• ESOPHAGEAL CANCER •

# Production of a human single-chain variable fragment antibody against esophageal carcinoma

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# Abstract

**AIM:** To construct a phage display library of human singlechain variable fragment (scFv) antibodies associated with esophageal cancer and to preliminarily screen a scFv antibody against esophageal cancer.

**METHODS:** Total RNA extracted from metastatic lymph nodes of esophageal cancer patients was used to construct a scFv gene library. Rescued by M13K07 helper phage, the scFv phage display library was constructed. esophageal cancer cell line Eca 109 and normal human esophageal epithelial cell line (NHEEC) were used for panning and subtractive panning of the scFv phage display library to obtain positive phage clones. Soluble scFv was expressed in *E.coli* HB2151 which was transfected with the positive phage clone, then purified by affinity chromatography. Relative molecular mass of soluble scFv was estimated by Western blotting, its bioactivity was detected by cell ELISA assay. Sequence of scFv was determined using the method of dideoxynucleotide sequencing.

**RESULTS:** The size of scFv gene library was approximately  $9 \times 10^6$  clones. After four rounds of panning with Eca109 and three rounds of subtractive panning with NHEEC cells, 25 positive phage clones were obtained. Soluble scFv was found to have a molecular mass of 31 ku and was able to bind to Eca109 cells, but not to HeLa and NHEEC cells. Variable heavy (V<sub>H</sub>) gene from one of the positive clones was shown to be derived from the  $\gamma$  chain subgroup IV of immunoglobulin, and variable light (V<sub>L</sub>) gene from the  $\kappa$  chain subgroup I of immunoglobulin.

**CONCLUSION:** A human scFv phage display library can be constructed from the metastatic lymph nodes of esophageal cancer patients. A whole human scFv against esophageal cancer shows some bioactivity.

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against esophageal carcinoma. *World J Gastroenterol* 2004; 10(18): 2619-2623 http://www.wjgnet.com/1007-9327/10/2619.asp

# INTRODUCTION

Esophageal cancer is one of the most common malignancies in China with a relatively high mortality rate. In recent years, antibodymediated tumor immunoscintigraphy and immunotherapy have been used in the diagnostic and therapeutic approaches of cancers<sup>[1,2]</sup>. However, most antibodies are of murine origin, and repeated administration can induce human anti-mouse antibodies (HAMA). In addition, intact antibody is too large to penetrate into tumor masses, its application is limited. To overcome such deficiencies, many kinds of humanized antibodies including human-murine chimeric antibody and small molecular antibodies have been developed, but they are still of murine origin.

Recently, the emergence of genetically engineered antibodies and phage display libraries of human antibody fragments from immune or naïve donors has enabled the production of human antibody fragment targeting cancers<sup>[3]</sup>. In the present study, phage antibody library techniques were used to construct a human phage single-chain Fv antibody library from metastatic lymph nodes of esophageal cancer patients. To obtain a single chain Fv AD09, panning and subtractive panning were performed with human esophageal cancer cell line (Eca109) and normal human esophageal epithelial cell line (NHEEC) respectively. Soluble AD09 was expressed in *E.coli* HB2151 and purified by affinity chromatography using anti-E tag antibody, its bioactivity was then detected by cell ELISA assay.

# MATERIALS AND METHODS

## Cell culture

Human esophageal carcinoma cell line Eca109 (Cytology Institute of Chinese Medical Academy, Beijing) and HeLa cell line (Shanghai Cytology Institute, China) were cultured at 37 in RPMI1640 medium supplemented with 100 mL/L fetal calf serum (Hyclone, USA) in a humidified atmosphere of 50 mL/LCO<sub>2</sub>. Normal human esophageal epithelial cell (NHEEC) line was a primary cell line from a 20-wk conception fetus cultured in RPMI1640 with 200 mL/L fetal calf serum.

# Primer design

Primer sequences were created as previously described<sup>[4]</sup> with some modifications in PCR assembly part (Table 1B). We designed complementary coding sequences for a peptide linker at the 5'-end of  $J_H$  forward primers and the 3'-end of human V kappa (or lambda) back primers to optimize the diversity and efficiency of ligation. The primers were synthesized by Sunbiotech Company (Beijing, China) and the sequences are shown in Table 1. Sequences were given using the IUPAC nomenclature of mixed base (R = A or T, K = G or T, Y = C or T, S = G or C, H = A or C or T, N = A or C or G or T).

# Library construction

Metastatic lymph nodes of 5 esophageal cancer patients were

A. Primary PCRs	
Human V <sub>H</sub> back primers (sens	se)
HuV <sub>H</sub> laBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuV <sub>H</sub> 2aBACK	5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuV <sub>H</sub> 3aBACK	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuV <sub>H</sub> 4aBACk	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
	5-GAG GIG CAG CIG IIG CAG ICI GC-3
I forward primore (anti-sone	3-CAG GIA CAG CIG CAG CAG ICA GG-3
J <sub>H</sub> forward primers (anti-sens)	5) 5'-TCA CCA CAC CCT CAC CAC CCT CCC.3'
Hulu3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJ <sub>u</sub> 4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJ <sub>H</sub> 6FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
Human V kappa back primer	s (sense)
HuV κ laBack	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
HuV к 2aBack	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuV к 3aBack	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
HuV κ 4aBack	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
HuV κ 5aBack	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuV κ 6aBack	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'
Human J kappa forward prin	ner (anti-sense)
HuJ κ 1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJ κ 2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJ κ 3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJ κ 4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJ κ 5FOR	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'
Human V lambda back prime	ers (sense)
HuV λ 1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
HuV λ 2BACK	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
HuV λ 3aBACK	5-TCC TAT GTG CTG ACT CAG CCA CC-3
HuV λ 3bBACK	5-TCT TCT GAG CTG ACT CAG GAC CC-3
HuV & 4BACK	5 - CAC GIT ATA CIG ACT CAA CCG CC-3
HUV A SBACK	5 - CAG GUT GTG UTC AUT CAG CUG TU-3
HUV A BBACK	5 - AAT TH AIG CIG ACT CAG CCC CA-3
Human J lambda forward pri	mers (anti-sense)
HUJ & IFOR	
$\mathbf{R} = \mathbf{P} \mathbf{C} \mathbf{P}$ assombly	J-ACC TAA AAC GGT GAG CTG GGT CCC-3
Hu I Linker primers	
Hul 1 21 inker	5' ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^ ^
Hul. 21 inkor	5' ACACCCACCTCCCCCTCAACCCCCTCAACACCCCT CACCACCCT CACCACCCCTC ?'
Hul. 4-51 inker	5'-ACACCCACCTCCCCCTCAACCCCCCCCCCCCCCCCCCC
Hul6I inker	5'-AGAGCCACCTCCGCCTCGACCGCCTCCACCTGAGGAGACGGT GACCGTGGTCCC-3'
I inker-Hu V x primers	
Linker-HuV $\kappa$ 2-3-6 BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAWRTTGTGHTGACKCAGTCTCC-3'
Linker-HuV K 1-4 BACK	5'-oTTCAGCCGGAGGTGGCTCTCGCCGCTCGCCGGATCGGACATCSWGATGACCCAGTCTC C-3'
Linker- HuV ĸ 5 BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAAACGACACTCACG CAGTCTCC-3'
Linker-Hu V $\lambda$ primers	
Linker-HuV $\lambda$ 1-2BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGTCTGYSYTGACKCAGCCKS C-3'
Linker-HuV λ 3BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGTCYTMTGWGCTGACTCAGSMMCC-3'
Linker-HuV $\lambda$ 4-5BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCASGYTRTRCTSACTCARCCGYC-3'
Linker-HuV λ 6BACK	5'-GTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAATTTTATGCTGACTCAGCCC CA-3'
C. Reamplification with prime	ers containing restriction sites
Human $V_{H}$ back (Sfi) primers	(sense)
HuV <sub>H</sub> 1aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG-3'
HuV <sub>H</sub> 2aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTCAACTTAAGGGAGTCTGG-3'
HuV <sub>H</sub> 3aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG-3'
HuV <sub>H</sub> 4aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG-3'
HuV <sub>H</sub> 5aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGTTGCAGTCTGC-3'
HuV <sub>H</sub> 6aBACKSfi	5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG-3'
Human J kappa forward (No	t) primers (anti-sense)
HuJ κ 1FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATTTCCACCTTGGTCCC-3'
HuJ κ 2FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATCTCCAGCTTGGTCCC-3'
HuJ κ 3FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATATCCACTTTGGTCCC-3'
HuJ κ 4FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTGATCTCCACCTTGGTCCC-3'
HuJ κ 5FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACGTTTAATCTCCAGTCGTGTCCC-3'
Human J lambda forward (N	ot) primers (anti-sense)
HuJ λ 1FORNot	5'-GAGTCATTCTCGACTTGCGGCCGCACCTAGGACGGTGACCTTGGTCCC-3'
HuJ λ 2-3FORNot	5 -GAGTUATTCTCGACTTGCGGCCGCACCTAGGACGGTCAGCTTGGTCCC-3'
HuJ λ 4-5FORNot	5 -GAGIUATTUTUGAUTTGUGGUUGCAUTTAAAAUGGTGAGCTGGGTUCC-3'

Table 1	Oligonucleotide	primers used fo	or PCR of human	immunoglobulin g	enes
	- <b>A</b>				

collected for total RNA extraction (TRizol, Gibco BRL, UK). First-strand cDNA synthesis was performed in the presence of 40 U RNase inhibitor, 200 U Superscript II transcriptase (Gibco BRL, UK). The sample was finally treated with 2 U RNase H for 30 min at 37 °C and stored at -20 °C until use.

IgG-specific variable heavy ( $V_H$ ) and light ( $V_L$ ) chain gene fragments were amplified using Pyrobest PCR system (TarkaRa Biotechnology, Dalian, China) for 30 cycles (at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 1 min), with each forward oligonucleotide primer and one of the back primers (Table 1A). The fragments were isolated from a 15 g/L agarose gel with the QIAex kit (QIAgen, Germany). Then fragments were used as templates for PCR amplification to extend a linker,  $V_H$  fragments used human  $J_H$ -linker primers and human  $V_H$  back primers,  $V_L$ fragments used linker-human V $\kappa$  primers (or linker-human V $\lambda$ primers) and human J $\kappa$  forward primers (or human J $\lambda$  forward primers), respectively.

The amplified V<sub>H</sub>-linker and V<sub>L</sub>-linker PCR products were combined in a SOE-PCR reaction mixture. First, approximately 100 ng each of  $V_{H}$ -linker and  $V_{L}$ -linker was assembled by PCR without primers in which the short regions of complementarities built into the ends of primers drove hybridization of various fragments. An initial denaturation step (at 94 °C for 5 min) was followed by five cycles (at 94 °C for 1 min, at 60 °C for 1 min and at 72 °C for 1.5 min) in the absence of primers. After the outer primers containing restriction sites (Table 1C) were added, 30 cycles (at 94 °C for 30 s, at 60 °C for 1 min and at 72 °C for 1.5 min) were performed. These were gel-purified, digested with SfiI and NotI (TarkaRa Biotechnology, Dalian), cloned into the vector pCANTAB 5E (Amersham Pharmacia Biotech, Sweden) and transformed into electrocompetent E.coli TG1 (Amersham Pharmacia Biotech, Sweden). After electroporation, cells were plated on SOBAG medium (containing 20 g/L glucose and 100 mg/mL ampicillin) in 20 dishes and incubated overnight at 30 °C. The clones were scraped off the plates in 50 mL 2×YT medium with 100 mL/L glycerol and subsequently stored at -70 °C. Plasmid DNA was prepared from 10 randomly selected clones using Qiagen plasmid minikit (Qiagen, Germany). PCR and a SfiI/Not I double digestion reaction were performed to identify the positive insert clones.

#### Rescue of phagemid libraries

Ampicillin-resistant colonies were scraped into 2×YT medium and superinfected by M13K07 (Amersham Pharmacia Biotech, Sweden) helper phage. After an overnight induction in 2×YTAK medium without glucose, the phage preparation was precipitated in 40 g/L PEG/0.5 mol/L NaCl and resuspended in 10 g/L PBS.

#### Panning and subtractive panning of phage antibody library

To screen the positive phage clones, live Eca109 cell line as the antigen was used for panning. Live NHEEC was used for subtractive panning. The panning procedure was carried out essentially as described previously<sup>[5]</sup>. After four rounds of panning and three rounds of subtractive panning, the unabsorbed phages were amplified.

## Cell ELISA assay with phage

To detect the scFv-phage recombinant antibody, cell ELISA was performed. Eca109 cells  $(5\times10^4)$  as antigens were grown in 96-well plates at 37 °C for 24h, then fixed with 25 g/L glutaradehyde and blocked with 10 g/L BSA. This was followed by incubation with scFv-phage at 37 °C for 2 h. After washed three times with PBS, HRP/anti-M13 monoclonal conjugate (1:5 000) (Amersham Pharmacia Biotech, Sweden) was added into wells with scFv-phage and they were incubated for 1 h at 37 °C. After washed again as above, 1×ABTS substrate solution was added, and incubated in darkness for 30 min and the reactions were read at 405 nm. PBS was used as negative control. The absorbance

reading for the positive was 0.2 or above at least three times higher than that for the negative control.

#### Expression and purification of soluble scFv

To produce soluble scFvs, strongly positive recombinant phage clones were used to infect log-phage *E.coli*.HB2151 (Amersham Pharmacia Biotech, Sweden). Expression of soluble scFv was induced by adding isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L and the cultures grown overnight at 30 °C. The induced culture was centrifuged at 1 500 r/min for 20 min. Cell pellets were resuspended in 2% of culture volume ice-cold 1×TES. Subsequently, 3% of culture volume ice-cold 0.2×TES was added, the mixture was incubated on ice for 30 min to induce a mild osmotic shock. The contents were centrifuged at 12 000 g for 10 min. The supernatant, containing the soluble antibodies from the periplasm, was transferred to the clean tubes and stored at -20 °C.

Soluble scFvs from periplasm were purified by affinity chromatography. Anti-E tag antibody (Amersham Pharmacia Biotech, Sweden) was covalently coupled to a protein G column (Amersham Pharmacia Biotech, Sweden) and soluble scFvs were selected by binding to anti-E tag antibody. After washed with 20 mmol/L phosphate buffer, pH 7.0, +0.5 g/L NaN3, scFvs were eluted from the column with 0.1 mol/L glycine-HCl, pH 3.0, and neutralized immediately with 1 mol/L Tris/HCl, pH 8.2, +0.5 g/L NaN3. Column fractions were assayed and positive fractions were pooled and stored at -70 °C. The expressed soluble scFv proteins were analyzed by 120 g/L sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and confirmed by Western blotting. Purity and concentration of proteins were determined with Bradford assay.

#### Cell ELISA assay for activity of soluble scFv

To detect the activity of soluble scFv, HRP/anti-E tag antibody (Amersham Pharmacia Biotech, Sweden) was used. Eca109 cells, HeLa cells and NHEEC cells ( $5 \times 10^4$ ) were used as antigens. The cell ELISA assay procedure was performed as described above.

#### Sequencing

Plasmid DNA was prepared from recombinant clones using the Qiagen plasmid minikit (Qiagen, Germany). Nucleic acid sequencing was carried out on the ABI PRISM 377 DNA sequencer by the method of dideoxynucleotide sequencing. DNA and deduced amino acid sequence were compared with NCBI database.

# RESULTS

#### Library construction and panning

The presence of  $V_H$  and  $V_L$  gene fragments obtained by RT-PCR was confirmed by electrophoresis, with their sizes being approximately 350 bp (Figure 1 A). The scFv genewas assembled successfully. Its size was about 750 bp (Figure 1B).



**Figure 1** V<sub>H</sub> and V<sub>L</sub> and scFv fragments in 20 g/L agarose gel electrophoresis with staining of ethdium bromide (EB). (A) V<sub>H</sub> and V<sub>L</sub> fragments, (B) scFv fragment, M: DGL 2 000 marker.

After the scFv gene repertoires were transformed into *E. coli* TG1 cells, approximately  $9 \times 10^6$  ampicillin-resistant clones grew. PCR and *Sfil/Not*I double digestion reactions showed the positive insert ratio was about 95% (19/20). Rescued by M13K07 helper phage, the recombinant phage antibody library (about  $9 \times 10^{11}$ cfu/mL) was constructed.

Four rounds of panning with Eca109 cells resulted in a 130-fold enrichment of tumor cell binding scFv-phage. After three rounds of subtractive panning with NHEEC cells, individual phage-displayed scFv fragments were tested for reactivity with Eca109 cells by cell ELISA. Of the 95 clones screened, 25 were positive. The highest  $A_{405}$  nm value was found in AD09 clone.

#### Expression and purification of soluble scFv

The soluble scFv was stably expressed in *E. coli* HB2151 transfected with AD09 positive phage clone. In pCANTAB 5E, the pel B signal peptide upstream from the scFv directed the expression to periplasmic compartment. The periplasmic extract of AD09 was run through anti-E tag antibody affinity chromatography column and soluble AD09 was eluted from the column as a single peak (data not shown). The expressed and purified AD09 was loaded on 120 g/L SDS-PAGE and analyzed by Western blot. This protein migrated with a molecular mass approximately 31 ku (Figure 2). The overall yield of soluble AD09 in *E. coli* flask culture was more than 0.55 mg/L. The purity was about 90%.



**Figure 2** Expression and purification of AD09. A: SDS-PAGE. Lane 1: Markers (Amersham Pharmacia Biotech, Sweden). Lane 2: Product of *E.coli* HB2151 without scFv gene. Lane 3: Expression of AD09 in *E.coli* HB2151 with induction of IPTG. Lane 4: Purified AD09 protein. B: Western blot. Lane 1: Markers. Lane 2: Purified AD09 protein.

# Determination of immunoreactivity of soluble scFv

The immunoreactivity of purified soluble AD09 was determined by ELISA. The result revealed that AD09 was highly specific and could bind to Eca109 cells, but not to HeLa and NHEE cells (Table 2).

Table 2 Immunore activity of soluble scFv determined by ELISA

Commle	$A_{405}$	<sub>nm</sub> (mean±SD)	
Sample	Eca109	HeLa	NHEE
Soluble scFv PBS	0.78±0.12 0.14±0.04	$\begin{array}{c} 0.21{\pm}0.09^{\rm d} \\ 0.13{\pm}0.08 \end{array}$	$\begin{array}{c} 0.23{\pm}0.07^{\rm b} \\ 0.15{\pm}0.02 \end{array}$

<sup>d</sup>*P*<0.001, <sup>b</sup>*P*<0.01 *vs* Eca109 group.

#### Sequence analysis

Sequencing of six randomly selected antibodies from the positive clones was performed with an ABI PRISM 377 DNA sequencer using pCANTAB5 sequencing primer set (Amersham Pharmacia Biotech, Sweden). The sequence f AD09 clone is shown in Figure 3. Compared using BLAST, both V<sub>H</sub> and V<sub>L</sub> had sequence similarities to the variable fragments of some known human antibodies. Alignment with the V<sub>H</sub> and V<sub>L</sub> sequences of Ig, blast analysis of immunoglobulin sequences showed that V<sub>H</sub> was the  $\gamma$  chain subgroup IV of human immunoglobulin and V<sub>L</sub> was the  $\kappa$  chain subgroup I of human immunoglobulin.

ATG	CCA	GCC	GGC	CAT	GGC	CGA	GGT	GC/	AGCI	r GGT	GCA	GTO	CTGC	CCA	GGA	CTG
Μ	Р	А	G	Н	G	R	G	Α	Α	G	Α	V	С	Р	G	L
GTG	AAG	CCT	TCG	GAG	ACC	CTG	TCC	CTC	ACC	TGC	ACT	GTC	TCT C	GGT	GGC1	TCC
V	Κ	Р	S	Е	Т	L	S	L	Т	С	Т	V	S	G	G	S
ATC	AGT	AGT	TAC	TAC	IGG .	AGC	TGG	ATC	CGG	CAG	CCC	CCA	GGG	AAG	GGA	CTG
Ι	S	S	Y	Y	W	S	W	Ι	R	Q	Р	Р	G	Κ	G	L
			CD	R1												
GAG	TGG	ATT	GGG	TAT	ATC	FAT 7	AC A	AGT	GGG	AGC /	ACC /	AAC'	TAC A	ACO	CCCT	CC
Е	W	Ι	G	Y	Ι	Y	Y	S	G	S	Т	Ν	Y	Ν	Р	S
												CD	R2			
CTC	AAG	AGT	CGA	GTC	ACC	ATA	TCA	GTA	GAC	ACG	TCC	AAG	AAC	CAC	TTC	TCC
L	Κ	S	R	V	Т	Ι	S	v	D	Т	S	Κ	Ν	Q	F	S
CTG	AAG	CTG	AGC	TCT	GTG	ACC	GCI	GCC	GAG	CACC	GCC	CGTO	G TAT	TAC	TGT	GCG
L	Κ	L	S	S	V	Т	Α	Α	D	Т	Α	V	Y	Y	С	А
AGA	GAC	GCG1	GCO	CGAG	G ATC	GC GC	TAC	A AT	C GG	GGG	T GC	TTT	T GAI	T ATC	TGG	GGC
R	E	R	Α	E	Μ	A	1	I	C	i C	i A	A I	F D	Ι	W	G
						C	DR3									
CAA	GGC	ACC	CACC	GTC	CACC	GT	CTC	CTC	A GG	ΓGG/	A GG	CGG	TTC	A GG	C GG.	A GGT
Q	G	Т	Т	V	Т	V	S	S	G	i G	G	i C	3 S	G	G	G
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GGC	TCT	GGC	GGT	GGC	GGA	TCC	GA	CATC	CIC	AIG	ACC	CAG	TCT	CCA	TCC	ICC
G	S	G	G	G	G	S	D	I	L	М	Т	Q	S	Р	S	S
		~~.			~~ .	~ . ~	. ~ .	-						~~.		
CIG	TCT	GCA	TCT	GIA	GGA	GAC	AGA	GIC	CACC	CATC	ACT	TGC	CGG	GCA	AGT	CAG
L	S	A	S	v	G	D	R	v	Т	1	Т	С	R	A	S	Q
				TAT	TTA		FOO		-	-			000		000	COT
AGC	ALL	AGC	AGC	IAI	TIA	AAL	IGG W	IAI	AG	AGI	AAA	D	GGG	AAA	GCC	UCI D
<u>s</u>	<u> </u>	3	3	I	L	IN	w	I	Q	Q	ĸ	Р	G	ĸ	А	Р
	CTC	OTO	ATC	TAT	CT	-	TCC	ACT	TTC	<b>C</b> A A	ACT	ccc	CTC	001	TCA	ACC
AAG	CIC	CIG	AIC	IAI	JCLO	JCA	ICC.	AGI	TIG	CAA	AGI	GGG	GIC	D	ICA	AGG
ĸ	L	L	1	I	A	А	3	<u> </u>	L	Q	5	G	v	Р	3	ĸ
TTC	ACT	ccc	ACT	CCA	тст	ccc		KZ A C A	r tite	ACT	CTC	ACC	ATC	100	ACT	CTC
E E	AGI	GGC	AGI c	GGA	. ICI	GGC	TAC	AUA D		ACI	UIC I	ACC	AIC.	AGC	AGI	T
	OCT	CAA	S CAT	UTTT	3 CCA	ACT	TAC	TAC	Г			I ACT	TAC	S ACT	3	
	D D	GAA	GAI	E	GCA	ACI	V	V	C	CAA	CAG.	AGI	IAC I	401.	T	10
Q	Р	E	D	г	А	1	r	r	C	Q	Q	3	<u> </u>	<u>5</u>	1	L
TAC	ACT	ттт 4		CAC	ccc	100		C CT	COM	г <b>л</b> тс		CC				
V	T	1110 E	G	CAG	GUU	ACC	AA		AU U A	I AIC	AAA V	D D	1 000	JOCC A	_ GCA	
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Figure 3 Sequence of AD09 gene.

# DISCUSSION

The discovery of hybridoma technology by Kohler and Milstein in 1975<sup>[6]</sup> has heralded a new era in antibody research and clinical development. However, until recently, there were few antibody-based products suitable for clinical trial. This delay can be largely explained by the fact that murine antibodies could trigger a human anti-mouse antibody response<sup>[7-9]</sup>. Human monoclonal antibodies seldom triggered a harmful immune response and have been used in cancer immunotherapy<sup>[10]</sup>. However, there are many difficulties in making human monoclonal antibodies by hybridoma technology. Phage display antibody library technique provides a powerful tool to produce human antibody. In this study, we selected a human single chain antibody against esophageal cancer from a phage displayed antibody library. It confirmed that this technique was feasible.

Since scFv could penetrate faster and deeper in solid tumors, scFv format of monoclonal antibody was selected. ScFv is a small antigen-binding antibody fragment consisting of  $V_H$  and  $V_L$  joined together by a flexible peptide linker. The main advantage of scFv over intact whole IgG or Fabs was its small size (one sixth of intact IgG), making it penetrate a solid tumor mass more rapidly and evenly<sup>[11,12]</sup>. In addition, the lack of constant regions decreased retention by Fc receptors found in most tissues and organs, which further reduced the side effects<sup>[13,14]</sup>. These characteristics rendered scFv an ideal vector for delivery of

agents such as radionuclide, enzyme, drugs or toxin *in vivo*<sup>[14,15]</sup>.

Normally, peripheral blood lymphocytes were the main source for the construction of antibody libraries<sup>[16]</sup>. However, it was inconvenient to isolate at least 200 mL of peripheral blood to obtain 10<sup>7</sup> B-lymphocytes for constructing a large antibody library<sup>[3]</sup>. There are thousands of B cells in metastatic peritumor lymph nodes which may be preimmunized by tumorassociated antigens in esophageal cancer patients. The preimmunized B cells are sufficient to construct a library, and can be directly used for screening recombinant antibodies, since the heavy and light chain genes have been rearranged and ligated to specific targets of tumor antigens. Therefore, it has become a better source of B cells for human antibody library construction<sup>[17]</sup>.

Recombinant antibodies in phage antibody library could be best captured with purified tumor antigens<sup>[18]</sup>. Unfortunately, esophageal cancer associated specific antigens have not yet been identified. Human esophageal cancer cell line, Eca109 could express human esophageal cancer-associated specific antigens<sup>[19]</sup>. In addition, live cells could facilitate the identification of antibodies better than fixed cells<sup>[5,20]</sup> since the antibodies bound to native rather than denatured antigens. Moreover, using live cancer cells to screen phage antibody library was a feasible method<sup>[20,21]</sup>. So we used live Eca109 as antigens to screen the phage antibody library, and live NHEEC cell line was used for subtractive panning to obviate the cross-reaction with normal human esophageal epithelial cells. This was done to facilitate subsequent expression cloning of corresponding antigens, as well as to enhance the therapeutic potential of the antibodies obtained.

Generation of large repertoires of scFv genes is a crucial step during phage antibody display. To construct a large scFv gene repertoire, a number of methods were used. (1) Multiple primers covering most of the immunoglobulin heavy and light chain variable genes were used. (2) To optimize the diversity and efficiency of ligation, linker sequences were designed in PCR assembly primers. These made the linker easily synthesized and increased the diversity of scFv genes. (3) Electroporation transformation was used to obtain an efficiency of  $10^{\circ}$ cfu/ug for pUC18 and  $3 \times 10^{7}$  cfu/ug for ligation products. (4) The quality of mRNA appeared essential in the PCR amplification step and in subsequent construction of the library. To preserve intact mRNA, DEPC and RNase inhibitor were used during total RNA extraction and cDNA synthesis.

To further identify the bioactivity of scFv, soluble scFv protein was expressed in *E.coli* HB2151 induced by IPTG. SDS-PAGE and Western blot showed that the molecular mass was about 31 ku, which was consistent with the theoretically predicted product. The soluble expression level of scFv in *E.coli* HB2151 was still low, but it was sufficient for bioactivity detection. Cell ELISA assays showed that the soluble scFv had esophageal cancer associated antigen-binding activity. Whether this scFv shows affinity and specificity for tissue *in vivo* remains to be determined. Finally, DNA sequencing determined that scFv had common characteristics shared by other known scFvs<sup>[3]</sup>.

Our study demonstrated that specific human antibodies against tumor-associated antigens could be selected from a phage library constructed from the metastatic lymph nodes of esophageal cancer patients. The approach did not depend on immunization procedures. Since the antibody is entirely of human origin, it is expected to be much less immunogenic than murine antibodies and more efficient in targeting tumor cell surface. It may also be used as research reagents or a starting point for the development of therapeutic antibodies.

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• GASTRIC CANCER •

# Pathobiological significance of vascular endothelial growth factor and Maspin expressions in human gastric carcinoma

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# Abstract

**AIM:** To investigate the correlation between expression of vascular endothelial growth factor (VEGF) and cell differentiation, invasion, metastasis and Maspin expression in gastric carcinoma.

**METHODS:** Formalin-fixed paraffin-embedded tissue specimens from 73 cases of gastric carcinoma were studied with SP immunohistochemistry, using anti-VEGF monoclonal antibody, and thirty-nine of them were studied using anti-Maspin monoclonal antibody. VEGF expression was compared with the clinical stage, lymph node metastasis, and Borrmann's and WHO's classification of gastric carcinoma.

**RESULTS:** The positive rate of VEGF expression was significantly higher in adjacent non-carcinoma epithelia (ANCE) than in non-metaplastic, non-carcinoma gastric epithelia (NMNCE), which were at least 4 cm distant from the primary tumor (P = 0.000,  $\chi^2 = 73.03$ ). The positive rate of VEGF expression was significantly higher in advanced gastric carcinoma (AGC) than in early gastric carcinoma (EGC) (P = 0.032,  $\chi^2 = 4.62$ ). The positive rate of VEGF expression in gastric carcinomas with lymph node metastases was significantly higher than that in those without metastasis  $(P = 0.006, \chi^2 = 7.47)$ . Maspin was weakly expressed in 16 out of 39 cases of NMNCE, and the positive immunoreaction was limited to gland cells of the stomach body. There was no significant correlation between the expression of VEGF and histological or gross classifications, and correlation between the expressions of VEGF and Maspin in gastric carcinoma (P = 0.648,  $\chi^2 = 0.21$ ).

**CONCLUSION:** Expression of VEGF is significantly correlated to the malignant biological behaviors of gastric carcinoma, but there is no significant correlation between the expression of VEGF and Maspin.

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# INTRODUCTION

Tumor angiogenesis is one of the most important biological features. It has been shown that tumor angiogenesis plays an important role in its growth, invasion, metastasis and recurrence<sup>[1-3]</sup>. Among the factors contributing to angiogenesis, VEGF is recognized as one of the most important molecules in the formation of new blood vessels. There is clinical and experimental evidence that VEGF plays a role in the progression of solid tumors, and its clinical significance in solid tumors has been demonstrated both immunohistochemically and quantitatively<sup>[4]</sup>. Many studies demonstrated that over-expression of VEGF participated the growth and metastasis of malignant tumors depended on angiogenesis<sup>[2,5]</sup>. VEGF increased the incidence rate of tumor metastasis by inducing tumor angiogenesis<sup>[5]</sup>. Some studies demonstrated that the level of VEGF expression was of prognostic value in predicting metastasis of various malignant solid tumors and the level of VEGF expression correlated with tumor progression in human brain cancers and experimental tumor models<sup>[6,7]</sup>. Hence, most studies in the field have focused on the regulation and inhibition of angiogenesis. The tumor suppressor gene Maspin, a unique member of the serpin super family, could inhibit cell motility, invasion, and metastasis in some cancers<sup>[8-10]</sup>. Although at present the molecular and biological mechanisms of the function of Maspin remain unknown, there is evidence that Maspin interacts with the p53 tumor suppressor pathway and may function as an inhibitor of angiogenesis in vitro and in vivo<sup>[11]</sup>. Pemberton et al.<sup>[12]</sup> demonstrated the presence of Maspin in epithelia of several normal human organs (such as prostate, thymus, testis, small intestine, and colon). We are interested whether the tumor suppression function of Maspin in mammary or pancreatic carcinoma can be also detected in gastric carcinoma.

In this study, VEGF expression was immunohistochemically investigated in non-metaplastic, non-carcinoma gastric epithelia (NMNCE), which were at least 4 cm distant from the primary tumor, adjacent non-carcinoma epithelia (ANCE) and gastric carcinoma, and compared with the pathobiological behaviors of gastric carcinoma in order to clarify the clinical and pathobiological significance of the expression of VEGF. The relationship between the expressions of VEGF and Maspin was also explored.

# MATERIALS AND METHODS

## Tissue specimens

Seventy-three surgically removed specimens of gastric carcinoma were collected from Cancer Institute, China Medical University. The age of patients ranged from 32 to 80 years, mean age was 55.2 years; Forty-eight were males and 25 females. Carcinomas were staged according to pathological characteristics including depth of tumor invasion, tumor location, Borrmann's classification, and status of lymph node metastasis. According to clinical staging, 24 cases were in early stage (early gastric carcinoma, EGC), 49 cases in advanced stage (advanced gastric carcinoma, AGC). According to metastasis status, 40 cases had lymph node metastasis (without lymph node metastasis), and 32 had not any metastasis. Seventy-three cases of gastric carcinoma were

studied with SP immunohistochemistry, using anti-VEGF monoclonal antibody, and thirty-nine of them were studied using anti-Maspin monoclonal antibody. Each specimen was classified according to the Borrmann's classification and WHO's histological classification criteria.

#### Immunohistochemistry

All specimens were fixed in 40 g/L formaldehyde solution and embedded in paraffin. Five µm Sections were cut and mounted onto glass slides. Mouse anti-human monoclonal antibody against VEGF (ready to use) was from Maixin Biotech (Fuzhou, China) and mouse anti-human monoclonal antibody against Maspin was from Novo Castro (Newcastle, England). Immunohistochemical staining was performed using SP method. For control, sections were proceeded with PBS (0.01 mol/L, pH 7.4) instead of the primary antibodies. Counterstaining was performed with haematoxylin.

#### Evaluation of VEGF and Maspin expression

Clearly brown staining restricted to cytoplasm was considered as positive reaction for VEGF or Maspin. Two experienced pathologists assessed the positive rate according to the percent of positive cells in counted cells from 5 randomly selected representative fields. To evaluate the expression of VEGF and Maspin, immunostaining was classified into two groups, corresponding to the percentage of immunoreactive cells. The cut-off point to distinguish negative from positive VEGF or Maspin expression was 20% of positive cells.

#### Statistical analysis

Statistical evaluation was performed by  $\chi^2$ -test to differentiate the rates between two groups. *P*<0.05 was considered statistically significant.

## RESULTS

None of NMNCE expressed VEGF. VEGF expression was significantly higher in ANCE than in NMNCE ( $P = 0.000, \chi^2 = 73.03$ ) (Table 1). Immunohistochemically, VEGF expression was significantly higher in AGC than in EGC (P = 0.032,  $\chi^2 = 4.62$ ). There was no correlation between expression of VEGF and histology typing or gross typing (Table 2). VEGF expression in gastric carcinoma with lymph node metastases was significantly higher than that in those without metastasis ( $P = 0.006, \chi^2 = 7.47$ ) (Table 3). Sixteen (41.0%) out of thirty-nine cases of NMNCE showed a weak Maspin expression that was limited to gland cells of the stomach body, while all gastric normal epithelia with intestinal metaplasia (GNEIM) strongly expressed Maspin (14/14) (Table 4). The positive rate of Maspin was 53.6% (15/28) in specimens of positive VEGF expression, whereas the positive rate of Maspin was 45.5% (5/11) in specimens of negative VEGF expression (Table5). There was no significant correlation between the expressions of VEGF and Maspin in gastric carcinoma (P = 0.648,  $\chi^2 = 0.21$ ) (Table 5).

**Table 1** VEGF expression in NMNCE, ANCE and gastric carcinoma (n = 73)

Tissue origin	n	VEGF e	xpression	Positive rate
lissue origin	11	-	+	(70)
NMNCE	73	73	0	-
ANCE	73	23	50	$68.5^{\mathrm{b}}$
Gastric carcinoma	73	14	59	<b>80.8</b> <sup>1</sup>

<sup>b</sup>*P* = 0.000 *vs* NMNCE (Yates corrected:  $\chi^2$  = 73.03), <sup>1</sup>*P* = 0.086 *vs* ANCE (Yates corrected:  $\chi^2$  = 2.93).

		VEGF ex	pression	Positive rate	
Туре	n	-	+	(%)	
Gross types					
EGC <sup>a</sup>	24	8	16	66.7	
Ι	4	0	4	100.0	
II	12	6	6	50.0	
III	7	2	5	71.4	
SS <sup>1</sup>	1	0	1	100.0	
AGC	49	6	43	87.8	
Bor. 0	3	0	3	100.0	
Bor. I	1	0	1	100.0	
Bor. II	6	0	6	100.0	
Bor. III	36	5	31	86.1	
Bor. IV	3	1	2	66.7	
Histological type					
Papillary adenocarcinoma	8	1	7	87.5	
Well-differentiated adenocarcinoma	3	2	1	33.3	
Moderately-differentiated adenocarcinoma	11	3	8	72.7	
Poorly-differentiated adenocarcinoma	30	5	25	83.3	
Undifferentiated carcinoma	3	1	2	66.7	
Signet-ring cell carcinoma	10	1	9	90.0	
Mucinous adenocarcinoma	7	0	7	100.0	
Carcinoid	1	1	0	-	

**Table 2** Relationship between VEGF expression and gross and histological types of gastric carcinoma (n = 73)

<sup>a</sup>P = 0.032 vs AGC ( $\chi^2 = 4.62$ ), There was no correlation between the expression of VEGF and histology typing or gross typing (P>0.05). <sup>1</sup>EGC SS (early gastric carcinomas of superficial spreading type).

	_	VEG	VEGF		Maspin		. 0/
Histological type	п	+	-	+ %	+	-	+ %
NMNCE	39	0	39	0	16ª	23	41.0
GNEIM	14	-	-	-	14	0	100.0
Gastric carcinoma							
Papillary adenocarcinoma	3	3	0	100.0	2	1	66.7
Well-differentiated adenocarcinoma	3	2	1	66.7	2	1	66.7
Moderately-differentiated adenocarcinoma	6	5	1	83.3	2	4	33.3
Poorly-differentiated adenocarcinoma	21	14	7	66.7	11	10	52.4
Undifferentiated adenocarcinoma	3	2	1	66.7	1	2	33.3
Signet ring-cell carcinoma	3	2	1	66.7	2	1	66.7
Total of gastric carcinoma	39	28	11	71.8	20	19	51.3

Table 4	VEGF and Maspin	expressions in NMNCE,	GNEIM and gastr	ic carcinoma

Maspin was weakly expressed in gland cells of the stomach body, while it was not expressed in superficial epithelial cells and pyloric gland of the stomach.

 
 Table 3 Relationship between VEGF expression and metasta sis of gastric carcinoma (n = 73)

Matastasis status	n	VEGF ex	Positive rate	
	n	-	+	(70)
No metastasis	32	12	20	$62.5^{\mathrm{b}}$
Lymph node metastasis	39	4	35	89.7
Liver metastasis	1	1	0	0
Ovary metastasis	1	0	1	100.0

<sup>b</sup>P = 0.006 vs lymph node metastasis ( $\chi^2 = 7.47$ ).

Table 5 Relationship between VEGF and Maspin expressions in gastric carcinoma

Gastric carcinoma	Maspin +	Maspin -	Total
VEGF +	15	13	28
VEGF -	5	6	11
Total	20	19	39

There was no significant correlation between the expression of VEGF and Maspin in gastric carcinoma (P = 0.648,  $\chi^2 = 0.21$ ).

# DISCUSSION

Ferrara<sup>[13]</sup> and his colleagues found that bovine pituitary follicular cells secreted a novel heparin-binding growth factor specific for vascular endothelial cells in 1989 and named it VEGF. VEGF is known to be a highly specific mitogen for endothelial cells which is almost specifically expressed in endothelial cells. VEGF might act as an autocrine and paracrine growth factor to induce the proliferation of tumor cells as well as tumor angiogenesis of tumor cells<sup>[14]</sup>.

Tumors require blood vessels for nutrient and oxygen supply to maintain their viability. In the first stage of growth, cloning proliferative phase does not need angiogenesis. To continue tumor expansion, additional blood supply was prerequisite, which was significantly correlated to tumor invasion, metastasis and recurrence<sup>[15-18]</sup>. It has been widely accepted that tumor angiogenesis was one of the most crucial steps in tumor invasion and metastasis. There was a close relationship between VEGF expression and depth of invasion, lymph node metastasis and five-year survival rate of patients, which was an independent prognostic factor. Our study showed that there was no significant relationship between VEGF expression and histological or gross types of gastric carcinomas.

Yonemura further demonstrated the correlation between VEGF-C expression and lymphatic invasion or lymph node

metastasis<sup>[19]</sup>. Tumors with high expression of VEGF-C had more remote lymph node involvement than those with low VEGF-C expression<sup>[7,19,20]</sup>. These results strongly suggested that cancer cells producing VEGF-C might induce proliferation and dilation of lymphatic vessels, resulting in the development of invasion of cancer cells into lymphatic vessels and lymph nodes. These results were consistent with recent reports that showed a positive correlation of VEGF-C levels with lymph node metastasis in gastric carcinoma.

A number of observations and animal trials have spurred extensive investigations of VEGF inhibitors as possible therapies for cancer. In tumor cell lines VEGF was an autocrine growth factor, so that inhibitors of VEGF or VEGF receptors (VEGFR) compromised the viability of tumor cells. Lastly, inhibition of VEGF or VEGFR signaling would inhibit both tumor angiogenesis and tumor cell growth and viability when there was evidence that VEGFR was expression in tumor cells<sup>[21,22]</sup>.

Our study showed that VEGF was positively expressed in 76.7% of gastric carcinomas, which was significantly higher than that in NMNCE. The result indicated that VEGF was upregulated and there might exist an autocrine mechanism of VEGF in gastric carcinoma. VEGF could promote tumor growth and metastasis by both direct and indirect pathways<sup>[23]</sup>.

Maspin, a member of the serpin family of protease inhibitors, is expressed in normal human mammary and prostate epithelial cells, and down-regulated during cancer progression. Biological studies demonstrated a tumor-suppressive role of Maspin, acting at the levels of tumor invasion and metastasis<sup>[8,12]</sup>. Maass<sup>[24]</sup> did not detect Maspin expression in any of 6 gastric cancer cells. Son<sup>[25]</sup> studied Maspin expression in 30 cases of human gastric adenocarcinoma using immunohistochemistry and reverse transcripted-polymerase chain reaction. Twenty-seven cases (90%) of gastric adenocarcinoma, regardless of histological type, and all cases of GNEIM showed diffuse and strong immunoreactivity to Maspin. Eighteen of 26 cases (69.2%) of NMNCE showed weak and focal immunoreactivity. The level of Maspin expression was higher in GNEIM and lower in NMNCE than in adenocarcinoma cases. Akiyama<sup>[26]</sup> examined Maspin expression and/or allele-specific methylation status in four gastric cancer cell lines, as well as normal, metaplastic, and carcinoma epithelia obtained from 50 gastric cancer patients. Three gastric cancer cell lines exhibiting Maspin overexpression showed hypomethylation on both alleles or a haploid allele. Dense and diffuse immunoreactivity to Maspin was observed in 40 (80%) of 50 gastric carcinomas and all GNEIM, but not in GNE without IM. Maspin gene promoter region of all GNE without IM was hypermethylated on both alleles whereas those with IM frequently represented the haploid type of hypomethylation status. Maspin mRNA was amplified from GNEIM and cancerous crypts but not from GNE without IM. These results suggested that demethylation at the Maspin gene promoter disrupted the cell-type-specific gene repression in both GNE and gastric cancer. In our study, 41.0% (16/39) of NMNCE showed a weak Maspin expression that was limited to gland cells of the stomach body, and 51.3% (20/39) of gastric carcinomas expressed Maspin. The positive rate of Maspin expression in NMNCE and in gastric carcinoma in our study was significantly lower than that in Son and Akiyama's study. We considered that the cut-off point made the different results. The reason why all GNEIM showed immunoreactivity to Maspin in all studies should been studied further. In addition, the role of Maspin gene and its encoding protein in tumorigenesis and progression of gastric cancer need to be investigated further.

In our study, Maspin and VEGF showed no correlation in gastric carcinomas. The precise roles of VEGF and Maspin in cancer tumorigenesis, invasion, and metastasis should be studied further. The relationship between expression of VEGF and Maspin in gastric cancer needs to be proved by amplifying samples.

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• GASTRIC CANCER •

# Effects of mifepristone on proliferation of human gastric adenocarcinoma cell line SGC-7901 *in vitro*

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# Abstract

**AIM**: To explore the effects of mifepristone, a progesterone receptor (PR) antagonist, on the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro* and the possible mechanisms involved.

**METHODS:** In situ hybridization was used to detect the expression of PR mRNA in SGC-7 901 cells. After treatment with various concentrations of mifepristone (2.5, 5, 10, 20  $\mu$ mol/L) at various time intervals, the ultrastructural changes, cell proliferation, cell-cycle phase distribution, and the expression of caspase-3 and Bcl-X<sub>L</sub> were analyzed using transmission electron microscopy (TEM), tetrazolium blue (MTT) assay, <sup>3</sup>H-TdR incorporation, flow cytometry, and reverse transcription-polymerase chain reaction (RT-PCR).

**RESULTS:** Mifepristone markedly induced apoptosis and inhibited cell proliferation of PR- positive SGC-7 901 cells revealed by TEM, MTT assay and <sup>3</sup>H-TdR incorporation, in a dose- and time-dependent manner. The inhibitory rate was increased from 8.98% to 51.29%. Flow cytometric analysis showed mifepristone dose-dependently decreased cells in S and G<sub>2</sub>/M phases, increased cells in G<sub>0</sub>/G<sub>1</sub> phase, reduced the proliferative index from 57.75% to 22.83%. In addition, mifepristone up-regulated the expression of caspase-3, and down- regulated the Bcl-X<sub>L</sub> expression, dose-dependently.

**CONCLUSION:** Mifepristone effectively inhibited the proliferation of PR-positive human gastric adenocarcinoma cell line SGC-7 901 *in vitro* through multiple mechanisms, and may be a beneficial agent against human adenocarcinoma.

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# INTRODUCTION

Gastric adenocarcinoma is the second most common cancer with the second highest mortality rate<sup>[1,2]</sup>. Presently, there is still no effective treatment means for patients with advanced gastric adenocarcinoma<sup>[3,4]</sup>. Chemotherapy or radiation therapy has generally shown some clinical response but little survival advantage and is not tolerated in many patients<sup>[5,6]</sup>. Therefore, there is a need to identify other therapeutic agents against the tumor.

Mifepristone is a progesterone receptor (PR) antagonist that has been widely used as the first- line drug for the termination of early pregnancy<sup>[7]</sup>. Interestingly, recent studies have proved that mifepristone could effectively inhibit the proliferation of PR-positive breast cancer<sup>[8-10]</sup>, ovarian cancer<sup>[11,12]</sup>, endometrial cancer<sup>[13]</sup>, and prostate cancer<sup>[14]</sup> cells without serious side effects and drug resistance. However, the effects of mifepristone on gastric adenocarcinoma are still unknown. Therefore, the present study was undertaken to explore the effects of mifepristone on the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro*. Results showed that mifepristone effectively inhibited the proliferation of cultured SGC-7 901 cells *in vitro* through multiple mechanisms.

# MATERIALS AND METHODS

# Cell culture and treatment

Human gastric adenocarcinoma cell line SGC-7 901, obtained from Wuhan University Type Culture Collection (Wuhan, China), was routinely maintained in phenol red-free RPMI1640 (Gibco BRL, Grand Island, NY) containing 100 mL/L fetal bovine serum (Hyclone, Logan, UT),  $10^5$  U/L penicillin and 100 mg/L streptomycin at 37 °C in a humidified atmosphere with 50 mL/L CO<sub>2</sub> in air. When cells were grown to approximately 50% confluence, medium was replaced with serum-free RPMI1640. After 24 h, fresh media containing 2.5, 5, 10, 20 µmol/L mifepristone (Sigma Chemical Co., St Louis, MO) were added, respectively. Control cells were treated with the same volumes of vehicle (ethanol). Unless otherwise indicated, the cells were harvested after 96 h of incubation.

# In situ hybridization analysis of PR

The expression of PR mRNA in SGC-7 901 cells was detected by in situ hybridization (ISH) using an ISH detection kit for PR (Boster, Wuhan, China) according to the manufacturer's instructions. Unless otherwise stated, all steps were performed at room temperature. Briefly, after 24 h of culture on the RNAsefree slides, cells were washed 3 times with phosphate buffered saline (PBS, pH7.4), fixed with 40 g/L paraformaldehyde in PBS containing 0.1 g/L diethylpyrocarbonate (DEPC-water) for 30 min, washed 3 times with 0.01 mol/L PBS, and then incubated with 5 mL/L hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After being rinsed with 0.01 mol/L PBS, cells were digested with proteinase K (10 g/mLin 0.01 mol/L PBS) at 37 °C for 15 min. Further washes with 0.5 mol/L PBS were performed before pre-hybridization for 3 h at 37 °C in the pre-hybridization solutions in a humidified environment. Hybridization was then performed with digoxigeninlabeled cRNA antisense probe (5 g/mL) overnight at 37 °C in a moist chamber. Subsequently cells were washed for 10 min with 2 SSC (1 SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH7.0), followed by 0.5 SSC for 15 min, and finally 0.2 SSC for

15 min. After treatment with blocking reagent for 30 min, cells were incubated with biotin-labeled mouse anti-digoxigenin antibody at 37 °C for 1 h, washed 4 times, for 5 min each time, with 0.5 mol/L PBS, and then treated with SABC solutions at 37 °C for 20 min. Then cells were washed 3 times, for 5 min each time, with 0.5 mol/L PBS, incubated with biotin-labeled peroxidase (POD) at 37 °C for 20 min, washed three time with 0.5 mol/L PBS. Finally, cells were visualized with 3,3- diaminobezidine (DAB), counterstained with hematoxylin, dehydrated, cleared, mounted with neutralgum, and examined under an microscope. Brown-yellow deposits indicated the sites of hybridization. PR-positive breast cancer tissues were used as positive control, and probes were replaced by PBS as negative control.

## Ultrastructural analysis

Harvested cells were washed 3 times with PBS, fixed for 2 h with 2.5 g/L glutaraldehyde in PBS, and then post-fixed for 2 h at 4 °C with 1 g/L OsO<sub>4</sub> in PBS. Cells were dehydrated using gradually increasing concentrations of ethanol from 50% to 100%, and then embedded in Epon 812. The ultra-thin sections (60 nm) were stained with uranyl acetate and lead citrate prior to examination at 50 kV with a Hitachi 600 transmission electron microscrope (Hitachi Corp., Tokyo, Japan).

#### MTT assay

SGC-7 901 cells were seeded into 96-well plates at a density of  $1 \times 10^{5}$ /mL in RPMI1640. After 96 h of incubation with various concentrations of mifepristone, cell proliferation was measured by MTT (Sigma) reduction assay as described previously<sup>[15]</sup>. Absorbance at 570 nm ( $A_{570nm}$ ) was assayed. The inhibitory rate (IR) of SGC-7 901 cells was calculated according to the equation as following: IR (%) = ( $A_{570nm}$  in control group- $A_{570nm}$  in mifepristone-treated group)/ $A_{570nm}$  in control group×100%.

## <sup>3</sup>H-thymidine (<sup>3</sup>H-TdR) incorporation

Cells were incubated at various time intervals without or with various concentrations of mifepristone, followed by treatment with 10  $\mu$ Ci <sup>3</sup>H-TdR (Amersham, Arlington Heights, IL) for an additional 6 h. Then, cells were washed twice with 100 mL/L trichloroacetic acid by centrifugation and resuspension, and were continuously incubated for 30 min at 60 °C with 0.5 mL of NaOH (0.3 mol/L). Finally, the cell lysates were collected, and the radioactivity was measured by a liquid scintillation counter (Beckman LS1 801, USA).

#### Cell cycle analysis by flow cytometry

The harvested cells were fixed with 700 mL/L ethanol at -20 °C for 30 min, and then stained with propidium iodide (Sigma) for 30 min in the dark. The stained cells were analyzed in a FACS Calibur flow cytometer (Becton Dickinson Labware, Lincoln Park, NJ) with excitation wavelength of 488 nm. The resulting histograms were analyzed by program MODFIT for cell distribution in cell cycle phase. Proliferative index (PI) was calculated according to the formula: PI (%) = (S+G\_2/M)/(G\_0/G\_1+S+G\_2/M)×100%.

# Measurement of caspase-3 activity

Total proteins were extracted from harvested cells as described previously<sup>[16]</sup>, and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). An equal amount of cellular protein from extract of each group was added to a final volume of 100  $\mu$ L of reaction mixture containing 0.2 mmol/L of a colorimetric caspase-3 substrate, acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA; Calbiochem, San Diego, CA), followed by incubation at 30 °C for 10 min. Free p-nitroaniline ( $\rho$ NA) released upon enzymatic cleavage was detected at 405 nm using a microplate reader (Bio-Rad). Caspase-3 activity correlated with the concentration of free

ρNA generated in the reaction. Purified caspase-3 (Calbiochem) was used as positive control, whereas caspase-3 inhibitor I (Ac-DEVD- CHO, Calbiochem) was used as negative control.

#### RT-PCR analysis for BcI-X<sub>L</sub>

Total RNA was extracted from the cells using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. Two milligrams of total RNA were used for reverse transcription in a total volume of 20  $\mu$ L with the SuperScript preamplification system (Promega, Madison, MI). Aliquots of 2 µL cDNA were subsequently amplified in a total volume of 50  $\mu$ L using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primers for Bcl-X<sub>L</sub> were 5'-AGGCAGGCGATGAGTTTGAAC-3' and 5'-GAACCACCACCAGCCACA GTCA-3', respectively. The sense and antisense primers for -actin used as an internal control were 5'-ATCTGGCACC ACACCTTCTACAATGAGCT GCG-3' and 5'-CGTCA TACTCCTGCTTGCTGATCCACAT CTGC-3', respectively. The cycling conditions were 94 °C for 2 min, followed by 30 cycles of 92 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 1 min and a final extension of 72 °C for 5 min. PCR products were separated on the 15 g/L agarose gel stained with ethidium bromide (EB) and viewed under ultraviolet light.

# Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical analysis was performed using the Student's *t* test and the chi-square test. *P*<0.05 was considered statistically significant.

# RESULTS

# Expression of PR mRNA

*In situ* hybridization analysis showed that PR mRNA was highly expressed in the cultured SGC-7 901 cells, which was mainly localized in the cytoplasm of the cells (Figure 1).



**Figure 1** *In situ* hybridization (ISH) analysis of progesterone receptor (PR) expression in human gastric adenocarcinoma cell line SGC7 901 (ISH, ×1000).

#### Morphological changes

To assess the effect of mifepristone on the ultrastructural changes of SGC-7 901 cells, transmission electron microscopic analysis was performed. Results revealed that mifepristone dose-dependently induced apoptosis, which was especially remarkable at the  $20 \,\mu$ mol/L concentration (Figure 2B). However, the irregular and enlarged nuclear, multiple nucleoli and increased nucleus-to-cytoplasm ratio were clearly seen in the cells of control group (Figure 2A).

# MTT assay and <sup>3</sup>H-TdR incorporation

After 96 h of incubation with 2.5, 5, 10, 20  $\mu$ mol/L mifepristone, MTT assay revealed  $A_{570nm}$  was markedly decreased in a dosedependent manner, and the inhibitory rate (IR) of SGC-7 901

		Cell cycle				
Concentration (µmoi/ L)	A <sub>570nm</sub> (IVI I I )	$G_0/G_1$	G <sub>0</sub> /G <sub>1</sub> S		Caspase-3 activity (U)	
0	1.125±0.048	$42.25{\pm}4.20$	$35.68 \pm 3.98$	22.07±3.01	1.28±0.28	
2.5	$1.024 \pm 0.030$	$49.47 \pm 5.68$	$30.82 {\pm} 4.36$	$19.71 \pm 2.41$	$2.79 {\pm} 0.36$	
5	$0.896 {\pm} 0.035$	$52.23 \pm 6.22$	$28.68 {\pm} 3.64$	19.01±1.36	$5.04 {\pm} 0.29$	
10	$0.678 {\pm} 0.026$	$65.80{\pm}5.63$	$25.93 {\pm} 3.01$	8.27±1.10	$9.46 {\pm} 0.20$	
20	$0.548 {\pm} 0.031$	$77.16 \pm 8.25$	$15.54 {\pm} 2.54$	$7.29{\pm}0.82$	$15.23 \pm 0.41$	

 Table 1
 Effects of mifepristone on cell proliferation, cell-cycle phase distribution and caspase-3 activity of SGC7 901 cells in vitro (mean±SD)

cells by mifepristone was 8.98%, 20.36%, 39.73% and 51.29%, respectively (Table 1). Figure 3 shows that <sup>3</sup>H-TdR incorporation into DNA of SGC-7 901 cells was significantly decreased in a dose- and time-dependent manner.



