invasion and frequent regional lymph node metastasis at presentation. Spread of malignant tumors is a multi-step process and many of the stages of tumor invasion require degradation or breakdown of the extracellular matrix and connective tissue surrounding the tumor cells. The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases collectively capable of degrading essentially all components of extracellular matrix (ECM)<sup>[1-3]</sup>, and there is considerable evidence to indicate that individual MMPs have an important role in tumor invasion and metastasis<sup>[4-7]</sup>.

At present, more than 20 members of the human MMP gene family are known, and they are classified into subgroups of collagenases, stromelysins and gelatinases based on their structure and substrate specificity. MMP-9 is one of the gelatinases that mainly degrades type IV collagen, which is the main component of basement membrane. Then the activity of MMP-9 is suggested to be associated with the disruption of basement membrane and also to play a very important role in the distant metastasis potential of carcinoma cells through vessel permeation. Recently, some studies have indicated that MMP-9 is associated with the ESCC<sup>[8,9]</sup>. However, there is still lack of comprehensive analysis for the expression of MMP-9 in ESCC in large sections of examples.

In this study, immunohistochemistry (IHC) was performed to detect the expression of MMP-9 and the relationship of its expression to clinicopathological parameters was analyzed. We found that the expression of MMP-9 correlates with cancer cell differentiation, vessel permeation and lymph node metastasis, and hence might be a novel biomarker for the diagnosis and treatment of ESCC.

## MATERIALS AND METHODS

### Patients and tissue samples

Paraffin-embedded tumor specimens from 208 patients with ESCC who were surgically treated in Peking University School of Oncology from July 1996 to November 2002 were used for IHC analysis, 162 males and 46 females, aged 38-78 years (mean 60 years).

### Immunohistochemistry

The 4- $\mu$ m sections were dewaxed in xylene, rehydrated in alcohol, and immersed in 3% hydrogen peroxide for 10 min

to suppress endogenous peroxidase activity. Then antigen retrieval was performed by microwave treatment (650 W) of the sections for 10 min in 0.01 mol/L sodium citrate buffer (pH 6.0). After being rinsed for 5 min×3 times in PBS, the sections were incubated for 18 h at 4 °C with a mouse antihuman MMP-9 antibody (Novocastra Laboratories, 15W2, 1:20) diluted in PBS. After washing for 5 min×3 times in PBS, the sections were incubated with horseradish peroxidase-labeled goat antimouse immunoglobulin (DAKO, K4001) for 1 h at room temperature. After 3 additional washes, peroxidase activity was developed with diaminobenzidine (DAB) at room temperature. Finally, the sections were counterstained in Mayer's hematoxylin.

Immunostaining signals were scored by two independent observers. The scores were calculated as the number of stained cells divided by the total number of carcinoma cells. Unequivocal staining of the cytoplasm in more than 10% of carcinoma cells was considered positive.

### Statistical analysis

SPSS 10.0 software was used to perform the  $\chi^2$  test to

analyze the relationship between the expression of MMPs and clinicopathological parameters. *P* value less than 0.05 was considered statistically significant.

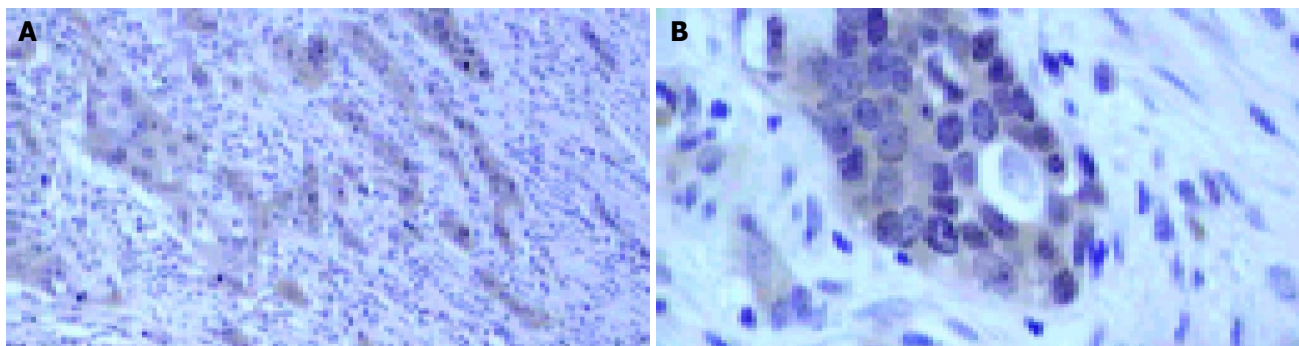
## RESULTS

### Expression of MMP-9 in ESCC detected by IHC

The percentage of positive cases for MMP-9 detected by IHC is 49.0% and Figure 1 shows the representative results. The expression of MMP-9 is mainly located in the cytoplasm of cancer cells. And the immunostaining showed a tendency to be stronger in deeply invading nests especially in invasive fronts. Weak staining was seen in stromal cells and no staining was seen in normal esophageal mucosa.

### Relationship of MMP-9 expression and clinicopathological parameters

The expression of MMP-9 was positively correlated with poorer differentiation, existence of vessel permeation and lymph node metastasis, but had no relationship with other parameters (Table 1).



**Figure 1** Immunohistochemical staining of MMP-9 in ESCC. A: Strong immunostaining can be seen in deeply invading nests of ESCC, weak immunostaining can be seen in stromal cells (×100); B: Positive staining locates in the cytoplasm of ESCC cells (×400).

**Table 1** Relationship of the MMP-9 expression and clinicopathological parameters

Clinicopathological parameters		MMP-9 expression		$\chi^2$	<i>P</i>
		Positive cases (%)	Negative cases (%)		
Age (yr)	<60	44 (49.4)	45 (50.6)	0.010	0.921
	>60	58 (48.7)	61 (51.3)		
Sex	Male	74 (45.7)	88 (54.3)	3.308	0.069
	Female	28 (60.9)	18 (39.1)		
Tumor location	Upper	8 (50.0)	8 (50.0)	1.484	0.476
	Middle	69 (51.9)	64 (48.1)		
	Lower	25 (42.4)	34 (57.6)		
Tumor cell differentiation	Well	25 (32.5)	52 (67.5)	13.503	0.001
	Middle	46 (59.7)	31 (40.3)		
	Poor	31 (57.4)	23 (42.6)		
Vessel permeation	Absent	75 (45.2)	91 (54.8)	4.896	0.027
	Present	27 (64.3)	15 (35.7)		
Tumor invasion (T)	T1	8 (57.1)	6 (42.9)	1.305	0.521
	T2	19 (42.2)	26 (57.8)		
	T3	75 (50.3)	74 (49.7)		
Lymph node metastasis (N)	N0	45 (41.7)	63 (58.3)	4.885	0.027
	N1	57 (57.0)	43 (43.0)		
TNM stage	I	7 (58.3)	5 (41.7)	7.139	0.068
	Ia	38 (39.6)	58 (60.4)		
	Iib	7 (46.7)	8 (53.3)		
	III	50 (58.8)	35 (41.2)		

## DISCUSSION

It is widely accepted that cancers develop and progress through the accumulation of various genetic alterations. ESCC is one of the most aggressive carcinomas and the postoperative outcome remains unsatisfactory<sup>[10]</sup>. But it is recognized that some patients who undergo a curative operation do gain a long-term survival, even though the carcinoma has reached an advanced stage<sup>[11]</sup>. That is, the widely used TNM staging cannot accurately predict the prognosis in all the patients. So it is important to study some useful biologic markers as indicators for the malignant potential<sup>[12]</sup>. MMPs have attracted more and more attention because of their important function in tumor invasion, angiogenesis and metastasis. In the present study, we detected the expression of MMP-9 to figure out the role of MMPs in ESCC.

### **Expression of MMP-9 is elevated in ESCC especially at the invasive front**

The results of IHC for MMP-9 showed that the immunostaining was mainly located in the cytoplasm of cancer cells especially the cancer cells at the invasive front. There were weak expressions in stromal cells and no expression in non-cancerous mucosa. This expression status showed that the expression of MMP-9 was elevated in carcinoma tissues compared to non-cancerous mucosa, which indicates that MMP-9 may play a role in the development of ESCC. The high expression of MMP-9 at the invasive front may enforce the degradation of the ECM and then facilitate the invasion or metastasis of tumor cells. Some authors suggest that the expression of MMPs in stromal cells may exert more important function<sup>[13,14]</sup>, but others do not think so<sup>[3,4]</sup>. In our study, it was found that the expression of MMP-9 was mainly located in the cytoplasm of cancer cells, which indicates that the MMP-9 expressed in cancer cells may play a more important role.

### **Expression of MMP-9 is related to the clinicopathological parameters of ESCC**

MMP-9 is important in many aspects of biology; ranging from cell proliferation, differentiation and remodeling of the ECM, these events may be the reasons for MMP-9 expression being related to the clinicopathological parameters.

Poorly differentiated ESCC has high MMP-9 expression. This may explain why poorly differentiated ESCC has high malignant tendency. High expression of MMP-9 can provide the cancer cell more chances to invade and metastasize.

MMP-9 expression is positively correlated with the existence of vessel permeation (vascular or lymphatic). There may be two reasons for this. One is, MMP-9 mainly degrades type IV collagen, which is the principal component of basement membrane. So the high expression of MMP-9 may facilitate the cancer cells to penetrate the vessel membranes and then to enter the blood stream or metastasize to lymph nodes. Kim *et al.*<sup>[15]</sup> found that MMP inhibitors can block intravasation of tumor cells and only MMP-9-expressing cells are able to enter the blood stream, which indicates that MMP-9 is required for intravasation and hematogenous spread of tumor cells *in vivo*. The other reason is that MMP-9 expression can promote the tumor angiogenesis, which can provide oxygen and nutrition for cancer cells, and promote the

formation of cancer emboli. Bergers *et al.*<sup>[16]</sup> suggested that MMP-9 can induce angiogenesis by releasing sequestered VEGF.

High expression of MMP-9 is positively correlated with lymph node metastasis. The mechanism may be the same as that mentioned above. Many authors reported that lymph node metastasis is an independent prognostic factor of ESCC<sup>[17,18]</sup>, and MMP-9 may affect the prognosis by affecting the lymph node metastasis.

On the other hand, the expression of MMP-9 has no relationship with the depth of tumor invasion, which may be due to the fact that the main substrate of MMP-9 is type IV collagen and not type I collagen. Type I collagen is the main component of connective tissue, and the tumor invasion to the deeper layer is always accompanied with the lysis of type I collagen. So, maybe collagenases play an important role in the tumor invasion depth.

### **MMP-9 might be a novel biomarker for ESCC**

In our study, MMP-9 was highly expressed in poor prognostic potential patients, weakly expressed in good prognostic potential patients and did not express in non-cancerous mucosa. This expression pattern lends considerable support to the likelihood that MMP-9 might be acting as a novel biomarker for the diagnosis and treatment of ESCC. Now many potential inhibitors of MMPs, including MMP-9, are assessed for anticancer properties such as synthetic low-molecular weight MMPis and bryostatin compounds, but there are still some uncertainties and a definite conclusion awaits more studies<sup>[19-21]</sup>.

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• BRIEF REPORTS •

# Activation of STAT3 signaling in human stomach adenocarcinoma drug-resistant cell line and its relationship with expression of vascular endothelial growth factor

Li-Fen Yu, Ying Cheng, Min-Min Qiao, Yong-Ping Zhang, Yun-Lin Wu

Li-Fen Yu, Ying Cheng, Min-Min Qiao, Yong-Ping Zhang, Yun-Lin Wu, Department of Gastroenterology, Ruijin Hospital of Shanghai Second Medical University, Shanghai 200025, China  
Supported by Shanghai Education Committee Foundation, No. 024119114

Correspondence to: Dr. Li-Fen Yu, Department of Gastroenterology, Ruijin Hospital of Shanghai Second Medical University, 197 Ruijin Road, Shanghai 200025, China. [graceyu1028@sohu.com](mailto:graceyu1028@sohu.com)  
Telephone: +86-21-64370045-665242 Fax: +86-21-64373909  
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## Abstract

**AIM:** To investigate the difference in activation of STAT3 signaling between two human stomach adenocarcinoma cell lines: 5-fluorouracil resistant cell line and its parental cell line, and to evaluate its relationship with the expression of vascular endothelial growth factor (VEGF).

**METHODS:** Western blot and electrophoretic mobility shift assay (EMSA) were used to detect the expression of phospho-STAT3 protein and constitutive activation of STAT3 in two human stomach adenocarcinoma cell lines, 5-fluorouracil resistant cell line SGC7901/R and its parental cell line SGC7901, respectively. The mRNA expression of VEGF was analysed by semi-quantitative RT-PCR. The expressive intensity of VEGF protein was measured by immunocytochemistry.

**RESULTS:** The expressions of phospho-STAT3 protein and constitutive activation of STAT3 between two human stomach adenocarcinoma cell lines were different. Compared with the parental cell line SGC7901, the STAT3-DNA binding activity and the expressive intensity of phospho-STAT3 protein were lower in the drug-resistant cell line SGC7901/R. The expression levels of VEGF mRNA and its encoded protein were also decreased in drug-resistant cell line.

**CONCLUSION:** Over-expression of VEGF may be correlated with elevated STAT3 activation in parental cell line. Lower VEGF expression may be correlated with decreased STAT3 activation in resistant cell line, which may have resulted from negative feedback regulation of STAT signaling.

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**Key words:** Stomach adenocarcinoma; Vascular endothelial growth factor; STAT3 protein; Antineoplastic Drug Resistance

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## INTRODUCTION

As one of the signal transducers and activators of transcription (STAT) family members, STAT3 has been correlated with positive regulation of cell growth and is highly activated in cancer cells<sup>[1,2]</sup>. Activation of STAT3 signaling regulates the expression of numerous genes involved in growth control and survival. Many studies have shown that numerous genes encoding *Bcl-x<sub>L</sub>*, *Mcl-1*, *cyclins D1/D2*, and *c-Myc* proteins are downstream targets of STAT3<sup>[3-6]</sup>. Recent studies have indicated that constitutive STAT3 signaling induces VEGF expression and tumor angiogenesis<sup>[7-11]</sup>. The expression of VEGF antigen in gastric cancer cells can serve as a pertinent predictive factor for hematogenous invasion or metastasis, and the importance of it has been proven and widely studied<sup>[12-14]</sup>. In addition, the resistance to 5-fluorouracil (5-FU) is a main obstacle in gastric cancer chemotherapy. However, the correlation between STAT3 and VEGF has not been studied in drug resistant cell line. In this study, we aimed to investigate the different activation of STAT3 signaling in two human stomach adenocarcinoma cell lines, 5-fluorouracil resistant cell line and its parental cell line, and evaluate its relationship with the expression of VEGF.

## MATERIALS AND METHODS

### Reagents

Nuclear and cytoplasmic extraction reagents and bovine serum albumin (BCA) protein assay kit were purchased from Pierce (Rockford, IL, USA). EasyTides adenosine 5'-triphosphate [ $\gamma$ -<sup>32</sup>P] was purchased from PerkinElmer (Boston, MA, USA). MicroSpin™ G-25 column and hybond-C membrane were purchased from Amersham Biosciences (Piscataway, NJ USA). TRIzol reagent and MagicMarker Western standard were purchased from Invitrogen (Carlsbad, California, USA). AMV reverse transcription system ( $A_{3500}$ ), Taq DNA polymerase, dNTP, PCR marker and EMSA kit were purchased from Promega (Madison, WI, USA). Monoclonal anti- $\beta$ -actin was purchased from Sigma. Polyclonal rabbit anti-human phospho-Tyr705-

STAT3 was obtained from Cell Signaling (Beverly, MA, USA). Goat anti-rabbit IgG-AP was purchased from Santa Cruz (California, USA). Monoclonal mouse anti-human VEGF antibody was obtained from Fujian Maixin Co (Fujian, China). DAKO Envision system HRP (DAB) was purchased from DAKO (Produktionsvej, Glostrup, Denmark).

### Cell lines and culture

Two human gastric adenocarcinoma cell lines, 5-fluorouracil resistant cell line SGC7901/R and its parental cell line SGC7901 were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All of the cells were grown in RPMI 1640 supplemented with 100 mL/L fetal bovine serum in a humidified atmosphere containing 50 mL/L CO<sub>2</sub> at 37 °C. The final inducing dosage of 5-fluorouracil was 1 g/L.

### Western blot analysis

Both the extraction of nuclear protein of two cell lines and the contents of nuclear protein were determined by the kits according to the manufacturer's instruction. A total of 100 µg of nuclear extracts was loaded onto SDS-polyacrylamide gel and blotted onto hybond-C membranes by electrophoresis. Equal protein sample loading was monitored by hybridizing the same membrane filter with an anti-β-actin antibody. MagicMarker was used for molecular weight determinations. The membranes were rinsed in TBS and blocked for 1 h at room temperature with 50 g/L fat free milk powder in TBS. The membranes were then incubated with 1:400 dilution of polyclonal antibodies against phospho-STAT3 at room temperature for 2 h. The proteins were detected by incubating the strips in alkaline phosphatase-conjugated anti-rabbit IgG antibody for 1 h at room temperature. The Quantity one software was used to analyse the scanned protein bands.

### Electrophoretic mobility shift assay (EMSA) for STAT3-DNA binding activity

The STAT3-DNA binding activity was assessed by EMSA using the nuclear extract from the two cell lines. The sense strand that binds activated STAT3 protein was 5' TCG ACA TTT CCC GTA AAT C 3' (synthesized by Shanghai Shenggong Co). Double-stranded oligonucleotide was end-labeled with [γ-<sup>32</sup>P] ATP using a T4 polynucleotide kinase according to the manufacturer's instruction. The final concentration of probe was 1.75 pmol/L. The labeled probes were then purified by G-25 spin columns. One microliter of <sup>32</sup>P-labeled STAT3 oligonucleotide was added to each reaction. For STAT3 specific test, a 150-fold unlabeled STAT3 probe was applied as a competitor. The final volume of reaction was 20 µL, including 10 µg of nuclear extract and 5×binding buffer. The reactions were placed on ice for 30 min. The 45 g/L nondenaturing acrylamide gel was pre-run in 1×TBE buffer at 25 mA for 60 min. After loading of the samples, the gel was run at room temperature in 1×TBE buffer at 25 mA for 90 min. The gel was dried on a gel dryer, then exposed to x-ray film overnight at -80 °C with intensifying screen. The protein-DNA complex was detected by autoradiography. The Quantity one software was used to analyse the scanned EMSA gel bands.

### Semi-quantitative RT-PCR

Total RNA was extracted from the two cell lines with a single step method using the TRIzol reagent according to the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed using 2 µg of total RNA by reverse transcription system (*A<sub>3500</sub>*) according to the manufacturer's instructions. Polymerase chain reaction was then conducted for 26, 28, 30 and 35 cycles in a thermal controller (Programmable Thermal Controller; MJ Research, Inc.) and the optimal cycle number for quantification (30 cycles) was determined. The primer annealing for STAT3 was carried out at 57 °C for 0.5 min. The sequences of PCR primers were as follows: for VEGF, 5' TGC ATT CAC ATT TGT TGT GC 3' (sense), and 5' AGA CCC TGG TGG ACA TCT TC 3' (antisense, a 200 bp product); for β-actin, 5' AAG GAT TCC TAT GTG GGC 3' (sense), and 5' CAT CTC TTG CTC GAA GTC 3' (antisense, a 532 bp product). β-actin mRNA levels were used as internal controls. Then 8 µL of each PCR product was electrophoresed on 20 g/L agarose gels, and the intensities of the specific bands were analyzed by the software of Quantity one.

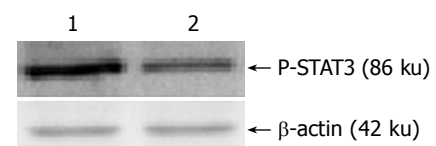
### Immunocytochemistry

DAKO Envision system HRP (DAB) was used for immunocytochemical staining of VEGF. Two kinds of cells were fixed in 40 g/L paraformaldehyde. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 30 mL/L hydrogen peroxide for 10 min at room temperature. After washed with PBS for 5 min, slides were blocked with normal serum for 20 min, followed by incubation with the primary antibody for VEGF (a mouse anti-human monoclonal antibody diluted at 1:50), overnight at 4 °C. As negative controls, PBS was used to replace primary antibody. After rinsed with PBS for 5 min, slides were incubated with the secondary antibody for 30 min and washed again. Chromogen was developed with 3, 3'-diaminobenzidine (DAB). All of the slides were lightly counterstained with hematoxylin for 10 s before dehydration and mounting. The slides were examined under a microscope (BX-50, Olympus Company). The software of Spot version 3.5.2 was used to analyze the images.

## RESULTS

### Western blot analysis of phospho-STAT3 protein

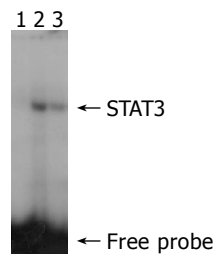
We performed Western blot with phospho-STAT3 antibody. This antibody reacts with the phosphorylated tyrosine 705 residue that is indicative of STAT3 activation<sup>[15]</sup>. The expression levels of the phospho-STAT3 proteins were markedly reduced in drug-resistant cell line SGC7901/R when compared with the parental cell SGC7901 (Figure 1).



**Figure 1** Western blot analysis of phospho-STAT3 in different cell lines. Lane 1: SGC7901 cell line; lane 2: SGC7901/R cell line.

### The level of STAT3-DNA binding activity

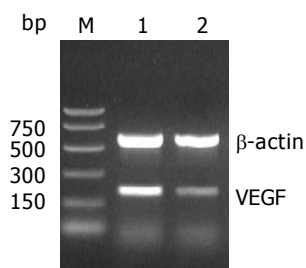
The result of EMSA showed that STAT3-DNA binding activities were different between the two cell lines (Figure 2). The STAT3-DNA binding activity was lower in the drug-resistant cell line SGC7901/R, as compared with the parental cell lines SGC7901, suggesting that the constitutive activation of STAT3 was decreased in the drug-resistant cell line.



**Figure 2** EMSA analysis of STAT3 DNA-binding activity in different cell lines. Lane 1: Competitive probe; lane 2: SGC7901/R cell line; lane 3: SGC7901 cell line.

### Expression of VEGF mRNA

The semi-quantitative RT-PCR showed that the levels of VEGF mRNA were markedly decreased in SGC7901/R cell line when compared with the SGC7901 cell line (Figure 3).



**Figure 3** Identification of VEGF mRNA by RT-PCR in different cell lines. M: Ladder marker; lane 1: SGC7901 cell line; lane 2: SGC7901/R cell line.

### Detection of VEGF protein by immunostaining

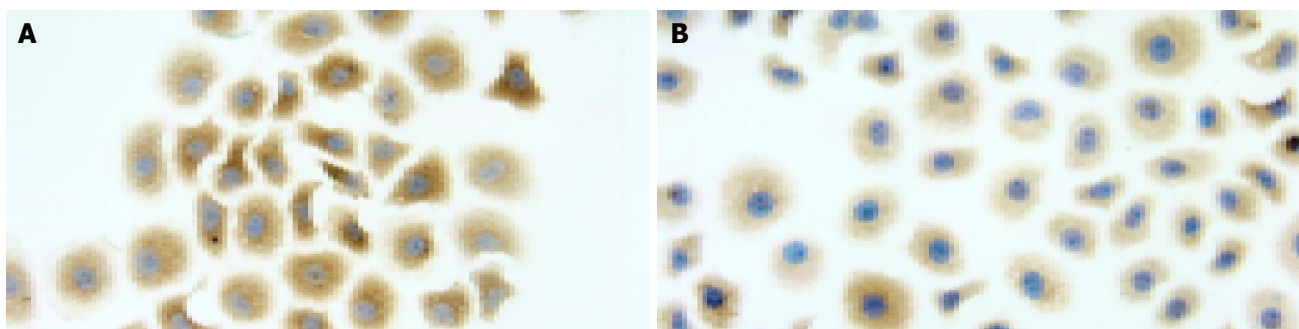
Compared with parental cell line SGC7901, lower expression

of VEGF in cytoplasm was observed in the drug-resistant cell line SGC7901/R (Figure 4).

## DISCUSSION

The STAT factors function as downstream effectors of cytokine and growth factor receptor signaling. STAT signaling is critical for normal cellular processes, such as embryonic development, organ genesis and function, innate and adaptive immune function, regulation of cell differentiation, growth, and apoptosis. Studies have demonstrated constitutive activation of STAT, in particular STAT1, STAT3, and STAT5, in a large number of diverse human tumor cell lines. The function of Stat1 has been associated with growth suppression rather than malignant transformation and thus can be considered a potential tumor suppressor. However, STAT3 and STAT5, have been demonstrated to directly contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis. A variety of cellular mechanisms of multi-drug resistance (MDR) have been identified in human drug-resistant cell lines. These include P-glycoprotein (Pgp), multi-drug resistance-associated protein (MRP), lung resistance-related protein (LRP), glutathione-transferase (GST-pi), DNA topoisomerase II alpha (Topo II alpha) and apoptosis, *etc.* Catlett-Falcone *et al.*<sup>[16]</sup> found that constitutive activation of STAT3 signaling conferred resistance to apoptosis in human U266 myeloma cells. But inhibition of STAT3 signaling led to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. These studies suggested that tumor cells lacking STAT activation were typically more tolerant to the pharmacological doses used in these experiments<sup>[17]</sup>.

A main chemotherapeutic drug for patients with progressive gastric cancer 5-fluorouracil is one of the pyrimidine antagonists. A research on 5-FU resistant human gastric cancer cell line showed that SGC7901/R was an acquired resistant cell line<sup>[18]</sup>. Besides highly resistant to 5-FU, the drug-resistant cell line was cross-resistant to several drugs. SGC7901/R cell line was 12.77, 271 and 14.04-fold more resistant to cytotoxic action of DNR, CDDP and MMC, respectively. So the 5-FU resistant cell line has the characteristics of multi-drug resistance (MDR). The drug resistance mechanisms of 5-FU resistant cell line were related not only to the alteration of thymidylate synthase (TS) and thymidine kinase (TK), which play key role in the



**Figure 4** Immunocytochemical staining of VEGF in two human stomach adenocarcinoma cell lines (DAB×400). A: parental cell line SGC7901; B: drug-resistant cell line SGC7901/R.

pathway of pyrimidine synthesis, but also to the P-glycoprotein (P-gp) coded by *mdr-1* genes.

The present study originally investigated the relationship between STAT3 and MDR in the drug-resistance cell line. Western blot was firstly used to detect the expression of phospho-STAT3 protein. The result demonstrated that the expression of phospho-STAT3 protein was lower in the drug-resistant cell line SGC7901/R as compared with its parental cell line SGC7901. The EMSA assay provides a simple and rapid method for detecting DNA-binding protein. This method has been used widely in the study of sequence-specific DNA-binding proteins, such as transcription factors. The result of EMSA in our study showed that STAT3 DNA-binding activities were different between the two cell lines. The STAT3 DNA-binding activity was decreased in the drug-resistant cell line. These evidence suggested that drug-resistant cells which had lower level of STAT activation was more tolerant to the drugs of chemotherapy.

Numerous studies on STAT signal pathway have shown that signals usually generated at cell surface receptors. Upon binding of cytokines to cognate receptors on the surface of cells, receptors dimerize and thereby activate receptor-associated tyrosine kinases such as JAKs. Receptor-bound STAT subsequently become tyrosine phosphorylated either by receptor intrinsic or associated tyrosine kinase activities. Once phosphorylated, STAT dimerizes via reciprocal phosphotyrosine-SH2 domain interactions. Within minutes, the dimers translocate to the nucleus, where they interact with other transcriptional modulators bound to specific promoter sequences and induce gene expression. Wei *et al*<sup>[19]</sup> found that more than 80% of the human pancreatic cancer cell lines exhibited constitutively activated STAT3, with STAT3 activation correlated with the VEGF expression level. Immunohistochemical studies have indicated that VEGF over-expression coincides with elevated STAT3 activation in human pancreatic cancer specimens. Blockade of activated STAT3 via ectopic expression of dominant-negative STAT3 significantly suppressed VEGF expression, angiogenesis, tumor growth, and metastasis *in vivo*. Furthermore, constitutively activated STAT3 directly activated the VEGF promoter, whereas dominant-negative STAT3 inhibited the VEGF promoter. These results indicated that STAT3 directly regulated VEGF expression and hence angiogenesis, growth, and metastasis of human pancreatic cancer. The present study demonstrated that compared with the parental cell line SGC7901, both the constitutive activation of STAT3 and the expressive intensity of phospho-STAT3 protein were lower in drug resistant cell line. The expression levels of VEGF mRNA and its encoded protein were also decreased in drug-resistant cell line. These results suggested that over-expression of VEGF might be correlated with elevated STAT3 activation in parental cell line and the lower VEGF expression is correlated with decreased STAT3 activation in resistant cell line.

A number of modulators of STAT signaling pathways have been described<sup>[20,21]</sup>, and the suppressor of cytokine signaling has received a great deal of focus. The suppressors of cytokine signaling (SOCS) family consist of eight proteins, CIS and SOCS1-SOCS7, which were discovered to suppress STAT signaling by binding to and inhibiting JAKs<sup>[22-24]</sup>. Some

of these proteins were transcriptionally regulated by STAT themselves, suggesting that STAT could negatively regulate their own phosphorylation state. SOCS1 could play a key role in the negative regulation of interferon-gamma signaling and in T cell differentiation<sup>[25]</sup>. It has been demonstrated to block Tel-JAK2-mediated transformation of hematopoietic cells<sup>[26]</sup>. Recently, a deletion on chromosome 16p that contains SOCS1 was found in 48% of primary hepatocellular carcinomas, raising the possibility that inactivation of this gene might participate in hepatocarcinogenesis<sup>[27]</sup>. SOCS2 (-/-) mice were 30-40% larger than wild-type mice, demonstrating that SOCS2 might be a critical regulator of postnatal growth<sup>[24]</sup>. SOCS-3, which is known to inhibit STAT3 and STAT5 activation, has been shown to be critical in the negative regulation of fetal liver hematopoiesis<sup>[28]</sup>. The biological role of other SOCS proteins remains to be determined. The protein inhibitors of activated STAT (PIAS) represent another group of proteins that normally serve to decrease DNA activation by blocking STAT DNA binding activity<sup>[29]</sup>. Reports have demonstrated that over-expression of PIAS3 suppresses gene transcription mediated by STAT3<sup>[30]</sup>. The present study demonstrated that both the STAT3-DNA binding activity and the expressive intensity of phospho-STAT3 protein were lower in drug-resistant cell line. It can therefore be speculated that negative regulators of STAT signaling might be correlated with the decreased STAT3 activation in drug-resistant cell line.

In summary, our study demonstrates different activation of STAT3 signaling in these two human stomach adenocarcinoma cell lines, 5-fluorouracil resistant cell line and its parental cell line. The knowledge of STAT signaling pathway has proved to be of even broader benefit for illustrating the multi-drug resistance mechanisms of gastric cancer. The lower VEGF expression may be correlated with decreased STAT3 activation in drug-resistant cell line. Because the negative regulators of STAT signaling might play important roles in the control of tumor incidence and/or progression, further studies should be done in order to illustrate their regulation for tumors' drug-resistance. The fact that STAT3 regulates the key angiogenic factor, VEGF, makes STAT3 a critical transcription factor in tumor development and progression. The parent cell line SGC7901, which is sensitive to the drugs of chemotherapy, has elevated constitutive activation of STAT3 and over-expression of VEGF, so targeting at STAT3 signaling may represent a novel approach to control the angiogenesis and growth of human gastric cancer.

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• BRIEF REPORTS •

## Characterization of *CagA* variable region of *Helicobacter pylori* isolates from Chinese patients

Yong-Liang Zhu, Shu Zheng, Qin Du, Ke-Da Qian, Ping-Chu Fang

Yong-Liang Zhu, Qin Du, Ke-Da Qian, Department of Gastroenterology, Second Hospital of Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China  
Shu Zheng, Cancer Institute, Second Hospital of Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China  
Ping-Chu Fang, Department of Microbiology, Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China  
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Correspondence to: Professor Shu Zheng, Jiefang road 88#, Hangzhou 310009, Zhejiang Province, China. zhengshu@zju.edu.cn  
Telephone: +86-571-87783868

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### Abstract

**AIM:** To characterize the *CagA* variable region of *Helicobacter pylori* isolates from Chinese patients.

**METHODS:** DNA fragments in *CagA* variable region were amplified and sequenced respectively from genomic DNA of 19 isolates from patients with gastric cancer and 20 isolates from patients with chronic gastritis. The tendency of phosphorylation in tyrosine(s) of *CagA* proteins was evaluated subsequently by phosphorylation assay *in vivo* and *in vitro* respectively.

**RESULTS:** About 97.44% (38/39) *H. pylori* isolates possessed *CagA* gene. *CagA*<sup>+</sup> strains contained 2-4 tandem five-amino-acid motifs EPIYA but only one EPIYA had repeated sequence in *CagA* variable region in different isolates. There was no significant difference between the number of EPIYA motifs in *H. pylori* from patients with different diseases. However, only tyrosine site in EPIYA within repeated sequence could be phosphorylated by AGS cells *in vivo* although all tyrosine sites in EPIYA could be phosphorylated *in vitro*.

**CONCLUSION:** *CagA* in Chinese has no functional difference in perturbing cellular signal pathway among different *H. pylori* isolates.

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**Key words:** *Helicobacter pylori*; *cag* pathogenicity island; *CagA*; Tyrosine phosphorylation

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### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) has been recognized as the causative agent of chronic gastric inflammation, which can progress further to a variety of diseases such as peptic ulcer and mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma<sup>[1-3]</sup>. Type I isolates of *H. pylori* possess a major disease-associated genetic component, the *cag* pathogenicity island (*PAI*) which encodes a type IV secretion apparatus and virulence factors such as the immunodominant *CagA* protein<sup>[4,5]</sup>. Recent studies have demonstrated that the *CagA* protein is translocated from the bacterium into the host cell and induces reorganization of the host cell actin cytoskeleton, activation of Rho GTPases Rac1 and Cdc42, recruitment of transcription factors NF- $\kappa$ B and AP-1, activation of proto-oncogenes *c-fos* and *c-jun* and release of cytokines and chemokines<sup>[6-10]</sup>. Stein *et al*<sup>[11]</sup> have confirmed that the C-terminal half of *CagA* (namely *CagA* variable region) is phosphorylated in tyrosine sites by *c-src*/lyn protein kinase family once translocated into eukaryotic cells. Higashi and coworkers<sup>[12]</sup> found a src homology 2 (SH2) containing tyrosine phosphatase 2 (SHP-2) was an intracellular target of *CagA* protein. SHP-2 is an important mediator molecule in several cell signal transductions and rearrangement of the actin cytoskeleton which plays a crucial role in inducing the scattering ('hummingbird') phenotype in gastric epithelial cells (AGS)<sup>[12,13]</sup>. Phosphorylated *CagA* disturbs signal transductions by binding to SHP-2 in host cells<sup>[10]</sup>. However, *CagA* protein varies in size in different strains, this size variation raises an intriguing possibility that the biological activity of *CagA* can vary from one strain to the next, which may influence the pathogenicities of different strains<sup>[14-16]</sup>. China is one of the nations where the infection incidence of *H. pylori* is among the highest in the world and more than 90% isolated strains possess *PAI* gene, the biological activity of *H. pylori* virulence factor *CagA* isolated from Chinese remains unclear so far<sup>[17,18]</sup>. In the present report, we cloned and sequenced the variable region of *CagA* DNA fragments in 19 strains from patients with gastric cancer and 20 strains from patients with chronic gastritis. Further, we evaluated the ability of *CagA* protein to phosphorylate in tyrosine(s).

### MATERIALS AND METHODS

A total of 39 *H. pylori* clinical strains including 19 strains from patients with gastric cancer and 20 strains from patients with chronic gastritis were isolated from the biopsy specimens in the Second Affiliated Hospital of Zhejiang University College of Medicine between October 2000 and

April 2002. The diagnosis obtained by endoscopy and histology was recorded for all patients from whom the strains were isolated. There were 22 males and 17 females, with their age ranging 36–68 years (median age 48.6 years).

### Isolation and culture of *H. pylori* strains

Gastric mucosa supernatant was directly cultured in the PYL plate (Biomerux Company, France) and incubated in microaerophilic atmosphere for 4 d at 37 °C. Bacterial clones were identified by both biochemical (including rapid urease reaction, peroxidase test and Gram's staining) and serological reactions. Following primary isolation, the bacteria were inoculated into Columbia agar (Biomerux Company, France) with 50 mL/L frozen-melting sheep blood, 100 mL/L fetal bovine serum, and Skirrow's antibiotic supplement (Biomerux Company, France) in a microaerophilic atmosphere for 5 d at 37 °C, washed 3 times with 100 mmol/L PBS, pH7.4. *H. pylori* genomic DNA was extracted according to the instructions of bacterial genome extraction kit (Sangon Engineering Biotechnology Company, Shanghai, China).

### Amplification of DNA fragments of *CagA* variable region in clinical isolates

A total of 39 DNA samples were amplified by high fidelity PCR master (Roche Company, Germany) with upstream primer: 5'-GACGAGCCTATTTATGCT-3' and downstream primer: 5'-GCCTCATCAAAATCAATTGT-3'. The reaction mixture was incubated in a thermal cycler. PCR conditions were as follows: denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 30 s, and extension at 72 °C for 30 s, with a final extension reaction for 7 min<sup>[14]</sup>. Samples were analyzed on an 1% agarose gel in 1×Tris base-EDTA buffer. DNA fragments were excised and purified from gel with kit for DNA sequence respectively.

### Construction of pcDNA3.1(+) and pET15b expression vectors containing *CagA* DNA fragments with 2, 3 and 4 EPIYAs as well as their mutants respectively

Genomic DNA containing *CagA* fragments with 2, 3 and 4 EPIYAs from the above clinical isolates were used as templates, respectively. Two subfragments (about 800 bp) from *CagA* variable region in each strain could be amplified by high fidelity overlapped PCR with primers containing *Bam*HI or *Xba*I<sup>[19]</sup>. The upstream primer was 5'-TCGGATCCAATTGGAGAGCAGAA-3' and the downstream primer: 5'-GCTAGGCTCCAAAGCGGCCGCTCTTGCTTCCTTACTAG-3' for construction 1. The upstream primer was 5'-ATGCCTGGACCAAGAGCGGCCGCTTTCGATC-3' and the downstream primer was 5'-GACTCGAGTTAAGATTTTGGAAACCAC-3' for construction 2. After ligation at *Not*I site, the PCR products were digested with double restriction endonuclease *Bam*HI/*Xba*I, and an 1 509 bp *CagA* fragment was cloned into pcDNA3.1(+) mammalian expression vector (Invitrogen Company, USA). The mutants (referred to as EPIFA) of *CagA* variable region in which tyrosine in the repeated sequence was mutated to phenylalanine were produced by using a Chameleon site-directed mutagenesis kit (Stratagene Company, USA, data

not shown), including EPIFA with 1, 2 and 3 EPIYAs, respectively. The procedure was strictly performed under the manufacturer's instructions. pcDNA3.1(+) containing *CagA* or mutants was constructed as template, wild and mutated *CagA* fragments with EPIYA(s) were subsequently subcloned into *Nde*I/*Bam*HI sites of pET15b expression vectors (Novagen Company, USA) and BL21 DE3 plysS *E. coli* component cells (Novagen Company, USA) were transformed. The recombinant proteins were induced by IPTG at the concentration of 0.4 mmol/L for 2 h, and purified by the His.Bind purification kit (Novagen Company, USA) for evaluation of the phosphorylation at tyrosine site(s) of *CagA* protein *in vitro*.

### Phosphorylation assay

**Phosphorylation assay *in vivo*** Human AGS gastric epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen Company, USA) before transfection. Cells were transfected with pcDNA3.1(+) expression vectors containing *CagA* or mutants with 2–4 EPIYA motifs respectively by Lipofectamine 2000 reagent (Invitrogen Company, USA) according to the manufacturer's protocols. Fifty milligrams of plasmid was transfected into AGS cells by 40 µL of Lipofectamine 2000 reagent. Cells were harvested 72 h after transfection and lysed in lysis buffer containing 1 mmol/L EDTA, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µmol/L leupeptin, 1 µmol/L pepstatin. The cell lysate was mixed with anti-*CagA*-antibody-sepharose 4B gel (a generous gift from Dr. Yi-Xiong Wang in Right Biotechnology, China) at 4 °C for 60 min, gently mixed, washed 3 times with 100 mmol/L PBS, pH7.4, denatured with Tris base-chloride buffer containing 100 mmol/L dithiothreitol, centrifuged at 13 000 g for 20 min. The supernatant collected was subjected to electrophoresis and transferred to a nitrocellulose membrane filter (Hybond Company, USA), blocked with 5% milk powder, incubated in at 1:250 diluted monoclonal antibodies against phosphorylated tyrosine (Y99, Santa Cruz Company, USA) at 37 °C for 2 h, washed 5 times. Then 1:100 diluted biotinylated sheep antibodies against mouse (Maxim Biotechnology Company, USA) were added and washed 5 times, and incubated with streptavidin-peroxidase conjugate (Maxim Biotechnology Company, USA) for 60 min. Finally, the color was developed by ECL substrate.

**Phosphorylation assay *in vitro*** Nonidet P40-soluble fraction of AGS cells was performed as described by Stein *et al.*<sup>[11]</sup>. Ten milligrams of *CagA* or mutant recombinant proteins with EPIYAs was incubated with 10 µL of cell lysate in a total reaction volume of 40 µL 250 mmol/L Tris-Cl buffer (pH7.4) containing 100 Ci gamma-<sup>32</sup>P ATP (Perkin Elmer company, USA), 62.5 mmol/L MnCl<sub>2</sub>, 312.5 mmol/L MgCl<sub>2</sub>, 5 mmol/L EGTA, 1 mmol/L sodium-o-vanadate. Phosphorylation was carried out at 30 °C for 10 min and the mixed solution was subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was exposed at 4 °C for 24 h, respectively.

## RESULTS

### Amino acid sequence of *CagA* variable region

Thirty-eight of 39 isolates possessed *CagA* gene and the positive rate of *CagA*<sup>+</sup> was 97.44% (38/39). The nucleotide sequences of *CagA* were quite similar among the 38 strains with *CagA*<sup>+</sup>. However, the variation in the number of repetitions of the 5-amino acid EPIYA sequence in the 3' region of *CagA* was observed. Amino acid sequences were found to be 2-4 tandem five-amino-acid motifs EPIYA between each of which, there was an interval of 13-44 amino acids in *CagA* variable region. The case of *CagA* protein with 3 tandem EPIYAs was most common in all clinical isolates. There were 3 types of *CagA* variable region among the 38 isolates, namely *CagA* with 2 EPIYAs, *CagA* with 3 EPIYAs and *CagA* with 4 EPIYAs (Figure 1). To explore the relationship between the number of EPIYAs in isolates and disease outcomes, we adopted  $\chi^2$  test to evaluate the correlation and found that there was no significant difference in the number of tandem five-amino-acid motif EPIYAs in isolates from gastric cancer and chronic gastritis (Table 1),  $P>0.05$ . It implied that the variation of the number of EPIYAs might not relate to the disease outcome (gastric cancer *vs* chronic gastritis). Subsequently the analysis of sequences showed that each *CagA*<sup>+</sup> strain contained only one repeated sequence in the C-terminal half of *CagA* protein, namely genetic structure of EPIYA-TIDDL-FPLKRHDKVEDLSKV (this EPIYA was usually referred to as D1 region, TIDDL as D2 region, FPLKRHDKVEDLSKV as D3 region<sup>[11]</sup>). There were two obviously differences in this structure compared with Western standard strain NCTC11637. One difference was found in the number of repeated sequences. There was only one repeated sequence in each Chinese isolate compared with three repeated sequences in NCTC11637. The other was found in the key amino acid sequences in D2 region of repeated sequences. The amino acid sequence in D2 region was DFD in each *H. pylori* from Chinese patients, and was DDL in NCTC11637 and other Western isolates<sup>[11,14]</sup>. The variation of two amino acids in D2 region conferred Chinese *CagA* protein to more perfectly match the SHP-2 consensus motif (PY-S/T/A/V/I-X-V/I/L-X-W/F) than the Western strains<sup>[20]</sup>. Hence, we speculated that Chinese *CagA* protein might be more able to activate SHP-2 molecules.

### Tyrosine phosphorylation sites in EPIYA

We used the phosphorylation assay *in vivo* and *in vitro* to identify whether and which tyrosine site(s) in EPIYA could be phosphorylated. In phosphorylation assay *in vitro*, *CagA* recombinant proteins with 2, 3 and 4 EPIYAs as well as their mutants EPIFA with 1, 2 and 3 EPIYAs could be labeled with <sup>32</sup>P respectively. Moreover, *CagA* protein with 4 EPIYAs became more marked with <sup>32</sup>P than that with 2 or 3 EPIYAs and their mutants with 1, 2 and 3 EPIYAs (Figure 2: A-F). It implied that the tyrosine phosphorylation site in EPIYA both within and outside the repeated sequences of *CagA* recombinant proteins could be phosphorylated by AGS cell lysate *in vitro*, and *CagA* protein with 4 EPIYAs became more phosphorylated than that with 2 or 3 EPIYAs and their mutants. However, only one tyrosine site in EPIYA within the repeated sequences could be phosphorylated and no tyrosine in EPIYA outside the repeated sequences could

```

CZU-1: EPIYAQVNKKKAGQVARINKIASAGKGVGGFGGVGRSASPE
CZU-2: EPIYAQVNKKKAGQVASPEEPIYAQVARKVSVKIDQLNEAT
CZU-3: EPIYAQVNKKKTGQATSPEEPIYAQVNKKKTGQVASPE-----
NCTC11637EPIYAKVNKKKAGQAASLEEPIYA-----

CZU-1: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
CZU-2: SAINRKIDRINKIASAGKGVGGFGGVGRSASPEEPIYAQVAKK
CZU-3: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKV-----
Repeat1 D1  D2  D3

CZU-1: -----
CZU-2: VSAKIDQLNEATSAINRKIDRINKIASAGKGVGGFGGVGRSASPE
CZU-3: -----
NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKVG-----
Repeat2 D1  D2  D3

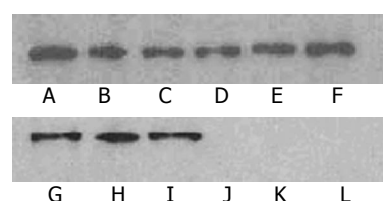
CZU-1: -----
CZU-2: EPIYATIDFDEANQAGFPLRRSAAVNDLSKV-----
CZU-3: -----
NCTC11637:EPIYATIDDLGGPFPLKRHDKVEDLSKV-----
Repeat3 D1  D2  D3
    
```

**Figure 1** Amino acid sequence characteristics of *CagA* variable region. CZU-1,2,3: Isolates from Chinese patients stand for the three types of *CagA* variable region respectively; CZU-1: *CagA* with 2 EPIYAs; CZU-2: *CagA* with 3 EPIYAs and CZU-3: *CagA* with 4 EPIYAs. The red letters stand for the deletion of amino acids in CZU-1 and the letters underline the mean extra amino acids in CZU-3 when compared to CZU-2. NCTC11637 was a Western standard strain.

**Table 1** Comparison of the number of tandem five-amino-acid motif EPIYAs in strains from gastric cancer and gastritis

	Case	EPIYA (n)		
		2	3	4
Gastritis	20	4	13	3
Gastric cancer	18	3	12	3

$\chi^2$  test,  $\chi^2 = 0.301$ ,  $P>0.05$ .



**Figure 2** Tyrosine phosphorylation sites in EPIYA *in vitro* and *in vivo*. A-F: tyrosine phosphorylation *in vitro*. Lane A: *CagA* with 4 EPIYAs; lane B: *CagA* with 3 EPIYAs; lane C: *CagA* with 2 EPIYAs; lane D: EPIFA with 1 EPIYA; lane E: EPIFA with 2 EPIYAs; lane F: EPIFA with 3 EPIYAs. lane G-L: tyrosine phosphorylation *in vivo*. G: *CagA* with 4 EPIYAs; H: *CagA* with 3 EPIYAs; I: *CagA* with 2 EPIYAs; lane J: EPIFA with 3 EPIYAs; lane K: EPIFA with 2 EPIYAs; lane L: EPIFA with 1 EPIYA.

be phosphorylated *in vivo* by AGS cells when transfected pcDNA3.1(+) mammalian expression vectors containing *CagA* with 2, 3 and 4 EPIYAs as well as EPIFA with 1, 2 and 3 EPIYAs, since the mutants of EPIFA with 1, 2 and 3 EPIYAs could not react with antibody to phosphorylated tyrosine in transfected AGS cells (Figure 2: G-L). Moreover, *CagA* protein with 4 EPIYAs did not become more phosphorylated than that with 2 or 3 EPIYAs. The outcomes were similar to that of infection of AGS cells with *H. pylori* bacteria containing *CagA* with 2, 3 and 4 EPIYAs, respectively

(data not shown). Hence, we speculated that the number of EPIYA motifs might not relate to the ability of tyrosine to phosphorylate on *CagA* protein *in vivo*, which could only be determined by the number of EPIYA repeated sequences. As a result, this outcome raised an intriguing possibility that the ability of Chinese *CagA* to phosphorylate in tyrosine was equal to one another in different isolated strains of type I because each *CagA* protein contained only one repeated sequence in clinical isolates from Chinese patients with either gastric adenocarcinoma or gastritis.

## DISCUSSION

The correlation between *H pylori* as a class I carcinogen and gastric adenocarcinoma is not completely clarified and has attracted much attention so far. *CagA* gene as a marker for *cag* pathogenicity island associated with more virulent *H pylori* strains may be one of the most important contributors in the process of gastric carcinogenesis, because the antibody titer of *CagA* is much higher in gastric carcinoma than in non- gastric carcinoma and the detectable rate of *CagA* antibody in human beings is significantly higher in high occurrence region of gastric cancer than in low occurrence area. *CagA*<sup>+</sup> strains can significantly increase the risk for developing severe gastritis and gastric carcinoma compared with *CagA*<sup>-</sup> *H pylori* strains<sup>[21-23]</sup>. A research suggests that the development of more prominent gastritis and severe atrophy in *CagA*<sup>+</sup> patients is an indicator for the importance of *CagA* rather than *H pylori* load<sup>[24]</sup>, indicating that *CagA* protein plays an important role in pathogenesis of *H pylori*. In general, *CagA* from different *H pylori* has a significant difference because of the number of repeated sequences of EPIYA in C-terminal half of *CagA* although N-terminal of *CagA* protein is quite conserved. Hence, the highlight in study of *CagA* is whether this diversity of *CagA* is correlated with disease outcomes. Azuma *et al.*<sup>[15]</sup> found that there were more EPIYAs in *H pylori* from atrophic gastritis and gastric cancer than in *H pylori* from gastritis. The frequencies of genotypes of *CagA* with more than 4 EPIYAs were significantly higher in atrophic gastritis than in duodenal ulcer. One-third of strains with more than 4 EPIYAs were in gastric cancer. They concluded that *H pylori* infection with the *CagA* genotype with more than 4 EPIYAs might correlate with the pathogenesis of atrophic gastritis and gastric cancer<sup>[15]</sup>. In contrast, *CagA* protein varied in the number (from 1 to 3) of repeated sequences of EPIYA in different strains isolated from Western countries<sup>[11,16]</sup>. We demonstrated in this work that each Chinese *CagA* variable region contained 2-4 tandem EPIYA motifs and only one EPIYA had repeated sequences. There was no significant difference between the number of EPIYA motifs in *CagA* variable regions in *H pylori* isolated from patients with either gastritis or gastric carcinoma. There were three types of *CagA* variable region among the 38 isolates, namely *CagA* with 2 EPIYAs, *CagA* with 3 EPIYAs and *CagA* with 4 EPIYAs. But no *CagA* with more than 4 EPIYAs or deletion of *cagE* was observed in these Chinese *H pylori* isolates. It appears that there is a difference in the 3'-variable region of *CagA* of *H pylori* isolates from Chinese and Japanese<sup>[15]</sup>.

The ability of *CagA* protein to phosphorylate in tyrosine

(s) is the premise of *CagA* protein perturb cellular signal pathway and induction of cellular response. Higashi and coworkers<sup>[12]</sup> have found that a tyrosine phosphatase -2 containing c-src homology 2 is an intracellular target of tyrosine-phosphorylated *CagA* protein. The phosphorylase activity of SHP-2 by binding to tyrosine-phosphorylated *CagA* protein has a close relationship with the "hummingbird" phenotype of AGS cells. Besides SHP-2 as an intracellular target of tyrosine-phosphorylated *CagA* protein, *CagA* protein could interact with Grb2 and phosphorylate *CagA* protein which is indispensable for the Grb2 binding<sup>[25]</sup>, resulting in the activation of the Ras/MEK/ERK pathway and cell proliferation. Hence, the ability of phosphorylated *CagA* protein could reflect the level of biological function of *CagA* and might play a key role in the pathogenicity of *H pylori*. Except that one tyrosine site in repeated sequences is phosphorylated after *CagA* DNA is transfected into gastric epithelial cells, no other tyrosine sites in EPIYA outside the repeated sequences could be phosphorylated *in vivo* although all the tyrosine sites in EPIYA could be phosphorylated *in vitro*, indicating that sequence diversity of tyrosine phosphorylation motifs on *CagA* is not related to the presence of tyrosine phosphorylation. The reason is that the membrane tethering of *CagA* protein is necessary for the tyrosine-phosphorylated EPIYA in host cells. The other obvious difference that might influence the phosphorylation ability of *CagA* is the amino acid sequence in D2 region in repeated sequences<sup>[16]</sup>. Each Chinese isolate is DFD which could more perfectly match the SHP-2 consensus motifs than DDL in NCTC11637 and other Western isolated strains. It implies that Chinese *CagA* has a much stronger ability to perturb cell signal pathway. Above all, the fact raises an intriguing possibility that Chinese *CagA* has no functional difference in perturbing cellular signal pathway among different isolates of type I although there is a diversity in C-terminal half of *CagA*.

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• BRIEF REPORTS •

# Gene expression profile in rat small intestinal allografts after cold preservation/reperfusion

Shu-Feng Wang, Qi Liang, Guo-Wei Li, Kun Gao

Shu-Feng Wang, Department of General Surgery, First Hospital, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China  
Qi Liang, Department of Anesthesiology, Xi'an traditional Chinese Hospital, Xi'an 710001, Shaanxi Province, China  
Guo-Wei Li, Department of General Surgery, Second Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China  
Kun Gao, School of Life Science, Xi'an Jiaotong University, Xi'an 710049, Shaanxi Province, China  
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Correspondence to: Dr. Shu-Feng Wang, Department of General Surgery, First Hospital, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. dawnwsf@sina.com  
Telephone: +86-29-85324147 Fax: +86-29-82655730  
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## Abstract

**AIM:** To determine the changes of gene expression profile in small intestinal allografts in rats after cold preservation/reperfusion, and to identify the genes relevant to cold preservation/reperfusion injury.

**METHODS:** Heterotopic segmental small bowel transplantation was performed in six rats with a sham operation and they were used as controls. Total RNA was extracted from the allografts (experimental group) and normal intestines (control group) 1 h after cold preservation/reperfusion, and then purified to mRNA, which was then reversely transcribed to cDNA, and labeled with fluorescent Cy5-dUTP and Cy3-dUTP to prepare hybridization probes. The mixed probes were hybridized to the cDNA microarray. After high-stringent washing, the fluorescent signals on cDNA microarray chip were scanned and analyzed.

**RESULTS:** Among the 4 096 target genes, 82 differentially expressed genes were identified between the two groups. There were 18 novel genes, 33 expression sequence tags, and 31 previously reported genes. The selected genes may be divided into four classes: genes modulating cellular adhesion, genes regulating cellular energy, glucose and protein metabolism, early response genes and other genes.

**CONCLUSION:** A total of 82 genes that may be relevant to cold preservation/reperfusion injury in small intestinal allografts are identified. Abnormal adhesion between polymorphonuclears and endothelia and failure in energy, glucose and protein metabolism of the grafts may contribute to preservation/reperfusion injury. The functions of the novel genes identified in our study need to be clarified further.

**Key words:** Small intestinal allografts; Cold preservation; Reperfusion Injury; Gene Expression Profiling

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## INTRODUCTION

Primary dysfunction of small intestinal grafts induced by rejection and cold preservation/reperfusion injury, is still one of the main obstacles in clinical small intestinal transplantation<sup>[1-6]</sup>. The cold preservation/reperfusion injury of small intestinal grafts, an unspecific injury, may enhance the immunogenicity of the grafts, and exacerbate the degree of acute or chronic rejection, thus influence the survival and function of the grafts<sup>[7]</sup>. Therefore, better understanding of molecular mechanism in the course of cold preservation/reperfusion is beneficial for searching for new methods that could decrease the graft injury. However, previous studies only investigated the relationship between one or a few genes or proteins and cold preservation/reperfusion injury by Northern hybridization or RT-PCR. Thus, the overall changes in intra-graft gene expression profile are not clearly identified. The aim of this study was to determine the gene expression profile of rat small bowel allograft after cold preservation/reperfusion injury, and to identify the genes relevant to cold preservation/reperfusion injury by means of cDNA microarray.

## MATERIALS AND METHODS

### Animal preparation

Healthy male Sprague-Dawley (SD) rats, weighing  $280 \pm 20$  g, provided by Medical Experiment Animal Center, Xi'an Jiaotong University, were housed in standard animal facilities, fed with commercially available rat chow and tap water *ad libitum* for 1 wk before test to acclimatize the laboratory, and maintained on a 12-h light/dark cycle.

### Experimental design

SD rats were used as donors and recipients. They were randomly divided into two groups: sham operation group (S group,  $n = 6$ ), in which animals were only subjected to anesthesia and laparotomy and left nephrectomy, and heterotopic segmental small bowel transplantation group (SBT group,  $n = 12$ ), in which the donor and recipient were paired according to the similar body weight.

### Heterotopic small bowel transplantation

Heterotopic segmental small bowel transplantation was

performed with a technique modified from that described by Monchik and others<sup>[8]</sup>. All animals were fasted for 12 h, but had free access to water before operation. They were anesthetized with pentobarbital sodium solution (2%, 35 mg/kg) intraperitoneally. After perfused *in situ* with cold lactated Ringer's solution, the small intestine 5 cm distal to Treitz ligament was harvested from each donor rat with a vascular pedicle including portal vein (PV) and aorta segment with superior mesenteric artery, and stored at 4 °C in lactated Ringer's solution for 1 h. After left nephrectomy, the left renal vein and abdominal aorta of the recipient rat were isolated. The graft was revascularized by end-to-side anastomosis between donor aorta and recipient aorta using the continuous 9-0 non-traumatic nylon sutures and end-to-side anastomosis between donor PV and left renal vein of the recipient using a cuff technique. The recipient's own intestine was left intact. During transplantation, mean warm ischemia time of graft was less than 1 min, and cold preservation time of graft did not exceed 60 min.

### Gene chip preparation

Four thousand and ninety-six target cDNA clones were used in the cDNA chip (provided by Biostar Gene Ltd, Shanghai, China). These genes were amplified through polymerase chain reaction (PCR) using universal primers and purified with a standard method. The quality of PCR was monitored by agarose electrophoresis. Target genes were dissolved in 3×standard saline citrate (SSC) spotting solution, and spotted on silylated slides (Telechem, Inc., USA) by Cartesian 7 500 spotting Robotics (Cartesian, Inc., USA). After spotting, the slides were hydrated for 2 h, dried for 0.5 h at room temperature, cross-linked under UV light and then treated with 0.2% sodium dodecyl sulfate (SDS), H<sub>2</sub>O and 0.2% NaBH<sub>4</sub> for 10 min, respectively. The slides were then dried in the cold and ready for use.

### Probe preparation

In SBT group, the small intestinal grafts were cut 3 cm long and kept in liquid nitrogen immediately after successful revascularization and 1 h after reperfusion of the small intestinal grafts. Samples taken from the same anatomic sites in the S group were kept in liquid nitrogen. Total RNA in different groups was extracted by a single step method. UV light analysis and electrophoresis detection showed the good quality of purified RNA. mRNA was isolated and purified using an oligotex mRNA Midi kit (Qiagen, Inc., California, USA). The fluorescein-labeled cDNA probes were prepared through retro-transcription and purified according to the method of Schena *et al*<sup>[9]</sup>. Probes from normal small intestine in S group were labeled with Cy3-dUTP, and those from small intestinal graft in SBT group with Cy5-dUTP. The probes were mixed, precipitated by ethanol and resolved in 20 µL hybridization solution (5×SSC+0.2% SDS).