**Figure 2** Transmission electron microscopic photographs of the SGC7901 cells cultured for 96 h in the absence(A) or the presence of 20  $\mu$ mol/L mifepristone (B) *in vitro* (TEM,  $\times$ 2000). Arrows indicate apoptotic bodies which were formed in the cells of mifepristone-treated group.



**Figure 3** Effect of various concentrations of mifepristone on the <sup>3</sup>H-TdR incorporation of SGC7 901 cells at various time intervals *in vitro*.

#### Cell-cycle phase distribution

The effect of mifepristone on the cell-cycle phase distribution of SGC-7 901 cells was determined by flow cytometry. After treatment with mifepristone, there was a strong dose- dependent decrease in the percentage of S- and  $G_2/M$ -phase cells, and with a concomitant increase in the percentage of cells in the

 $G_0/G_1$  phases of the cell cycle (Table 1). Additionally, there is a significant decrease in the proliferative index (PI) of the mifepristone-treated cells (50.53%, 47.69%, 34.20% and 22.83%) as compared with control group (57.75%, *P*<0.01).

#### Expression of caspase-3 and Bcl-X<sub>L</sub>

As shown in Table 1, mifepristone significantly up-regulated the activity of caspase-3 as compared with that in control group. Figure 4 shows the results of RT-PCR analysis for Bcl- $x_L$  mRNA expression. Results indicated that mifepristone dose-dependently inhibited the expression of Bcl- $X_L$  in the SGC-7 901 cells.



**Figure 4** RT-PCR analysis of  $Bcl-X_L$  mRNA expression in the SGC7 901 cells cultured for 96 h in the absence or the presence of various concentrations of mifepristone *in vitro*. Lanes 1-5: Marker (bp), contro l, 5, 10, 20  $\mu$ mol/L mifepristone, respectively.

# DISCUSSION

Accumulating evidence demonstrates that PR level is closely associated with proliferation, invasion and metastasis of human gastric adenocarcinoma, as well as prognosis of patients<sup>[17-19]</sup>. Therefore, there has been increasing interest in the development of antiprogestins for tumor treatment. Mifepristone has been proved to be a potent and effective PR antagonist by competing with progesterone for PR binding, followed by binding to progesterone response element (PRE)<sup>[20]</sup>. In the present study, we proved that mifepristone effectively inhibited the proliferation of human gastric adenocarcinoma cell line SGC-7 901 *in vitro* through induction of apoptosis and arresting the cell cycle progession.

Previous studies<sup>[21]</sup> found that the determination of PR levels was primarily used as a marker of a tumor's responsiveness to mifepristone. To determine the expression of PR mRNA in the SGC-7 901 cells, *in situ* hybridization was performed. We found that PR mRNA was highly expressed in cultured SGC-7 901 cells. Meanwhile, the result is in agreement with the work of Cui *et al.*<sup>[22]</sup>, who reported that the concentrations of PR protein in the cytoplasm and nuclei of cultured SGC-7901 cells were 20.3 fmoL/mg and 22.7 fmoL/mg, respectively, revealed by dextran-coated charcoal (DCC) assay. Thus, we speculate that the growth inhibitory effects of mifepristone in our study might be mediated, at least in part, by PR. In our study, TEM, MTT assay and <sup>3</sup>H-TdR incorporation were used to evaluate the effect of mifepristone on the proliferation of SGC-7 901 cells *in vitro*. We found that mifepristone exerted significantly anti-proliferative effect on cultured SGC-7 901 cells *in vitro* in a dose- and time-dependent manner. The results are in agreement with those of previous studies on other tumor cell lines *in vitro*<sup>[9-11,23]</sup>.

Although a number of studies have proved that mifepristone has the growth inhibitory effects on tumor cells, but the mechanisms remain unknown. These theoretically could be related with the changes of the dynamics of cell proliferation. The hypothesis was demonstrated by the work of Thomas et al.<sup>[24]</sup>, who proved that mifepristone inhibited the proliferation of the MCF-7 human breast cancer cells by arresting them in the  $G_0/G_1$  phase of the cell cycle. Recently, Peters *et al.*<sup>[25]</sup> reported that mifepristone up-regualted the expression of cell cycle protein p21<sup>WAF/cip1</sup> in medroxyprogesterone acetate-induced ductal mammary adenocarcinoma. Thus, we further explored the effects of mifepristone on the cell cycle of SGC-7 901 cell using flow cytometry. Results showed that mifepristone markedly increased the proportion of cells in  $G_0/G_1$ , and simultaneously decreased the percentage of cells in S- and  $G_2/M$ -phase. Taken together, it seems reasonable to conclude that the growth inhibitory effects of mifepristone on SGC-7901 cells is partially due to an accumulation of cells in  $G_0/G_1$  phase.

To explore whether or not the apoptosis-related genes contributed to the inhibitory effect of mifepristone on the SGC-7 901 cells, we assessed the activity of caspase-3, an executioner of apoptosis<sup>[26,27]</sup>, and mRNA level of Bcl-X<sub>L</sub>, an anti-apoptotic gene. Our findings showed that mifepristone dose-dependently up-regulated caspase-3 activity and down-regulated Bcl-X<sub>L</sub> mRNA expression. This result is supported by previous studies on prostate cells. El Etreby *et al.* reported that mifepristone significantly induced apoptosis in LNCaP prostate cancer cells in a time- and dose-dependent manner through down-regulation of Bcl-2 protein and induction of caspase-3 activity. Collectively, it is possible that the activation of caspase-3 and the degradation of Bcl-X<sub>L</sub> are partially responsible for the antiproliferative effects of mifepristone on cultured SGC-7 901 cells.

In conclusion, the study demonstrated that mifepristone exerted marked antiproliferative effect on the PR-positive SGC-7901 cells by inducing apoptosis, arresting cell cycle progression, up-regulating caspase-3 activity and down-regulating Bcl- $X_L$  mRNA expression. These results indicate that mifepristone may be a useful agent against human gastric adenocarcinoma although further studies are clearly needed to prove the possibility.

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LIVER CANCER •

# Mitotic cell death in BEL-7402 cells induced by enediyne antibiotic lidamycin is associated with centrosome overduplication

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# Abstract

**AIM:** Mitotic cell death has been focused on in tumor therapy. However, the precise mechanisms underlying it remain unclear. We have reported previously that enediyne antibiotic lidamycin induces mitotic cell death at low concentrations in human epithelial tumor cells. The aim of this study was to investigate the possible link between centrosome dynamics and lidamycin-induced mitotic cell death in human hepatoma BEL-7402 cells.

**METHODS:** Growth curve was established by MTT assay. Cell multinucleation was detected by staining with Hoechst 33342. Flow cytometry was used to analyze cell cycle. Aberrant centrosomes were detected by indirect immunofluorescence. Western blot and senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining were used to analyze protein expression and senescence-like phenotype, respectively.

**RESULTS:** Exposure of BEL-7402 cells to a low concentration of lidamycin resulted in an increase in cells containing multiple centrosomes in association with the appearance of mitotic cell death and activation of SA- $\beta$ -gal in some cells, accompanied by the changes of protein expression for the regulation of proliferation and apoptosis. The mitochondrial signaling pathway, one of the major apoptotic pathways, was not activated during mitotic cell death. The aberrant centrosomes contributed to the multipolar mitotic spindles formation, which might lead to an unbalanced division of chromosomes and mitotic cell death characterized by the manifestation of multi- or micronucleated giant cells. Cell cycle analysis revealed that the lidamycin treatment provoked the retardation at G2/M phase, which might be involved in the centrosome overduplication.

**CONCLUSION:** Mitotic cell death and senescence can be induced by treatment of BEL-7402 cells with a low concentration of lidamycin. Centrosome dysregulation may play a critical role in mitotic failure and ultimate cell death following exposure to intermediate dose of lidamycin. Liang YX, Zhang W, Li DD, Liu HT, Gao P, Sun YN, Shao RG. Mitotic cell death in BEL-7402 cells induced by enediyne antibiotic lidamycin is associated with centrosome overduplication. *World J Gastroenterol* 2004; 10(18): 2632-2636 http://www.wjgnet.com/1007-9327/10/2632.asp

# INTRODUCTION

Liver cancer is one of the most malignant tumors in the world<sup>[1,2]</sup>. Surgical resection is considered the most effective but not the most popular method for the treatment of hepatocellular carcinoma (HCC). Chemotherapy is indicated for a large member of HCC patients. Mitotic cell death is a cell death form different from apoptosis, on which has been focused in tumor therapy. It is also known as mitotic catastrophe or delayed reproductive death, and can be activated by radiation or antitumor agents at low doses or concentrations<sup>[3-5]</sup>. Mitotic cell death is frequently characterized by enlargement of cell volume, appearance of multi- or micronucleation, and arrest in G2/M phase of cell cycle. Finally, these cells underwent death. Thus far, little is known about the mechanism responsible for mitotic cell death. Some researchers considered that defects in mitotic machinery, such as multiple rounds of DNA synthesis without an intervening cytokinesis, and chromosome missegregation, might play a key role in the process of lethal nuclear fragmentation<sup>[6]</sup>. Previous reports have suggested that the absence or delay of the G1/S checkpoint and the subsequent absence of interphase apoptosis coupled to this checkpoint contribute to mitotic cell death<sup>[7,8]</sup>.

The centrosome, representing the major microtubule organizing centre in eukaryotic cells, contains a pair of centrioles surrounded by pericentriolar material. The centrosome duplicates once during each cell cycle. To complete the normal cell cycle, the centrosome duplication cycle and the centrosome quantity must be precisely regulated to couple the other events of cell cycle<sup>[9]</sup>. If centrosome replication deviates from cycles of DNA synthesis and mitotic division, an unsuccessful mitosis will come out with the features associated with the formation of aberrant centrosomes and multiple mitotic spindles, and unbalanced chromosome segregation<sup>[10]</sup>.

Enediyne antibiotics have been focused on their potent antitumor activity due to their unique ability to damage the DNA of tumor cells by inducing single strand (SSB) and/or double strand (DSB) breaks through free radical attacks on the deoxyribose moieties in DNA<sup>[11]</sup>. Lidamycin (also designated as C1027) is a member of the enediyne antibiotic family, which was isolated from a Streptomyces globisporus C1027 strain in China<sup>[12,13]</sup>. Lidamycin consists of a chromophore and an apoprotein, and the former has the ability to attack DNA, whereas the latter plays the role as a protecting protein<sup>[14]</sup>. The biological effects induced by lidamycin and ionizing radiation are similar<sup>[11]</sup>. Previous reports have shown that lidamycin is highly cytotoxic toward tumor cells<sup>[14-16]</sup>. As an attempt to investigate the mechanisms of lidamycin-induced mitotic cell death in human hepatoma BEL-7402 cells, we treated cells with lidamycin at low concentrations, and discovered centrosome overduplication, multipolar mitotic spindle formation, multinucleation, delayed reproductive death and changed patterns of protein expression associated with the regulation of proliferation and apoptosis. These results indicate that mitotic cell death in BEL-7402 cells induced by lidamycin is associated with centrosome overduplication independently of mitochondria pathway.

# MATERIALS AND METHODS

# Chemical

Lidamycin was generously provided by Professor Lian-Fang Jin from our institute, and stored at -20 °C as a 100  $\mu$ mol/L stock solution in 9 g/L NaCl solution.

## Cell culture

Human hepatoma BEL-7402 cells (obtained from the Key Laboratory of Cell Proliferation and Regulation Biology of the Ministry of Education, Beijing Normal University) were cultured in DMEM (Gibcol BRL) supplemented with 100 mL/L fetal bovine serum (HyClone), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37 °C in the presence of 50 mL/L CO<sub>2</sub>.

#### Growth curve assay

Growth curves establishments were performed at a 5-d interval as previously described<sup>[17]</sup> with some modifications. Totally  $2.0 \times 10^3$  cells were seeded into 96-well plates and then treated with lidamycin for 2 h. A 12 µL MTT (5 mg/mL) was added to each well before assay and incubated for an additional 4 h at 37 °C, followed by treatment with 100 µL of 0.01 mol/L HCl-100 g/L SDS overnight. The value at each time point was read on a Microplate Reader (Model 550, Bio-Rad) at  $\lambda_{570nm}$ .

#### Cell multinucleation shown by staining with Hoechst 33342

Subconfluent cells were continuously incubated for 72 h after exposure to lidamycin for 2 h, and then were stained by the DNA-specific fluorescent dye Hoechst 33342 (2 µg/mL) (Sigma) for 15 min at 37 °C. Next, cells were washed once, kept in PBS, and observed using a fluorescence microscope (BH2 system, Olympus) equipped with a  $\lambda_{455mm}$  filter.

#### Flow cytometry

Cells were exposed to 0.5 nmol/L lidamycin for 2 h and then incubated in fresh, drug-free medium. Following a 3-d incubation, cells including the floating and the attached were harvested and washed with cold PBS twice. Cell suspensions were fixed in 700 mL/L ethanol at 4 °C overnight. Next, the fixed cells were washed twice in PBS and incubated with 50  $\mu$ g/mL RNase (Sigma) for 30 min at 37 °C. Samples were then stained with 50  $\mu$ g/mL propidium iodide (Sigma) in the dark at 4 °C for 30 min, and analyzed on a fluorescence-activated cell sorter (EPICS XL, Coulter).

#### Indirect immunofluorescence

Cells were grown on coverslips. After 3 d following 2-h lidamycin treatment, the cells were washed in PHEM buffer (60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA and 2 mmol/L MgCl<sub>2</sub>) twice briefly, and incubated with a permeabilization buffer (5 mL/L Triton X-100 in PHEM buffer) for 90 s. Then, the cells were fixed in 37 g/L paraform in PHEM buffer for 15 min at room temperature. After washed in PBS 3 times, the cells were incubated with a blocking solution (50 g/L defatted dry milk and 0.5 mL/L Tween-20 in PBS) for 30 min and used for indirect immunofluorescence. The primary antibodies included anti-αtubulin monoclonal antibody (Zymed) and anti-centrin polyclonal antibody<sup>[18]</sup> (kindly provided by Professor Da-Cheng He, the Key Laboratory of Cell Proliferation and Regulation Biology of the Ministry of Education, Beijing Normal University). Rhodamine-labeled goat anti-mouse antibody (Zymed) and Fluoresceinisothiocyanate-labeled goat anti-rabbit antibody (Zymed) were used as second antibodies. The microscope slides were mounted with glycerol mounting medium (900 mL/L glycerol and 100 mL/L PBS) and observed under a laser-scanning

microscope (IX-70 system, Olympus). The cell with three or more centrosomes was considered aberrant.

#### Senescence-associated $\beta$ -galactosidase (SA- $\beta$ -gal) staining

Cells were treated with 0.1 nmol/L or 0.5 nmol/L lidamycin for 2 h and continuously maintained for 72 h. The attached cells were fixed in 5 mL/L glutaraldehyde and stained for SA- $\beta$ -gal activity using X-gal at pH 6.0 as previously described<sup>[19]</sup>.

#### Western blot analysis

Cells incubated with lidamycin at 37 °C for 2 h and then allowed to recover for 72 h at 37 °C were harvested and washed in PBS. The cells were lysed on ice in lysis buffer (100 mmol/L Tris, pH 6.8, 25 g/L SDS, 100 mL/L β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 mL/L glycerol) for 10 min, followed by ultrasonication. The cell lysates were cleared by centrifugation, and the protein concentration was estimated using the Bradford method with bovine serum albumin as a standard. Western blot analysis was performed as a protocol described previously<sup>[20]</sup>. In brief, equal amounts of protein were electrophoresed on SDS-polyacrylamide gel (Fluka) and transferred onto a nitrocellulose membrane (Hybond-P, Amersham Pharmacia) for blotting with primary antibodies including anti-Bax (N-20, Santa Cruz), anti-Smac (a kind gift from Dr. Xiao-Dong Wang, University of Texas Southwestern Medical Center, Dallas, USA), anti-cyclin B1 (GNS-1, Santa Cruz), anti-p16 (16P04, NeoMarkers), anti-Rb (C-15, Santa Cruz), anti-p53 (DO-1, Santa Cruz), anti-p21 (F-5, Santa Cruz), and anti-actin (I-19, Santa Cruz) antibodies. Secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia). Enhanced chemiluminescence (ECL Western Blot Kit, Amersham Pharmacia) was used according to the manufacturer's instructions.

# RESULTS

#### Growth inhibition induced by lidamycin in BEL-7402 cells

Inhibition of cell growth and proliferation was measured by the MTT test. Exposing BEL-7402 cells to 0.1 and 0.5 nmol/L lidamycin resulted in a dose-dependent inhibition of cell growth (Figure 1).



**Figure 1** Effects of lidamycin treatment on BEL-7402 cells growth. Cells were seeded in 96-well plates and treated with 0.1 nmol/L or 0.5 nmol/L lidamycin for 2 h. Growth curves were established indirectly by detecting reactions with MTT. The value of each time point was derived from three samples.

# Atypical chromatin condensation and multinucleation induced by lidamycin in BEL-7402 cells

With 0.1 nmol/L lidamycin for 2 h, followed by a 72-h incubation in drug-free medium, the treated BEL-7402 cells displayed a unique and atypical chromatin condensation characterized by appearance of small "dots" representing segregated condensed chromatin without apoptotic bodies (Figure 2B). Moreover, we did not observe detachment of these cells from the monolayer during the process of chromatin condensation. After 72-h incubation, multinucleation (three or more nuclei), one of the



**Figure 2** Induction by lidamycin of atypical chromatin condensation and multinucleation in BEL-7402 cells, determined by staining with the fluorescent dye Hoechst 33342 at 72 h after exposure to 0.1 nmol/L and 0.5 nmol/L lidamycin for 2 h. A: Untreated BEL-7402 cells; B: 0.1 nmol/L lidamycin treated BEL-7402 cells; C: 0.5 nmol/L lidamycin treated BEL-7402 cells.



**Figure 3** Effects of lidamycin on cell cycle. BEL-7402 cells were treated with 0.5 nmol/L lidamycin for 2 h, then washed and fed with fresh medium. After 72 h of incubation, the cells were stained with propidium iodide and sorted by flow cytometry. A,B: Untreated BEL-7402 cells; C, D: 0.5 nmol/L lidamycin treated BEL-7402 cells. The changes indicated in dot density maps were in accordance with those displayed in histograms.



**Figure 4** Immunofluorescence analysis of centrosomes and mitotic spindles in BEL-7402 cells at 72 h after treatments with 0.5 nmol/L lidamycin for 2 h. Cells grown on coverslips were fixed in paraform and doubly labeled with antibodies to  $\alpha$ - tubulin (red) and centrin (green). Localization of centrosome on superimposed image showed yellow. A: Untreated BEL-7402 cells; B,C: 0.5 nmol/L lidamycin treated BEL-7402 cells. Arrows in B,C indicated the cells with multiple centrosomes, multipolar mitotic spindle or chromosome missegregation.



**Figure 5** Phase contrast images of SA- $\beta$ -gal stained BEL-7402 cells. At 72 h after incubation with lidamycin for 2 h, attached cells were fixed with 5 mL/L glutaraldehyde and stained for SA- $\beta$ -gal activity for 16 h. A: Untreated BEL-7402 cells; B: 0.1 nmol/L lidamycin treated BEL-7402 cells; C: 0.5 nmol/L lidamycin treated BEL-7402 cells. The photographs were taken at 200-fold magnification (25  $\mu$ m scale bars).

main features of mitotic cell death, occurred at 0.5 nmol/L lidamycin-treated BEL-7402 cells (Figure 2C).

# *Changes of cell cycle progression and DNA polyploidy induced by lidamycin in BEL-7402 cells*

The biochemical and cytological changes of multinucleated

giant cells remain poorly understood. To further characterize the etiology of mitotic cell death, we analyzed the cell cycle and DNA content of the lidamycin-treated BEL-7402 cells by flow cytometry. The cells were exposed to a low concentration of lidamycin for 2 h. At 72 h after treatment, ~23.7% of BEL-7402 cells arrested in G2/M phase, and the cells with >4N DNA content were detected at 32.0% (Figure 3C).

# Centrosome overduplication, multipolar spindle formation and unbalanced division induced by lidamycin in BEL-7402 cells

Immunofluorescence microscopy revealed that the abnormalities of multiple centrosomes and multipolar mitotic spindles markedly increased in BEL-7402 cells induced by lidamycin at a low concentration. Co-staining with antibodies to centrin and  $\alpha$ -tubulin indicated that the increased centrosomes were localized at each pole of the multiple spindles, and the cells not deriving from equal division appeared (Figure 4B, C). However, to untreated cells, these defects rarely displayed (Figure 4A).

# Induction by lidamycin of senescence-like phenotype in BEL-7402 cells

The induction of SA- $\beta$ -gal activity and mitotic cell death were thought to be independent events<sup>[21]</sup>. To investigate the effects of mitotic cell death on senescence-like phenotype of the lidamycintreated cells, we observed SA- $\beta$ -gal expression, a senescence marker, at 72 h after lidamycin treatment. The treated cells showed phenotypic changes that resembled features of normal senescence, including enlarged and flattened morphology, increased granularity, vacuolization, and enhanced SA- $\beta$ -galpositive cells (Figure 5). Moreover, the induction of senescencelike phenotype in BEL-7402 cells was increased dose-dependently.

# Alterations of cell cycle related proteins in lidamycin-treated BEL-7402 cells

To understand some molecular changes that led to mitotic cell death, cell cycle proteins were analysed by Western blot analysis in lidamycin-treated cells. In BEL-7402 cells, lidamycin induced an increase in the levels of cyclin B1, p16 and pRb, meanwhile a decrease in expression of p53 and p21 (Figure 6).



**Figure 6** Western blot analysis of BEL-7402 cells. Cells were harvested at 72 h after 2-h lidamycin treatment, and total proteins (30 µg/lane) were resolved by 8 to 15% SDS-PAGE and transferred onto nitrocellulose, and Western blot was performed using the indicated antibodies. Lane A, untreated BEL-7402 cells; lane B, 0.1 nmol/L lidamycin treated BEL-7402 cells.

# Alterations of apoptosis related proteins in lidamycin-treated BEL-7402 cells

To confirm that mitotic cell death induced by lidamycin was distinguished from typical apoptosis, and detect the correlation between them, proteins associated with apoptosis were examined. Bax is a regulator of apoptosis<sup>[20]</sup>. Smac, an inhibitor of caspase suppressors, promotes cytochrome c-induced activation of caspases by sequestering the inhibitor of apoptosis protein family<sup>[22]</sup>. As is shown in Figure 6, the protein levels of Bax and Smac declined in lidamycin-treated BEL-7402

cells. We were unable to detect the proteolytically activated caspase-3 and caspase-9, and found no significant alterations of the blots representing caspase-3 precursor and caspase-9 precursor after exposure to low concentrations of lidamycin in BEL-7402 cells (data not shown).

# DISCUSSION

HCC is one of the most common malignant neoplasms in the world<sup>[1]</sup>. The incidence of HCC in China exceeds 100 000 per year, and at least 110 000 HCC-related deaths occur every year. Lidamycin is a highly potent cytotoxic antitumor agent. We treated human hepatoma BEL-7402 cells with lidamycin at concentrations in the nanomolar range, and assessed the potential role of centrosome in lidamycin-induced mitotic cell death. The results indicated a series of abnormal events including centrosome overduplication, formation of multipolar mitotic spindles, multinucleation, and eventual mitotic catastrophe. In this study, we have described for the first time the association between centrosomes and enediyne antibiotics-induced cell death in human hepatoma cells.

Thus far, the modes of cell death induced by lidamycin can be divided into two classes, one is apoptosis, and the other is mitotic cell death<sup>[5,23]</sup>. It has been reported that lidamycin can act directly as an endonuclease without dependence on caspase activities and is considered as an apoptosis-mimetic agent, at high concentrations<sup>[24]</sup>. Cells exposed to low concentrations of lidamycin lost reproductive integrity due to inappropriate entry into mitosis, and apparently exhibited the morphological and biochemical changes associated with mitotic cell death: enlarged cell shape, multi- or micronucleation, accumulation of karyotypic abnormalities, and a G2/M arrest<sup>[5]</sup>. Apoptosis, mitotic cell death and irreversible cell cycle arrest may all contribute to cell death after lidamycin treatment. The exact mechanism of mitotic cell death is unclear, and only a few studies have attempted to elucidate the effects of lidamycin on cells at moderate concentrations<sup>[5,11]</sup>. In the present study, we used low-dose lidamycin to treat BEL-7402 cells, and mitotic cell death was observed predominantly after treatment. We demonstrate that lidamycin can induce multiple centrosomes which may be responsible for the assembly of multipolar mitotic spindles and the chromosomes missegregation. Most of the cells containing multiple nuclear fragments were temporarily viable but reproductively dead. However, in some cases, the cells undergoing mitotic death initiated endocycles, restituted mitosis and finally survival<sup>[8]</sup>. We plan to continue this study to confirm the link between lidamycininduced mitotic cell death and centrosome overduplication by using centrosome inhibitors and vectors containing antisense mRNAs to centrosome related proteins.

Because centrosome duplication was closely associated with DNA replication, cytokinesis and cell cycle regulation<sup>[25,26]</sup>, we analyzed the cell cycle progression of lidamycin-treated BEL-7402 cells to detect relationship between centrosome dysregulation and cell cycle distribution as well as to confirm the appearance of multinucleated cells, which is one of the main features of mitotic cell death. We found that lidamycin induced centrosome overduplication associated with induction of G2/M arrest. Previous reports showed that centrosome replication could dissociate from DNA synthesis cycle and mitotic division<sup>[27]</sup>, and cell cycle block in G2/M phase might be related to abnormal centrosome accumulation<sup>[10]</sup>, and endocycles starting from G2/M arrest could produce endopolyploid cells<sup>[7]</sup>. We suppose that lidamycin-induced DNA replication cycle retardation in BEL-7402 cells could be helpful to trigger centrosome overduplication.

Analysis of gene expression may provide further insights into the molecular mechanisms mediating mitotic cell death. Cyclin B1, a component of the mitosis-promoting factor, plays an important role in G2/M regulation by forming a complex with p34cdk1 to phosphorylate various substrates necessary for mitosis. The cells with the morphological features of mitotic catastrophe frequently undergo up-regulation of cyclin B1 level<sup>[28,29]</sup>, which is consistent with our results. Bax expression is a regulator of apoptosis. Ordinarily action of Bax facilitates apoptosis<sup>[30]</sup>. However, our present study demonstrated that the levels of Bax and Smac both decreased along with no proteolytic activation of caspase-3 and caspase-9, and no DNA ladder was obtained (data not shown) after lidamycin treatments, which suggested that the mitochondrial apoptosis pathway might not be activated. The undetectable typical apoptosis is not caused by Bcl-2 involvement in BEL-7402 cells exposed to low concentrations of lidamycin (data not shown) and some other genes might play an essential role in this response. p21 is a p53regulated protein. p53 inhibition was shown to increase mitotic death<sup>[31]</sup>. We noticed decreased levels of p53 and p21 in BEL-7402 cells after lidamycin treatment. However, the expression of p16 and pRb proteins was upregulated in BEL-7402 cells, which might explain the increased intensity of staining for SA- $\beta$ -gal in lidamycin-treated cells, since p16 is closely related to induction of senescence-like phenotype<sup>[32]</sup>. Based on data presented here, we propose that the G2/M arrest of BEL-7402 cells may not be mediated by a classically driven cell cycle checkpoint mechanism correlated with p53. From a therapeutic standpoint, centrosome dysregulation might provide a valuable anticancer target. Further study to identify signaling pathways to mitotic cell death in tumor cells and normal somatocytes would help to improve the efficacy of HCC therapy with a low systemic toxicity.

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LIVER CANCER •

# Safety of *Curcuma aromatica* oil gelatin microspheres administered *via* hepatic artery

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# Abstract

**AIM:** To evaluate the safety of *Curcuma aromatica* oil gelatin microspheres (CAO-GMS) infused via hepatic artery against primary liver cancer.

**METHODS:** The safety of CAO-GMS was evaluated in view of its acute toxicity in rats, long-term toxicity in Beagle dogs and general pharmacology in rats and mongrel dogs.

**RESULTS:** The 50% lethal dose (LD<sub>50</sub>) of CAO-GMS infused via the hepatic artery was 17.19 mg/kg, and the serum biochemical indices of dying rats after the administration changed markedly while those of survived rats did not. Subsequent pathological examination of the tissues from the dead rats indicated improper embolism. Similar edema and small necrotic foci in the hepatic lobule were found in the hepatic tissue of rats receiving 10 and 5 mg/kg CAO-GMS and GMS 60 d after the last administration, while not in the rats of the blank control group, indicating that microspheres infused via the hepatic artery may induce irreversible liver damage dose-dependently. General pharmacological study showed that the activities (posture and gait), respiration frequency, blood pressure or heart rate of the dogs were not affected by CAO-GMS, nor were salivation, tremor or pupil changes of the rats observed or their balancing ability compromised, suggesting CAO-GMS infused via the hepatic artery did not significantly affect the nervous, respiratory and cardiovascular systems.

**CONCLUSION:** CAO-GMS embolization administered via the hepatic artery is safe but undesired embolization induced by vascular variation should be given due attention in its clinical application. Individualized embolization dosage and super-selective catheterization technique are recommended to avoid undesired embolism and reduce complications.

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# INTRODUCTION

Curcuma aromatica oil (CAO) is the oil mixture extracted from

Curcuma aromatica consisting of multiple effective ingredients, such as  $\beta$ -elemene and curcumol<sup>[1-7]</sup>. Researchers have demonstrated that CAO, with relatively low toxic effects, is the main anti-neoplasm ingredient of Curcuma aromatica<sup>[8-12]</sup>, which can be used for therapy against neoplasmic diseases, especially liver neoplasm<sup>[13-19]</sup>. Clinically, CAO has been applied in interventional therapy administered via the hepatic artery, and produced favorable effects in patients with primary liver cancer<sup>[20-24]</sup> and experimentally, in rats with transplanted liver tumor as well<sup>[25]</sup>. As an oil preparation, CAO is often difficult to achieve total embolization in interventional therapy with far too short retention time in the neoplastic lesions. CAO microsphere is suggested to be ideal for increasing the retention time, enhancing the inhibitory effects on cancer cells, and also for blocking the trophic vessels for the hepatoma, so to produce dual effects of chemotherapy and embolization. Therefore, we employed the microsphere degradation technique to entrap the CAO into the gelatin medium and prepared CAO-GMS<sup>[26]</sup>, which has shown potent therapeutic efficacy against transplanted liver cancer in rats<sup>[27-29]</sup> and from which the released CAO exhibited marked dose-dependent inhibition of the growth of human hepatoma cell line SMMC-7721<sup>[30]</sup>.

CAO-GMS is a preparation of traditional Chinese herbal drugs possible for quality control<sup>[26]</sup>. Considering its special pharmaceutical form and administration route, we adopted the interventional procedures described by Lindell *et al.*<sup>[31]</sup> similar to clinical situations, so as to study CAO-GMS in view of its acute toxicity, long-term toxicity and general pharmacology in rats and dogs. The results may help evaluate the safety of the interventional therapy with CAO-GMS embolization administered via the hepatic artery in the treatment of primary liver cancer and provide experimental evidence for its potential clinical application.

# MATERIALS AND METHODS Drugs and reagents

CAO-GMS (50-100  $\mu$ m in diameter, Batch No.: 971006), 8% CAO and blank gelatin microspheres (50-100  $\mu$ m in diameter, Batch No.: 97050) were provided by the Department of Pharmaceutics, Shenyang Pharmaceutical University. Sodium carboxylmethyl cellulose (CMC-Na) was purchased from Guangzhou Chemical Reagents and Glassware Co. (Batch No.: 950808). The microspheres were suspended in 3 g/L CMC-Na solution for use.

# Animals

A total of 104 SD rats (clean animals, including 52 males and 52 females weighing 220-300 g, Certification No.: 152) used in this study were purchased from Shanghai Sino-British SIPPR/BK Lab Animal Co. Ltd. Twenty-one mongrel dogs (conventional animals, 11 males and 10 females, weighing 13.5-21.0 kg, Certification No.: 19980610) were purchased from Guangzhou Shima Experimental Animal Company and quaranteed by the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine. Eighteen Beagle dogs (specific pathogenfree animals, 9 males and 9 females, weighing 12.5-13.1 kg, Certification No.: 97A025) were purchased from the Experimental Animal Center of Guangzhou Medicine Industry Institute.

Six-channel physiological recorder (Plugsys, Hugo Sachs Elektonik, Germany), blood pressure sensor (Isotec, Hugo Sachs Elektonik, Germany), thermosensitive recorder (WR500, Isotec, Hugo Sachs Elektonik, Germany), digital subtraction angiography-interventional therapy apparatus (DSA, Siemens, Germany), automated blood analyzer (Counter-5, Japan), automated blood coagulation analyzer (ACT-3000 plus, Germany), clinical biochemical analyzer (CL-7200, Japan) and electrocardiograph (ECG-6511, Japan) were used in this study.

# Acute toxicity in rats

Seventy-four SD rats were randomly divided into 7 groups including CAO-GMS-1 group (n = 10) receiving CAO-GMS 5 mg/kg, CAO-GMS-2 group (n = 12) receiving CAO-GMS 10 mg/kg, CAO-GMS-3 group (n = 14) receiving CAO-GMS 20 mg/kg, CAO-GMS-4 group (n = 12) receiving CAO-GMS 40 mg/kg, GMS control group (n=10) receiving gelatin microspheres 40 mg/kg, blank control group (n = 8) receiving 3.5 mL/kg CMC-Na (3 g/L) in a volume equivalent to that of injected microspheres and ZT group (n = 8) receiving 3.2 mg/kg CAO. All rats were anesthetized with 45 mg/kg intraperitoneal pentobarbital injection prior to drug administration following the method described by Lidell et al.<sup>[31]</sup> (Figure 1). In brief, a catheter was inserted through the gastroduodenal artery to the proper hepatic artery, the distal end of the gastroduodenal artery was ligated, and the common hepatic artery as well as the right branch of the proper hepatic artery was temporarily blocked to receive the agents through the left branch of the proper hepatic artery. The general activities, mortality and survival time of the rats were consecutively observed and recorded for 14 d after drug administration. Blood samples of the dying rats were collected and determined for ALT, AST, total bilirubin (T-BIL), direct bilirubin (D-BIL), blood creatinine (BCr) and blood urea nitrogen (BUN). Blood samples were also taken from the survived rats on d 14 for determination of the above indices. Autopsy was performed on the dead rats and the vital organs such as the liver, spleen, lung and kidney etc. were pathologically examined.



Figure 1 Administration method (from Lindell<sup>[31]</sup>).

#### Long-term toxicity in Beagle dogs

Eighteen Beagle dogs were randomly divided into 4 groups including CAO-GMS high-dose group (CAO-GMS-H, 5 dogs) receiving 15 mg/kg CAO-GMS every 4 wk, CAO-GMS low-dose group (CAO-GMS-L, 5 dogs) receiving 7.5 mg/kg CAO-GMS every 4 wk, GMS control group (4 dogs) receiving 15 mg/kg gelatin microspheres every 4 wk, blank control group (4 dogs) receiving 10 mL/kg saline in the same volume as that of the microspheres every 4 wk. Each dog was anaesthetized with 30 mg/kg pentobarbital, and a catheter was super-selectively inserted into the hepatic artery through the femoral artery puncture under perspective monitor. The hepatic artery was examined by intra-arterial digital subtraction angiography with

the injection of 8 mL meglumine diatrizoate (760 g/L) at the rate of 2 mL/s, 150 Psi by a high-pressure syringe. The microspheres of specified doses were mixed with the contrast media and slowly injected. To prevent improper embolism due to reflux of the liquid, the injection rate was strictly controlled. The catheter was washed with saline after the injection, and angiography of the hepatic artery was performed for confirmation of the range and degree of embolization. The catheter was withdrawn after the surgery, and the puncture pressed for 15 min and bandaged with pressure. The procedures were repeated twice every 4 wk. Half of the dogs in each group were sacrificed on d 30 after the last treatment and the other half on d 60. The tissue samples were fixed in 40 g/L neutral formaldehyde, processed by the standard histological techniques, and stained with hematoxylin and eosin (HE) for light microscopic examination. The liver, kidney, lung, adrenal, pancreas, stomach, duodenum, ileum, colon, prostate, brain, spinal cord, heart, spleen, sternum (bone and marrow), bladder, uterus, ovary, thyroid, thymus and testis of the dogs were all examined pathologically to assess the long-term toxicity, reversible toxicity and delayed toxicity of CAO-GMS.

#### General effect on nervous system of rats

Thirty rats were randomly divided into 3 groups including highdose CAO-GMS group (CAO-GMS-H, 12 rats) receiving 10 mg/kg CAO-GMS, low-dose CAO-GMS group (CAO-GMS-L, 10 rats) receiving 5 mg/kg CAO-GMS, and blank control group (8 rats) receiving 3.5 mL/kg CMC-Na (3 g/L, in equivalent volume). The administration of the agents followed the procedures described above. The general activities (posture and gait), salivation, tremor or pupil changes of rats were recorded. Balance test of rotarod model was performed on d 3, 5, 7 and 14, respectively, in which the rats were put on a 90-cm-long rod 2.5 cm in diameter to observe the dropping frequency. The rats dropping more than 3 times were defined to have incoordination with nervous system impairment.

#### General effect on respiratory and cardiovascular systems of dogs

Twenty-one mongrel dogs were randomly divided into 4 groups, namely high-dose CAO-GMS group (CAO-GMS-H, 6 dogs receiving 0.10 g CAO-GMS), low-dose CAO-GMS group (CAO-GMS-L, 6 dogs receiving 0.03 CAO-GMS), CAO group (4 dogs receiving 6.4  $\mu$ L/kg CAO) and blank control group (5 dogs) receiving 10 mL/kg CMC-Na (3 g/L, in equivalent volume). Under anesthesia with pentobarbital, the blood pressure of the dogs was measured with catheterization through the common carotid artery (connected by Isotec blood pressure sensor). Two-lead electrocardiogram and respiratory frequency were also recorded. Thirty minutes later, the dogs were given specified agents with the method described above. The respiratory frequency, blood pressure and electrocardiogram of the dogs before and 30, 60, 120, 180 min after administration were measured using six-channel physiology recorder.

#### Statistical analysis

Data were expressed as mean $\pm$ SD. The 50% lethal dose (LD<sub>50</sub>) was calculated with Bliss method. The biochemical indices were assessed by multiple ANOVA. Changes in blood pressure, respiratory frequency and heart rate before and after administration of the agents within the same group were assessed by paired *t* test using SPSS 10.0 statistical software. The difference was considered significant when *P* value was less than 0.05.

# RESULTS

# Acute toxicity in rats

All rats in CAO-GMS-4 group fell in a state of drowsiness with

hair erection and died within 24 h after administration of the agent. Death of the rats in CAO-GMS-3 group occurred 24-48 h after the administration, with the survived rats exhibiting signs of fatigue, hair erection, and emaciation accompanied by reduced activity and anorexia; 2 rats developed ascites and survived until d 14. The rats in CAO-GMS-2 group also showed fatigue within the first 2 d after administration of the agent but resumed normal condition on d 3. The rats in CAO-GMS-1, blank control and CAO group did not show signs of discomfort. The rats of GMS group exhibited changes similar to those of CAO-GMS-4 group. LD<sub>50</sub> of CAO-GMS embolization via the hepatic artery was 17.19 mg/kg, with the 95% confidential interval of 13.08-23.67 mg/kg (Table 1).

Compared with those of the blank control group, all the dying rats of the CAO-GMS groups showed marked increase in ALT, AST, T-BL, D-BL, BCr and BUN (Table 2), but none of the survived rats showed marked changes in these indices (Table 3).

The tissue samples of the rats in CAO-GMS and GMS groups showed damage of hepatic tissues, as were mostly manifested in CAO-GMS-3, 4 groups and GSM group. The rats in CAO-GMS-3 and -4 groups were found to have hemorrhagic damage in lung tissues and necrosis of renal tubules, indicating dosedependent damage of the kidney and lung by CAO-GMS. We identified traces of the microspheres in a tissue sample of CAO-GMS-2 group, suggesting that the toxic effects on kidney tissues in CAO-GMS-1, -2, -3, -4 groups arose from improper

 $\label{eq:table1} \textbf{Table 1} \ \ \textbf{Deaths of the rats after administration of CAO-GMS}$ 

embolization of the renal artery. The tissue samples from the 4 groups showed dose-independent damage of the spleen tissues. Abnormal changes were not observed in vital organs in blank control and CAO groups.

We found that CAO-GMS induced untoward responses and death of the rats dose-dependently. Since the rats in blank control and CAO groups showed no untoward response as those in GMS group did, we presumed that GMS, instead of CAO, caused renal and hepatic injuries. The most probable cause of the renal damage might be undesired embolization of the renal artery by GMS.

# Long-term toxicity in Beagle dogs

The dogs of CAO-GMS-H, CAO-GMS-L and GMS groups showed necrotic foci in the hepatic tissue with color attenuation 1-2 mm in diameter. The blank control group showed no marked change in the color and texture of the hepatic tissue.

Figure 2 displays the pathological changes in hepatic tissues of the dogs. Two dogs in CAO-GMS-H group exhibited whole lobule coagulation necrosis in the hepatic tissue with total destruction of the normal structures. The remaining hepatic tissue exhibited extensive moderate cellular edema with occasional cellular inflammatory edema in the necrotic region. In a dog died during the experiment, whole lobule coagulation necrosis was observed in the hepatic tissue, which was also seen in two dogs sacrificed on d 30 with mild cellular edema in the remaining hepatic tissue and destruction of normal structures.

Group	Number of rats	Dosage (mg/kg)	Number of dead rats	Mortality (%)	Peak time of death occurrence (h)
CAO-GMS-1	10	5	0	0	-
CAO-GMS-2	12	10	4	33.33	24-48
CAO-GMS-3	14	20	8	57.14	24-48
CAO-GMS-4	12	40	12	100	<24
GMS	10	40	7	71.42	24-48
Blank control	8	$3.5^{1}$	0	0	-
CAO	8	3.2	0	0	-

<sup>1</sup>Measured in mL/kg.

Table 2 Changes in biochemical indices of the dying rats receiving CAO-GMS (mean±SD)

Group	Nurmber of rats	ALT (U/L)	AST (U/L)	T-BiL (mmol/L)	D-BiL (µmol/L)	BCr (µmol∕L)	BUN (mmol/L)
CAO-GMS-1	0	-	-	-	-	-	-
CAO-GMS-2	4	$200{\pm}54^{\mathrm{b}}$	$805{\pm}533^{\rm b}$	$12.0\pm5^{ m b}$	$46.0{\pm}90^{\rm b}$	$50.0{\pm}34^{\mathrm{b}}$	$20.0{\pm}12^{a}$
CAO-GMS-3	8	$186{\pm}89^{\mathrm{b}}$	$700{\pm}522^{\mathrm{b}}$	$12.4{\pm}8.6^{\rm b}$	$59.0{\pm}88^{\mathrm{b}}$	$49.0{\pm}43^{\mathrm{b}}$	$20.0{\pm}14^{a}$
CAO-GMS-4	12	$241{\pm}289^{\mathrm{b}}$	$715\pm614^{\mathrm{b}}$	$15.0{\pm}19^{\mathrm{b}}$	$78.0{\pm}162^{\rm b}$	$58.0{\pm}43^{\mathrm{b}}$	$22.0 \pm 13^{a}$
GMS	7	$232{\pm}168^{\rm b}$	$685{\pm}503^{\rm b}$	$13.8{\pm}9.6^{\rm b}$	$54.0{\pm}126^{\rm b}$	$52.0{\pm}36^{\mathrm{b}}$	$20.0{\pm}13^{a}$
Blank control	0	38±11	$278 \pm 114$	$4.5 \pm 1.3$	$3.2{\pm}1.6$	$38.7 {\pm} 3.5$	8.6±11.8
CAO	0	-	-	-	-	-	-

<sup>a</sup>*P*<0.05; <sup>b</sup>*P*<0.01 *vs* blank control.

Table 3 Changes in biochemical indices of survived rats receiving CAO-GMS (mean±SD)

Group	Nurmber of rats	ALT (U/L)	AST (U/L)	T-BiL (mmol/L)	D-BiL (µmmol/L)	BCr (µmmol∕L)	BUN (mmol/L)	
CAO-GMS-1	10	37.7±5.5	$310\pm96$	$5.4{\pm}1.4$	$3.8{\pm}1.4$	$40.3 \pm 5.2$	$8.42{\pm}0.69$	
CAO-GMS-2	8	$39.2 \pm 7.7$	$301 \pm 74$	$5.2 \pm 1.7$	$3.6{\pm}1.8$	$41.8 {\pm} 9.3$	$10.00{\pm}2.2$	
CAO-GMS-3	6	$39.0 \pm 10$	$314 \pm 59$	$7.4{\pm}4.7$	$4.5 {\pm} 2.9$	$39.0 \pm 13$	$9.20{\pm}2.9$	
CAO-GMS-4	0	-	-	-	-	-	-	
GMS	3	$46.0 \pm 6.5$	$265 \pm 114$	$4.0 \pm 1.5$	$2.9{\pm}1.6$	$35.0{\pm}1.4$	$7.60{\pm}1.4$	
Blank control	8	$38.0 \pm 11$	$278 \pm 114$	$4.5 \pm 1.3$	$3.2{\pm}1.6$	$38.7 \pm 3.5$	$8.60{\pm}1.8$	
CAO	8	$36.0 \pm 7$	$307 \pm 84$	$4.8 {\pm} 1.9$	$3.2 \pm 1.7$	$39.4{\pm}2.4$	$8.00 \pm 1.6$	



**Figure 2** Long-term toxicity of CAO-GMS on the liver of dogs. A: CAO-GMS-H group receiving 15 mg/kg CAO-GMS every 4 wk; B: CAO-GMS-L group receiving 7.5 mg/kg CAO-GMS every 4 wk; C: GMS group receiving 15 mg/kg GMS every 4 wk; D: Blank control group receiving 10 mL/kg saline every 4 wk.

Two dogs of CAO-GMS-L group developed extensive moderate to severe hepatic cellular necrosis. The central region of the liver lobules showed moderate cellular edema with fatty degeneration and foci of coagulation necrosis, where neutrophilic leukocyte infiltration could be seen. Two dogs sacrificed on d 30 had extensive moderate cellular necrosis in the hepatic tissue and small necrotic foci, with ruptured normal structure.

In GMS group, two dogs were found to have whole lobular coagulation necrosis in the hepatic tissue where normal structure was disrupted. The other regions of the liver lobules showed moderate cellular edema with lymphocyte infiltration in the necrotic region. Two dogs sacrificed on d 30 exhibited extensive mild cellular necrosis in the hepatic tissue and spotty necrosis of the hepatic cells.

In the blank control group, mild cellular edema in the hepatic tissue was found in 2 dogs, with disrupted normal structure. Two dogs sacrificed on d 30 exhibited mild cellular necrosis in the hepatic tissue.

Pathological examination of the other vital organs showed no abnormal changes in the morphology of the kidney, lung, adrenal, pancreas, stomach, duodenum, ileum, colon, prostate, brain, spinal cord, heart, spleen, sternum (bone and marrow), bladder, uterus, ovary, thyroid, thymus and testis.

The above results demonstrated that CAO-GMS infused via the hepatic artery may cause irreversible ischemic necrosis of the liver without visible damage on other organs. Delayed toxic response was not evident. The toxic effects on liver were identical with those observed in study of the acute toxicity. Partly due to the super-selective catheterization technique employed in this study, no improper embolization was found, indicating the importance of the technique in the clinical application for reducing complications.

#### Effects on nervous system of rats

The rats receiving 5 or 10 mg/kg CAO-GMS or 3 g/L CMC-Na showed emaciation, reduced activity and anorexia in the first 2 postoperative days, but resumed normal conditions on d 3, probably due to operative injury. No pupil abnormality or tremor was observed. All rats dropped less than 3 times in the balance test of rotarod model, indicating that CAO-GMS did not affect the balancing ability of the rats.

#### Effects on respiratory and cardiovascular systems of dogs

The effects of CAO-GMS on respiratory frequency, blood pressure and heart rate are shown in Table 4. CAO infused via the hepatic artery produced no marked effects on blood pressure and heart rate of the dogs (P>0.05). The respiratory frequency of the dogs within 3 h after administration of the agent was lowered, but not statistically (P>0.05 vs indices before embolization).

CAO-GMS or blank GMS produced no marked effects on blood pressure and heart rate of the dogs (P>0.05, Tables 5, 6).

 $\label{eq:table_transform} \mbox{Table 4} \mbox{ Changes in respiration, rates blood pressure and ECG in dogs at different time points after CAO (6.4\,\mu L/kg) interventional therapy (mean \pm SD)$ 

Time point	Numer of note	Description for success	Heart rate (heat (min)	Blood pressure (mmHg)		
Time point	(breath/min)		Heart rate (beat/ mm)	Systolic	Diastolic	
Preoperation	4	17.0±10.2	$198.5 \pm 38.4$	147.8±8.2	116.0±11.5	
30 min postoperative	4	$15.0 \pm 7.0$	$193.5 \pm 36.1$	$141.5 \pm 8.2$	$114.8 \pm 9.7$	
60 min postoperative	4	$16.0 \pm 8.5$	$189.3 \pm 27.1$	$140.5{\pm}8.9$	$115.8 \pm 9.3$	
120 min postoperative	4	$17.8 \pm 9.5$	$190.5 \pm 23.6$	$141.5 \pm 3.3$	$117.0 \pm 5.0$	
180 min postoperative	4	$13.8{\pm}6.0$	$189.0{\pm}28.4$	$143.5{\pm}5.8$	$119.0{\pm}6.3$	

Time point	Decer	Number of note	Deanination from on an	I I continato	Blood pressure (mmHg)		
Time point	Dosage	Number of rats	(/min)	(/min)	Systolic	Diastolic	
Preoperation	0.10	6	$19.0 \pm 5.2$	219.0±22.7	$142.5 \pm 9.0$	112.7±9.1	
	0.03	6	$13.5 {\pm} 4.9$	$196.0 \pm 28.5$	$144.7 \pm 12.2$	$116.2 \pm 9.8$	
30 min postoperative	0.10	6	$20.5 {\pm} 6.7$	$214.7{\pm}19.1$	$142.7 \pm 7.3$	$113.0 \pm 8.6$	
	0.03	6	$14.0{\pm}4.9$	198.3±17.7	$146.3 \pm 11.3$	$118.8 \pm 12.6$	
60 min postoperative	0.10	6	$19.7 \pm 7.7$	$211.5 \pm 18.7$	$142.3 {\pm} 6.4$	$113.5 \pm 8.1$	
	0.03	6	$12.3 \pm 3.4$	$194.0 \pm 16.6$	$144.0 \pm 10.7$	$119.2 \pm 13.6$	
120 min postoperative	0.10	6	$19.5 \pm 7.8$	$211.7 \pm 25.0$	$142.3 \pm 10.0$	$114.0 \pm 10.7$	
	0.03	6	$15.3 {\pm} 5.5$	$193.7{\pm}20.2$	$145.0 \pm 9.1$	$118.8 {\pm} 9.2$	
180 min postoperative	0.10	6	$20.3 \pm 8.9$	$209.3 \pm 30.2$	$141.5 \pm 12.3$	$114.5 \pm 13.1$	
	0.03	6	$16.8{\pm}6.8$	$193.7{\pm}21.6$	$143.8 {\pm} 9.5$	$119.2 \pm 9.5$	

**Table 5** Changes in respiration, blood pressure and ECG in dogs at different time points after CAO-GMS interventional therapy (mean±SD)

**Table 6** Changes in respiration, blood pressure and ECG in dogs at different time points after GMS (0.1 g) interventional therapy(mean±SD)

Time point	Number of rate	Pospiration fraguency	Hoart rate	Blood pressu		
Time point	Number of fats	(/min)	(/min)	Systolic	Diastolic	
Preoperation	5	$20.4{\pm}6.2$	$186.6 \pm 28.3$	$133.4{\pm}16.0$	$109.4 \pm 16.9$	
30 min postoperative	5	$18.0{\pm}5.8$	$178.2 {\pm} 28.6$	$139.8 {\pm} 20.0$	$116.0 {\pm} 14.9$	
60 min postoperative	5	$18.2 \pm 5.8$	$179.0 \pm 29.2$	$143.8 {\pm} 20.7$	$117.2 \pm 17.4$	
120 min postoperative	e 5	$17.4 \pm 3.8$	$183.8 \pm 34.1$	$146.0 \pm 17.7$	$121.0 \pm 18.9$	
180 min postoperative	e 5	$18.0 {\pm} 4.1$	$180.0 \pm 37.5$	$142.6{\pm}20.0$	$116.8 \pm 19.7$	

The respiratory frequency of the dogs within 3 h after the administration was lowered, but not significantly (P>0.05 vs indices before embolization).

# Effects on respiratory blood pressure and heart rate graphs

The recorded graphical data also indicated the same results (Figure 3) as above.



**Figure 3** Effects of CAO-GMS infused via the hepatic artery on respiration, blood pressure and heart rate of dogs. A: Blank control group (with 3 g/L CMC-Na, 10 mL/kg); B: CAO-GMS group (with CAO-GMS, 0.1 g); C: CAO-GMS group (with CAO-GMS, 0.03 g); D: CAO group (with CAO, 6.4  $\mu$ L/kg); 1: Before administration; 2: 60 min after administration; 3: 180 min after administration.

## DISCUSSION

Transarterial chemoembolization (TACE) is most commonly adopted for palliative treatments in patients with hepatocellular carcinoma (HCC)<sup>[32,33]</sup>, but the shrinkage of neoplasm comes at the price of sacrificing the hepatic function and in the longterm effects, it does not significantly differ from conservative therapy<sup>[34]</sup>. Improvement in the operative technique and in the agents used for interventional therapy is therefore much desired to produce better anti-tumor effect with low toxicity and liver protection. By producing CAO-GMS, we take full advantage of CAO of its anti-tumor, immune-enhancing effects with low-toxicity<sup>[12-26]</sup>. To enhance the effect of embolizing the trophic vessels for hepatoma, CAO was coupled with biodegradable microsphere (50-100  $\mu$ m in diameter).

Previous studies demonstrated that CAO-GMS exhibited marked inhibitory effects on transplanted liver cancer in rats, which were attributed to the blocking of trophic vessels of the hepatoma by the microspheres and the therapeutic effects of CAO released slowly from the degraded microspheres<sup>[27-30]</sup>.

In this study, CAO-GMS was not found to affect respiration, blood pressure and heart rate, *etc.*, nor did it elicit acute or long-term toxic response, but at high doses, CAO-GMS embolization via the hepatic artery might induce significant increase in AST and ALT as well as irreversible ischemic necrosis of the liver. This was a sign of hyperreactive acute liver injury, as was similar to the clinical effect of other chemotherapeutic microspheres at high doses<sup>[35,36]</sup>, and predominantly responsible for its untoward, or even lethal effects.

CAO-GMS or blank GMS administered via the hepatic artery in normal animals caused embolization in the kidney and untargeted liver lobes, leading to the death of animals, which, however, did not happen with CAO or saline. This result indicates that CAO alone normally does not induce the adverse effects, which arise from undesired embolization of CAO-GMS as with other chemotherapeutic microspheres<sup>[36,37]</sup>. Based on these facts, we concluded that the most probable reason for the acute hyperreactive response was the sudden accumulative embolization in the liver or undesired embolization as in the renal artery.

The dose adopted for embolization and the operation technique are crucial for clinical application of CAO-GMS. Most researchers preferred the use of computed tomography (CT) to determine individualized dosage rather than a conventionally fixed dosage, along with super-selective catheterization techniques to reduce reflux embolization of the microspheres. These techniques were believed to enhance the safety of the CAO- GMS interventional therapy.

In general, the results of this study demonstrated that CAO-GMS is fairly safe for interventional therapy but individual difference should be fully considered in its clinical application. Clinicians are advised to determine the embolization dosage and interventional method individually to avoid the undesired embolization caused by the microspheres. We propose the use of superselective catheterization technique to precisely block the trophic artery of the hepatoma, and the exact dosage of CAO-GMS should be determined according to the size, location and trophic vessels of the tumors to prevent undesired embolization or complications after the interventional therapy.