### Hybridization

Hybridization probes and gene chip were denatured in a 95 °C bath for 5 min. The probes were added on the chip and covered with a glass. The chip was hybridized in a sealed chamber at 60 °C for 15-17 h. After removing the cover

glass, the chip was washed in 2×SSC+0.2%SDS, 0.1×SSC+0.2%SDS and 0.1×SSC, 10 min each, respectively, and then dried at room temperature.

### Fluorescence scanning and analysis

The chip was scanned with a Scan Array 4 000 scanner (General scanning Inc., USA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using ImaGene 3.0 software (BioDiscovery, USA). The fluorescent intensity of each spot at the two wavelengths represented the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Cy3 and Cy5 overall intensity was normalized and corrected by a coefficient according to location ratios of the housekeeping genes. The criteria for screening out each differentially expressed gene were defined: (1) The absolute value of the natural logarithm of the signal ratio of Cy5/Cy3 was greater than 0.69; or the ratio of Cy5/Cy3>2.0 or <0.5, or (2) one of the signal values of Cy3 and Cy5 was greater than 800.

## RESULTS

### Verification of gene chip by the hybridization system

The microarray consisted of 4 096 sequences of rats including full length and partial complementary DNA (cDNA). Its standards of quality control included 20 housekeeping genes as positive controls, 16 plant genes as negative controls and, 19 spotting solutions as blank controls. The results of this study completely accorded with the above standards of quality control, which indicated that there was no contamination during the process of study.

### Scatter-plot of hybridization signals

The scatter plot, derived from Cy3 and Cy5 fluorescent signal values, revealed a dispersed distribution pattern (Figure 1). Every spot represented a gene. Most spots were found on an almost 45° diagonal line. Certain yellow spots were distributed beyond the 45° diagonal line, which indicated the existence of some differentially expressed genes between normal small intestine and small intestinal graft. The genes with a remarkably different expression distributed on the upper and lower sides of the 45° diagonal line, representing the up-regulation and down-regulation, respectively. Red spots showed signal differences between 0.5 and 2 folds, which indicated the non-differentially expressed genes close to the 45° diagonal line.

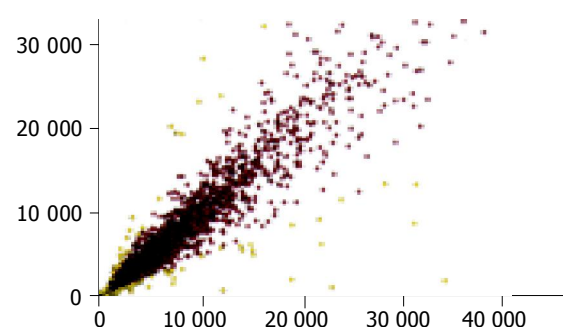
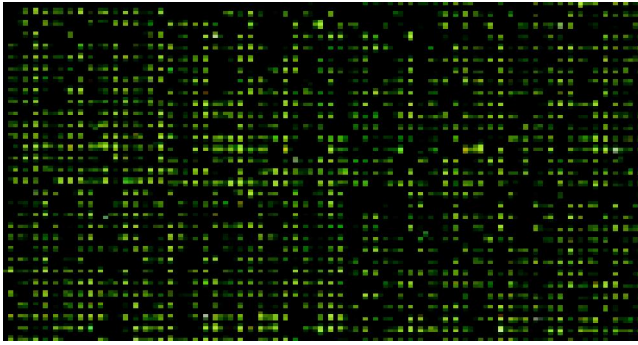


Figure 1 Scatter plots of gene expression pattern.



**Figure 2** Merged image of hybridizing signals on gene chip.

### Gene expression by scanning analysis

The fluorescent scanning profile of gene expression was merged by computer to produce the Figure 2. Yellow spots indicated no significant difference in gene expression between normal small intestine (Cy3) and that of small intestinal graft (Cy5). Green spots indicate that gene expression was higher in normal small intestine than in small intestinal grafts, whereas red spots indicate the opposite.

Overall, 82 differentially expressed genes between normal small intestines and small intestinal grafts were detected. Among them, 31 genes with GenBank identity numbers included 17 down-regulation genes and 14 up-regulation genes (Table 1). Expression sequence tags (EST) counted to 33. In addition, there were 18 novel genes, eight up-regulated genes and 10 down-regulated genes. The functions of these novel genes need to be investigated further.

## DISCUSSION

Small bowel transplantation has been advocated as the preferred therapy for patients with irreversible intestinal failures such as short bowel syndrome<sup>[10-14]</sup>. However, primary graft rejection and cold preservation/reperfusion damage involved in loss of small intestine function after transplantation are still the major obstacles to small bowel transplantation. At present, in addition to theory of free radical species and theory of calcium overload, the mechanism of cold preservation/reperfusion injury has not yet completely elucidated. In order to modulate this non-specific injury, it is necessary to investigate the molecular and genetic mechanism of cold preservation/reperfusion injury. In this study, we identified some genes that may be relevant to cold preservation/reperfusion damage by use of cDNA microarray. The selected genes could be divided into four classes: genes modulating cellular adhesion, genes regulating cellular energy, glucose and protein metabolism, early response genes and other genes.

In physiological conditions, the repelling action between the vascular endothelia and polymorphonuclears flowing in the blood vessels guarantees the microcirculatory perfusion. Our experiments showed that the expression of cell adhesion regulator genes (U76714) and receptor-typed protein-tyrosine-phosphatase (NM-013080) with function of inhibiting cell adhesion decreased 1 h after cold preservation/reperfusion of grafts, whereas the expression of DORA protein (AJ223184), an adhesion molecule of immunoglobulin superfamily, and

**Table 1** Differentially expressed genes with GenBank identity number (*n* = 31) as screened by the gene chip

GenBank ID	Definition	Ratio Cy5/Cy3
M 15327	Rat alcohol dehydrogenase mRNA	0.050
U 76714	Cell adhesion regulator mRNA	0.233
NM-016998	Carboxypeptidase mRNA	0.243
NM-024391	17-beta hydroxysteroid dehydrogenase mRNA	0.291
NM-019158	Aquaporin 8 mRNA	0.304
NM-017272	Aldehyde dehydrogenase family mRNA	0.337
NM-013098	Glucose-6-phosphatase 1 mRNA	0.361
NM-031639	Discs, large homolog 3 mRNA	0.396
NM-022407	Aldehyde dehydrogenase subfamily A1 mRNA	0.406
X05080	Beta-globin	0.422
M83143	Beta-galactoside-alpha 2,6-sialyltransferase mRNA	0.429
AF 154572	ERG2 protein mRNA	0.436
NM-031351	Attractin mRNA	0.450
NM-013080	Protein tyrosine phosphatase, receptor-type mRNA	0.450
NM-031565	Carboxylesterase 1 mRNA	0.461
NM-031579	Protein tyrosine phosphatase 4a1 mRNA	0.465
NM-053297	Pyruvate kinase 3 mRNA	0.479
NM-054008	Rgc 32 protein mRNA	2.053
NM-020538	Arylacetamide deacetylase mRNA	2.056
M 20406	P450 II B mRNA	2.083
NM-053523	Herpud 1 mRNA	2.085
AJ 223184	DORA Protein	2.210
NM-022392	Growth response protein mRNA (CL-6)	2.489
NM-012771	Lysozyme mRNA	2.491
X 68312	Ig rearranged mu-chain C region	2.495
NM-017268	Hmgcs1 mRNA	2.497
NM-053372	Secretory leukocyte protease inhibitor mRNA	2.796
AJ 238392	Sulfotransferase K2 mRNA	2.876
AF 146518	Amino peptidase A short variant mRNA	2.883
NM 017211	Selectin, endothelial cell ligand mRNA	2.949
NM-022251	Aminopeptidase A mRNA	3.302

selectin family (NM-017211) increased. Thus, these changes may induce the adhesion of polymorphonuclears to the endothelia, and initiate the adhesion cascade reaction. Adhesion of polymorphonuclears to the endothelia and the recruitment of the cells into the grafts are essential for the development of cold preservation/reperfusion injury. In the process, selectins play an important role<sup>[15,16]</sup>. The results of cDNA microarray showed that 1 h after cold preservation/reperfusion selectins revealed up-regulation, which directed polymorphonuclears accumulation in the grafts and activated polymorphonuclears to release inflammatory mediators and proteases. Injury to the graft may occur through the following ways. Firstly, adhesion of polymorphonuclears to the endothelia causes the damage and physical obstruction of capillaries, such as the no reflow phenomenon, which injures the graft further. Secondly, a great amount of free radical species is produced through the respiratory burst of polymorphonuclears, which destroy the structure of membrane. Thirdly, polymorphonuclears secrete proteolytic enzymes such as elastase that could decompose the graft. Therefore, anti-

adhesion therapy is likely to be a new approach to relieve/inhibit cold preservation/reperfusion injury of small intestinal transplant.

Our study showed that the expression of cytochrome P450 (M20406) of respiratory chain of biological oxidation increased in the course of cold preservation/reperfusion of small intestinal grafts, whereas that of attractin (NM-031351), which controls homeostasis of cell energy metabolisms, decreased, suggesting that there is a failure in energy and substance metabolism of cells<sup>[17,18]</sup>. The injury of capillaries mediated by adhesion cascade reaction made the grafts in the state of relative anoxia during reperfusion since the biological oxidation of glucose mainly depended on glycolysis. In our study, pyruvate-kinase (NM-053297), the key enzyme in the course of glycolysis, expressed downward, which hindered glycolysis. In the meantime, the expression of glucose-6-phosphatase (NM-013098), the key enzyme in the course of glyconeogenesis, was also down regulated, which inhibited glyconeogenesis. Although the expression of aminopeptidase A (AF146518, NM-022251), which hydrolyzes the peptide chains, increased, the expression of carboxypolypeptidase A (NM-016998), which hydrolyzes the peptide bonds and releases amino acids, expressed downward, resulting in the failure of peptide or protein utilization by the cells. In addition, due to the action of the oxygen-free radical, peroxide lipid produced in the course of reperfusion of small intestinal grafts usually degrades, and forms some aldehyde compounds like malonic aldehyde, which easily causes the occurrence of additional response with cell proteins, leading to the cross-linking of intra-molecules or inter-molecules of proteins and subsequently resulting in coagulation. In our study, aldehyde dehydrogenases (NM-022407 and NM-017272), which oxidize and decompose the aldehyde, expressed downward, which may aggravate the harm to the cells<sup>[19,20]</sup>. Therefore, there is not only the disturbance of biological oxidation of glucose or proteins but also the failure of catabolism of toxic products, such as the aldehyde during the reperfusion of small intestinal grafts.

Early response genes of cells, a kind of important mediated genes, determine the final response mode of cells stimulated in the external environment, namely expression of the function-relevant gene downstream<sup>[21]</sup>. Our study showed that early response genes of cells, growth response protein (CL-6 or NM-022392) and Rgc32 protein (NM-054008) expressed upward whereas protein tyrosine phosphatase (PTPase or NM-031579) expressed downward during cold preservation/reperfusion of small intestinal grafts. Rgc32 protein increases the activation of CDC2 kinase that cell cycle depends on, and impels the synthesis of cellular DNA to enter the S phase, indicating a function of activating cell cycle. CL-6 also plays an important role in cell proliferation. Therefore, the cold preservation/reperfusion of intestinal grafts triggers cell proliferation of small intestine and promotes the recovery of injury. This conclusion coincides with those obtained from previous studies at the single gene level<sup>[22]</sup>.

Our study also indicates that endogenous protection in small intestinal grafts is limitedly initiated through adjustment of the expression mode of genes, which may relieve the reperfusion injury. This finding has seldom been observed in previous studies. Aquaporin 8 (NM-019158) expressed

downward, which could lessen cell edema caused by relative lack of oxygen<sup>[23]</sup>. Secretory protease inhibitor of leukocyte (NM-022407) expressed upward, which could suppress protease released by adhesion cascade reaction activated by endothelia and polymorphonuclears, and lessen the hydrolysis of proteins<sup>[24]</sup>.

In conclusion, our results using cDNA microarray are consistent with previous studies at the single gene level. Moreover, there are changes in the gene expression mode after the cold preservation/reperfusion of small intestinal grafts on more concrete links, which enable us to further understand the molecular mechanisms of the cold preservation/reperfusion injury. Furthermore, the functions of many new genes identified in our study in the cold preservation/reperfusion injury of small intestinal grafts need to be clarified further.

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• BRIEF REPORTS •

# Hybrid artificial liver support system for treatment of severe liver failure

Jian-He Gan, Xia-Qiu Zhou, Ai-Lan Qin, Er-Ping Luo, Wei-Feng Zhao, Hong Yu, Jie Xu

Jian-He Gan, Ai-Lan Qin, Er-Ping Luo, Wei-Feng Zhao, Jie Xu, Department of Infectious Diseases, First Hospital affiliated to Suzhou University, Suzhou 215006, Jiangsu Province China  
Xia-Qiu Zhou, Hong Yu, Department of Infectious Diseases, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China  
Supported by Health Office of Jiangsu Province Science Research Foundation, No. WK2002 (for important subject) and H200145 (for natural science)

Correspondence to: Dr. Jian-He Gan, Department of Infectious Diseases, First Hospital affiliated to Suzhou University, Suzhou 215006, Jiangsu Province, China. ellen2111@sina.com.cn  
Telephone: +86-512-65223637-8385

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**CONCLUSION:** The HALSS established by us is effective in supporting liver function of patients with severe liver failure.

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**Key words:** Liver failure; Hybrid artificial liver support system; Hepatocytes

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## Abstract

**AIM:** To construct a novel hybrid artificial liver support system (HALSS) and to evaluate its efficacy in patients with severe liver failure.

**METHODS:** Hepatocytes were isolated from suckling pig by the modified Seglen's method. Isolated hepatocytes were cultured in a spinner flask for 24 h to form spheroids before use and the functions of spheroids were detected. HALSS consisted of a plasma separator, a hemo-adsorba and a bioreactor with hepatocytes spheroids in its extra-fiber space. HALSS was applied to 10 patients with severe liver failure. The general condition and the biochemical indexes of the patients were studied just before and after the treatment.

**RESULTS:** The number of cells per liver was about  $2.4 \times 10^{10}$  (mean,  $3.1 \pm 1.5 \times 10^{10}$ ). The cell viabilities were more than 95%. After 24 h of spheroid culture, most hepatocytes formed spheroids. The levels of albumin and urea in the medium of spheroid culture were higher than those in supernatant of petri dish culture ( $P = 0.0015$  and  $0.0001$ , respectively). The capacity of albumin production and urea synthesis remained stable for more than one wk and declined rapidly after two weeks *in vitro*. In HALSS group, the duration of HALSS treatment was 6-10 h each time. All patients tolerated the treatment well without any fatal adverse reaction. After HALSS treatment, the general condition, psychic state, encephalopathy and hepatic function of the patients were improved. The survival rate of the HALSS group, Plasmapheresis group and control group was 30% (3/10), 20% (2/10) and 0% (0/10), respectively ( $P = 0.024$ ). Two weeks after treatment, Tbil and ALT decreased and the PTA level elevated in HALSS group and plasmapheresis group ( $P$  value:  $0.015$  vs  $0.020$ ,  $0.009$  vs  $0.012$  and  $0.032$  vs  $0.041$ , respectively). But there was no significant change of blood albumin concentration before and after treatment in HALSS group and Plasmapheresis group.

## INTRODUCTION

Liver is an important organ with complex functions, including gluconeogenesis, synthesis of blood proteins, amino acid metabolism, urea synthesis, lipid metabolism, drug detoxification, waste removal, and immune and hormonal modulation<sup>[1]</sup>. Many people suffer from liver diseases, especially severe hepatitis, in China, and most of the cases give rise to widespread hepatic necrosis with little hepatocyte regeneration. Currently, the only available treatment is liver transplantation. However, liver transplantation faces acute shortages of donors worldwide and the patients treated with liver transplantation are subjected to the lifetime risks of graft rejection and immunosuppression<sup>[2,3]</sup>. The liver has a remarkable capacity for regeneration. But a minimum critical mass of hepatocyte is required to support homeostasis while regeneration progresses after liver damage. Without this critical mass, liver failure supervenes and regeneration is impaired<sup>[4]</sup>. Artificial liver support systems, such as plasma exchange, hemodialysis and hemofiltration, have been proposed as a temporary liver support that allows the native liver to regenerate<sup>[5-7]</sup>. But these treatment do not perform many complex functions for survival. Hybrid artificial liver support system (HALSS) is an extracorporeal circulation system comprising of a bioreactor with highly differentiated hepatocytes in hollow fiber space<sup>[8]</sup>. The large number of highly differentiated hepatocytes is the key to construct HALSS. It has become increasingly evident that three-dimensional rather than monolayer growth is particularly important for maintaining differentiated hepatocyte function in culture. One means of establishing three-dimensional hepatocyte growth is the creation of multicellular spheroid aggregates<sup>[8-12]</sup>. Here we developed a HALSS that contained a hollow fiber bioreactor inoculated with porcine hepatocyte spheroids and applied it to 10 patients with severe liver

failure. The efficacy of the HALSS for treatment of severe liver failure was evaluated.

## MATERIALS AND METHODS

### Animals

Chinese suckling pigs weighing about 4.0 kg were supplied by Laboratory Animal Center of Chinese Academy of Sciences.

### Isolation and spheroid culture of hepatocytes

Hepatocytes were isolated from suckling pigs by modified two-step *in situ* collagenase perfusion method described by Seglen<sup>[13]</sup>. The liver was perfused at 4 °C with Hanks solution containing 40 mg/L dexamethasone (Sigma-Aldrich, the USA) through the portal vein to rinse whole blood. At the end of the first perfusion, the liver was excised gently from the surrounding tissue and the isolated liver was placed on the stage of perfusion apparatus. Then the liver was circularly perfused with Hanks solution containing 0.5 g/L collagenase IV (Sigma-Aldrich, America) at 37 °C, which was equilibrated with 950 mL/L O<sub>2</sub> and 50 mL/L CO<sub>2</sub>. The softened liver was gently lifted and placed in ice-cold minimum essential medium (MEM) (GIBCO™, Invitrogen). After dissociation of the liver capsule by scissor, the liver was shaken gently in the medium until it was reduced to only connective tissue. The resulting cell suspension was filtered through 50 µm sterile stainless steel meshes. The hepatocytes were washed three times at 500 r/min for 1 min each.

The hepatocyte suspension was adjusted to  $5 \times 10^6$ /mL in warmed dulbecco's modified eagle medium (D-MEM) culture medium supplemented with 0.1 µmol/L dexamethasone, 10 µg/L hepatocyte growth factor (HGF), 20 µg/L epidermal growth factor (EGF),  $1 \times$  insulin-transferrin-selenium solution (GIBCO™, Invitrogen), 10 mg/L linoleic acid, 200 mmol/L glutamine, 50 mL/L fetal bovine serum, 0.1 µg/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 50 pmol/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 15 mmol/L HEPES, 125 mg/L penicillin and 100 mg/L streptomycin. The cells suspension was then placed in a 50 mL/L CO<sub>2</sub> and 950 mL/L air humidified incubator at 37 °C and immediately stirred with a magnetic stirrer at 120 r/min. Air from the incubator was blown into the flask from the open side of the spinner flask by an air pump. The medium was changed every 24 h. Albumin secretion and urea synthesis were measured in the medium of the spheroid culture according to the method described by Chen *et al.*<sup>[14]</sup> with the petri dish culture at the same density as control.

### Configuration of bioartificial liver

The HALSS was constructed as shown in Figure 1. The formed spheroid hepatocytes containing  $1-2 \times 10^{10}$  highly differentiated primary porcine hepatocytes in DMEM medium were inoculated into the outer space of the hollow fibers of the bioreactor (Languna Hills). A hepatocytes reservoir was designed to connect to the extra-fiber compartment of the bioreactor just as described by Ding *et al.*<sup>[15,16]</sup>. Blood was removed from the patient through a double lumen catheter in superficial femoral vein at a rate of 100 mL/min and run through a plasma separator. The

separated plasma passed a hemo-adsorba 160 for removing bilirubin and toxin, and then it was run through the intra-fiber compartment of bioreactor. The reacted plasma was then reconstituted with red blood cells and returned to the patient via the venous cannula.

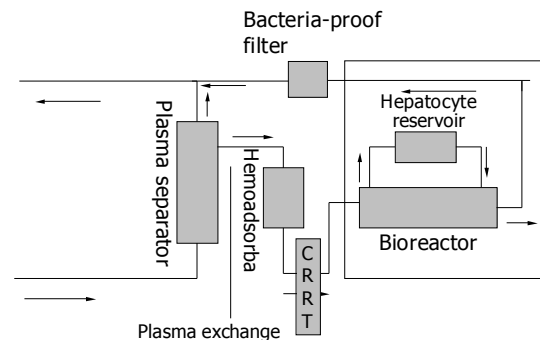


Figure 1 Schematic diagram of HALSS.

### Clinical treatment

All patients were at the end stage of severe liver failure according to the diagnostic standard defined in Xi'an meeting in China in 2000. Ten patients (including 7 males and 3 females, and 8 patients were infected with hepatitis B and 2 patients were infected with hepatitis E) with severe liver failure were assigned to HALSS group. Ten patients (2 females, 8 males) were assigned to Plasmapheresis group. And another ten patients were assigned to control group. There was no significant difference in symptoms and biochemical index between the two groups (Table 1).

Table 1 Clinical feature of patients

	HALSS group (n = 10)	Plasmapheresis group (n = 10)	Control group (n = 10)
Sex			
Male	3	2	3
Female	7	8	7
Average age (yr)	39.5	41.5	40.5
Range (yr)	35-56	27-60	30-58
Encephalopathy	4	2	1
Ascites	8	6	7
Hepatorenal syndrome	9	7	7
Biochemical index			
Tbil (µmol/L)	499.5±25.1	519.4±137.5	496.3±93.2
Dbil (µmol/L)	227.5±19.0	269.6±118.4	286.5±107.3
ALT (U/L)	347.6±126.5	303.2±139.4	336.2±139.6
ALB (g/L)	29.8±0.4	29.9±5.3	30.9±5.3
PTA	17.5±1.2	22.8±6.5	21.5±4.4

Tbil: Total bilirubin, Dbil: Direct bilirubin, ALT: Alanine aminotransferase, PTA: Prothrombin activity.

### Biochemical analysis

Blood samples were obtained at the start and end of the treatment. Blood alanine aminotransferase (ALT), total bilirubin (Tbil) and albumin (ALB) were determined with

an automatic biochemical analyzer. Prothrombin activity (PTA) was detected according to manufacturer's instructions.

### Statistical analysis

Results were expressed as mean $\pm$ SD. Paired *t* test was used (SPSS10.0 software) to compare the data. *P*<0.05 was considered statistically significant.

## RESULTS

### Cell morphology and viability

The number of cells per liver was about  $2.4 \times 10^{10}$  (mean,  $3.1 \pm 1.5 \times 10^{10}$ ). The cell viabilities were more than 95% (Figure 2). After 24 h of spheroid culture, most hepatocytes formed spheroids (Figure 2).

### Metabolites secretion in culture

The functions about albumin production and urea synthesis are shown in Figure 3. The levels of albumin and urea in the medium of spheroid culture were higher than those in supernatant of petri dish culture (*P* = 0.0015, 0.0001, respectively). The capacity of albumin production and urea synthesis was constant for more than one week and declined rapidly after two weeks.

### Clinical effect of HALSS

In HALSS group, the duration of HALSS treatment was 6-10 h each time. All patients tolerated the treatment well without any fatal adverse reaction. After HALSS treatment, general condition, psychic state, encephalopathy and hepatic function of the patients were improved (Table 2). The

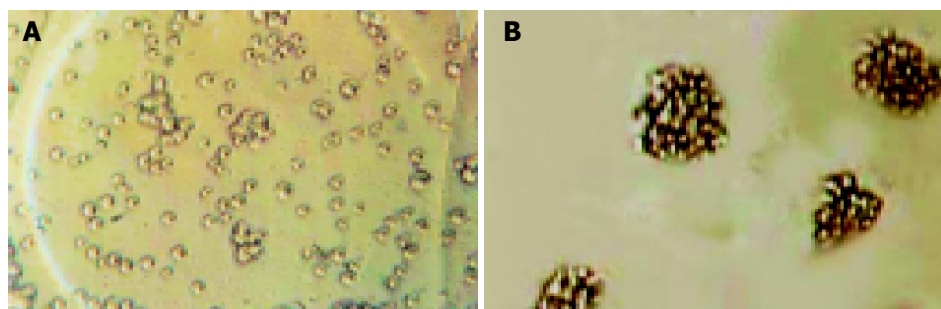
survival rate of the HALSS group, Plasmapheresis group and control group was 30% (3/10), 20% (2/10) and 0% (0/10), respectively (*P* = 0.024). Two weeks after treatment, TBil and ALT decreased and the level of PTA elevated in HALSS group and Plasmapheresis group (*P* value is 0.015 *vs* 0.020, 0.009 *vs* 0.012 and 0.032 *vs* 0.041, respectively). But there was no significant change of blood albumin concentration before and after the HALSS treatment (Figure 4).

**Table 2** General condition of patients at two weeks after treatment

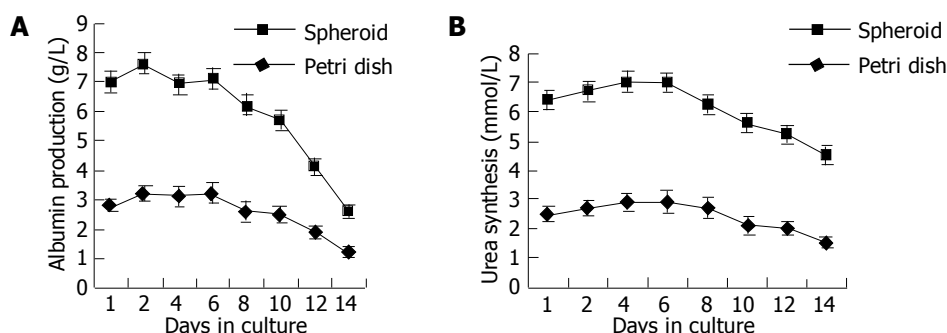
	HALSS group ( <i>n</i> = 10)		Plasmapheresis group ( <i>n</i> = 10)		Control group ( <i>n</i> = 10)	
	Pre-	Post-	Pre-	Post-	Pre-	Post-
Encephalopathy	4	2	2	2	1	2
Ascites	8	3	6	3	7	9
Hepatorenal syndrome	9	3	7	3	7	9

## DISCUSSION

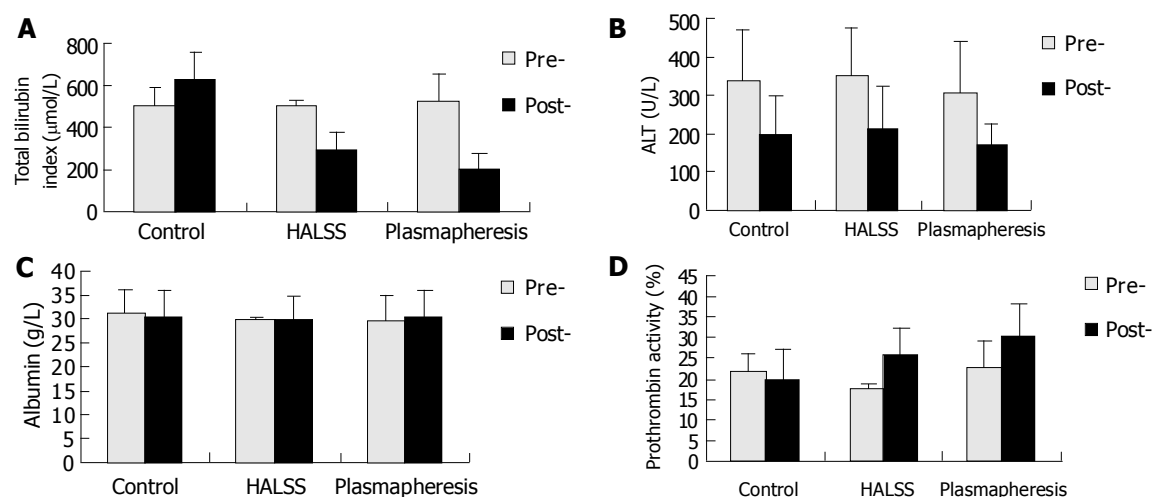
Acute or acute-on-chronic liver failure is a life-threatening condition. Most of the cases are caused by viral hepatitis, such as hepatitis B and C, in China. The mortality of liver failure is very high, more than 70%<sup>[17]</sup>. Liver transplantation is an effective therapy for many severe hepatic failure, but the number of patients who can benefit from this procedure is limited by the availability of donor organs<sup>[17,18]</sup>. Alternative therapy including liver cell



**Figure 2** Morphology of hepatocytes. A: freshly isolated single hepatocytes stained with trypan blue ( $\times 200$ ); B: spheroid formation after 24 h of spheroid culture ( $\times 100$ ).



**Figure 3** Spheroid cultivation of hepatocytes at a density of  $5 \times 10^5$ /mL cells. Control hepatocytes were cultivated in a petri dish at the same density. A: albumin production; B: urea synthesis.



**Figure 4** Changes of total bilirubin, ALT, albumin and PTA index in patients in HALSS group, Plasmapheresis group and control group. Pre-: index before treatment. Post-: index at two weeks after treatment.

transplantation and artificial livers has been developed. The non-bioartificial livers consisted of haemofiltration, hemoperfusion, plasma exchange and molecular adsorbents recycling system<sup>[6,19-21]</sup>. These devices could remove toxic factors, including ammonia, endotoxin, mercaptans and endogenous inhibitor neurotransmitters. But some factors, which are involved in hepatic regeneration, such as interleukin-6, tumor necrosis factor- $\alpha$ , hepatocyte growth factor and albumin-bilirubin complex might also be removed<sup>[22]</sup>. And they cannot replace the complex functions of liver such as synthetic and metabolic functions. On this condition, bioartificial liver has been developed to support liver functions temporarily<sup>[4,15,16,23,24]</sup>.

The large numbers of highly differentiated primary hepatocytes are demanded for configuration of bioartificial liver. Primary human hepatocytes divide much less readily *in vitro* even under optimal culture conditions<sup>[25]</sup>. Cell lines and stem cells are still under study, and they cannot be used for clinical treatment currently<sup>[11,26]</sup>. Primary porcine hepatocytes are the most common cellular components of current bioartificial liver devices<sup>[27]</sup>. Hepatocytes in monolayer culture lose their functions quickly *in vitro* and the density of hepatocytes in monolayer cannot satisfy the needs for configuration of bioreactor. Hepatocytes multicellular aggregates that have a tissue-like structure expressed highly differentiated functions<sup>[24,28]</sup>. Spheroidal aggregates of hepatocytes are known to exhibit better hepatocyte functions for a longer time than hepatocytes produced by monolayer culture<sup>[10,29]</sup>. Our data showed that functions of albumin production and urea synthesis of spheroidal hepatocytes were better than that of monolayer cultured hepatocytes ( $P < 0.05$ ). The functions of spheroidal hepatocytes were stable more than one week in the medium supplemented with growth factors and hormones, but they declined quickly after two weeks *in vitro*. The density of spheroid culture was enough to meet the needs of large numbers of hepatocytes for configuration of bioartificial liver.

Blood from the patients with severe liver failure has many toxins in it, which are harmful to living hepatocytes<sup>[30]</sup>. In HALSS, the blood from the patients was detoxified with a

hemo-adsorba 160 and continuous renal replacement therapy (CRRT) before it was run through a bioreactor. To ensure that enough hepatocytes were used, a hepatocyte reservoir was added to the system.

Data from clinical study showed that HALSS improved general condition, encephalopathy and biochemical indexes, including ALT, TBil and PTA of patients. But the albumin level was not changed, which was in agreement with the previous study by Ding *et al.*<sup>[6]</sup>. The survival rate of HALSS treatment group was also higher than that of the Plasmapheresis group and control group (30% *vs* 0%). It suggests that the HALSS significantly prolongs the survival time of the patients.

On the basis of the above mentioned results, the HALSS established by us is effective for the treatment of severe liver failure.

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• BRIEF REPORTS •

# An analysis: Colon cancer mortality in Tianjin, China, from 1981 to 2000

Yao-Gang Wang, Ke-Xin Chen, Guang-Lin Wu, Feng-Ju Song

Yao-Gang Wang, Tianjin University, Tianjin 300060, China  
Ke-Xin Chen, Feng-Ju Song, Department of Epidemiology,  
Tianjin Cancer Institute and Hospital, Tianjin 300060, China  
Guang-Lin Wu, Department of Epidemiology, Tianjin Medical  
University, Tianjin 300070, China

Correspondence to: Ke-Xin Chen, Department of Epidemiology,  
Tianjin Cancer Institute and Hospital, Huanhu Xi Road, Tiyan Bei,  
Hexi District, Tianjin 300060, China. wangygyt@yahoo.com  
Telephone: +86-22-23340123-5226

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## Abstract

**AIM:** To analyze the data from Tianjin Cancer Registry of mortality due to colon cancer from 1981 to 2000 in Tianjin, China.

**METHODS:** Tumors diagnosed in this study were coded according to ICD-9. Mortality rates were calculated by sex and calendar year of diagnosis.

**RESULTS:** Seventy point four percent of colon cancer deaths occurred in the age group of 55-79 years and the mortality rate reached its peak in the age group of 75-80 years. The average age at death was 64.10 years. An ascending trend was observed in the mean age of death due to colon cancer from 1981 through 2000. However, as for the sex ratio, there was no clear trend exhibited. During 1981-2000, the total number of deaths was 2147, 1041 males and 1106 females. The mean mortality rate of colon cancer was 3.04/100 000. The mortality caused by colon cancer ascended from 1981 to 2000.

**CONCLUSION:** The epidemic trend of colon cancer in Tianjin and its risk factors and prevention should be studied further.

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**Key words:** Colon cancer; Mortality trend; Cancer registry

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## INTRODUCTION

Colon cancer and rectum cancer are distinct to some extent in their epidemiology<sup>[1,2]</sup>. Colon cancer is one of the

most common cancers in the Western and industrialized nations<sup>[3-6]</sup>. By the year 1996, it had remained an important subject of research in China for more than 20 years. Mortality due to colon cancer has increased gradually, partly as a result of the changes of life style and nutrition. From the early 1970s to the late 1980s, the highest rate of increase in cancer was observed in colon cancer<sup>[7]</sup>. Therefore, it appears especially critical to gather and analyze the data concerning the incidence and mortality of colon cancer. This study analyzed the data about the mortality as a result of colon cancer in the urban areas of Tianjin during 1981-2000. The results provide insights into the etiology and prevention of colon cancer.

## MATERIALS AND METHODS

### Cancer deaths

The data concerning cancer mortality were provided by Tianjin Cancer Registry. As mandated by Tianjin Health Bureau, it is the duty of the medical institutions to fill cards to report their individual cancer cases to Tianjin Cancer Registry. The cards should include the name, birthday, gender, address, tumor site, date of diagnosis, age at diagnosis, diagnostic method of cases, and the date and age at death for cases to be registered. The Registry is responsible for the coding, checking, sorting, and analyzing of the reported cards. As for cases with information omissions, family visits were made to compensate for such lost data. Cases in this study were constant inhabitants in urban Tianjin, coded according to ICD-9.

### Population data

Tianjin Public Safety Bureau provided the population data.

### Statistical analysis

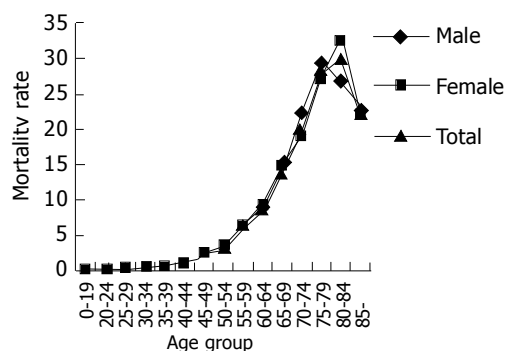
Software packages SAS 8.0 and SPSS 10.0 were employed to calculate the mortality rate, standardized rate, and mean age at death. A linear regression model was established for the trend of mortality.

## RESULTS

### Age distribution of mortality

A total of 2 147 cards were reported during 1981-2000 concerning dead cases of colon cancer, all of which included a definite age at death. The number of deaths increased with age among people under 55 years, but decreased for those over 75 years. The group aged between 55 and 79 years accounted for 70.4% of the total cases. Mortality rates varied greatly among different age groups, increasing

moderately and remaining at a relatively low level before reaching age 50, but increased dramatically after 50 years of age and reached its peak after 70-80 years of age (Table 1, Figure 1).



**Figure 1** Age-specific rate of colon cancer mortality in Tianjin, from 1981 to 2000.

**Table 1** Age distribution of colon cancer mortality in Tianjin during 1981-2000

Age at death	Male			Female			Total		
	Death prop (%) rate			Death prop (%) rate			Death prop (%) rate		
0-	2	0.2	0.02	2	0.2	0.02	4	0.2	0.02
20-	4	0.4	0.14	4	0.4	0.15	8	0.4	0.14
25-	15	1.4	0.43	8	0.7	0.24	23	1.1	0.34
30-	25	2.4	0.69	21	1.9	0.60	46	2.1	0.64
35-	29	2.8	0.83	23	2.1	0.67	52	2.4	0.75
40-	32	3.1	1.10	31	2.8	1.07	63	2.9	1.08
45-	48	4.6	2.10	63	5.7	2.71	111	5.2	2.41
50-	58	5.6	2.99	68	6.1	3.39	126	5.9	3.19
55-	115	11.0	6.53	119	10.8	6.37	234	10.9	6.44
60-	143	13.7	9.11	145	13.1	8.70	288	13.4	8.88
65-	166	15.9	13.27	190	17.2	14.85	356	16.6	14.05
70-	187	18.0	22.12	164	14.8	18.77	351	16.3	20.40
75-	139	13.4	29.31	145	13.1	27.44	284	13.2	28.29
80-	58	5.6	26.83	90	8.1	32.64	148	6.9	30.07
85-	20	1.9	22.47	33	3.0	21.86	53	2.5	22.07
Total	1041	100.0	2.92	1106	100.0	3.15	2147	100.0	3.04

Prop: proportion; rate: 1/100 000.

### Trend for mean age at death

The average age at death during 1981-2000 was 64.10 years (male: 63.57 years; female: 64.59 years), which was higher in females than in males, but the difference was statistically insignificant between sexes. The mean age at death (from 62.85 years in 1981 to 66.61 years in 2000) increased by 3.76 years during the two decades. This trend was almost the same for both the sexes. Linear regression further confirmed this trend (Tables 2, 3 and Figure 2A).

### Trends for sex ratio of colon cancer mortality

Sex ratio of colon cancer mortality fluctuated around 1.0 during 1981-2000, ranging from 0.65 in 1984 to 1.40 in 1991, but no clear trend was observed in these 20 years. Linear regression model was statistically insignificant:  $t = 1.622$ ,  $P = 0.122$  (Tables 4, 5 and Figure 2B).

### Mortality rate and its trend

Two thousand one hundred and forty-seven new colon cancer deaths were reported in Tianjin during 1981-2000 of which 1041 were male and 1106 were female. Mortality rates of colon cancer were calculated on the basis of population data and the corresponding number of new deaths reported. The average crude mortality rate over these 20 years was 3.04/100 000 (male: 2.92/100 000; female: 3.15/100 000), while the average age standardized rate was

**Table 2** Trend of mean age at death due to colon cancer in Tianjin, from 1981 to 2000

Year	Male			Female			Total		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
1981	58.70	13.94	2.20	66.31	8.48	1.22	62.85	11.85	1.26
1982	57.81	16.49	2.92	64.42	14.24	2.17	61.60	15.49	1.79
1983	59.12	11.69	1.83	64.15	14.14	1.83	62.11	13.37	1.33
1984	59.53	16.50	2.92	59.00	16.86	2.43	59.21	16.61	1.86
1985	62.64	12.63	1.84	61.75	16.66	2.63	62.23	14.54	1.56
1986	59.28	15.70	2.31	62.76	12.37	1.67	61.18	14.02	1.40
1987	57.63	16.24	2.40	62.49	13.36	2.14	59.86	15.10	1.64
1988	63.57	10.99	1.70	60.56	12.28	1.74	61.93	11.75	1.22
1989	61.65	14.97	2.04	62.04	15.03	2.22	61.83	14.92	1.49
1990	63.85	13.50	1.87	63.78	13.71	1.87	63.81	13.54	1.32
1991	63.39	13.04	1.73	65.75	12.06	1.91	64.36	12.63	1.28
1992	64.65	12.88	1.84	65.05	12.30	1.62	64.87	12.51	1.21
1993	67.83	10.77	1.49	64.16	13.25	1.62	65.76	12.32	1.13
1994	64.17	12.16	1.38	65.89	12.91	1.48	65.02	12.53	1.01
1995	65.13	12.85	1.56	65.07	11.20	1.50	65.10	12.09	1.09
1996	65.21	12.25	1.50	65.81	11.99	1.56	65.49	12.08	1.08
1997	64.56	14.19	1.80	69.82	12.56	1.47	67.41	13.54	1.17
1998	67.41	10.90	1.40	65.19	11.66	1.48	66.29	11.30	1.02
1999	68.06	13.44	1.67	66.29	13.51	1.63	67.15	13.45	1.16
2000	67.22	11.69	1.65	66.13	14.79	1.86	66.61	13.46	1.27
Total	63.57	13.52	0.42	64.59	13.31	0.40	64.10	13.42	0.29

Comparison of mean age at death between sexes:  $t = -1.75$ ,  $P = 0.0796$ .

**Table 3** Linear regression analyses for mean age at death of colon cancer

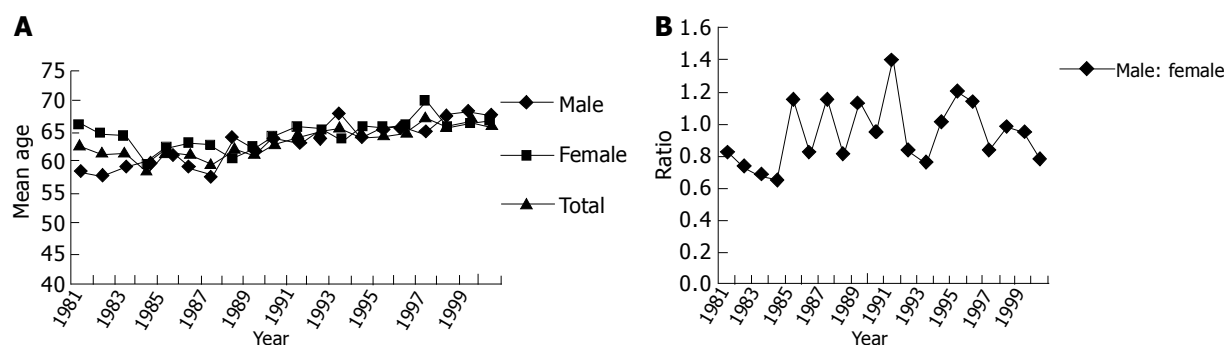
Year of diagnosis	B	SE	STD B	t	P	95%CI
Male	0.436	0.050	0.900	8.735	0.000	0.331, 0.541
Female	0.237	0.060	0.683	3.970	0.001	0.112, 0.363
Total	0.336	0.042	0.882	7.943	0.000	0.247, 0.425

**Table 4** Trend for sex ratio of colon cancer mortality in Tianjin from 1981 to 2000

Year	Ratio	Year	Ratio	Year	Ratio	Year	Ratio
1981	0.81	1986	0.82	1991	1.40	1996	1.13
1982	0.73	1987	1.15	1992	0.83	1997	0.84
1983	0.67	1988	0.82	1993	0.77	1998	0.98
1984	0.65	1989	1.15	1994	1.01	1999	0.94
1985	1.15	1990	0.94	1995	1.20	2000	0.79

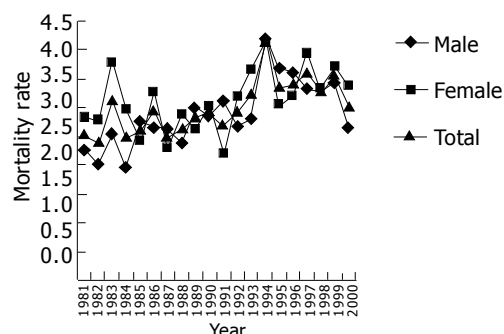
**Table 5** Linear regression analyses for sex ratio of colon cancer mortality

Variable	B	SE	STD B	t	P	95%CI
Year of diagnosis	8.857E-03	0.008	0.260	1.140	0.269	-0.007, 0.025



**Figure 2** Trend for mean age (A) and sex ratio (B) of colon cancer mortality in Tianjin from 1981 to 2000.

2.49/100 000 (male: 2.49/100 000; female: 2.48/100 000). The difference is statistically insignificant between sexes. Linear regression takes the year of death as independent variable and mortality rate as dependent variable that suggests an ascending trend apparent in colon cancer mortality during 1981-2000 (from 2.55/100 000 in 1981 to 3.02/100 000 in 2000) representing about a 18.43% increase over these 20 years, annually 0.89% (Tables 6, 7 and Figure 3).



**Figure 3** Mortality trend of colon cancer in Tianjin during 1981-2000.

**Table 6** Mortality rate of colon cancer in Tianjin during 1981-2000

Year	Male			Female			Total		
	Deaths	Rate	ASR	Deaths	Rate	ASR	Deaths	Rate	ASR
1981	40	2.28	2.55	48	2.83	3.10	88	2.55	2.78
1982	32	2.03	2.07	43	2.78	2.81	75	2.40	2.49
1983	41	2.55	2.56	60	3.80	3.71	101	3.17	3.21
1984	32	1.95	1.93	48	2.98	2.88	80	2.46	2.43
1985	47	2.78	2.75	40	2.42	2.22	87	2.61	2.49
1986	46	2.68	2.43	55	3.28	3.04	101	2.97	2.77
1987	46	2.64	2.32	39	2.29	2.01	85	2.47	2.18
1988	42	2.37	2.08	50	2.89	2.64	92	2.63	2.36
1989	54	3.01	2.92	46	2.62	2.26	100	2.82	2.51
1990	52	2.86	2.48	54	3.03	2.49	106	2.94	2.49
1991	57	3.12	2.59	40	2.23	1.77	97	2.68	2.19
1992	49	2.67	2.30	58	3.21	2.44	107	2.94	2.35
1993	52	2.82	2.24	67	3.68	2.74	119	3.24	2.52
1994	78	4.21	3.24	76	4.15	2.98	154	4.18	3.11
1995	68	3.67	2.84	56	3.05	2.14	124	3.36	2.46
1996	67	3.62	2.64	59	3.21	2.42	126	3.41	2.53
1997	62	3.35	2.47	73	3.97	2.58	135	3.66	2.55
1998	61	3.27	2.35	62	3.35	2.19	123	3.31	2.24
1999	65	3.48	2.42	69	3.72	2.36	134	3.60	2.38
2000	50	2.66	1.77	63	3.38	2.09	113	3.02	1.92
Total	1 041	2.92	2.49	1 106	3.15	2.48	2 147	3.04	2.49

Comparison of mean mortality between sexes:  $\chi^2 = 0.075$  (df = 2145),  $P > 0.05$ .

**Table 7** Parameter estimation for linear regression of colon cancer mortality

Year of diagnosis	B	SE	STD B	t	P	95% CI
Male	6.880E-02	0.016	0.709	4.267	0.000	0.035, 0.103
Female	4.294E-02	0.019	0.469	2.252	0.037	0.003, 0.083
Total	5.598E-02	0.013	0.702	4.185	0.001	0.028, 0.084

## DISCUSSION

As shown in this study, 2147 new deaths of colon cancer were reported in Tianjin from 1981 to 2000. The average age-standardized mortality rate during the two decades was 2.49/100 000 (2.49/100 000 for males and 2.48/100 000 for females). Available reports suggest that the highest mortality rate of colon cancer for males was 17.1/100 000 observed in Hungary, and 14.2/100 000 for females in New Zealand during 1988-1992<sup>[8]</sup>. Both were approximately six times the rates in Tianjin.

Although colon cancer mortality in Tianjin is insignificant in comparison with that of industrialized nations, it had experienced an 18.43% increase over these 20 years, a growth from 2.55/100 000 in 1981 to 3.02/100 000 in 2000. Certain Western countries have already made progress in controlling colon cancer. For example, in Canada, colon cancer mortality decreased from 13.9/100 000 to 13.1/100 000 for males and from 11.9/100 000 to 9.4/100 000 for females during the 1980s. In the same period, the Swedish colon cancer mortality descended from 10.4/100 000 to 9.9/100 000 for males and from 9.1/100 000 to 8.1/100 000 for females<sup>[9]</sup>. Therefore, the trend of colon cancer mortality is apparently unfortunate in Tianjin. It is imperative to curb the pace of such an increase in colon cancer deaths for the sake of humanity.

Although the constant increase in colon cancer mortality may be directly due to the increase in colon cancer incidence, it may be that its primary cause is the change in lifestyle and environment. A large body of evidence indicated that several dietary and lifestyle factors were likely to have a major influence on the risk of colon cancer. Physical inactivity, excess body weight, and a central deposition of adiposity are consistent risk factors. Over-consumption of energy is likely to be one of the major contributors to the high incidence rates of colon cancer in Western countries. Red meat, processed meats, and perhaps refined carbohydrates could contribute to the risk<sup>[8]</sup>. Immigrant epidemiology can well establish the relation between environment and colon

cancer incidence. For example, Blacks in America have 10-20 times more incidence than Blacks in Africa. Male and female American Chinese have an incidence of 10 times and 3-4 times, respectively than those in Shanghai<sup>[10]</sup>.

This study also showed that the mortality of colon cancer was only slightly higher in females than in males, and this difference was statistically insignificant. Trend analysis for sex ratio showed no clear change in sex distribution of colon cancer mortality, suggesting that males and females share almost the same risk of dying of colon cancer. On the other hand, age distribution analysis found that older people had a higher mortality rate than younger people, especially after age 50, the mortality rate increased exponentially. The group aged 55-79 years accounted for 70.4% of total deaths. Trend analysis for mean age at death proposed an even worse situation for the old, since the mean age has been increasing. Then, it is self-evidently true that the older people are the high-risk group of colon cancer mortality. In such cases, in addition to effective treatment for colon cancer, screening of the group with high risk would be effective to prevent potential deaths of colon cancer. It has been reported that annual fecal occult blood tests can reduce the incidence and mortality rate of colorectal cancer<sup>[11-13]</sup>. Furthermore, there is evidence that screening not only reduces the incidence of colorectal cancer, but also the mortality, which is substantially reduced after removal of the precursor lesions<sup>[14,15]</sup>.

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# Analysis of prognosis on patients with severe viral hepatitis using the model for end-stage liver disease

Zhi-Hong Weng, Shu-Qing Cai

Zhi-Hong Weng, Shu-Qing Cai, Department of Infectious Diseases, Xiehe Hospital, Tongji Medical College, Huazhong Science and Technology University, Wuhan 430022, Hubei Province, China  
Correspondence to: Zhi-Hong Weng, Department of Infectious Diseases, Xiehe Hospital, Tongji Medical College, Huazhong Science and Technology University, Wuhan 430022, Hubei Province, China. wengzh2004@yahoo.com.cn  
Telephone: +86-27-85726135 Fax: +86-27-85356369  
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## Abstract

**AIM:** To study the practical use of the model for end-stage liver disease (MELD) on clinic and assess its validity by the concordance (C)-statistic in predicting the prognosis of the patient with severe viral hepatitis.

**METHODS:** One hundred and twenty-one patients were divided into plasma exchange group and non-plasma exchange group, and were graded with MELD formula. The death rate was observed within 3 mo.

**RESULTS:** Eighty-one patients died within 3 mo (35 cases in PE group, 46 cases in non-PE group). The mortality of patients in PE group whose MELD score between 20-30 and 30-40 were 31.6% and 57.7%, respectively, but in non-PE cases they were 67.6%, 81.3% respectively. There was significant difference between PE group and non-PE group ( $P < 0.05$ ). However, the mortality of patients whose MELD score higher than 40 were 93.3% in PE group and 100% in non-PE group and there was no significant difference between the two groups ( $P = 0.65 > 0.05$ ). The optimal cut-off values of MELD to predict the prognosis of patients were 30 in PE group whose sensitivity, specificity and C-statistic were 80.0%, 52.0% and 0.777, but in non-PE group they were 25, 82.6%, 86.7% and 0.869, respectively.

**CONCLUSION:** The MELD score can act as a disease severity index for patients with severe viral hepatitis, and the mortality of the patient increases with the increase of the MELD score. The MELD can accurately predict the short-term prognosis of patients with severe viral hepatitis.

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**Key words:** Prognosis; Severe Viral Hepatitis; Model for end-stage liver disease

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## INTRODUCTION

Viral hepatitis is one of the diseases endangering people's health worldwide, especially patients with severe viral hepatitis (severe hepatitis in short) with bad prognosis and a high mortality. Despite exploitation of new medicines and continuous advances in supporting therapy, mortality of patients undergoing conservative therapy of internal medicine is still reaching 60-80%<sup>[1]</sup>. It is important to establish a good method to estimate the prognosis of severe hepatitis, assess the severity of disease and development direction afterwards in order to select reasonable and effective therapy on the basis of relative clinical information.

These years, the establishment of Artificial Liver Support System (ALSS) provides important methods for the treatment of severe hepatitis, of which plasma exchange has become a relatively mature method in many hospitals. However, in the absence of unity of the index to estimate the therapy effect, people lack uniform cognition about the clinical effect of plasma exchange.

This study uses the model for end-stage liver disease (MELD)<sup>[2]</sup> to predict the short-term(3 mo) prognosis of patients with severe viral hepatitis and assess its practical value on clinic.

## MATERIALS AND METHODS

### Patients

One hundred and twenty-one hospitalized patients with severe hepatitis diagnosed according to the diagnostic standards recommended in Xian National Hepatopathy Meeting in 2000<sup>[3]</sup> from April of 2001 to August of 2003 were recruited for the study. We collected the case history of the 121 patients and they were divided into plasma exchange group and non-plasma exchange group, with 60 cases in PE group and 61 cases in non-PE group. There is no statistically significant difference between the two groups. Patients with autoimmune hepatitis, drug hepatitis, toxic hepatitis, alcoholic hepatitis and fatty liver were excluded. Comparison of the clinical statistics between the two groups is shown in Table 1.

### Combined therapy

The basic management of the two groups were the same: bed rest; venous infusion with liver-protecting medicine; transfusing blood product such as albumin and plasma; supplying energy and vitamin; maintaining electrolytic and acid-base homeostasis; preventing complications. PE group was appended with plasma exchange therapy and times of treatments were according to the state of each patient.

**Table 1** Patients' demographics

	PE group	Non-PE group
Sex (male/female)	53/7	58/3
Age (yr)		
Median (range)	42 (18-65)	40 (18-68)
Etiology (cases)		
B	45	41
B+E	13	16
B+C	2	4
Clinical type (cases)		
Subacute severe hepatitis	9	6
Chronic severe hepatitis	51	55
Laboratory parameters		
Serum total bilirubin (μmol/L)	588.88	514.99
(Range)	(217.0-1 015.5)	(293.0-1 140.3)
Serum creatinine (μmol/L)	131.63	142.09
(Range)	(43.3-401.8)	(42.0-945.0)
INR <sup>1</sup>	3.18	2.88
(Range)	(1.90-6.60)	(1.91-7.20)

<sup>1</sup>International normalized ratio (INR).

### Plasma exchange therapy

Patients were kept in plasma exchange therapy's room under strict disinfection and temperature control. A dual lumen catheter placed percutaneously into the femoral vein used as a blood access. Heparin was added to prevent clotting. Plasma exchange device of M-25 made in the USA was used. The blood flow rate in PE circuit was 10-20 mL/min. The duration of PE was 3 to 4 h. Some volumes of plasma (3 000-3 500 mL) per PE were exchanged and replaced with homotypic fresh frozen plasma.

### Test indices and scores

Function index of liver and kidney and international normalized ratio of the 121 patients tested in our hospital clinical laboratory were studied, and test data of the subsequent day after plasma exchange was compared with the data before treatment. Developments of liver disease were observed during 3-mo follow-up period from the day of hospitalization

(recovery and discharge from hospital, transit to receiving liver transplantation or death for instance).

The formula for the MELD score is: score of MELD<sup>[4]</sup> =  $3.8 \times \ln [\text{Total bilirubin (mg/dL)}] + 11.2 \times \ln (\text{INR}) + 9.6 \times \ln [\text{creatinine (mg/dL)}] + 6.4 \times (\text{etiology: 0 if cholestatic or alcoholic, 1 otherwise})$ . MELD score (integer) can be obtained from the official web site of American Mayo Clinic research center [www.mayoclinic.org/gi-rst/mayomodel 5.html](http://www.mayoclinic.org/gi-rst/mayomodel 5.html) by inputting the patients' clinical data.

### Statistical methods

Comparisons between groups were performed using *t* test, the rates were compared by  $\chi^2$  test and Fisher's exact test. The validity of MELD model in predicting the prognosis was measured by the C-statistic, which was equivalent to the area under receiver operating characteristic curve (ROC curve)<sup>[5]</sup>. Besides, the optimal cut-off values of MELD to discriminate between deceased and surviving patients with severe hepatitis were calculated by ROC curves.

Statistically, a C-statistic between 0.8 and 0.9 indicates excellent diagnostic accuracy and a C-statistic greater than 0.7 is generally considered a useful test. For prognostic models, a C-statistic of 0.9 or greater is seldom seen<sup>[6,7]</sup>. For all analyses *P* < 0.05 was considered statistically significant. Data were analyzed with the SPSS software package for Windows.

## RESULTS

### Comparison of clinical biochemical data and MELD score between PE group and non-PE group

Scores obtained by the two groups according to MELD formula and MELD scores, of all the cases were 20 or above. Both groups were categorized into three subsets:  $20 \leq \text{MELD} < 30$ ,  $30 \leq \text{MELD} < 40$ ,  $\text{MELD} \geq 40$  (Table 2).

The changes in clinical biochemical index and MELD score of the PE group after several times of plasma exchange are shown in Table 3.

There were significant decreases in the concentrations of the serum total bilirubin, INR and MELD score between,

**Table 2** Comparison of clinical biochemical data and MELD score between PE group and non-PE group before treatment (mean±SD)

	20≤MELD<30		30≤MELD<40		MELD≥40	
	PE group	Non-PE group	PE group	Non-PE group	PE group	Non-PE group
Bilirubin (μmol/L)	561.66±148.92	463.28±169.69	606.40±150.60	526.44±148.00	600.77±140.90	677.79±221.18
INR	2.13±0.62	2.34±0.67	3.54±1.16	4.14±1.24	4.01±1.58	3.58±1.69
Creatinine (μmol/L)	70.53±18.69	72.51±26.11	106.45±37.31	101.97±65.85	237.12±136.64	523.01±289.10
MELD score	24.7±3.1	24.2±3.0	34.7±2.2	32.4±7.1	42.7±1.7	48.6±5.3

**Table 3** Changes in related data of the PE group between before and after the plasma exchange (mean±SD)

	20≤MELD<30		30≤MELD<40		MELD≥40	
	Before PE	After PE	Before PE	After PE	Before PE	After PE
Bilirubin (μmol/L)	561.66±148.92	242.01±134.39 <sup>b</sup>	606.40±150.60	260.85±64.65 <sup>b</sup>	600.77±140.90	289.70±147.50 <sup>b</sup>
INR	2.13±0.62	1.55±0.28 <sup>a</sup>	3.54±1.16	1.59±0.22 <sup>a</sup>	4.01±1.58	2.40±1.52 <sup>a</sup>
Creatinine (μmol/L)	70.53±18.68	88.00±21.28	106.45±37.31	124.78±41.22	237.12±136.64	228.38±132.04
MELD score	24.7±3.1	19.7±3.5 <sup>a</sup>	34.7±2.2	22.7±3.7 <sup>a</sup>	42.7±1.7	28.4±5.3 <sup>a</sup>

<sup>a</sup>*P* < 0.05, after PE vs before PE; <sup>b</sup>*P* < 0.01, after PE vs before PE.

before and after the plasma exchange treatments (Table 3). Serum creatinine level also changed after plasma exchange therapy, but without statistically significant difference ( $P>0.05$ ).

### Relation between mortality and MELD score

The transformations of the disease of the two groups were observed from the day of hospitalization. The mortality and MELD score of all patients within 3 mo are shown in Table 4.

**Table 4** Three-month death rates

	$20 \leq \text{MELD} < 30$	$30 \leq \text{MELD} < 40$	$\text{MELD} \geq 40$
Non-PE group	67.6% (25/37)	81.3% (13/16)	100% (8/8)
PE group	31.6% (6/19) <sup>a</sup>	57.7% (15/26) <sup>c</sup>	93.3% (14/15) <sup>1</sup>

$\chi^2 = 6.58$ , <sup>a</sup> $P < 0.05$ , PE group *vs* non-PE group;  $\chi^2 = 3.97$ , <sup>c</sup> $P < 0.05$ , PE group *vs* non-PE group; <sup>1</sup> $P = 0.65$ , PE group *vs* non-PE group.