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COLORECTAL CANCER •

# Histone acetylation regulates *p21<sup>WAF1</sup>* expression in human colon cancer cell lines

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# Abstract

**AIM:** To investigate the effect of histone acetylation on regulation of  $p21^{WAF1}$  gene expression in human colon cancer cell lines.

**METHODS:** Two cell lines, Colo-320 and SW1116 were treated with either trichostatin or sodium butyrate. Expressions of  $p21^{WAF1}$  mRNA and protein were detected by real-time RT-PCR and Western blotting, respectively. Acetylation of two regions of  $p21^{WAF1}$  gene-associated histones and total cellular histones were examined by chromatin immunoprecipitation assay and Western blotting.

**RESULTS:** Trichostatin or sodium butyrate re-activated  $p21^{WAF1}$  transcription resulted in up-regulated  $p21^{WAF1}$  protein level in colon cancer cell lines. Those effects were accompanied by an accumulation of acetylated histones in total cellular chromatin and  $p21^{WAF1}$  gene-associated region of chromatin.

**CONCLUSION:** Histone acetylation regulates *p21*<sup>WAF1</sup> expression in human colon cancer cell lines, Colo-320 and SW1116.

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# INTRODUCTION

Cell cycle progression is controlled by various cyclin-dependent kinases (CDKs), whose activation is carefully regulated at multiple levels including the induction and degradation of cyclin protein, CDKs phosphorylation by cyclin-activating kinase, and the induction of CDK inhibitors (CDKIs)<sup>[1]</sup>. CDKI  $p21^{WAF1}$  was first cloned and characterized as an important effector that acts to inhibit CDK activity in p53 mediated cell cycle arrest in response to various agents<sup>[2]</sup>. Increased expression of  $p21^{WAF1}$  may play a crucial role in the G<sub>1</sub>/S phase arrest induced in transformed cells, and may prevent the progression of neoplasia<sup>[3]</sup>. Histone acetylation is emerging as a major regulatory mechanism thought to modulate gene expression by altering the accessibility of transcription factors to DNA and recent studies suggest that these alterations may also be important in the process of neoplasia formation<sup>[4]</sup>. The level of histone acetylation depends on the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). An important approach that has been used to study the function of chromatin acetylation is the use of specific inhibitors of HDAC. Trichostatin A<sup>[5,6]</sup> (TSA, a hybrid polar compound of specific inhibitor) and sodium butyrate<sup>[4]</sup> (a short chain fatty acid produced in human colon by bacterial fermentation of carbohydrate) were reported to inhibit HDAC activity.

Previously it was revealed that acetylation of gene-associated histone or total cellular histone alone regulated  $p21^{WAFI}$  expression in colon cancer cell lines<sup>[7,8]</sup>. We have shown<sup>[9]</sup> that TSA or sodium butyrate induced G<sub>1</sub> phase cell cycle arrest was linked to increased expression of  $p21^{WAFI}$ . However, little is known about the regulation of acetylation of both gene-associated histone and total cellular histone on  $p21^{WAFI}$  expression in human colon cancer. It is as yet not clear about the effect of histone acetylation on  $p21^{WAFI}$  protein in Colo-320 and SW1116 cell lines. Therefore, in the present study, we further investigated whether TSA and sodium butyrate induced overexpression of  $p21^{WAFI}$  resulted from hyperacetylation of gene-associated histones and histones in total cellular chromatin in two human colon cancer cell lines, Colo-320 and SW1116.

# MATERIALS AND METHODS

## Cell culture

Human colon cancer-derived cell lines Colo-320 and SW1116 were obtained from Shanghai Institute of Biochemistry and Cell Biology, SIBS, China and Shanghai Second Medical University Ruijin Hospital, respectively. Colo-320 and SW1116 cells were maintained in RPMI 1640 supplemented with 100 mL/L heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37 °C in a 50 mL/L CO<sub>2</sub> incubator.

## Treatment of cells with TSA or sodium butyrate

Colon cancer cell lines were exposed to 1  $\mu$ mol/L TSA or 5 mmol/L sodium butyrate (Sigma, St. Louis, MO) alone for 24 h, as described by Siavoshian *et al.*<sup>[10]</sup>. The control cultures were treated simultaneously with phosphate-buffered saline (PBS) or alcohol (control for TSA treatment, because TSA can only be dissolved in alcohol).

# Western blotting of acetylated histones and p21WAF1

Colo-320 cells were cultured as described below with or without treatment. Cells were recovered by centrifugation, washed twice with ice-cold PBS, and resuspended for lysis in 1 mL buffer A (10 mmol/L HEPES, pH 7.4, 10 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 1 µg/mL protease inhibitors, 0.25 g/L NP40) for 15 min with rotation at 4 °C and the nuclear pellet was resuspended in 100 µL buffer B (20 mmol/L HEPES, pH 7.4, 420 mmol/L NaCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L EDTA,

250 mL/L glycerol, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 1 µg/mL protease inhibitors) for 30 min, then the soluble nuclear protein was collected by centrifugation. Fifty  $\mu g$  (for acetylated histone H3 and H4) or 150 µg (for p21<sup>WAF1</sup> protein) of nuclear extracts was boiled in loading buffer (125 mmol/L Tris-HCl, pH 6.8, 40 g/L SDS, 200 g/L glycerol, 0.05 g/L bromphenol blue) for 5 min and then loaded onto a 150 g/L SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membrane (0.45  $\mu$ m). The following antibodies were used: rabbit polyclonal antibody against acetylated histone H3 or H4 (Upstate Biotechnology, Lake Placid, NY) and goat polyclonal antibody against p21<sup>WAF1</sup> (C19, Santa Cruz, California). The bindings of antibodies were detected using ECL-system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and membranes were then exposed to Kodak BioMax film for 1 min. Antibody against  $\beta$ -actin (Sigma) in Western blot was used as a control for protein concentration.

# Real-time RT-PCR for p21<sup>WAF1</sup> mRNA

mRNA level of  $p21^{WAF1}$  was measured using a real-time quantitative PCR system. Total RNA samples from SW1116 and Colo-320 cells with or without treatment were prepared by TriZol Reagent. Gene-specific TaqMan probes and PCR primers were designed using Primer Express software (PE Biosystems, Foster City, CA). The sequence for forward and reverse primers and the probe are shown in Table 1. Triplicate PCR reactions were prepared for each cDNA sample. PCR consisted of 40 cycles of 95 °C denaturation (15 s) and 60 °C annealing/extension (60 s). Thermal cycling and fluorescent monitoring were performed using an ABI 7 700 sequence analyzer (PE Biosystems). The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample, and the average Ct of triplicate samples was calculated. To determine the quantity of gene-specific transcripts present in treated cells cDNA relative to untreated cells, their respective Ct values were first normalized by subtracting the Ct value obtained from the  $\beta$ -actin control ( $\triangle$ Ct = Ct FAM-Ct VIC). The concentration of gene-specific mRNA in treated cells relative to untreated cells was calculated by subtracting the normalized Ct values obtained with untreated cells from those obtained with treated samples ( $\triangle \triangle Ct = \triangle Ct$  of treated cells-DCt of untreated cells), and the relative concentration was determined  $(2^{-\triangle \triangle Ct})$ . Altered mRNA expression was defined as 3-fold difference in the expression level in cells after the treatment relative to that before treatment<sup>[11]</sup>.

# Chromatin immunoprecipitation (ChIP) assay

A ChIP assay kit from Upstate Biotechnology was used

Tabl	e 1	Sequence	of	primers	and	probes	for	real	l-time	PCR
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according to the manufacturer's protocol and Richon's report<sup>[12]</sup>. Colo-320 cells that were either treated with 1 µmol/L TSA or 5 mmol/L sodium butyrate for 24 h or untreated were plated at a density of  $10{\times}10^6\,/T25$  flask. Formaldehyde was then added to the cells to a final concentration of 10 g/L, and the cells were incubated at 37 °C for 10 min. The medium was removed, and the cells were suspended in 1 mL of ice-cold PBS containing protease inhibitors [1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin and 1 µg/mL pepstatin A, Boehringer Mannheim]. Cells were pelleted, resuspended in 0.2 mL of SDS lysis buffer, and incubated on ice for 10 min. Lysates were sonicated. The majority of DNAs ranged from 200 bp to 1 000 bp. Debris was removed from samples by centrifugation for 10 min at 15 000 g at 4 °C in a microcentrifuge. An aliquot of the chromatin preparation (200 µL) was set aside and designated as the input. Supernatants were 10-fold diluted in ChIP dilution buffer containing the protease inhibitors as above, and 80 µL of a salmon sperm DNA/protein A-agarose beads was added and incubated for 30 min at 4 °C with rocking. Beads were pelleted by centrifugation, and supernatants were placed in tubes with 10 µg of antibody against acetylated histone H3 or H4, or normal rabbit IgG, and incubated overnight at 4 °C with rotation. Salmon sperm DNA/Protein A-agarose beads (60 µL) was added, and samples were rocked for 1 h at 4 °C. Protein A complexes were centrifuged and washed 5 times for 5 min each with low salt buffer, high salt buffer, LiCl buffer and TE buffer, respectively. Immune complexes were eluted twice with 250 µL of elution buffer (10 g/L SDS/0.1 mol/L NaHCO<sub>3</sub>) for 15 min at room temperature. NaCl (5 mol/L, 20  $\mu L)$  was added to the combined eluate, and the samples were incubated at 65 °C for 4 h. EDTA, Tris-HCl, pH 6.5, and proteinase K were then added to the samples at a final concentration of 10 mmol/L, 40 mmol/L, and  $0.04\,\mu g/\mu L$ , respectively, and the samples were incubated at 45 °C for 1 h. Immunoprecipitated DNA (both immunoprecipitation samples and input) was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR.  $p21^{WAF1}$ -specific primers were used to carry out PCR. Sequences of two sets of primers for  $p21^{WAF1}$  PCR and PCR condition are shown in Table 2. The first set primer was used to amplify -576 to -293 and the second set primer was used to amplify -51 to +77 of  $p21^{WAF1}$  promoter and exon 1, which contained the transcription factor E2A binding sites.

# RESULTS

# HDAC inhibitors resulted in accumulation of acetylated core histones H3 and H4

Western blotting showed that before incubation with TSA or

Gene	Primer (forward) $(5 \rightarrow 3')$	Primer (reverse) (5 <sup>,</sup> →3')	Probe	GenBank accession number
p21 <sup>WAF1</sup>	CTG GAG ACT CTC	GGA TTA GGG CTT	ACG GCG GCA GAC	NM_078467
	AGG GTC GAA	CCT CTT GGA	CAG CAT GA	
β-actin	CTG GCA CCC AGC	GGA CAG CGA	ATC ATT GCT	BC016045
	ACA ATG	GGC CAG GAT	CCT CCT GAG	

Table 2 Sequence of primers and program of PCR for ChIPs

Primers	Sense (5 <sup>·</sup> →3')	Antisense (5 <del>'→</del> 3')	Size of product and PCR condition	GenBank accession number
γ-actin	GGA CCT GGC	GTG GCC ATC TCC	153 bp 95 °C 5 min 95 °C 1 min,	
	TGG CCG GGA CC	TGC TCG AA	56 °C 1 min, 72 °C 1 min, 35 cycles	
<i>p21</i> <sup>WAF1</sup> (P1)	CGT GGT GGT GGT	CTG TCT GCA	296 bp 95 °C 5 min 95 °C 1 min,	U24170
	GAG CTA GA	CCT TCG CTC CT	58 °C 1 min, 72 °C 1 min, 35 cycles	
p21 <sup>WAF1</sup> (P2)	GGT TGT ATA	CTC TCA CCT CCT	128 bp 95 °C 5 min 95 °C 1 min,	U24170
	TCA GGG CCG	CTG AGT GC	58 °C 1 min, 72 °C 1 min, 35 cycles	

sodium butyrate, the levels of acetylated H3 and H4 in colo-320 cells were low. Incubation with HDAC inhibitors resulted in the accumulation of acetylated histones H3 and H4 (Figure 1).



**Figure 1** Western blotting of acetylated histones H3, H4 and p21<sup>WAF1</sup> protein in Colo-320 cells. Lane 1: Control (PBS); lane 2: Control (alcohol); lane 3: TSA 1  $\mu$ mol/L, 24 h; lane 4: NaBu 5 mmol/L, 24 h.

# Either TSA or sodium butyrate induced re-expression of p21<sup>WAF1</sup> mRNA and protein

To understand the change of  $p21^{WAF1}$  expression level following HDAC inhibitors treatment, we examined accumulation of mRNA and protein by RT-PCR and Western blotting. As shown in Figures 1 and 2,  $p21^{WAF1}$  mRNA and protein were activated after treatment of TSA and sodium butyrate. In addition, Colo-320 cells had an initial increase in  $p21^{WAF1}$  expression to a higher level than that in SW1116 cells.



Figure 2 RT-PCR showed either TSA or sodium butyrate induced overexpression of p21<sup>WAF1</sup> mRNA in human colon cancer cell lines Colo-320 and SW1116. Lane 1: Control (PBS); lane 2: Control (alcohol); lane 3: TSA 1  $\mu$ mol/L, 24 h; lane 4: NaBu 5 mmol/L, 24 h.



Figure 3 HDAC inhibitors induced accumulation of acetylated histones H3 and H4 in chromatin associated with  $p21^{\rm WAF1}$ 

gene. A: PCR products for the 1st set primer of p21WAF1; B: PCR products for the 2nd set primer of p21<sup>WAF1</sup>; C: The figure in A and B was scanned and quantified by using IMAGE analysis software. The ratio between input DNA and precipitated DNA was calculated for each treatment and primer set. The increase after treatment with either TSA or sodium butyrate was calculated from indicated ratios. PCR products of the 2 nd primer were not visualized on agarose gel before treatment with HDAC inhibitor, so it could not be calculated. Input DNA, lanes 1-4: Soluble chromatin was immunoprecipitated with antiacetylated histone H3 antibody; lanes 5-8: Soluble chromatin was immunoprecipitated with antiacetylated histone H4 antibody; lanes 9-12: Normal rabbit serum as negative control; lanes 13-16, 1, 5, 9, 13: PBS control; lanes 2, 6, 10, 14: Alcohol control; lanes 3, 7, 11, 15: TSA 1 µmol/L, 24 h; lanes 4, 8, 12, 16: NaBu 5 mmol/L, 24 h.

# p21<sup>WAF1</sup> gene-associated histone H3 was hyperacetylated in colon cancer cells treated with TSA or sodium butyrate

To determine whether histone acetylation reflected  $p21^{WAF1}$  transcription and the functional interaction between  $p21^{WAF1}$  and TSA or sodium butyrate treatment, ChIPs-PCR was performed. As shown in Figure 3, the densities of bands of  $p21^{WAF1}$  gene-associated acetylated histones H4 and H3 were higher in chromatin extracted from Colo-320 cells treated with either TSA or sodium butyrate than that from cells mock treated, either the first or the second set PCR primer.

Taken together, TSA or sodium butyrate activated the transcription of  $p21^{WAF1}$  through acetylation of histones H4- and H3-associated  $p21^{WAF1}$  promoter.

# DISCUSSION

Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering chromatin structure<sup>[13]</sup>. Acetylation of core nucleosomal histones is regulated by the opposing activities of HATs and HDACs. The latter catalyze the removal of an acetyl group from the  $\varepsilon$ -amino group of lysine side chains of histones H2A, H2B, H3 and H4, thereby reconstituting the positive charge in lysine. Transcriptionally silent chromatin is composed of nucleosomes in which the histones have low levels of acetylation of lysine residues at their amino-terminal tails<sup>[14,15]</sup>. Acetylation of histone neutralizes the positive charge in lysine residues and disrupts nucleosome structure, allowing unfolding of the associated DNA, access by transcription factors, and changes in gene expression. Chromatin fractions enriched in actively transcribed genes are also enriched in the more highly acetylated isoforms of the core histones<sup>[16]</sup>. HDAC inhibitors appear to be selective with regard to the genes whose expression is altered<sup>[17]</sup>.

Total cellular histone acetylation is also involved in the regulation of gene expression. Several studies<sup>[18]</sup> indicated that the effect of HDAC inhibitors on gene transcription was associated with an increased accumulation of acetylated histones H3 and H4 in total cellular chromatin. However, Lee's group<sup>[19]</sup> showed an accumulation of acetylated histores H3 and H4 in total cellular chromatin after treatment with HDAC inhibitor (MS-275), but no change in the level of histone acetylation in chromatin-associated TGF- $\beta$  I receptor gene. Therefore, we wanted to know whether HDAC inhibitor affected the acetylation level of histones in both gene-associated and total cellular chromatin. The data from ChIP and Western blotting suggested that  $p21^{WAF1}$  transcription was dependent upon acetylation at the level of chromatin, since the level of p21<sup>WAF1</sup> promoter amplified from acetylated histone H3- or H4associated chromatin was greater in chromatin isolated from HDAC inhibitor-treated cells than that from untreated cells. Accumulation of acetylated p21<sup>WAF1</sup>-assocaited histones induced by HDAC inhibitors was higher than that in total cellular chromatin, although there was accumulation of

acetylated histones H3 and H4 in total cellular chromatin.

It is noteworthy that, the level of acetylated histones H3 and H4 at the domain containing the transcriptional start site in  $p21^{WAF1}$  promoter and the binding sites of E2A was significantly higher than that at another domain or total cellular chromatin analyzed. The result of our observations suggested that  $p21^{WAF1}$ expression could be activated by histone acetylation of its transcription start domain in promoter. Therefore, a possible mechanism involved the binding of transcription factor E2A to  $p21^{WAF1}$  promoter at transcription start site of acetylated  $p21^{WAF1}$ gene-associated histones H3 and H4, and the enhancement of  $p21^{WAF1}$  gene transcription. It is known that histone acetylation can be targeted to specific promoters by gene-specific activator. E2A transcription factor belongs to the basic helix-loop-helix family of proteins<sup>[20]</sup>, which contains a conserved basic region responsible for DNA binding and a helix-loop-helix domain for dimerization<sup>[21]</sup>. E2A binds  $p21^{WAF1}$  at the domain nearby TATA box in the promoter. TATA box-independent transcription of the  $p21^{WAF1}$  promoter has been previously reported<sup>[22]</sup>. The proximal  $p21^{WAF1}$  promoter contains a TATA box<sup>[23]</sup>. Some reports indicated that *p21<sup>WAF1</sup>* was up-regulated by E2A binding to HTLV-1-infected T cells<sup>[20]</sup>. Moreover, overexpression of E2A proteins, such as E47 has been shown to induce  $p2I^{WAF1}$ promoter activity independent of p53 binding sites<sup>[24,25]</sup>.

Also, we showed that the levels of  $p21^{WAF1}$  mRNA and protein in colon cancer cells were very low, even difficult to detect before treatment. In Colo-320,  $p21^{WAF1}$  mRNA was increased by 27.1-fold and 17.15-fold after 1 µmol/L TSA and 5 mmol/L sodium butyrate treatment, respectively. Accordingly, the protein level of  $p21^{WAF1}$  was elevated. Similar effects were shown in SW1116 cells (data not shown). Our data about TSA or sodium butyrate inducing  $p21^{WAF1}$  mRNA and protein expression are consistent with previous reports<sup>[12]</sup>.

In summary, this study demonstrated that HDAC inhibitor, TSA or sodium butyrate, activated the expressions of  $p21^{WAF1}$  mRNA and protein, and this increased expression was associated with an accumulation of acetylated histones in total cellular chromatin and the chromatin of  $p21^{WAF1}$  gene in these two colon cancer cell lines. It has been shown that  $p21^{WAF1}$  expression is reduced in adenomas and colorectal carcinomas. Our observations support the claim for the therapeutic potential of HDAC inhibitors in the treatment of colorectal carcinoma, because there is probably no mutation of the  $p21^{WAF1}$  gene in colorectal cancer.

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• COLORECTAL CANCER •

# Clinical features and mismatch repair gene mutation screening in Chinese patients with hereditary nonpolyposis colorectal carcinoma

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# Abstract

**AIM:** Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominantly- inherited cancer-susceptibility syndrome that confers an increased risk for colorectal cancer and a variety of other tumors at a young age. It has been associated with germline mutations in five mismatch repair (MMR) genes (hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6/GTBP). The great majority of germline mutations were found in hMSH2 and hMLH1. The purpose of this study was to analyze the clinical features of Chinese HNPCC patients and to screen hMSH2 and hMLH1 gene mutations.

**METHODS:** Twenty-eight independent Chinese families were collected, of which 15 met Amsterdam criteria I and 13 met the Japanese clinical diagnosis criteria. The data were recorded including sex, site of colorectal cancer (CRC), age of diagnosis, history of synchronous and/or metachronous CRC, instance of extracolonic cancers, and histopathology of tumors. Peripheral blood samples were collected from all pedigrees after formal written consents were signed. PCR and denaturing high-performance liquid chromatography (DHPLC) were used to screen the coding regions of hMSH2 and hMLH1 genes. The samples showing abnormal DHPLC profiles were sequenced by a 377 DNA sequencer.

**RESULTS:** One hundred and seventy malignant neoplasms were found in one hundred and twenty-six patients (multiple cancer in twenty-three), including one hundred and twentyseven CRCs, fifteen gastric, seven endometrial, and five esophageal cancers. Seventy-seven point eight percent of the patients had CRCs, sharing the features of early occurrence (average age of onset, 45.9 years) and of the right-sided predominance reported in the literature. In Chinese HNPCC patients, gastric cancer occurred more frequently, accounting for 11.9% of all cancers patients and ranking second in the spectrum of HNPCC predisposing cancers. Synchronous CRCs occurred less frequently, only accounting for 3.1% of the total CRCs. Twenty percent of the colorectal patients had metachronous CRCs within 10 years after operation. Eight hMSH2 or hMLH1 gene sequence variations were found in twelve families, including the first Mongolian kindred with a hMSH2 gene mutation.

CONCLUSION: HNPCC is characterized by an early-age

onset, proximal predominance of CRC, multiple metachronous CRCs, and an excess of extra-colonic cancers. Frequent gastric cancer occurrence and less synchronous CRCs are the remarkable features in Chinese HNPCC patients. DHPLC is a powerful tool in hMSH2 and hMLH1 gene mutation screening. hMLH1 gene mutations, especially of the first nine exons, have been found more common than hMSH2 gene mutations in Chinese patients. Three of seven mutations have been found to be novel, and the germline G204X nonsense mutation in the third exon of hMSH2 has become the first MMR gene mutation found in Chinese Mongolian people.

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# INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC, or Lynch syndrome) is an autosomal dominantly-inherited cancersusceptibility syndrome. It is estimated that HNPCC may account for 5-10% of the total colorectal cancers (CRC) worldwide<sup>[1]</sup>. In Western countries, patients inheriting this predisposition are at a particularly high risk of developing CRC and endometrial cancer at a young age, and also at an increased risk of developing various other types of tumors, such as ovarian, uroepithelial, small intestine, biliary tract, stomach, brain, and skin cancers<sup>[2]</sup>. Five mismatch repair (MMR) genes (hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6/GTBP<sup>[3-8]</sup>) have been known to be involved in this cancer susceptibility. Currently, more than 300 different mutations have been described in these genes, which account for approximately 500 HNPCC kindreds in the world<sup>[9]</sup>. hMSH2 and hMLH1 germline mutations were found to be responsible for more than 90% of the HNPCC families<sup>[10]</sup> (http://www.nfdht.nl/). Therefore, identification of the mutational incidence and spectrum of hMSH2 and hMLH1 genes is important. Identifying the clinical features of HNPCC in China, which might have some differences from those reported in Western countries, will facilitate its diagnosis and treatment. We described the clinical features and the results of mutation screening of both hMSH2 and hMLH1 genes in 28 HNPCC families registered in our hospital. We think that Chinese HNPCC patients have some unique clinic features and MMR gene defects.

# MATERIALS AND METHODS

## Clinical Data

Subjects were selected from 28 independent Chinese families from January 1992 to August 2003. Among these families, 15 met the Amsterdam criteria  $I^{[11]}$ . The criteria were as follows: (1) Three or more relatives had histologically-verified CRC, one of them was a first degree relative to the other two relatives; (2) At least two successive generations were affected; (3) One or

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more CRC cases were diagnosed under 50 years of age; and (4) Familial polyposis of the colon was excluded. The Japanese clinical diagnosis criteria for HNPCC<sup>[12]</sup> were used for the other 13 highly-suspected families that did not fully meet the Amsterdam criteria I. Families that met the following A or B were also clinically diagnosed as having HNPCC: A: a case with three or more CRCs within the first-degree relatives; B: a case with two or more CRCs within the first-degree relatives meeting one of the following criteria: age at onset of CRCs being under 50 years, right colon involvement, synchronous or metachronous multiple CRCs, or associated with synchronous or metachronous extracolorectal malignancies.

Detailed family and medical histories were obtained through interview with the proband, and a home visit to extended family members and an extensive review of medical records if available. Peripheral blood samples were collected from all participants after formal written consents were signed.

Eligible HNPCC families were registered and family members were followed up intensively. All patients were reviewed by telephone or outpatient visit at regular intervals. Data concerning sex, site of CRC, age of diagnosis, history of synchronous and/or metachronous CRC, instance of extracolonic cancers, and histopathology of tumors were documented and thoroughly verified.

#### DNA extraction and PCR amplification

Genomic DNA was isolated from peripheral blood lymphocytes according to the salting-out procedure<sup>[13]</sup>. The entire hMLH1 and hMSH2 coding region and the splice junctions were amplified by PCR according to Weber methods<sup>[14]</sup> with minor modifications.

#### DHPLC analysis

DHPLC analysis was performed on a Transgenomic WAVE system (Transgenomic Inc.) identical with that described previously<sup>[15]</sup>. Briefly, PCR products (25 µL) were denatured for 5 min at 95 °C and then gradually reannealed by decreasing sample temperature from 95 °C to 45 °C over a period of 30 min to form homo- and/or heteroduplexes. Crude PCR product  $(7-10 \,\mu\text{L})$ was loaded on the DHPLC column and eluted with a linear acetonitrile gradient at a flow rate of 0.9 mL/min. Gradient parameters were determined based on size and G-C content of the amplicon. Generally, an analysis took approximately 7 min, including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 mol/L triethylammonium acetate pH 7.0 (TEAA) with (buffer B) or without (buffer A) 250 mL/L acetonitrile. The temperature for heteroduplex analysis was primarily established by using the DHPLC melting algorithm WAVE Maker<sup>TM</sup> of the WAVE<sup>TM</sup> instrument. The final temperature and denaturing condition for optimal resolution of homoduplexes and heteroduplexes of each fragment were experimentally determined.

#### DNA sequencing

PCR products displaying a double DHPLC peak indicating existence of heteroduplex were purified with micron microconcentrator filters (Amicon, Beverly, MA) to remove unwanted reagents from the PCR reaction and to concentrate the final products, which were then sequenced by a 377DNA sequencer. All mutations were sequenced in both directions and confirmed in other family members.

#### RESULTS

#### Statistics on patients and tumors

A total of 28 kindreds were studied, all of them met the Japanese clinical diagnosis criteria and 15 of them met the Amsterdam I criteria. There were 9 Lynch syndrome I families, in which only colorectal cancers were found, and 19 Lynch syndrome II families, which were characterized by concurrent extracolonic malignancies.

One hundred and seventy malignant neoplasms were found in 126 patients (multiple cancer in twenty-three), including 127 CRCs; 15 gastric, 7 endometrial, 5 esophageal, 2 skin, 2 pancreatic, 2 lung, 1 breast, 1 cervical, 1 ovarian, 1 hepatic, and 1 biliary cancers; 1 gastric leiomyosarcoma, 1 liposarcoma, 1 bone sarcoma, 1 leukemia, and 1 brain glioblastoma. In the present group, 77.8% of the patients had CRCs and 74.7% of the cancers were colorectal ones. There were 45 metachronous CRCs and 4 synchronous CRCs, accounting for 35.4% and 3.1% of the total CRCs, respectively. Right-sided colon cancers constituted 52.9% of the total tumors, and 70.9% of CRCs. Individuals suffering from gastric cancer amounted to 11.9% of total patients.

The average age of malignant neoplasm onset in all the patients was 47.0 years and the ratio of males to females was 1.2:1. Individuals developed CRCs at an average age of 45.9 years. Sixty-two point seven percent of colorectal tumors developed under 50 years of age, 33.3% under 40 years of age and less than 4% occurred above the age of 70 years. In the 28 pedigrees, the average age of tumor occurrence in the first, second, third, and fourth degree was 59.6, 50.0, 44.0, and 31.8 years, respectively.

Eighty-five percent of the patients received radical operations. The remaining patients received chemotherapy, irradiation, and traditional Chinese medicine treatment. Twenty percent of colorectal patients had metachronous CRCs within 10 years after the first operation and required re-operations.

#### hMSH2 and hMLH1 mutation results

Thirteen double peak profiles displayed in DHPLC were found among 28 probands of all the pedigrees. Finally, 12 probands were identified with a varying DNA sequence by sequencing, of which 7 developed different mutations and 5 had the same hMSH2 polymorphism (Table1 and Figure1). We also examined the relatives of affected probands for the same mutations, and found that cancers and mutations were co-segregated in all affected pedigees.

Table 1 hMSH2/hMLH1 gene sequence variations identified by sequencing

Sample No.	Gene/Exon	Point of mutation	Mutation result	Reported previously by
231	hMSH2/3	g.²610G>T,GGA →TGA	G204X, Truncated protein (nonsense mutation)	None
10,12,15,16,28	hMSH2/10	g.1661+12A>G	In intron, polymorphism	Scott et al. <sup>[16]</sup>
26	hMSH2/14	g.2211-2 A>C	Truncated protein (splice point mutation)	None
11	hMLH1/3	g.265 G>T,GAG →TAG	E89X, Truncated protein (nonsense mutation)	Wang et al.[17]
14	hMLH1/6	g.545+3 A>G	Truncated protein (splice point mutation)	Pensotti et al.[18]
8	hMLH1/8	g.655 A>G,ATC →GTC	I219V (missense mutation)	Tomlinson et al.[19]
25	hMLH1/8	g.677 G>A,CGA →CAA	R226Q (missense mutation)	None
18	hMLH1/9	g.790+1 G>A	Truncated protein (splice point mutation)	Cunningham <sup>[20]</sup>

<sup>1</sup>The first Mongolian family with hMSH2 gene mutation in China. <sup>2</sup>G refers to genomic DNA.



Figure 1 Sequencing graphs of mutations.

# DISCUSSION

HNPCC was characterized by an early onset of colorectal cancers (proximal predominance, with 70% proximal to the splenic flexure), multiple synchronous and metachronous CRCs (about 18.1% and 24.2% respectively<sup>[21]</sup>), and an excess of certain extracolonic cancers<sup>[22]</sup>. In our study, patients with HNPCC developed CRC at an average age of 45.9 years, much earlier than the general population in China. According to our data, right-sided colon cancers amounted to 52.9% of the total cancers, and 70.9% of CRCs, similar to those reported in Western countries. Moreover, we also found the phenomenon of "generation anticipation", that is, the later the generation was, the earlier the CRC developed. The fact that the family members

tended to be diagnosed early in the follow-up was an explanation for this phenomenon. Another reason for this phenomenon might be that there were carriers who harbored mutated MMR genes but did not become penetrant. Further studies should be carried out.

We found that the following two features were different from those reported in Western countries. Though Chinese patients had a high incidence of metachronous CRCs, synchronous cancer occurred quite rarely, only 3.1% in this study. Our previous studies also had a similar conclusion<sup>[20]</sup>, and the reason for the rare incidence of synchronous cancer in Chinese HNPCC patients remains unclear. The other striking feature was that gastric cancer was the second most common



**Figure 2** Pedigree of the first Mongolian family with hMSH2 gene mutation in China. ● Cancer patient, ○ Normal person, ④ Mutational gene carrier, SKC: skin cancer, RC: rectal cancer, CC: colonic cancer, EC: endometrial cancer, SC: stomach cancer.

cancer in Chinese HNPCC families, amounting to 11.9% of all cancers patients, much higher than the reported incidence in Western countries<sup>[23,24]</sup>. Endometrial cancer ranked third, amounting to only 5.6%, and was followed by esophagus carcinoma. In Western countries, however, the second most commonly-seen tumor was endometrial cancer<sup>[22,25]</sup>. In Japan and Korea, gastric cancer also occurred more frequently<sup>[26]</sup>. We suppose that these features may represent the ethnic and geographical characteristics that may have some diagnostic significance in China and/or Asia.

The diagnosis of HNPCC depends on the detection of MMR genes. Single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and direct sequencing have been used in MMR gene defect screening. Using DHPLC, we found a specificity of 92.3%, and recommended it as a hMSH2 and hMLH1 gene mutation screening method. In our study, 5 of 15 families (proband sample NO. 23, 11, 14, 8, 25), who met the Amsterdam I criteria, were found to have mutations and its mutation detection rate is 33.3%. But these criteria could only identify large families where the gene defect was highly penetrant and many small families were inappropriately excluded. In this study, 2 of 13 families (proband sample NO. 26, 18), which did not meet Amsterdam I criteria but fulfilled the Japan criteria, were also found to have mutations. Though the MMR gene defect rate of the latter is comparatively low (only 25%), it has been recommended as the clinical diagnosis criteria in HNPCC patients.

The mutations found in these families were compared with those described already in the human gene mutation database (HGMD) (http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html). To our knowledge, apart from mutation of sample 11 (hMLH1, g. 265 G>T,GAG→ TAG, E89X ) reported before<sup>[17]</sup>, mutations of samples 14, 8, and 18 and the polymorphism of hMSH2 (g.1661+12A>G) were also reported previously<sup>[18-20]</sup>. The other three mutations are novel. All the seven mutations resulted in an impaired capacity in MMR, which were consistent with their associations with penetrant tumors in the families. The probability of polymorphism of hMSH2 (g.1661+12A>G) being pathogenic was very small. Though there were no distinct "hot spot" mutations, we still noticed that hMLH1 gene mutations, especially of the first nine exons, were more common than hMSH2 gene mutations in China. Similarly, Baba<sup>[12]</sup> and Yuan et al.[27] described that hMLH1 gene mutations were more

frequently seen in Asia. So, it is worthwhile to initiate MMR gene mutation screening from the first nine exons of hMLH1 gene.

In this study, we identified the first Mongolian family with hMSH2 gene mutation in China. The pedigree is shown in Figure 2. In the large family, the nonsense alteration 610 (genomic DNA)  $G \rightarrow T$  at codon 204 in exon3 of hMSH2 resulted in the substitution of stop codon TGA for glycine codon GGA. This mutation co-segregated with the disease in the family. In five phenotypic normal family members, the same mutated gene was found in the germline. These carriers remain to be followed up intensively.

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• COLORECTAL CANCER •

# Comparative study of proteome between primary cancer and hepatic metastatic tumor in colorectal cancer

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# Abstract

**AIM:** To identify the differential proteins associated with colorectal cancer genesis and hepatic metastasis.

**METHODS:** Hydrophobic protein samples were extracted from normal colorectal mucosa, primary cancer lesion and hepatic metastatic foci of colorectal cancer. With twodimensional electrophoresis and image analysis, differentially expressed protein spots were detected, and the proteins were identified by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and peptide mass fingerprint analysis.

**RESULTS:** Significant alterations of the proteins in number and expression levels were discovered in primary cancer and hepatic metastatic foci, the expression of a number of proteins was lost in 25-40 ku, but protein spots was increased in 14-21ku, compared with normal mucosa. Nine differentially expressed protein spots were identified. Three proteins expressed in normal mucosa, but lost in primary cancer and hepatic metastasis, were recognized as calmodulin, ribonuclease 6 precursor and mannosidase- $\alpha$ . Proapolipoprotein was expressed progressively from normal mucosa to primary cancer and hepatic metastasis. The differentially expressed protein of beta-globin was found in normal mucosa and hepatic metastatic tumor, but lost in primary cancer lesion. Cdc 42, a GTP-binding protein, was identified in hepatic metastasis. The protein spots of C4 from primary cancer, M7 and M9 from hepatic metastasis had less homology with the proteins in database.

**CONCLUSION:** Variations of hydrophobic protein expression in colorectal cancer initiation and hepatic metastasis are significant and can be observed with two-dimensional electrophoresis. The expression of calmodulin, ribonuclease 6 precursor and mannosidase- $\alpha$  is lost but the expression of proapolipoprotein is enhanced which is associated with colorectal cancer genesis and hepatic metastasis. Cdc 42 and beta-globin are expressed abnormally in hepatic metastasis. Protein C4, M7 and M9 may be associated with colorectal cancer genesis and hepatic metastasis.

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http://www.wjgnet.com/1007-9327/10/2652.asp

# INTRODUCTION

Colorectal cancer genesis and metastasis are complex processes involving multiple changes in gene and protein expression<sup>[1-5]</sup>. The liver is a common site of metastasis from colorectal cancer<sup>[6-8]</sup>. Hepatic metastasis caused severe and fatal effects on patients who underwent radical excision for large intestine primary carcinoma<sup>[9-12]</sup>. The success of metastatic hepatic cancer treatment is strongly dependent on early diagnosis and understanding of the molecular mechanisms and biological behaviors of colorectal cancer, especially its infiltration and metastasis. To unravel these alterations, genome and proteome approaches for the identification of qualitative and quantitative changes in gene and protein compositions provide theoretic and technical support<sup>[13-16]</sup>. Our study was focused on the identification of differential expression proteins between primary colorectal cancer foci and hepatic metastasis with proteome approach. Hydrophobic proteins including membrane proteins play important roles in cellular signal transduction. Identification of the proteins is helpful to understand the molecular biological mechanisms of colorectal carcinogenesis and hepatic metastasis and to select tumor markers for colorectal cancer.

# MATERIALS AND METHODS

# Tissue sample collection

Samples of normal colorectal mucosa, primary cancer lesion and hepatic metastasis were collected from 12 colorectal cancer patients aged 36-68 years including 6 males and 6 females. The samples were stored in liquid nitrogen. Pathology examination was performed for all the specimens and the histological types consisted of moderately and poorly differentiated adenocarcinoma, signet-ring cell carcinoma and undifferentiated carcinoma, 4 cases in each type.

# Protein sample preparation

A set of samples were taken from the same patient, 0.9 g of each, including normal colorectal mucosa, primary cancer and hepatic metastatic tumor. The samples were washed with PBS and then immediately ground by a liquid nitrogen cooled mortar, and homogenized in protease inhibitor buffer (cocktail formula: phenylmethylsulfonyl fluoride 40 µg/mL, ethylenediamine tetraacetic acid 1 mmol/L, peptide inhibitin 0.7 µg/mL, leupeptin  $0.5 \,\mu g/mL$ ). Protein extraction was performed with Molloy procedure. Lysis buffer I (Tris 40 mmol/L, pH8.8) was added, stirred and mixed by an ultrasonic disintegrator. The mixture was centrifuged at 105 000 g for 1 h. Yellow lipids were discarded from the supernatant and the middle layer liquid was transferred and dried with a freezing dryer. The pellet was solubilized in lysis buffer II (Urea 8 mol/L, Tris 10 mmol/L, CHAPS 40 g/L, DTT 65 mmol/L) and centrifuged. The supernatant was dried and stored as *idem*. The pellet was solubilized in lysis buffer III (urea 5 mol/L, thiocarbamate 2 mol/L, SB3-10 20 g/L, 0.2 g, CHAPS 20 g/L, TBP 2 mmol/L), then the procedure was repeated once more. The proteins extracted with fractional procedure were stored at -20 °C.

# Two-dimensional gel electrophoresis and image analysis

The first dimension of isoelectric focusing (IEF) was performed

in immobilized pH gradient (IPG) gel strips with nonlinear immobilized pH 3-10 gradient. Three hundred  $\mu g$  of protein sample from each tissue specimen of normal mucosa, primary cancer and hepatic metastatic tumor was loaded respectively. IPG strips were placed onto the rehydration buffer (urea 8 mol/L, CHAPS 40 g/L, TBP 2 mmol/L, IPG buffer 3 g/L) and added into the sample solution without trapping air bubbles. IEF with a low voltage (30 V, followed by 60 V and 200 V) was carried out according to the programmed settings. After IEF was terminated, the IPG strips were equilibrated for 20 min in equilibration buffer. The second dimension of SDS-PAGE was performed on vertical systems. IPG strips were loaded and run on a 125 g/L acrylamide SDS-PAGE gel in electrode buffer (Tris 0.025 mol/L, glycine 0.192 mol/L, SDS 1 g/L, pH8.3). Electrophoresis was performed with a current of 30 mA/gel for 15 min, then at maximum settings of 60 mA/gel for 4 h. The temperature of the cooling plate was set at 20 °C. After SDS-PAGE, the gels were stained with silver nitrate. Proteins were visualized by silver-staining and then scanned using Scan Prisa 640 UT. Two DE image computer analysis was carried out with Melanie 3.0 software (GeneBio, Geneva). Isoelectric points and molecular weights of individual proteins were evaluated with polypeptide SDS-PAGE-standards. Differential protein spots among normal colorectal mucosa, primary cancer lesion and hepatic metastasis were picked out by comparison of 2-DE images with Melanie 3.0 analysis.

# Mass spectrometry and peptide mass fingerprint analysis

Selected differential protein spots were excised from 2-DE gels and transferred to a 96-well plate ready for trypsin digestion. The gel pieces were washed successively in water, Tris-HCl (20 mmol/L pH 8), Tris-HCl (20 mmol/L pH 8)/500 g/L acetonitrile and finally 1 000 g/L acetonitrile, and dried. The dried gel pieces were incubated in trypsin solution for 16 h at 37 . The resultant peptide mixture was extracted with 20  $\mu$ L 5 g/L TFA/300 g/L

acetonitrile, followed by extraction with  $20 \,\mu\text{L} 5 \,\text{g/L}$  TFA/600 g/L acetonitrile.  $\alpha$ -cyano-4- hydroxycinnamic acid was used as matrix solution. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry analysis were performed with MALDI-TOF mass spectrometer (Bruker, USA). The spectrum was obtained and recorded. Proteins were identified by searching NCBI and SwissProt databases using Profound peptide mass fingerprinting retrieval software (http://129.85.19.192/profound\_bin/WebProFound.exe?FORM = 1). Protein identities were assigned if at least four peptide masses were matched within a maximum of 100 ppm error spread across the data set and the candidate agreed with the estimated pl and molecular weight from the 2-DE gel.

#### Statistical analysis

Experimental data were analysed statistically with Crosstab chi-Square test and Student's *t*-test using SPSS 10.0. *P*<0.05 was considered statistically significant.

# RESULTS

# 2-DE image analysis of protein spots in matched sets of colorectal cancer

The hydrophobic protein profiles including partial membranous proteins from colorectal normal mucosa, primary cancer and metastatic foci in liver are displayed in Figures 1A-C. Comparing the 2-DE protein images of the three tissues, we found that the number of protein spots and protein expression level were significantly changed in primary cancer and hepatic metastatic lesion. Under the same experimental conditions,  $390\pm28$  protein spots and  $206\pm22$ ,  $236\pm19$  spots were found in normal colorectal mucosa and in primary cancer and hepatic metastasis, respectively. Compared with normal colorectal mucosa, the number of protein spots in primary cancer and metastatic tumor was significantly different t = 53.116, t = 33.399,



Figure 1 Silver-stained two-dimensional electrophoretic images of hydrophobic proteins from (A) Normal colon mucosa, (B) Primary colon cancer lesion, (C) Hepatic metastasis.



**Figure 2** MALDI-TOF mass spectrometry and peptide mass fingerprint analysis of the differential protein spots (A) N2 protein spot from normal colon mucosa, (B) M6 protein spot from hepatic metastasis.

Spot No.	Accession (NCBInr)	Theoretical pI	Theoretical $M_{\rm r}$	Length (AA)	Protein name
N1	1CDL_B	4.0	16.56	147	Calmodulin complexed with calmodulin-binding peptide
N2	NP_003721	6.7	29.46	256	Ribonuclease 6 precursor
N3	XP_040720	8.4	32.76	287	Hypothetical protein XP_040720
C4		4.5	22.48	217	
C5	AAA51747	5.4	28.94	249	Proapolipoprotein
M6	AAA88054	6.8	15.96	147	Beta-globin
M7		5.5	22.57	203	
M8	XP_010554	6.8	21.25	191	Similar to cell division cycle 42
M9		4.5	14.72	127	

**Table 1** Identification of nine differentially expressed proteins by peptide mass fingerprint and matching with proteins in databases

The difference of protein spot number between hepatic metastatic tumor and primary cancer was also significant (t = 24.407, P < 0.01).

# Peptide mass fingerprinting of differential protein spots from 2-DE gels

Nine differential protein spots of the 2-DE gels were analysed using mass spectrometry. Three protein spots, N1, N2, N3, were taken from normal colorectal mucosa. C4 and C5 spots were from primary cancer lesions and four spots, M6, M7, M8, M9, from hepatic metastatic cancer. Molecular weight and isoelectric points of the nine protein spots were determined according to the standard molecular markers and peptide mass fingerprint analysis, and the data are shown in Table 1 and Figure 2.

# Identification of differential expression proteins of colorectal cancer and hepatic metastasis

The peptide mass fingerprints obtained from the nine differential protein spots were compared to fingerprints obtained by theoretical cleavage of protein sequences in databases and the protein identities were assigned. Protein spots of N1, N2 and N3 from normal colorectal mucosa represented calmodulin, ribonuclease 6 precursor and hypothetical protein XP\_040720, respectively. The expression of three proteins was lost in primary cancer and hepatic metastatic foci. Protein C5, matching to proapolipoprotein, expressed progressively from normal mucosa to primary cancer and hepatic metastatic tumor. Protein spot M6 was observed in normal mucosa and in hepatic metastatic cancer, but lost in primary carcinomas, being recognized as beta-globin. Expressed in hepatic metastasis, M8 was identified with cell division 42 (GTP-binding protein), but not found in normal mucosa and primary cancer lesion. Differential protein spots of C4 from primary carcinomas, M7 and M9 from hepatic metastasis were not identified by peptide mass fingerprint analysis because their peptide mass fingerprints had less homology with the known proteins in databases.

# DISCUSSION

The initiation and hepatic metastasis of colorectal cancer involved multiple gene and protein alterations<sup>[17-19]</sup>. Understanding the molecular basis of the disease is of great significance for its early detection and treatment. In this study, the proteome approach was applied to the identification of differential proteins between primary colorectal cancer lesion and its hepatic metastasis. We used 2-DE to isolate and analyze the set of hydrophobic proteins from normal colorectal mucosa, primary

cancer and hepatic metastatic tumor. It was of clinical importance to identify the differential expression proteins that had potentiality of being tumor markers and anticancer targets.

By comparison with 2-DE images, significant differences of protein expression were found in normal mucosa, primary cancer and hepatic metastasis, and the number and distribution of protein spots changed noticeably in the range of pH 4.0-9.0. Compared with normal mucosa, a number of protein spots with a molecular weight of 25-40 ku were lost in primary cancer and hepatic metastasis, but proteins with a molecular weight of 14-21 ku were observed in the same pH range. What the differential proteins were and what functions they performed in colorectal carcinogenesis and in hepatic metastasis attracted our attention. We identified nine protein spots and studied their roles in the course of initiation and hepatic metastasis of colorectal cancer.

Proteins of calmodulin (N1), ribonuclease 6 precursor (N2) and hypothetical protein XP\_040720 (N3) were expressed in normal colorectal mucosa, but lost in primary cancer lesion and in hepatic metastasis. It indicated the loss of proteins was associated with colorectal cancergenesis and hepatic metastasis. Calmodulin could regulate the concentration of calcium ions in cells and had important effects on normal cellular functions. Ca<sup>2+</sup> regulation was necessary for cell differentiation and apoptosis. Combination of calcium ions and the receptors could influence cell signal transduction that controls cell differentiation and division. Low concentration of calcium ions made cell division easily, and the high concentration was advantageous to cell differentiation. Calcium ions promoted cancer cells into apoptosis. Thus, calcium was regarded as a chemoprophylaxis agent for colorectal cancer<sup>[20,21]</sup>. We propose loss of calmodulin expression is connected with initiation and hepatic metastasis of colorectal cancer.

Ribonuclease 6 precursor protein belongs to the Rh/T2/Sglycoprotein class of extracellular ribonucleases and the gene is present in a single copy in the human genome and has been mapped to 6q27. This has been found to be a region of the human genome prone to rearrangements associated with several human malignancies<sup>[22]</sup>. The family of the proteins possesses the function of ribozyme and self-splicing. They catalyze breaking of RNA, synthesis of polypeptide bonds and nucleotides. The protein can disintegrate DNA fragments, regulate cell biological behaviors and cell division. Loss of ribonuclease 6 precursor expression would facilitate carcinogenesis and infiltration. Ribozyme has been found useful in anticancer therapy<sup>[23]</sup>. The loss of ribonuclease 6 precursor expression in primary colorectal cancer and hepatic metastasis could provide an experimental interpretation of ribozyme treatment.

XP\_040720, a hypothetical protein is now defined as a
member of mannosidase- $\alpha$  class 1A. The protein is located in Golgi complex and participates in N-glycoprotein synthesis and oligosaccharide processing. It could play an important role in synthesis of membranous proteins and receptor proteins<sup>[24]</sup>. But we do not know what functions the protein performs in colorectal carcinogenesis and metastasis. It could be regarded as a differentiation-related protein in normal mucosa cells, and loss of the protein expression is a dedifferentiation phenotype in the primary cancer lesion and hepatic metastasis, which is still lack of evidence.

Loss of the three protein expressions was considered to be connected with colorectal cancer initiation and hepatic metastasis. However proapolipoprotein expression was found stepwisely increased from normal mucosa to primary cancer and hepatic metastasis, and enhanced expression of the protein was in association with colorectal cancer. Proapolipoprotein was hydrolyzed by the signal peptidase and propeptidase, through which apolipoprotein was generated<sup>[25]</sup>. Apolipoprotein is a carrier of lipids and regulates many cellular functions. It was found that apolipoprotein had an antiapoptosis effect and was related with carcinogenesis and progression. Enhanced expression of apolipoprotein has been reported in hepatoma<sup>[26]</sup>. Our study provided an evidence of apolipoprotein in colorectal cancer.

A differentially expressed protein in our study was beta-globin. Its expression was found in normal mucosa and hepatic metastasis, but lost in primary cancer lesion. We suggested beta-globin was an associated protein with hepatic metastasis. The gene family of beta-globin consists of five functional genes and is located on chromosome 11. The family members expressed in order of the genes as they were arranged on the chromosome during various developmental stages. Beta-globin gene was activated and expressed continuously in late stage of pregnancy<sup>[27,28]</sup>. Study on regulation of beta-globin showed that the variations of transcription frequency and cycle of phasic and specific expression of beta-globin family genes in developmental regulation were more important than changes of the gene transcription speed and expression quantity<sup>[29]</sup>. Understanding the mechanisms of beta-globin regulation is helpful to researches on cell growth, carcinogenesis and progression. The cycle variation of beta-globin expression from the normal mucosa to primary cancer and hepatic metastasis implied genetic recombination and regulation changes in hepatic metastasis of colorectal cancer. It was reported that beta-globin expression was induced by treatment of chemotherapy agents in some cancer cells. This might indicate that the expression of beta-globin increased drug resistance of cancer cells and facilitated hepatic metastasis.

It has been found that Cdc 42, a differential protein expressed in hepatic metastasis, is a Rho-related member of the Ras superfamily, and acts as a GTP-binding protein<sup>[30,31]</sup>. The protein performs the function of a molecular switch to control a diversity of cellular processes, and regulates cytoskeleton actin recombination, cell polarity and cell movement. Enhanced expression of Cdc 42 might facilitate cell division and accelerate cancer cell growth and proliferation, as well as interrupt signal transduction of apoptosis<sup>[32]</sup>. Invasive behavior of cancer cells was reinforced by enhanced Cdc 42 expression through regulating cellular skeleton, cell adhesiveness and neovascularization. Therefore, Cdc 42 has been regarded as an associated protein in hepatic metastasis of colorectal cancer.

C4 expressed in primary cancer, M7 and M9 in hepatic metastasis, had low homology with the proteins known in database, and the three proteins were not identified. They manifested the possibility to be new proteins associated with colorectal cancer. Sequencing of the proteins and study of their functions are needed to help understand the mechanisms of colorectal cancergenesis and hepatic metastasis.

In summary, we identified 9 differentially expressed proteins that were associated with colorectal cancer genesis and hepatic metastasis. Relations of these proteins with colorectal cancer were not or seldom reported before. The differential proteins will help understand the mechanism of colorectal cancer genesis and hepatic metastasis. The results prove that proteome study represents a very useful and promising tool in discovering new tumor markers and anticancer targets of colorectal cancer.

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COLORECTAL CANCER •

# Polymerase synthesis and potential interference of a small-interfering RNA targeting hPim-2

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#### Abstract

**AIM:** To synthesize three small-interference RNAs (siRNAs) by T7 RNA polymerase-catalyzed reaction, and to investigate their efficacy on modulating the expression of serine/threonine kinase Pim-2 in human colon cancer cell line.

**METHODS:** siRNA I, II and III were synthesized by T<sub>7</sub> RNA polymerase-directed *in vitro* transcription, then transfected into human colon cancer cells SW-480. After incubation for 6 h at 37 , 100 mL/L FBS in RPMI 1640 was substituted in each well. After the transfection was repeated twice to three times in each kind of siRNA, hPim-2 mRNA and protein expression were measured by RT-PCR and Western blotting, respectively.

**RESULTS:** Compared to the control group, after transfected for 48 h with hPim-2 siRNA I, II and III, the relative inhibition rates of hPim-2 mRNA expression in colon cancer cells were 65.4% (P<0.05), 46.2% (P<0.05) and 56.1% (P<0.05), respectively. The protein level of hPim-2 was decreased at 72 h compared to the untransfected cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II, III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively.

**CONCLUSION:** The *in vitro* transcribed siRNAs can be useful for silencing oncogene hPim-2 expression specifically and efficiently. This may open a new path toward the use of siRNAs as a gene-specific therapeutic tool.

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#### INTRODUCTION

RNAi is an evolutionarily conserved mechanism known to control insects, plants, and mammalian cells<sup>[1-4]</sup>. In this process, introduced double-stranded RNAs (ds-RNAs) silence gene was expressed through specific degradation of their cognate mRNAs<sup>[5,6]</sup>. Importantly, RNAi can be achieved in mammalian cells following transfection of synthetic 21- and 22-nucleotide (nt) small interfering (si) RNAs, indicating that RNAi may serve as a powerful tool to block the expression of target genes

specifically<sup>[7-11]</sup>.

Pim-2 is a member of a family of serine/threonine protein kinases that consists of two other members, Pim-1 and Pim-3, and it exists at high concentrations in many tumor cells<sup>[12,13]</sup>. Though it was identified 20 years ago, its function that maintains the cell size and its role in the survival of cancer cells have been just determined recently<sup>[14,15]</sup>. It is believed to be a cancer-causing gene, or oncogene. Here, we sought to use siRNA-targeting hPim-2 to determine whether this technique could be used to specifically inhibit hPim-2 expression.

#### MATERIALS AND METHODS

#### T<sub>7</sub> siRNA synthesis

siRNAs selection was based on the characterization of siRNA by Elbashir et al.<sup>[16]</sup>. Three hPim-2 siRNA sequences are given in Figure 1. For in vitro transcription, 40-nt DNA template oligonucleotides were designed to produce 21-nt siRNAs. siRNA sequences of the form GN<sub>17</sub>CN<sub>2</sub> were selected for each target. Uridines in the last two nt form the 3' overhang of the siRNA duplex. The template and a 19-nt T<sub>7</sub> promoter (GGT AAT ACG ACT CAC TATA) were synthesized by Applied Biosystems 393 DNA synthesizer and purified by OPC (Perkin-Elmer, Foster city, CA). The oligonucleotide-directed mutagenesis of small siRNA transcription with T<sub>7</sub> polymerase is as follows: for each transcription reaction, 1 nmoL of each oligonucleotide was annealed in 50 µL of TE buffer (10 mmol/L Tris-HCl pH 8.0, and 1 mmol/L EDTA) by heating at 95 °C; after 5 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50  $\mu$ L of transcription mixture: 1×T<sub>7</sub> transcription buffer (40 mmol/L Tris-HCl pH 7.9, 6 mmol/L MgCl<sub>2</sub>, 10 mmol/L DTT, 10 mmol/L NaCl and 2 mmol/L spermidine), 1 mmol/L rNTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RNase (Life Technologies) and 100 U T<sub>7</sub> RNA polymerase (Fermentas) containing 200 pmoL of the dsDNA as template. After incubation at 37 °C for 3 h, 1 U RNase free-DNase (Promega) was added at 37 °C for 30 min. Sense and antisense 21-nt RNAs (single strand RNA, ssRNA) generated in separate reactions were annealed by mixing both crude transcription reactions, incubating at 37 °C overnight to obtain "T7 RNA polymerase synthesized small interfering double-strand RNA (T<sub>7</sub> siRNA, dsRNA)". The mixture (100  $\mu$ L) was then extracted with TE-saturated (pH 4.5) phenol:chloroform:isoamyl alcohol (25:24:1), purified with chloroform:isoamyl alcohol(24:1), isopropanol and 0.2 mol/L sodium acetate (pH5.2). The pellet was washed once with 750 mL/L ethanol, dried, and resuspended in 50  $\mu$ L of water.