The study shows that the mortality of the patients with the MELD score between 20-30 and 30-40 in PE group was obviously lower than that of non-PE group. However, the mortalities of the patients with MELD score reaching or exceeding 40 in PE group and non-PE group were 93.3% and 100% respectively, and  $P$  value was 0.65 under Fisher's exact test which means the difference between the two groups was of no statistical significance.

### Prognostic analysis using the cut-off value of MELD

Using different MELD scores as cut-off value to discriminate between deceased and surviving patients, true/false positive value, true/false negativity value, and corresponding sensitivity and specificity can be obtained from four-fold table analysis of diagnostic experiment (Table 5).

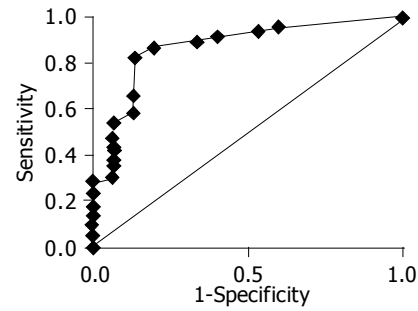
**Table 5** Four-fold table analysis of diagnostic experiment

Real prognosis	Predicted prognosis	
	Number of deaths	Number of survivors
Number of deaths	Value of true positive <sup>a</sup>	Value of false negative <sup>b</sup>
Number of survivors	Value of false positive <sup>c</sup>	Value of true negative <sup>d</sup>

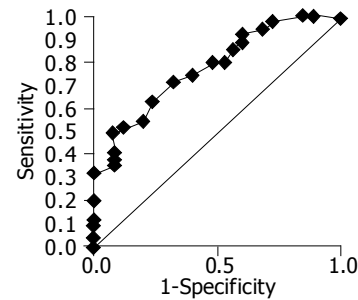
Sensitivity =  $a/(a+b) \times 100\%$ , specificity =  $d/(c+d) \times 100\%$ .

The cut-off values with the best sensitivity and specificity in predicting a 3-mo survival for MELD score were calculated using ROC curve, which gained from ROC software package with sensitivity as vertical axis and 1-specificity as horizontal axis (Figures 1, 2). The more the area under the curve, the higher the diagnostic validity<sup>[7]</sup>.

Figure 1 shows the ROC curve estimating the prognosis of the non-PE group within 3 mo through MELD model. ROC analysis software can output the following results: Area under the curve is 0.869; SE is 0.055;  $P$  is less than 0.0001; 95% CI is 0.760-0.978; the optimal cut-off value is 25; corresponding sensitivity is 82.6% and specificity is 86.7%.



**Figure 1** ROC curve of PE group determined by MELD model, area under the broken line is 0.5 standing for no discrimination.



**Figure 2** ROC curve of PE group determined by MELD model, area under the broken line is 0.5 standing for no discrimination.

Figure 2 demonstrates the ROC curve estimating the prognosis of the PE group within 3 mo through MELD model. The following results can be obtained from the ROC analysis software: Area under the curve is 0.777; SE is 0.059;  $P$  is less than 0.0001; 95% CI is 0.661-0.892; the optimal cut-off value is 30; corresponding sensitivity is 80.0% and specificity is 52.0%.

## DISCUSSION

MELD scoring system was originally developed to access the short-term prognosis of patients with cirrhosis hypertension undergoing the transjugular intrahepatic portosystemic shunt (TIPS) procedure by Mayo Clinic research center. MELD scoring system uses serum creatinine, total bilirubin, INR for prothrombin time and etiology of hepatopathy as indices and calculates the data through mathematical formula. Those patients with high score are of bad prognosis and greater possibility of death in short term. Besides using objective indices, this scoring system employs the renal function as an independent variable in estimating the prognosis of the hepatopathy, while other models don't.

A European study<sup>[8]</sup> showed that 6-mo survival sensitivity, specificity and C-statistic of using MELD score of 14 as the cut-off value to discriminate between deceased and surviving patients with hepatic cirrhosis is 75%, 72% and 0.82, respectively. The MELD scoring system showed nice discriminant ability. The study also demonstrated that MELD score correlates with the degree of liver functional impairment.

Recently, scholars both at home and abroad made a lot of research and study about predicting the prognosis of the severe hepatitis, but most of them are limited to univariate

analysis. Some multivariate analysis cannot be widely used in clinic due to the complexity of their indices. Most scholars discussed the plasma exchange therapy, about its improvement of symptom, mend of biochemical index and increase of survival rate, but the therapeutic effect reported by different research institutions has not been consistent with each other due to the absence of the objective of the index. The establishment of MELD scoring system solved these problems primitively.

The purpose of this study is to evaluate the ability of the MELD scoring system in discriminating the prognosis of the patients with severe hepatitis in short term (within 3 mo), and its validity in prediction is also analyzed by ROC curve, which gives out the optimal cut-off value of MELD score to discriminate between deceased and surviving patients in 3 mo.

The mortalities of patients in non-PE group whose MELD score in 20-30, 30-40 and  $\geq 40$  are 67.6%, 81.3%, 100%, respectively and the C-statistic is 0.869, which is nearer to the result of Kamath *et al*<sup>[2]</sup>. In PE cases they are 31.6%, 57.7% and 93.3%, respectively. MELD score can act as a severity index for the severe hepatitis, and mortality in 3 mo increases as the MELD score increases. The mortality of the patients in PE group with MELD score in 20-30 and 30-40 is significantly lower than that of non-PE group ( $P < 0.05$ ), thus plasma exchange therapy can increase the short-term survival rate of some patients with severe hepatitis.

Therapeutic effect of internal medicine for patients with MELD score reaching or exceeding 25 is not so well with bad prognosis in 3 mo. Plasma exchange or other artificial liver support system such as molecular absorbing recirculating system and liver transplantation should be applied early. Plasma exchange is of little effect for the patients whose MELD score is reaching or exceeding 40 and liver transplantation may be the only way to save their lives.

The following conclusions can be drawn from this study: the MELD score can act as a disease severity index for patients with severe viral hepatitis, and the mortality of the

patient increases with the increase of the MELD score. The MELD can accurately predict the short-term prognosis of patients with severe viral hepatitis, and it can be popularized in clinic. Clinical doctors can rely on MELD score for reference in determining whether the patients with severe hepatitis need plasma exchange or other therapy of artificial liver, even liver transplantation and its treatment occasion, and avoiding waste of medical resources. Further studies will be required to research and develop more exact quantified method in estimating liver function, increase the prediction vitality of clinical model, in order to predict the possibility of survival/fatality of individual patient.

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• BRIEF REPORTS •

# Tumor metastasis and the reciprocal regulation of heparanase gene expression by nuclear factor kappa B in human gastric carcinoma tissue

Hou-Jun Cao, Yong Fang, Xing Zhang, Wen-Jun Chen, Wen-Peng Zhou, Hong Wang, Lin-Bo Wang, Jin-Min Wu

Hou-Jun Cao, Yong Fang, Xing Zhang, Wen-Jun Chen, Wen-Peng Zhou, Hong Wang, Lin-Bo Wang, Jin-Min Wu, Center of Oncology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China

Correspondence to: Professor Jin-Min Wu, Center of Oncology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, Zhejiang Province, China. srshwu@mail.hz.zj.cn  
Telephone: +86-571-86090073 Fax: +86-571-86044817

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## Abstract

**AIM:** To investigate whether NF- $\kappa$ B is activated in human gastric carcinoma tissues and, if so, to study whether there is any correlation between NF- $\kappa$ B activity and heparanase expression in gastric carcinoma.

**METHODS:** NF- $\kappa$ B activation was assayed by immunohistochemical staining in formalin-fixed, paraffin-embedded specimens from 45 gastric carcinoma patients. Electrophoretic mobility shift assay (EMSA) method was used for nuclear protein from these fresh tissue specimens. Heparanase gene expression was quantified using quantitative RT-PCR.

**RESULTS:** The nuclear translocation of RelA (marker of NF- $\kappa$ B activation) was significantly higher in tumor cells compared to adjacent and normal epithelial cells [(41.3 $\pm$ 3.52)% vs (0.38 $\pm$ 0.22)%,  $t = 10.993$ ,  $P = 0.000 < 0.05$ ; (41.3 $\pm$ 3.52)% vs (0 $\pm$ 0.31)%,  $t = 11.484$ ,  $P = 0.000 < 0.05$ ]. NF- $\kappa$ B activation was correlated with tumor invasion-related clinicopathological features such as lymphatic invasion, pathological stage, and depth of invasion ( $Z = 2.148$ ,  $P = 0.032 < 0.05$ ;  $\chi^2 = 8.758$ ,  $P = 0.033 < 0.05$ ;  $\chi^2 = 18.531$ ,  $P = 0.006 < 0.05$ ). NF- $\kappa$ B activation was significantly correlated with expression of heparanase gene ( $r = 0.194$ ,  $P = 0.046 < 0.05$ ).

**CONCLUSION:** NF- $\kappa$ B RelA (p65) activation was related with increased heparanase gene expression and correlated with poor clinicopathological characteristics in gastric cancers. This suggests NF- $\kappa$ B as a major controller of the metastatic phenotype through its reciprocal regulation of some metastasis-related genes.

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**Key words:** Nuclear factor kappa B; Heparanase gene; Metastasis; Gastric carcinoma

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Wang LB, Wu JM. Tumor metastasis and the reciprocal regulation of heparanase gene expression by nuclear factor kappa B in human gastric carcinoma tissue. *World J Gastroenterol* 2005; 11(6): 903-907

<http://www.wjgnet.com/1007-9327/11/903.asp>

## INTRODUCTION

Gastric cancer is one of the most common malignant neoplasms in China and its incidence is gradually increasing in recent years. The clinical outcome for gastric cancer patients is still very poor with a 5-year survival rate of only 20% in all stages<sup>[1]</sup> and only 40% of the patients respond to surgical intervention<sup>[2]</sup>.

NF- $\kappa$ B is involved in the regulation of apoptosis, tumor progression, and responses to chemotherapy and ionizing radiation<sup>[3]</sup>. But its role in the process of gastric cancer metastasis has not been examined in detail. NF- $\kappa$ B is a transcriptional activator of MMP-9 and uPA<sup>[4]</sup>, supports a role of the NF- $\kappa$ B signal transduction pathway in the metastatic process. However, little information is available concerning the activity of NF- $\kappa$ B in gastric carcinoma, one of the most aggressive types of cancer.

Degradation of basement membrane and extracellular matrix structures are important features of invasion and metastasis in gastric cancer<sup>[5]</sup>. Human heparanase, an endoglycosidase, specifically involved in cleaving heparan sulfate, has been cloned recently, and it has been reported that this gene is functionally related to the invasion and metastasis of cancer cells<sup>[6]</sup>. Based on previous reports, we speculate that heparanase may be involved in the invasion and metastasis of gastric cancer cells. In the present study, we examined heparanase expression of the gastric cancer patients using quantitative RT-PCR.

It is recently reported that heparanase expression is regulated by NF- $\kappa$ B in tumor cell lines<sup>[7]</sup>, suggesting an important role for the NF- $\kappa$ B signal transduction pathway in the metastatic process. However, little information is available concerning NF- $\kappa$ B activation and whether it regulates the expression of heparanase expression in gastric carcinoma.

In the present study, we aimed to investigate whether NF- $\kappa$ B was constitutively activated in gastric carcinoma tissues. We also analyzed the correlation between NF- $\kappa$ B activity and clinicopathological features and whether heparanase expression was regulated by NF- $\kappa$ B activation in gastric carcinoma.

## MATERIALS AND METHODS

### *Clinical specimens*

Forty-five gastric carcinoma patients who gave informed consent before surgical treatment were enrolled into the present study at Sir Run Run Shaw Hospital between 2000 and 2004. All the normal, adjacent and tumor specimens, including 3 cases of metastatic liver tissues, were obtained from surgically resected tissues in the operating room, and stored in liquid nitrogen until use. The nuclear proteins were extracted immediately. Tissues were fixed in 10% formalin and embedded in paraffin for H&E staining and immunohistochemical staining. All specimens were classified histologically. The clinical pathological characteristics were evaluated according to the guidelines of the Union Internationale Contre le Cancer.

### *Immunohistochemistry*

Immunostaining was performed as described previously with a moderate modification<sup>[8]</sup>. Briefly, antigen retrieval on paraffin sections was performed by heating three times in a 10 mmol/L citrate buffer solution (pH 6.0) in a microwave. Slides were then probed with primary (anti-RelA p65; 1:200; sc-109; Santa Cruz Biotechnology, Santa Cruz, CA), and incubated with secondary antibodies (RelA, goat anti-rabbit immunoglobulin). Antibody binding was detected with a combination of DAB (40 mg/150 mL in PBS; Sigma Biotechnology, USA) and 0.06% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The number of nuclear-positive cells was counted and found to be 200 cells for each section. Nuclear staining, which indicated nuclear translocation of RelA, was considered as the marker of NF- $\kappa$ B activation.

### *Nuclear protein extraction*

Normal, adjacent and tumor specimens were collected in the operating room and homogenized in hypotonic buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.1% NP40, and 5% protease inhibitor (0.2 mmol/L DTT, 10 mmol/L benzamidine, 7 ng/L leupeptin, 50 ng/L soybean trypsin inhibitor, 2 ng/L aprotinin, 2 ng/L antipain, 0.7 ng/L pepstatin, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride)] immediately. Homogenized tissues were incubated on ice for 10 min and extraction of nuclear contents was performed as described previously<sup>[9]</sup>. The protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). The nuclear extracts were stored at -80 °C until use.

### *Electrophoretic mobility shift assay (EMSA)*

To further prove the results of immunostaining, NF- $\kappa$ B nuclear translocation was analyzed by EMSA for nuclear protein extracts of carcinomas, adjacent and normal tissues as described previously<sup>[9]</sup>. Nuclear protein extracts (10  $\mu$ g in each assay) were incubated with the binding buffer [60 mmol/L HEPES (pH 7.5), 180 mmol/L KCl, 15 mmol/L MgCl<sub>2</sub>, 0.6 mmol/L EDTA, and 24% glycerol], poly (dI-dC) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and <sup>32</sup>P-labeled double-stranded oligonucleotides containing the binding motif of NF- $\kappa$ B (Promega Corp., Madison, WI) for 30 min at 37 °C. The sequence of the double-stranded oligomer used for EMSA was 5'-AGTTGAGGGGACTTT

CCCAGGC-3'.

### *Quantitative RT-PCR*

Total RNA was extracted from carcinoma, adjacent and normal tissues by the TRIzol reagent. The primer sequences for amplification of heparanase gene were 5' AGA CCT TTG GGA CCT CAT GGA 3' (forward) and 5' GCA ACT TTG GCA TTT CTT ATC ACA A 3' (reverse), and the probe was 5' FAM- CAG GAA GTT CAC TGG GCT TGC CAG CTT TCT CA -TRAM 3'. The primer sequences for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were 5' CTT AGC ACC CCT CCC CAA G 3' (forward) and 5' GAT GTT CTG GAG AGC CCC G 3' (reverse) and the probe was 5' FAM- CAT GCC ATC ACT GCC ACC CAG AAG A -TRAM 3'. Expected RT-PCR product sizes were 179 bp for heparanase gene and 150 bp for GAPDH. PCR conditions consisted an initial denaturation step for 5 min at 94 °C, followed by 40 cycles of amplification (denaturation for 30 s at 94 °C, annealing for 15 s at 62 °C, and a final extension for 10 min at 72 °C). The RT-PCR products were sequenced in Tumor Research Institute of Zhejiang University, and the sequences we obtained coincided with the sequences available in GenBank [accession number BC083511 (for GAPDH) and accession number BC051321 (for heparanase)]. The RT-PCR products were also visualized on 2.0% agarose gels stained by ethidium bromide. Based on the fact that the C<sub>T</sub> (threshold cycle) value was inversely proportional to the log value of the original copy number of the target sequence, heparanase C<sub>T</sub>/ GAPDH C<sub>T</sub> was used to evaluate the mRNA expression level in different tissues.

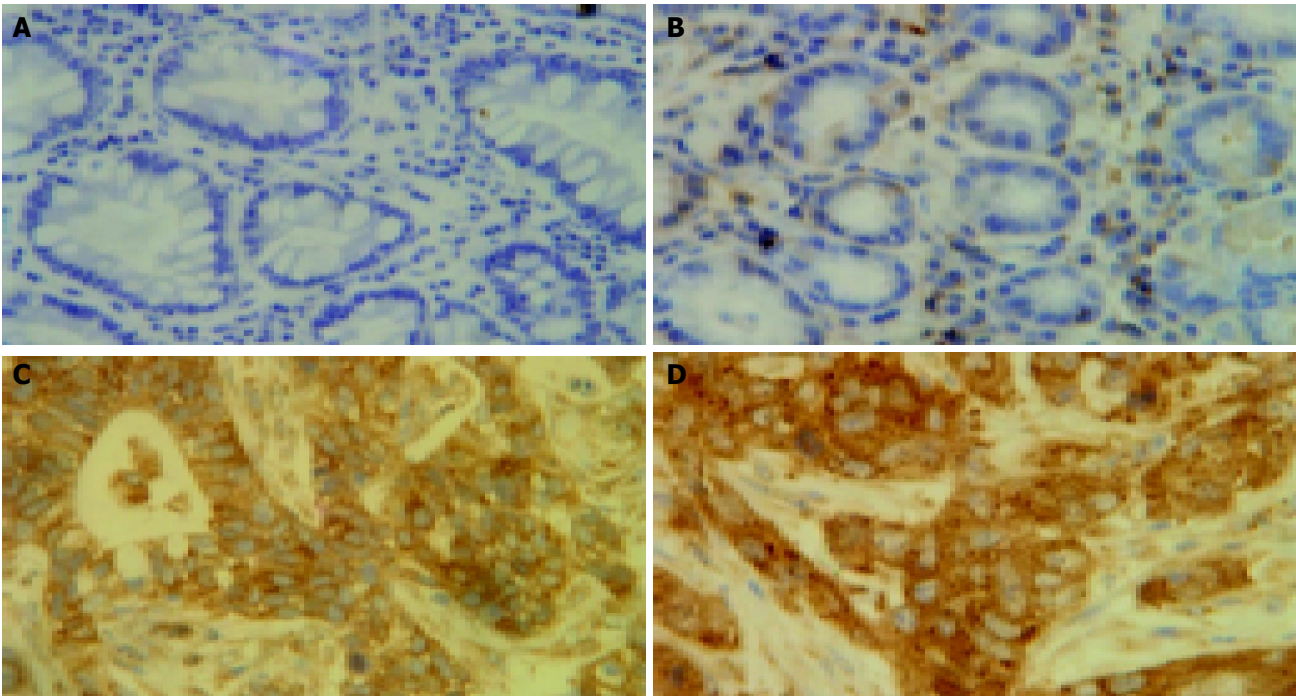
### *Statistical analysis*

All statistical analyses were performed with SPSS Statistical Software (release 12.0) on the windows workstation. The difference of the mean staining rate among tumor, adjacent and normal cells was analyzed by *t*-test for paired data. The correlation between the RelA nuclear staining rate and the clinical parameters was analyzed by Rank Sum test. The correlation between RelA nuclear staining and heparanase gene expression was analyzed by Spearman's test (one-sided). *P* value <0.05 was considered as significant.

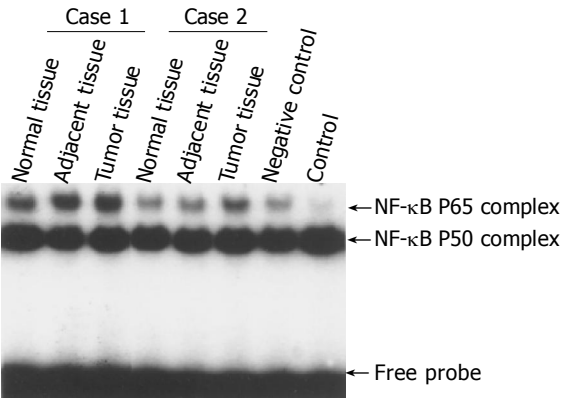
## RESULTS

### *RelA staining in gastric carcinoma specimens*

To ascertain whether NF- $\kappa$ B was activated in gastric carcinoma specimens, an immunohistochemical analysis of RelA was performed in formalin-fixed, paraffin-embedded gastric carcinoma specimens (Figure 1). RelA (P65) staining was significantly enhanced in the nuclei of the tumor cells compared to adjacent and normal epithelial cells. RelA staining was markedly higher in the metastatic tissues of the liver in the 3 cases compared to the gastric tumor specimens. The percentages of RelA (P65) staining were (41.3 $\pm$ 3.52)%, (0.38 $\pm$ 0.22)%, and (0 $\pm$ 0.31)% in tumor, adjacent and normal epithelial cells, respectively. The nuclear staining rates were statistically higher in tumor cells compared with those in adjacent and normal epithelial cells (*t* = 10.993, *P* = 0.000; *t* = 11.484, *P* = 0.000).



**Figure 1** Comparison of nuclear staining rates statistically higher in tumor cells with those in adjacent and normal epithelial cells after staining of RelA. A: Normal epithelial cells with no nuclei staining; B: Adjacent epithelial cells with no nuclei staining; C: Cancer tissues with strong nuclei staining; D: Cancer metastasis to the liver with specially enhanced nuclei staining ( $\times 400$ ).



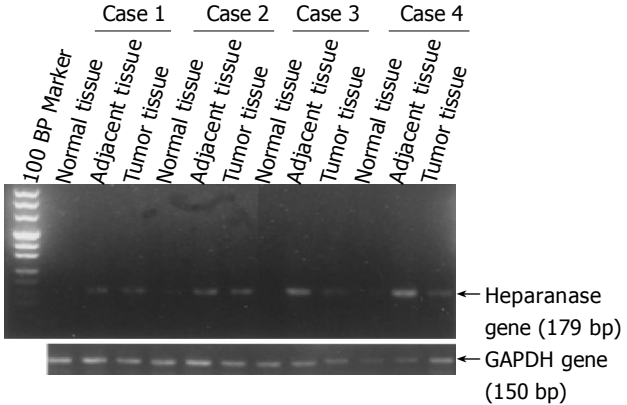
**Figure 2** Increased NF- $\kappa$ B RelA (p65) DNA binding activities in tumor specimens compared with that in adjacent and normal tissue of 2 typical cases.

**Increased NF- $\kappa$ B DNA binding activity in gastric carcinoma specimens**

EMSA was used to confirm the increased nuclear translocation of RelA (p65) in gastric carcinoma tissues. Figure 2 showed significantly increased NF- $\kappa$ B RelA (p65) DNA binding activity in tumor tissue compared to adjacent and normal tissues of 2 typical cases.

**NF- $\kappa$ B activation and clinical pathological features**

The correlations between RelA nuclear staining and the clinical pathological parameters in the 45 gastric carcinoma specimens were analyzed statistically as described in “Materials and Methods”. As shown in Table 1, NF- $\kappa$ B activation was correlated with lymphatic invasion ( $Z = 2.148$ ,  $P = 0.032$ ), pathological stage ( $\chi^2 = 8.758$ ,  $P = 0.033$ ), and depth of invasion ( $\chi^2 = 18.531$ ,  $P = 0.006$ ).



**Figure 3** The significantly higher heparanase mRNA levels in gastric cancer specimens than those in adjacent and far normal specimens of 4 typical cases.

**Heparanase expression and correlation between NF- $\kappa$ B activation and heparanase expression in gastric carcinoma specimens**

We examined the expression of heparanase in gastric carcinoma specimens by RT-PCR (Figure 3) and quantitative RT-PCR, since the expression of heparanase was thought to be mediated by NF- $\kappa$ B. Heparanase mRNA levels in gastric cancer tissues were significantly higher than those in adjacent and normal tissues (data not included in this paper and will be published in another paper). Correlation analysis between NF- $\kappa$ B activation and heparanase expression with one-sided Spearman’s test showed positive relation ( $r = 0.194$ ,  $P = 0.046$ ).

**DISCUSSION**

Previously, our studies have shown the activation of NF- $\kappa$ B

**Table 1** Correlations between the expression of NF- $\kappa$ B and clinical pathological features of gastric cancers

Clinical pathological indices	Cases	Mean rank	Value	Asymp. sig. (two-sided)	Test
Depth of invasion	45		$\chi^2 = 18.531$	0.006 <sup>a</sup>	Kruskal-Wallis <i>H</i>
Mucosa	4	52.83			
Submucosa	2	14.50			
Superficial muscular	3	19.50			
Deep muscular	2	5.50			
Serosa	30	32.50			
Out of serosa	4	5.83			
Lymphatic permeation	45		$Z = 2.148$	0.032 <sup>a</sup>	Mann-Whitney <i>U</i>
Have	3	5.50			
No	42	33.37			
Stage			$\chi^2 = 8.758$	0.033 <sup>a</sup>	Median test
I	5	35.00			
II	5	39.50			
III	20	30.90			
IV	15	34.40			
Histological grading	45		$\chi^2 = 8.920$	0.014 <sup>a</sup>	Kruskal-Wallis <i>H</i>
Well	8	18.30			
Middle	15	31.60			
Poor	22	37.21			

<sup>a</sup>*P* < 0.05.

in various cultured cell lines and several carcinoma tissues. NF- $\kappa$ B activation was usually evaluated on the basis of nuclear translocation of RelA (p65) and/or NF- $\kappa$ B 1 (p50) using EMSA and/or immunohistochemical analysis<sup>[10]</sup>. In this study, NF- $\kappa$ B RelA (p65) was constitutively activated in gastric carcinoma tissues by immunohistochemical analysis through quantified staining of nuclear RelA (p65) to evaluate NF- $\kappa$ B activation (Figure 1). The results were further proved by EMSAs in several cases. The significance of NF- $\kappa$ B activation in carcinoma tissues remained unclear until now. Here, we analyzed the relationship between NF- $\kappa$ B activation, as estimated by nuclear translocation of RelA, and the clinicopathological parameters. Our data proved that NF- $\kappa$ B activation was correlated with clinicopathological parameters such as lymphatic invasion, pathological stage, and depth of invasion (*P* = 0.032, *P* = 0.033 and *P* = 0.006, separately) in gastric carcinoma tissues (Table 1). These results supported the correlation between NF- $\kappa$ B activation and aggressiveness of gastric carcinomas, which is consistent to reports from other investigators<sup>[11,12]</sup>. Recent studies also showed that NF- $\kappa$ B is constitutively activated in several other tumors such as pancreatic cancer and breast cancer<sup>[13,14]</sup>. Interestingly, RelA (p65) staining was especially enhanced in the metastatic specimens of the liver, suggesting that NF- $\kappa$ B activation might contribute to the metastasis to distant organs. Since only 3 cases of liver metastatic specimens were collected in this study, additional specimens would need to be collected for future studies.

Specific enzymes produced and activated by cancer cells contributed to the degradation of extracellular matrices and basement membranes. Overexpression of heparanase was thought to contribute to tumor invasion, metastasis<sup>[15]</sup>, and inflammatory reactions<sup>[16]</sup>, and the enzyme was moderately up-regulated especially in metastatic cancers<sup>[17]</sup>.

In our previous study, the heparanase mRNA levels in gastric cancer specimens were significantly higher than those in adjacent and normal tissues, while there was no significant difference between the adjacent and normal tissues. The heparanase mRNA levels in gastric cancer tissues were significantly correlated with age, invasive depth, differentiation status, tumor size, metastasis, blood vessel invasion, lymphatic vessel invasion and nerves invasion (data were not shown in this paper and will be published in another paper). However, the mechanism leading to the overexpression of heparanase gene in cancer cells remains unclear.

Recently, it was reported that heparanase expression was regulated by NF- $\kappa$ B in tumor cell lines<sup>[7]</sup>. NF- $\kappa$ B modulated the expression of extracellular matrix proteinases such as MMPs, and the blockage of the NF- $\kappa$ B signal pathway resulted in the down-regulation of MMP-9 and heparanase. However, there is little information on the relationship between the NF- $\kappa$ B activity and the expression of heparanase gene in gastric cancer tissue.

To explore this relationship, the correlation between NF- $\kappa$ B activation and the heparanase gene expression of the invasion-related factor in tumor cells was analyzed by immunohistochemistry and quantitative RT-PCR separately. The results showed that there is a positive correlation between NF- $\kappa$ B activation and the heparanase expression in gastric carcinoma. However, specimens, showing a high heparanase expression, were not always detected corresponding to high NF- $\kappa$ B activation. Thus, this mechanism needs further study. We planned to block the NF- $\kappa$ B/I $\kappa$ B signal transduction pathway in a gastric cancer cell line using either mutant I $\kappa$ B or siRNA of I $\kappa$ B, to study whether the expression of heparanase gene could be down-regulated or not, and to determine how it affects the metastatic activity of tumor cells.

In conclusion, we found that the level of nuclear translocation of NF- $\kappa$ B was higher in gastric carcinoma cells than in adjacent and normal epithelial cells. There is a significant correlation between NF- $\kappa$ B RelA (p65) activation and the expression of heparanase gene. In the future, this positive relationship will be further studied to learn whether blocking the NF- $\kappa$ B signal transduction pathway can inhibit heparanase expression in gastric cancer cell lines. NF- $\kappa$ B and/or heparanase could be potential targets for anti-invasion therapies of gastric carcinoma.

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• BRIEF REPORTS •

# Comparison of probiotics and lactulose in the treatment of minimal hepatic encephalopathy in rats

Lin Jia, Mei-Hua Zhang

Lin Jia, Department of Digestive Diseases, Affiliated First People's Municipal Hospital, Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China  
Mei-Hua Zhang, Department of Digestive Diseases, Affiliated First People's Municipal Hospital, Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China  
Correspondence to: Dr. Lin Jia, Department of Digestive Diseases, First People's Municipal Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China. gzejalin@hotmail.com  
Telephone: +86-20-81628877 Fax: +86-20-81628809  
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## Abstract

**AIM:** To compare the efficacy of probiotic preparation Golden Bifid and lactulose on rat experimental model of minimal hepatic encephalopathy (MHE) induced by thioacetamide (TAA).

**METHODS:** MHE was induced by intraperitoneal injection of TAA (200 mg/kg) every 24 h for two consecutive days. Thirty-six male MHE models were then randomly divided into 3 groups: TAA group ( $n = 12$ ) received tap water *ad libitum* only; lactulose group ( $n = 12$ ) and probiotics group ( $n = 12$ ) were gavaged, respectively with 8 mL/kg of lactulose and 1.5 g/kg of probiotic preparation Golden Bifid (highly concentrated combination of probiotic) dissolved in 2 mL of normal saline, once a day for 8 d (from the 5<sup>th</sup> d before the experiment to the 3<sup>rd</sup> d of the experiment). The latency of brainstem auditory evoked potentials (BAEP) I was used as an objective index of MHE. The incidence of MHE, the level of serum endotoxin, ammonia, liver function and histological grade of hepatic injury of rats were examined individually.

**RESULTS:** There were no overt HE and rat deaths in 3 groups. The incidence of MHE, the levels of blood ammonia and endotoxin in TAA group, which were 83.3% (10/12),  $168.33 \pm 15.44$  mg/dL and  $0.36 \pm 0.04$  EU/mL, respectively, were significantly higher than those in lactulose group, which were 33.3% (4/12),  $110.25 \pm 7.39$  mg/dL and  $0.19 \pm 0.02$  EU/mL, and probiotics group, which were 33.3% (4/12),  $108.58 \pm 10.24$  mg/dL and  $0.13 \pm 0.03$  EU/mL respectively ( $P < 0.001$ ). It showed that either probiotics or lactulose could significantly lower the level of hyperammonemia and hyper-endotoxemia, lighten centrilobular necrotic areas as well as inflammatory reaction in the liver of rats, normalize the latency of BAEP, and decrease the incidence of MHE. However, no significant differences were observed between these two

groups ( $P > 0.05$ ).

**CONCLUSION:** Probiotic compound Golden Bifid is at least as useful as lactulose for the prevention and treatment of MHE. Probiotic therapy may be a safe, natural, well-tolerated therapy appropriate for the long-term treatment of MHE.

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**Key words:** Minimal hepatic encephalopathy; Probiotics; Lactulose

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## INTRODUCTION

Hepatic encephalopathy (HE) is a common and serious complication of liver diseases, ranging from minimal disease (grade 0) to hepatic coma (grade IV)<sup>[1-2]</sup>. Minimal hepatic encephalopathy (MHE) is a term that describes patients with chronic liver disease who have no clinical symptoms of brain dysfunction, but perform substantially worse on psychometric tests compared to healthy controls<sup>[3-5]</sup>. Although the exact pathogenesis of HE still remains unknown, the products of gut flora metabolism, such as ammonia, endotoxin, and benzodiazepine-like substances have been universally recognized as critical in recent years<sup>[6-10]</sup>.

Present treatment strategies, including lactulose and poorly absorbable antibiotics, may not be the optimal therapy for all HE patients due to their side effects and patients' poor compliance with therapy. Being viable bacteria given orally to improve health, probiotics were found to have multiple mechanisms of action to disrupt the pathogenesis of HE, and probiotic therapy was supposed to be the ideal strategy for HE<sup>[6,7]</sup>.

Probiotic compound Golden Bifid is a highly concentrated combination of probiotic containing bifidobacteria, lactobacilli and a mixture of *Streptococcus thermophilus* strains<sup>[6]</sup>. Based on the MHE model of rats established in 2004<sup>[11]</sup>, the present study was conducted to further compare the efficacy of Golden Bifid and lactulose on rat experimental MHE induced by thioacetamide (TAA).

## MATERIALS AND METHODS

### Induction of MHE model and its grouping

A total of 36 male Sprague-Dawley rats (Experimental

Animal Center of Sun Yat-Sen University) weighing 220–250 g were used. MHE was induced by intraperitoneal injection of TAA (200 mg/kg in normal saline, purity >99%, Shanghai Central Chemical Factory) every 24 h for two consecutive days<sup>[11]</sup>. Rats were fed with regular chow and water *ad libitum* in cages placed in a room with a 12-h light/dark cycle and constant humidity and temperature (25 °C).

MHE models were then randomly divided into 3 groups: TAA group ( $n = 12$ ) received tap water *ad libitum* only; lactulose group ( $n = 12$ ) and probiotic group ( $n = 12$ ) were gavaged respectively with 8 mL/kg of lactulose (Duphalac®, Solvay Pharmaceuticals B.V.) and 1.5 g/kg of Golden Bifid (highly concentrated combination of probiotic, provided by Shuangqi Pharmaceutical Co., Inner Mongolia, China) dissolved in 2 mL of normal saline, once a day for 8 d (from the 5<sup>th</sup> d before the experiment to the 3<sup>rd</sup> d of the experiment).

At the 3<sup>rd</sup> d of the experiment, brainstem auditory evoked potentials (BAEP), the serum level of endotoxin and ammonia, and liver function tests were carried for a quantitative evaluation of the efficacy of the Golden Bifid and lactulose treatment.

### Diagnosis of HE and MHE in rats

The behavioral manifestations of hepatic encephalopathy in the rats that received intraperitoneal injection of TAA evolved through four stages: (1) lethargy; (2) mild ataxia; (3) lack of spontaneous movement, loss of righting reflex, but positive response to tail pinch, and (4) coma, no response to tail pinch<sup>[12]</sup>. If TAA-treated rats showed one of the manifestations, it could be diagnosed as overt HE. Otherwise, evoked potentials of rats should be tested to confirm the diagnosis of MHE.

In our previous studies, the latency of BAEP of healthy rats was used as an objective index of MHE, and the average value of the latency of BAEP I in healthy rats  $\pm 1.96$  SD (1.45 ms) was regarded as normal value. MHE was diagnosed if the test score of the latency of BAEP I of rats was above 1.45 ms. Incidence of MHE and HE was recorded<sup>[11]</sup>.

### Analysis of serum endotoxin, ammonia and liver function tests

All the blood samples for the endotoxin determination were stored in endotoxin-free tubes. The serum was pretreated with perchloric acid for removal of the possible inhibitors to limulus amebocyte lysate (LAL). Serum level of endotoxin was determined by LAL test with LAL kits (purchased from Yihua Medical Technology Co., Shanghai, China).

Liver function tests, including total bilirubin (TB), albumin, serum glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvic transaminase (SGPT) were measured using a biochemical autoanalyzer (Hitachi Co., Tokyo, Japan). Tail vein ammonia was measured using a blood ammonia detector (Model AA-4120, Kyoto Daiichi Kagaku Company, Japan).

### Hepatic histopathology of rats

Liver tissue samples from the right major liver lobe were obtained, fixed in formalin and embedded in paraffin. Five-

micron thin tissue sections were obtained and stained with hematoxylin and eosin for microscopic examination. Histological grade of hepatic injury was determined by a semi-quantitative method based on the criteria described below: Grade 0: normal liver, grade 1: edema in the liver cell, grade 2: changes as balloon in liver cell, grade 3: necrosis as dots in the liver cell, inflammation cells in the portal vein section, grade 4: necrosis as small pieces.

Two pathologists who had no knowledge of the sample sources and each other's assessment examined the stained slides independently.

### Statistical analysis

All the values were expressed as the mean  $\pm$  SD. One-way ANOVA was used to check the differences among them.  $\chi^2$  test was used to check the differences of the incidence of MHE among the groups. When  $P$  was less than 0.05, the difference was considered statistically significant. Software SPSS10.0 was used in all statistical analyses.

## RESULTS

### Probiotics effects on the incidence of MHE

There were no overt HE and rat deaths in 3 groups. The incidence of MHE in TAA group was 83.3% (10/12), if 1.45 ms was regarded as the normal value of latency of BAEP I of rats (Table 1). Administration of lactulose and probiotics could significantly normalize the latency of BAEP, and decrease the incidence of MHE (33.3% and 33.3% respectively,  $P < 0.05$ ), however, there was no difference in the incidence of MHE between lactulose group and probiotics group ( $P > 0.05$ ).

### Probiotic effects on endotoxin, ammonia and liver function tests

The levels of blood ammonia and endotoxin in TAA group were  $168.33 \pm 15.44$   $\mu$ g/dL and  $0.36 \pm 0.04$  EU/mL respectively, which were significantly higher than those in lactulose group, which were  $62.25 \pm 7.63$   $\mu$ g/dL and  $0.07 \pm 0.02$  EU/mL, and probiotics group, which were  $62.25 \pm 7.63$   $\mu$ g/dL and  $0.07 \pm 0.02$  EU/mL, respectively ( $P < 0.05$ ). It revealed that lactulose and probiotics could significantly improve the ammonemia and endotoxemia in MHE models, as presented in Table 1.

**Table 1** Incidence of MHE, serum level of endotoxin and ammonia in three groups

Group	Latency of BAEP I (ms)	Incidence of MHE (%)	Level of endotoxin (EU/mL)	Level of ammonia ( $\mu$ g/dL)
TAA	$1.52 \pm 0.07$	83.3 (10/12)	$0.36 \pm 0.04$	$168.33 \pm 15.44$
Lactulose	$1.43 \pm 0.04^a$	33.3 (4/12) <sup>a</sup>	$0.13 \pm 0.02^a$	$110.25 \pm 7.39^a$
Probiotics	$1.42 \pm 0.09^a$	33.3 (4/12) <sup>a</sup>	$0.13 \pm 0.03^a$	$108.58 \pm 10.24^a$

<sup>a</sup> $P < 0.05$  vs TAA group.

Serum ALT, AST, albumin and TB concentrations in lactulose and probiotic groups showed slight improvement compared with those in TAA group ( $P > 0.05$ ) (Table 2).

However, there were no significant differences between lactulose group and probiotic group ( $P>0.05$ ).

**Table 2 Serum content of ALT, AST, albumin and TB in three groups**

Group	ALT (IU/L)	AST (IU/L)	Albumin (g/L)	TB ( $\mu$ mol/L)
TAA	137.00 $\pm$ 20.55	430.75 $\pm$ 60.25	31.27 $\pm$ 1.26	14.97 $\pm$ 2.76
Lactulose	132.58 $\pm$ 18.95	421.17 $\pm$ 60.00	31.95 $\pm$ 0.96	12.23 $\pm$ 1.07
Probiotics	128.00 $\pm$ 16.21	409.67 $\pm$ 63.02	32.23 $\pm$ 1.07	13.76 $\pm$ 2.18

### Probiotic effects on hepatic histopathology of rats

The control livers showed a normal lobular architecture with central veins and radiating hepatic cords. Two consecutive intraperitoneal injections of TAA (200 mg/kg) at 48 h time intervals caused severe pathological damages such as inflammation, dot necrosis or patchy necrosis. Administration of lactulose and probiotics could significantly lighten centrilobular necrotic areas as well as inflammatory reaction in rats subjected to TAA ( $P<0.05$ ). Semi-quantitative hepatic injury staging scores are shown in Table 3.

**Table 3 Hepatic histopathology of rats in three groups**

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
TAA	0	0	0	10	2
Lactulose	1	6	3	1 <sup>a</sup>	0
Probiotics	1	7	2	2 <sup>a</sup>	0

<sup>a</sup> $P<0.05$  vs TAA group.

## DISCUSSION

According to a consensus statement about HE in 1998, the study of HE has been greatly hindered by the lack of properly designed therapeutic trials<sup>[1,3]</sup>. The exact pathogenesis of HE remains uncertain, but is almost certainly multifactorial. Gut-derived nitrogenous substances are universally acknowledged to play a major role. Specifically, many nitrogenous products of gut flora metabolism including ammonia, endotoxin, benzodiazepine-like substances, or mercaptans are implicated in the genesis of HE<sup>[6-10]</sup>. Consequently, treatment of HE generally aims to reduce the production and absorption of intestinal toxins such as modifying the quantity and type of protein intake, reducing the intestinal transit time, and the proteolytic flora and increasing the saccharolytic flora<sup>[6,7,13-16]</sup>.

Presently, lactulose is considered to be the 'standard therapy' for HE due to its efficacy. However, lactulose has an unpleasant taste and causes flatulence, diarrhea, abdominal pain or intestinal malabsorption, which does not contribute to the improvement of patients' quality of life<sup>[17-19]</sup>. *Pai et al*<sup>[20]</sup> showed that only 20% patients favored the taste of lactulose, and 30% patients complained of meteorism and flatulence, and 20% patients complained of nausea. A high rate of dropouts (21-31%) was reported in some studies when treated with lactulose at a dose titrated to pass 2-3 semi-soft stools a day<sup>[18,19]</sup>. Therefore, lactulose may not be the optimal therapy for all HE patients due to

side effects, cost, and relatively poor compliance with therapy, particularly for the long-term treatment of MHE. Clearly, safe and well-tolerated alternatives are needed. Since probiotic is a safe, natural, well-tolerated therapy appropriate for long-term use, probiotic therapy is supposed to be the ideal strategy for HE, and has been gradually accepted worldwide in recent years<sup>[6]</sup>.

A number of studies have been performed using several strains of fermentative lactic-producing bacteria in order to modify the composition of gut flora. These trials employed high doses of non-urease-producing bacteria, either *Lactobacillus acidophilus* or *Enterococcus fecium* SF68. The effects of probiotics on HE have been demonstrated in many studies and no adverse effects were reported<sup>[7,16,21,22]</sup>. In a carefully conducted randomized controlled study, either short-term or long-term administration of SF68 in compensated patients with cirrhosis could enhance tolerance to protein load, lower ammonia levels, and improve neurological symptoms in patients with HE, and was at least as useful as lactulose for long-term treatment of chronic grades 1-2 of HE. It had no adverse effects, and in contrast to lactulose, treatment can be interrupted for 2 wk without losing the beneficial effects<sup>[7]</sup>. However, these above studies were limited to therapy with single aforementioned probiotic product and treatment of overt HE. Therefore, *Solga*<sup>[6]</sup> further proposed a hypothesis that probiotic compound may be superior to the single one, and probiotic compound VSL#3, which contains viable, lyophilized bifidobacteria, lactobacilli and a mixture of *Streptococcus thermophilus* strains might be ideally suited to HE<sup>[23,24]</sup>.

Unfortunately, no useful animal models exist for MHE study<sup>[6]</sup>. We used the latency of BAEP I as an objective index of MHE, and established animal model of MHE induced by injection of small dose of TAA in rats for the first time. The MHE model had a high level of ammonemia and endotoxemia, and its incidence was 83.3%<sup>[11]</sup>. The efficacy of probiotics and lactulose on rat experimental MHE has not been elucidated before.

In the current study, Golden Bifid served as a representative of probiotic compound VSL#3<sup>[23,24]</sup>. We used the well-established MHE model to compare the efficacy of Golden Bifid and lactulose, and tried to reason Solga's hypothesis. The results demonstrated that either Golden Bifid or lactulose could significantly lower the level of hyperammonemia and hyper-endotoxemia, lighten centrilobular necrotic areas as well as inflammatory reaction in the liver of rats, normalize the latency of BAEP, and decrease the incidence of MHE. However, there were no significant differences between lactulose group and Golden Bifid group. Probiotics may exhibit efficacy in the treatment of MHE by decreasing total ammonia in the portal blood, as well as in the uptake of other toxins, reducing inflammation and oxidative stress in hepatocytes leading to increased hepatic clearance of ammonia and other toxins<sup>[6]</sup>.

In conclusion, probiotic compound Golden Bifid showed excellent effects in lowering the level of ammonemia and endotoxemia, improving hepatic histopathology of rats, and decreasing the incidence of MHE. It was as effective as lactulose in the prevention and treatment of MHE. Our study agrees with Loguerio's conclusions on chronic HE

and confirms Solga's hypothesis for the first time. The probiotic therapy is a safe, effective, and well-tolerated strategy for HE, especially appropriate for long-term treatment of MHE.

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• BRIEF REPORTS •

## Alcohol dehydrogenase: A potential new marker for diagnosis of intestinal ischemia using rat as a model

Upendra R Gumaste, Mukund M Joshi, Devendra T Mourya, Pradip V Barde, Ghanshyam K Shrivastav, Vikram S Ghole

Upendra R Gumaste, Ghanshyam K Shrivastav, Vikram S Ghole, Division of Biochemistry, Department of Chemistry, University of Pune, Pune 411007, India

Devendra T Mourya, Pradip V Barde, Microbial Containment Complex, National Institute of Virology, Sus-Road, Pashan, Pune, Pune 411008, India

Mukund M Joshi, Department of Surgery, B J Medical College, Pune 411001 and Joshi Clinic and Health Sciences Foundation, Pune 411005, India

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Correspondence to: Vikram Ghole, Division of Biochemistry, Department of Chemistry, University of Pune, Pune 411007, India. vsghole@chem.unipune.ernet.in

Telephone: +91-20-25601225 Fax: +91-20-25691728

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in portal and heart blood within 1 h of SMA occlusion without increase in SGPT in heart blood, suggests that the origin of ADH is from ischemic intestine and not from liver. Similarly, raised ADH levels were found in DA and IVC as well. IVC blood does represent peripheral blood sample. A raised level of ADH in test animals confirms it to be a potential marker in the early diagnosis of Ii.

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**Key words:** Intestinal ischemia; ADH; Biochemical parameter

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### Abstract

**AIM:** Intestinal ischemia (Ii) is an abdominal emergency due to blockade of the superior mesenteric artery resulting in 60-100% mortality if diagnosed late. Changes in several biochemical parameters such as D (-)-lactate, Creatinine kinase isoenzymes and lactate dehydrogenase suggested for early diagnosis, lack specificity and sensitivity. Therefore a biochemical parameter with greater sensitivity needs to be identified.

**METHODS:** Wistar male rats were randomly assigned into two groups; control sham operated ( $n = 24$ ) and ischemic test ( $n = 24$ ) group. Superior mesenteric arterial occlusion was performed in the ischemic test group for 1 h. Alcohol dehydrogenase (ADH) was estimated in blood from portal vein, right ventricle of heart, dorsal aorta (DA) and inferior vena cava (IVC). The Serum glutamic acid pyruvate transaminase (SGPT) was also estimated in blood from portal vein and right ventricle of heart.

**RESULTS:** A significant increase ( $P < 0.001$ ) in the levels of ADH in both portal blood as well as heart blood of the test group ( $232.72 \pm 99.45$  EU and  $250.85 \pm 95.14$  EU, respectively) as compared to the control group ( $46.39 \pm 21.69$  EU and  $65.38 \pm 30.55$  EU, respectively) were observed. Similarly, increased levels of ADH were observed in blood samples withdrawn from DA and IVC in test animals ( $319.52 \pm 80.14$  EU and  $363.90 \pm 120.68$  EU, respectively) as compared to the control group ( $67.68 \pm 63.22$  EU and  $72.50 \pm 58.45$  EU, respectively). However, in test animals there was significant increase in SGPT in portal blood ( $P = 0.054$ ) without much increase in heart blood.

**CONCLUSION:** Significant increase in the levels of ADH

### INTRODUCTION

Acute mesenteric ischemia is an abdominal emergency due to inadequate tissue perfusion. Consequence of late diagnosis is a multi-system organ dysfunction syndrome, which may lead to death<sup>[1]</sup>. The mortality rate due to intestinal ischemia (Ii) ranges between 60-100%. Yet there is no non-invasive method to diagnose Ii in the early stage. There are several attempts to analyze a biochemical marker for the diagnosis of Ii; however, no suitable marker has been identified till date.

The measurement of plasma D (-)-lactate, amylin level, serum concentration of cytosolic beta-glucosidase (CBG) and diamine oxidase (DAO) may be useful to assess the intestinal injury<sup>[2-6]</sup>. Preliminary findings suggest that human intestinal fatty acid binding protein (hIFABP) may serve as a diagnostic marker for early intestinal mucosal damage<sup>[7,8]</sup>. The results suggest that plasma and peritoneal lactic acid (LA) are sensitive indicators in the early diagnosis of bowel ischemia in contrast to serum CK, which is not a useful indicator<sup>[9,10]</sup>. Glutathione-S-transferase ( $\alpha$ -GST) monitoring is also a useful tool for the diagnosis of Ii<sup>[11]</sup>.

Association of Alcohol dehydrogenase (ADH) (EC 1.1.1.1) levels with various pathological conditions has already been suggested<sup>[12-14]</sup>. It has been reported that endotoxemia occurs in Ii<sup>[15]</sup>. Gastrointestinal barrier dysfunction in Ii has also been reported<sup>[16-18]</sup>. Recently, involvement of endotoxin in up-regulation of ADH gene from liver reported by Mezey *et al*<sup>[19]</sup> (2003) along with the above-mentioned findings, prompted us to look for ADH levels in Ii. ADH is a major enzyme catalyzing the biological oxidation of ethanol in mammals<sup>[20]</sup>. Five protein subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\pi$  and  $\chi$ ) combine into three classes of isoenzymes. The class IV ( $\sigma$ ) ADH

encoded by ADH7 is a key enzyme in the metabolism of retinol to retinoic acid (RA)<sup>[21,22]</sup>. Rat ADH3 and human class I (ADH) are analogous and mainly localized in liver. Rat ADH2 and human  $\chi$ -ADH (class III) are analogous and ubiquitously found in all tissues. The correspondence between rat ADH-1 and human  $\pi$ -ADH (class II) is not clear. Human  $\pi$ -ADH has been detected exclusively in liver while rat ADH 1 is found in eye, stomach, lung and other rat organs but not in liver<sup>[23]</sup>. The wide tissue distribution of ADH is already established<sup>[23,24]</sup>. In an attempt of qualitative determination of ADH in animals suffering from Ii, we found significant increase in ADH enzyme activity. Thus further studies were therefore conducted to investigate the association of increase in ADH enzyme activity with SMA occlusion.

## MATERIALS AND METHODS

Eight weeks old rats (Wistar strain) weighing between 150-200 g were fasted 18 h before conducting the experiments. Two groups were assigned, sham-operated control ( $n = 24$ ) and one-hour ischemic test group ( $n = 24$ ). Midline laprotomy was performed on both the groups after giving ether anesthesia. The control group of animals was sham-operated in order to trace the superior mesenteric artery (SMA) while in test group the SMA was flush ligated. Both the groups were kept under observation for 1 h.

After 1 h the blood was withdrawn from portal vein, right ventricle of heart, dorsal aorta (DA) and inferior vena cava (IVC). The serum was separated immediately and stored at  $-40^{\circ}\text{C}$  till the assay was performed. The serum glutamic acid pyruvate transaminase (SGPT) was assayed in serum sample drawn from portal vein and heart while alcohol dehydrogenase (ADH) was assayed in samples drawn at all points mentioned above within 24 h of blood withdrawal. Alcohol dehydrogenase assay was performed by the method of Skursky *et al.*<sup>[25]</sup>. The substrate p-nitroso N, N dimethyl aniline (NDMA) was prepared in the laboratory<sup>[26]</sup>. Both kinetic as well as one-point incubation method were performed. Concentration of ADH is

expressed in terms of  $\mu\text{M}$  of NDMA reduced per min per mL of serum.

The enzyme kinetic assays were carried out in 96 well flat bottom micro titer plates and 7.5  $\mu\text{L}$  of serum was added to each well. To this 132.5  $\mu\text{L}$  of substrate solution was added and the plates were read immediately at 440 nm in kinetic mode for 10 min on the kinetic reader Softmax Pro, Version 4 (Molecular devices, USA). SGPT assay was carried out using 'Infinite GPT' kit (Accurex Biomedical Pvt. Ltd, India).

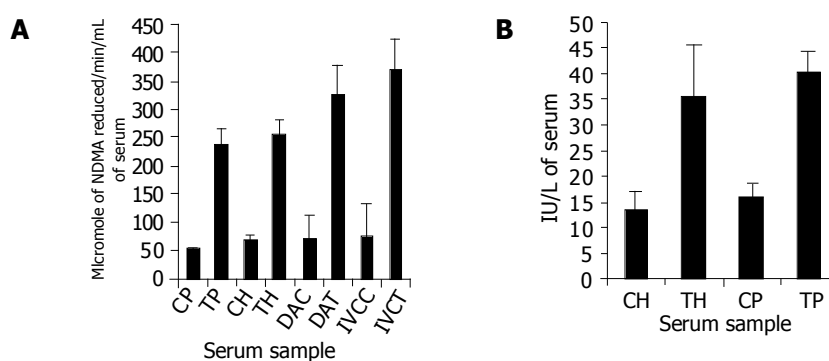
Intestinal tissue of about 10 cm in length and a small part of liver were removed immediately after the end of the experiment and fixed in 10% formal saline. The sections were stained with hematoxylin/eosine for histopathological investigation. The grades of the injury to the intestine were defined as mentioned by Khurana *et al.*<sup>[27]</sup> briefly: grade 0-normal. Grade 1- subepithelial blebs with small areas of apical epithelial loss; stroma and crypts normal; vessels are patent. Grade 2- total loss of villous epithelium but crypts and stroma are normal; vessels are patent. Grade 3- total villous loss with some crypts involved; stroma normal; vessels are patent. Grade 4- all crypts involved, stroma is normal, but vessels patent and remain patent. Grade 5- all Grade 4 changes with stromal necrosis and/or evidence of vessel thrombosis.

Student *t* test was used for statistical analyses of data using 'Quick-stat' software.

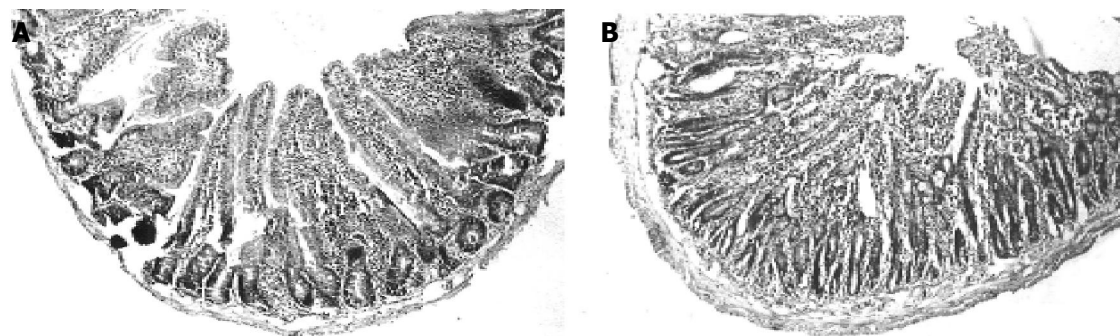
## RESULTS

The levels of SGPT and ADH are depicted in (Figure 1). The values of SGPT in portal blood in test and control animals were  $39.93 \pm 7.99$  IU/L and  $15.70 \pm 4.83$  IU/L, respectively. SGPT levels in samples drawn from heart were  $35.27 \pm 18.11$  IU/L and  $13.04 \pm 6.87$  IU/L, respectively in test and control animals. The increase in SGPT levels in heart blood of test animals was not significant as compared to that in portal blood of test animals ( $P = 0.054$ ).

There was nearly 5 times increase in the levels of ADH in blood from portal vein, DA and IVC; however, heart blood showed a rise of about 4 times in test group as compared



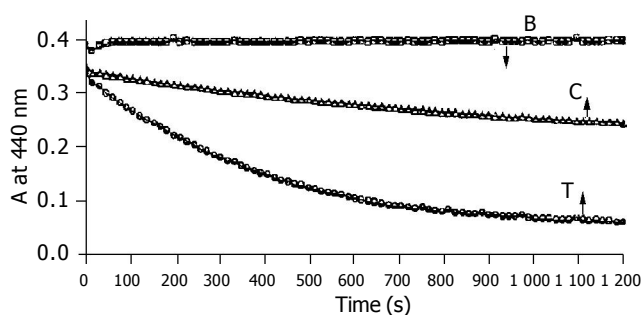
**Figure 1** Alcohol Dehydrogenase Assay By Skursky *et al* (A) and SGPT Assay (B) performed in heart blood and portal blood of 1 h ischemic test and sham operated control group. A: Alcohol dehydrogenase assay performed by Skursky *et al* method. CH and TH represent the blood withdrawn from heart of control group and test group, respectively. CP and TP represent the blood withdrawn from portal vein of control group and test group, respectively. DAC and DAT represent the dorsal aorta blood sample from control and test group, respectively while IVCC and IVCT represent the blood sample from inferior vena cava of control and test group, respectively. The EU expressed in terms of micromoles of NDMA reduced per min/L of serum; B: SGPT assay. CH and TH represent the blood withdrawn from heart of control group and 1 h ischemic test group, respectively. CP and TP represent the blood withdrawn from portal vein of control group and 1 h ischemic test group, respectively.



**Figure 2** Histology of intestine from rats. A: C- control sham operated group; 0 grade injury i.e. no damage to villi and crypts; B: t-test one-hour intestinal ischemic group; Total villous loss with some crypts involved; stroma normal. Vessels are patent (i.e. grade 3 injury).

to control group. The levels of ADH in portal blood of test group and control group were  $232.72 \pm 99.44$  EU and  $46.38 \pm 21.69$  EU, respectively whereas those in heart blood of test and control were  $250.85 \pm 95.14$  EU and  $65.37 \pm 30.55$  EU, respectively. Similarly there was an increase in DA and IVC blood in test group ( $319.52 \pm 80.14$  EU and  $363.90 \pm 120.68$  EU, respectively) as compared to control group ( $67.68 \pm 63.22$  EU and  $72.50 \pm 58.50$  EU, respectively). The increase in ADH levels at all the points in test animals were highly significant as compared to control group ( $P < 0.001$ ). The kinetic data of blood sample taken from heart of the test animal and control animal also support this fact (Figure 3). Kinetic data also reconfirmed the finding that the levels of ADH at all the points mentioned above were significantly higher in test group as compared to control group.

It was interesting to note that histological studies in liver tissue from control and test animal do not show any apparent damage. Grade three injuries were observed in intestines of the test animals those who had suffered 1-h SMA occlusion (Figure 2).



**Figure 3** Kinetic assay of Alcohol dehydrogenase from serum carried out at  $30^{\circ}\text{C}$ . (B) Showing practically no auto-reduction of NDMA in the absence of serum, (C) reduction of NDMA in the presence of serum drawn from the heart of control animal after 1 h of sham operation, (T) reduction of NDMA in presence of serum drawn from the heart of test animal after 1 h SMA occlusion.