#### Cell culture

Human colon cancer cell line SW-480 was obtained from Chinese National Cancer Institute. The cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 100 mL/L fetal bovine serum (GIBCO BRL, Grand Island, NY),100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37 °C with 50 mL/L CO<sub>2</sub>.

#### Transfection with siRNA oligonucleotides

Cells were seeded the day before the experiment in 6-well plates at a density of  $1.5 \times 10^5$  per well to be 50% confluent on the day

hPim-2-I Target mRNA	5′	AAGUUUGCCCAGUUCCCUUCC	3′	
siRNA	5′	GUUUGCCCAGUUCCCUUCCUU	3′	Sense strand
	3′	UUCAAACGGGUCAAGGGAAGG	5′	Antisense strand
hPim-2-II Target mRNA	5′	AAGACAUAAACCAAGUUUGCC	3′	
siRNA	5′	GACAUAAACCAAGUUUGCCUU	3′	Sense strand
	3′	UUGUGUAUUUGGUUCAAACGG	5′	Antisense strand
hPim-2-III Target mRNA	5′	AAGUUGUUCCCAUUUUGAGCC	3′	
siRNA	5′	GUUGUUCCCAUUUUGAGCCUU	3′	Sense strand
	3′	UUCAACAAGGGUAAAACUCGG	5′	Antisense strand

Figure 1 Sequences of 21-nt siRNA duplex that were used to target at hPim-2.

of the experiment. Transfection of the RNA oligonucleotides was performed using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer to result in a final RNA concentration of 50 nmol/L. After transfection (incubation for 6 h at 37 °C), cells were washed with PBS and incubated in fresh culture medium until additional analyses.

#### Analysis of hPim-2 mRNA by RT-PCR

After transfection, total RNA was isolated using TRIZOL (Invitrogen) by a single-step phenol-extraction. Subsequent RT-PCR was performed (RT-PCR kit, Promega, Madison, WI.). Briefly, first strand cDNA was synthesized using an Oligo (dT)<sub>15</sub> primer at 42 °C for 30 min. PCR for hPim-2 and β-actin was performed in a single reaction of 20 µL volume. The latter served as a control following 28 cycles of denaturing at 95 °C for 45 s, annealing at 58 °C for 40 s, and extending at 72 °C for 40 s. Under this PCR condition, the amplification showed linearity as was determined experimentally (data not shown). PCR products were run on a 30 g/L agarose gel and visualized by ethidium bromide staining, and the intensities were then measured by scanning the gel with Gel Doc 1000 (Bio-Rad, Hercules, CA). Inhibition of hPim-2 mRNA was calculated according to the following formula:

Inhibition percentage = 
$$\frac{(1 - A_{\text{sample}} \times A_{0 \text{control}})}{A_{\text{control}} \times A_{0 \text{sample}}} \times 100\%.$$

 $A_{\text{sample}}$ : the intensity of hPim-2 PCR product in cells transfected with siRNA and Lipofectamine;  $A_{0\text{sample}}$ : the intensity of hPim-2 PCR product in cells transfected with Lipofectamine alone;  $A_{\text{control}}$ : the intensity of  $\beta$ -actin product in cells transfected with siRNA and Lipofectamine;  $A_{0\text{control}}$ : the intensity of  $\beta$ -actin product in cells transfected with Lipofectamine alone.

#### Analysis of hPim-2 protein

The expression levels of hPim-2 protein in cells transfected with siRNAs were measured by scanning the density of bands on Western blotting. The expression level of hPim-2 mRNA was analyzed by the method described above. After 72 h of transfection, cells were lysed in RIPA buffer [10 mmol/L Tris-HCl (pH7.4), 10 g/L deoxycholate, 10 g/LNP40, 150 mmol/L NaCl, 1 g/L SDS, 0.2 mmol/L phenylmethyl sulfonyl fluoride, 1  $\mu$ g/mL aprotinin and 1 µg/mL leupeptin] for 30 min on ice. The lysates were centrifuged at 15 000 r/min for 15 min to remove debris. Equal amounts (30  $\mu$ g) of proteins were separated by 120 g/L SDS-PAGE and transferred onto PVDF membrane (Hybondpolyvinylidene difluoride membranes, Amersham Biosciences). The transferred membrane was incubated with anti-hPim-2 goat polyclonal or anti- $\beta$  actin rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and followed with peroxidase-linked secondary antibody. Finally, the

immunoreactive proteins were detected by an ECL-plus detection kit (Amersham Biosciences) and scanned by Gel Doc 1000 (Bio-Rad), and the inhibition percentage (%) was calculated according to the following formula: inhibition percentage =  $(1-A_{sample}/A_{control}) \times 100$ .

#### **Statistics**

The data were expressed as mean±standard deviation (mean±SD). Statistical analysis was performed by Student's-*t*-test (two tailed). All data represented at least two independent experiments.



Figure 2 Strategy to generate T7 siRNA.

#### RESULTS

#### Synthesis of siRNA by in vitro transcription

To generate siRNAs by *in vitro* transcription, we designed the strategy presented in Figure 2. Target sequences for siRNA were identified by scanning the length of the hPim-2 gene with AA sequences. The AA and downstream 19 nucleotides were recorded and compared to an appropriate genome database to eliminate any sequences with significant homology to other genes. Those sequences that appear to be specific are the potential siRNA target sites. Besides, it is noteworthy that  $T_7$  RNA polymerase can transcribe a template efficiently if only the first nucleotide of the RNA transcript is G. Thus, the design of  $T_7$  siRNAs requires that the sequence starts with a G and has a C at position 19 (GN<sub>17</sub>CN<sub>2</sub>) to allow annealing with the complementary RNA, which also starts with a G<sup>[17,18]</sup>. The  $T_7$  promoter oligonucleotide is invariant and common to any target gene. A 40 mer DNA oligonucleotide template was synthesized

by a 21 mer oligonucleotide encompassing the  $T_7$  promoter with complementary sequence preceded by two additional nucleotides (reading the sequence 5'----3'). Following transcription reactions, sense and antisense transcriptions were annealed, ethanol precipitated and yielded what we refer to as  $T_7$ siRNAs. The integrity of the transcriptions was checked on a 30 g/L agarose gel (Figure 3).



**Figure 3** Lane 1: T7 *in vitro* transcribed single-strand RNA. Lane 2: annealed double-strand DNA template. Lane 3: hybridized double-strand small interference RNA.

#### Effect of siRNAs on hPim-2 expression

The mRNA level of hPim-2 was determined by semi-quantitative RT-PCR. A 237-bp DNA fragment of hpim-2 gene and a 317-bp DNA fragment of  $\beta$ -actin gene were amplified by RT-PCR with specific primers, respectively. As shown in Figure 4A, mRNA expression level of hPim-2 was decreased when compared to the uninduced cells, while the mRNA level of  $\beta$ -actin as the control was almost unchanged. As shown in Figure 4B, after transfection with hPim-2 siRNA I, II and III and compared with the

levels of  $\beta$ -actin, the relative inhibition rates of hPim-2 mRNA expression were 65.4% (*P*<0.05), 46.2% (*P*<0.05) and 56.1% (*P*<0.05) in colon cancer cells, respectively.

In order to verify the decrease in mRNA expression, which corresponded to the decreases at protein levels, Western blotting was performed. Figure 5A shows that the protein level of hPim-2 was decreased at 72 h compared to the uninduced cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II and III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively (Figure 5B).

#### DISCUSSION

Oncogene overexpression has been implicated in the development and progression of a variety of human cancers and, therefore, provides a potential target for cancer gene therapy<sup>[19-22]</sup>. For years, research has been focused on effective tools to specifically down-regulate oncogene overexpression such as antisense oligonucleotide strategy. However, there has been only limited success because of the lack of specificity and potency for this method. For example, screening of more than 20 oligomers is usually required before identifying one antisense that functions effectively, and the dose required for inhibiting gene expression is often not much different from the doses that lead to nonselective toxicity<sup>[23-25]</sup>.

Recent progress of RNAi techniques has demonstrated the potential to overcome those limitations. The selection of targeting sequences of RNAi is less restricted, once the site is identified, sense and antisense oligonucleotides with 3'-UU overhangs can be designed, so the success rates of producing effective duplexes are higher. Just like in this experiment, siRNAs were designed complimentary to three different regions of the corresponding Pim-2 mRNA, and each of them has different level of inhibition efficacy, the suppression of hPim-2



**Figure 4** Inhibitory effects of siRNA on mRNA level of hPim-2. A: Electrophoresis of RT-PCR products of hPim-2 gene and  $\beta$ -actin gene in colon cancer cells transfected with siRNA I,II,III. B: Quantitation of inhibitory percentage of hPim-2 mRNA in transfected cells.Each level of PCR product of hPim-2 gene was. quantitated and normalized to the level of  $\beta$ -actin. Inhibitory rate was calculated by comparing to the control cells.The results were expressed as means±SD from independent experiments. *P*<0.05 *vs* the cells transfected with lipofectamine alone.



**Figure 5** Inhibitory effects of siRNA on protein level of hPim-2. A: Western blot analysis of hPim-2 protein in colon cancer cells transfected with siRNA I, II, III. B: inhibitory percentage of hPim-2 protein in transfected cells compared to the control cells.Each level of hPim-2 protein was quantitated.Inhibitory rate was calculated by comparing to the control cells. The results were expressed as mean±SD from independent experiments. *P*<0.05 vs the cells transfected with lipofectamine alone.

gene expression by these siRNAs directed at different sites varied from 45-65%. This indicates that screening potential target of RNAi is much more easy.

Besides, our results demonstrate that in vitro transcribed siRNA can effectively down-regulate oncogene expression with great efficiency. It has been suggested that siRNA may inhibit gene expression through diverse effects, inhibition of mRNA can occur through the formation of a nuclease complex called RISC (RNA-induced silencing complex) that targets and cleaves mRNA which is complementary to the siRNA. The damaged mRNA may deteriorate through the action of the RNAdependent RNA polymerase (RdRP), producing new siRNAs to target other mRNA. This incessant waterfall-like amplification can produce RNA interference effect at a very small dose, and inhibit the protein translation quickly and efficiently<sup>[26-30]</sup>. In our experiment, the dose required for inhibiting Pim-2 gene expression was 50 nmol/L, far below the dose required for the antisense oligonucleotide<sup>[31]</sup>, indicating that siRNA synthesized by the in vitro transcription strategy can suppress the hPim-2 gene expression sensitively.

Here, we used the *in vitro* transcription method for the synthesis of siRNAs by  $T_7$  RNA polymerase and transferred them into cells. The main advantage of this technique is its simplicity. It provides a reproducible and highly efficient means to inhibit the target gene expression. Human Pim-2 gene, a regulated transcriptional apoptotic inhibitor, has a novel role in promoting cell autonomous survival. Over-expression of Pim-2 allows the tumour cells to ignore or become insensitive to boosters of the immune system<sup>[14]</sup>. Application of Pim-2-directed siRNA can significantly reduce Pim-2 mRNA and protein levels efficiently. Our next step is to try to manipulate the action of Pim-2 with siRNA, so that we can interfere with the survival of cancer cells.

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• VIRAL HEPATITIS •

# Effect of lamivudine treatment on plasma levels of transforming growth factor $\beta_1$ , tissue inhibitor of metalloproteinases-1 and metalloproteinase-1 in patients with chronic hepatitis B

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Abstract

AIM: Transforming growth factor (TGF)- $\beta_1$ , metalloproteinase (MMP)-1 and its tissue inhibitor (TIMP)-1 are considered predictive biomarkers of chronic hepatitis activity and fibrosis. The aim of this study was to evaluate the effect of lamivudine treatment on the plasma levels of these peptides in patients with chronic hepatitis B.

**METHODS:** TGF- $\beta_1$ , MMP-1 and TIMP-1 plasma concentrations were measured with an enzyme immunoassay in 40 patients treated with lamivudine for 48 wk. Elimination of HBV-DNA and HBV antigens was evaluated 24 wk after treatment completion.

**RESULTS:** Baseline TGF- $\beta_1$  (29.6±2.2 ng/mL) and TIMP-1 (1 578±93 ng/mL) significantly exceeded normal values (18.3±1.6 ng/mL and 1 102±67 ng/mL respectively). Lamivudine treatment resulted in a significant decrease of TGF- $\beta_1$  and TIMP-1 during treatment with an increase after 24 wk of treatment. Pretreatment MMP-1 levels (6.7±0.7 ng/mL) were significantly lower than normal values (11.9±0.9 ng/mL) and increased during treatment and follow-up. A significant correlation was noted between TGF- $\beta_1$  or TIMP-1 and aminotransferases as well as fibrosis scored in liver biopsy specimens. There were no statistically significant differences of TGF- $\beta_1$ , TIMP-1 and MMP-1 between four groups at baseline, 24 and 48 wk of treatment. TGF- $\beta_1$  and TIMP-1 levels increased significantly in non-responders and normalized in responders at wk 72. MMP-1 also normalized in responders and decreased to values significantly lower than normal in non-responders.

**CONCLUSION:** These findings support the role of TGF- $\beta_1$ , TIMP-1 and MMP-1 in the pathogenesis of chronic hepatitis B. Because of their association with hepatic injury and antiviral treatment efficacy, determination of these peptides may be useful in disease management.

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#### INTRODUCTION

Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) is considered a pivotal inducer of liver fibrosis, acting mostly through activation of hepatic stellate cells (HSCs), which are the main source of extracellular matrix (ECM) proteins<sup>[1-3]</sup>. The effect of TGF- $\beta_1$ on liver fibrosis is at least in part related to stimulation of the tissue inhibitor of metalloproteinases-1 (TIMP-1), which affects metalloproteinases (MMP) activity and is responsible for inhibition of ECM proteins breakdown<sup>[4]</sup>. Apart from a profibrogenic role, TGF- $\beta_1$  inhibits DNA synthesis serving as a terminator of regenerative cell proliferation and induces apoptosis of hepatocytes in normal liver and during regression of liver hyperplasia<sup>[5]</sup>. On the other hand, TGF- $\beta$ may inhibit stellate cells apoptosis and promote their survival, which are at least in part a result of anti-apoptotic effect of TIMP-1<sup>[6]</sup>. Additionally TGF- $\beta_1$  exerts regulatory, mostly immunosuppressive effects on the immune system. Since hepatitis B virus (HBV) infection is related to an immune response, cell proliferation and fibrosis, modulation of TGF- $\beta_1$  might affect the course of chronic viral hepatitis B<sup>[7,8]</sup>. The possible role of TGF- $\beta_1$ , TIMP-1 and MMP-1 as predictive biomarkers of chronic hepatitis activity and progression was supported by recent clinical studies<sup>[9-16]</sup>, which demonstrated their association with hepatic function impairment or fibrosis, but did not evaluate their effect of antiviral treatment. We undertook this study to evaluate the effect of lamivudine, the most widespread antiviral medication for chronic HBV infection, on plasma TGF- $\beta_1$ , TIMP-1 and MMP-1 levels in patients with chronic hepatitis B.

#### MATERIALS AND METHODS

#### Patients

Ethical approval for the study was obtained from the Bioethical Committee of the Medical Academy of Bialystok. Informed consent was obtained from 40 patients (13 females and 27 males, mean age: 45±3 years) with chronic hepatitis B, who were included into the protocol of lamivudine (Zeffix<sup>TM</sup>, Glaxo-Smith-Kline) treatment. Normal values of TGF- $\beta_1$ , TIMP-1 and MMP-1 were collected from 13 healthy volunteers (5 females and 7 males, mean age: 47±2 years). The diagnosis of chronic hepatitis B was confirmed by the presence of HBs and HBe antigens with stable elevated alanine aminotransferase (ALT) activity for at least 6 mo. Additionally the disease activity was confirmed by the presence of viral replication and evaluation of liver biopsy specimens performed by means of the Hepafix System (Braun, Melsungen, Germany). Paraffin-embedded biopsy specimens were stained and evaluated using the Scheuer scoring system<sup>[17]</sup>. Patients received 100 mg of lamivudine daily for 48 wk. Plasma levels of TGF- $\beta_1$ , TIMP-1 and MMP-1 were measured before treatment and at wk 24, 48 (end of the treatment) and 72. These results were compared to standard laboratory indices of liver injury. To evaluate treatment efficacy patients were divided into four groups with respect to elimination of HBV antigens and HBV-DNA at week 72 (24 wk after

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completion of treatment). Criteria for inclusion into particular groups were as follows: group I (complete response): HBsAg (-), HBeAg (-), HBV-DNA (-); group II: HBsAg (+), HBeAg (-), HBV-DNA (-); group III: HBsAg (+), HBeAg (-), HBV-DNA (+); group IV (no response): HBsAg (+), HBeAg (+), HBV-DNA (+).

#### Methods

Venous blood for plasma TGF- $\beta_1$ , TIMP-1 and MMP-1 was collected on ice using tubes with EDTA. Samples for TGF- $\beta_1$ were immediately activated with acetic acid and urea and assayed with ELISA using recombinant human TGF- $\beta$  soluble receptor type II (T $\beta$ RII) as a solid phase precoated onto a microplate (Quantikine®, R&D Systems Inc., Minneapolis, USA) as we described previously<sup>[18]</sup>. TIMP-1 and MMP-1 were assayed by the two-site ELISA sandwich technique (Amersham Pharmacia Biotech, Little Chalfont, Buckinghampshire, England) using specific antibodies as a solid phase. MMP-1 assay recognises total human MMP-1, ie. free and complexed with TIMP-1. TIMP-1 assay recognises total human TIMP-1, including free and complexed with any metalloproteinases bound to the solid phase. TIMP-1 or MMP-1 bound to the solid phase, were detected by peroxidase labelled antibodies. There is no cross-reactivity between TIMP-1 and MMP-1 in these assays.

Liver function tests: ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT) activity and bilirubin concentration were measured using a Cobas Mira instrument (Roche) and the prothrombin index (PI) was determined using Kselmed K-3002 (Poland).

#### Statistics

Values were expressed as the mean $\pm$ SE. The significance of the difference was calculated by two-tailed Student's *t* test. For correlation analysis the Pearson product moment correlation was performed. Values of *P*<0.05 were considered as statistically significant.

#### RESULTS

Plasma concentrations of TGF- $\beta_1$  and TIMP-1 were determined before lamivudine treatment (mean: 29.6±2.2 ng/mL and 1 578±93 ng/mL respectively) and significantly exceeded mean normal values (18.3±1.6 ng/mL and 1 102±67 ng/mL respectively). Treatment resulted in a significant decrease by wk 24 and a further decline at the end of the treatment. However 24 wk after treatment completion mean values of both TGF- $\beta_1$ and TIMP-1 increased (Table 1). In contrast, mean MMP-1 baseline level (6.7±0.7 ng/mL) was almost half the normal value (11.9±0.9 ng/mL) and increased during treatment. After completion of treatment the levels still remained lower than normal (Table 1). There was a significant positive correlation between TGF- $\beta_1$ , TIMP-1 and ALT or AST (Table 2). A significant correlation was also demonstrated between TIMP-1 and ALP or GGT, but there was no association between MMP-1 and the biochemical indices of liver injury (Table 2). As demonstrated in Figure 1 a significant positive correlation (r = 0.435; P < 0.01) was observed between TGF- $\beta_1$ and TIMP-1. There was no correlation between TGF- $\beta_1$  and MMP-1 (r = 0.069), or between TIMP-1 and MMP-1 (r = -0.143).

As demonstrated in Table 3 scored degree of hepatic inflammation and fibrosis was similar before and after the treatment. Histologic observations in biopsies performed before the treatment showed a significant correlation between the degree of fibrosis and plasma TGF- $\beta_1$  or TIMP-1 levels (Table 4). There was no association between scored inflammation or fibrosis and classical biochemical parameters of liver injury. **Table 1** Plasma concentrations of TGF- $\beta_1$ , TIMP-1 and MMP-1 during treatment with lamivudine (mean±SE)

	N	Time	Time since treatment beginning (wk)				
	values	0	24	48	72		
TGF-β <sub>1</sub>	18.3±1.6	$29.6{\pm}2.2^{1}$	$22.6 \pm 1.2^2$	$21.9{\pm}1.5^{2}$	24.1±2.4		
TIMP-1	1 102.0±67	$1 578.0 \pm 93^{1}$	1 235.0±67 <sup>2</sup>	$927.0{\pm}50^{\scriptscriptstyle 2}$	1 215.0±98 <sup>2</sup>		
MMP-1 (ng/mL)	$11.9{\pm}0.9$	$6.7{\pm}0.7^{1}$	$8.3{\pm}0.5^{1}$	$9.6{\pm}0.5^1$	$9.7{\pm}0.7{}^2$		

Statistical significance in comparison with normal  $^{\rm l}$  and baseline  $^{\rm 2} values.$ 

**Table 2** Correlation expressed by *r*-value and its significance between analyzed biochemical indices and TGF- $\beta_1$ , TIMP-1 or MMP-1 in chronic hepatitis B patients treated with lamivudine

	mean±SE	$TGF-\beta_1$	TIMP-1	MMP-1
Bil (mg%)	$1.02 \pm 0.06$	0.049	0.119	-0.059
ALT (U/L)	$74.50 \pm 5.9$	0.201ª	$0.247^{\mathrm{a}}$	0.005
AST (U/L)	$60.10 \pm 5.2$	0.244 <sup>a</sup>	0.269 <sup>a</sup>	-0.029
ALP (U/L)	$96.10 \pm 3.8$	0.114	0.295 <sup>a</sup>	-0.071
GGT (U/L)	$47.70 \pm 4.9$	0.062	$0.247^{\mathrm{a}}$	-0.102
PI (%)	$87.30{\pm}1.5$	-0.109	0.006	-0.065

Bil: bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyltranspeptidase; PI: prothrombin index.  ${}^{a}P$ <0.05 vs other groups.

**Table 3** Values of scored degree of hepatic inflammation and fibrosis in liver biopsy specimens obtained from patients before and after lamivudine treatment (mean±SE)

	Before the treatment	After the treatment
Portal inflammation	2.6±0.1	$2.5{\pm}0.2$
Lobular inflammation	$1.9{\pm}0.1$	$1.8 \pm 0.1$
Fibrosis	$2.0\pm0.2$	$1.8 \pm 0.2$

**Table 4** Correlation expressed by *r*-value and significance between scored degree of hepatic inflammation or fibrosis and plasma levels of TGF- $\beta_1$ , TIMP-1 and MMP-1 in patients with chronic hepatitis B before lamivudine treatment

	Inflam	Inflammation	
	Portal	Lobular	FIDFOSIS
TGF-β <sub>1</sub>	0.207	0.179	0.391ª
TIMP-1	0.224	0.319	0.620 <sup>a</sup>
MMP-1	0.009	-0.094	-0.135

<sup>a</sup>*P*<0.05 *vs* others.

The majority of patients (23-57.5%) did not respond to treatment and were classified into group IV. A complete response (group I), with elimination of HBs and HBe antigens as well as HBV-DNA, was observed in 5 patients (12.5%). Six patients (15%) demonstrated a partial response (group II), and another six eliminated HBeAg with detectable viral replication (group III). Evaluation of baseline liver function tests and efficacy of treatment showed no statistically significant differences between the four groups. As demonstrated in Table 5, at the end of the treatment, patients in groups III and IV had significantly higher ALT activities than patients in group I. This difference was statistically significant in respect to both ALT and AST activities, 24 weeks after the treatment completion (Table 5). Histologic pictures did not demonstrate significant differences between groups in liver biopsies preformed before

	ALT (U/l)			AST (U/L)				
	0	24	48	72	0	24	48	72
Group I	87.0±24.5	$25.2 \pm 5.0$	23.0±4.8	$23.2{\pm}4.9$	79.8±15.9	$30.2{\pm}6.7$	$28.0{\pm}6.4$	27.0±6.1
Group II	$108.5 \pm 53$	$32.5 \pm 9.5$	$31.9 \pm 7.5$	$31.7 \pm 5.7$	$66.7 \pm 24.8$	$44.2 \pm 12.7$	$32.1 \pm 4.8$	$29.8 \pm 3.8$
Group III	$127.0 \pm 43.4$	$38.7 \pm 8.2$	78.0±30.1ª	$88.2\pm34^{b}$	$117 \pm 47.2$	$39.2 \pm 8.8$	$60.5 {\pm} 15.9$	$63.5 \pm 22^{a}$
Group IV	$101.0 \pm 11.8$	$78.6 \pm 8.3$	$69.2{\pm}6.3^{\mathrm{b}}$	$68.5{\pm}4.2^{\rm b}$	$65.9{\pm}6.4$	$65.2{\pm}21.2$	$59.7{\pm}8.4$	$58.7{\pm}3.9^{\mathrm{a}}$

**Table 5** Activities of alanine and aspartate aminotransferases during the treatment (0, 24 and 48 wk) and 24 wk after its completion (week 72) in four groups (mean±SE)

Statistical significance in comparison with group I (complete response to treatment): <sup>a</sup>P<0.05, <sup>b</sup>P<0.001 vs other groups.

and after the treatment. During treatment TGF- $\beta$ 1, TIMP-1 and MMP-1 plasma levels were similar among all patients before divided into the response-related groups (Figures 2, 3, 4). And there were no statistically significant differences in their concentrations between groups at baseline, 24 and 48 weeks of treatment, either Although at week 48 the mean TGF- $\beta_1$  in the non-responder groups (III and IV) tended to increase, whereas in groups I and II normalized, the difference was not significant (Figure 2). However 24 weeks after treatment completion (wk 72), the difference between TGF- $\beta_1$  levels in groups I and IV (17.5±0.6 and 26±1.8 ng/mL respectively) became significant (P = 0.04). Moreover the concentration in group IV was significantly (P = 0.009) higher than normal values. TIMP-1 mean concentrations were similar among all groups during the treatment period and normalized at week 48 (Figure 3). However 24 wk later, values in group IV (1 350±70 ng/mL) were significantly higher than normal, as well as that in group I (900±131 ng/mL). MMP-1 levels increased in all groups during the treatment. At wk 72, MMP-1 almost normalized in responders and slightly decreased in non-responders (Figure 4). The difference between MMP-1 concentrations in group IV after treatment completion (9.1±0.8 ng/mL) and normal values was statistically significant (P = 0.037).



Figure 1 Correlation between plasma TGF- $\beta_1$  and TIMP-1 in patients with chronic hepatitis B treated with lamivudine.



**Figure 2** Mean TGF- $\beta_1$  plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (wk 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.



**Figure 3** Mean TIMP-1 plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (wk 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.



**Figure 4** Mean MMP-1 plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (week 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.

#### DISCUSSION

The pivotal role of TGF- $\beta_1$  in fibrogenesis was finally proved in transgenic mice with an overexpression of TGF- $\beta_1$ , which caused an increase of TGF- $\beta_1$  plasma levels up to 700 ng/mL and a marked upregulation of TIMP-1 gene expression<sup>[19-21]</sup>. Chronic liver injury leading to fibrosis displayed diminished ECM degradation mainly through TIMP induction and consequent MMP inhibition<sup>[4]</sup>. As demonstrated recently by Nie *et al.* in the process of hepatic fibrosis, fibroblasts and myofibroblasts are the major cells that express TIMP-1 and TIMP-2, and their gene expressions correlate with degree of hepatic fibrosis.

The most important factor that affects the measurement of TGF- $\beta_1$  in human is the preparation of samples with minimal contamination from platelets which are an important source of this cytokine<sup>[22]</sup>. The Quantikine ELISA System is recommended because of the relatively quick and simple activation with acid and urea, which disrupt the majority of TGF- $\beta_1$  complexes.

As we have demonstrated previously, TGF- $\beta_1$  correlates significantly with the degree of liver insufficiency in humans with liver cirrhosis<sup>[18]</sup>. Moreover both TGF- $\beta_1$  and TIMP-1 measured in plasma of patients with chronic hepatitis B demonstrate a significant correlation with the degree of hepatocyte injury and fibrosis in liver biopsy specimens<sup>[10]</sup>. We confirmed these observations in the present study. As demonstrated by Yoo *et al.*<sup>[7]</sup> HBV antigens induce expression of TGF- $\beta_1$  in the early stages of infection. According to Lee *et al.*<sup>[8]</sup> HBV encoded pX protein enhances transcriptional activity and response to TGF- $\beta$  by amplification of Smadmediated signaling, which can also contribute to HBV-associated liver fibrosis. Therefore the stimulatory effect of HBV antigens on TGF- $\beta_1$  seems to be an important mechanism of liver fibrosis in addition to TGF- $\beta$  release caused by hepatocyte injury.

Association between circulating or tissue TGF-B and liver fibrosis has also been confirmed in HCV infection<sup>[10,11]</sup>. Recent studies of Neuman et al.<sup>[23]</sup>, Chen et al. and Lu et al.<sup>[16]</sup> demonstrated a similar relationship between different chronic liver diseases including primary biliary cirrhosis and alcoholic liver disease and TGF- $\beta_1$ . Our previous study showed that the positive predictive value of TGF- $\beta_1$  plasma levels exceeding the upper normal range reached 96% for liver cirrhosis<sup>[18]</sup>. As demonstrated recently by Zhang et al. serum level of TIMP-1 and TIMP-1/MMP-1 could be used as the indices for the diagnosis of hepatic fibrosis in chronic hepatitis B. According to Boeker et al.<sup>[9]</sup>, measurement of plasma TIMP-1 detected cirrhosis with a 100% sensitivity but lower specificity. These observations were also confirmed by the association between hepatic fibroproliferation and expression of hepatic TIMP and MMP mRNA<sup>[12]</sup>. Walsh et al.<sup>[15]</sup>, who studied liver histology in patients with chronic hepatitis C, underlined the high sensitivity of TIMP-1 and TIMP-2 in detecting advanced liver disease. According to Nie et al., there was a significant correlation between circulating and liver levels of TIMP-1 in cirrhotics, so its measurement in plasma might be useful in fibrosis management. These observations indicate the usefulness of both TGF- $\beta_1$  and TIMP-1 as possible early noninvasive biomarkers for liver fibrosis.

The rate of HBeAg seroconversion in our study (27.5%) was in range (18-32%) demonstrated by numerous authors in previous research with lamivudine treatment<sup>[24-26]</sup>. In this study we confirmed the association between the degree of hepatocyte injury or liver fibrosis and plasma TGF- $\beta_1$  or TIMP-1 levels in patients with chronic hepatitis B. Aminotransferases, which are classical surrogate markers of hepatic injury, demonstrated similar association with the outcomes of the treatment. However, in contrast to TGF- $\beta_1$  and TIMP-1 their activities were not related to liver fibrosis. As the levels of TGF- $\beta_1$ , TIMP-1 and MMP-1 showed a similar change in all groups during therapy, it was unclear whether this was a direct effect of lamivudine on their expressions or an effect caused by HBV inhibition. However, their levels at 24 wk after therapy completion demonstrated their association with infection activity. This relationship was noted in respect to TGF- $\beta_1$ , which increased in group IV (non-responders) at the end of treatment. The effect on TIMP-1 was probably secondary to TGF- $\beta_1$ . The inhibitory activity of excessive TIMP-1 accumulation might be the reason for the decreased MMP-1 plasma concentration. These results are in accordance with our previous findings, demonstrating the strong association between TGF- $\beta_1$  or TIMP-1 plasma levels and scored hepatic fibrosis evaluated in biopsy specimens of patients with chronic hepatitis B and C<sup>[10]</sup>. The findings of increased TGF- $\beta_1$  and TIMP-1 accompanied by an elevation in plasma carboxyterminal cross-linked telopeptide of type 1 procollagen (ICTP), indicative of type I collagen degradation. We suggested that collagenolytic mechanisms preceded TGF- $\beta_1$ /TIMP-1

dependent stimulation of liver fibrosis<sup>[10]</sup>. Low MMP-1 plasma levels before treatment in the present study is consistent with this observation. It is also in accordance with the report by Murawaki et al.[13], who demonstrated a decrease in MMP-1 concentration during histological progression of chronic hepatitis. Moreover, a significantly decreased baseline plasma MMP-1, followed by an increase during treatment, supports the role of TGF- $\beta_1$ /TIMP-1 dependent mechanism of liver fibrosis in patients with active chronic hepatitis B. Similar effects on MMP-1 and TIMP-1 were observed by Ninomiya et al.<sup>[14]</sup> in patients with chronic hepatitis C who showed improvement of liver histology after treatment with interferon-alfa. Reduced progression of liver fibrosis during treatment of chronic hepatitis B with lamivudine has been demonstrated in numerous trials<sup>[24-28]</sup>. Downregulation of TGF- $\beta_1$ /TIMP-1 causing an increase of MMP-1 activity should be considered as the probable mechanism underlying this effect of lamivudine. However, to confirm this hypothesis further research should be focused on the MMP-1 activity measurement.

These findings support the role of TIMP-1 and MMP-1 balance in the TGF- $\beta_1$  dependent mechanism of liver fibrosis related to HBV infection. The association of TIMP-1, MMP-1 and TGF- $\beta_1$  with hepatic injury and antiviral treatment efficacy suggest their possible usefulness in chronic hepatitis B management. Elevated TGF- $\beta_1$  plasma concentrations during antiviral therapy may indicate medication failure.

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• VIRAL HEPATITIS •

# Establishment and assessment of two methods for quantitative detection of serum duck hepatitis B virus DNA

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#### Abstract

**AIM:** To establish and assess the methods for quantitative detection of serum duck hepatitis B virus (DHBV) DNA by quantitative membrane hybridization using DHBV DNA probe labeled directly with alkaline phosphatase and fluorescence quantitative PCR (qPCR).

**METHODS:** Probes of DHBV DNA labeled directly with alkaline phosphatase and chemiluminescent substrate CDP-star were used in this assay. DHBV DNA was detected by autoradiography, and then scanned by DNA dot-blot. In addition, three primers derived from DHBV DNA S gene were designed. Semi-nested primer was labeled by AmpliSensor. Standard curve of the positive standards of DHBV DNA was established after asymmetric preamplification, semi-nested amplification and on-line detection. Results from 100 samples detected separately by alkaline phosphatase direct-labeled DHBV DNA probe with dot-blot hybridization and digoxigeninlabeled DHBV DNA probe hybridization. Seventy samples of duck serum were tested by fluorescent qPCR and digoxigeninlabeled DHBV DNA probe in dot-blot hybridization assay and the correlation of results was analysed.

**RESULTS:** Sensitivity of alkaline phosphatase direct-labeled DHBV DNA probe was 10 pg. The coincidence was 100% compared with digoxigenin-labeled DHBV DNA probe assay. After 30 cycles, amplification products showed two bands of about 180 bp and 70 bp by 20 g/L agarose gel electrophoresis. Concentration of amplification products was in direct proportion to the initial concentration of positive standards. The detection index was in direct proportion to the quantity of amplification products accumulated in the current cycle. The initial concentration of positive standards was in inverse proportion to the number of cycles needed for enough quantities of amplification products. Correlation coefficient of the results was (0.97, P<0.01) between fluorescent qPCR and dot-blot hybridization.

**CONCLUSION:** Alkaline phosphatase direct-labeled DHBV DNA probe in dot-blot hybridization and fluorescent qPCR can be used as valuable means to quantify DHBV DNA in serum.

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#### INTRODUCTION

Duck hepatitis B virus (DHBV) infection model is an important referenced animal model for studying HBV infection and viral replication and evaluating antiviral agents<sup>[1-8]</sup>. HBV DNA level in serum is an important evidence for assessing antiviral effect. At present, it is difficult to accurately define the quantity of HBV DNA, though many methods<sup>[9-15]</sup> such as competitive PCR, Chiron Quantiplex branched DNA (bDNA) assay, Abbott Genostics solution hybridization assay and Digene Hybrid Capture System have been reported which are not suitable for clinical application because of time- and cost-consuming process. HBV DNA quantitative PCR has been used in clinical detection<sup>[16-18]</sup>, but no reports were presented about DHBV DNA quantitative PCR. With the development of non-isotopelabeled nucleic acid probes, especially chemiluminescentlabeled probes<sup>[19-21]</sup>, the probe-sensitivity has been improved obviously. In order to establish a quick, sensitive and specific method for quantitative detection of DHBV DNA in serum, we detected DHBV DNA in serum sample with alkaline phosphatase direct-labeled DHBV DNA probe with dot-blot hybridization and fluorescent qPCR.

#### MATERIALS AND METHODS

#### Materials

DHBV serum was obtained from Institute of Viral Hepatitis, Chongqing University of Medical Sciences, Chongqing, China. pBR325 containing DHBV DNA sequence was extracted, digested with EcoRI and recovered according to the kit protocol. Then quantification was made with DU<sup>R</sup> Series 600. DNA fragment diluted with water to a series concentrations of  $2.76 \times 10^6$  to  $2.76 \times 10^1$  copies/µL was used as positive standard. Reagents and instruments such as alkaline phosphatase direct-labeled kit (Amersham Pharmacia Biotech), Wizard Plus Midipreps DNA purification system (Promega), agarose gel DNA extraction kit (Roche), DU<sup>R</sup> Series 600 (Beckman), scanning apparatus (Vuego Scan, Brisa-620ST), AmpliSensor kit (Biotronics), Taq DNA polymerase (Roche), AG-9 600 Analyzer and Amplicor monitor (Biotronics) were used in the study. Primers derived from DHBV DNA S gene were 5'-TGGCCTAATCGGATTACTGG-3' (Limited primer, 20 bp), 5'-CCTGGGCATCCCCACGGGCAGG-3' (Excess primer, 22 bp) and 5'-GGGACGCGCGCTTTCCAAGATACTG-3' (Semi-nested primer, 25 bp). The three primers were synthesized by Seagon, Shanghai.

#### Methods

Detection of serum DHBV DNA with a membrane hybridization assay using alkaline phosphatase direct-labeled DHBV DNA probe DHBV DNA fragment was diluted to a concentration of 10 ng/µL and labeled referring to alkaline phosphatase directlabeling protocol. The probe can be stored in 50 mL/L glycerol at -20 °C for up to six months. DHBV DNA in the concentration series (100 ng, 10 ng, 1 ng, 100 pg, 10 pg) was dotted on nitric fibrous membrane in order to determine the probe sensitivity. The specificity was detected with human serum, duck serum and rat serum. The serum sample (40 µL) was dotted on nitric fibrous membrane and 1 mol/L NaOH was used for DNA degeneration. The membrane was baked at 80 °C for 2 h and prehybridized for at least 15 min, then 100 ng of the labeled probe was added to the buffer used for the prehybridization stage to hybridize at 55 °C overnight in a shaking water bath. The membrane was washed with the primary wash buffer for 20 min at 55 °C and with secondary wash buffer for 10 min at room temperature. The excess secondary wash buffer was drained from the blots and the membrane was placed on a clean, flat surface. Detection reagent was piped onto the blots (30-40 µL/cm<sup>2</sup>) for 2-5 min. The DNA blots were placed side up in the film cassette and a sheet of autoradiography film was placed on top of the blots. The cassette was closed and exposed for 1 h at room temperature. The blots on the film were scanned with scanning apparatus and analysed with Discovery Series Quantity One [BioRad, volume (mm<sup>3</sup>) = intensity×Area].

**Fluorescent qPCR for serum DHBV DNA ligation** Five×coupling reagent 10  $\mu$ L, 5×AmpliSensor 10  $\mu$ L, semi-nested primer 0.5  $\mu$ g, water 30  $\mu$ L were added to a microcentrifuge tube at room temperature. The tube was incubated for 90 min at 37 °C, then the reaction was terminated by adding 50  $\mu$ L of deionized water and heating to 90 °C for 5 min to denature the enzymes. The ligated product was labeled as 5×AmpliSensor primer stock.

Ligation efficiency Two of the sample wells (5×AmpliSensor primer 1 µL, 1×PCR buffer 4 µL and PCR extension mixture 5 µL per well), two for negative control and two for standard (5×AmpliSensor primer 0.1 µL, 1×PCR buffer 4.9 µL and PCR extension mixture 5 µL per well), two for blank control (water 5 µL, PCR extension mixture 5  $\mu$ L per well) were designated. The negative control served as a control of baseline signal, whereas the standard was a reference of maximum energy transfer. The reaction mixture was heated to 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, and then 20 °C for 30 s to equilibrate the signal. The fluorescence was read under the coupling mode using AG-9 600 Analyzer. Upon finishing, data were saved as baseline reading. One unit of Taq DNA polymerase was added to both the sample and standard wells and the reactions were subjected to three additional thermal cycles. Each cycle consists of 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C. At the end of the cycling, reactions were cooled down to 20 °C for 30 s. The fluorescence reading was repeated under the same mode. When the data were saved, the coupling efficiency was displayed on the screen

Asymmetric preamplification Length of amplification fragment was 182 bp. Ten×PCR buffer 110  $\mu$ L, 40 mmol/L MgCl<sub>2</sub> 110  $\mu$ L,  $10 \times dNTP 110 \mu L$ ,  $10 \times primer 110 \mu L$ ,  $10 \times terminal primer 110 \mu L$ ,  $H_2O$  330 µL were added. PCR master mix is intended for 100 reactions. The wells for negative (addition of  $5 \mu L H_2O$ ), Apex, standards (addition of 5 µL positive standards), sample (addition of  $5 \,\mu\text{L}$  sample) were designated. PCR master mix  $5 \,\mu\text{L}$ was added into each well except the well designated as Apex, to which 10 µL of 1×PCR buffer instead of 0.5 U Taq DNA polymerase was added. PCR cycle was repeated for 25 times: 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C. At the end of the cycle, the reaction was at 72 °C for 30 s and cooled down to 4 °C for 2 min. Semi-nested PCR and on-line detection A 700-bp fragment was amplified as follows: The 5×AmpliSensor primer stock 66 µL,  $10 \times PCR$  buffer 33 µL, 40 mmol/L MgCl<sub>2</sub> 33 µL and H<sub>2</sub>O 198 µL were added into a 0.5 mL microcentrifuge tube to make the AmpliSensor mix. AmpliSensor mix 5 µL was aliquoted to each reaction. PCR cycles were: At 94 °C for 20 s, at 55 °C for 30 s and at 72 °C for 30 s. The reaction was cooled for 30 s at 20 °C to was activated to reveal the quantitative data of each sample. Assessment of the two methods Alkaline phosphatase directlabeled DHBV DNA probe with dot-blot hybridization was compared with digoxigenin-labeled DHBV DNA probe with dotblot hybridization for 100 samples, and then the correlation of 70 samples between fluorescent qPCR and dot-blot hybridization with digoxigenin-labeled probe was analysed.

#### RESULTS

#### DHBV DNA fragment

The recovered DHBV DNA fragment after digestion of *Eco*RI was about 3.0 kb and the concentration was 28  $\mu$ g/mL ( $A_{260}/A_{280} = 1.9$ ) (Figure 1).



**Figure 1** Detection of *Eco*RI-digested pBR325 and recovered long fragment by 10 g/L agarose gel electrophoresis. Lane 1: Molecular marker; lane 2: pBR325 digested with *Eco*RI; lane 3: Long fragment of pBR325 (DHBV DNA).

# Sensitivity and specificity of alkaline phosphatase direct-labeled probe

Sensitivity of alkaline phosphatase direct-labeled probe was 10 pg without nonspecific signs (Figure 2, Table 1).



Figure 2 Sensitivity of alkaline phosphatase direct-labeled probe.

## *Comparison of alkaline phosphatase direct-labeled probe and digoxigenin-labeled DHBV DNA probe*

Compared with digoxigenin-labeled DHBV DNA probe, the detection sensitivity of alkaline phosphatase direct-labeled probe was 100% (60/60), the specificity was 100% (40/40) and the coincidence was 100% (80/80) (Table 2).

 Table 1
 Results of serum samples from different genera detected by alkaline phosphatase direct-labeled probe

Genera	Samples ( <i>n</i> )	DHBV DNA positive samples ( <i>n</i> )	DHBV DNA negative samples ( <i>n</i> )
Duck serum	20	10	10
Human serum	20	0	20
Rat serum	20	0	20

*Ligation efficiency of semi-nested PCR and AmpliSensor assay* The detection indices of semi-nested PCR and AmpliSensor assay for sample wells were 424.79 and 551.28, respectively. A "+" status was assigned to samples with ligation efficiency greater than 70. The primer could be used in asymmetric preamplification.

 Table 2
 Comparison of the results of 100 serum samples

 detected with different probes

Probe	Positive samples ( <i>n</i> )	Negative samples ( <i>n</i> )
Digoxigenin-labeled probe	60	40
Alkaline phosphatase	60	40
direct-labeled probe		

#### Detection of DHBV DNA positive standard with qPCR

After 30 cycles, amplification products showed two bands of about 180 bp and 70 bp by 2% agarose gel electrophoresis. The concentration of amplification products was in direct proportion to the initial concentration of the positive standards (Figure 3).



**Figure 3** Detection of products of DHBV DNA positive standard with qPCR by 20 g/L agarose gel electrophoresis. Lane l: Negative control; lanes 2-7: DHBV DNA positive standard  $(2.76 \times 10^6 - 2.76 \times 10^1 \text{ copies}/\mu\text{L})$ ; lane 8: DNA marker.



Figure 4 Amplification index standard curves of DHBV DNA positive standard.



**Figure 5** Concentration standard curve of DHBV DNA positive standard.

**Standard curves of DHBV DNA positive standards with qPCR** The detection index curves Std1-Std5 showed that the detection index was in direct proportion to the quantities of amplification products at the current cycle (Figure 4). The concentration curve showed that the initial concentration of the positive standards was in inverse proportion to the number of cycles needed for enough quantities of amplification products and the correlation coefficient between initial concentration and number of cycles was 0.992 (Figure 5).

#### Detection of serum DHBV DNA

Seventy serum samples of ducks were tested by fluorescent qPCR and digoxigenin-labeled DHBV DNA probe with dot-blot hybridization, and the correlation of results was analysed. Correlation coefficient of the results was 0.97 (P<0.01) between the two methods. DHBV DNA could not be found by dot-blot hybridization, but it could be detected by qPCR when DHBV DNA loads <10<sup>2</sup> copies/mL (Table 3).

 Table 3 Correlation analyses of DHBV DNA quantitative results of 70 serum samples detected by two methods

Serum samples (n)	qPCR (mean±SD) (copy/mL)	Dot-blot hybridization using digoxigenin-labeled probe (mean±SD) (volume, mm <sup>3</sup> )
10	$(2.37{\pm}1.70){ imes}10^2$	-
10	(2.44±1.71)×10 <sup>3</sup>	$1 \ 155 \pm 25$
10	$(4.47 \pm 1.96) \times 10^4$	$1 241 \pm 32$
10	$(5.02\pm2.14) \times 10^{5}$	$1 \ 353 \pm 27$
10	$(1.83 \pm 1.03) \times 10^{6}$	$1 \ 428 \pm 25$
10	(4.81±2.61)×10 <sup>7</sup>	$1 550 \pm 40$
10	$(5.16\pm2.72)\times10^8$	$1748 \pm 118$

#### DISCUSSION

Though probes labeled with radioisotope are still used to detect nucleic acids because of its high sensitivity and complete operation rule established many years ago, the safety and halflife of radioisotope must be taken into account. At present, labeling and detecting with non-radioisotope have made significant progress, and the sensitivity of the probe labeled with chemiluminescence has reached 50 fg. Two methods of labeling with non-radioisotope were available in the present study: non-direct-labeling such as random primed DNA-labeling with digoxigenin-dUTP<sup>[22]</sup> and direct-labeling such as DNAlabeling with alkaline phosphatase<sup>[23]</sup>. DNA-labeling with alkaline phosphatase is a new method with advantages of quickness, convenience and high sensitivity. Some procedures such as long time incubation with antibody and blocking reagent and elution can be omitted. The probe can be detached from hybridizing membrane and therefore the membrane can be used to hybridize with another probe sensitively and conveniently. Furthermore, alkaline phosphatase may be detected with chemiluminescence (dioxetane) and autoradiography or photoscanning apparatus. Chen et al.<sup>[21]</sup> reported that the sensitivity of dot-blot hybridization with Epstein-Barr virus probe coupled with alkaline phosphatase was 4 pg, which is similar to our results that the sensitivity of dot-blot hybridization with DHBV probe labeled by alkaline phosphatase was 10 pg. It is necessary to optimize labeling method of alkaline phosphatase for improving the sensitivity of the probe. This study showed that the alkaline phosphatase direct-labeled probe could be used to evaluate antiviral activity in DHBV infection animal model.

AmpliSensor-PCR is a real-time, quantitative tool for PCRbased detection. The assay is based on the principle that fluorescence resonance energy transfer can be used to detect duplex formation between complementary nucleic acid strands. If the two complementary strands are labeled with donor and

acceptor fluorophores, respectively, fluorescence resonance energy transfer between the fluorophores is facilitated when the strands are base-paired, or eliminated when the base-pair is disrupted. In this way, the extent of energy transfer can be used to measure the amount of duplex formation between the fluorophore-labeled oligonucleotide duplexes, thereby the extent of duplex formation mediated DNA polymerase. The measured fluorescence intensity is in proportion to the quantity of AmpliSensor duplex left at the end of each amplification cycle. The decrease in the fluorescence intensity correlates proportionately to the initial target dosage and the extent of amplification. The signal-generating mechanism of AmpliSensenor assay is tightly linked to the priming event. However, unlike typical PCR, where single-stranded primers are constantly subject to the possibility of nonspecific priming, the AmpliSensor primer is sequestered in a double-stranded form and stabilized against random priming. The AmpliSensor is a quasi-stable signal duplex of two oligonucleotides each labeled with an energy donor and acceptor fluorophore, respectively. Two strands of the AmpliSensor are unequal in length with the long strand 5' overhanging the short one by 7 nucleotides (5'-GCGTCCC-3'). For effective ligation, the seminested primer should encompass at its 5' end a "hook" sequence, that is, 5'-GGGACGC-3', complementing the overhang of the AmpliSensor. The fluorescence signal correlates to the overall energy transfer efficiency in a predictable, sequence-specific manner, and the amplified product can be monitored directly. As a simple, homogeneous assay for PCR quantification, AmpliSensor technology is target-specific, and it is amenable to the normalization of signal loading and sample loading. Thus it can reliablly count the amplified product while it accounts for the efficiency difference among reactions.

At present, fluorescence quantitative PCR has been applied in the fields of clinical diagnosis for hepatitis B and C, evaluating and monitoring of antiviral effect, prediction of interferon effect, *etc*.<sup>[24,25]</sup>.

DHBV and HBV are two members of the hepadnavirus family of viruses. They both display similar molecular biological character and 40% nucleic acid sequence homology. We usually make use of DHBV model to learn human hepatitis B about its infection and replication strategies or antiviral effect and to screen new anti-HBV drugs. In previous studies, dot-blot hybridization, Southern blotting and *in situ* hybridization with digoxigeninlabeled probe were used to detect DHBV DNA in serum or liver, but the advanced AmpliSensor-PCR and a membrane hybridization assay with alkaline phosphatase direct-labeled DHBV DNA probe could provide more objective, more valuable experimental data for developing new anti-HBV drugs and elucidating pathogenesis of hepatitis B.

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VIRAL HEPATITIS •

### A vaccinia replication system for producing recombinant hepatitis C virus

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#### Abstract

**AIM:** To develop a cell culture system capable of producing high titer hepatitis C virus (HCV) stocks with recombinant vaccinia viruses as helpers.

**METHODS:** Two plasmids were used for the generation of recombinant HCV: one containing the full-length HCV cDNA cloned between T7 promoter and T7 terminator of pOCUS-T7 vector, and the other containing the HCV polyprotein open reading frame (ORF) directly linked to a vaccinia late promoter in PSC59. These two plasmids were co-transfected into BHK<sub>21</sub> cells, which were then infected with vTF7-3 recombinant vaccinia helper viruses.

**RESULTS:** After 5 d of incubation, approximately  $3.6 \times 10^7$  copies of HCV RNA were present per milliliter of cell culture supernatant, as detected by fluorescence quantitative RT-PCR (FQ-PCR). The yield of recombinant HCV using this cell system increased 100- to 1 000- fold compared to *in vitro*-transcribed HCV genomic RNA or selective subgenomic HCV RNA molecule method.

**CONCLUSION:** This cell culture system is capable of producing high titer recombinant HCV.

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#### INTRODUCTION

Hepatitis C virus (HCV) is a causative agent of acute and chronic liver diseases that infect millions of people worldwide<sup>[1]</sup>. There is no vaccine for prevention of HCV infection, and interferon is the only known therapeutic drug, yet it is only 10-20% effective alone and less than 50% effective when used in combination with ribavirin<sup>[2]</sup>. Overall, despite the epidemic significance of HCV infection, the development of anti-HCV drugs and vaccines has been impeded by the lack of functional cell culture systems capable of growing the virus on a large scale.

Several approaches have been taken for the propagation of HCV in cell cultures. One approach focuses on the identification of primary cells or cell lines that support replication of the virus. It has been reported that HCV isolated from infected patients is capable of infecting human primary hepatocytes<sup>[3-5]</sup>,

peripheral blood mononuclear cells<sup>[6]</sup>, and cell lines such as human T cell line HPBMa10-2, Daudi B cell line<sup>[7-9]</sup> and various hepatocyte cell lines<sup>[10-12]</sup>. However, HCV replication in these cell culture systems is generally transient and very inefficient. A second approach is to transfect in vitro-transcribed HCV genomic RNA into human hepatoma cell line, Huh7<sup>[13]</sup>. Although it was reported that the transfected cells produced infectious viral particles, the replication efficiency of this method was very poor, and moreover, many research groups have reported failure to generate detectable HCV virions using this method. Recently, Lohmann et al. recovered high levels of selective subgenomic HCV RNA molecules from transfected Huh-7 cells. These replicons were derived from a cloned full-length HCV (genotype 1b) consensus sequence with the C-p7 or C-NS2 regions removed and a neomycin phosphotransferase gene (neo) inserted downstream of the HCV internal ribosome entry site IRES<sup>[14-17]</sup>. However, HCV particles generated by this system may differ from the native virions, due to the alteration of fulllength HCV genomic RNA. Thus, researchers continue to search for a HCV replication system that will produce large-scale quantities of native virions.

Compared to the infection of cell lines with HCV-containing patient serum, the introduction of cloned viral genomes was superior because the inoculum was well-defined and could be generated in high quantities<sup>[18]</sup>. Here, we used recombinant vaccinia viruses as helper viruses to produce high-titer cloned HCV stocks in a cell culture system. This method has the advantages of being simple, highly efficient, and capable of producing large quantities of high-fidelity HCV particles.

#### MATERIALS AND METHODS

#### Plasmid construction

Plasmid pBRT703'X (NIHJ1) (generously provided by T. Suzuki), containing the full-length HCV cDNA, was cut with Hind III and inserted into vector pOCUS-T7 (plasmid pOCUS containing a T7 terminator) between *Eco*R I and Hind III sites, resulting in insertion of the HCV sequence between bacteriophage T7 promoter and terminator sequences to create plasmid pT7HCV. Next, plasmid O. pMKC1A (HCV) was cut with *Eco*R I and Hind III and inserted into PSC59 between *Eco*R I and Stu I sites to generate plasmid pVHCV, which contained the HCV polyprotein open reading frame (ORF) linked to a vaccinia late promoter. The correct sequences of pT7HCV and pVHCV were confirmed by restriction enzyme digestion and DNA sequencing.

#### **HCV production**

One day prior to use, BHK<sub>21</sub> cell cultures ( $5 \times 10^5$  cells per well) were seeded in 6-well plates and incubated in Dulbecco's modified Eagle's medium (DMEM) with high levels of glucose (4.5 g/L) supplemented with 100 mL/L fetal calf serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). Cultures were incubated overnight in a 37 °C CO<sub>2</sub> incubator. Cells were then transfected with either 5 µg pT7HCV, 5 µg pT7HCV plus 5 µg pVHCV, 5 µg pVHCV or control plasmid (pOCUS-T7) using the LIPOFECTAMINE<sup>TM</sup> 2000 reagent (Invitrogen) followed by infection with vTF7-3 vaccinia viruses<sup>[19]</sup>.

Two hours after inoculation, the inoculum was removed and the cells were cultured in a fresh medium in a  $30 \degree C CO_2$  incubator for 2-5 d.