## DISCUSSION

Our data reveals highly significant increase in the levels of ADH at all points as mentioned above, as compared to control group. There is evidence to suggest that the barrier

properties of the gastrointestinal mucosa are significantly in conditions like ischemia<sup>[16,17]</sup>. Measurement of plasma (or luminal) clearances of water-soluble molecules has proved to be a popular method for studying intestinal permeability<sup>18</sup>. Rise in ADH enzyme levels suggest that probably ADH leaks out from gastrointestinal lumen into the blood compartment due to an intestinal barrier dysfunction caused by Ii. Caglayan *et al*<sup>[28]</sup> (2002) studied ischemia-reperfusion in intestine in rabbits and estimated CK, LDH, AST and SGPT. They concluded that in ischemic condition there was a rise in AST, LDH and CK levels but there is no rise in the level of SGPT. With these findings they concluded that in ischemia of intestine, liver per se is not contributing into the increased levels of other enzymes and their primary source is injured intestine. It is a well-known fact that alcoholism causes liver damage, but Chrostek *et al*<sup>[29]</sup> has shown that in alcoholics the main source of serum ADH is gastrointestinal tract and not liver. Our study also reports significant increase in SGPT levels in portal blood without insignificant increase in heart blood in test animals. This is another supporting evidence that in our study, the intestine and not the liver is responsible for the rise of serum ADH levels in Ii. Data obtained from the histological findings also suggest that there was no damage of liver cells in the test animals.

Variation in SGPT levels in control heart blood and test heart blood was not found to be statistically significant. However, portal vein samples of the same animals showed significant increase of SGPT levels ( $P = 0.05$ ). This can be explained as the portal blood represents diffuse drainage from the intestine allowing higher concentration of SGPT due to ischemic intestine while such blood that enters subsequently into liver and in body-circulation gets diluted and final concentration would vary according to blood volume of each animal.

It seems that residual level of ADH in normal animal is around 46 EU and the rise due to ischemic insult shoots up to 232 EU in heart blood. Comparatively the levels of ADH in heart, DA and IVC show higher levels than blood withdrawn from portal vein, which is contradictory to the argument, made in relation to SGPT. The DA blood represents the blood, which supplies blood to organs and tissues of lower part of the body while IVC blood represents peripheral blood circulation and collection of blood from

all organs and tissues from distal part of the body. When closely compared, the levels of ADH obtained in test animals are  $232.72 \pm 99.44$  EU in portal blood,  $250.85 \pm 95.14$  EU in heart blood,  $319.52 \pm 80.14$  in DA blood and  $363.90 \pm 120.68$  in IVC blood, imply that point of collection of sample does not affect the level of detection of ADH. On the contrary the higher levels of ADH at different points highlight their utility towards diagnosis of Ii as only tissue damaged in this process was the intestine. However, this fact can be explained by hypothesizing that there may be some unknown compounds or factors, which might be getting released by intestinal tissue into the blood, which may interact with other tissues allowing additional ADH release in the blood. The endotoxemia occurs following severe injuries in the intestine in Ii<sup>[15]</sup>. It is also known that bacterial endotoxin up-regulates the ADH gene in liver<sup>[19]</sup>. However, we believe, the duration of 1-h ischemia is insufficient to up-regulate the ADH gene in liver and cause increased ADH enzyme in blood. The increase in ADH levels is equally significant in portal blood as well as all other points (Figure 1) in SMA-ligated test animals, which suggests that it cannot be due to up-regulation of ADH gene in the liver by endotoxin. The investigation of such factor will be an interesting approach but presently beyond the scope of this paper.

ADH is also termed as s-nitroso glutathione (GSNO) terminase involved in the elimination of aldehyde resulting from lipid peroxidation, thereby constituting a defense mechanism against cytotoxic aldehydes generated by lipid peroxidation<sup>[30]</sup>. Since there is generation of localized free radicals, nitric oxide, lipid peroxides and possible production of GSNO in ischemic intestine<sup>[31]</sup>, it is surmised that the increased levels of ADH may be involved in neutralization of lipid peroxidation product and degradation of GSNO.

ADH1 and ADH4 are expressed throughout the gastrointestinal tract and exhibit retinol dehydrogenase activity, which is responsible for the generation of retinoic acid (RA). RA is an essential modulator of epithelial development, differentiation and aging of vascular smooth muscle cells<sup>[30]</sup>. Due to the occurrence of apoptosis and large amount of cell destruction of enterocytes in ischemia, the role of ADH in generation of enterocytes and preparedness of tissue for recovery cannot be neglected.

The significant increase in the levels of ADH in portal and heart blood along with similar increase in DA blood and IVC blood within 1 h of SMA occlusion without increase in SGPT in heart blood suggests that the origin of ADH is from ischemic intestine. This also rules out the possibility of direct pathophysiological involvement of liver. In fact, the histopathological examination of liver tissue from test samples reconfirms the above findings. Since IVC blood sample, which represents peripheral blood, has shown that increased levels of ADH in test animals confirms the potential of serum ADH in early diagnosis of Ii. Even in clinical settings femoral blood sample can be taken very effectively. Therefore, we feel its clinical utility is much more prominent; however, clinical trials are required in order to confirm this finding and utility in day to day clinical practices.

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• BRIEF REPORTS •

## Establishment of a new pig model for auxiliary partial orthotopic liver transplantation

Cheng-Hong Peng, Liu-Bin Shi, Hong-Wei Zhang, Shu-You Peng, Guang-Wen Zhou, Hong-Wei Li

Cheng-Hong Peng, Hong-Wei Zhang, Guang-Wen Zhou, Hong-Wei Li, Transplantation Center, Ruijin Hospital, Shanghai 200025, China

Liu-Bin Shi, Transplantation Center, Huashan Hospital, Shanghai 200040, China

Shu-You Peng, Department of General Surgery, the Second Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310009, Zhejiang Province, China

Correspondence to: Professor Cheng-Hong Peng, Transplantation Center, Ruijin Hospital, 147 Second Ruijin Road, Shanghai 200025, China. [chhpeng@medmail.com.cn](mailto:chhpeng@medmail.com.cn)

Telephone: +86-21-64370045-666043 Fax: +86-21-64333548

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with such advantages as simple vessel processing, quality anastomosis, less postoperative hemorrhage and higher success rate, effectively prevents ischemia reperfusion injury of the host liver and deserves to be spread.

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**Key words:** Auxiliary partial orthotopic liver transplantation; Model pig

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### Abstract

**AIM:** To establish a new pig model for auxiliary partial orthotopic liver transplantation (APOLT).

**METHODS:** The liver of the donor was removed from its body. The left lobe of the liver was resected *in vivo* and the right lobe was used as a graft. After the left lateral lobe of the recipient was resected, end-to-side anastomoses of suprahepatic inferior vena cava and portal vein were performed between the donor and recipient livers, respectively. End-to-end anastomoses were made between hepatic artery of graft and splenic artery of the host. Outside drainage was placed in donor common bile duct.

**RESULTS:** Models of APOLT were established in 5 pigs with a success rate of 80%. Color ultrasound examination showed an increase of blood flow of graft on 5<sup>th</sup> d compared to the first day after operation. When animals were killed on the 5<sup>th</sup> d after operation, thrombosis of hepatic vein (HV) and portal vein (PV) were not found. Histopathological examination of liver samples revealed evidence of damage with mild steatosis and sporadic necrotic hepatocytes and focal hepatic lobules structure disorganized in graft. Infiltration of inflammatory cells was mild in portal or central vein area. Hematologic laboratory values and blood chemical findings revealed that compared with group A (before transplantation), mean arterial pressure (MAP), central venous pressure (CVP), buffer base (BB), standard bicarbonate (SB) and K<sup>+</sup> in group B (after portal vein was clamped) decreased ( $P < 0.01$ ). After reperfusion of the graft, MAP, CVP and K<sup>+</sup> restored gradually.

**CONCLUSION:** Significant decrease of congestion in portal vein and shortened blocking time were obtained because of the application of *in vitro* veno-venous bypass during complete vascular clamping. This new procedure,

### INTRODUCTION

Orthotopic liver transplantation has become an accepted form of treatment for chronic liver failure, acute liver failure, primary hepatic malignancy not treatable by conventional resection and inborn errors of metabolism due to a liver-based enzyme defect but without parenchymal liver disease<sup>[1]</sup>. In the middle of the 1990s, with the development of reduced-size liver transplantation, split liver transplantation and partial-living liver transplantation, much attention was paid to auxiliary partial orthotopic liver transplantation (APOLT)<sup>[2,3]</sup>. APOLT, in which a reduced-size graft orthotopically replaces the resected lobe of the recipient, has been introduced with acceptable results for patients with fulminant hepatic failure (FHF)<sup>[4,5]</sup>. It has great advantages over standard orthotopic liver transplantation. In APOLT, both the reduced native liver and the reduced graft can obtain optimal accommodation, and a position close to the right atrium may improve hepatic venous outflow of the graft<sup>[6]</sup>. On the other hand, if the graft is destroyed by some events such as severe rejection or if primary nonfunction occurs, the remnant native liver can sustain the patient's life and emergency re-transplantation is not necessary<sup>[6,7]</sup>. Furthermore, when the diseased native liver regenerates with its function fully restored, the withdrawal of immunosuppressive therapy is then possible<sup>[5,8]</sup>, which will decrease the incidence of drug-related side effects and neoplasia, and monitoring requirements<sup>[9]</sup>. Also, in the preserved native liver future gene therapy is still possible<sup>[10]</sup>.

However, there are few studies of APOLT using large animal models. In this experimental study, we attempted to establish a new pig model of APOLT and evaluated the changes of intraoperative systemic hemodynamics and graft function in the model.

## MATERIALS AND METHODS

### Animal selection

Ten healthy male or female domestic pigs, weighing 22-27 kg, were randomly selected to be either donor group ( $n = 5$ ) or recipient group ( $n = 5$ ). Another 5 animals, weighing 75-150 kg, were used as blood supply group. Prior to surgical procedure, the pigs were fasted for 12 h, but were fed water *ad libitum*.

### Surgical procedures

Anesthesia was induced by ketamine intramuscularly with a dose of 20 mg in the donor or recipient. After intubation, inhalation anesthesia was maintained by pentothal sodium. For donor operations, we used a midline laparotomy. The infrahepatic inferior vena cava (IVC), common bile duct, portal vein (PV) and hepatic artery (HA) were dissected and exposed. The hepatic artery was kept in continuity with the celiac trunk and abdominal aorta up to the iliac bifurcation. The whole liver was perfused *in situ* with 4 °C cold kidney preservation solution via abdominal aorta (1 500 mL) and portal vein (2 000 mL). Once perfusion was finished, the harvested graft was removed and immersed in a basin filled with cold kidney preservation solution at 4 °C. During bench surgery the gallbladder was resected and the liver splitting was performed with the technique of curettage and aspiration by use of PMOD (Peng's Multifunctional Operative Dissector). The liver was divided along the right side of the

middle hepatic vein and the right branches of the PV. The right liver was used as a partial graft and stored at 4 °C in the same solution bath. During the donor preparation, another surgical team performed the recipient operation. The recipient animal was intubated, anesthetized, and an internal jugular vein catheter was placed for blood sampling, intravenous infusion and central venous pressure (CVP) monitoring. A second catheter was placed in the carotid artery to monitor blood pressure during the procedure. When heparin was intravenously infused with a bolus dose of 2 mg/kg, left hepatectomy was performed and the spleen was resected and removed. Before all hepatic vessels of the recipient were interrupted, an extracorporeal veno-venous bypass (splenic vein and femoral vein to extra jugular vein) was established and the graft (right liver) was placed into the position of left lobe of recipient. The following anastomoses were then made: donor suprahepatic inferior vena cava end-to-side to host suprahepatic inferior vena cava, donor portal vein end-to-side to host portal vein, donor hepatic artery end-to-end to host spleen artery. The donor's common bile duct was intubated for outside drainage and bile was collected with an extracorporeal bag. The time consumed in suprahepatic inferior vena cava and portal vein anastomoses between donor and recipient was 15-20 min and 10-15 min, respectively. After operation, the donor's infrahepatic inferior vena cava was ligated. Heat and cold ischemia time were 0 min and  $60 \pm 4.0$  min, respectively. Figure 1A-E shows our APOLT

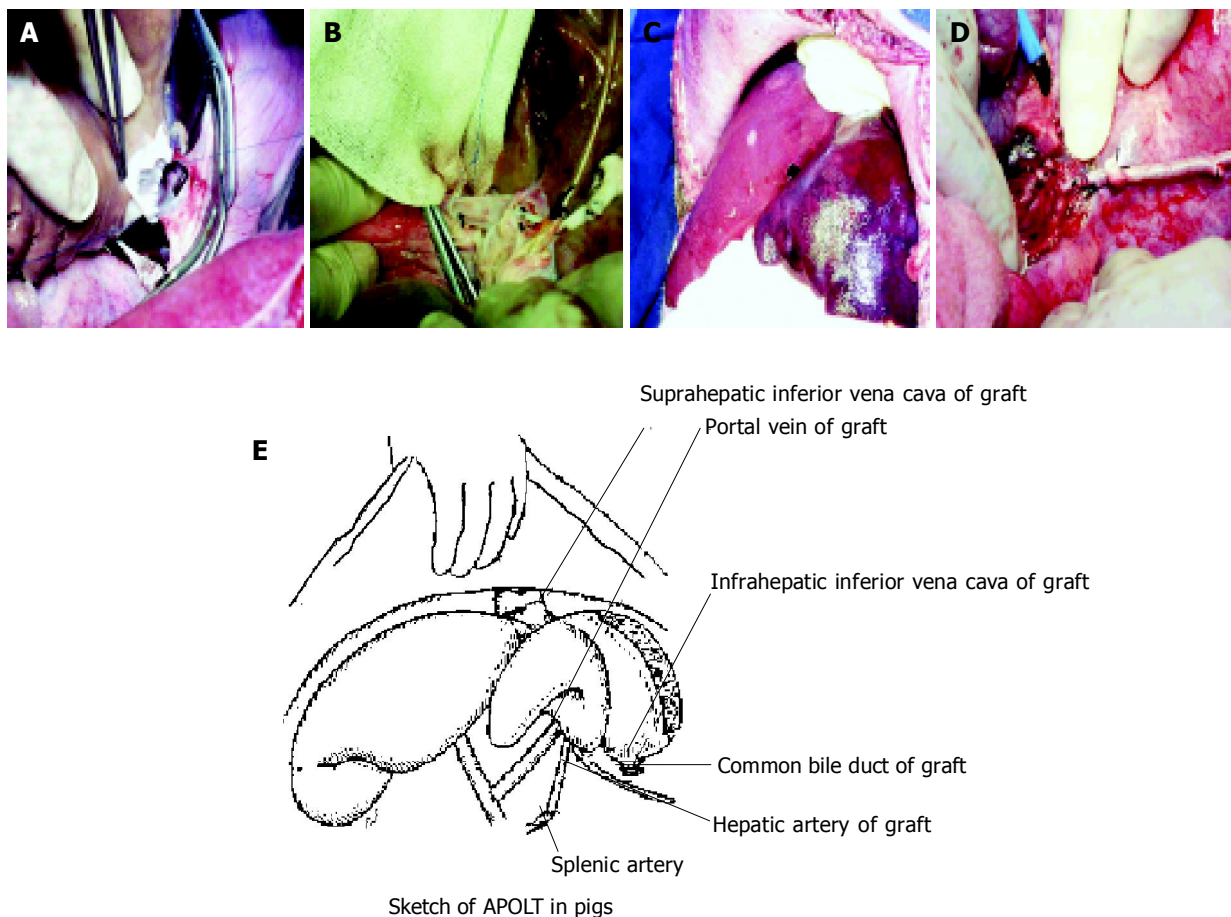


Figure 1A-E Surgical procedure of APOLT in pigs using right lobe graft.

method using the right liver graft. Immunosuppressant was not administered. Antibiotics were administered for three days after operation. During transplantation, arterial blood pressure and central venous pressure were recorded. Buffer base (BB), standard bicarbonate (SB) and pH of arterial blood sample were measured in order to assess the impact of operation on the animals. Color ultrasound examinations were performed 5 d after operation on selected recipients and autopsies were made on all of the recipients. Wedge biopsy specimens of every pig were obtained and stained with hematoxylin-eosin and then examined.

### Statistical analysis

Data were given as mean $\pm$ SE. Analysis of variance and *q* test were used to compare the change of hemodynamics and acid-base balance of the recipients in three groups. *P*<0.05 was considered statistically significant.

## RESULTS

Five recipient pigs were operated up on. Four of five (80%) survived for five days, one recipient could not recover from anesthesia, probably due to hyperkalemia and severe acidosis. The hemodynamics of the other four recipients recovered rapidly and stabilized after graft reperfusion. Color ultrasound examination showed an increase of blood flow of graft on 5<sup>th</sup> d compared to the first day after operation. When animals were killed on the 5<sup>th</sup> d after operation, thrombosis of hepatic vein (HV) and portal vein (PV) were not found. Histopathological examination of liver samples made at this time revealed evidence of damage with mild steatosis and sporadic necrotic hepatocytes and focal hepatic lobules structure disorganized in graft, especially in a pericentral venous area. Infiltration of inflammatory cells was mild in portal or central vein area.

Hematologic laboratory values and blood chemical findings revealed that mean arterial pressure (MAP), central venous pressure (CVP), buffer base (BB), standard bicarbonate (SB) and K<sup>+</sup> decreased when suprahepatic inferior vena cava, infrahepatic inferior vena cava and hepatic hilus were clamped. After reperfusion of the graft, MAP, CVP and K<sup>+</sup> restored gradually but BB, SB decreased further (Table 1).

**Table 1** Changes of hemodynamics and acid-base balance in APOLT (*n* = 5)

	MAP (mmHg)	CVP (cmH <sub>2</sub> O)	BB (mmol/L)	SB (mmol/L)	K <sup>+</sup> (mmol/L)
A	92.5 $\pm$ 11.8	5.6 $\pm$ 2.1 <sup>a</sup>	42 $\pm$ 7.0	21 $\pm$ 3	4.17 $\pm$ 1.08
B	68.5 $\pm$ 16.8 <sup>b</sup>	4.3 $\pm$ 1.4 <sup>a</sup>	38 $\pm$ 4.3 <sup>b</sup>	18 $\pm$ 3.4 <sup>b</sup>	3.19 $\pm$ 0.36 <sup>b</sup>
C	71.3 $\pm$ 15.4 <sup>b</sup>	4.3 $\pm$ 1.9	31 $\pm$ 4.0 <sup>b</sup>	11 $\pm$ 3.7 <sup>b</sup>	5.78 $\pm$ 1.64 <sup>b</sup>

Note: Before transplantation (A), after the portal vein was clamped (B), after reperfusion of the graft (C); MAP: mean arterial pressure, CVP: central venous pressure, BB: buffer base, SB: standard bicarbonate <sup>a</sup>*P*<0.05 vs group C; <sup>b</sup>*P*<0.01 vs group A.

## DISCUSSION

Recent advances in clinical liver transplantation have revealed that auxiliary partial orthotopic liver transplantation (APOLT)

is a feasible approach to treat patients with fulminant hepatic failure. Compared to standard orthotopic liver transplantation, APOLT shows us such advantages as the supporting role of remnant native liver against destroyed graft, withdrawal of immunosuppressant and application of small-for-size transplantation, in which remnant native liver can support insufficient graft function until the graft has regenerated sufficiently<sup>[5-8,11]</sup>. However, there are many unresolved problems in the clinical application of APOLT. The focus problem concerned by many authors was the functional competition resulted from the portal blood flow struggle between the graft and the native liver and the necessity of preemptive transaction of the portal branch to the native liver at the time of transplantation<sup>[12-14]</sup>. Other problems include deleterious effects of the necrotic liver on the graft function<sup>[15]</sup> and the difficulty in predicting regeneration of the remnant native liver before operation<sup>[16,17]</sup>. Thus, it is very important to establish stable experimental models using large animals to clarify these problems.

Welch first performed auxiliary total liver transplantation in 1955 and the first human auxiliary heterotopic liver transplantation was performed by Absolon in 1964. Since then auxiliary liver transplantation became an important branch of liver transplantation especially in treatment of fulminant hepatic failure. Recently, van Hoek reported that the best survival result, after liver transplantation for patients with acute liver failure, could be achieved after APOLT rather than HALT and orthotopic liver transplantation<sup>[18]</sup>. They also reported that 62% of all patients after auxiliary liver transplantation were free of immunosuppression within 1 year after transplantation. APOLT has been increasingly spread and developed well in Western countries<sup>[18-20]</sup>. Therefore, it is important to establish a perfect experimental model using large animals for improving the surgical skills of the modern APOLT team. Among the large animal models, pigs are considered the most realistic choice due to their low cost and availability<sup>[21]</sup>. The purpose of this study was to establish a new and stable pig model of APOLT and observe the changes of intraoperative hemodynamics and graft function in this model.

Taniguchi<sup>[22]</sup> reported their swine model of APOLT and preliminary data of the model for treatment of ischemic liver injury in 1998, but the operation pattern was very complicated. The characteristic anatomical structure of pigs, quite different from that of humans, lies in its short extrahepatic vena and the intrahepatic bifurcation of the hepatic veins, which lead to difficult anastomoses of hepatic vessels. Our model's advantages were as follows: (1) Operation was simple and safe. After the graft (right liver) was placed into the position of left lobe of recipient, the following anastomoses were then made: donor suprahepatic inferior vena cava end-to-side to host suprahepatic inferior vena cava, donor portal vein end-to-side to host portal vein, donor hepatic artery end-to-end to host spleen artery. The donor common bile duct was intubated for outside drainage. Furthermore, use of extracorporeal veno-venous bypass before the interruption of all hepatic vessels can markedly decrease the congestion of portal vein and obtain stable hemodynamics. (2) Under the premise of skilled performance of vascular anastomoses, centrifugal pump was employed

in extracorporeal veno-venous bypass. The anhepatic phase was controlled within 30 min and ischemia reperfusion injury of the host liver can be effectively prevented (3) Suture was performed on the section of the donor liver to prevent hemorrhage. End-to-side anastomoses of suprahepatic inferior vena cava were performed between the donor liver and recipient liver. Because of the simple vessel processing, unobstructed stoma and little postoperative hemorrhage could easily be achieved.

One of the puzzles of partial liver transplantation is hemorrhage on the section of the donor liver. The quantity of intraoperative bleeding is regarded as a significant risk factor relating to early mortality after liver transplantation. Blood coagulation disorder after reperfusion, incomplete surgical hemostasis and blood leakage at stoma are the main reasons for hemorrhage. In our experiment, hepatic parenchyma was split with forceps fracture method when the donor liver trimming and resection of liver lobes were performed *in vitro*. Hepatic vein and left branch of portal vein were sutured with Prolene thread under direct view. We recognized that hemorrhage on the section of the donor liver would not happen during and after operation if the graft had vitality. The heparin consumption of pigs was twice as much as that of human when thorough heparinization was obtained. The related check results still stayed at normal levels even if widespread blood permeation had taken place. Therefore, the proper administration of heparin and hemostatic drugs was a key procedure to prevent blood coagulation disorders.

In APOLT, the reperfusion of graft and the metabolic changes during operation consequentially lead to relevant hemodynamic change. Our experiment proved that there were certain rules about hemodynamic change in APOLT: Hemodynamics was stable before the onset of new liver's blood circulation. MAP decreased and HR speeded up after onset of blood circulation of portal vein of donor liver (compared to preoperation) ( $P < 0.01$ ). The change of CVP was inapparent. We believe that the abrupt decrease of returned blood volume was the main reason for hemodynamic change at this phase. After such treatments as accelerating fluid replacement and blood transfusion, MAP and HR could restore to stability. Plenty of toxin released from recipient liver due to reperfusion injury, hypothermia and plenty of fluid containing potassium in liver or kidney preservation solution, *etc.*, usually resulted in metabolic acidosis and hyperpotassemia after the onset of new liver's blood circulation. One of the key procedures, that had to be stressed in this model, was the correcting of metabolic acidosis and hyperpotassemia.

The severity of congestion in portal vein system was relevant to two factors in APOLT. One was portal vein's blockage period. Pigs were considered to be sensitive to the congestion of portal vein system blockage. Obvious intestinal mucosa injury took place 45 min after blockage<sup>[22]</sup>. Another was retention blood flow in portal vein system. Pigs have short limbs and strong gastrointestinal tract. Blood flow in portal vein is plentiful. Severe congestion in portal vein system would certainly lead to whole body's hemodynamic disorder. The exsanguine liver period of recipient group all lasted less than 40 min. The changes of MAP, CVP,  $K^+$ ,

PCO<sub>2</sub>, BB and SB took place after the blockage of hepatic hilus, suprahepatic and infrahepatic inferior vena cava (that was anhepatic phase). But the change gradually restored as portal vein was reperfused.

Our experiments proved we have established a new operative and ideal large animal model of APOLT. We predict this model may contribute to experimental or clinic APOLT research.

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• CASE REPORT •

# Inflammatory pseudotumor of the liver and peripheral eosinophilia in autoimmune pancreatitis

Naoki Sasahira, Takao Kawabe, Akira Nakamura, Kenji Shimura, Haruhisa Shimura, Ei Itobayashi, Manabu Asada, Yasushi Shiratori, Masao Omata

Naoki Sasahira, Takao Kawabe, Masao Omata, Department of Gastroenterology, University of Tokyo, Tokyo 113-8655, Japan  
Akira Nakamura, Kenji Shimura, Haruhisa Shimura, Ei Itobayashi, Manabu Asada, Department of Gastroenterology, Asahi General Hospital, Chiba, Japan  
Yasushi Shiratori, Department of Gastroenterology, University of Okayama, Okayama, Japan

Correspondence to: Naoki Sasahira, M.D., Department of Gastroenterology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. sasahira-ky@umin.ac.jp  
Telephone: +81-3-3815-5411

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## Abstract

**AIM:** Inflammatory pseudotumor (IPT) of the liver is a rare benign lesion, the etiology of which remains obscure. It is not associated with any particular diseases apart from phlebitis and Crohn's disease.

**METHODS:** A middle-aged male with hepatic IPT and peripheral eosinophilia associated with autoimmune pancreatitis (AIP) was selected for this study and review of literature.

**RESULTS:** A 59-year-old male was admitted with obstructive jaundice, marked eosinophilia ( $1\,343/\text{mm}^3$ ) and hypergammaglobulinemia ( $4\,145\text{ mg/dL}$ ). Imaging techniques revealed dilatation of the intrahepatic bile duct, stenosis of the common bile duct with diffuse wall thickening, gallbladder wall thickening, irregular narrowing of the pancreatic duct, and swelling of the pancreatic parenchyma. Multiple liver masses were also demonstrated and diagnosed as IPT by biopsy specimens. Six months later, the abnormal features of the biliary tree remarkably improved by the oral administration of prednisolone, and the liver masses disappeared. The swelling of the pancreatic head also improved. The peripheral eosinophil count normalized. IPT associated with AIP, as we know, has not been reported in the literature. The clinical features of the present case mimicked those of pancreatic cancer with liver metastasis. This case deserves to be documented to prevent misdiagnosis of similar cases.

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**Key words:** Inflammatory pseudotumor; Autoimmune pancreatitis

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## INTRODUCTION

Inflammatory pseudotumor (IPT) is a rare benign lesion histologically characterized by the presence of a heterogeneous population of inflammatory cells, particularly plasma cells, macrophages and fibroblasts, as well as areas of fibrosis and necrosis. Pack *et al*<sup>[1]</sup> first described IPT of the liver in 1953. Despite numerous subsequent descriptions in the literature<sup>[2-5]</sup>, IPT remains difficult to diagnose, causing major problems particularly for differential diagnosis especially that with malignant liver tumor. There are neither specific signs in imaging techniques, nor conclusive biochemical tests. Histological examinations are always required for confirmation of the diagnosis. The etiology of IPT remains obscure to date, especially in the absence of documented evidence of its having associations with any particular diseases apart from phlebitis<sup>[6]</sup> and Crohn's disease<sup>[7]</sup> shown in a few reports.

For our study, we selected a middle-aged male patient with hepatic IPT and peripheral eosinophilia associated with autoimmune pancreatitis (AIP). AIP is a recently recognized disorder and new clinical entity associated with irregular narrowing of the pancreatic ducts and swelling of the parenchyma<sup>[8-10]</sup>. AIP is difficult to diagnose differentially from pancreatic cancer. When liver tumors are found in AIP, there is a possibility of a misdiagnosis with pancreatic cancer with liver metastasis. Thus, the documentation of this case may be of value for the prevention of misdiagnosis in similar cases.

## CASE REPORT

A 59-year-old man was admitted with obstructive jaundice. He had no remarkable familial or personal medical histories. He had taken no drugs prior to admission. Physical examination on admission revealed no abnormal findings except for the jaundice.

Abnormal laboratory findings were as follows: erythrocyte sedimentation rate (ESR) 60 mm/hr, CRP 0.6 mg/dL, white blood cell count  $10\,100/\text{mm}^3$  with 13.3% eosinophils ( $1\,343/\text{mm}^3$ ), serum IgE 1481 IU/mL, IgG 4145 mg/dL, total bilirubin 11.6 mg/dL, ALP 655 IU/L. Serum amylase,

ALT and AST were not elevated. CEA and CA19-9 remained within normal range. Autoantibodies were negative except for rheumatoid factor. Antibodies for hepatitis virus and HIV were negative. Stool specimens showed no ova or parasites.

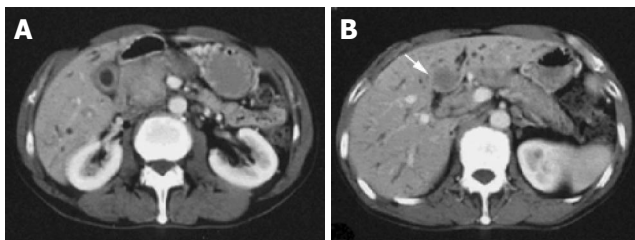
Ultrasonography and CT scan revealed dilatation of the intrahepatic bile duct, common bile duct stenosis with diffuse wall thickening, gallbladder wall thickening and swelling of the pancreas (Figure 1). Multiple liver masses were also demonstrated as low echogenic areas by ultrasonography (Figure 2), and they were not enhanced by contrast medium on CT. Magnetic resonance cholangiopancreatography (MRCP) revealed the stricture of the common bile duct and the markedly dilated intrahepatic bile duct (Figure 3). Endoscopic retrograde cholangiopancreatography (ERCP) was not successful, but the brush cytology specimens obtained from the distal bile duct and distal pancreatic duct contained considerable numbers of eosinophils though malignancy was not indicated by the cytology tests.

Percutaneous biopsy of the liver masses was performed

under ultrasonographic guidance. Histology showed mixed inflammatory infiltrates composed of lymphocytes, histiocytes, plasma cells, and a prominent number of eosinophils. There was no evidence of epithelial, mesenchymal or lymphoid malignancy (Figure 4).

Prednisolone (40 mg/d) was administered orally. Four weeks later, the bile duct stricture, common bile duct wall thickening and gallbladder wall thickening improved remarkably. The liver masses resolved. The swelling of the pancreatic head also improved. The steroid dose was tapered gradually, and 6 mo later he took 5 mg of prednisolone. At this point, the biliary and pancreatic abnormal findings had completely disappeared (Figure 5), and the peripheral eosinophil count had also become normal (Figure 6).

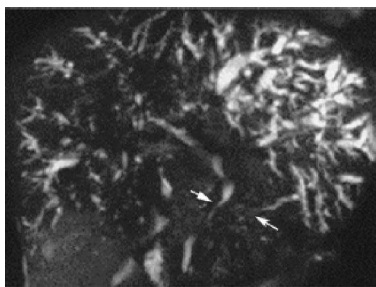
Two years after remission of the disorder, serum immunoglobulin IgG4 was assayed. The serum level of IgG4 remained markedly elevated to 1 810 mg/dL. The patient, on a maintenance regimen of 5 mg of prednisolone, was in good health, and the features of the disorder remained resolved.



**Figure 1** A: CT demonstrated dilatation of the intrahepatic bile ducts, swelling of the pancreatic head, thickening of the gallbladder wall; B: CT revealed the liver mass, which was not enhanced by contrast medium.



**Figure 2** Ultrasonography demonstrated hypoechoic liver mass.



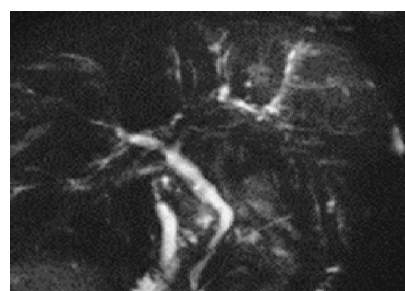
**Figure 3** MRCP showed stricture of the common bile duct with marked dilatation of the intrahepatic bile duct and irregular narrowing of the pancreatic duct.



**Figure 4** Biopsy specimens from the liver mass showed lymphocytes, histiocytes, plasma cells, and a number of eosinophils. It did not contain normal liver tissue or malignant cells.



**Figure 5** CT revealed that the pancreatic swelling almost resolved 1 mo after the treatment. Liver masses decreased.



**Figure 6** MRCP demonstrated the normalized bile duct 6 mo after the treatment.

## DISCUSSION

The clinical features of the present case were similar to those of pancreatic cancer with obstructive jaundice and liver metastasis. The liver masses were diagnosed as inflammatory pseudotumor (IPT) by histological examination of biopsy specimens. IPT is a benign lesion with the presence of a heterogeneous population of inflammatory cells, particularly plasma cells, macrophages and fibroblasts, as well as areas of fibrosis and necrosis. However, it is difficult to distinguish this lesion from malignant tumor. The diagnostic challenge of IPT is emphasized by the fact that most of the reported cases were diagnosed by surgical procedures including either wedge resection or lobectomy. In such intricate cases as the present one, careful attention must be paid so that misdiagnosis and resultant inappropriate treatment can be avoided.

The most conspicuous finding in the laboratory tests was the marked peripheral eosinophilia. This might suggest a diagnosis of hypereosinophilic syndrome (HES). HES is diagnosed when the following criteria are met: Persistent eosinophilia ( $1\ 500$  eosinophils/ $\text{mm}^3$ ) for at least 6 mo or death before 6 mo with signs and symptoms of HES disease; exclusion of other recognized causes of eosinophilia; organ system involvement or dysfunction attributable to eosinophil infiltration or not otherwise explained<sup>[11]</sup>. In the present case, the peripheral eosinophil count was lower than  $1500/\text{mm}^3$ , and the condition persisted for only 2 mo. Thus, a case like this should not be diagnosed as HES, despite the fact that possible causes of eosinophilia such as parasitic infection had been excluded. Nonetheless, it cannot be ruled out that eosinophilia might play a role in the pathogenesis of the clinical features presented.

Another conspicuous feature was obstructive jaundice caused by biliary stricture with common bile duct wall thickening. We first considered that this obstructive jaundice was associated with eosinophilic cholangitis (EC). Butler first reported EC in 1985, which was associated with obstructive jaundice, lymphadenopathy and peripheral eosinophilia, and showing marked eosinophilic infiltration in the gallbladder and cystic duct as well as mild infiltration in the liver<sup>[12]</sup>. Subsequently, a number of cases with similar conditions were reported under various nomenclatures, including primary biliary sclerosing cholangitis with eosinophilic infiltration and eosinophilic gastroenteritis with biliary involvement as well as EC. In our patient, an additional feature was the swelling of the pancreas, whereas pancreatic involvement was never observed in the reported cases with EC<sup>[13-21]</sup>. Furthermore, the mean age of the patients with EC was 34 years (range 20-48), while our patient was 59. Our patient showed marked hypergammaglobulinemia (IgG  $4\ 145$  mg/dL), a finding not reported in EC cases except for one with mild serum IgG elevation of  $1\ 700$  mg/dL<sup>[14]</sup>. Thus, it was hardly conceivable that the present case belonged to a category of eosinophilic cholangitis or the like.

It is in this regard that we think of autoimmune pancreatitis (AIP) for a disorder manifesting the aforesaid features. AIP is a recently recognized unique form of chronic pancreatitis with irregular narrowing of the pancreatic duct and swelling of the pancreatic parenchyma<sup>[8-10]</sup>. This condition was initially described as chronic pancreatitis associated with

hypergammaglobulinemia by Sarles *et al*<sup>[18]</sup> in 1961. Since then, AIP has been described mainly in Japanese literature. Multiple definitions are given for this condition, including primary inflammatory sclerosis of pancreas, sclerosing pancreatitis, lymphoplasmacytic sclerosing pancreatitis, autoimmune sclerosing pancreatitis and sclerosing pancreato-cholangitis. As those terms indicated, the affected organs are not limited to the pancreas. The biliary tree is the most frequent location involved<sup>[19]</sup>. Okazaki *et al*<sup>[10]</sup> reported that obstructive jaundice was observed in 40% of their series. The increase of peripheral eosinophil count is often observed in AIP<sup>[9]</sup>. Furthermore, the age of AIP patients was reported to be an average of 59-61 years<sup>[10,20]</sup>, clearly higher than that of eosinophilic cholangitis.

Hamano *et al*<sup>[21]</sup> reported that patients with AIP had high serum IgG4 concentrations, the finding of which is usually observed in only a limited number of conditions, including autoimmune skin diseases such as pemphigus vulgaris and pemphigus foliaceus. Recently, the IgG4 assay has been recognized as the most useful test for the diagnosis of AIP. The present case showed a high serum IgG4 concentration of  $1\ 810$  mg (normal range  $<135$  mg/dL).

Based on our findings, the diagnosis of this case was AIP with IPT, a condition not previously described in the literature. Recently, IPT was associated with an immune reaction<sup>[22,23]</sup>. It is entirely possible that IPT is associated with AIP.

To date, several treatment approaches, including antibiotics<sup>[24]</sup>, nonsteroidal anti-inflammatory drugs<sup>[25]</sup>, have been described for IPT of the liver. The spontaneous resolution of the lesion after a few months has been reported<sup>[26]</sup>. The prognosis of this lesion is considered favorable in the majority of cases. The liver mass of our patient was successfully treated with prednisolone. However, there had been a few cases having undergone surgical resection or liver transplantation<sup>[27-29]</sup>. Some of them could have avoided surgery if preoperative diagnosis was certainly made.

In conclusion, a case of hepatic IPT and peripheral eosinophilia in AIP was reported. The present case deserves to be documented. This is helpful in avoiding misdiagnosis and inappropriate treatment in similar cases.

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• CASE REPORT •

## Undifferentiated (embryonal) sarcoma of liver in adult: A case report

Chao-Liu Dai, Feng Xu, Hong Shu, Yong-Qing Xu, Yong Huang

Chao-Liu Dai, Feng Xu, Yong-Qing Xu, Yong Huang, Department of Hepatobiliary Surgery, the 2<sup>nd</sup> Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China  
Hong Shu, Department of Pathology, the 2<sup>nd</sup> Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China

Correspondence to: Professor Chao-Liu Dai, Department of Hepatobiliary Surgery, the 2<sup>nd</sup> Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China. daicl-sy@163.net

Telephone: +86-24-83955052 Fax: +86-24-23926097

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### Abstract

We report a case of undifferentiated (embryonal) sarcoma of the liver (UESL), which showed cystic formation in a 20-year-old man with no prior history of any hepatitis or liver cirrhosis. He was admitted with abdominal pain and a palpable epigastric mass. The physical examination findings were unremarkable except for a tenderness mass and the results of routine laboratory studies were all within normal limits. Abdominal ultrasound and computed tomography (CT) both showed a cystic mass in the left hepatic lobe. Subsequently, the patient underwent a tumor excision and another two times of hepatectomy because of tumor recurrence. Immunohistochemical study results showed that the tumor cells were positive for vimentin, alpha-1-antichymotrypsin (AACT) and desmin staining, and negative for alpha-fetoprotein (AFP), and eosinophilic hyaline globules in the cytoplasm of some giant cells were strongly positive for periodic acid-Schiff (PAS) staining. The pathological diagnosis was UESL. The patient is still alive with no tumor recurrence for four months.

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**Key words:** Liver undifferentiated(embryonal) sarcoma

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### INTRODUCTION

Undifferentiated (embryonal) sarcoma of the liver (UESL) is a rare malignant hepatic tumor with poor prognosis that often presents in the pediatric population between the ages

of 6 and 10 years, and rarely occurs in adults. UESL, first documented by Stocker and Ishak<sup>[1]</sup> in 1978, has been recognized as a unique clinicopathologic entity. This tumor usually appears on CT and ultrasound as a predominantly solid mass with or without cystic areas. We report a case of primary UESL, which showed cystic formation in an adult.

### CASE REPORT

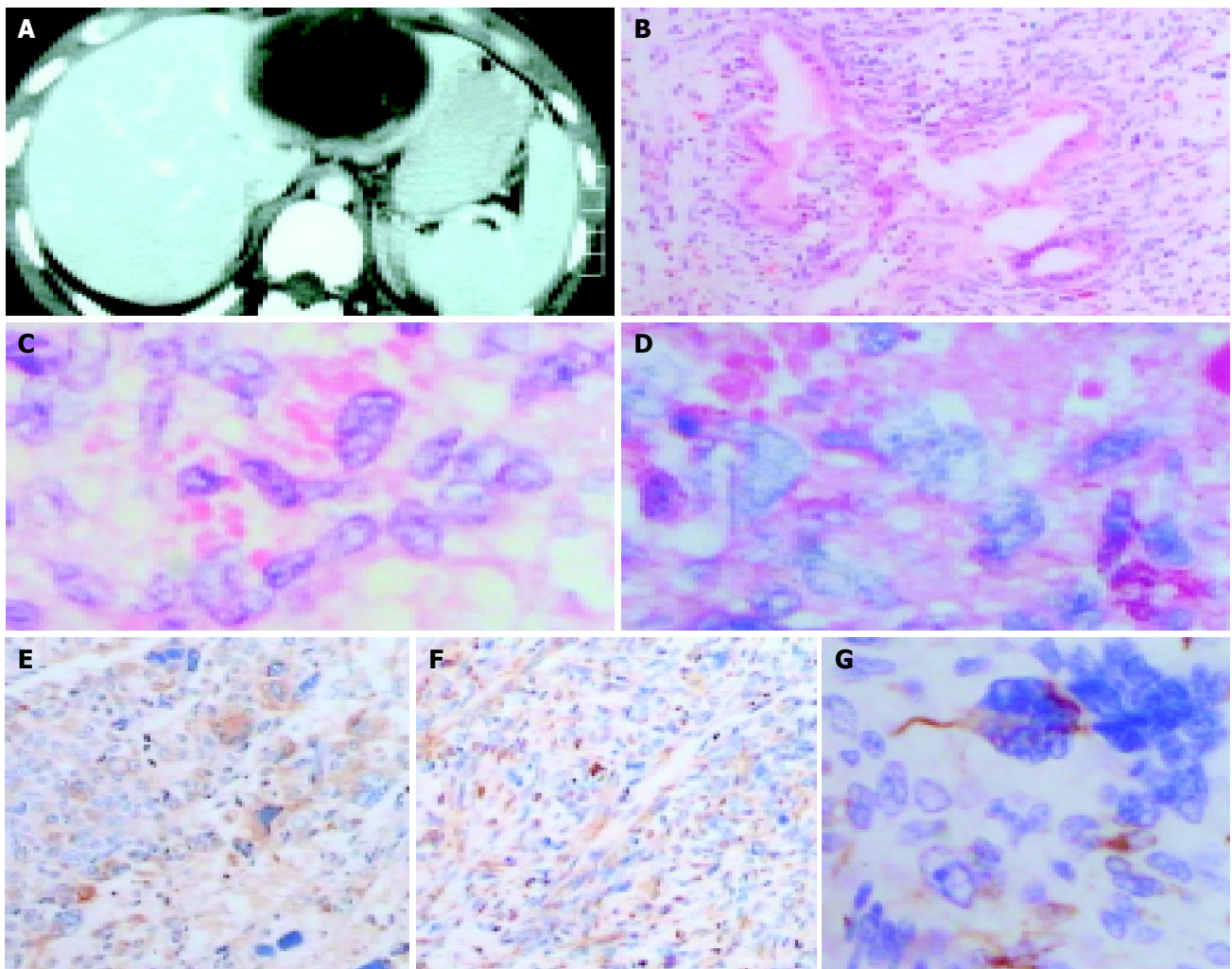
A 20-year-old male was presented with a palpable epigastric mass and upper abdominal pain and of 5 d' duration. Five days ago, he abruptly suffered from intermittent sharp pain located at slightly-left side below the xiphoid process, which was aggravated on deep breath or raise of abdominal pressure but relieved at quiet rest, with no predisposing cause, and a fist-like mass was palpated in the epigastrium. He was admitted due to a cystic mass in the left lobe of the liver, revealed by ultrasonography on October 16, 2000. He was previously in good health. His family and past histories were not contributory. Physical examination on admission disclosed that his body temperature was 36.4 °C, pulse rate was 84 beats/min, respiratory rate was 16 breaths/min and blood pressure was 130/80 mmHg. He was in his right senses and well-nourished. Yellow skin, icteric sclera, spider naevi and palmar erythema were not noted. Cardiopulmonary examination was unremarkable. The upper abdomen was slightly swollen, but the abdomen was soft. A tender, smooth-faced mass, measuring 6 cm×6 cm, was palpable at slightly left side of the mid-epigastric abdomen. He felt a little pain when we percussed the right hypochondrium. Laboratory data showed liver function test was normal, hepatitis markers of HAV, HBV, HCV, etc. were negative and AFP was 55 ng/mL. The chest radiograph showed no pulmonary abnormality. Abdominal sonography showed that an 8.4 cm×5.9 cm multiloculated cystic lesion with a few internal septations, a peripheral integrated capsule and papillary protrusion extending inward the cystic cavity was located in the left lobe of the liver. The thickest portion of the wall of the cyst was 1.28 cm. These findings supported the diagnosis of a cystic occupied lesion of the left lobe of the liver. Abdominal unenhanced computed tomography (CT) showed that a 6 cm×7 cm mass in the left hepatic lobe with a few internal septations projected to the anterior abdominal wall, and the mass was predominantly hypodense with an attenuation value of 9-25 HU. On dynamic contrast CT, there was a thick-walled mass with marked enhancement of its septations and capsule with an attenuation value of 9-44 HU (Figure 1A). No other abnormality was seen in the rest of the liver or in other abdominal viscera. On account of these features, a cystic occupied lesion of the

left lobe of the liver was suspected. So the initial diagnosis was hepatic cyst.

After preoperative preparation, the patient underwent an exploratory laparotomy under general anesthesia. During operation, a 10 cm×9 cm×6 cm cystic mass with ruptured capsule and abdominal invasion, which was located at the left exterior lobe of the liver, was noted, and there were also large quantities of blood clots and about 100 mL of bloody fluid near the mass. The right hepatic lobe was normal and no other tumor was found in the abdominal cavity. Bloody fluid was aspirated with fine-needle from the mass that contained not only fluid, but also a lower proportion of solid ingredients. A portion of neoplastic wall of biopsy was sent to pathology laboratory for frozen section, and the pathological report suggested the mass was hepatic cyst and microscopically some cells were atypia. Subsequently, hepatic segmentectomy of S2, S3 was performed because we considered the mass might be malignant. The pathological gross specimen showed the tumor

was a multilocular cystic mass with mastoid protuberances in the intracavity on cut section. On microscopic examination postoperatively, the tumor consisted of inhomogeneous glandular cavities with some secretion. The tumor cells with giant nuclei deeply stained were spindle shaped, polygonal and predominantly atypical. The cells were distributed diffusely (Figure 1B). Pathological diagnosis was malignant tumor of the liver (adenofibrosarcoma?).

The epigastric pain recurred in July 2002. Abdominal sonography and CT both showed the mass was a cystic tumor, and was close to the previous resection margin of the left hepatic lobe. Subsequently, the patient underwent an intervention of hepatic segmentectomy of S4. At surgery, the cystic cavity of the tumor with inhomogeneous wall was full of bloody fluid and its anterior part adhered to abdominal wall. Postoperative pathology revealed that most of cystic wall was organized, and there were only a few spindle shaped, polygonal atypical cells and no adenoid structure.



**Figure 1** The appearance of UESL in enhanced CT, H&E stain, PAS staining and immunohistochemical assay. A: Enhanced CT scan of primary tumor. It shows a well encapsulated, multiloculated cystic mass in left hepatic lobe containing internal septations and mastoid protuberances in the intracavity, and the mass projects to the anterior abdominal wall; B: Microphotograph of primary tumor showing adeniform structure surrounded by predominantly atypical, pleomorphic sarcomatous cells (H&E stain, ×100); C: Eosinophilic hyaline globules seen in the cytoplasm of some tumor cells (H&E stain, ×400); D: Eosinophilic hyaline globules showing strongly positive for periodic acid-Schiff (PAS) staining (×400); E: Immunohistochemical assay revealing spindle shaped, polygonal, multinucleated or macronuclear cells with positive staining for vimentin (SP, ×100); F: Immunohistochemical feature presenting some anaplastic tumor cells with positive staining for alpha-1-antichymotrypsin (AACT) (SP, ×100); G: Immunohistochemical assay showing many pleomorphic tumor cells with positive staining for desmin (SP, ×400).

During follow-up, a tumor was discovered in the right upper abdomen in July 2003. Abdominal ultrasonography and CT both showed a solid-to-cystic lesion followed by surgical resection of the tumor. Gross appearance of the lesion on section was reddish white fish-meat-like and the cystic cavity with inhomogeneous wall was filled with bloody fluid. Postoperative histopathology on microscopic examination showed that the cells with frequent abnormal mitosis were spindle shaped, polygonal, multinucleated or macronucleus, and variable numbers of eosinophilic hyaline globules in the cytoplasm of some giant cells were seen (Figure 1C), which were strongly positive for periodic acid-Schiff (PAS) staining (Figure 1D). There were mucinous degeneration of mesenchyme and no adenoid structure. Immunohistochemical study (SP method) results showed that the tumor cells were positive for vimentin (Figure 1E), alpha-1-antichymotrypsin (AACT) (Figure 1F) and desmin staining (Figure 1G), and negative for alpha-fetoprotein. The pathological diagnosis was UESL.

The postoperative course was uneventful with no adjuvant chemotherapy and then the patient was discharged. He has been receiving regular follow-up examinations at the outpatient department and is still alive with no tumor recurrence for four months.

## DISCUSSION

Cystic-occupied lesions of liver were classified according to pathogenesis by Henson<sup>[2]</sup> in 1956 into four types: (1) congenital cyst of liver, including solitary (single) and diffuse forms; (2) traumatic cyst; (3) inflammatory hepatic cyst, including specific and nonspecific ones; and (4) tumorous hepatic cyst, including benign and malignant forms. Cystadenoma and cystadenocarcinoma are the main forms of tumorous hepatic cyst. Moreover, ischemic necrosis followed by cystic change, attributed to rapid growth of the tumor, may occur at the central part of the mass of primary hepatocarcinoma. Hepatic sarcoma is of low incidence in malignant tumors of the liver, and those that present cystic change are especially rare.

UESL, also called malignant mesenchymoma of the liver, is a rare tumor that most often presents in late childhood (6-10 years old) but infrequently in adults. Less than 50 cases of UESL in adults have been reported within 40 years up to 2003 in the world-wide literature<sup>[3-8]</sup>. Grossly, it is usually a large, solitary and well-circumscribed mass with variable areas of hemorrhage, necrosis and cystic degeneration<sup>[1]</sup>. Some lesions may be with a capsule or pseudocapsule. Microscopically, the tumor is predominantly composed of a mixture of highly atypical spindle-shaped and giant cells<sup>[8]</sup>. The presence of malignant fibrous histiocytoma-like, rhabdomyosarcoma-like or fibrosarcoma-like areas can be seen. Tumor cells arrange loosely or densely within myxoid matrix. The larger cells often contain numerous intracytoplasmic eosinophilic hyaline globules that are strongly positive for PAS staining, and caryomitosis can be seen. Scattered hyperplastic or degenerating bile duct-like structures surrounded by tumor cells are seen in most cases, and considered to be the residue of normal bile duct or, much more likely, be the constituent of the tumor<sup>[9-11]</sup>. But the pathohistologic feature in this case suggested that duct-like

structure might be present in primary tumor, but not in secondary tumor. We drew a conclusion that the duct-like structure in this case might be the residue of normal bile duct. On immunohistochemical analysis of documentary reports, the tumor cells were positive for vimentin, alpha-1-antichymotrypsin (AACT), alpha-1-antitrypsin ( $\alpha$ 1AT), lysozyme, desmin, smooth muscle actin and cytokeratin<sup>[8-12]</sup>. The peculiar combination of phenotypical features has led a couple of authors to postulate a histogenetic relationship with primary hepatic embryonal rhabdomyosarcoma<sup>[11]</sup>, and others to suggest that the tumor could originate from mesenchymal hamartoma<sup>[13,14]</sup>.

UESL has no specific clinical features. The most common clinical presentation includes hepatomegaly, an upper abdominal mass, which was accompanied by abdominalgia or not, and weight loss. Occasionally, tumoral spontaneous rupture may result in intraperitoneal hemorrhage due to rapid growth of the tumor. Different from primary carcinoma of the liver, UESL has no relation to hepatitis or liver cirrhosis, no disturbance of hepatic function or elevation of AFP. CT and magnetic resonance image (MRI) features of cystic change are usually different from that of solid-to-cystic change revealed by sonography or upon macroscopy<sup>[15]</sup>. The cystic change may be the result of hemorrhage or necrosis during tumor growth. In our case, both the primary and the first recurrent tumor mainly displayed cystic change, but the second recurrent mass presented with solid-to-cystic change and solid type predominance. Bloody fluid in the cystic cavity of the primary or recurrent tumor mass suggested that cystic degeneration had relation to the presence of hemorrhage or necrosis in the tumor. But, on the other hand, the inner wall of the cystic cavity was rather smooth, and we could deduce that the tumor had a tendency to develop a cyst spontaneously. The clinical diagnosis of UESL is rather difficult, so is the differential diagnosis, such as malignant fibrous histiocytoma. The ultimate diagnosis relies on pathological examination. Immunohistochemical and electron microscopic examinations are supposed to be carried out if it is difficult to evaluate tumoral histogenesis. In our case, the definite diagnosis was not made until PAS staining and immunohistochemical examination were applied to the second recurrent tumor mass. Radical resection of the tumor is the optimal treatment of choice<sup>[16]</sup>. The prognosis for UESL has been poor to date and majority of the patients died of tumor recurrence or metastasis within two years after operation<sup>[1,17,18]</sup>. Recent researches have shown that pre- and/or post-operative systemic chemotherapy (with cisplatin, adriamycin, cyclophosphamide) and/or radiotherapy, when necessary, can remarkably improve patients' survival<sup>[19,20]</sup>. It has been reported that a patient has been free from tumor for 20 years postoperatively.

In conclusion, through the diagnosis and treatment of the case, several points are worth recommending. First, we need to extend our understanding of cystic change of malignant tumor of liver, such as sarcoma. Hepatic cystic mass with abrupt abdominalgia, which should be paid special attention to, usually suggests spontaneous rupture or intra-tumor hemorrhage. Second, we should study imaging features of hepatic cystic lesion comprehensively in

differential diagnosis of malignant mass or not. In our case, CT and sonography both revealed the primary tumor with complete capsule, heterogeneous cystic wall with thickness, a few internal septations of cystic cavity, papillary protrusion extending inward the cavities and especially prominent protrusion of parenchyma of the tumor attributed to the outward growth. These imaging features are seldom seen in benign cysts, such as congenital solitary hepatic cyst. Third, we should carry out timely excision of non-traumatic cyst suspected of malignancy when bloody fluid was drawn out with fine needle under the direction of ultrasound or revealed inside cyst during operation. Fourth, frozen section, during operation, is of great value for ascertaining the nature of tumor. But for fear of false-negative results, the material should be precisely harvested from the proper tissue. And last, post-operative follow-up, is necessary for the patients with hepatosarcoma as well as for those with primary carcinoma of liver. Once there is an evidence of recurrence, resection of the tumor wherever feasible should be performed in most cases.

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**Andrew Kenneth Burroughs, Professor**

Department of Liver Transplantation and Hepatobiliary Medicine, Royal Free Hospital, Pond Street Hampstead, London NW3 2QG, United Kingdom

**Xian-Ming Chen, M.D.**

Mayo Medical School, Clinic and Foundation, 200 First Street, SW, Rochester, MN 55905, United States

**Zong-Jie Cui, Professor**

Institute of Cell Biology, Beijing Normal University, Beijing 100875, China

**Abraham Rami Eliakim, Professor**

Gastroenterology, Rambam Medical Center, Technion School of Medicine, P.O.B.9602, Haifa 31096, Israel

**Xue-Gong Fan, Professor**

Xiangya Hospital, Changsha 410008, China

**Fu-Lian Hu, Professor**

Department of Gastroenterology, Peking University First Hospital, 8 Xishiku St, Xicheng District, Beijing 100034, China

**Robert J Korst, M.D.**

Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United States

**Jie-Shou Li, M.D.**

Academician of Chinese Academy of Engineering, Department of General Surgery, General Hospital of Nanjing Command Area, 305 East Zhongshan Road, Nanjing 210002, Jiangsu Province, China

**Robin G Lorenz, Associate Professor**

Department of Pathology, University of Alabama at Birmingham, 845 19th Street South BBRB 730, Birmingham, AL 35294-2170, United States

**Sasa Markovic, Professor**

Head, Department of Gastroenterology, University Clinical Center Ljubljana, 2 Japljeva 1525 Ljubljana, Slovenia

**Chun-Yang Wen, M.D.**

Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

**Yuan Wang, Professor**

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

**Benjamin Wong, M.D.**

Department of Medicine, University of Hong Kong, Pokfulam Road, Pokfulam, Hong Kong, China

**Wai-Man Wong, M.D.**

Department of Medicine, University of Hong Kong, St Paul's Hospital, 2 Eastern Hospital Road, Causeway Bay, Hong Kong, China

**Hong Xiao, Professor**

Managing Director, Acta Pharmacologica Sinica, 294 Taiyuan Road, Shanghai 200031, China

**Yuan Yuan, Professor**

Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

**Man-Fung Yuen, Associate Professor**

Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong, China

**Michael Zenilman, Professor**

Department of Surgery, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn NY, United States

**Mu-Jun Zhao, M.D.**

Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

**Zhi-Rong Zhang, Professor**

West China School of Pharmacy, Sichuan University, 17 South Renmin Road, Chengdu 610041, Sichuan Province, China

**Xiao-Hang Zhao, Professor**

State Key Laboratory of Molecular Oncology, Cancer Institute of Chinese Academy of Medical Sciences, 17 Panjiayuan, Chaoyangqu, Beijing 100021, China

**Shu Zheng, Professor**

Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, China

## 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

**Canadian Digestive Disease Week Con-  
ference**  
February 26-March 6, 2005  
www.cag-acg.org  
Banff, AB

**International Colorectal Disease  
Symposium 2005**  
February 3-5, 2005  
info@icds-hk.org  
Hong Kong

**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

**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 106<sup>th</sup>  
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 Sym-  
posium 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 Thera-  
peutic Endoscopy**  
September 10-12, 2005  
www.alfamedical.com  
Theodor Bilharz Research Institute

**EASL 2005 the 40<sup>th</sup> 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 106<sup>th</sup>  
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**  
October 20-25, 2006  
Venetian Hotel, Las Vegas, Nevada

## Instructions to authors

### GENERAL INFORMATION

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#### Key words

Please list 3-10 key words that could reflect content of the study.

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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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Data which is not statistically significant should not be noted. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 (*P*>0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P*<0.05 and <sup>d</sup>*P*<0.01 are used; Third series of *P* values can be expressed as <sup>e</sup>*P*<0.05 and <sup>f</sup>*P*<0.01. Other notes in tables or under

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.

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- 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 biliary 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>

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### Statistical data

Present as mean±SD and mean±SE.

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

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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<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub> not 5% CO<sub>2</sub>; 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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Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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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	1NHCl	1 mol/L HCl	
24	1NH <sub>2</sub> SO <sub>4</sub>	0.5 mol/L H <sub>2</sub> SO <sub>4</sub>	
25	4rd edition	4 <sup>th</sup> edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku, 18 500u or M <sub>r</sub> 18 500	
28	25 g·kg <sup>-1</sup> /d <sup>-1</sup>	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·L <sup>-1</sup>	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	GeneBank	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 <sub>260</sub>	A <sub>260</sub>	"OD" has been abandoned.
43	Oneg/L	One microgram per liter	At the beginning of a sentence
44	A <sub>260 nm</sub> <sup>b</sup> P<0.05	A <sub>260 nm</sub> <sup>a</sup> P<0.05	A should be in italic. In Table, no note is needed if there is no significance in statistics: <sup>a</sup> P<0.05, <sup>b</sup> P<0.01 (no note if P>0.05). If there is a second set of P value in the same table, <sup>c</sup> P<0.05 and <sup>d</sup> P<0.01 are used for a third set: <sup>e</sup> P<0.05, <sup>f</sup> P<0.01.
45	*F=9.87, <sup>§</sup> F=25.9, <sup>#</sup> F=67.4	<sup>1</sup> F=9.87, <sup>2</sup> F=25.9, <sup>3</sup> F=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
57	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 <sup>3</sup>	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$\bar{x} \pm s$	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 <i>et al</i>	
72	EcoRI	EcoRI	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	