#### Determination of HCV virions in cell culture supernatants

HCV virion RNA was used as an indicator for HCV virion production. Accordingly, cell culture supernatants were filtered through a 0.22  $\mu$ m filter to remove residual cells and vaccinia viruses. Ten units of RNase A and 20 units of DNase I were added to 200  $\mu$ L of each filtrate, and samples were incubated at 37 °C for 1 h for complete digestion of RNA and DNA. Protease K was then added to a final concentration of 100  $\mu$ g/mL, and extraction of HCV genomic RNA was carried out using the high pure viral RNA kit (Roche). The resultant RNA was treated with DNase I and used as a template for RT-PCR (GIBCO BRL). For detection of positive strand HCV genomic RNA, a 416 bp fragment of the 5' region (nt 346 to 761) and a 488 bp fragment of the 3' region (nt 9378 to 8891) were amplified by RT-PCR. The primers used for reverse transcription and 5' and 3' region PCR reactions are shown in Table 1.

PCR amplification consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Amplified fragments were confirmed by 10 g/L agarose gel electrophoresis, ethidium bromide staining and visualization under UV light.

#### Immunofluorescence and Western blot analysis of HCV proteins

For immunofluorescence,  $BHK_{21}$  cells were grown on glass coverslips for 48 h and fixed after washed 3 times with PBS in an ice-cold mixture of 9:1 acetone and methanol. After 10 min of incubation at -20 °C, the cells were washed 3 times with PBS and incubated for 1 h in 1F buffer (PBS, 30 g/L bovine serum albumin and 1 g/L Triton X-100) at 4 °C. A mixture of NS3- and NS5a-specific mouse monoclonal antibodies (Biodesign) was added at a dilution of 1:100 in 1F buffer. After 1 h, the cells were washed 3 times with PBS followed by incubation with a mousespecific antibody conjugated with fluorescein isothiocyanate (Sigma) in 1F buffer. Coverslips were washed 3 times with PBS and mounted on glass slides, and the cells were examined under a fluorescence microscope.

For Western blot analysis, cells were lysed with a buffer containing 10 g/L sodium deoxycholate, 1 g/L sodium dodecyl sulfate (SDS), 10 g/L Triton X-100, 10 mmol/L Tris, 140 mmol/L NaCl, 1 mmol/L phenylmethylsulfony1 fluoride (PMSF), 2 µg of aprotinin per mL, and  $2 \mu g$  of leupeptin per mL (pH 8.0). The cell lysate was cleared of cell debris and nuclei by low-speed centrifugation (15 min at 15 000 g, 4 °C). A fraction of the supernatant was denatured by heating for 4 min at 100 °C in SDS sample buffer. The samples were electrophoresed on SDS-100/L polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then incubated overnight in blocking buffer (PBS containing 5 mL/L Tween 20 and 50 mL/L milk powder), and the mixture of NS3- and NS5a-specific mouse monoclonal antibodies was added at a dilution of 1:100 in blocking buffer for 1h. After washed 3 times with 5 mL/L Tween 20 in PBS, the membranes were then incubated with a mousespecific antibody conjugated with peroxidase (Sigma) in blocking buffer for 1 h, washed 3 times as described above, and bound antibodies were detected by DAB.

#### Immunoelectron microscopy

Transfected cells were grown in 6-well plates for 48 h. After washed 2 times with ice-cold PBS (pH 7.4), the cells were fixed in Petri dishes for 30 min in a mixture of 40 g/L formaldehyde, 2.5 g/L glutaraldehyde, and 2 mL/L picric acid in PIPES buffer. The fixed cells were washed 3 times with PBS, then cell pellets were embedded in 40 g/L melted agarose and cut into small blocks, which were stained in 10 g/L uranyl acetate dissolved in water for 30 min at 4 °C. The blocks were further dehydrated in ethanol and embedded in Epon-Araldite. Ultrathin sections were mounted on nickel grids without supporting films. All labeling was conducted on grids according to the method of Sparkman and White<sup>[20]</sup>. Grids were etched with 10 g/L sodium periodate in water for 15 min, washed 3 times with PBS, and incubated with 30 g/L BSA in PBS for 30 min. The grids were then incubated for 1 h with a mixture of NS3- and NS5a-specific mouse monoclonal antibodies (diluted 1:100 in 10 g/L BSA-PBS) or in 10 g/L BSA-PBS only. After washed 5 times with PBS, the sections were treated for 1 h with goat anti-mouse antibody conjugated with colloidal gold (10-nm diameter, diluted 1:100 in PBS). Samples were rinsed five times with PBS. After counterstained with uranyl acetate and lead citrate, the sections were examined with an electron microscope.

#### Quantification virion RNA copy numbers

Fluorescence quantitative RT–PCR (FQ-PCR) was used to determine the amount of HCV virion RNA in cell culture supernatants treated with RNase A and DNase I as described above. Hepatitis C virus fluorescence polymerase chain reaction (PCR) diagnostic kit (Da Am Company, China) was used as directed to quantify virion RNA. Samples from ten transfections were detected by FQ-PCR.

#### RESULTS

Recombinant plasmids pT7HCV and pVHCV were confirmed by restriction endonuclease digestion. As expected, pT7HCV generated two fragments of 6.7 kb and 4.6 kb when digested with *Eco*R I, and pVHCV generated two fragments of 8 kb and 4.4 kb when digested with *Xh*o I. DNA sequencing confirmed that the 3' and 5' sequences were as expected.

RT-PCR was used to detect HCV virion RNA in the cell culture supernatant as a measure of virion production. To ensure that the detected HCV RNA was the product of virions, we used RNase A treatment to remove HCV RNA that might release into the culture medium, as the virion envelope could protect the desired HCV RNA from digestion. To ensure that the fragments detected by RT-PCR did not come from the HCV DNA, the cell culture supernatants were also treated with DNase I. To ensure that the detected HCV RNA included the entire genome, the 5' and 3' ends of HCV genome were both amplified by RT-PCR. Positive samples amplified a 416 bp DNA fragment from the 5' UTR and a 488 bp DNA fragment from the 3' UTR. Transfection of BKH<sub>21</sub> cells with either pT7HCV or pT7HCV plus pVHCV resulted in samples that were positive for both the 3' and 5' fragments amplified by RT-PCR but not PCR alone. HCV RNA was not detected following transfection with pVHCV alone or with the control plasmid. These results confirmed that HCV virions existed in the culture supernatant of BKH<sub>21</sub> cells

Table 1	Primers	for	RT-PCR	2
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Oligodeoxynucleotide	5' region	3' region
Reverse transcription primers	agccctggcagcgccccctagggggggcgcc (nt 791 to 762)	gggggatggcctattggcctggagtgt (nt 9414 to 9388)
PCR primer 1	gcacaaatccaaaaccccaaagaaaaa (nt 346-372)	cgctcatcggttggggagcaggtagatg (nt 9378 to 9351)
PCR primer 2	gacgagcggaatgtaccccatgaggtcggc (nt 761-732)	ccatcettetageccaggageaacttga (nt 8891 to 8918)



Figure 1 Immunofluorescent analysis of HCV nonstructural proteins expressed in  $BHK_{21}$  cells using a mixture of NS3- and NS5a-specific mouse monoclonal antibodies. A: cells transfected with pT7HCV; B: cells cotransfected with pT7HCV and pVHCV; C: cells transfected with pVHCV.

transfected with pT7HCV or pT7HCV plus pVHCV.

Expression of HCV nonstructural proteins in the infected cells was detected by immunofluorescence and Western blot analysis using NS3- and NS5a-specific mouse monoclonal antibodies. BKH<sub>21</sub> cells transfected with pT7HCV, pT7HCV plus pVHCV or pVHCV were positive for immunofluorescence, though to varying degrees. Cells transfected with pVHCV showed the strongest signal, and cells transfected with pT7HCV showed the weakest (Figure 1). Expression of HCV nonstructural proteins in BKH<sub>21</sub> cells was further confirmed by Western blot analysis. Nonstructural proteins NS3 and NS5a were detected as bands of -70 and 56 ku, respectively. Western blot analysis also showed that the expression of nonstructural proteins was highest in cells transfected with pVHCV and lowest in cells transfected with pT7HCV (Figure 2).



Figure 2 Western blotting of HCV gene products expressed in BHK<sub>21</sub> cells probed with a mixture of NS3- and NS5a-specific mouse monoclonal antibodies. 1: cells transfected with control plasmid (pOCUS-T7); 2: cells transfected with pVHCV; 3: cells cotransfected with pT7HCV and pVHCV; 4: cells transfected with pT7HCV.

NS3- and NS5a-specific mouse monoclonal antibodies were used to determine the subcellular localization of HCV proteins in cells transfected with pT7HCV plus pVHCV. Specific, though weak, signals were found in cytoplasm and vesicles (Figure 3), which were consistent with the results of Pietscmann *et al.*<sup>[16]</sup>, who reported that antisera monospecific for NS3, NS4b, NS5a or NS5b resulted in weak specific signals that were difficult to interpret. Our immunoelectron microscopy results identified positively stained small particles, about 40 to 60 nm in diameter, in cytoplasm and vesicles (Figure 3). The morphology and size of these particles were consistent with previous predictions regarding the HCV particles<sup>[21-24]</sup>. No virion-like structures were identified in cells transfected with control plasmid and/or infected with vTF7-3 vaccinia alone.

Next, FQ-PCR was used to quantify the virion production in supernatants of cells transfected with pT7HCV, pT7HCV plus pVHCV, pVHCV or the control plasmid. The copy number of HCV RNA per milliliter of culture supernatant 2, 3, 4 and 5 days after inoculation are shown in Table 2.



**Figure 3** Immunoelectron microscopy of HCV-like virions in BHK21 cells cotransfected with pT7HCV plus pVHCV and stained with mixed NS3- and NS5a-specific mouse monoclonal antibodies. Virions about 40 to 60 nm in diameter (arrows) were identified in cytoplasm and vesicles. Bar: 500 nm (A) and 200 nm (B). c: cytoplasm; lu: lumen; n: nucleus.

**Table 2** Virion RNA quantitation in cell culture supernatantsas measured by fluorescence quantitative RT-PCR (RNA copies per mL)

Culture time		RNA copies		
Culture lime	PT7HCV	PT7HCV+ PvHCV	PvHCV	Control plasmid
48 h	$1.12{\pm}0.18{\times}10^{5}$	$3.76 \pm 0.22 \times 10^{5}$	0	0
72 h	$4.08{\pm}0.20{\times}10^{5}$	$8.00{\pm}0.28{\times}10^{5}$	0	0
96 h	$1.28{\pm}0.27{\times}10^{6}$	$7.68{\pm}0.22{\times}10^{6}$	0	0
120 h	$3.24{\pm}0.25{\times}10^{6}$	$3.60{\pm}0.18{\times}10^7$	0	0

#### DISCUSSION

Since HCV virions contain only genomic RNA and structural proteins, transfecting host cells with *in vitro* synthesized HCV viral genomic RNA could result in generation of HCV virions if the host cells were able to translate mRNA and properly processed the resulting polyprotein<sup>[13]</sup>. However, because of low transfection efficiency, the short lifetime of viral RNA in the cytoplasm, and poor translation efficiency, viral production was generally very low by the RNA transfection method.

In this study, we used the vaccinia viral replication machinery to produce HCV virions in cell culture. The vaccinia expression system was previously tried for the production of HCV virions in cell culture. Selby et al. transfected Ost7-1 cells with a plasmid containing a cDNA of HCV genomic RNA downstream of a T7 promoter. The transfected cells were then infected with a recombinant vaccinia virus containing a T7 polymerase gene. Although HCV polyprotein was synthesized in Ost7-1 cells and correctly processed into individual viral proteins, no HCV virions were generated<sup>[25]</sup>, perhaps because the researchers did not place a T7 terminator downstream of HCV cDNA. Without terminator, transcripts synthesized by T7 RNA polymerase were heterogeneous concatemers that were too large to be packaged into a HCV virion. To correct this problem, Mizuno et al. cloned HCV cDNA between a T7 promoter and a T7 terminator<sup>[21]</sup>, resulting in the expression of both structural and nonstructural proteins in HeLa G cells, and the appearance of HCV core antigen-positive particle-like structures in cytosol and cisternae of the endoplasmic reticulum (ER). However, these particles were not tested for the presence of HCV RNA.

For identification of recombinant HCV virions, we detected the expression of HCV nonstructural proteins NS3 and NS5a in the supernatant of transfected cells. This has been reported by Mizuno et al. who detected the expression of structural proteins in HeLa G cells transfected with the full-length HCV genome sequence<sup>[21]</sup>. Next, we used RT-PCR to detect the presence of positive strand HCV genomic RNA. Following digestion of HCV RNA from blocked cells, and residual plasmid DNA, RT-PCR of fragments from the 5' (nt 346 to 761) and 3' (nt 9378 to 8891) regions of HCV RNA showed that virions contained the entire sequence. This was in contrast to the report of Baumert et al., who reported that HCV-like particles produced in insect cells using a recombinant baculovirus containing cDNA of HCV structural proteins contained various shortened HCV RNAs<sup>[22]</sup>. Finally, we observed the expression of HCV proteins and virion-like structures using immunoelectron microscopy.

In this new culture system, cells were transfected with two plasmids. One contained the HCV genomic RNA-coding region between upstream T7 promoter and downstream T7 terminator, transcripts synthesized by bacteriophage T7 RNA polymerase would have a defined size. The other plasmid contained the open reading frame (ORF) of HCV polyprotein directly linked to a vaccinia late promoter. The doubly transfected cells were subsequently infected with vTF7-3 recombinant vaccinia viruses containing a T7 RNA polymerase gene under the control of a vaccinia promoter. Thus, T7 RNA polymerase was synthesized in the infected cells and in turn transcribed plasmid DNA encoding HCV genomic RNA. Meanwhile, vaccinia RNA polymerase transcribed DNA encoding HCV polyprotein. After polyprotein was processed, the resulting viral proteins packaged HCV genomic RNA and assembled it into virions, which were then released from cells via the secretory pathway. In the system, we took the advantage of the unique properties of vaccinia viruses. Vaccinia virus replicates entirely in cytoplasm and uses its own enzymes to replicate DNA and systhesize 5' capped and 3' polyadenylylated mRNA. Vaccinia DNA polymerase is able to replicate plasmid DNA in cytoplasm, increase the number of DNA copies, and transcribe cytoplasmic DNA that is linked to a vaccinia promoter. Meanwhile, the viral capping enzyme and poly(A) polymerase add a 5' cap and 3' poly(A) tail to the transcribes. The resulting mRNA can be translated by the host cell translation machinery. Beacuase HCV genomic RNA is synthesized by T7RNA polymerase of vaccinia while mRNA for the viral proteins is synthesized by vaccinia enzymes, RNA synthesis will not be restricted as RNA replication that is catalyzed by HCV enzymes.

For practical examination of this new system, we transfected BHK<sub>21</sub> cells with pT7HCV and pVHCV respectively, or cotransfected the cells with pT7HCV plus pVHCV for comparison. After 5 d of culture, we detected approximately 3.24±0.25×106 copies of HCV RNA per mL of supernatant from cells transfected with pT7HCV and  $3.60\pm0.18\times10^7$  copies of HCV RNA per mL from cells cotransfected with pT7HCV plus pVHCV. HCV RNA was not detected in cells transfected with pVHCV alone. These results suggest that cotransfection with pT7HCV plus pVHCV could increase virion production. Evidence from a previous study with in vitro-transcribed HCV RNA showed that although transfection of cells with genomic HCV RNA could result in the production of HCV virions, cotransfection with both genomic RNA and 5' capped RNA in which 5'UTR is deleted could increase virion production<sup>[13]</sup>. We hypothesize that it may be possible to further increase the production of viral particles by slight alterations of pT7HCV, including addition of mutations in the NS5a or NS3 regions (e.g., serine to isoleucine at NS5 position 1179) to enhance replication<sup>[26-29]</sup>, or addition of a hairpinribozyme cassette right after the end of the 3' untranslated region<sup>[30-32]</sup>, which may also increase the titer of the resulting viral particles. However, the experimental benefits of these changes have yet to be determined.

Indeed, it also remains to be seen whether recombinant HCV virions identified in the present study are infectious. Evidence from a study with *in vitro*-transcribed full-length and subgenomic HCV RNA showed that recombinant viral particles were infectious and replication competent<sup>[13]</sup>. To determine whether the virions produced by our new method are infectious, future work may include purification of the particles by sucrose and CsCl gradient centrifugation according to Baumert *et al.*<sup>[22]</sup>, to rule out interference by residual vaccinia viruses. Alternatively, we might generate a defective vaccinia virus that lacks D13L gene, which is essential for HCV production. Repression of D13L gene expression had no effect on viral replication and viral protein expression but could block formation of progeny virions<sup>[33]</sup>.

In conclusion, the yield of recombinant HCV using our cell system increased 100- to 1000-fold compared to *in vitro*-transcribed HCV genomic RNA method or selective subgenomic HCV RNA molecule method. This culture system may enable us to provide academic and medical researchers with high quality HCV preparations as well as to generate attenuated HCV for vaccine development. Furthermore, it may be useful as a system for future drug screening and vaccine selection.

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• H pylori •

# Construction of prokaryotic expression system of *ItB-ureB* fusion gene and identification of the recombinant protein immunity and adjuvanticity

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#### Abstract

**AIM:** To construct *ltB-ureB* fusion gene and its prokaryotic expression system and identify immunity and adjuvanticity of the expressed recombinant protein.

METHODS: The ureB gene from a clinical Helicobacter pylori (H pylori) strain Y06 and the ItB gene from Escherichia coli (E. coli) strain 44851 were linked into ItB-ureB fusion gene by PCR. The fusion gene sequence was analyzed after T-A cloning. A prokaryotic recombinant expression vector pET32a inserted with ItB-ureB fusion gene (pET32a-*ItB-ureB*) was constructed. Expression of the recombinant LTB-UreB protein (rLTB-UreB) in E. coli BL21DE3 induced by isopropylthio- $\beta$ -D-galactoside (IPTG) at different concentrations was detected by SDS-PAGE. Western blot assays were used to examine the immunoreaction of rLTB-UreB by a commercial antibody against whole cell of H pylori and a self-prepared rabbit anti-rUreB serum, respectively, and determine the antigenicity of the recombinant protein on inducing specific antibody in rabbits. GM<sub>1</sub>-ELISA was used to demonstrate the adjuvanticity of rLTB-UreB. Immunoreaction of rLTB-UreB to the UreB antibody positive sera from 125 gastric patients was determined by using ELISA.

**RESULTS:** In comparison with the corresponding sequences of original genes, the nucleotide sequence homologies of the cloned ItB-ureB fusion gene were 100%. IPTG with different dosages of 0.1-1.0 mmol/L could efficiently induce pET32a-ltB-ureB-E.coli BL21DE3 to express the rLTB-UreB. The output of the target recombinant protein expressed by pET32a-ureB-E.coli BL21DE3 was approximately 35% of the total bacterial proteins. rLTB-UreB mainly presented in the form of inclusion body. Western blotting results demonstrated that rLTB-UreB could combine with the commercial antibody against whole cell of *H pylori* and anti-rUreB serum as well as induce rabbit to produce specific antibody. The strong ability of rLTB-UreB binding bovine GM<sub>1</sub> indicated the existence of adjuvanticity of the recombinant protein. All the UreB antibody positive sera from the patients (125/125) were positive for rLTB-UreB.

CONCLUSION: A recombinant prokaryotic expression

system with high expression efficiency of the target fusion gene *ltB-ureB* was successfully established. The expressed rLTB-UreB showed qualified immunogenicity, antigenicity and adjuvanticity. All the results mentioned above laid a firm foundation for further development of *H pylori* genetically engineered vaccine.

Yan J, Wang Y, Shao SH, Mao YF, Li HW, Luo YH. Construction of prokaryotic expression system of *ltB-ureB* fusion gene and identification of the recombinant protein immunity and adjuvanticity. *World J Gastroenterol* 2004; 10(18): 2675-2679

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#### INTRODUCTION

In China, gastritis and peptic ulcer are the most prevalent gastric diseases and gastric cancer is one of the malignant tumors with high morbidities<sup>[1]</sup>. Helicobacter pylori (H pylori), a microaerophilic, spiral and Gram-negative bacterium, is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world's populations<sup>[2]</sup>. Most infected individuals are asymptomatic. However, in some subjects, H pylori infection causes acute, chronic gastritis or peptic ulceration. Furthermore, the infection is also a high risk factor for the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma<sup>[3-8]</sup>. Recently, direct evidence of carcinogenesis of the microbe in an animal model has been presented<sup>[9-11]</sup>. Immunization against the bacterium represents a cost-effective strategy to prevent H pylori-associated common peptic ulcer diseases and to reduce the incidence of global gastric cancers<sup>[12]</sup>. However, no vaccines preventing H pylori infection have been commercially available so far.

Previous studies revealed many protective protein antigens of the microbe such as UreB, HpaA, FlaA, CagA, VacA  $etc^{[13-18]}$ . Among these protein antigens, UreB, one of the four subunits of an urease produced by almost all the isolated strains of *H pylori*, has been demonstrated to have the strongest antigenicity and protection in all known proteins of *H pylori*<sup>[13,19,20]</sup>. *ureB* gene, responsible for encoding UreB with 569 amino acid residues, is a highly conserved nucleotide sequence with a similarity of approximately 95% in different *H pylori* isolates<sup>[21-23]</sup>. These data strongly indicate that UreB can be used as an excellent antigen candidate for *H pylori* vaccine.

Since a genetically engineered vaccine composed of a single protein antigen usually showed a low immunization effect, it is necessary to increase immunogenicity of the antigen by co-administration with an appropriate adjuvant. *Escherichia coli* (*E. coli*) heat-labile toxin B subunit (LTB) and cholera toxin B subunit (CTB) were well-confirmed mucosal adjuvants<sup>[24-28]</sup>. However, some of the previous studies demonstrated that the mucosal adjuvanticity of LTB was stronger than that of CTB<sup>[26,29]</sup>. Furthermore, CTB activates Th2 pathway, and induces IL-4, a

cytokine closely related to IgE-mediated allergic reaction, but LTB mainly stimulates Th1 pathway<sup>[26,30]</sup>.

In order to simplify the procedure steps and further reduce cost in H pylori vaccine production, we constructed ltB-ureB fusion gene and its recombinant prokaryotic expression system. The immunogenicity, antigenicity and adjuvanticity of the expressed target recombinant protein (rLTB-UreB) were examined. The results of this study would benefit the mass production of H pylori UreB-associated genetically engineered vaccine at a lower cost.

#### MATERIALS AND METHODS

#### Materials

Both the *ureB* gene from a clinical *H pylori* strain Y06 and the ltB gene from E. coli strain 44851 (offered by National Institute for the Control of Pharmaceuticals and Biological Products of China) was cloned by our laboratory<sup>[31]</sup>. A plasmid *pET32a* (Novagen, Madison, USA) and E. coli BL21DE3 (Novagen, Madison, USA) were used as the expression vector and host cell, respectively. Primers for PCR amplification were synthesized by BioAsia (Shanghai, China). Taq-plus high fidelity PCR kit and restriction endonucleases used were purchased from TaKaRa (Dalian, China). The T-A Cloning Kit, DNA Agarose Gel Purification Kit and sequencing service were provided by BBST (Shanghai, China). DAKO (Glostrup, Denmark) and Jackson ImmunoResearch (West Grove, USA) supplied rabbit antiserum against whole cell of H pylori, HRPlabeling sheep anti-rabbit IgG and anti-human IgG antibodies, respectively. The UreB antibody positive serum samples from 125 H pylori infected patients with gastritis or ulcer were stored at -70 °C in our laboratory<sup>[31]</sup>.

#### Methods

**Extraction of DNA templates** *E. coli* DH5  $\alpha$  strains respectively containing plasmid *pUCm-T- ureB*, *pUCm-T-ltB* were cultured in LB medium. The two plasmids were extracted by alkaline-denature method and then purified by DNase-free RNase treatment and routine phenol-chloroform method described by Sambrook *et al.*<sup>[32]</sup>. The obtained DNA extracts were dissolved in TE buffer and their concentrations as well as purity were measured by ultraviolet spectrophotometry<sup>[32]</sup>. The *pUCm-T-ureB* DNA was further digested with restriction endo- nucleases *EcoR* V and *Xho* I at 37 °C for 3 h. The target fragment of *ureB* gene was separated by agarose gel electrophoresis and then recovered by DNA Agarose Gel Purification Kit.

Amplification of *ureB* and *ltB* gene The sequence of *ltB* sense primer was: 5'-CCGGATATCATGAATAAAGTAA AATGTTA-3'(EcoR V). The sequence of antisense primer linking the 3'-end of *ltB* gene and the 5'-end of *ureB* gene was: 5'-AGAAACATATTCTTTTCTGCTAATGTTTTCCATA CTGATTGCCGC-3'. Total volume per PCR was 100 µL containing 2.5 mol/L each dNTP, 250 nmol/L each of the two primers, 15 mol/L MgCl<sub>2</sub>, 2.5 U Taq-plus polymerase, 100 ng pUCm-T-ltB DNA template and 1×PCR buffer (pH8.3). Parameters for PCR of *ltB* gene were: at 94 °C for 5 min, ×1; at 94 °C for 30 s, at 48 °C for 30 s, at 72 °C for 45 s, ×10; at 94 °C for 30 s, at 48 °C for 30 s, at 72 °C for 50 s (an addition of 5 s for each of the following cycles), ×20; finally at 72 °C for 7 min, ×1. The results of PCR were observed under UV light after electrophoresis in 15 g/L agarose pre-stained with ethidium bromide. The expected size of target amplification fragment from *ltB* gene was 375 bp. The target fragment in the gel was recovered by using DNA Agarose Gel Purification Kit.

**Construction of** *ltB-ureB* **fusion gene by PCR** Total volume per tube was 90 µL containing all the PCR reagents mentioned above but not the primers, 100 ng of the recovered *ltB* DNA fragment with a cohesive end and 400 ng of the recovered *ureB* 

DNA fragment were added. Parameters for the following PCR were : at 94 °C for 5 min, ×1; at 94 °C for 30 s, at 45 °C for 30 s, at 72 °C for 150 s, ×10; at 72 °C for 10 min, ×1. After this PCR, the two fragments of *ureB* and *ltB* produced a complex fragment of *ureB-ltB* dependent on the cohesive end in the *ltB* fragment, which would be used as a template for the next PCR. The sense primer for *ureB-ltB* amplification was as previously mentioned. The sequence of antisense primer was: 5'-CGACTCGAGGAA AAT GCTAAAGAGTTGTGC-3'(Xho I). The 250 nmol/Leach of the two primers was added into each of the tubes. Parameters for *ltB-ureB* amplification were: at 94 °C for 3 min, ×1; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 180 s, ×10; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 195 s (an addition of 15 s for each of the following cycles), ×15; at 72 °C for 12 min, ×1. Examination of the results of this PCR and recovery of the target fragment were the same as described above. The expected size of target amplification fragment from *ureB-ltB* fusion gene was 2 070 bp. T-A cloning, sequencing and subcloning of *ureB-ltB* fusion gene The *ltB-ureB* amplification DNA fragment was cloned into plasmid vector pUCm-T (pUCm-T-ltB-ureB) by using T-A Cloning Kit according to the manufacturer's instruction. The recombinant plasmid was amplified in *E. coli* DH5 $\alpha$  and then extracted by Sambrook's method<sup>[32]</sup>. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. Two plasmids pUCm-T-ltB-ureB and pET32a in two different strains of E. coli DH5a after amplified in LB medium were extracted and then digested with EcoR V and Xho I, respectively<sup>[32]</sup>. The fragment *ltB-ureB* and *pET32a* were recovered and then ligased. The recombinant expression vector pET32a -ltB-ureB was transformed into E. coli BL21DE3, and the expression system was named as pET32a-ltB-ureB-E.coli BL21DE3. The *ltB-ureB* fragment inserted in *pET32a* was sequenced again.

**Expression of the target recombinant protein** pET32a-ltB-ureB-E.coli BL21DE3 was rotatively cultured in LB medium at 37 °C induced by isopropylthio- $\beta$ -D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol/L. The supernatant and precipitate were separated through centrifugation after the bacterial pellet was ultrasonically broken (300 V, 3×5 s). The molecular mass and output of the target recombinant protein (rLTB-UreB) were measured by SDS-PAGE.

**Identification of immunoreactivity and antigenicity of rLTB-UreB** The expressed rLTB-UreB was collected by Ni-NTA affinity chromatography. The commercial rabbit antiserum against whole cell of *H pylori* or rabbit anti-rUreB serum prepared in our previous study and HRP-labeling sheep anti-rabbit IgG were used as the first and second antibodies, respectively, to determine the immunoreactivity of rLTB-UreB by Western blot. Rabbits were immunized with rUreB to prepare the antiserum and Western blot was applied again to determine the antigenicity of rLTB-UreB.

**GM<sub>1</sub>-ELISA** GM<sub>1</sub>-ELISA was used to demonstrate the adjuvanticity of rLTB-UreB. Briefly, 40-well plates were coated by bovine GM<sub>1</sub> (Sigma) and then added with rLTB-UreB. The rabbit anti-rLTB-UreB serum was used as the first antibody (1:100 dilution) and the commercial HRP-labeling sheep anti-human IgG (1:4 000 dilution) was applied as the second antibody. Each of the first antibody dilutions contained four wells. Negative controls without addition of rLTB-UreB with four repeated wells were set up and their mean  $A_{490}$  value plus 3-fold *SD* values were used as the positive standard for each of the tested wells<sup>[33]</sup>.

**ELISA** By using rLTB-UreB as coated antigen at the concentration of  $20 \mu g/mL$ , each of the UreB antibody positive serum samples from the 125 patients (1:400 dilution) as the first antibody and HRP-labeling sheep anti-human IgG (1:4 000 dilution) as the second antibody, the immunoreaction of rLTB-UreB to the specific antibody in the sera were detected by

ELISA. In this assay, six UreB antibody negative serum samples were used as the control and the positive standard was similar to that in the  $GM_1$ -ELISA.

#### Statistical analysis

The nucleotide sequence of the cloned *ltB-ureB* fusion gene was compared for homologies with the original sequences<sup>[31]</sup> by using a molecular biological analysis software.

#### RESULTS

#### PCR

The target fragments of *ureB*, *ltB* and *ltB-ureB* genes with the expected sizes are shown in Figure 1.



**Figure 1** Target amplification fragments of *ltB* and *ureB* genes and *ltB-ureB* fusion gene. Lane 1: 250 bp DNA marker (BBST); Lanes 2, 4 and 6: Blank controls; Lanes 3 and 5: Target amplification fragments of *ltB* gene and *ltB-ureB* fusion gene, respectively; Lane 7: Target recovered fragment of *ureB* gene from *pUCm-T-ureB* after digestion with both *EcoR* V and *Xho* I.

#### Nucleotide sequence analysis

The homologies of the nucleotide sequences of the cloned *ltB-ureB* fusion gene compared with the original *ltB and ureB* gene sequences were  $100\%^{[31]}$ . The nucleotide and putative amino acid sequences of the *ltB-ureB* fusion gene are shown in Figure 2.

#### Expression of target fusion protein

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol/L could efficiently induce the expression of rLTB-UreB in *pET32a-ltB-ureB-E.coli* BL21DE3 system. The product of rLTB-UreB was mainly presented in the ultrasonic precipitate and the output was approximately 35% of the total bacterial proteins (Figure 3).

#### Immunoreactivity and antigenicity of rLTB-UreB

Commercial rabbit antibody against the whole cell of *H pylori* could combine with rLTB-UreB and induce rabbit to produce specific antibody as confirmed by Western blotting (Figure 4), respectively.

#### GM<sub>1</sub>-ELISA

Since the mean±SD of  $A_{490}$  of the negative control in the four repeated wells was 0.28±0.09, the positive reference value was 0.55. The mean±SD of  $A_{490}$  of the tested wells was 1.29±0.10, indicating that rLTB-UreB had the ability of binding to bovine GM<sub>1</sub>.

#### ELISA

Since the mean±SD of  $A_{490}$  values of the six UreB antibody negative serum samples was  $0.17\pm0.03$ , the positive reference value for the specific antibody detection in patients' sera was 0.26. According to the reference value, 100% (125/125) of the tested patients' sera were positive for the antibodies against rLTB-UreB with an  $A_{490}$  value ranging from 0.37-1.98.

1: ATGAATAAAGTAAAATGTTATGTTTTATGTTATGTGCGCGTTACTATCCTCTCTATGTGCACAC 1: M N K V K C Y V L F T A L L S S L C A Y 61: GGAGCTCCCCAGTCTATTACAGAACTATGTTCGGAATATCGCAACACACAAATATATACG 21: G A P Q S I T E L C S E Y R N T Q I Y T 181: ATTACATTTAAGAGCGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACATATAGAC 61: I T F K S G A T F Q V E V P G S Q H I D 241: TCCCAAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAATCACATATCTGACCGAG 81: S Q K K A I E R M K D T L R I T Y L T E 301: ACCAAAATTGATAAATTATGTGTATGGAATAATAAAACCCCCAATTCAATTGCGGCAATC 101: T K I D K L C V W N N K T P N S I  $\overrightarrow{A}$   $\overrightarrow{A}$   $\overrightarrow{I}$ 421: AAAGTGAGATTGGGCGATACAGACTTGATCGCTGAAGTAGAACATGACTACACCATTAT 141: K V R L G D T D L I A E V E H D Y T I Y 481: GGCGAAGAGCTTAAATTCGGTGGCGGTAAGACTTTGAGGGAAGGCATGAGCCAATCCAAC 161: G E E L K F G R G K T L R E G M S Q S N 541: AACCCTAGCAAAGAAGAACTGGATTTAATCATCACCACTAACGCTTTAATCGTGGATTACACC 181: N P S K E E L D L I I T N A L I V D Y T 661: GGTAACAAAGACATGCAAGATGGCGTTAAAAACAATCTTAGCGTGGGTCCTGCTACTGAA 221: G N K D M Q D G V K N N L S V G P A T E 781: ATCTCCCCCCAACAAATCCCTACAGCTTTTGCAAGCGGTGTAACAACCATGATTGGTGGC 261: I S P Q Q I P T A F A S G V T T M I G G 841: GGAACTGGTCCTGCTGATGGCACTAACGCAACCACTATCACTCCAGGCAGAAGAAACTTA 281: G T G P A D G T N A T T I T P G R R N L 901: AAATGGATGCTCAGAGCGGCTGAAGAATATTCCATGAACTTAGGTTTCTTAGCTAAAGGT 301: K W M L R A A E E Y S M N L G F L A K G 961: AACACTTCTAACGATGCGAGCTTAGCCGATCAAATTGAAGCCGGTGCGATTGGTTTTAAA 321: N T S N D A S L A D Q I E A G A I G F K 1141: GACACTATGGCAGCCATTGCCGGACGCACTATGCACACTTTCCACACTGAAGGCGCTGGT 381: D T M A A I A G R T M H T F H T E G A G 1201: GGCGGACACGCTCCTGATATTATATAAAGTGGCCGGCGAACAACATTCTGCCCGCTTCC 401: G G H A P D I I K V A G E H N I L P A S 1261: ACTAACCCCACTATCCCTTTCACTGTGAATACAGAAGCAGAACACATGGACATGCTTATG 421: T N P T I P F T V N T E A E H M D M L M 1321: GTGTGCCACCACTTGGATAAAAGCATTAAAGAAGATGTTCAGTTCGGTGATTCAAGGATC 441: V C H H L D K S I K E D V Q F A D S R I 1381: CGCCCTCAAACTATTGCGGCTGAAGACACTTTGCATGACATGGGGATTTTCTCCATCACT 461: R P Q T I A A E D T L H D M G I F S I T 1441: AGTTCTGACTCTCAAGCTATGGGTCGTGTGGGTGAAGTTATCACTAGAACTTGGCAAACA 481: S S D S Q A M G R V G E V I T R T W Q T 1501: GCTGACAAAAACAAAAAAGAATTTGGCCGCTTGAAAGAAGAAGAAAAAGGCGATAACGACAAC 501: A D K N K K E F G R L K E E K G D N D N 1561: TTCAGGATCAAACGCTACTTGTCTAAATACACCATTAACCCAGCGATCGCTCATGGGATT 521: F R I K R Y L S K Y T I N P A I A H G I 1621: AGCGAGTATGTAGGTTCTGTAGAAGTGGGCAAAGTGGCTGACTTGGTATTGTGGAGTCCA 541: S E Y V G S V E V G K V A D L V L W S P 1681: GCATTCTTTGGCGTGAAACCCAACATGATCATCAAAGGCGGGTTCATTGCGTTAAGTCAA $561:\ A\ F\ F\ G\ V\ K\ P\ N\ M\ I\ I\ K\ G\ G\ F\ I\ A\ L\ S\ Q$ 1741: ATGGGCGATGCGAACGCTTCTATCCCTACCCCACAACCAGTTTATTACAGAGAAATGTTC 581: M G D A N A S I P T P Q P V Y Y R E M F 1801: GCTCATCATGGTAAAGCCAAATACGATGCAAACATCACTTTTGTGTCTCAAGCGGCTTAT 601: A H H G K A K Y D A N I T F V S Q A 1861: GACAAAGGCATTAAAGAAGAATTAGGGCTTGAAAGACAAGTGTTGCCGGTAAAAAATTGC 621: D K G I K E E L G L E R Q V L P V K N C 1921: AGAAACATCACTAAAAAAGACATGCAATTCAACGACACTACCGCTCACATTGAAGTCAAT 641: R N I T K K D M Q F N D T T A H I E V N 1981: CCTGAAACTTACCATGTGTTCGTGGATGGCAAAGAAGTAACTTCTAAACCAGCCACTAAA 661: P E T Y H V F V D G K E V T S K P A T K 

**Figure 2** Nucleotide and putative amino acid sequences of *ltB-ureB* fusion gene. Note: Underlined areas are sense, linking and antisense primers, respectively. The framed area is the sequence from plasmid *pET32a.* "\*" means stop codon.



**Figure 3** rLTB-UreB expression induced with different dosages of IPTG. Lane 1: Protein marker (Shanghai Shisheng); Lane 2: Blank control; Lane 3: Non-induced with IPTG; Lanes 4-6: Induced with 0.1, 0.5 and 1.0 mmol/L IPTG, respectively; Lanes 7 and 8: Bacterial precipitate and supernatant induced with 0.5 mmol/L IPTG, respectively.



**Figure 4** Western blotting of rLTB-UreB with rabbit antibody against whole cell of *H pylori* and rUreB- and rLTB-UreB-immunized rabbit antisera. Lanes 1, 3 and 5: rLTB-UreB with rabbit antibody against whole cell of *H pylori*, and rUreB- and rLTB-UreB-immunized rabbit antisera, respectively; Lanes 2, 4 and 6: Corresponding blank controls.

#### DISCUSSION

The selection of antigenic targets is critical in the design of *H pylori* vaccine. A large number of published data showed that UreB might be the most definitive antigen candidate for *H pylori* vaccine<sup>[13,19-23]</sup>. On the other hand, LTB is found to be the most efficient mucosal adjuvant with few possibility of inducing allergic reaction<sup>[24-30]</sup>. So UreB and LTB should be the optimal antigen and adjuvant for developing orally taken *H pylori* vaccine, respectively.

In the present study, *ltB-ureB* fusion gene was obtained by using three PCRs and the nucleotide sequence of the gene showed absolutely the same as the corresponding ones. This data indicated that the method used for constructing fusion gene was highly efficient and of high fidelity.

SDS-PAGE performed in this study confirmed that the constructed prokaryotic expression system *pET32a-ltB-ureB-E.coli* BL21DE3 could produce rLTB-UreB with high efficiency even when the concentration of IPTG was as low as 0.1 mmol/L. The inclusion body as a major form of rLTB-UreB and higher output (35% of the total bacterial proteins) of the recombinant protein was beneficial to industrial production.

The results of Western blotting in this study demonstrated that the rLTB-UreB could combine with both the commercial antibody against whole cell of *H pylori* and rabbit anti-rUreB serum. And this recombinant protein was able to efficiently induce rabbit to produce specific antibody. Furthermore, all the UreB antibody positive serum samples from 125 patients confirmed by our previous studies could recognize rLTB-UreB. In the reports, the adjuvanticity of LTB was based on the binding ability to  $GM_1$  receptor on the surface of cell<sup>[27-33]</sup>. In this study, the strong binding to  $GM_1$  receptor of rLTB-UreB was confirmed by  $GM_1$ -ELISA. Therefore, rLTB-UreB with qualified immunoreactivity, antigenicity and adjuvanticity could be used to develop *H pylori* genetically engineered vaccine at lower costs.

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• BASIC RESEARCH •

# Gene expression differences of regenerating rat liver in a short interval successive partial hepatectomy

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#### Abstract

**AIM:** To identify the genes expressed differentially in the regenerating rat liver in a short interval successive partial hepatectomy (SISPH), and to analyze their expression profiles.

**METHODS:** Five hundred and fifty-one elements selected from subtractive cDNA libraries were conformed to a cDNA microarray (cDNA chip). An extensive gene expression analysis following 0-36-72-96-144 h SISPH was performed by microarray.

**RESULTS:** Two hundred and sixteen elements were identified either up- or down-regulated more than 2-fold at one or more time points of SISPH. By cluster analysis and generalization analysis, 8 kinds of ramose gene expression clusters were generated in the SISPH. Of the 216 elements, 111 were up-regulated and 105 down-regulated. Except 99 unreported genes, 117 reported genes were categorized into 22 groups based on their biological functions. Comparison of the gene expression in SISPH with that after partial hepatectomy (PH) disclosed that 56 genes were specially altered in SISPH, and 160 genes were simultaneously upregulated or down-regulated in SISPH and after PH, but in various amount and at different time points.

**CONCLUSION:** Genes expressed consistently are far less than that intermittently; the genes strikingly increased are much less than that increased only 2-5 fold; the expression trends of most genes in SISPH and in PH are similar, but the expression of 56 genes is specifically altered in SISPH. Microarray combined with suppressive subtractive hybridization can in a large scale effectively identify the genes related to liver regeneration.

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#### INTRODUCTION

In the liver regeneration (LR) after partial hepatectomy (PH), a great deal of genes is involved, and varied in the different phases of LR<sup>[1-5]</sup>. Peak of DNA synthesis appears at 24 h and two small peaks occur at 36 h and 48 h after PH<sup>[6]</sup>. Despite numerous related papers, the molecular mechanism of LR has not been thoroughly elucidated<sup>[7-16]</sup>. To explore the hepatic regeneration mechanism, a 0-36-72-96-144 h short interval successive partial hepatectomy (SISPH) model was established in 2001, and has been proved an important tool for studying specific gene expression at various crucial points of LR<sup>[17-19]</sup>. To uncover unknown differential display genes relevant to LR, the method of subtractive suppression hybridization (SSH) was used, and a bulk of up-regulated and down-regulated expressed sequence tags (ESTs) in the regenerating rat liver of 0-36-72-96-144 h SISPH were obtained. With development of cDNA microarray technology, genomewide expression of thousands of genes can be simultaneously analyzed facilitating differential expression monitoring of a large number of activated or suppressed genes under various biological conditions. To further display their expression variation in the LR, an in-house cDNA microarray was successfully performed to identify gene expression profiles in regenerating liver following the SISPH. Relevant information was achieved by data analysis of Microsoft Excel and GeneSpring.

#### MATERIALS AND METHODS

#### Short interval successive partial hepatectomy of rats

Male and female Sprague-Dawley (SD) rats, aged 10-12 wk and weighing 200-220 g, were raised in Experimental Animal Center of Henan Normal University. According to Xu *et al.*, lobule external sinister and lobus centralis sinister, lobus dexter, lobus centralis, and lobus caudatus were removed subsequently at four time points of 0, 36, 72, 96 h of 0-36-72-96-144 h SISPH<sup>[20]</sup>.

#### Sample preparation and RNA extraction

The removed liver lobes were rinsed in cold 1×PBS and immersed in -80 °C refrigerator for RNA and protein extraction. Total RNA was isolated from frozen liver lobes according to the manual of Trizol kit of Invitrogen. In brief, 50-100 mg liver tissue was homogenized in 1 mL Trizol reagent containing phenol and guanidinium isothiocyanate/cationic detergent, followed by phenol-chloroform extraction and isopropyl alcohol precipitation. The quantity and integrity of total RNA were examined by ultraviolet spectrometer and denaturing formaldehyde agarose electrophoresis stained by ethidium bromide (EB).

#### Subtracted cDNA library construction and screening

cDNA subtractive libraries were generated from total RNA by PCR-Select TM cDNA Subtraction kit (Clontech) following the manufacturer's instruction. Briefly, total RNA was reverse transcribed to double cDNA strands and digested with restriction enzymes, followed by subtractive hybridization with drivers and testers. Finally with suppression PCR, differential expression sequence tags were performed to construct subtractive cDNA library, which was cloned into T-vector (Promega) and screened by PCR with nest primer 1 and 2.

#### Sequence analysis

The base sequence assay of ESTs was carried out according to the current protocols in molecular biology. All sequences were determined for both strands. Comparison analysis of the selected sequences was conducted with the DNAman and the National Center for Biotechnology Information (www.ncbi.nlm. nih.gov) GenBank database.

#### cDNA microarray construction

cDNA fragments amplified by polymerase chain reaction (PCR) with nested PCR primer 1 and primer 2, and purified by NaAc/ isopropanol were spotted onto glass slides (Biostar) with the help of ProSys-5 510A spotting machine according to designed project. Then the gene chips were ready by hydrating, blocking and drying. Totally 1 152 elements (double spot chip) including 50 control systems (8 negative control, 12 blank control, 30 internal control) and 551 target genes to be studied comprised 8 submatrixes (12\*12) occupying 9 mm×18 mm (Biostar).

#### Fluorescence-labeled cDNA preparation

RNA isolated from rat livers before SISPH served as a reference for all cDNA microarray analyses. Total denatured RNA was reverse transcribed with Cy3-conjugated dCTP (control group) and Cy5-conjugated dCTP (test group) (Amersham-Pharmacia Biotech) using MMLV reverse transcriptase (Promega) with olig (dT) primer. After bath incubation for 2 h, labeled buffer I and II were subsequently added to the reaction. The control group and test group were mingled together symmetrically and stored in the dark for use.

#### Hybridization and scanning

The glass slices were prehybridized at 42  $^{\circ}$ C for 5-6 h in hybridization buffer containing freshly cooked shared salmon sperm DNA. The labeled denatured probe was hybridized against cDNA microarrays with an overnight (16-18 h) incubation at 42  $^{\circ}$ C. The slides were then washed twice with 2×SSC containing 5 g/L SDS for 5 min at room temperature, once with 0.2×SSC containing 5 g/L SDS at 60  $^{\circ}$ C for 10 min, and finally with 0.2×SSC at 60  $^{\circ}$ C for 10 min. After that, the slices were photographed. Hybridized images were scanned by a fluorescence laser scanning device, Gene Pix 4 000 A (Axon Instruments, Inc., Foster City, CA). At least two hybridizations were performed at each time point. In addition, a semiquantitative inspection of the hybridization results was performed for (1) green signal (down regulation); (2) yellow signal (no obvious regulation); and (3) red signal (up regulation).

#### Data analysis

The cy3 and cy5 signal intensities were quantified by Gene Pix Pro 3.0 software (Axon Instruments, Inc., Foster City, CA). Subsequently, we normalized the obtained numerical data with classical linear regression techniques. In brief, quantified cy3 and cy5 signal intensities were obtained when foreground signal intensities were deducted by background signal intensities and cy5 signal intensities were replaced by 200 when it was <200. When Ri (Ri=cy5/cy3) was between 0.1 and 10, Ri was taken logarithms base natural to generate Ri'[log (Ri)] and ND was taken by EXP (R) (averaged Ri'). The modified cy3\* was generated when ND was multiplied by cy3, and was replaced by 200 when it was <200. The ratio was expressed as cy5/ cy3\*. Therefore, we selected genes whose ratio was more than 2 or less than 0.5 representing a 2-fold difference in expression level. To analyze the selected gene expression data, we applied  $\kappa$ -means cluster analysis, and performed

GeneMaths hierarchical clustering to appraise the number of groups. Whole analyses were executed with Microsoft Excel (Microsoft, Redmond, WA) and GeneSpring (Silicon Genetics, San Carlos, CA).

#### RESULTS

## Category and expression changes of genes related to rat liver regeneration

Among the tested 551 genes, 216 were identified to be altered by more than 2-fold in intensity at least at one time point in the 0-36-72-96-144 h SISPH. Of the 216 identified genes, 111 were up-regulated and 106 were down-regulated. Ninety-nine of these 216 genes were unreported genes and the other 117 were reported, of which quite a few genes had not been reported to be involved in LR. Based on the functions and the time points at which they showed maximum up- or down-regulation, those reported genes were respectively involved in stress response, glycometabolism, fat and stearoyl metabolism, oxidation and reduction response, regulation-proteins, glycoproteins, lipidproteins, nucleolar proteins, receptors, factors, hemoglobins, immunological proteins, chaperonins, cytoskeletons, marker proteins, amino acid enzymes, proteolytic enzymes, proteinase inhibitors, phosphorylases, phosphatases, synthases and transferases (Table 1).

#### Gene expression differences at various time points of the 0-36-72-96-144 h SISPH

The gene expression profiles at different time points were generalized at 36, 36-72, 36-96, 36-144, 72, 72-96, 72-144, 96, 96-144, 144 h, and it was found that at 36 h of SISPH, 17 genes were up-regulated and 2 were down-regulated; at the time points of 36 h and 72 h of SISPH, 3 genes were up-regulated and 3 downregulated; at the time points of 36 h and 96 h of SISPH, only 2 genes were up-regulated; at the time points of 36 h and 144 h of SISPH, 32 genes were up-regulated and 23 genes downregulated, which is the largest group at all time points of SISPH; at 72 h of SISPH, 13 genes were down-regulated and 12 upregulated. At the time points of 72 h and 96 h of SISPH, 5 genes were down-regulated and 4 up-regulated; at the time points of 72 h and 144 h of SISPH, 14 genes were up-regulated and 21 down-regulated; at 96 h of SISPH, one gene were up-regulated and 6 down-regulated; at the time points of 96 h and 144 h of SISPH, 10 genes were down-regulated and 7 up-regulated; at 144 h of SISPH, 11 genes were up-regulated and 31 downregulated. Briefly, the LR of 0-36-72-96-144 h SISPH involved 216 elements, of which, 111 were up-regulated and 105 were down-regulated (Figure 1).

#### *Gene expression level in the regenerating rat liver of 0-36-72-96-144 h SISPH*

According to the up-regulated and down-regulated intensity of genes in the 0-36-72-96-144 h SISPH, we categorized the genes into 3 groups: (1) 105 genes were down-regulated by less than 50%; (2) 93 genes were up-regulated by 2-5 fold; (3) 18 genes were strongly up-regulated by more than 5-fold (Figure 2).

# Hierarchical cluster analysis of genes expressed in the liver regeneration

The expression profile of the 216 genes altered by more than 2fold in intensity at least at one time point in the 0-36-72-96-144 h SISPH was emanative to the last time point, indicating that at 144 h of SISPH, the liver regeneration has not been completed yet (Figure 3A). We undertook hierarchical clustering of 5 time points (0, 36, 72, 96 and 144 h) of SISPH using GeneSpring software and discovered that gene expression profiles had no similarity at the four time points (Figure 3B).



144 h





Figure 1 Gene expression differences in the regenerating rat liver of 0-36-72-96-144 h SISPH.



Figure 2 Expression level of genes in the regenerating rat liver of 0-36-72-96-144 h SISPH.



**Figure 3** Cluster analysis of 216 elements. A: The difference of their intensity was identified more than two-fold at least at one time point. B: A hierarchical clustering of five time points indicated that the genes at these time points hardly had a common expression profile.



Figure 4 Cluster analysis of gene expression profiles identified by cDNA microarray. These genes were classified into 8 clusters by the  $\kappa$ -means method.

No.	Gene description	Fold difference	No.	Gene description	Fold difference
Unrej	ported genes	0.2	*93 94	RP24-176A1 RP24-347B22	2.4
2	CG31759-PA	2.0 3.2	95	RP32-28p17	0.4
4	CH230-1155H3	0.3 2.6	90 97	DNA segment of Chr 1	3.9
5 6	CH230-155H3 CH230-186B23	0.1	98 99	12 d embryo liver cDNA 13 d embryo liver cDNA	2.2 0.5
7 8	CH230-206C20 CH230-329A5	0.5	Stress	s response	19.6
9 10	CH230-372C24 CH230-403C20	0.3	100	Petaxin	2.6
*11	CH230-404C20 CH230-404C20	2.4 4.0	102 103	Angiotensinogen (Agt) Kininogen	5.8 8.0
12	CH230-4L11 CH230-7A22	0.1	104	T-kininogen	16.1
14 *15	Citb585c7 CTD-2328C19	2.5	Glyco 105	Aldolase B	0.4
*16 17	FLJ20356 KIAA1230	2.8	*106 107	C-reactive protein Glycerol 3-phosphate dehydrogenase (Gpd3)	2.6 0.5
18 *19	LOC119392 LOC311304	2.0	108 *109	Isocitrate dehydrogenase 1 (Idh1) Maize aldolase	0.4 0.5
*20	LRRP Aa1-018 LPRP Aa1027	2.8 4.0	*110	3-phosphoglycerate dehydrogenase	17.6
*22	LRRP Aa1-076	2.6 2.4	Fatty : 111	and stearoyl metabolism Malonyl-CoA decarboxylase	0.4
*23 24	LRRP Aa1-114 LRRP Aa2-020	0.5	112 113	NAD(P) dependent steroid hydrogenase P450 cholesterol 7- g -hydroxylase (P450 VII)	0.5 3.0
25 *26	LRRP Aa2-066 LRRP Aa2-111	2.5	114	Prostaglandin D2 synthase 2 (Ptgds2) Retinol dehydrogenase 11	2.0
27 28	LRRP Aa2-174 LRRP Aa2-296	3.1	116	3-alpha-hydroxysteroid dehydrogenase	0.3
29	LRRP Ab1-021 LPRP Ab1-046	4.9 2.9	<b>Oxida</b> 117	ation and reduction response	0.2
31	LRRP Ab1-108	3.0 3.4	118	Alcohol dehydrogenase (ADH) Cytochrom P450 15-beta (Cym2c12)	0.4
32 *33	LRRP Ab1-114 LRRP Ab1-119	2.1	120	Cytochrome b	0.5
34 35	LRRP Ab1-152 LRRP Ab1-216	4.7	121 122	Cytochrome D5 (Cyb5) Cytochrome P450	0.4
36 37	LRRP Ab1-331 LRRP Ab1-334	3.6	123 124	Cytochrome P450 (PNCN inducible, Cyp3A1) Cytochrome P450 2E1	0.4 0.1
38	LRRP Ab2-001 LPRD Ab2-001	0.5	125 *126	Cytochrome P450, 2c39 (Cyp2c39) CytochromeP450, 2b19 (Cyp2b15)	0.4
40	LRRP Ab2-008	2.1 2.0	127	CytP450 arachidonic acid epoxygenase (cyp 2C23)	0.2
*41 42	LRRP Ab2-018 LRRP Ab2-034	3.0 0.4	120	Paraoxonase 1 (Pon1)	0.1
*43 *44	LRRP Ab2-057 LRRP Ab2-079	2.0	*130 *131	Peroxiredoxin 1 (Prdx1) Plasma selenoprotein P1 (Sepp1)	0.4 2.8
*45 46	LRRP Ab2-093 LRRP Ab2-095	2.1 4.6	132 Barryl	Selenium-dependent glutathione peroxidase	0.2
47	LRRP Ab2-132 LPPD Ab2-142	0.4 2.0	133	II-protein with tetratricopeptide repeats 3	2.3
40 49	LRRP Ab2-225	0.3 0.5	*134 135	Glu-Pro dipeptide repeat protein RAKb	0.4 0.5
*50 51	LRRP Ab2-255 LRRP Ab2-379	3.1	Glyco	Proteins	0.1
*52 53	LRRP Ab2-390 LRRP Ab2-402	0.4	130	Fibrinogen, gamma polypeptide (Fgg)	7.2
54 55	LRRP Ac1-060 LRRP Ac1-163	0.5	138 139	Fibronectin 1 (Fn1) Histidine-rich glycoprotein (Hrg)	5.0 0.4
56 57	LRRP Ac1177 LRRP Ac1.233	0.4 3.3	140 141	Myelin-associated glycoprotein (L-MAG) TRAM1	0.3 2.6
58	LRRP Ac1873	7.1 3.8	142	UDP-glucuronosyltransferase 2B3 (Udpgt)	0.3
59 60	LRRP Ac2-061 LRRP Ac2-125	0.2	Lipid- 143	- <b>proteins</b> Apolipoprotein C-I (Apoc1)	0.4
61 62	LRRP Ac2-143 LRRP Ac2-193	3.1	144 145	Apolipoprotein C-II Apolipoprotein C-III	0.3 0.4
63 64	LRRP Ac2-202 LRRP Ac2-223	2.1	146 147	C57BĹ/6J Fatty acid binding protein 1 (Fabp1)	2.2
*65 *66	LRRP Ac2-256	0.5	148	Plasma retinol-binding protein (PRBP)	0.5
*67	LRRP Ac2-202 LRRP Ac2-200 LPDP Be1 647	0.4 3.9	Nucle	colar proteins	0.2
69	LRRP Bm403207	7.9 0.4	150 Recen	RNase A family 4	0.4
70 71	LRRP Cc1-27 LRRP Cc1-8	2.6	151 *152	Cocoa protein Colsi SNAP recentor member 1 (Cosr1)	5.5
72 *73	LRRP Cc1-9 LRRP Da1-10	2.1	153	Nuclear receptor subfamily 0, mem 2 (Nr0b2)	0.4 0.2
74 75	LRRP Da1-24 LRRP Da1-6	2.9	Factor	rype i interieukin i receptor (iiiii)	6.2
*76	LRRP Da2-19 LRPP Da2-19	2.6	155	Angiogenin Angiopoletin-like 3	0.3
*78	LRRP Da2-4	2.3 0.4	157	Early growth response factor 1 (Egr1)	0.5 2.4
*80	MGC 38937	2.6 3.5	158	Insulin-like growth factor I	2.5 0.5
81 82	RIKEN 1110061A24 RIKEN 1300002A08	0.4	160 Hemo	Neuropeptide Y (Npy) pelobins	3.9
*83 *84	RIKEN 1500012D08 RIKEN 2310045J23	0.5	161	Hemoglobin, alpha 1 (Hba1)	0.3
85 *86	RIKEN 2810051A14 RIKEN 4930408O21	0.5	Immu	inological proteises	0.3
87	RP11-281N10 PD22 105K1	0.5 3.9	163 164	Achaete-scute complex homolog-like 1 (Ascl1)	0.3
89	RP23-23501	0.4 0.5	165 *166	Immunoglobulin C kappa	8.6 0.2
91 91	RP23-417P22	0.4 0.5	167	JE/MCP-1	5.5 6.1
92 Chan	RP23-480P21		191 192	Alpha-1-macroglobulin Contrapsin-like protease inhibitor (CPi-26)	3.1
*168 *160	DnaJ (Hsp40), subfamily B, mem 11 (Dnajb11)	2.6	193	Leuserpin-2 (Serpind1)	14.1 0.5
Cytos	kelets	0.5	Phosp	bhorylases	4.6
170 *171	Actin gamma Actin beta (Actb)	2.7	*195	CDK103 CDK110	2.5
172	Clathrin, heavy polypeptide (Hc) (Cltc)	2.7	197	Mss4 protein Pho associated kinace bate (Pock1)	0.5 3.6
*174	Mutant beta-actin (beta-actin)	0.4 3.4	199	Thymidylate kinase (dTMP kinase)	0.5 2.8
*175 Mark	Ribosomal protein S12 (Rps12)	2.2	Phosp 200	ohatases Pyrophosphatase/phosphodiesterase 1(Eppp1)	
176	ATP-binding cassette, sub-family C	0.3	201	Phosphatase 1 (GL-subunit)	4.2 0.2
*178	CD44 antigen (Cd44)	2.2	*203	Secreted phosphoprotein 1 (Spp1)	3.0 2.2
179 180	Pregnancy-zone protein (Pzp) Serum amyloid a-5 protein	4.7 45.1	204 Synth	orr-giucose-i-prosphate	0.4
181	Subchromosomal transferable fragment 4	0.5	*205	ATPase synthase subunit 6	2.3
182	Cytosolic aspartate aminotransferase	5.1	206 *207	Glutamyl-prolyl-tRNA synthetase (Eprs)	3.3 2.6
183 *184	r nenyialanine nydroxylase (Pah) Tissue-type transglutaminase (Tgm2)	0.4 2.8	Trans	ferases	
185 Brot	2-hydroxyphytanoyl-CoA lyase (Hpcl2)	0.4	208 209	Carnitine O-octanoyltransterase (Crot) Glutathione S-transferase 1 (Mgst1)	0.3 0.5
* rote *186	Alpha/beta hydrolase domain containing protein 1	2.9	*210 211	Glutathione S-transferase Y(b) subunit Glutathione S-transferase, alpha 1 (Gsta1)	0.4
187 *188	Catnepsin C (Ctsc) Proteasome ( macropain subunit, beta type 6 Psmb6)	0.4	212	Glutathione S-transferase, type 3 (Yb3) (Gstm3)	0.4
Prote	inase inhibitors		213 214	Sialyltransferase 1 (Siat1)	4.4
190	Alpha-2-macroglobulin (A2m)	0.4 8.1	215 216	Sulfotransferase K2 UDP-glucuronosyltransferase 2, mem 5 (Ugt2b5)	0.2

Table 1 The genes related to liver regeneration altered in 0-36-72-96-144h SISPH (\*genes specially altered in SISPH)

#### Table 2 The comparison of difference of gene expression in SISPH with that after in PH

	Fold difference			Fold difference	
Gene description -	SISPH	РН	Gene description	SISPH	
Unreported genes	515111	111		515111	111
AW558171	0.2	0.3	Cytochrome P450 (PNCN inducible, Cyp3A1)	0.4	0.2
CG31759-PA	2.0	2.9	Cytochrome P450 2E1	0.1	0.1
CH230-155H3	0.3	0.3	Cytochrome P450, 2039 (Cyp2039) CytP450 arachidonic acid enoxygenase (cyp 2023)	0.4	0.1
CH230-155H3 CH230-186B23	2.6	2.2	Flavin-containing monooxygenase 1 (Fmo1)	0.1	0.1
CH230-206C20	0.2	0.2	Paraoxonase 1 (Pon1)	0.1	0.2
CH230-329A5	0.5	0.3	Selenium-dependent glutathione peroxidase	0.2	0.4
CH230-372C24	0.1	0.1	Regulation-proteins		
CH230-403C20 CH220 4I 11	0.3	0.2	RAKb	2.3	0.2
CH230-7A22	4.0 0.1	4.5 0.1	Clyconrotains	0.5	0.2
Citb585c7	0.3	0.2	Alpha-1-B glycoprotein (A1bg)	0.1	0.1
KIAA1230	2.8	2.6	Fibrinogen, gamma polypeptide (Fgg)	0.1	0.1 7.2
LOC119392 L DDD A 21027	2.3	2.1	Fibronectin 1 (Fn1)	5.0	7.2
LRRP Aa2-020	4.0	0.4	Histidine-rich glycoprotein (Hrg)	0.4	0.1
LRRP Aa2-066	0.4	0.4	TRAM1	0.3	7
LRRP Aa2-174	0.1	0.1	UDP-glucuronosyltransferase 2B3 (Udpgt)	2.6	5.1 0.3
LRRP Aa2-296 I DDD Ab1 021	3.1	2.1	Lipid-proteins	0.5	0.5
LRRP Ab1-046	2.9	0.5	Apolipoprotein C-I (Apoc1)	0.4	3 3
LRRP Ab1-108	3.0	2.9	Apolipoprotein C-II	0.3	0.3
LRRP Ab1-114	3.4	4.2	Apolipoprotein C-III	0.4	0.5
LRRP Ab1-152	0.5	0.4	Eatty acid binding protein 1 (Fabp1)	2.2	7.3
LRRP Ab1-331	4.7	2.2	Plasma retinol-binding protein (PRBP)	0.2	0.3
LRRP Ab1-334	3.6	2.7	Transthyretin-related protein (TTN)	0.3	0.4
LRRP Ab2-001	0.5	0.2	Nucleolar proteins	0.2	010
LRRP Ab2-001	0.5	0.2	RNase A family 4	0.4	0.2
I RRP Ab2-008	2.1	2.1	Receptors		
LRRP Ab2-095	4.6	3.1	Cocoa protein	5.5	4.6
LRRP Ab2-132	0.4	0.1	Nuclear receptor subfamily 0, mem 2 (Nr0b2)	0.2	0.2
LRRP Ab2-143	2.0	3.3		6.2	7.8
LRRP AD2-223 I RRP Ab2-379	0.3	0.3	Factors Angiogenin		
LRRP Ab2-402	0.4	0.1	Angiopoietin-like 3	0.3	0.2
LRRP Ac1-060	2.7	0.4,2.3	Early growth response factor 1 (Egr1)	0.5	0.2
LRRP Ac1-163	0.5	0.4	Eukaryotic translation initiation factor 4A1	2.5	3.8
LRRP Ac1-233	0.4	0.4, 5.5	Insulin-like growth factor I	0.5	0.5
LRRP Ac1873	7.1	6		3.9	18.2
LRRP Ac2-061	3.8	7.6	Hemoglobins Hemoglobin, alpha 1 (Hba1)		
LRRP Ac2-125	0.2	0.3	Hemoglobin beta chain (Hbb)	0.3	0.3
LRRP Ac2-143	4.0	3.3 2.3	Immunological proteises	0.3	0.3
LRRP Ac2-202	4.1	2.5	Achaete-scute complex homolog-like 1 (Ascl1)	0.2	0.4
LRRP Ac2-223	2.1	2.4	Complement component 5 (C5)	0.5	0.4
LRRP Ba1-647	3.9	3.2	Immunoglobulin Č kappa	0.2	0.2
I RRP Cc1-97	7.9	5.7 2.2	JE/MCP-1	6.1	4
LRRP Cc1-8	2.6	2.3	Cytoskelets		
LRRP Cc1-9	4.7	2.5	Actin gamma Clathrin, hoavy, polypoptido (Hc) (Cltc)	2.7	4.7
LRRP Da1-24	2.8	3.5	Karvopherin (importin) alpha 2	2.7	3.3
LRRP zbs559	2.9	2.0	Marker proteins	0.4	0.4
RIKEN 1110061A24	3.5	3	ATP-binding cassette, sub-family C	0.2	0.4
RIKEN 1300002A08	0.4	0.3, 2.4	Pregnancy-zone protein (Pzp)	4.7	2.4
RIKEN 2810051A14	2.0	2.7	Serum amyloid a-5 protein	45.1	90.5
RP23-195K1	3.9	2.4	Subchromosomai transferable fragment 4	0.5	0.3
RP23-235O1	0.4	0.2	Amino acid enzymes		
RP23-35D4	0.5	0.4, 2.8	2-hydroxyphytanovl-CoA lyase (Hpcl2)	5.1	5.3
RP23-41/P22 RP93-480P91	0.4	0.1	Proteolytic enzyme	0.4	0.4
RP24-347B22	3.0	2.2	Cathepsin C (Ctsc)	0.4	0.4
RP32-28p17	0.4	0.3	Proteinase inhibitor	0.4	0.4
Adult male liver cDNA	6.4	0.1	Alpha-1 microglobulin/bikunin (Ambp)	0.4	91
13 d embryo liver cDNA	3.9	0.1 5 9	Alpha-2-macroglobulin (A2m)	8.1	21.3
Strass response	0.0	0.0	Alpha-1-macroglobulin	3.1	2
Alpha-1 major acute phase protein prepentide	13.6	6.2	Leuserpin-2 (Serpind1)	14.1	6.6
Petaxin	2.6	2.2	Serine protease inhibitor 1	0.5	0.2
Angiotensinogen (Agt)	5.8	8.4	Phosphorylases	4.0	J
Kininogen T kininogen	8.0	3.4	CDK110	0.5	0.5
Charamatabaliam	10.1	5.5	Mss4 protein	3.6	2.1
Aldolase B	0.4	0.3	Thymidylate kinase (dTMP kinase)	2.8	3
Glycerol 3-phosphate dehydrogenase (Gpd3)	0.5	0.3	Phosphatases		
Isocitrate dehydrogenase 1 (Idh1)	0.4	0.3	Pyrophosphatase/phosphodiesterase 1(Enpp1)	4.2	6.6
Fatty and stearoyl metabolism			Phosphatidylserine-specific phospholipase A1	0.2	0.2
Malonyl-CoA decarboxylase	0.4	0.3	Synthase	3.0	2.8
NAD(P) dependent steroid hydrogenase	0.5	0.4	Carbamyl phosphate synthetase I	0.0	9.0
r 450 cholesterol 7- a -nyaroxylase (P450 VII) Prostaglandin D2 synthase 2 (Ptgds2)	3.0 2.0	0.5, 2.3	Transferases	3.3	2.9
3-alpha-hydroxysteroid dehydrogenase	0.3	0.2	Carnitine O-octanovltransferase (Crot)	0.9	0.2
Oxidation and reduction response			Glutathione S-transferase 1 (Mgst1)	0.5	0.5
Acyl-coA oxidase	0.2	0.4, 2.7	Glutathione S-transferase Y(b) subunit	0.4	0.3
Alcohol dehydrogenase (ADH)	0.4	0.1, 2.4	Gutathione S-transferase, alpha 1 (Gsta1)	0.1	0.1
Cytochrom P450 15-beta (Cyp2c12)	0.1	0.2	Sialyltransferase 1 (Siat1)	0.4	0.3
Cytochrome b5 (Cvb5)	0.3	0.5	Sulfotransferase K2	4.4 0.2	2.6 0.3
Cytochrome P450	0.2	0.2	UDP-glucuronosyltransferase 2, mem 5 (Ugt2b5)	0.4	0.3



**Figure 5** Category of the 216 elements. Based on the results of the cluster analysis, eight distinct temporal patterns were designated. A: Immediate induction, B: Middle induction, C: Late induction, D: Consistent induction, E: Immediate suppression, F: Middle suppression, G: Late suppression, H: Consistent suppression.

To facilitate the visualization and interpretation of the gene expression program presented in this very large body of data, we used the method of  $\kappa$ -means to order genes on the basis of similarities in their expression patterns and displayed the results in a compact graphical format, generating 8 kinds of ramose gene expression clusters (Figure 4). We then categorized the selected elements into 8 distinct temporal induction or suppression patterns immediate induction, middle induction, late induction, consistent induction, immediate suppression, middle suppression, late suppression, consistent suppression (Figure 5).

#### Comparison of gene expression in SISPH with that after PH

Comparison of gene expression profile in SISPH with that after PH revealed that 56 genes were specially induced by SISPH, and the expression of 160 genes was altered simultaneously with the same trend in both SISPH and PH, but the time points of their expression and degree of up-regulation and down-regulation were different (Table 2).

#### DISCUSSION

This study found that 111 genes were up-regulated in the 0-36-72-96-144 h SISPH, suggesting that they could promote the liver growth, development and regeneration. It was also found that a large number of genes were related to positive and negative acute phase reaction to the successive hepatectomy, which suggests that these genes might regulate the balance of cell proliferation and death in the acute-phase response.

In the 25 genes up-regulated to reach the highest level at 36 h

of 0-36-72-96-144h SISPH, 20 genes were decreased immediately to control level after the peak of 36 h, but 5 kept a high level until 144 h of SISPH. Among them, 3-phosphoglycerate dehydrogenase (PGDH) was reported to catalyze the first step in serine biosynthesis and was increased in regenerating liver<sup>[21-23]</sup>. Prostaglandin D2 synthase 2 was confirmed to play an important role in reproduction as a PGD2-producing enzyme and a retinoid transporter<sup>[24,25]</sup>. Phosphoprotein 1 was involved in regulation of hepatocyte proliferation in LR<sup>[26]</sup>. The maximum expression of these genes at 36 h of SISPH showed that they could regulate hepatocyte multiplication after the peak of DNA replication in LR.

In the 27 genes up-regulated to reach the highest level at 72 h of 0-36-72-96-144 h SISPH, 12 of them declined gradually to control level at 96-144 h, and 6 did not decline until 144 h of SISPH, of which eIF4A1 was reported to control melanoma cell proliferation by over expression<sup>[27]</sup>, whose up-regulation was assumed to accelerate protein synthesis at 72 h of SISPH. Actin  $\gamma$  played specific roles in the growth of liver parenchymal cells in the LR of SISPH<sup>[28]</sup>. Cocoa extract could protect against early alcohol-induced liver injury in the rat<sup>[29]</sup>, whose conduction at 72 h was presumed to be involved in relieving hepatocytes from alcohol damage in LR of SISPH. Alpha-2-macroglobulin (A2M) was confirmed to reduce paracrine-and autocrinestimulated extracellular matrix synthesis by scavenging TGFbeta<sup>[30]</sup>. The successive induction of alpha 2-macroglobulin, a multifunctional binding protein with protease and cytokine scavenging properties<sup>[31]</sup>, may restrain protein degradation and termination of TGF- $\beta$  in LR. The increase of HSP40 at 72 h means that lots of newly synthesized proteins need to correctly

fold with help of HSP40 in LR of SISPH.

In the 11 genes up-regulated to reach the highest level at 96 h of 0-36-72-96-144 h SISPH, cytochrome P450 cholesterol 7-alphahydroxylase (CYP7) is confirmed to regulate the protein modeling and the mRNA level in response to multiple physiological activities, including liver cholesterol synthesis, bile acid feedback inhibition, and diurnal rhythm<sup>[32,33]</sup>. The conduction of CYP7 at 96 h is supposed to relate with cholesterol synthesis and hormone regulation in LR of SISPH.

In the 48 genes up-regulated to reach the highest level at 144 h of 0-36-72-96-144 h SISPH, plasma fibronectin was decreased in favor of LR impairment<sup>[34-36]</sup>, its expression at 144 h indicated that fibronectin-mediated function between the cells and the extracellular matrix was active in LR of SISPH.  $\alpha$ -1macroglobulin, serine protease inhibitor 1, angiotensinogen (Agt), fibrinogen  $\gamma$ , pregnancy-zone protein (Pzp) were always up-regulated from 36 h to 144 h of 0-36-72-96-144 h SISPH, suggesting that they are necessary for inhibiting proteolysis and facilitating cell growth and connection at these time points of SISPH.  $\alpha$ -1 major acute phase protein (alpha 1-MAP) is one of the cysteine protease inhibitors<sup>[37]</sup>. Complement component 5 can increase hepatic glycogenolysis by a prostanoid-mediated intercellular communication between Kupffer cells and hepatocytes<sup>[38]</sup>. Fc- $\gamma$  receptor III is responsible for IgGdependent cell cytotoxicity and production of several cytokines and chemokines and involved in macrophage inflammatory protein  $1\alpha$  (MIP-1alpha) and neutrophil influx<sup>[39-41]</sup>. JE/MCP-1 is known as a CC chemokine attracting monocytes, basophils and T-lymphocytes<sup>[42,43]</sup>. Serum amyloid A-5 (SAA-5) is a major acute-phase protein synthesized and secreted mainly by the liver<sup>[44]</sup>, and is increased in response to acute inflammation in LR of SISPH. T-kininogen and kininogen are promoters to IL-6 as LR signal. These genes were always up regulated from 36 h to 144 h of 0-36-72-96-144 h SISPH, suggesting that they are necessary for relinquishing inflammation and promoting growth in whole SISPH.

This study found that 105 genes were suppressed in 0-36-72-96-144 h SISPH and a large number of them were related to energy metabolism, suggesting that they restrain LR by various paths, and that the need for energy in LR of SISPH is not as important as for other demand, which is different after PH.

Eight genes were suddenly down-regulated at 36 h after SISPH, including histidine-rich glycoprotein (HRG), apolipoprotein C-I (Apo C-I), retinol-binding protein (PRB), cytochrome P450 3A1 (Cyp3A1), RNase A family 4, carnitine O-octanoyltransferase (Crot), cytochrome b5 (Cyb5), etc. Histidine-rich glycoprotein (HRG) is confirmed an abundant serum exhibitive protein in diverse biological systems, whose combination with zinc could be used as an antidote for heparin<sup>[45,46]</sup>. Therefore, the downregulation of HRG at 36 h indicated that the increased activity of heparin is essential for LR of SISPH. Apo C-I is known associated with the lipid surface of the plasma chylomicron, VLDL, and HDL subfractions, and reverse transfer from VLDL to HDL and to SBV<sup>[47]</sup>, acting as a major plasma inhibitor of cholesteryl ester transfer protein and phospholipase inhibitor<sup>[48,49]</sup>. From the above evidence, a low level of Apo C-I at 36 h is supposed to facilitate lipoprotein linkage to LDL receptor, LDL receptorrelated protein, and VLDL receptor, as well as fatty acid uptake of hepatocytes in LR of SISPH. Cyp3A1 enzymes belong to the most abundant subfamily of the cytochrome P-450 system that is predominantly found in the liver where they metabolize numerous drugs and endogenous substances such as oestrogens<sup>[50]</sup>. The down-regulation of cyp 3A1 suggested that the harm induced by hepatectomy was presumably distinct from that by drugs and endogenous substances in rat liver.

Twenty-nine genes were suppressed and had a minimum expression at 72 h in after SISPH. Among them, angiopoietin-like protein 3 (Angptl3) is reported to activate lipolysis in adipocytes as a vascular endothelial growth factor by response to the liver X receptor (LXR)<sup>[51]</sup>. The extensive suppression of angiopoietinlike protein 3 mRNA at 72 h suggested that the activity of lipolysis of hepatocytes was very low in LR of SISPH. Acyl-CoA can play many important roles in numerous biochemical reactions, such as tricarboxylic acid cycle, glycoxylate bypass, fatty acid synthesis. The mRNA level of acyl-coA oxidase was first dropped to meet the condition and later increased to eliminate over expressed acyl-CoA in LR of SISPH.

Hpcl 2 was expressed at 96 h in SISPH, and involved in the carbon-carbon bond cleavage as peroxisomal pyrophosphatedependent enzyme during  $\alpha$ -oxidation of 3-methyl-branched fatty acids<sup>[52,53]</sup>. Down-regulation of Hpcl 2 can protect phytanic acid against being broken down, which may store energy during LR of SISPH. Fmo1 can lead to the decrease of cytochrome P- $450^{[54]}$ , which was repressed at 96 h to accommodate electronic environment for hepatocyte multiplication in LR of SISPH.

Retinoic acid is known necessary for the maintenance of many lining epithelia of the body, whereas retinol dehydrogenase can catalyze the first step in retinoic acid biosynthesis<sup>[55]</sup>. Its suppression at 144 h after SISPH demonstrates that retinoic acid is not necessary in late phase of LR. In normal liver the activity of ADH is in excess, while in regenerating rat liver, the rate of ethanol elimination may be limited by the activity of alcohol dehydrogenase in SISPH<sup>[56]</sup>. Cathepsin C (Ctsc) and dipeptidyl aminopeptidase I are regarded to play an important role in protein degradation and the activation of proenzyme in rat liver<sup>[57]</sup>. The down-regulation of cathepsin C may be due to the indispensability of peptide for protein construction in LR of SISPH. Hepatectomy is reported to decrease liver cytochrome P450 levels by inducing heme oxygenase and inhibiting ALA synthase activities<sup>[58]</sup>, which was inhibited at 144 h to regulate the oxidation reaction of hepatocytes in LR of SISPH. Glutathione S-transferase (GST) is a family of conjugative enzymes that catalyze neucleophilic addition of tripeptide glutathione to xenobiotics carcinogens and endogenous lipophilic compounds<sup>[59]</sup>. It was manifested that xenobiotics carcinogens and endogenous lipophilic might produce some uncertain toxic effect on LR of SISPH. Glutathione S-transferase type 3 (Yb3) mRNA was always hampered, implying that over accumulation of Yb3 could lead to contrary reaction. Fatty binding protein is well known to transfer fat from cytoplasm to nuclear or membrane, and fatty acid elongase 1 (rELO1) catalyzes short chain fat transition to long chain fat. The repression of its mRNA in SISPH indicates that long chain fatty acid was not in badly need until 144 h in LR of SISPH. Leuserpin-2 (Sperpind1) was confirmed to participate in complement activation in fibrinolysis and inflammatory response<sup>[60]</sup>, which was continuously repressed in SISPH, suggesting that it can regulate inflammatory response to improve severely injured hepatocytes in LR of SISPH. Myelin-associated glycoprotein (MAG)-binding activity of novel sulfated GM1b, high-affinity ligands for neural singles is important to nervous system regeneration<sup>[61]</sup>. The repression of MAG at 144 h of SISPH may result in mild damage of hepatocytes and nerve system in late phase of LR.

In conclusion, further experiments will be done by using sham surgical rats as control, so as to confirm which genes reported in this paper are related to surgical operation, and which are really related to liver regeneration.

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• BASIC RESEARCH •

### Protective effect of melatonin against liver injury in mice induced by *Bacillus Calmette-Guerin* plus lipopolysaccharide

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#### Abstract

**AIM:** To investigate the effects and mechanisms of melatonin on immunological liver injury in mice.

**METHODS:** A model of liver injury was induced by tail vein injection of *Bacillus Calmette Guerin* (BCG) and lipopolysaccharide (LPS) in mice. Kupffer cells and hepatocytes were isolated and cultured according to a modified two-step collagenase perfusion technique. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and nitric oxide (NO), content of malondiadehyde (MDA), activity of superoxide dismutase (SOD), were measured by biochemical methods. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) activity was determined by RIA. Interleukin (IL)-1 activity was measured by thymocyte proliferation bioassay. Hepatic tissue sections were stained with hematoxylin and eosin and examined under a light microscope.

**RESULTS:** Immunological liver injury induced by BCG+LPS was successfully duplicated. Serum transaminase (ALT, AST) activities were significantly decreased by melatonin (0.25, 1.0, 4.0 mg/kg bm). Meanwhile, MDA content was decreased and SOD in liver homogenates was upregulated. Furthermore, pro-inflammatory mediators (TNF- $\alpha$ , IL-1, NO) in serum and liver homogenates were significantly reduced by melatonin. Histological examination demonstrated that melatonin could attenuate the area and extent of necrosis, reduce the immigration of inflammatory cells. In in vitro experiment, TNF- $\alpha$  was inhibited at the concentrations of 10<sup>-8</sup>-10<sup>-6</sup> mol/L of melatonin, while IL-1 production of Kupffer cells induced by LPS (5  $\mu$ g/mL) was decreased only at the concentration of 10<sup>-6</sup> mol/L of melatonin, but no effect on NO production was observed. Immunological liver injury model in vitro was established by incubating hepatocytes with BCG- and LPS-induced Kupffer cells. Activities of ALT, TNF- $\alpha$ , IL-1, and MDA in supernatant were significantly increased. Melatonin had little effect on the level of ALT, but reduced the content of TNF- $\alpha$  and MDA at concentrations of 10<sup>-7</sup>-10<sup>-5</sup> mol/L and decreased the content of IL-1 at concentrations of 10<sup>-6</sup>-10<sup>-5</sup> mol/L.

**CONCLUSION:** Melatonin could significantly protect liver injury in mice, which was related to free radical scavenging, increased SOD activity and pro-inflammatory mediators.

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Xu SY. Protective effect of melatonin against liver injury in mice induced by *Bacillus Calmette-Guerin* plus lipopolysaccharide. *World J Gastroenterol* 2004; 10(18): 2690-2696 http://www.wignet.com/1007-9327/10/2690.asp

#### INTRODUCTION

Viral infection, alcoholic or drug toxicity, or any other factors that cause damage to hepatocytes elicit an inflammatory reaction in the liver. The damaged hepatocytes, their membrane components, metabolites of toxic agents, and infiltrating inflammatory cells are the activators of Kupffer cells. Kupffer cells, the resident macrophages in the sinusoids of the liver, play significant roles in immunomodulation, phagocytosis, and biochemical attack<sup>[1,2]</sup>. It has been reported that Kupffer cells to be activated are considered the key evidence in the pathophysiology of many animal models of hepatic injury<sup>[3-8]</sup>. Activated Kupffer cells contribute to the onset of liver injury by producing and releasing cytotoxic agents such as pro-inflammatory cytokines including tumor necrosis factor alpha (TNF- $\alpha$ )<sup>[9,10]</sup>, interleukin-1 (IL-1)<sup>[9]</sup>, interleukin-6 (IL-6) and active oxygen<sup>[11]</sup>, nitric oxide (NO)<sup>[8]</sup> which damage sinusoidal endothelial cells and hepatocytes. Previous studies have shown that elimination of Kupffer cells by gadolinium chloride<sup>[5]</sup> and TNF- $\alpha$  antibodies are effective on reducing hepatic damage in vivo.

Ferluga et al. first reported the P. acnes-primed LPS-induced hepatic shock model in mice in 1978<sup>[12]</sup>, and three years later Ferluga also reported that LPS injection subsequent to the priming of Bacillus Calmette-Guerin (BCG) provoked the same hepatic injury in mice<sup>[13]</sup>. Since then, many investigators have tried to explain the mechanism of the above two similar models<sup>[14,15]</sup>. It is known that P. acnes or BCG priming and LPS challenge in mice cause massive liver injury, which consists of priming and eliciting phases. P. acnes or BCG priming induces mononuclear cell infiltration into the liver lobules and granuloma formation<sup>[16]</sup>. The subsequent LPS injection elicits acute and massive hepatic injury, with a concomitant release of various cytokines and active free radicals<sup>[17-21]</sup>. Thus, in immunological liver injury model induced by BCG and LPS, hepatocyte damage is presumably caused by immunological mechanisms. Pro-inflammatory cytokines and active free radicals produced by activated Kupffer cells play an important role in the progress of the resulted liver injury. This experimental immunological liver injury has frequently been used as a model for testing and developing new drugs<sup>[12,13,22-24]</sup>.

The hormone melatonin (N-acetyl-5-methoxytryptamine) is synthesized by pineal gland. Melatonin participates in many important physiological functions, including anti-inflammation<sup>[25]</sup> and immunoregulation<sup>[26,27]</sup>, as well as acting as a broad spectrum antioxidant<sup>[28-30]</sup>. In addition, melatonin protects against liver injury induced by endotoxin shock<sup>[31,32]</sup> and ischemia/reperfusion<sup>[33,34]</sup> in rats through its antioxidant action. The purpose of this study was to investigate whether exogenous melatonin would protect against BCG- and LPS-induced immunological liver injury in mice via antioxidative and immunoregulatory mechanisms. Meanwhile, the direct effect of melatonin on isolated Kupffer cells or co-cultured Kupffer cells and hepatocytes *in vitro* was studied.
#### MATERIALS AND METHODS

#### Materials

Melatonin, purchased from Sigma Chemical Co. (St Louis, Mo, USA), was dissolved in 9 g/L saline with absolute ethanol (</=0.1 mL/L) and stored at -20 °C. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT), RPMI 1640, Hepes buffer, collagenase IV, Pronase E, DNase, Nycodenz and LPS from *Escherichia coli* were obtained from Sigma Chemical Co (St Louis, Mo, USA). Commercial kits used for determining lipid peroxidation and superoxide dismutase (SOD) activity were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China). Other chemicals used in these experiments were of analytical grade from commercial sources.

#### Animals

Male Sprague-Dawley (SD) rats ( $300\pm20$  g), C57BL/6J mice ( $18\pm2$  g) and male Kunming mice ( $20\pm2$  g), obtained from the Animal Department of Anhui Medical University, were maintained on a 12 h light/ dark cycle from 6AM to 6PM in a controlled environment ( $20\pm1$  °C). Animals were housed in plastic cages with free access to food and water. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Institute of Clinical Pharmacology, Anhui Medical University.

#### Establishment of immunological liver injury model<sup>[8,13]</sup>

A 2.5 mg dose of BCG (viable bacilli) suspended in 0.2 mL saline was injected via the tail vein into each animal, and 10 d later, 7.5  $\mu$ g LPS dissolved in 0.2 mL saline was injected. Mice were anesthetized with ether, then sacrificed by cervical dislocation 16 h after LPS injection and trunk blood was collected into heparinized tubes (50 U/mL) and centrifuged (1 500 r/min, 10 min, 4 °C). Serum was aspirated and stored at -70 °C until assayed as described below. The liver was also removed and stored at -70 °C until required.

#### Drug treatment

*In vivo* experiment, the animals were equally divided into 5 groups randomly, including normal, model control and melatonin groups (3 different doses). Mice in melatonin groups were received daily doses of 0.25, 1.0 or 4.0 mg/kg bm of melatonin using an 18-gauge stainless steel animal feeding needle for 10 d prior to LPS injection. Mice in normal and model control group were only fed the same volume of vehicle.

In *in vitro* experiment, after Kupffer cells were isolated from normal rat, the cells were divided into 7 groups randomly, including control cells, cells added with LPS (5  $\mu$ g/mL) alone, cells added with LPS (5  $\mu$ g/mL) simultaneously with melatonin (5 different concentrations including 10<sup>-9</sup>, 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup> mol/L). Every group had triplicate wells and the experiment above was repeated twice.

In another *in vitro* experiment, after Kupffer cells were isolated from BCG priming rat and hepatocytes isolated from normal rat, the two types of cells were divided into 7 groups randomly, including control cells, cells added with LPS ( $5 \mu g/mL$ ) alone, cells added with LPS ( $5 \mu g/mL$ ) simultaneously with melatonin (5 different concentrations including  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$  mol/L). Every group had triplicate wells and the experiment above was repeated twice.

#### Isolation and culture of Kupffer cells and hepatocytes

*In vitro* experiment, the direct effect of melatonin on Kupffer cells was studied by isolating Kupffer cells from the livers of normal rat. Rat liver perfusion was performed using a modification of the two-step collagenase perfusion technique introduced by previous studies<sup>[35,36]</sup>.

In brief, the rat liver was perfused through the portal vein with D-Hank's until blood free, and then with Hank's containing 0.5 g/L collagenase IV. The latter was administered by recirculation until the vessels were digested (up to 20 min). The liver was then scraped using a cell scraper, filtered through a 100-µm filter, and stirred in Hank's containing 2.5 g/L pronase and 0.05 g/L DNase for 20 min at 37 °C. After three times of centrifugation and washing at  $300 \times g$  for 10 min at 4 °C in Gey's balanced salt solution (GBSS), cells were centrifuged in an 180 g/L Nycodenz gradient at 2 500  $\times$  g for 20 min. Kupffer cells were carefully sucked by cusp-straws at the pearl layer inderphase. At last, purified Kupffer cell fractions were obtained by centrifugal elutriation. The viability of Kupffer cells prepared was more than 95% as determined by trypan blue exclusion. The purity of Kupffer cells was greater than 90% based on a peroxidase activity assay<sup>[37]</sup>.

Kupffer cells, obtained as described above, were washed with Hanks' and resuspended in RPMI 1640 medium containing antibiotics (penicillin, 100 U/mL; streptomycin, 100 mg/mL), 2 mmol/L glutamine, and 100 mL/L fetal calf serum. One-milliliter aliquots containing  $1 \times 10^6$  cells were added to 24-well culture plates. The cells were incubated for 60 min in a humidified atmosphere containing 50 mL/L CO<sub>2</sub> at 37 °C. Nonadherent cells were removed, and adherent cells were washed twice with PBS. The cells at a density of  $1 \times 10^6$  /mL were incubated with different concentrations of melatonin ( $10^9$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-5}$  mol/L). They ( $1 \times 10^6$ /well) were cultured for 48 h with 5 µg/mL LPS and the supernatants were collected and TNF- $\alpha$  and NO concentration measured with the methods described below.

In *in vitro* liver injury model, BCG induced Kupffer cells were isolated from the livers of the rats which were injected via the tail vein with 3 mg of BCG 10 d before, at the same time hepatocytes isolated from normal rat. The hepatocytes at a density of  $1 \times 10^9$  /mL were incubated with different concentrations of melatonin ( $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$  mol/L) and BCG-induced Kupffer cells ( $1 \times 10^6$ /well). They were co-cultured for 48 h with 5 µg/mL LPS and the supernatants were collected.

#### Measurement of serum ALT and AST

Serum ALT and AST were determined using commercial kits produced by Jiancheng Institute of Biotechnology (Nanjing, China). Their activities were expressed as an international unit (U/L).

#### Measurement of NO in serum and cell culture supernatants

Nitric oxide (NO) in Kupffer cells was measured by a microplate assay using Griess reagent, which produces a chromophore with the nitrite<sup>[38]</sup> Briefly, 100  $\mu$ L of cell culture supernatants was removed and incubated with 100  $\mu$ L of Griess reagent (10 g/L sulfanilamide and 1 g/L N-1-naphthylethylenediamine dihydrochloride in 25 mL/L phosphoric acid) in a 96-well plate. The plate was incubated for 10 min at room temperature. Nitrite production was quantified spectrophotometrically using an automated colorimetric procedure. Absorbance at 540 nm was measured using a microplate reader (Bio-Tek, USA). The nitrite concentration was calculated by comparing samples with standard solutions of sodium nitrite produced in the culture medium. All samples were assayed in triplicate. Results were expressed as  $\mu$ mol/L.

#### Measurement of MDA and SOD in liver homogenates

Livers were thawed, weighed and homogenized with Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (1 000 r/min, 10 min, 4 °C) and the supernatant was used immediately for the assays of MDA and SOD. MDA and SOD were determined following the instructions of the kit. In brief, MDA in liver tissue was determined by the thiobarbituric

acid method<sup>[39]</sup>. All samples were assayed in triplicate. The content of MDA was expressed as nmol per gram liver tissue. The assay for total SOD was based on its ability to inhibit the oxidation of oxyamine by the xanthine-xanthine oxidase system. The red product (nitrite) produced by the oxidation of oxyamine has an absorbance at 550 nm. One unit (U) of SOD activity was defined as the quantity that reduced the absorbance at 550 nm by 50%. All samples were assayed in triplicate. Results were expressed as U per gram liver tissue.

#### Measurement of TNF- $\alpha$ in serum and cell culture supernatants

Serum was assayed according to procedures described by the instruction of the commercial kits. The standard curve of TNF- $\alpha$  measured was between 2 and 160 pg/mL.

#### Bioassay of IL-1 activity in serum and cell culture supernatants

IL-1 activity was measured by mouse thymocyte activation assayed by MTT (Sigma) reduction<sup>[40]</sup>. MTT was dissolved in sterile PBS to a concentration of 5 mg/mL and stored in the dark at 4 °C for up to 1 wk. Immediately before use, stock MTT was filtered (0.22 µm) to remove any formazan precipitate. Thymocytes  $(2 \times 10^{6} / \text{well})$  from mice were cultured for 48 h in 96-well plates containing RPMI 1640 medium supplemented with 5  $\mu$ g/mL concanavalin A and 0.1 mL collected supernatants in triplicate. Three hours before the termination of culture, cells were pulsed with MTT stock (20  $\mu L/well$ ), returned to 37  $^\circ C$  and incubated for another 3 h. The plates were centrifuged for 10 min at 1000 gto cell pellets and MTT formazan products. The supernatant was carefully aspirated without disturbing the pellets, and formazan was solubilized by addition of isopropanol (100  $\mu$ L/200  $\mu$ L supernatant). Insoluble material was then removed by centrifugation for 10 min at 1 000 g. The solubilized formazan in isopropanol was collected and distributed into 12-well flatbottom ELASA plates at a final volume of 100 µL/well. Plates were read at 570 nm in EL 301 Strip Reader (Bio-Tek, USA) within 1 h of addition of isopropanol. Values were expressed as mean absorbance (A) of triplicate wells.

#### Histological analysis

Formalin-fixed specimens were embedded in paraffin and stained with hematoxylin and eosin for conventional morphological evaluation. After decapitation of rats, small liver specimens were placed in 100 mL/L formalin solution and processed routinely by embedding in paraffin. Tissue sections (4-5  $\mu$ m) were stained with hematoxylin and eosin and examined under light microscope (Olympus). An experienced histologist who was unaware of the treatment conditions made histological assessments.

#### Statistical analyses

All values were presented as mean $\pm$ SE. Statistical analysis of the data for multiple comparisons was performed by one-way analysis of variance (ANOVA) followed by Duncan's test. For a single comparison, the significance of differences between means was determined by the *t*-test, A level of *P*<0.05 was accepted as statistical significant.

#### RESULTS

#### Effect of melatonin on serum ALT and AST

Activities of both serum AST and ALT, indices of hepatic cell damage, were significantly higher in BCG- and LPS-induced group than in the control group. Melatonin (0.25, 1.0, 4.0 mg/kg bm) significantly reduced the activities of serum AST and ALT (Table 1).

#### Effect of melatonin on liver homogenate MDA and total SOD

Liver homogenate malondialdehyde (MDA) content in BCG-

and LPS-induced group was significantly higher than that in the control group while liver total SOD activity was lower. Melatonin (0.25, 1.0, 4.0 mg/kg bm) significantly attenuated MDA generation and increased liver total SOD activity (Table 2).

**Table 1** Effects of melatonin on serum ALT and AST activities in immunological liver injury in mice (n = 10, mean±SE)

Group	Dose (mg/kg bm)	ALT (U/L)	AST (U/L)
Normal		$28.2 \pm 8.8$	$26.8 \pm 8.5$
Model		$224{\pm}40^{\rm d}$	$205{\pm}40^{\rm d}$
Melatonin	0.25	187±21 <sup>a</sup>	$129 \pm 13^{b}$
	1.0	$119 \pm 41^{b}$	$124\pm36^{b}$
	4.0	$163{\pm}44^{\mathrm{b}}$	$162\pm33^{a}$

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 *vs* model group; <sup>d</sup>P<0.01 *vs* normal control group.

**Table 2** Effects of melatonin on MDA level and SOD activity of liver homogenates of immunological injury mice (n = 10, mean±SE)

Group	Dose (mg∕kg bm)	MDA (nmol/g tissue)	SOD (U∕g tissue)
Normal		92±26	297±22
Model		$439{\pm}25^{\rm d}$	$202.46{\pm}26^{\rm d}$
Melatonin	0.25	$335{\pm}37^{\mathrm{b}}$	$266 \pm 71^{b}$
	1.0	$332{\pm}28^{\mathrm{b}}$	$273 \pm 31^{b}$
	4.0	$346{\pm}17^{\mathrm{b}}$	$244{\pm}27^{\mathrm{b}}$

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs model group; <sup>d</sup>P<0.01 vs normal control group.

### Effect of melatonin on serum and liver homogenate TNF- $\alpha$ , IL-1 and NO concentration

As shown in Table 3, when mice were first injected with BCG and then challenged with LPS, the levels of TNF- $\alpha$ , IL-1 and NO were elevated significantly. Melatonin (0.25, 1.0 and 4.0 mg/kg bm) obviously reversed these effects. Similarly, in liver homogenates of immunological injury mice the levels of above three indices increased and melatonin (0.25, 1.0 and 4.0 mg/kg bm) significantly inhibited the production of TNF- $\alpha$  and NO while melatonin (4 mg/kg bm) inhibited IL-1(Table 4).

**Table 3** Effects of melatonin on serum TNF- $\alpha$ , IL-1 and NO levels in immunological liver injury mice (n = 10, mean±SE)

Group	Dose (mg/kg bm)	TNF-α (ng/L)	IL-1 (A <sub>570 nm</sub> )	NO (μmol/L)
Normal		1.61±0.76	0.107±0.001	9.82±1.92
Model		$4.80{\pm}1.61^{\rm d}$	$0.242{\pm}0.03^{\rm d}$	$73 \pm 19^{\rm d}$
Melatonin	0.25	$3.42{\pm}1.08^{a}$	$0.183 {\pm} 0.07^{a}$	$55 \pm 18^{\mathrm{a}}$
	1.0	$3.26{\pm}0.73^{\rm a}$	$0.131 {\pm} 0.04^{\rm b}$	$28{\pm}14^{\rm b}$
	4.0	$2.64{\pm}1.19^{\rm b}$	$0.158{\pm}0.04^{\rm b}$	$56 \pm 19^{a}$

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 *vs* model group; <sup>d</sup>*P*<0.01 *vs* normal control group.

### Effect of melatonin on NO, TNF- $\alpha$ and IL-1 production of isolated Kupffer cells

In vitro, melatonin at the concentrations of  $10^{-8}$ - $10^{-6}$  mol/L was able to inhibit directly the production of TNF- $\alpha$  while only at the concentrations of  $10^{-6}$  mol/L decreased IL-1 production of Kupffer cells co-cultured with LPS (5 µg/mL). However, melatonin had no effect on NO production (Table 5).

**Table 4** Effects of melatonin on TNF- $\alpha$ , IL-1 and NO levels of liver homogenates in immunological injury mice (n = 10, mean±SE)

Group	Dose (mg/kg bm)	TNF-α (ng/L)	IL-1 (A <sub>570 nm</sub> )	NO (µmol/L)
Normal		3.1±8.3	0.15±0.03	9.5±2.7
Model		$26.6{\pm}2.3^{\rm d}$	$0.41{\pm}0.03^{\rm d}$	$45.0{\pm}9.8^{\rm d}$
Melatonin	0.25	$13.9 \pm 2.9^{b}$	$0.38 {\pm} 0.13$	$25.6 \pm 9.5^{\mathrm{b}}$
	1.0	$12.4{\pm}2.9^{\mathrm{b}}$	$0.22{\pm}0.05^{\rm b}$	17±11 <sup>b</sup>
	4.0	$17.9{\pm}4.8^{\rm b}$	$0.35{\pm}0.04^{\rm b}$	$24{\pm}10^{\rm b}$

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01vs model group; <sup>d</sup>P<0.01 vs normal control group.

**Table 5** Effect of melatonin on TNF- $\alpha$ , IL-1 and NO released from cultured normal Kupffer cells of rats (n = 10, mean±SE)

Group	Concentration melatonin (mo	n of TNF-α l/L) (ng/L)	IL-1 (A <sub>570 nm</sub> )	NO (µmol/L)
КС		$4.25 {\pm} 0.79$	$0.137 \pm 0.03$	13.65±0.21
KC+LP	S	$11.21 \pm 1.63^{d}$	$0.233{\pm}0.03^{\rm d}$	$18.85{\pm}0.88^{\rm d}$
KC+LP	S 10 <sup>-9</sup>	$9.96 {\pm} 0.24$	$0.238 {\pm} 0.02$	$19.08 {\pm} 0.40$
+melato	onin			
	10-8	$9.31 \pm 0.53^{a}$	$0.223 {\pm} 0.02$	$19.13{\pm}0.46$
	10-7	$8.35{\pm}0.82^{\rm b}$	$0.201 {\pm} 0.01$	$18.90{\pm}0.77$
	10-6	$8.01{\pm}0.38^{\rm b}$	$0.177 \pm 0.01^{b}$	$18.65 \pm 0.61$
	10-5	$9.74 {\pm} 0.39$	$0.216{\pm}0.02$	$18.70 \pm 0.57$

KC: Kupffer cells; <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 *vs* model group; <sup>d</sup>*P*<0.01 *vs* normal control group.

#### Effect of melatonin on liver injury in mice in vitro

Immunological liver injury model *in vitro* was established by hepatocytes incubated with BCG- and LPS-induced Kuppfer cells. Activities of ALT, TNF- $\alpha$ , IL-1 and MDA in supernatant

were significantly increased. Melatonin had little effect on the level of ALT, but at concentrations of  $10^{-7}$ - $10^{-5}$  mol/L it reduced the content of TNF- $\alpha$  and MDA while at concentrations of  $10^{-6}$ - $10^{-5}$  mol/L it decreased the content of IL-1 (Table 6).

#### Histological results

In normal group, there was no pathological abnormality. Liver parenchyma was in good morphology and hepatocytes were arranged around the central vein. No congestion and inflammation were observed in the sinusoids (Figure 1A).

In model group, there was a severe pathological abnormality. Hepatocytes were with marked vacuolization, moreover, hepatocytes dot necrosis, striped necrosis, bridging necrosis appeared and inflammatory cells arranged around the necrotic tissue. Congestion in liver sinusoids was significant with scattered infiltration of inflammatory cells (Figure 1B).

In melatonin-treated group, the area and extent of necrosis attenuated and the immigration of inflammatory cells reduced. Liver parenchyma was well preserved with radially arranged hepatocytes around the central vein. Regular sinusoidal structures were seen without congestion (Figure 1C).

#### DISCUSSION

Injection of BCG followed by LPS is useful for the creation of experimental models of acute hepatic damage<sup>[13,16,22,41]</sup>. In the present study, immunological liver injury in mice was successfully induced by BCG and LPS and the model *in vitro* also duplicated. On this basis, administration of melatonin *in vivo* resulted in marked reduction of acute liver injury, as demonstrated by significant reduction of serum transaminase concentration and amelioration of severe hepatic pathological abnormalities. Meanwhile, melatonin decreased MDA content and increased total SOD activity in liver homogenates. Furthermore, melatonin significantly reduced TNF- $\alpha$ , IL-1 and NO production in serum and liver homogenates. The *in vitro* experiment also supported

**Table 6** Effects of melatonin on MDA, TNF- $\alpha$  and IL-1 released from co-cultured normal rat hepatocytes and Kupffer cells activated by BCG (n = 5, mean±SE)

Group	Concentration of melatonin (mol/L)	ALT (U/L)	MDA (nmol/L)	TNF-α (ng/L)	IL-1 (A <sub>570 nm</sub> )
KC+HC		37.1±3.7	0.506±0.029	4.50±0.78	0.16±0.03
KC+HC+LPS		$81.4\pm6.4$ d	$0.849 {\pm} 0.062^{\rm d}$	$11.36{\pm}1.6^{\rm d}$	$0.25{\pm}0.03^{\rm d}$
KC+HC+LPS+Melatonin	10 <sup>-9</sup>	$82.4 \pm 4.3$	$0.809 \pm 0.015$	$10.13 \pm 0.23$	$0.26{\pm}0.02$
	10-8	$79.6 \pm 5.4$	$0.789 {\pm} 0.036$	$9.49 {\pm} 0.52$	$0.24{\pm}0.02$
	<b>10</b> <sup>-7</sup>	$74.9 {\pm} 4.2$	$0.706 {\pm} 0.014^{ m b}$	$8.54{\pm}0.81^{\rm b}$	$0.22 {\pm} 0.01$
	10-6	$72.9 {\pm} 6.6$	$0.676 {\pm} 0.043^{ m b}$	$8.21{\pm}0.37^{\mathrm{b}}$	$0.19{\pm}0.01^{a}$
	10-5	$83.8 \pm 3.3$	$0.69{\pm}0.04^{\rm b}$	$8.39{\pm}1.22^{\rm b}$	$0.20{\pm}0.01^{\rm b}$

KC: Kupffer cell; <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs model group; <sup>d</sup>*P*<0.01 vs normal control group.



**Figure 1** Histological results of tissues stained with hematoxylin and eosin under light microscope. A: Normal control group; B: Model group; C: Melatonin-treated group.

the direct protective role of melatonin in suppression of Kupffer cell function. Although melatonin had no direct protection on hepatocytes injury *in vitro* induced by BCG and LPS, it could inhibit the production of MDA and pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1. Based on the current results, we propose that the mode of melatonin's hepatic protective action is, at least in part, related to its antioxidative and immunoregulatory properties.

As it is well known, melatonin, the chief secretory product of the pineal gland, was found to be a multi-faceted free radical scavenger and antioxidant. It detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, singlet oxygen, peroxynitrite anion and nitric oxide<sup>[29,30]</sup>. In both in vitro and in vivo experiments, melatonin has been found to protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes, e.g., the carcinogen safrole, LPS, carbon tetrachloride, ischemia-reperfusion, amyloid-protein, and ionizing radiation<sup>[42,43]</sup>. Melatonin also has been reported to stimulate the activities of enzymes and increase gene expression that improves the total antioxidative defense capacity of the organism, *i.e.*, SOD, glutathione peroxidase, glutathione reductase<sup>[44,45]</sup>. Moreover, recent studies indicate that melatonin is effective on inhibiting oxidative liver damage. Calvo et al. found that melatonin protected against ANIT-induced liver injury with cholestasis in rats, and suggested that this protective effect was likely due to its antioxidative properties and above all to its capacity to inhibit liver neutrophil infiltration, a critical factor in the pathogenesis of ANIT-induced liver injury<sup>[46]</sup>. Melatonin also could dose-dependently reduce liver lipid peroxide content in CCl<sub>4</sub> treated rats. This indicated that melatonin exerted a therapeutic effect on CCl<sub>4</sub>-induced acute liver injury in rats, possibly through its antioxidant action. Melatonin plays a cytoprotective role in the liver insulted by ischemia and reperfusion by virtue of its ability to prevent hepatic malfunction and inhibit the generation of free radicals and accumulation of neutrophils in the damaged hepatic tissue<sup>[35]</sup>. In the present study, the effects of melatonin on immunological liver injury model were firstly investigated. The results showed that melatonin decreased MDA content in liver homogenates, meanwhile, SOD activity rose significantly. Those results are in accordance with the findings of melatonin's antioxidant properties.

A growing body of evidence suggests that nitric oxide (NO) may also modulate different experimental liver injuries. The role, that NO plays in the process of liver injury has been the subject of active debate. In vitro and in vivo data suggest that NO may act to protect tissue by virtue of its ability to react with and decompose superoxide radical<sup>[47-49]</sup>. It has also been suggested that NO may act to modulate the activity of certain transcription factors such as NF-KB<sup>[50]</sup>. Although some studies demonstrate that NO may act to limit or down-regulate liver injury, there are other reports suggesting that NO may actually promote hepatocellular damage possibly due to the formation of strongly oxidizing species peroxynitrite<sup>[51]</sup>. Much of the controversy may be related to the use of non-specific inhibitors of different NOS isoforms and the concentration of NO<sup>[52]</sup>. Melatonin is reported to inhibit peroxynitrite induced oxidative reactions. Additionally, melatonin under or near physiological concentration inhibits the prooxidative enzyme nitric oxide synthase (NOS) activity<sup>[14,49]</sup> and thereby influences NO production. It was also reported that melatonin had protective effect in an endotoxic and non-septic shock partly related to prevention of NO overproduction. The present study showed that melatonin significantly inhibited serum NO and did not directly inhibit Kupffer cells and generate NO in vitro. The results demonstrated that melatonin might modulate the generation of NO at a whole body level but had no direct effect on Kupffer cells. It seems

likely that melatonin regulated NO production to a certain extent, but more studies should be carried out to clarify it.

As it is well known, TNF- $\alpha$  is a multifunctional cytokine mostly secreted by inflammatory cells and is involved in numerous pathological states. TNF- $\alpha$  is considered to be a common early effector molecule for liver injury, in addition to its direct cytotoxic effects, this cytokine is able to induce chemokines, macrophage chemotactic protein-1 and adhesive molecules, vascular-cell adhesion molecule-1, which are key to inflammation and consequent liver damage<sup>[9-11]</sup>. Prevention of liver injury has been observed upon neutralization of TNF- $\alpha$  with anti-TNF- $\alpha$  antibody, prevention of translation of primary RNA transcript of TNF- $\alpha$ by antisense oligonucleotide and interaction of TNF- $\alpha$  with soluble TNF-α receptors<sup>[53]</sup>. Although IL-1 itself does not exert damage on liver, its elevation could stimulate inflammatory cells to excrete many other cytokines including TNF- $\alpha$ , IL-6 and IL-8. Our results suggest that the elevation of inflammatory cytokines including TNF- $\alpha$  and IL-1 in serum, liver homogenates and Kupffer cell culture supernatants contributes to the mechanisms of immunological liver injury.

It is now well recognized that melatonin plays an important immunoregulatory role. Shin et al. found that low levels of Bacillus anthracis were known to induce release of cytokines such as TNF- $\alpha$ , and thereby exposure of melatonin (10<sup>-7</sup>-10<sup>-6</sup> mol/L) to anthrax lethal toxin-treated macrophages also decreased the release of TNF- $\alpha$  to extracellular medium as compared to the control<sup>[54]</sup>. Sacco found that administration of melatonin to mice (5 mg/kg bm, s.c. 30 min before or simultaneously with LPS) inhibited serum TNF- $\alpha$  levels by 50-80% and improved survival of mice treated with a lethal dose of LPS. Melatonin did not increase serum corticosterone and did not modify the elevation of serum corticosterone levels by LPS or by IL-1. Furthermore, it exerted its inhibitory effect in adrenalectomized or hypophysectomized mice also, indicating that its effect is independent of the hypothalamus-pituitary-adrenal axis<sup>[55]</sup>. It was previously reported melatonin had a protective role in LPSinduced septic shock by suppressing pro-inflammatory cytokines, prostaglandins and NO production<sup>[31,32]</sup>. However, other studies indicated melatonin did not alter cytokines including TNF- $\alpha$  and IL-1 secretion by LPS-stimulated macrophages<sup>[56]</sup>. The inconsistencies in the literature suggested that the effect of melatonin on macrophages was complex. In our experimental condition, we found that in in vitro experiments melatonin at the concentrations of 10<sup>-8</sup>-10<sup>-6</sup> mol/L directly inhibited production of TNF- $\alpha$  while only at the concentrations of 10<sup>-6</sup> mol/L decreased IL-1 production of Kupffer cell co-cultured with LPS (5 µg/mL). In in vitro liver injury model, we also found that melatonin at the concentrations of 10-7-10-5 mol/L reduced the content of TNF- $\alpha$  while at concentrations of 10<sup>-6</sup>-10<sup>-5</sup> mol/L decreased the content of IL-1. Even more, in vivo experiment showed that melatonin at the dose between 0.25 mg/kg bm and 4.0 mg/kg bm significantly inhibited serum TNF- $\alpha$  and IL-1 level. Thus suppression of TNF- $\alpha$  and IL-1 could be one of the means by which melatonin attenuated immunological liver injury in mice.

Therefore, the protective effects of melatonin on immunological liver injury might relate to free radical scavenging, increased content of SOD, decreased expression of procytokines.

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BASIC RESEARCH •

### Effects of endothelin-1 on hepatic stellate cell proliferation, collagen synthesis and secretion, intracellular free calcium concentration

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#### Abstract

AIM: To explore the effects of endothelin-1(ET-1) on hepatic stellate cells (HSCs) DNA uptake, DNA synthesis, collagen synthesis and secretion, inward whole-cell calcium concentration ( $[Ca^{2+}]_i$ ) as well as the blocking effect of verapamil on ET-1-stimulated release of inward calcium ( $Ca^{2+}$ ) of HSC *in vitro*.

**METHODS:** Rat hepatic stellate cells (HSCs) were isolated and cultivated. <sup>3</sup>H-TdR and <sup>3</sup>H-proline incorporation used for testing DNA uptake and synthesis, collagen synthesis and secretion of HSCs cultured *in vitro*; Fluorescent calcium indicator Fura-2/AM was used to measure [Ca<sup>2+</sup>]; inward HSCs.

**RESULTS:** ET-1 at the concentration of  $5 \times 10^{-8}$  mol/L, caused significant increase both in HSC DNA synthesis (2 247±344 cpm, P<0.05) and DNA uptake (P<0.05) when compared with the control group. ET-1 could also increase collagen synthesis (P<0.05 vs control group) and collagen secretion (P<0.05 vs control group). Besides, inward HSC [Ca<sup>2+</sup>] i reached a peak concentration (422±98 mol/L, P<0.001) at 2 min and then went down slowly to165±51 mol/L (P<0.01) at 25 min from resting state (39±4 mol/L) after treated with ET-1. Verapamil (5 mol/L) blocked ET-1activated [Ca2+]i inward HSCs compared with control group (P<0.05). Fura-2/AM loaded HSC was suspended in no Ca<sup>2+</sup> buffer containing 1 mol/L EGTA, 5 min later, 10<sup>-8</sup> mol/L of ET-1 was added,  $[Ca^{2+}]_i$  inward HSCs rose from resting state to peak 399 $\pm$ 123 mol/L, then began to come down by the time of 20 min. It could also raise  $[Ca^{2+}]_i$  inward HSCs even without Ca<sup>2+</sup> in extracellular fluid, and had a remarkable dose-effect relationship(P<0.05). Meanwhile, verapamil could restrain the action of ET-1(P < 0.05).

**CONCLUSION:** Actions of ET-1 on collagen metabolism of HSCs may depend on the transportation of inward whole-cell calcium.

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#### INTRODUCTION

Hepatic fibrosis associated with the activation of hepatic stellate cells (HSCs), the major source of extracellular matrix (ECM) proteins<sup>[1]</sup>. It is generally believed that HSCs are the main cells producing ECM, from resting state to active myofibroblasts, which is the key point of formation and development of hepatic fibrosis<sup>[2-6]</sup>. Endothelin-1(ET-1) is currently known as a polypeptide with a stronger activity to contract blood vessel. So, based on prophase researches<sup>[7-9]</sup>, we chose ET-1 to observed its direct effect on DNA ingestion and synthesis as well as collagen synthesis and secretion of HSCs in cultivating. Meanwhile, as we know that Ca<sup>2+</sup> is an important intracellular messenger, relate to HSC proliferation and ECM synthesis<sup>[10-13]</sup>. The effects of ET-1 on regulation and intracellular [Ca<sup>2+</sup>]<sub>i</sub> of HSCs isolated and cultivated *in vitro* were studied.

#### MATERIALS AND METHODS MATERIALS

**Animals** Wistar male rats, weighting (450±50) g, were provided by Shanghai Experimental Animals Center of Chinese Academy of Sciences.

**Reagents** ET-1, calcium fluorescence probes Fura-2/AM, Triton X-100, pronase, trypsin, DMEM, DAB-H<sub>2</sub>O<sub>2</sub> were from Sigma; verapamil from Knoll; collagenase from Medical Industry Academy of Shanghai; RPMI 1640 from Gibco; HEPES from EMK; <sup>3</sup>H-L-proline from Academy of Atomic Energy in China (66.6 GBq/mmoL, radioactivity purity>90%). <sup>3</sup>H-TdR was from Institute of Atomic Energy in Shanghai (814 GBq/mmoL, radioactivity purity >95%).

#### Methods

Isolation and cultivation of rat HSCs Rat HSCs were isolated referring to Knook<sup>[14-17]</sup>. Rats were anaesthetized with pentobarbitone (200 mg/kg) by abdominal injection, then heparin sodium (10 mg/kg) was injected into the caudal vein. The abdominal cavity was opened and portal vein and dorsal vein were exposed. Blood was released through vein and D-Hank's solution was perfused (20-25 mL/min) until pale yellow appeared. Liver was taken out and undergone extracorporeal circulation when perfusion fluid was changed to GBSS containing 0.5 g/L pronase E, 0.5 g/L collagenase and 10 mmoL HEPES. Circle perfusion was performed for 30 min (15 mL/min). Liver was taken out and cut to pieces, then put into GBSS containing 0.25 g/L pronase E, 0.25 g/L collagenase and 10 mmol/L HEPES, shocked at 37 °C for 30 min, little suspended deposit was put in culture media on the top of three-layer density gradient centrifugation fluid containing 80 g/L and 130 g/L metrizamide, 2 800 r/min centrifugation for 20 min, Cells were sucked between top layer and 80 mL/L density layer. DMEM containing 200 mL/L calf serum was used to regulate the number of cells to  $1 \times 10^{5}$ /mL.

**DNA and collagen synthesis of HSCs** HSCs in 2 to 4 th generation were digested by pancreatin and cultured with DMEM supplemented with 100 g/L calf serum and 100 mL/L horse serum. Cells were adjusted to  $1 \times 10^{-8}$ /mL and inoculated on a 48-well plate, cultured for 24 h, then different concentration of ET-1 and the same dosage of drug was added, respectively

and triplicated for each concentration. <sup>3</sup>H-TdR and <sup>3</sup>H-proline were used to assay the incorporation.

**HSC ingestion of DNA** <sup>3</sup>H-TdR 18.5 GBq/mmoL was added at 10, 20, 30 and 60 min respectively, washed 3 times with PBS of  $1 \times 10^5$  mmol/L, centrifuged 1 000 r/min 10 min, the top layer fluid was removed, 2 mL of 100 g/L TCA was added and centrifuged 1000 r/min 10 min again. Top layer fluid was collected and deposited, washed 3 times with 800 mL/L ethanol at 4 °C. The top layer fluid was removed and dried in vacuum. 1 mol/L NaOH was added to lyse the deposit and 1N HCl was used to adjust pH to 7.0 Radioactivity of specimens was measured on Beckman scintillation counter.

**Collagen secretion of HSC** In experiment of <sup>3</sup>H-TdR, before transferred to  $F_{49}$  filter paper, 1 mL culture media was taken out and put into a tube. A 5 mmol/L acetic acid was used to adjust pH to 2 to 3, then 25 µL of 2.5 g/L pepsin was added to digest. A 50 µL of proline was added at 4 °C for 3 h, l, 1.2 mol/L trichloroacetic acid was fixed for 2 h, transferred to  $F_{4a}$  filter paper, closed with saline, 0.6 mol/L trichloroacetic acid was used again, then bleached with anhydrous alcohol, baked at 80 °C. Radioactivity of specimens was measured on YSJ-75 liquid scintillation counter.

 $[Ca^{2+}]_i$  in Fura-2/AM loaded HSC HSCs were cultured on a rectangle glass when HSCs grew and covered the glass. Then cells were taken out of the glass and RPMI 1640 containing Fura-2/AM (10 nmol/L) was added to incubate at 37 °C for 50 min, D-Hank's solution was used to wash extracellular free Fura-2/AM and incubated for another 30 min, 1 g/L trypsin was used to digest the cells and the number of cells was adjusted to  $10^6/mL$  by buffer.

**Fluorescence spectrum** About 2 mL of Fura-2/AM loaded HSCs was suspended for the test with a fluorescence spectrophotometer. Raster (EX) 5 nm, radiate raster (EM) 10 nm were excited at a middle scan speed (32 mm/min), excitation light scan ranged 300-400 nm, emission light scan ranged 440-540 nm.

**Intracellular fluorescence intensity** Fluorescence intensity F was detected first (laser wave-length 340 nm, EX 5 nm, emission wave-length 510 nm, EM 10 nm), then different concentrations of ET-1 and verapamil and EGTA (last concentration 8 mmol/L) were added for the detection of minimum fluorescence intensity (F min).

**Calculation of**  $[Ca^{2+}]_i$  Intracellular  $[Ca^{2+}]_i$  (nmol/L) = kd (F-F min)/(F max-F). Kd is a dissociation constant to Fura-2/Ca<sup>2+</sup> compound which equals to 224 nmol/L.

**Statistical analysis** Variance homogeneity tests were used to make comparisons.

#### RESULTS

#### HSC activity

Trypan blue staining revealed an activity above 90% for HSCs. The purity of HSCs was more than 80% assessed by fluorescence microscope. The nuclei of HSC were stained blue among the desmin-positive satellite cells.

#### Effect of ET-1 on HSC DNA synthesis

As shown in Table 1, ET-1 could accelerate  ${}^{3}$ H-TdR incorporation into HSCs and HSC DNA synthesis and proliferation (*P*<0.05), in a concentration-dependent manner.

#### Effect of ET-1 on HSC ingested <sup>3</sup>H-TdR

ET-1 could accelerate the rate of HSC ingested DNA, the rate increased with the time prolonged (P < 0.05 or P < 0.01, Figure 1).

#### Effect of ET-1 on HSC collagen synthesis

ET-1 could accelerate <sup>3</sup>H-Proline incorporation into HSCs and

collagen synthesis at the concentration of  $5 \times 10^{-8}$  mol/L (*P*<0.05), in a concentration-dependent manner.



Figure 1 Effect of ET-1 on HSC ingeste <sup>3</sup>H-TdR.

**Table 1** Effects of ET-1 on HSC DNA synthesis, collagen synthesis and secretion (cpm, mean±SD)

Group	DNA synthesis	Collagen Synthesis	Collagen secretion
Control	1 370±113	2 167±454	$1 \ 431 \pm 389$
ET 10 <sup>-9</sup> mol/L	$1 \hspace{0.1in} 489 {\pm} 305$	$2\ 206{\pm}725$	$1 \hspace{0.15cm} 528 {\pm} 242 \\$
ET 5×10 <sup>-8</sup> mol/L	1 986±457 <sup>a</sup>	$2\ 698{\pm}304^{a}$	$1 903 \pm 552^{a}$
ET 10 <sup>-8</sup> mol/L	$2 247 \pm 344^{a}$	$2 876 \pm 396^{a}$	$2 \ 087 \pm 128^{a}$
ET 10 <sup>-7</sup> mol/L	$4 \ 015 \pm 102^{a}$	$3  056{\pm}401^{a}$	$2\ 794{\pm}397^{\rm b}$

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 *vs* control group.

#### Effect of ET-1 on HSC collagen secretion

As shown in Tablet 1, ET-1 could remarkably accelerate HSC collagen secretion compared with the control group (P<0.05).

#### Effect of ET-1 (10<sup>8</sup> mol/L) on intracellular $[Ca^{2+}]_i$

As shown in Figure 2, when ET-1 was added to the suspension of Fura-2/AM loaded HSCs and kept for 25 min (n = 3),  $[Ca^{2+}]_i$  in HSCs rose from (39±4) mol/L (resting state) to (165±51) mol/L (P<0.01) and rose to peak (422±98) mol/L (P<0.001) after another 2 min, then it began to go down slowly and remained a higher concentration even after another 18 min compared with the resting  $[Ca^{2+}]_i$  (P<0.01). It suggested that the effect of ET-1 on  $[Ca^{2+}]_i$  in HSCs could be divided into 2 phases, a fast phase (I P) and a slow phase (II P).



**Figure 2** Effect of ET-1 on [Ca<sup>2+</sup>]i peak value in HSCs.

#### Effect of ET-1 on peak concentration of [Ca<sup>2+</sup>], in HSCs

As shown in Figure 3,  $[Ca^{2+}]_i$  in HSCs was in a ET-1 concentration-dependent manner. No change of  $[Ca^{2+}]_i$  occurred in HSCs when ET-1 was less than  $10^{-11}$  mol/L.  $[Ca^{2+}]_i$  reached its peak in a ET-1-dose-dependent manner when ET-1 was greater than  $10^{-9}$  mol/L.



Figure 3 Effect of ET-1 on [Ca<sup>2+</sup>]i in HSCs (10<sup>-10</sup>mol/L).

#### Blocking effect of verapamil

As shown in Figure 4, calcium channel blocking agent verapamil (5  $\mu$ mol/L) could significantly restrain I P and II P effects on  $[Ca^{2+}]_i$  in HSCs excited by ET-1 compared with control group (*P*<0.05). Fura-2/AM loaded HSCs suspended in Ca<sup>2+</sup>-free buffer containing 1 mol/L EGTA made  $[Ca^{2+}]_i$  in HSCs raise from resting state to peak (399±123) mol/L, then go down to (49±17) mol/L at the time of 20 min when first treated with 10<sup>-8</sup> mol/L of ET-1, suggesting that Ca<sup>2+</sup>-free buffer had no remarkable effect on I P of  $[Ca^{2+}]_i$  in HSCs excited by ET-1 but completely blocked II P.



Figure 4 Effect of ET-1 increase [Ca<sup>2+</sup>]i in HSCs.

#### DISCUSSION

HSCs were first detected by Ito and Nemoto in 1952, which provided a new way to study episode mechanism of hepatic fibrosis and deepened the cognition of hepatic fibrosis from an angle of source cells of collagen production<sup>[2-6,15,16]</sup>. HSCs is also named Ito cell, VitA storing cell, liver antrum around cell, fat-storing cell, and is one of the liver interstitial cells. The main function of HSC is to store and metabolize VitA. It has been found to be able to synthesize and secrete ECM and synthesize collagenase<sup>[2-6]</sup>. When hepatic fibrosis occurred, HSC turned into fibroblasts or myofibroblasts that were the cause of liver synthesis of ECM. This change of HSC was called activation or conversion<sup>[2,3]</sup>. It has been certificated that interstitial cells especially HSCs are the main cells which producte collagen when hepatic fibrosis occurs. So it has become a central link in hepatic fibrosis occurrence mechanism.

ET distributes widely in liver and portal vein system, and has important biological effects on liver<sup>[18-24]</sup>. This experiment showed that ET could remarkably accelerate HSC proliferation, DNA synthesis, collagen synthesis and secretion. It is thus clear that ET-1 had double roles during hepatic fibrosis, accelerating not only HSC synthesis of collagen but also selective excretion of collagen. Besides<sup>[25,26]</sup>, endothelial cells in hepatic sinusoid secrete endothelins that can activate HSCs. It has been reported that ET could raise  $[Ca^{2+}]_i$  in smooth muscle cells<sup>[27-29]</sup>. This study showed that ET-1 could raise  $[Ca^{2+}]_i$  in HSCs and appeared double phase reaction, fast phase and slow phase. Both phases had a dose-dependent manner. It turns out that when the cells are at resting state, if there is extracellular  $Ca^{2+}$ , the  $[Ca^{2+}]_i$  in HSCs will be higher than that without extracellular  $Ca^{2+}$ . ET-1 can remarkably raise  $[Ca^{2+}]_i$  in HSC with or without extracellular  $Ca^{2+}$ . It implies that ET-1 can accelerate HSC release of intracellular  $Ca^{2+}$ .

Three different ways have been found to elevate  $[Ca^{2+}]_i^{[30-33]}$ . Plenty of calcium flows into cell through Ca<sup>2+</sup> channel, Ca<sup>2+</sup>-ATP enzyme or Na<sup>+</sup>-Ca<sup>2+</sup> changing system is restrained which can transfer Ca2+ out of cells; Ca2+ storing systems such as mitochondrion and endoplasm increase Ca2+. We used Ca2+free buffer and found it had no effect on  $[Ca^{2+}]_I$  in I P in HSC excited by ET-1 but could block [Ca<sup>2+</sup>]<sub>I</sub> in II P. It implies that elevated  $[Ca^{2+}]_I$  in I P is caused by increased  $Ca^{2+}$  stored in cells, while elevated [Ca2+]I in II P is caused by Ca2+ flowing out of cells. It has been currently accepted by some of scholars that the raise of Ca<sup>2+</sup> in HSC is through the way of phospholipase C (PLC)-inositol triphosphate (IP<sub>3</sub>)- diacylgcerol (DAG)<sup>[34-51]</sup>. ET-1 excites PLC on cell membrane through G protein that makes 4,5-biphosphate inositol divide into IP<sub>3</sub> and DAG-IP<sub>3</sub>. Mitochondrion, endoplasm and sarcoplasm that make Ca<sup>2+</sup> in cell release to cytoplasm and increase free [Ca<sup>2+</sup>]i in cells. IP<sub>3</sub> works only a very short time, and is quickly converted to IP<sub>4</sub> by special enzymes. So peak I P lasts for a very short time, but  $IP_4$  can accelerate the opening of Ca<sup>2+</sup> channel on cell membrane, which makes an increase of calcium flowing out of cells and at last results in a fast raise of  $[Ca^{2+}]i$  in cells.

Physiological and pathological significance of elevated free  $Ca^{2+}$  in HSCs excited by ET is still not clear. Maybe it could participate the series of signals in cells and physiological effect of  $ET^{[25,27]}$ . In conclusion, ET-1 can remarkably accelerate HSC proliferation, collagen synthesis and secretion, increase of  $[Ca^{2+}]_i$  in HSC and of release of  $Ca^{2+}$  in cells, thus accelerating proliferation of fibrous tissues and repair of injury tissues.

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• BASIC RESEARCH •

## Proteomics to display tissue repair opposing injury response to LPS-induced liver injury

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#### Abstract

**AIM:** To examine the protein expression alterations in liver injury/repair network regulation as a response to gut-derived lipopolysaccharide (LPS) treatment, in order to anticipate the possible signal molecules or biomarkers in signaling LPS-related liver injury.

**METHODS:** Male BALB/c mice were treated with intraperitoneal (i.p.) LPS (4 mg/kg) and sacrificed at 0, 6, 24 and 30 h to obtain livers. The livers were stained with hematoxylin and eosin for histopathologic analyses. Total liver protein was separated by two-dimensional gel electrophoresis (2-DE). The peptide mass of liver injury or repair related proteins were drawn up and the protein database was searched to identify the proteins.

**RESULTS:** Observations were as follows: (1) TRAIL-R2 was down regulated in livers of LPS-treated mice. TNFAIP1 was significantly up regulated at 6 h, then down- regulated at 24, 30 h with silent expression during senescent stage. (2) The amount of metaxin 2 and mitochondria import inner membrane translocase subunit TIM8a (TIMM8A) was increased upon treatment with LPS. (3) P34 cdc2 kinase was significantly up-regulated 30 h after LPS administration with silent expression during senescent, 6, 24 h treated stage. (4) The amount of proteasome activator 28 alpha subunit (PA28), magnesium dependent protein phosphatase (MDPP) and lysophospholipase 2 was decreased 6 h after LPS treatment but recovered or up-regulated 24 and 30 h after LPS treatment.

**CONCLUSION:** LPS-treated mouse liver displaying a timedependent liver injury can result in expression change of some liver injury or repair related proteins.

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#### INTRODUCTION

Human intestinal tract accumulates Gram-negative bacteria that supply lipopolysaccharide (LPS). Gut-derived LPS reaches the first target organ liver through portal venous flow and results in hepatic clearance or tissue damage. Recent studies have shown that peritoneal macrophages in cirrhotic patients may secret angiogenic products when stimulated by LPS and accumulate in liver injury<sup>[1-3]</sup>. In vivo experiments also indicate LPS may cause liver injury in normal subjects and exaggerate liver injury in alcoholic liver disease<sup>[4-7]</sup>. That is, chronic liver diseases suffering from subsequent LPS attack would result in much severer clinical findings. The precise mechanisms underlying LPS-treated liver injury remain unclear. Several studies of liver protein expression exposed to some hepatotoxic agents have revealed certain changes which illustrated the pathogenesis of liver injury in some extent<sup>[2,4,8,9]</sup>. However, most reported researches focused only on some partial signaling or effecter molecules on a limited view<sup>[4,5,7]</sup>. Protein expression changes of liver exposed to LPS remains to be established.

The goal of the present study was to dynamically examine protein expression alterations in liver tissues from mice exposed to LPS administration, in order to anticipate of the possible signal molecules or biomarkers in signaling LPS-related liver injury.

#### MATERIALS AND METHODS

#### Reagents

Ultra pure reagents for polyacrylamide gel preparation were obtained from Bio-Rad. Eighteen cm immobilized pH gradient (IPG) strips (pH3-10 L), IPG buffer and dry strip cover fluid were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonate (CHAPS), glycine, ammonium persulphate (APS), TEMED, trifluoroacetic acid were obtained from Amresco (Solon, OH, USA). Dithiothreitol (DTT), PMSF, Iodoacetamide, urea, thiourea,  $\alpha$ -cyano-4-hydroxycinnaic acid (CCA), TPCK-Trypsin, *E.Coli* LPS 0111:B4 were purchased from Sigma Chemical (St Louis, MO, USA). Acetonitrile was HPLC grade. Ten  $\mu$ L ZipTip<sup>TM</sup>C18 tip were purchased from Millipore Company (USA).

#### Animal treatment protocol

Male BALB/c mice (supplied by Laboratory Research Animal Center, 2<sup>nd</sup> Xiangya Hospital, Central South University), 6 wk of age and weighing 19-21 g, were used. The animals were housed individually in mouse gang cages in cleaning-grade controlled room. The Hunan Association accredited the house facility for Accreditation of Laboratory Animal Care, and all animal handling procedures were in conform to the Guide for the Care and Use of Laboratory Animals. Four groups of five mice each received intra-peritoneal (i.p) RPMI-1640 medium treatment (control) and LPS (diluted in RPMI-1640 medium) 4 mg/kg for 6, 24, 30 h, respectively. Mice were killed by decapitation, liver samples were removed and flash-frozen in liquid nitrogen and kept at -70 °C until proteomics analysis. For histopathologic research, liver slices from the right lateral lobes were immersion-fixed in 40 g/L neutral buffered formaldehyde,

routinely processed to  $5-\mu m$  thick paraffin sections, stained with hematoxylin and eosin (HE) and examined by light microscopy.

#### Sample preparation<sup>[10]</sup>

Liver samples were homogenized in eight volumes of 8 mol/L urea, 2 mol/L thiourea, 40 g/L CHAPS, 20 g/L Triton X-100, 5 g/L DTT, 1 mmol/L PMSF, 1 mmol/L EDTA, 40 mmol/L Tris. Homogenates were centrifuged at 42 000 g at 20 °C for 1 h (LE-80 K ultracentrifuge, Type 90 rotor, Beckman). Supernatant was removed, divided into several aliquots and stored at -70 °C until to be used. Protein concentration of each sample was measured by Bradford method<sup>[11]</sup>.

#### Two-dimensional gel electrophoresis<sup>[11,12]</sup>

The first-dimensional isoelectrical focus (IEF) was performed on precast 18 cm IPG strips at 20 °C with a maximum current setting of 50 µA/strip using an Amersham Pharmacia IPGphor IEF unit. The strips were rehydrated at 30 V for 16 h in 350  $\mu$ L samples containing 8 mol/L urea, 20 g/L CHAPS, 20 mmol/L DTT, 5 g/L IPG buffer. Eight hundred µg proteins was applied to each IPG strip. After rehydration, IEF run was carried out using the following conditions: 500 V, 500 Vh; 1 000 V, 1 000 Vh; 8 000 V, 40 000 Vh. Then, the strips were subjected to a two-step equilibration. The first was with an equilibration buffer consisting of 6 mol/L urea, 300 g/L glycerol, 20 g/L SDS, 50 mmol/L Tris (pH8.8) and 10 g/L DTT. The second equilibration buffer was the same, only with DTT substituted by 25 g/L iodoacetamide. After the strips were transferred onto the second-dimensional 0.75 mm thick 125 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, the strips were sealed in place with 7.5 g/L agarose. Electrophoresis was carried out at a constant current of 25 µA/gel at 12 °C until bromophenol blue reached the edge of gels.

#### Silver staining

Gels were fixed in 400 mL/L ethanol, 100 mL/L acetic acid in water for 30 min, and then sensitized with 2 g/L sodium thiosulfate for 30 min. After the gels were rinsed twice with water for 5 min each, they were incubated in 2.5 g/L silver nitrate for 20 min. After the silver nitrate was discarded and rinsed with three changes of distilled water for 5 min each, the gels were visualized in 25 g/L sodium carbonate in 0.004% formalin until the desired intensity was attained. Then the gels were incubated with 14.6 g/L EDTA disodium dihydrate for 10 min to stop the development. The staining procedure was performed by three rinses with water for 5 min each.

#### Quantitative gel pattern analysis

Stained 2-D gels were scanned on a UMAX powerLook III scanner (Amersham Biosciences). Image analysis and Group Wise statistical comparisons were performed using the Image Master 2D v3.01 software (Amersham Pharmacia). The protein spot intensity was arbitrarily calculated by integrating the optical density over the spot area to search for treatment-related protein change. The amount of protein was marked by volumes ( $A \times area$ ) and expressed as mean±SD. Statistical difference was analyzed by analysis of Variance (ANOVA). The cutoff of quantitative protein changes between control and treated groups was made at P < 0.05.

**Protein digestion and protein identification by MALDI-TOF-MS**<sup>10,12]</sup> **Destaining** Protein spots of interest were excised from the 2-D gels and washed 3 times in distilled water, destained with 15 mmol/L potassium ferricyanide in 50 mmol/L sodium thiosulfate, dehydrated in acetonitrile and finally dried in a centrifugal vaporizer.

Reduction and alkylation Gel pieces were incubated in 100 mmol/L

 $NH_4HCO_3$  by adding 10 mmol/L DTT at 57 °C for 1 h. After they were cooled, 100 mmol/L  $NH_4HCO_3$  with 55 mmol/L iodoacetamide was added and incubated at room temperature in dark for 30 min. Supernatant was removed and samples were washed twice in 100 mmol/L  $NH_4HCO_3$  and dehydrated in 1 000 g/L acetonitrile, finally dried in a centrifugal vaporizer.

**In-gel digestion** Gel pieces were rehydrated in a digestion buffer containing 0.1 mg/mL TPK-trypsin for 30 min. After excess buffer was discarded, the gel pieces were covered with digestion buffer and proteins were in-gel digested for 24 h at 37 °C. **Deionization** Digested peptides were pipetted out and into to make them attached in a 10  $\mu$ L ZipTip<sup>TM</sup>C18 tip, washed twice with 1mL/L trifluoroacetic acid.

MALDI-TOF-MS for protein identification The matrix solution was saturated in 500 g/L acetonitrile, 1 g/L TFA in water. Peptides were eluted with 5 µL MALDI matrix, 2.0 µL mixed solution was loaded to the sample plate target and allowed to air-evaporate. MALDI experiments were performed on a Perspective Biosystem Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystems Voyager system 4307, USA) equipped with delayed ion extraction. Data were acquired in the delayed ion extraction mode using a 20 KV accelerating voltage and a 100 ns extraction delay time. Dual microchannel plate detection was utilized in the reflector mode with the ion signal recorded using a transient digitizer. The performance of the mass spectrometer produced sufficient mass resolution to produce isotopic multiplet for each ion species below a mass-to-charge (m/z) of 3 000. All MALDI mass spectra were internally calibrated using masses from two trypsin autolysis products (monoisotopic masses 2163.05 and 2273.15). The software package Protein Prospector (prospector. ucsf.edu) was used to identify protein spots. The mouse nonredundant (NR) database Genpept.5.1.2002 was used in searches. The search parameters used were as follows: Cysteines modification mode as carbamidomethylation, maximum allowed peptide mass error 50 ppm, more than four peptide mass hits required for protein match, up to one enzymatic missed cleavage. A restriction was placed on species of origin, pI (experimental pI+/- 1.00 pH unit) and protein mass (experimental molecular mass +/- 50%).

#### RESULTS

#### General health and histopathology

All subjects were inspected for general health before, during and after LPS exposure. Throughout the study, no animal experienced mortality that was determined to be related to the exposure. Nine of 10 animal that were treated by exposure to LPS for 24, 30 h experienced visible signs of laziness and retardation. Light microscopic examination of HE sections from LPS-treated mice revealed that a large number of inflammatory cells infiltrated portal areas with additional focal hepatocellular necrosis 24, 30 h after i.p. LPS (4 mg/kg)<sup>[13]</sup>.

<b>Table 1</b> Relative amount of 8 injury/repair related liver <b>p</b>	oro-
teins in response to LPS treatment (mean±SD)	

		LPS				
Liver protei	in	6 h	24 h	30 h		
1	9 800±32	1 130±41	absent	absent		
2	$9 952 \pm 21$	$11 \ 020 \pm 26$	34 060±19	$29 800 \pm 24$		
5	absent	38 040±18	$19~500{\pm}42$	$20\ 540{\pm}34$		
6	$12 \ 420 \pm 24$	absent	$15 500 \pm 32$	$17\ 200{\pm}30$		
9	absent	absent	$1\ 200{\pm}35$	$41 \hspace{0.15cm} 500{\pm}29$		
13	absent	$4 \hspace{0.15cm} 500{\pm}42$	$8 \hspace{0.1in} 900{\pm}44$	$10\ 500{\pm}32$		
447	43 200±21	$3\ 200{\pm}35$	absent	$54\ 400{\pm}32$		
524	absent	absent	absent	$9~500{\pm}23$		

Protein NO.	MOWSE score	Masses matched (	Coverage %) (%)	Protein $M_{\rm r}$	Protein PI	Accession NO.	Protein name
1	36.2	4	27	42 677	6.5	Q9QZM4	TRAIL receptor 2
2	56.3	5	45	35 646	6.0	P47802	Metaxin 2
5	29.5	4	32	36 134	8.0	O70479	TNF- $\alpha$ induced protein 1
6	47.9	4	26	28 673	5.7	P97371	Proteasome activator 28-alpha subunit (PA28)
9	76.1	4	20	34 191	7.8	P11440	P34 cdc2 kinase
13	25.3	4	26	11 043	5.1	Q9WVA2	Mitochondria import inner membrane translocase subunit TIM8 A (TIMM8A)
447	23.2	4	22	42 795	5.0	452526	Magnesium dependent protein phosphatase (MDPP)
524	37.3	4	25	24 794	6.7	P47713	Lysophospholipase 2

Table 2 Results from search of Genpept.5.1.2002 database for Musculus protein sequences with tryptic peptide masses



**Figure 1** Partial 2-D images of mouse liver proteins from  $(a_1, a_2)$  a control animal and animals treated with 4 mg/kg i.p LPS  $(b_1, b_2)$  6 h,  $(c_1, c_2)$  24 h,  $(d_1, d_2)$  30 h. The spots representing proteins whose levels changed following LPS treatment are shown. a1, b1, c1, d1 represent the relative same partial map after different LPS-treated onset. a2, b2, c2, d2 represent another relative same partial map.



**Figure 2** Peptide mass fingerprint of MDPP (spot447). The spot was in-gel digested with trypsin. After desalted, the peptide mixture was analyzed by MALDI-TOF-MS. All MALDI mass spectra were internally calibrated using masses from two trypsin autolysis products (monoisotopic masses 2 163.05 and 2 273.15).

#### 2-DE and relative abundance change

The resolution in 2-DE silver-stained gels resulted in approximately 1000 spots. The volumes ( $A \times area$ ) and coordinates of each spot (pI and MW) were determined to select the fine right proteins

among candidates. There were significant alterations in 40 protein spots following LPS treatment when compared with each other, P < 0.01. These 40 proteins were identified by MALDI-TOF-MS (data not shown). Figure 1 (A-D) shows the location of 8 injury/repair related proteins in illustrated partial 2-DE maps of control and LPS-treated mice, respectively. Table 1 summarizes the relative amount of these considered proteins. The amount of protein 1 was 9 800 in the control and downregulated to 1 130 in 6-h group, absent in 24-, 30-h group. Protein 5 was significantly up-regulated 6 h after LPS treatment, then down-regulated 24 h and 30 h after LPS treatment with silent expression in the control. Proteins 2 and 13 were increased upon treatment with LPS. Protein 9 was significantly up-regulated 30 h after LPS treatment with silent expression in control, 6, 24 h treated stage. Proteins 6, 447 and 524 were decreased in amount 6 h after LPS treatment, recovered and up-regulated 24 and 30 h after LPS treatment.

#### Identity of injury/repair related proteins

The proteins were cut from 2-DE gels and subjected to tryptic digestion. The 8 proteins analyzed by peptide mass fingerprinting yielded searchable masses and resulted in the identification (Table 2). Figure 2 indicates the peptide mass fingerprint of MDPP (spot447) as a representative.

#### DISCUSSION

It has been proved that gut-derived LPS provokes liver inflammation

with focal hepatocyte injury and increases the sensitivity of hepatotoxicity to carbon tetrachloride, concanavalin A, D-galactosamine and ethanol induced liver damage<sup>[4-6,14]</sup>. The role of LPS signaling pathway in the initiation and early propagation of immune response has been deeply demonstrated<sup>[8,9,15]</sup>. In this study, liver injury occurred apparently 24 h after LPS administration. It indicated that LPS could promote hepatotoxicity by indirect mechanisms. Some immediate signaling or effecter molecules may play a pivotal role in this delayed injurious response.

In our research, TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), TNF- $\alpha$  inducible endothelial protein 1 (TNFAIP1) and mitochondria apoptosis signaling related proteins metaxin 2, mitochondria import inner membrane translocase subunit TIM8a (TIMM8A) were demonstrated to be involved in the initiation of liver injury of LPS-treated mice and consistent with the histological change of liver tissue.

A recent research showed that LPS administration could result in elevated circulating TNF- $\alpha$  and up-regulation of gene transcription for TNF- $\alpha$  as early as 2 h after administration<sup>[16]</sup>. Then, TNF- $\alpha$  related apoptosis pathway played a control role in mediating the tissue injury. TRAIL was recently reported to promote apoptosis of carcinogenic cells by binding to the transmembrane receptors. The resulting death-inducing signaling complex consisting of TRAIL, TRAIL-R2 and caspase-8, initiated the subsequent cascade of Caspases mediating apoptosis<sup>[17,18]</sup>. The down-regulation of TRAIL-R2 in livers of LPS-treated mice could result in rather a protective mode of normal hepatocytes. The induction of TNFAIP1 has been shown to be apoptosis-specific, suggesting that TNF- $\alpha$  related apoptosis is triggered in liver cells of LPS-treated mice at earlier stage<sup>[19]</sup>.

Metaxin 2 is bound to the cytosolic face of mitochondria outer membrane by its interaction with membrane-bound metaxin 1, and this complex may play a role in protein import into mammalian mitochondria. The requirement of metaxin in TNFinduced cell death has been confirmed by *in vitro*<sup>[20,21]</sup>. It has been found that TIMM8A exists in the mitochondria intermembrane space which mediates the import and insertion of inner membrane proteins<sup>[22-24]</sup>. These two mitochondria proteins are required for normal physiological balance. Treatment-mediated changes of these proteins were likely to associated with mitochondria apoptosis. How mitochondria related pro- or antiapoptotic proteins are involved in liver injury needs to be further demonstrated.

Tissue repair associated proteins, p34 cdc2 kinase and proteasome related proteins, showed some controversial expression in response to LPS-treated liver injury. p34 cdc2 kinase belongs to the Ser/Thr family of protein kinases. The sequential activation and inactivation of p34 cdc2 kinase of eukaryotic cells are required for it to entry into mitosis and to exit from it. In addition, a sustained activation of it during mitotic arrest has been associated with anti-microtubule agentsinduced apoptosis<sup>[25,26]</sup>. In this study, we found significant histopathological changes in mice livers 24 h after LPS treatment. p34 cdc2 kinase of mice livers was significantly up-regulated r 30 h after LPS administration with silent expression during senescent, 6-, 24-h treated stage. It indicated that the enhanced expression of p34 cdc2 kinase was subsequently present to severe hepatic damage. It is likely that up-regulation of p34 cdc2 kinase in LPS-treated mice was more reflective of mitogenic response rather than antimitogenic activities. That is, cell division and tissue repair occurred as events opposing to tissue injury and could limit the extent of liver injury. It seemed possible that p34 cdc2 kinase could act by promoting mitosis and tissue repair, thus contributing to tissue repair response to LPS-treated liver injury.

The biological implications of amount in abundance of proteosome related proteins in mice liver response, including proteasome activator 28 alpha subunit (PA28), magnesium dependent protein phosphatase (MDPP) and lysophospholipase 2, which decreased in amount 6 h after LPS treatment but recovered or up-regulated 24 and 30 h after LPS treatment, were even less clear. Previous study demonstrated that PA28 could bind to and activate the proteasome by the alpha subunit<sup>[27]</sup>. MDPP is a new member of the halo acid dehalogenase family which has been found to be competent to catalyzing dephosphorylation of tyrosine-phosphorylated proteins<sup>[28]</sup>. Lysophospholipase 2 was implicated in the initiation of inflammatory response by mediating cell division and differentiation<sup>[1,29]</sup>.

It is concluded that proteosome related proteins play a major role in the initiation of tissue repair program opposing to tissue damage. While the function, pathway interactions and ultimate biological outcomes of these changes in protein expression remains to be explained by further research.

In summary, the current investigation used 2-DE to characterize the proteome of LPS-treated mice liver that displayed a time-dependent liver injury in some liver injury or repair related proteins. In this study, 8 liver injury or repair related proteins were identified whose expression levels were altered at different time points after LPS treatment. TNF related apoptosis induced by LPS treatment could provoke an initiation response in tissue repair.

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• BASIC RESEARCH •

# Effects of platelet-derived growth factor and interleukin-10 on Fas/Fas-ligand and Bcl-2/Bax mRNA expression in rat hepatic stellate cells *in vitro*

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#### Abstract

**AIM:** To investigate the effects of platelet-derived growth factor(PDGF) and interleukin-10 (IL-10) on Fas/Fas-ligand and Bcl-2/Bax mRNA expressions in rat hepatic stellate cells.

**METHODS:** Rat hepatic stellate cells (HSCs) were isolated and purified from rat liver by *in situ* digestion of collagenase and pronase and single-step density Nycodenz gradient. After activated by culture *in vitro*, HSCs were divided into 4 groups and treated with nothing (group N), PDGF (group P), IL-10 (group I) and PDGF in combination with IL-10 (group C), respectively. Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was employed to compare the mRNA expression levels of Fas/FasL and Bcl-2/Bax in HSCs of each group.

**RESULTS:** The expression levels of Fas between the 4 groups had no significant differences (P>0.05). FasL mRNA level in normal culture-activated HSCs (group N) was very low. It increased obviously after HSCs were treated with IL-10 (group I) (0.091±0.007 vs 0.385±0.051, P<0.01), but remained the low level after treated with PDGF alone (group P) or PDGF in combination with IL-10 (group C). Contrast to the control group, after treated with PDGF and IL-10, either alone or in combination, Bcl-2 mRNA expression was downregulated and Bax mRNA expression was up-regulated, both following the turn from group P, group I to group C. Expression of Bcl-2 mRNA in group C was significantly lower than that in group P (0.126±0.008 vs 0.210±0.024, P<0.01). But no significant difference was found between group C and group I, as well as between group I and group P (P>0.05). Similarly, the expression of Bax in group C was higher than that in group P (0.513±0.016 vs 0.400±0.022, P<0.01). No significant difference was found between group I and group P (P>0.05). But compared with group C, Bax expressions in group I tended to decrease (0.449±0.028 vs 0.513±0.016, *P*<0.05).

**CONCLUSION:** PDGF may promote proliferation of HSCs but is neutral with respect to HSC apoptosis. IL-10 may promote the apoptosis of HSCs by up-regulating the expressions of FasL and Bax and down-regulating the expression of Bcl-2, which may be involved in its antifibrosis mechanism.

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#### INTRODUCTION

Liver fibrosis is a progressive pathological process involving multi-cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular space. It is generally accepted that hepatic stellate cells (HSCs) are central to the process of fibrosis as the major source of extracellular matrix (ECM) components<sup>[1-10]</sup>. Following acute or chronic liver tissue injury, HSCs undergo a process of activation towards a phenotype characterized by increasing proliferation, motility, contractility and synthesis of ECM components. Cytokines play an important role in the formation, development and reversibility of fibrosis<sup>[9-14]</sup>. Activated HSCs secrete many important cytokines through autocrine and paracrine, of which platelet-derived growth factor (PDGF) can activate secretory cells and those quiescent HSCs around<sup>[15,16]</sup> and promote the proliferation of HSCs<sup>[17]</sup>. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines by T helper type 1 T cells and mono/macrophages. Previous studies have shown that endogenous IL-10 has the ability to inhibit the inflammation in injured liver and block the advance of fibrosis<sup>[18-21]</sup>. Previous works by our group have demonstrated that exogenous IL-10 has an anti-fibrogenic function<sup>[22]</sup>. But the underlying mechanism remains obscure. In this study, in order to investigate the effects of IL-10 and PDGF on the proliferation and apoptosis of rat HSCs, culture-activated HSCs were treated with IL-10 and PDGF. Fas/FasL and Bcl-2/Bax mRNA expressions in each group were assayed by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis.

#### MATERIALS AND METHODS

#### Materials

Male Wistar rats, weighing 450-500 g, were provided by Shanghai Center for Laboratory Animals. Total RNA isolation kit was obtained from Jingmei Biotechnology Company of Shenzhen. Moloney murine leukemia virus (M-MLV) reverse transcriptase was purchased from Promega. PCR reagent and Dulbecco's modified Eagle's medium (DMEM) were respectively provided by Shanghai Biotechnology Company and GibcoB. PCR primers were synthesized by Shanghai Biotechnology Company.

#### Isolation, culture and evaluation of HSCs

HSCs were isolated from normal male Wistar rats by *in situ* digestion of collagenase and pronase and single-step density Nycodenz gradient as Ramm GA<sup>[23]</sup> and Friedman SL<sup>[24]</sup> previously described, and cultured in DMEM supplemented with 100 mL/L FBS. Desmin immunocytochemistry was employed to determine the

isolated HSCs' purity. HSCs were subcultured 4 d after primary culture. Alpha smooth muscle actin ( $\alpha$ -SMA) immunocytochemistry and electron microscope were employed to confirm that HSCs were activated by culture *in vitro* and transformed into myofibroblasts.

#### Intervention and division of HSCs

The subcultured HSCs were diluted to a concentration of  $5 \times 10^4$ /mL with DMEM containing 100 mL/L FBS and seeded onto the 24-well plastic tissue culture plates. When HSCs spread the plate fully, the culture medium was replaced with DMEM containing 10 mL/L FBS. After incubated for 24 h, HSCs were divided randomly into 4 groups: one as control group cultured in 1 mL DMEM containing 10 mL/L FBS, the other three were cultured in the same medium and treated with 20 ng PDGF or 20 ng IL-10, either alone or in combination, respectively. We named them group N, group P, group I and group C, respectively. Each group included 5 wells.

#### **RNA** extraction

Total RNA was extracted from the above treated HSCs after incubated for 24 h according to the RNA isolation kit instructions. The content and purity of total RNA were determined by spectrophotography.  $A_{260}/A_{280}$  of total RNA was between 1.8-2.0.

#### RT-PCR for Fas/FasL and Bcl-2/Bax

For RT-PCR, total RNA was reverse-transcripted using M-MLV reverse transcriptase and oligo (dT) at 37 °C for 60 min, followed by at 70 °C for 10 min. Approximately 2  $\mu$ g total RNA was used in each reverse transcription reaction and the final volume was 25  $\mu$ L.  $\beta$ -actin was used as internal control. The PCR reaction volume was 50 ul, including 5  $\mu$ L 10×PCR buffer, 2 mmol/L MgCl<sub>2</sub>, 1  $\mu$ L 10 mmol/L dNTP, 1  $\mu$ L 20 pmol/ $\mu$ L target gene sense and anti-sense primers, 1  $\mu$ L 20 pmol/ $\mu$ L  $\beta$ -actin primer pair, 2  $\mu$ L RT product, 1.5 U Tag DNA polymerase. The specific sets of primers and the target gene amplification conditions are shown in Table 1.

#### **Result determination**

PCR products were run on 20 g/L agarose gel eletrophoresis and visualized with ethidium bromide staining. Bio imagine system was used to detect the densities of bands of the PCR products. The ratio of target gene density to  $\beta$ -actin density was used to represent the relative levels of Fas/FasL and Bcl-2/Bax mRNA expressions. The semi-quantitative detection was analyzed 5 times repeatedly.

#### Statistical analysis

All data were expressed as mean±SE. The significance for the

difference between the groups was assessed with SPSS 10.0 by one-way ANOVA. P < 0.05 was considered statistically significant.

#### RESULTS

#### **Evaluation of HSCs**

Freshly isolated HSCs were round-shaped with many yellow droplets in cytoplasm. After cultured for 5-6 d, the spread cells showed a typical 'star'-like configuration. Desmin immunocytochemistry showed that the positive percentage was about 95% (Figure 1A), indicating that 95% of the isolated cells were HSCs.  $\alpha$ -SMA immunocytochemistry showed that 98% of the cells were  $\alpha$ -SMA positive (Figure 1B), indicating that most of the cells were activated. The myofilament could be seen in cytoplasm under the electron microscope, confirming that HSCs were activated and transformed into myofibroblasts after cultured *in vitro* (Figure 2).



Figure 1 Desmin and  $\alpha$ -SMA immunocytochemistry (SP, original magnification: ×100). A: Desmin immunocytochemistry of HSCs 7 d after isolation; B:  $\alpha$ -SMA immunocytochemistry of HSCs 7 after isolation.

Table 1 Primer sequences for PCR and amplification conditions for each target gene

Primer (base)	Sequence	Amplification conditions
Fas 414	5'-GAATGCAAGGGACTGATAGC-3'	Denaturation at 94 °C for 45 s,
	5'-TGGTTCGTGTGCAAGGCTC-3'	Annealing at 55 °C for 30 s and synthesizing
		at 72 °C for 1 min for 25 cycles
FasL 239	5'-GGAATGGGAAGACACATATGGAACTGC -3'	Denaturation at 94 °C for 45 s,
	5'-CATATCTGGCCAGTAGTGCAGTAATTC-3'	Annealing at 55 °C for 30 s and synthesizing
		at 72 °C for 1 min for 33 cycles
Bcl-2 525	5'-TATGATAACCGGGAGATCGTGATC-3'	Denaturation at 94 °C for 45 s,
	5'-GTGCAGATGCCGGTTCAGGTACTC-3'	Annealing at 60 °C for 30 s and synthesizing
		at 72 °C for 1 min for 33 cycles
Bax 310	5'-GACACCTGAGCTGACCTTGG-3'	Denaturation at 94 °C for 45 s,
	5'-GAGGAAGTCCAGTGTCCAGC-3'	Annealing at 60 °C for 30 s and synthesizing
		at 72 °C for 1 min for 30 cycles
β-actin 660	5'-CCAACCGTGAAAAGATGACC-3'	Changed according to different target genes
	5'-CAGGAGGAGCAATGATCTTG-3'	

All initial denaturations were at 94 °C for 5 min. Finally an additional extension step at 72 °C for 7 min was done.



**Figure 2** Activated HSCs under the electron microscope. The myofilament can be seen in the cytoplasm as the arrow point shows.



**Figure 3** Relative Fas/ FasL mRNA expression levels in HSCs of different groups assessed by RT-PCR. A: Relative Fas mRNA expression levels (P>0.05 between random two groups.); B: Relative FasL mRNA expression levels ( $^{a}P$ >0.05 vsgroup N,  $^{b}P$ <0.01 vs group N,  $^{d}P$ <0.01 vs group N.; Broup N: Normal group as control; group P: PDGF treated group; group I: IL-10 treated group; group C: Combined PDGF and IL-10 treatment group.



**Figure 4** RT-PCR results of Fas/FasL mRNA expression in HSCs of different groups. A: RT-PCR results of Fas mRNA expression; B: RT-PCR results of FasL mRNA expression; M: 100 bp DNA ladder (upper to lower: 1 000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp); Lane 1: Normal group as control; Lane 2: PDGF treatment group; Lane 3: IL-10 treatment group; Lane 4: Combined PDGF and IL-10 treatment group.



**Figure 5** Relative Bcl-2/Bax mRNA expression levels in HSCs of different groups assessed by RT-PCR. A: Relative Bcl-2 mRNA expression levels ( ${}^{b}P$ <0.01 *vs* group P, group I and group C, respectively;  ${}^{a}P$ >0.05 *vs* group I,  ${}^{c}P$ >0.05 *vs* group C,  ${}^{d}P$ <0.01 *vs* group P.). B: Relative Bax mRNA expression levels ( ${}^{b}P$ <0.01 *vs* group P, group I and group C, respectively;  ${}^{a}P$ >0.05 *vs* group C, the second provide t



**Figure 6** RT-PCR results of Bcl-2/Bax mRNA expression in HSCs of different groups. A: Bcl-2 mRNA expression. B: Bax mRNA expression. M: 100 bp DNA ladder (upper to lower: 1 000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp); Lane 1: Normal group as control; Lane 2: PDGF treatment group; Lane 3: IL-10 treatment group; Lane 4: Combined PDGF and IL-10 treatment group.

#### Effects of PDGF and IL-10 on Fas and FasL expressions in HSCs

Fas mRNA was expressed in HSCs of each group and the expression levels had no significant difference among the 4 groups, as shown in Figures 3A, 4A, indicating that neither PDGF nor IL-10 had effect on Fas mRNA expression in HSCs. As it could be informed from Figures 3B, 4B, FasL mRNA level in normal culture-activated HSCs (group N) was very low. It increased obviously after HSCs were treated with IL-10 (group I) (0.091±0.007 vs 0.385±0.051, P<0.01), but remained the low level after treated with PDGF alone (group P) or PDGF in combination with IL-10 (group C) (0.085±0.006, 0.101±0.008, respectively). The data suggested that IL-10 could improve FasL mRNA expression in culture-activated HSCs and PDGF could not. Furthermore, PDGF tended to abolish this effect of IL-10.

Effects of PDGF and IL-10 on Bcl-2 and Bax expressions in HSCs Bcl-2 and Bax mRNA were expressed in normal culture-activated HSCs. Both of their expression levels were significantly changed after treated with PDGF and IL-10, either alone or in combination. Bcl-2 mRNA expression was down-regulated and Bax mRNA expression was up-regulated, following the turn from group P, group I to group C. The expression of Bcl-2 in group C was significantly lower than that in group P (0.126±0.008 vs 0.210±0.024, P < 0.01). But no significant difference was found between group C and group I, as well as between group I and group P (0.210±0.024 vs0.166±0.017, 0.166±0.017 vs0.126±0.008, P>0.05) (Figures 5A, 6A). Similarly, the expression of Bax in group C was higher than that in group P (0.513±0.016 vs 0.400±0.022, P<0.01). No significant difference was found between group I and group P (0.400±0.022vs0.449±0.028, P>0.05). But compared with combined treatment group, Bax expressions in group I tended to decrease  $(0.449\pm0.028 vs 0.513\pm0.016, P = 0.045 < 0.05)$  (Figures 5B, 6B). These results showed that both PDGF and IL-10 promoted the Bax mRNA expression in HSCs and inhibited the Bcl-2 expression, but the differences of their effects were not significant. Intervention with PDGF and IL-10 seemed to be able to manifest effects on Bax expression than intervention alone. IL-10 showed similar influences on culture-activated HSCs and reactivated HSCs by PDGF.

#### DISCUSSION

It is generally accepted that hepatic stellate cells (HSCs) are central to the process of hepatic fibrosis. They are the major source of extracellular matrix and during fibrogenesis undergo an activation process characterized by increased proliferation and collagen synthesis<sup>[1-10,24]</sup>. So the activation, proliferation and apoptosis of HSCs have close relationship with the formation and development of liver fibrosis. To inhibit the activation and proliferation of the HSCs and promote their apoptosis has become the most important therapeutic approach for liver fibrosis<sup>[7-10,14,25-28]</sup>.

There is evidence that HSCs can be successfully isolated by *in situ* digestion of collagenase and pronase and singlestep density Nycodenz gradient<sup>[23,24,29]</sup>. Desmin is a marker for muscle cells and expressed by all muscle lineages including HSCs (either quiescent or activated) in the liver. Alpha smooth muscle actin ( $\alpha$ -SMA) is an intermediate filament protein that is expressed by activated HSCs and is widely accepted to be a marker of activation. Both of them were used to identify and quantify HSCs and their activation. The desmin immunocytochemistry result showed that the purity of the isolated HSCs by this method was satisfying (Figure 1A). The results of  $\alpha$ -SMA immunocytochemistry (Figure 1B) and electron microscope (Figure 2) confirmed that HSCs were activated and transformed into myofibroblasts after cultured *in vitro*.

PDGF, which is produced by HSCs, Kuffer cells and platelets, is a major mitogen for connective tissues and certain other cells. It was viewed as one of the most important growth factors serving as the matrix-bound cytokines<sup>[11]</sup> and plays an important role in the pathogenesis of liver fibrosis via promoting the activation and proliferation of HSCs<sup>[12,15,25,30-32]</sup>. The best characterized chemotactic factor for HSCs identified so far is the PDGF-BB<sup>[33-35]</sup> which is also known as the most potent mitogen for HSCs overexpressed during active hepatic fibrosis<sup>[36]</sup>. But there is also evidence that PDGF is proapoptotic for fibroblasts in conditions of low serum<sup>[37]</sup>. Saile B<sup>[38]</sup> reported that resting HSCs displayed no sign of apoptosis and spontaneous apoptosis became detectable in parallel with HSCs activation, suggesting that apoptosis might represent an important mechanism terminating proliferation of activated HSCs. He also found that Fas and Fas-ligand in HSCs became increasingly expressed during the course of activation. But our data demonstrated that PDGF alone had no effect on the expression of Fas and FasL during further activating the culture-activated HSCs, which was supported by Issa R<sup>[39]</sup>. Bax and Bcl-2 are known as the representatives of proapoptotic factor and contra-apoptotic factor of Bcl-2 family, respectively<sup>[40,41]</sup>. In our study, evidences showed that PDGF could promote Bax mRNA expression in HSCs and inhibited Bcl-2 mRNA expression as well, resulting in the apoptosis of HSCs<sup>[41]</sup>. All the above data demonstrated that PDGF can accelerate the apoptosis of HSCs through Bcl-2/Bax pathway in parallel with their proliferation<sup>[42]</sup>. In other words, PDGF may promote proliferation but is neutral with respect to HSCs apoptosis. But the proportion of apoptosis-inducing forces and apoptosis-inhibiting forces would determine that PDGFactivated HSCs tend to proliferate and increase<sup>[22]</sup>.

Cytokine interleukin-10 (IL-10), produced by lymphocytes and macrophages as well as cells within liver such as Kufffer cells, hepatocytes and HSCs, has profound inhibitory actions on macrophages and inflammation. The present studies showed that IL-10 had additional effects on connective tissue cells, such as HSCs and fibroblast. IL-10 could inhibit the activation of HSCs by inflammatory cells<sup>[43]</sup>, relieve the inflammation of liver<sup>[18,19,44]</sup>, suppress the function of NF- $\kappa B^{[45]}$  and affect the expression of collagen I and collagenase<sup>[20]</sup>, thus exerting an antifibrogenesis effect<sup>[46]</sup>. Failure for HSCs to sustain IL-10 expression might underlie pathologic progression to liver cirrhosis<sup>[18,20]</sup>. Our previous studies also implied that IL-10 had an antagonism on CCL<sub>4</sub>-induced rat hepatic fibrosis<sup>[22]</sup>. But the underlying mechanism remains obscure. In this study, our results showed that IL-10 could promote the expression of FasL and Bax mRNA in culture-activated HSCs and meanwhile could inhibit Bcl-2 mRNA expression, implying that IL-10 may induce the apoptosis of HSCs through binding FasL to Fas on the cell membranes of HSCs and increasing the proportion of Bax and Bcl-2. Saile B<sup>[38]</sup> found that apoptosis could be fully blocked by Fas-blocking antibodies in normal cells and HSCs already entering the apoptotic cycle, implying that Fas/FasL system is the key pathway for the apoptosis of HSCs. Our data, however, showed that Bax/Bcl-2 system was another important pathway involving in HSCs' apoptosis<sup>[40,41]</sup>. In short, IL-10 could promote the apoptosis of HSCs, which may be related to its mechanism of antifibrosis.

There is evidence that activated-HSCs could express IL-10 as well as its receptor<sup>[20,47]</sup>. In this study, PDGF had a similar effect to IL-10 on Bax/Bcl-2 mRNA expression in HSCs. This promotes us to hypothesize that PDGF may regulate the expression of Bax and Bcl-2 mRNA by affecting the expression of IL-10 in HSCs. But PDGF in combination with IL-10 did not show a satisfying synergistic action, thus we can not exclude the possibility that PDGF and IL-10 affect in different ways, and further works are demanded.

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CLINICAL RESEARCH •

# Effects of 24 h ultra-marathon on biochemical and hematological parameters

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#### Abstract

**AIM:** To analyze detailed changes in hematology and biochemistry tests parameters before and after a long-distance race in ultramarathon runners.

**METHODS:** Blood samples of 11 participants were obtained for standard analysis before, immediately after, two days after and nine days after the 2002 International Ultramarathon 24 h Race and the International Association of Ultrarunners (IAU) Asia 24 h Championship.

**RESULTS:** Total bilirubin (BIL-T), direct bilirubin (BIL-D), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) increased statistically significantly (P<0.05) the race. Significant declines (P<0.05) in red blood cell (RBC), hemoglobin (Hb) and hematocrit (Hct) were detected two days and nine days d after the race. 2 d after the race, total protein (TP), concentration of albumin and globulin decreased significantly. While BIL, BIL-D and ALP recovered to their original levels. High-density lipoprotein cholesterol (HDL-C) remained unchanged immediately after the race, but it was significantly decreased on the second and ninth days after the race.

**CONCLUSION:** Ultra-marathon running is associated with a wide range of significant changes in hematological parameters, several of which are injury related. To provide appropriate health care and intervention, the man who receives athletes on high frequent training program high intensity training programs must monitor their liver and gallbladder function.

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#### INTRODUCTION

Numerous reports nave been published on the effects on the body of endurance sports such as the 5 000 m, the 10 000 m, the marathon, cross-country running, rowing and cycling<sup>[1-14]</sup>. In recent years, more athletes have become involved in ultraendurance races, such as the iron-man triathlon, the 100 km race and the 24 h marathon, and a few investigations have addressed the related hematological and biochemical changes. Strenuous physical activities are becoming increasingly popular around the world, and this work may benefit participants in future competitions.

Some of these previous studies have involved staged races with long rest periods; while others made comparisons before and after competition. This study examines, athletes who have completed a long-distance run lasting 24 h, and detailing the related hematological and biochemical changes before, immediately after, two days after and nine days after the race.

#### MATERIALS AND METHODS

#### Anthropometric data

A 24 h ultra-marathon was held at Soo-Chow University on March 2, 2002. The runners ran around a 400 m oval track for 24 h, covering a distance of at least 100 km. The runners changed direction every 4 h. The temperature during the 24 h race ranged from 19.0 to 26.8 °C and the relative humidity ranged from 63 to 91%. The runners were permitted to rest and to ingest water and food freely. Ten males (10/36) and one female (1/1) participated in this study, having previously given their informed consent. Table 1 lists the anthropometric data.

**Table 1** Anthropometric data of the study population (*n* = 11)

	Range	Mean	Standard Deviation
Age (yr)	26.00-55.00	45.10	$\pm 2.64$
Height (cm)	155.00-177.00	166.80	$\pm 6.23$
Body weight (kg)	47.00-69.30	60.60	$\pm 9.69$
Distance completed (km)	106.70-194.40	158.60	$\pm 26.78$
BMI(body mass index)	19.56-22.12	21.79	$\pm 0.24$

#### Parameter of blood tests

20 mL blood samples were obtained from the antecubital vein 24 h before the race, immediately after the race, two days after the race and nine days after the race. The blood was analyzed in 1 h using an ABBOTT CELL DYN 3 000 autoanalyser (Abbott Diagnostics, Mountain View, CA., USA) and HITACHI 7150 autoanalyser (Hitachi High Techotologies, Tokyo, Japan).

Statistical significance of paired differences in means and standard deviations of the related hematological and biochemical changes among pre-race, immediately post-race, two days post-race, and nine days post-race values were calculated using by one-way ANOVA analysis. The level of significance was set at P<0.05.

#### RESULTS

#### Hb. Hct. platelet data

The pre-race red cell count, Hb and Hct levels were not significantly

immediately after the race, but were significantly reduced two and nine days after the race, being lowest at two days after the race. The mean cell volume was not significantly changed immediately after the race or on day two post-race, but was significantly increased by day nine post-race. Mean cell Hb concentration was significantly lower on day two than before the race, but had recovered on day nine.

Mean cell Hb and red cell distribution width remained unchanged at all times. The platelet concentration immediately after the race and on day nine following the race was increased significantly compared to pre-race values, but had decreased significantly on day two. (Table 2).

#### Total WBC and differential count

The white blood cell count was significantly increased at the end of the race and remained high until day nine. Moreover, the number of neutrophils was increased at the end of race but recovered two days later. Furthermore, the lymphocytes, eosinophils and basophils were decreased immediately after the race but recovered two days later. Finally, the number of monocytes was increased immediately after the race and on day two post-race, but returned to the pre-race level by day nine post-race. (Table 3).

#### Ferritin, TIBC

The ferritin level, total iron binding capacity (TIBC) and transferring saturation rose significantly immediately postrace along with ferritin level, and remained at the end higher on day two and nine. All of the parameters remained normal mean values. (Table 4).

#### Liver function tests

The BIL-T and BIL-D concentrations were significantly raised immediately after the race and normalized two days later. TP, albumin and globulin concentrations were unchanged immediately after the race but were significantly reduced on day two, recovering gradually after day nine, though TP and albumin remained below pre-race levels.

ALP, AST and ALT had increased significantly by the end of the race. ALP returned to its pre-race level after day two. Moreover, AST declined by day two and resumed its pre-race level by day nine. Furthermore, ALT continued to rise until day two and had recovered by day nine. Gamma glutamyl transferase ( $\gamma$ -GT) remained unchanged until the end of race and beyond the end of the event. Finally, LDH was significantly raised by the end of the race and was decreased on day two post-race, but remained above their pre-race level on day nine. (Table 5).

#### Lipid metabolism

Triglyceride (TG), cholesterol/low-density lipoprotein cholesterol (CHO/LDL-C) ratio and LDL-C were lower immediately after the race had finished. TG level and CHO/ LDL-C ratio recovered by day two post-race, while LDL-C recovered by day nine. Cholesterol was not significantly changed at the end of the race, but was significantly lower on day two.

HDL-C was highest immediately after the race but had reduced by day two and nine post-race. (Table 6).

Table 2 Changes in Hb, Hct, red cell parameters and platelet count before and after the race

	Pre-race	0 h post-race	2 d post-race	9 d post-race
Red cell count (×10 <sup>12</sup> /L)	$4.71 \pm 0.25^{ce}$	4.71±0.45	4.07±0.27	$4.42{\pm}0.21$
Hb (g/dL)	$14.63 \pm 0.91$ <sup>ce</sup>	$14.58 \pm 1.17$	$12.52 \pm 0.86$	$13.81 {\pm} 0.69$
Hct (%)	$42.34{\pm}2.73^{ce}$	$42.37 {\pm} 3.82$	$37.33 \pm 3.15$	$40.27{\pm}1.84$
Mean cell volume (fl)	$89.91 \pm 3.11^{e}$	$90.05 {\pm} 3.37$	$90.29 \pm 3.50$	$91.15 \pm 3.19$
Mean cell Hb (pg)	$31.09 \pm 1.23$	$31.02 \pm 1.44$	$30.90 \pm 1.29$	$31.22 \pm 1.34$
Mean cell Hb				
Concentration (g/dL)	$34.59 {\pm} 0.45^{ m e}$	$34.44 {\pm} 0.60$	$34.24 \pm 0.47$	$34.25 {\pm} 0.64$
Red cell distribution width (%)	$12.84{\pm}0.60$	$12.94 {\pm} 0.88$	$12.69 \pm 0.57$	$12.80 \pm 0.65$
Platelet (×10 <sup>9</sup> /L)	$235.45{\pm}47.27^{ m ace}$	$248.91{\pm}46.95$	$209.82 \pm 58.28$	$280.27 \pm 67.23$

 $^{a}P<0.05$  vs statistically significant when pre-race compared with 0 h post-race.  $^{c}P<0.05$  vs statistically significant when pre-race compared with 2 d post-race.  $^{e}P<0.05$  vs statistically significant when pre-race compared with 9 d post-race.

Table 3 Total and differential white cell counts before and after the race

	Pre-race	0 h post-race	2 d post-race	9 d post-race	
White cell count (×10 <sup>9</sup> /L)	$4.95{\pm}1.05^{\rm ace}$	$11.87 \pm 1.46$	5.83±1.09	$5.95{\pm}1.45$	
Neutrophils (%)	$56.02 \pm 6.69^{a}$	$76.43 {\pm} 6.28$	$57.66 {\pm} 7.28$	$57.93 {\pm} 9.16$	
Lymphocytes (%)	$33.10 \pm 6.94^{a}$	$14.47 {\pm} 4.82$	$30.89 {\pm} 6.42$	$32.15 \pm 8.07$	
Monocytes (%)	$7.83 \pm 3.58$	$8.21 {\pm} 2.84$	$9.34{\pm}2.77$	$7.15 \pm 1.91$	
Eosinophils (×10 <sup>9</sup> /L)	$2.07 \pm 1.01^{a}$	$0.20 {\pm} 0.19$	$1.75 \pm 0.76$	$1.80 \pm 1.20$	
Basophils (×10 <sup>9</sup> /L)	$0.96{\pm}0.19^{a}$	$0.68{\pm}0.24$	$0.82{\pm}0.26$	$0.99{\pm}0.28$	

 $^{a}P<0.05$  vs statistically significant when pre-race compared with 0 h post-race.  $^{c}P<0.05$  vs statistically significant when pre-race compared with 2 d post-race.  $^{e}P<0.05$  vs statistically significant when pre-race compared with 9 d post-race.

Table 4 Comparisons of parameters related to iron metabolism before and after the race

	Pre-race	0 h post-race	2 d post-race	9 d post-race	
Ferritin (µg/L)	$64.45{\pm}27.95^{\rm ae}$	$117.00 \pm 52.66$	$70.18{\pm}44.88$	$103.36 {\pm} 42.15$	
TIBC (µmol/L)	$361.00 \pm 31.38^{\mathrm{ae}}$	$372.18 {\pm} 30.93$	$357.64 \pm 35.43$	$356.36 \pm 30.75$	
Transferrin saturation (%)	$17.73 {\pm} 8.05^{ae}$	$31.09 \pm 13.32$	$19.27 \pm 11.62$	$29.18 \pm 11.70$	

<sup>a</sup>*P*<0.05 *vs* statistically significant when pre-race compared with 0 h post-race. <sup>c</sup>*P*<0.05 *vs* statistically significant when pre-race compared with 2 d post-race. <sup>e</sup>*P*<0.05 *vs* statistically significant when pre-race compared with 9 d post-race.

Table 5	Serum enz	zyme activity	before and	after the	ultra marathon race

	Pre-race	0 h post-race	2 d post-race	9 d post-race
BIL-T (µmol/L)	11.63±2.91ª	$25.65 \pm 9.75$	13.68±7.70	$12.14{\pm}4.10$
BIL-D (µmol/L)	$2.57{\pm}0.68^{a}$	7.01±2.91	$3.25 \pm 1.54$	$2.74{\pm}1.20$
TP (g/L)	$72.51 \pm 4.70^{ce}$	$72.50 {\pm} 6.21$	$66.14 \pm 3.90$	$67.00 {\pm} 4.91$
Albumin (g/L)	$44.82 \pm 2.83$ <sup>ce</sup>	$45.42 \pm 2.92$	$38.55 {\pm} 5.83$	$42.43 \pm 2.84$
Globulin (g/L)	27.53±2.51°	$27.25 \pm 3.74$	$25.56{\pm}2.01$	$27.53 \pm 2.42$
ALP (U/L)	$132.85 \pm 56.50^{a}$	$160.55 \pm 33.00$	$131.36 \pm 34.00$	$134.27 \pm 34.40$
AST (U/L)	$37.10 \pm 19.10^{\rm ac}$	$536.70 \pm 311.10$	$271.30 \pm 227.80$	$34.30 \pm 8.70$
ALT (U/L)	$35.10 \pm 13.10^{\rm ace}$	$118.40 \pm 75.10$	$126.00 \pm 68.30$	$50.50 \pm 18.90$
γ-GT (U/L)	$20.18 \pm 6.23$	$24.18 \pm 14.30$	$19.18 {\pm} 9.00$	$20.91 \pm 8.00$
LDH (U/L)	$367.50{\pm}105.60^{\rm ace}$	$1 \hspace{0.1in} 420.50 {\pm} 598.50$	$1 \ 120.30 \pm 605.10$	$582.70{\pm}207.90$

 $^{a}P<0.05$  vs statistically significant when pre-race compared with 0 h post-race.  $^{c}P<0.05$  vs statistically significant when pre-race compared with 2 d post-race.  $^{e}P<0.05$  vs statistically significant when pre-race compared with 9 d post-race.

Tabl	e 6 (	Changes in	parameters relate	d to	lipid	metabo	olism	before and	l afte	er th	e ult	ra marat	hon	race
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	Pre-race	u n post-race	z a post-race	9 a post-race
TG (mmol/L)	$0.95{\pm}0.27^{a}$	$0.67 {\pm} 0.28$	$0.84{\pm}0.32$	$1.09 {\pm} 0.55$
CHO (mmol/L	$4.87 \pm 1.06^{\circ}$	$4.63 {\pm} 1.09$	$4.13 {\pm} 0.57$	$4.51 \pm 0.50$
HDL-C (mmol/L)	$1.92{\pm}0.47$	$2.02 {\pm} 0.50$	$1.68 {\pm} 0.24$	$1.77 \pm 0.32$
LDL-C (mmol/L)	$2.51{\pm}0.78^{\rm ac}$	$2.30{\pm}0.72$	$2.02 {\pm} 0.49$	$2.24{\pm}0.39$
CHO/ HDL-C	$2.50{\pm}0.48^{\rm a}$	$2.30 {\pm} 0.37$	$2.40{\pm}0.38$	$2.60 {\pm} 0.43$

 $^{a}P<0.05$  vs statistically significant when pre-race compared with 0 h post-race.  $^{c}P<0.05$  vs statistically significant when pre-race compared with 2 d post-race.  $^{e}P<0.05$  vs statistically significant when pre-race compared with 9 d post-race.

#### DISCUSSION

Few studies have extensively addressed the hematological and biochemical changes in endurance runners. This investigation elucidates the effects of intensive exercise on athlete health and the findings can be used to help participants in future competitions.

Red cell count, Hb and Hct, three indicators of anemia, were normal before the race. Significant decreases was found by day two, consistent with the accelerated destruction of RBC in endurance athletes. The three indicators remained reduced between days two and nine; so-called sports anemia<sup>[15]</sup>, is not only caused by hemolysis owing to mechanical trauma but also by oxidative injuries of the red cells<sup>[16]</sup>. Under normal conditions, red cells with a mean life of 120 d are renewed at approximately 1% daily. However, this turnover rate increases following endurance training, as reflected in the participants in this study. The increased turnover rate is good for the athletes as the young red cells can carry oxygen more efficiently than the older cells.<sup>[17,18]</sup> The mean cell volume, mean cell hemoglobulin and mean cell hemoglobulin concentration remained normal throughout. The transient sports anemia was caused by reduced red cell numbers rather than red cell size or amount of Hb<sup>[19,20]</sup>. The change in platelets number was inconsistent with previous studies. The platelet count was higher at the end of the race and on day nine, but remained within the normal range, and no coagulopathy was detected. Further study can clarify the significance of the increase. The white cell count increased markedly race and subsequently declined. The initial increase followed from to a rise in peripheral reserves and was mostly associated with neutrophils. Neutrophilia and numbers of lymphocytes were related to catecholamine, cortisol and some chemotactic factors: transient immunological dysfunction may occur under such conditions<sup>[21,22]</sup>. This study found on decrease in absolute lymphocyte count and no signs of infection in a followup questionnaire administered 2 wk after the race.

The concentration of serum ferritin was significantly increased immediately after the race and on day nine post-race, owing to the acute phase response of the destruction of red cells, consistent with previous reports. Total iron binding capacity and transferrin saturation were markedly increased by the end of race, reflecting the acute release of iron.

BIL and BIL-D increased at the end of the race and normalized after day two, associated with hemolysis that follows from ultralong running. The hemolysis was related to a decline in haptoglobin concentration and structural changes in the red cell membranes<sup>[23,24]</sup>. AST, ALT,  $\gamma$ -GT, LDH and ALP all increased by the end of the race, implying damage to the skeletal muscle cells and hepatic cells. Serum BIL normalized by day two as red cell turnover reduced, but AST, ALT and LDH continued to exceed pre-race levels, representing a continued release of enzymes from the muscles and liver<sup>[25-27]</sup>.

Albumin is involved in protein synthesis by the liver. Albumin reduced significantly by day two, reflecting damage to the anabolic functioning of hepatic cells. TP fell after day two, mainly owing to the decrease in albumin and had not recovered by day nine. Despite the reduced protein level, no clinical pitting edema was found. The pre-race mean AST, ALT and LDH exceeded the normal range, possibly indicating chronic damage to the liver following long-term strenuous exercise.

Most hepatic function parameters displayed no correlation with age, except for negatively correlated globulin, reflecting the lower immunological functioning of older runners following endurance exercise<sup>[28,29]</sup>. AST, ALT and LDH were positively correlated with runner performance and unrelated to BIL and ALP, implying that changes in hepatobiliary parameters resulted mainly from damage to hepatic cells.2 Long-term regular exercise has been recognized to contribute to reducing cholesterol<sup>[30-32]</sup>, triglyceride and LDL-C and increasing HDL-C. In this study the lipid parameters of all of the participants were in the normal ranges, supporting the beneficial effect of rhythmic aerobic exercise. However, the effects of long-term ultra-endurance activities deserve further investigation.

Most fat is stored as triglyceride in fat and muscle cells. Plasma and muscular triglyceride were consumed equally during the first stage of endurance exercise, and subsequently the free fatty acid became the major source of energy explaining the reduction in triglyceride at the end of the race<sup>[33,34]</sup>. The TG and LDL-C were significantly lower at the end of the race and on day agreeing with previous reports. Cholesterol is a major risk factor for coronary artery disease and deserves extensive investigation. Cholesterol levels were not significantly changed at the end of the race, and were decreased by day two, but the cholesterol/HDL-C ratio, which is not affected by plasma volume, was significantly reduced by the end of the race. This phenomenon may result from the increase in HDL-C and the decrease in LDL-C.

Ultramarathon running is associated with numerous changes in hematological parameters, many of which are injury related. These changes should not be confused with indicators of disease. Increasing liver enzyme levels in runners indicated damage to liver cells, but increased BIL resulted from higher clearance rates of RBC. The liver damage was directly proportional to workload. Acute reduction of TG might result from the use of body fat as the major energy source. An ultralong endurance run effectively reduced LDL-C for two days post-race, but did not significantly change HDL-C.

Safety guidelines, protective equipment and prevention education are crucial to reducing sports injuries. Then, preventing liver and gall bladder injuries and ensuring safe health management program are necessary for ultramarathon athletes. In summary, efforts to minimize these injuries are warranted both to ensure the long-term health of runners and to reduce medical costs. The key to management of ultramarathon runner osteoporosis involves identifying the potential risk for osteoporosis and osteoporotic fracture, followed by measures that focus on reducing modifiable risk factors through helath management program<sup>[33-34]</sup>.

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#### • BRIEF REPORTS •

### Assessment of autonomic function in untreated adult coeliac disease

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#### Abstract

**AIM:** Some recent studies showed that alteration of upper-gut motility in coeliac disease may be related to dysfunction of autonomic nervous system. The aim of our study was to investigate whether autonomic nervous system was altered in untreated and unselected coeliac disease patients.

**METHODS:** We studied 8 untreated and consecutive coeliac disease patients (2 males and 6 females, age range 37±14.5 years). Histological evaluation of duodenal mucosa, anti-gliadin antibodies (AGA), antiendomysial antibodies (EMA) and anti-tTG antibodies and sorbitol H2 breath test were performed in all patients. Extrinsic autonomic neuropathy was assessed by the standardized measurement of cardiovascular reflexes (lying-to-standing, Valsalva manoeuvre, deep breathing, sustained handgrip). The results obtained were compared with a healthy, asymptomatic control group (6 males and 7females, age range 42.3±13.5 years).

**RESULTS:** Coeliac patients exhibited a lower increase of PAS as a response to isometric effort, a reduction of spectral power LF as a response to clinostatic position, but without statistical significance. Also they showed a lower tolerance to orthostatic position, associated with a latent disequilibrium of sympathetic-vagal balance, a relative prevalence of parasympathetic component of the autonomic function. However, these results were not statistically significant when compared with control group (P = n.s.). And they were unchanged after 6 and 12 mo of gluten-free diet.

**CONCLUSION:** This study failed to confirm a significant correlation between autonomic dysfunction and coeliac disease, yet we could not exclude a role of autonomic dysfunction in the genesis of systemic symptoms in some coeliacs.

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#### INTRODUCTION

Coeliac disease (CD) is the most common severe food intolerance in the Western world<sup>[1]</sup>. It is a clinical syndrome of intestinal malabsorption, a characteristic though not specific histological lesion involving total, subtotal or partial small bowel villous atrophy (predominating in its proximal segments). It is the result of sensitiveness to ingested gluten in genetically susceptible people with the subsequent immune reaction leading to small bowel inflammation<sup>[2]</sup>. The classical malabsorptive symptoms of diarrhoea and weight loss are only one aspect of the spectrum of manifestations of this relatively common disease<sup>[3,4]</sup>, since symptoms may be subtle and many patients have subclinical or silent disease<sup>[5,6]</sup>. A proper gluten-free diet (GFD) would lead to a clinical and histological improvement<sup>[7-9]</sup>. In particular, GFD played a key role in preventing nutritional deficiency, especially of micronutrients, and in reducing the risk of the development of intestinal malignancies<sup>[10]</sup>.

It is quite frequently seen in clinical practice that coeliac patients present gastrointestinal motor abnormalities<sup>[11]</sup>. It has been recently shown that alteration of upper-gut motility may be related to dysfunction of autonomic nervous system<sup>[12]</sup>. The aim of our study was to investigate whether autonomic nervous system (ANS) was altered even in untreated and unselected coeliac patients and to assess the effect of GFD on ANS dysfunctions.

#### MATERIALS AND METHODS

#### Patients

We studied 8 untreated, consecutive and unselected coeliac disease patients (2 males and 6 females, age range 37±14.5 years). Both the original<sup>[13]</sup> and revised<sup>[14]</sup> criteria for the diagnosis of CD were used in this study. CD was defined as a permanent gluten-sensitive enteropathy, primarily manifested by the presence of characteristic small intestinal lesions<sup>[15]</sup>. Small-bowel biopsy was performed in all patients, as well as esophagogastroduodenoscopy (Fujinon EG300 videogastroscope; Fujinon, Omiya, Japan). At least six small-bowel biopsies were obtained from the second part of the duodenum using a disposable biopsy forceps with spike (U.S. Endoscopy Inc., Mentor, Ohio, U.S.A.) and evaluated by haematoxylin/eosin staining. Histopathology was expressed according to the Marsh classification of 1992<sup>[16]</sup>: "infiltrative" lesions with >30 lymphocytes/100 epithelial cells were defined as Marsh type I, "infiltrative/hyperplastic" lesions as Marsh II and "partial (sub)total villous atrophy (VA) as type III. We subdivided the Marsh III type into partial VA (Marsh IIIa), subtotal VA (Marsh IIIb) and total VA (Marsh IIIc), according to Oberhuber's modified classification<sup>[17]</sup>.

Other possible causes of villous atrophy or duodenal damage, such as *Giardia Lamblia* infection, tropical sprue, collagenous sprue, food protein hypersensitiveness (cow's milks, eggs, fish, rice, chicken) were excluded, as well as other causes of inflammatory infiltration of duodenum, such as peptic duodenitis<sup>[17]</sup>.

#### Methods

IgA and IgG anti-gliadin antibodies (AGA) were measured in all patients by enzyme-linked immunosorbent assay (kit Alfagliatest, Eurospital, Trieste - Italy); the lower limit of positivity of IgA-class was 0.2 EU/mL and of IgG-class 10.0 EU/mL. IgA antiendomysial antibodies (EMA) were screened by the indirect immunofluorescent method on monkey oesophagus (kit Antiendomysium, Eurospital, Trieste - Italy). IgA anti-tissue transglutaminase antibodies were also screened by enzyme-linked immunosorbent assay using human recombinant tTG (kit Eu-tTG, Eurospital, Trieste - Italy); the lower limit of positivity of these antibodies was 7 UA/mL.

Also sorbitol H2 breath test (H2-BT) was performed. All patients were studied after an overnight fasting having been instructed to consume a meal of rice and meat; they were also requested not to smoke on the morning of the test day. End expiratory samples were collected before the patients drank the test solution (5 g of sorbitol in 150 mL of tap water) and every 30 min for 4 h. Hydrogen concentrations in each collected sample were measured with a breath-hydrogen analyzer (EC60 Gastrolyzer Breath Hydrogen Monitor, Bedfont Scientific Ltd, Upchurch - Kent, England [U.K.]). An increase in H2 concentration of at least 20 ppm over fasting baseline was considered positive for sorbitol malabsorption. The cut-off for calculating the validity of the test was shifted every 30 min, and a response operating characteristics (ROC) curve was plotted on the basis of the obtained results.

The clinical, endoscopic, histological and serological pattern of the studied coeliacs are described in Tables 1, 2.

Table 2 Non- invasive tests in the studied coeliac population

Patient No.	AGA IgA	AGA IgG	EMA	Anti-tTG	Sorbitol H2-B7
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	+	+
4	+	+	-	-	+
5	+	+	+	+	+
6	+	+	-	+	+
7	-	-	+	-	+
8	+	-	-	+	+

Abbreviations: AGA: Anti-gliadin antibodies; EMA: anti-endomysium antibodies; anti-tTG: anti-tissue transglutaminase; H2-BT: Hydrogen2-breath test. No patient had other associated organic (such as hereditary, toxic, infectious, inflammatory) or metabolic diseases.

Extrinsic autonomic neuropathy was assessed by the standardized measurement of cardiovascular reflexes. Tests were performed in the morning and at constant temperatures. Autonomic function was evaluated by means of electrocardiograph Personal 120 (Esaote Biomedica, Firenze, Italy) and a Finapress set (Ohmeda, Louisville, Colorado, USA). We evaluated the heart rate variation (R-R interval) and systolic (SBP) and diastolic (DBP) blood pressures in response to a variety of stimuli: lyingto-standing (evaluation of R-R variation with a 30.15 ratio); Valsalva manoeuvre (forced expiration for 15 s with a 40 mm Hg pressure); deep breathing (6 expiratory cycles throughout one minute, evaluating the average of differences between the maximal and minimal duration of R-R interval during three subsequent deep breath [DB3]); sustained handgrip (performing isometric sub-maximal work for 3 min and evaluating blood pressure and heart rate variations); and tilt tests. The results of each test were scored according to Ewing's modified criteria<sup>[18,19]</sup>.

The results obtained were compared with a healthy, asymptomatic control group (13 people, 6 males and 7 females, age range  $42.3\pm13.5$  years). All coeliac patients were reevaluated about the autonomic function 6 and 12 mo after GFD has started.

#### Statistical analysis

The Student's *t*-test for unpaired data was used, and data are presented as mean $\pm$ SD. *P*<0.05 was considered as statistically significant.

#### RESULTS

We noted that coeliac patients showed a lower increase of PAS in response to isometric effort. Moreover, they showed a reduction of spectral power LF in response to clinostatic position, but without statistical significance (Tables 3, 4).

Only one female patient (Table 5, patient number 8) showed symptomatic orthostatic hypotension (defined as SBP decrease >/= 20 mm Hg and DBP >/= 10 mmHg within 3 min in orthostatic position) as responsiveness to orthostatic position. The overall results of ANS in coeliacs showed a tendency

 Table 1
 Demographic, clinical, endoscopic and histological data of the coeliac patients

Patient No.	Sex	Age (yr)	Clinical finding	Endoscopic finding	Histology
1	F	71	Weakness, diarrhoea	Absence of Kerckring's folds	Marsh IIIc
2	Μ	58	Diarrhoea, weight loss	Absence of Kerckring's folds	Marsh IIIb
3	F	31	Aphtous stomatitis, IDA	Reduction of Kerckring's folds	Marsh IIIb
4	F	31	IDA	"Scalloping" of duodenal folds	Marsh IIIa
5	F	38	Diarrhoea, abdominal	Reduction of Kerckring's folds,	
			pain, weight loss	micronodular mucosa	Marsh IIIc
6	Μ	33	IDA	Reduction of Kerckring's folds	Marsh IIIa
7	F	24	IDA, Grave's disease	Reduction of Kerckring's folds	Marsh IIIa
8	F	32	IDA, weakness	Reduction of Kerckring's folds	Marsh IIIa

Abbreviations: IDA: iron-deficiency anaemia.

**Table 3** Evaluation of autonomic function in coeliac patients and control group

Group	LS	VR	DB	riangle PAS Handgrip	$\triangle$ PAD Handgrip	
Coeliac disease	$1.22 \pm 0.1$	$1.73 \pm 0.38$	$35.5 {\pm} 16.4$	$35.7{\pm}16.4$	$24.6 \pm 13.2$	
Control group	$1.24 {\pm} 0.19$	$1.62 {\pm} 0.25$	$32.5 {\pm} 7.67$	$55.5 {\pm} 24.2$	$27.1 \pm 11.1$	
Р	NS	NS	NS	NS	NS	

LS: Lying-to-standing; VR: Valsalva reaction; DB: Deep breathing.

 Table 4 Evaluation of Heart rate variance in coeliac patients and control group

Group	LF Clino	LF Ortho	HF Clino	HF Ortho	LF/HF Clino	LF/HF Ortho	
Coeliac disease	$35.9 \pm 18.1$	$73.5 \pm 15.3$	35.7±18.9	$13.21 \pm 9.3$	$1.56{\pm}1.85$	$8.63 {\pm} 6.34$	
Control group	$52.8 \pm 21.0$	$67.1 \pm 25.9$	$31.1 \pm 15.6$	$17.90 \pm 12.9$	$2.35{\pm}1.65$	$6.94{\pm}5.5$	
Р	NS	NS	NS	NS	NS	NS	

 Table 5
 Overall results of autonomic tests in coeliac patients

Patient No.	Basal SBP	Basal DBP	Ortho SBP	Ortho DBP	Clino. LF	Ortho. LF	Clino. HF	Ortho. HF	Clino. LF/HF	Ortho. LF/HF	LS	VR	DB	Syst. SH	Diast. SH	Syst. Tilt Test	Diast. Tilt Test
1	127	82	120	82	40.99	61.65	48.00	32.82	0.85	1.88	1.03	1.46	19.2	54	27	-9	7.00
2	130	87	135	87	21.41	60.74	30.09	10.73	0.71	5.66	1.37	1.90	35.9	36	14	-9	5.00
3	130	80	125	80	77.04	92.52	12.89	5.96	5.98	15.52	1.20	1.40	33.3	35	25	19	14.00
4	105	70	105	85	38.26	63.87	20.19	17.85	1.9	3.58	1.20	2.30	40.1	39	36	3	7.00
5	115	80	120	82	31.15	58.43	62.16	16.49	0.5	3.54	1.20	1.40	39.3	48	28	7	14.00
6	120	80	108	75	27.16	87.79	27.33	11.00	0.99	7.98	1.18	1.50	40.2	50	47	2	12.00
7	105	70	115	75	20.30	94.00	61.00	4.79	0.3	19.6	1.30	2.30	44.3	7	6	-7	15.00
8	115	70	80	60	30.93	68.81	23.81	6.06	1.3	11.35	1.30	1.60	31.8	17	14	-10	2.00
Mean	118.38	77.38	113.50	78.25	35.91	73.48	35.68	13.21	1.57	8.64	1.22	1.73	35.51	35.75	24.6	3 -0.50	9.50
SD	10.20	6.52	16.50	8.51	18.13	15.27	18.88	9.28	1.85	6.34	0.10	0.39	7.74	16.37	13.2	0 10.23	4.87

to lower systolic-diastolic values of blood pressure both in clinostatic position and in active and passive orthostatic positions. However, these results had no statistical difference compared with those of control group (Table 4).

The spectral analysis of heart rate variance (HRV), performed with autoregressive method, confirmed these findings. The autoregressive analysis of HRV showed an important, but not significant, reduction of the power LF according to a considerable reductive activity of sympathetic tone at rest with a relative prevalence of parasympathetic tone. Passive orthostatic, evaluated by tilt tests, induced a marked sympathetic response (with increase of spectral power LF) (Table 5).

All patients were re-evaluated about the autonomic function 6 and 12 mo after GFD has started. In none of them we noted change of the results, and the symptomatic orthostatic hypotension persisted in patient number 8 despite strict adherence to GFD.

#### DISCUSSION

In recent years a discrete frequency of autonomic neuropathy has been disclosed in coeliac patients<sup>[12,20]</sup>, similar to that reported in other functional gastrointestinal disorders, but lower than that described in diabetic subjects<sup>[21]</sup>. Pathogenetic factors involved in autonomic dysfunction in coeliac disease were unknown, and autoimmune damage or metabolic derangement have been hypothesized<sup>[22-24]</sup>.

We investigated our coeliac patients, exploring their autonomic function using the cardiovascular tests because they were easily available reproducible, and not very expensive<sup>[25,26]</sup>. Our study showed quite different results from those recent studies about autonomic function in coeliacs. In particular, they differed from Usai's study, which reported ANS abnormalities in 45% of coeliacs affected by upper gastrointestinal symptoms<sup>[12]</sup>. Thus, we can hypothesize that ANS abnormalities may play a role in upper gastrointestinal symptoms rather than in systemic symptoms. In fact we recorded ANS abnormalities only in 1/5 patient without gastrointestinal symptoms. On the other hand, it is noteworthy that we did not find ANS abnormalities in any of the 3 patients complaining about gastrointestinal symptoms (Table 5). However, a role of ANS dysfunction in some coeliac patients cannot be excluded, since the patient No. 8 of our studied population (a 32-year-old

female), the only one with ANS dysfunction, did not show dyspeptic symptoms but only systemic symptoms.

What a role could then the autonomic neuropathy play? Can it really play a role in the systemic symptoms in coeliac disease? We knew that several coeliac patients experienced weakness or chronic fatigue in clinical practice. In most cases it was related to malabsorption (such as iron-deficiency anaemia or folic acid deficiency), but in some cases autonomic neuropathy might be suspected. Our study unfortunately failed to demonstrate a significant autonomic dysfunction in CD; however, we cannot exclude that in some patients ANS dysfunction might play a role in the genesis of some systemic symptoms, such as weakness or chronic fatigue. In fact we noted that coeliac patients showed a lower tolerance to orthostatism, associated with a latent disequilibrium of sympathetic-vagal balance, ie, a relative prevalence of the parasympathetic component of the autonomic function. These alterations, and in particular the reduced tolerance to orthostatism, may explain the above mentioned symptoms in some cases, as we noted in patient No. 8 (Table 5).

But another very finding was that ANS dysfunction did not improve in this patient after GFD. It is difficult to explain why ANS did not improve after gluten withdrawal. We speculate that ANS dysfunction may be a two step process. In the first phase it may be gluten-related, and may improve after GFD. This phase may have a variable length, probably related to age, gender and time to gluten exposure. In the second phase it may be gluten - independent, probably related to autoimmune axonal aggression to autonomic nervous system, in which autoimmunity may perpetuate the neurological damage. Recent studies of Luostarinen et al.<sup>[27]</sup> may in part confirm this hypothesis. They showed that axonal neuropathy in CD might be also subclinical without any sign of malabsorption and it often persisted despite good compliance to GFD<sup>[27]</sup>. This hypothesis may justify the persistence of the orthostatic hypotension in patient 8 after six and twelve months of GFD, and it may also explain why some coeliacs experienced persistence or recurrence of chronic fatigue despite GFD. We consider that the recurrence of systemic symptoms is related to incidental gluten ingestion from unknown sources: the autonomic neuropathy, with consequent disequilibrium of sympathetic-vagal balance may be the cause of these systemic symptoms.

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• BRIEF REPORTS •

# Detection of frameshift mutations of *RIZ* in gastric cancers with microsatellite instability

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#### Abstract

**AIM:** To study the frameshift mutations of the retinoblastoma protein-interacting zinc finger gene *RIZ* in gastric cancer with microsatellite instability, and to identify two coding polyadenosine tracts of *RIZ*.

**METHODS:** Frameshift mutations at  $(A)_8$  and  $(A)_9$  tracts of *RIZ* were detected in 70 human gastric cancer (HGC) specimens by DHPLC and DNA sequencing. Microsatellite instability (MSI) status was assessed by two mononucleotide markers, BAT26 and BAT25, by means of denaturing high-performance liquid chromatography (DHPLC).

**RESULTS:** In 70 HGC samples, 8 (11.4%) were found positive for instabilities at BAT26 and BAT25. In 7 of the 8 cases with instabilities at both BAT26 and BAT25 (MSI-H), 1 was unstable at BAT26 but stable at BAT25. Frameshift mutations were identified in 4 (57.1%) of the 7 samples with MSI-H in the (A)<sub>9</sub> tract of *RIZ* without mutations in the (A)<sub>8</sub> tract. In contrast, frameshift mutations were found in neither of the polyadenosine tracts in 63 samples of MSI-L or MSI stable tumors. Pro704 LOH detection in 4 cases with frameshift mutations did not find LOH in these cases.

**CONCLUSION:** Frameshift mutations of *RIZ* may play an important role in gastric cancers with MSI.

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#### INTRODUCTION

Two major pathways of genomic instabilities have been recently recognized, namely the chromosomal instability pathway (CIN) and the microsatellite instability (MSI) pathway (MIN), the

former is characterized by the loss of heterozygosity (LOH) whereas the latter by MSI<sup>[1]</sup>. Some genes contain repetitive regions in their coding sequences that are often targets of MSI. Gastrointestinal tumors with DNA mismatch repair (MMR) defects often present MSI and harbor frameshift mutations in coding mononucleotide repeats of cancer-related genes. MSI (+) tumors arise from the defects in the MMR system through the mechanism presumably involving frameshift mutations of the microsatellite repeats within the coding regions of the affected target genes, whose function loss is believed to contribute to tumorigenesis<sup>[2-5]</sup>.

The retinoblastoma protein-interacting zinc finger gene *RIZ* is a candidate tumor suppressor gene locus on 1p36, a region that commonly harbors alterations in many types of human cancers<sup>[6]</sup>. The interaction of *RIZ* with the retinoblastoma protein (Rb) suggests its involvement in the control of proliferation by an alternative mechanism. *RIZ* gene encodes two protein products, RIZ1 and RIZ2, which differ for a motif present in the N-terminal domain, defined as the PR domain that was previously identified as a common motif in several transcription factors. RIZ1 contains a PR domain indicative of tumor suppressor function, whereas RIZ2 does not contain this motif<sup>17,8]</sup>. RIZ1 has the capacity to induce cell cycle arrest at G<sub>2</sub>/M phase and cell apoptosis, and suppresses tumorigenicity in nude mice<sup>[9-11]</sup>. A role for *RIZ* has been recently proposed in cell cycle arrest and cell apoptosis through a transcriptional repression mechanism<sup>[7,12]</sup>.

In MSI(+) tumors, *RIZ* was found to be affected by frequent frameshift mutations of one or two coding poly(A) tract, an (A)<sub>8</sub> tract at the coding nucleotide sequence 4273-4280 and an (A)<sub>9</sub> tract at 4462-4471 in exon 8. These mutations generate truncated RIZ1 proteins lacking the COOH-terminal PR-binding motif and are expected to have serious deleterious effects on the PR domainspecific function of RIZ1. *RIZ* plays an important role in hereditary tumors of the MIN pathway as suggested by the frequent frameshift mutations in HNPCC tumors<sup>[11]</sup>. The role of *RIZ* in gastric MSI(+) tumors remains to be investigated. In this study, we used denaturing high-performance liquid chromatography (DHPLC), a highly productive method, to rapidly detect frameshift mutations of (A)<sub>8</sub> and (A)<sub>9</sub> tracts and LOH of pro704 in gastric cancer specimens and to explore the role of *RIZ* gene in gastric carcinogenesis.

#### MATERIALS AND METHODS

#### Tissue specimen and DNA extraction

Gastric cancer samples and matched adjacent normal gastric tissues were obtained from 41 male and 29 female patients during surgical resection of the tumors with informed consent from the patients at Beijing Institute for Cancer Research, Beijing Cancer Hospital. The fresh samples were collected at the time of surgery and frozen at -80 °C. The sections from each specimen were examined by a pathologist. There were 39 intestinal-type tumors and 31 diffuse-type tumors. High-molecular weight genomic DNA was extracted by standard proteinase K digestion and phenol/ chloroform extraction<sup>[13]</sup>.

#### Primers and PCR

Primers used for  $(A)_8$  tract and  $(A)_9$  tract in the *RIZ* gene amplification

were as follows: *RIZ* A8-F5'-GAGCTCAGCAAAATGTCGTC-3',*RIZ*A8-R5'-CAAGTCGGCCTTCTGCTTTG-3';*RIZ*A9-F5'-TCTCACATCTGCCCTTACTG-3',*RIZ*A9-R5'-GTGATGAGT GTCCACCTTTC-3'. The *RIZ* Pro704 deletion polymorphism was assayed by PCR followed by DHPLC. The PCR primers were: RP145 5'-CCCAAGATAAACTAACTCCT-3', RP105 5'-ACTCCATGCTGGTGAGTC-3'.

The samples used for mutation screening and sequencing were amplified in 25  $\mu$ L reaction solution containing 50 ng genomic DNA, 0.4  $\mu$ mol/L sense and antisense primers for each tract, 200  $\mu$ mol/L dNTPs (Perkin-Elmer, Foster City, CA, USA), 0.2  $\mu$ L Taq polymerase (Ampli Taq Gold: Perkin-Elmer), and 2.0 mmol/L MgCl<sub>2</sub>. After an initial activation of the enzyme by denaturation at 95 °C for 9 min, PCR amplification was performed for 35 cycles in the following sequence: at 94 °C for 30 s, at optimized annealing temperature for 45 s, and at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The annealing temperature for various primer sets was: 58 °C for *RIZ* A<sub>8</sub> tract, 60 °C for *RIZ* A<sub>9</sub> tract, and 55 °C for pro704.

#### Mutation analysis

For examining the heteroduplex content, 50-100 ng of the PCR products were subjected to DHPLC (WAVE<sup>TM</sup> system, Transgenomic, USA) under partial denaturation condition. The mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (TEAA, pH 7.0) with or without 25% acetonitrile. The flow rate used in this study was 0.9 mL/min. The column temperatures for the PCR products were 57 °C for *RIZ* (A)<sub>8</sub> tract and 56 °C for *RIZ* (A)<sub>9</sub> tract. The PCR products were heated to 95 °C for 3 min followed by cooling to 25 °C over 45 min. Homozygous mutant DNA must be combined with the wild

type at the ratio of approximately 1:1 prior to hybridization.

#### LOH analysis

The pro704 PCR products were directly used without a denaturation and reannealing process under non-denaturing conditions on the WAVE<sup>TM</sup> system. The gradient of buffer B from 1 to 7 min was 49-55%. The column temperature was 50 °C and flow rate was 0.75 mL/min.

#### DNA sequencing

The PCR product was treated with exonuclease and shrimp alkaline phosphatase based on the protocol provided by the United States Biochemical and sequenced by the Mayo Clinic DNA sequencing facility. Sequencing reactions were performed in the GeneAmp PCR System 9600 with fluorescent terminations, and the products were analyzed on an ABI 377 sequencer (Perkin-Elmer, Foster City, CA, USA). All sequence alterations were confirmed by bidirectional sequencing of the PCR products generated by at least two independent reactions.

#### MSI analysis by DHPLC

MSI analysis by DHPLC was performed as described previously<sup>[14]</sup>. Briefly, the PCR products were examined by DHPLC under fully denaturing conditions. The flow rate used in this study was 0.9 mL/min, with the column temperature of 80 °C. The gradient of buffer B from 0.1 to 7.1 min was 30-51%.

#### RESULTS

A total of 70 HGC samples and their matched normal tissues were analyzed for MSI status by two mononucleotide markers,



**Figure 1** MSI analysis of 70 paired HGCs by DHPLC. Curves at the bottom in both Panels A and B represent normal DNA as indicated by the specification on the bottom right, and the rest curves represent tumor DNA in specified cases. Column: DNASep<sup>TM</sup>; mobile phase: 0.1 mol/L TEAA (pH 7.0); linear gradient: 30-51% B in 7 min; flow rate: 0.9 mL/min; temperature: 80 °C; detection: UV at 260 nm. Panel A: DHPLC chromatograms of HGCs at BAT26; Panel B: DHPLC chromatograms of HGCs at BAT25.



**Figure 2** Typical elution profiles of DHPLC analysis and sequencing. Panel A: DHPLC elution profiles for *RIZ*(A)<sub>9</sub> tract, column temperature: 56 °C, flow rate: 0.9 mL/min, mobile phase: 0.1 mol/L TEAA (pH 7.0), linear gradient: 51-60% B in 4.5 min, detected with UV at 260 nm; Panel B: Sequence tracings for the same samples. The upper panel is a normal control, and the lower is a frameshift mutation (1-bp deletion). The arrow indicates the mutant nucleotide; Panel C: DHPLC elution profiles for pro704 LOH, column temperature: 50 °C, flow rate: 0.75 mL/min, mobile phase: 0.1 mol/L TEAA (pH 7.0), linear gradient: 49-55% B in 7 min, detected with UV at 260 nm.

BAT26 and BAT25, by means of DHPLC. In 70 HGC samples, 8 (11.4%) were found to contain sequence variation at BAT26 and BAT25 and 7 of them were shown to be unstable at both BAT26 and BAT25 (Figure 1), classified as MSI-H. One was unstable at BAT26 but stable at BAT25.

Frameshift mutations of RIZ (A)<sub>9</sub> and (A)<sub>8</sub> tract were identified. HCT-116 cell line had been studied previously and frameshift mutations were identified in the polyadenosine tracts<sup>[11]</sup>, which served as the positive control in this study. In 7 samples with MSI-H, 4 (57.1%) were found to have mutations in the (A)<sub>9</sub> tract of *RIZ* by DHPLC and DNA sequencing, including 3 intestinaltype tumors and 1 diffuse-type tumor. All of them had a 1-bp deletion (Figure 2A, B). No mutations were detected in the (A)<sub>8</sub> tract. No mutations in the (A)<sub>9</sub> or (A)<sub>8</sub> tract were found in the 63 MSI-L or MSI stable samples.

To determine whether *RIZ* was also affected by chromosomal deletion in MSI(+) tumors, pro704 LOH studies were performed on 4 samples with frameshift mutations for which the matched normal DNAs were available. We observed that both the PCR products of the tumor DNA and matched normal DNA resulted in the same peak chromatogram on DHPLC under the condition used for DNA sizing at 50 °C. LOH was not found at the *RIZ* locus in these tumors (Figure 2C).

#### DISCUSSION

Approximately 10-15% of gastrointestinal tumors are caused by defective MMR<sup>[15]</sup>, characterized by the presence of tumor MSI (MSI-H) and the absence of protein expression for any of the various genes involved in DNA MMR including hMLH1, hMSH2, hMSH6 or hPMS2<sup>[16]</sup>. Gastric cancer with an MSI-H phenotype often harbors somatic frameshift mutations in the coding mononucleotide repeats of cancer-related genes. Frameshift mutations in TGF\BRII, BAX, IGFIIR and E2F4 genes are often observed in cancers exhibiting a high frequency of MSI<sup>[17]</sup>. Recently, the new candidate tumor suppressor gene, RIZ, which may be targeted for deletion, was identified. RIZ is a protein with two alternative forms of RIZ1 and RIZ2, which differ for a PR domain present in the N-terminal domain. The PR domain is necessary for the negative regulatory function of RIZ. Frameshift mutations in either  $(A)_8$  or  $(A)_9$  tract are thought to lead to Cterminal domain loss of the RIZ protein involved in PR binding. In this study, we detected frameshift mutations in RIZ (A)<sub>9</sub> tract, whereas no mutation was found in (A)<sub>8</sub> tract. All of the frameshift mutations here found in RIZ are assumed to lead to the production of the COOH-terminal domain-truncated protein, which is likely to seriously affect RIZ1 functions.

Frameshift in short mononucleotide tracts is common in gastrointestinal tumors of the microsatellite mutator phenotype (MMP). MSI is considered a hallmark of the mutator phenotype, and determination of MSI is critical for understanding tumor biology. Separation of HGCs based on their mutator phenotypes is an effective first step to allow the distinction of these two different pathways of carcinogenesis. In the present study, we analyzed MSI status by two mononucleotide markers, and detected RIZ mutations in 4 (57.1%) of the 7 MSI-H tumors but in none of the 63 MSI-L or MSI stable gastric cancers, indicating that these mutations are specific for MSI-H tumors that exhibit a tendency to accumulate frameshift mutations in reiterated sequence of the coding regions of cancer-related genes known to facilitate cancer development and progression. These mutations may contribute to cancer progression either by inactivating their tumor suppressor functions or acting as secondary mutator mutations in MMP(+) gastric tumors<sup>[18]</sup>. Our study has shown that MSI-H gastric cancers accumulate frameshift mutations in the RIZ gene. On the basis of our findings, we suppose that RIZ is a candidate target gene in MSI tumorigenesis.

Two kinds of genetic instability, MIN and CIN, have been

documented in colorectal cancers. To determine whether *RIZ* is also affected by chromosomal deletion in MSI-H cancers, we detected the pro704 LOH in the 4 cases with frameshift mutations but failed to find LOH at the *RIZ* locus in these tumors, suggesting that *RIZ* frameshift mutations are common in MSI(+) gastric cancers, whereas LOH is not. More extensive studies on gastric cancers are needed to clarify whether *RIZ* is affected by two different ways. In MSI(+) tumors (MIN pathway), frameshift mutations in the COOH-terminal interfere with the interactions between the C terminus of the protein and its PR domain. In MSI(-) tumors (CIN pathway), mutations or deletions of the PR domain of *RIZ1* may have similar effects.

In this study, we detected MSI status and LOH by DHPLC analysis. DHPLC has been described as a novel technology for the detection of gene mutations in inherited diseases or cancers and for the identification of single nucleotide polymorphisms (SNPs)<sup>[19]</sup>. The present study demonstrates the efficacy of DHPLC in analysis of the MSI status and LOH, which allows automated examination of MSI and LOH with considerable precision at relatively low cost, without any special labeling procedure.

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BRIEF REPORTS •

### Ovarian hormone modulates 5-hydroxytryptamine 3 receptors mRNA expression in rat colon with restraint stress-induced bowel dysfunction

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#### Abstract

AIM: To examine the effects of ovarian hormone on the expression of 5-hydroxytryptamine 3 receptors  $(5-HT_3R)$  in rat colon of restraint stress-induced bowel dysfunction.

**METHODS:** Twenty-four female Sprague-Dawley rats were randomly divided into three groups of 8 each: sham operation, ovariectomy (OVX) and ovariectomy with estrogen ( $E_2$ ) and progesterone (P) replacement therapy (OVX+ $E_2$ +P). The rats were subjected to 1-h restraint stress 4 wk after operation. The changes of defecation were monitored by collection of fecal pellets. The gonadal steroids were measured in duplicate by radioimmunoassay (RIA). The expression of 5-HT<sub>3</sub>R mRNA in the colon was studied by RT-PCR.

**RESULTS:** Compared with sham group and  $OVX+E_2+P$  group, OVX group showed increase in fecal pellets and decrease in the time of vitreous pellets excretion (P<0.01). Serum levels of  $E_2$  and P were suppressed in OVX group and restored following treatment with ovarian steroids (P<0.01), and the levels of 5-HT<sub>3</sub>R mRNA in the colon of ovariectomized rats were significantly increased, the expression of 5-HT<sub>3</sub>R mRNA was significantly decreased in hormone replacement therapy group (P<0.01).

**CONCLUSION:** Ovarian hormone plays a role in the regulation of 5-HT<sub>3</sub>R expressions in restraint stress-induced bowel dysfunction of rats. The interactions between ovarian steroids and gastrointestinal tract may have major pathophysiological implications in 5-HT-related disorders, such as irritable bowel syndrome (IBS).

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#### INTRODUCTION

IBS is characterized by intermittent or continuous abdominal pain and alterations in bowel patterns<sup>[1,2]</sup>. The syndrome is one of the most common gastrointestinal (GI) disorders seen in primary care and specialist practices<sup>[3,4]</sup>. There is a female- to-male ratio of 2/l in North American population studies of IBS<sup>[5]</sup>,

and epidemiologic surveys indicate that women seek health care services for IBS more frequently than men<sup>[4,6]</sup>. In menstruating women, symptoms are influenced by menstrual cycle. Peri- and postmenopausal women have a high prevalence of altered bowel function and IBS-like gastrointestinal complaints<sup>[7]</sup>. For many women, symptoms become amplified around the time of menses<sup>[8]</sup>. However, the exact mechanism (e.g. ovarian hormones, stress response) accounting for these cyclic changes is still unknown.

Studies suggested that the female sex steroid hormones, including estrogen and progesterone, could affect the myoelectric and mechanical activity of colonic smooth muscle *in vitro*. By geometric center method, estrogen and progesterone pretreatment of ovariectomized rats resulted in a significant decrease in colonic transit compared with untreated ovariectomized rats<sup>[9]</sup>.

5-HT<sub>3</sub>R is a ligand-gated ion channel and probably involved in the modulation of colonic motility and visceral pain in the gut<sup>[10]</sup>. Clinically, 5-HT<sub>3</sub>R antagonists are important in the treatment of symptoms in IBS and more effective on diminishing bowel pattern symptom in women as compared to men<sup>[11]</sup>. Rat with wrap restraint stress is an appropriate animal model to study stress-related colonic dysfunction like IBS<sup>[12]</sup>. Previous studies have suggested that restraint stress results in an increase of fecal pellet output in rats via peripheral 5-HT<sub>3</sub> receptors<sup>[13]</sup>.

Earlier research has shown that  $E_2$  can suppress gastric motility response to thyrotropin-releasing hormone (TRH) and restraint stress in conscious rats<sup>[14]</sup>, low dose of P (1 mg/kg) increased intestinal transit while higher dose (10-20 mg/kg) had no effect. Recently, it has been demonstrated that both forskolin and 17-beta-estradiol inhibit the function of 5-HT<sub>3</sub>R in a noncompetitive manner and that this inhibition is independent of cAMP levels<sup>[15]</sup>. Based on the research mentioned above, we suggested that this change, in part, be due to the increased activity of 5-HT<sub>3</sub>R innervating these organs. Therefore, we decided to assess whether both of these sex steroids could modulate mRNA expression of 5-HT<sub>3</sub>R in rat colon with restraint stress-induced colonic dysfunction and the potential mechanisms involved. Ovariectomized rats were treated with or without E<sub>2</sub> and P in combination, after that changes in fecal pellets and the time of vitreous pellets output were assessed. Furthermore, we used RT-PCR to determine whether there was a decrease in 5-HT<sub>3</sub>R mRNA expression after treatment with sex steroids.

#### MATERIALS AND METHODS

#### Animals

Twenty-four adult female Sprague-Dawley rats (weighing 220-250 g), purchased from the Animal Department of Tongji Medical College, Huazhong University of Science and Technology were housed individually in a light and temperature controlled room with light-dark cycles of 12:12 h, where the temperature  $(24\pm2 \text{ °C})$  and relative humidity (65-70%) were kept constant. The animals were fed on a standard pellet diet, and food was withdrawn overnight before surgery and emptying experiments, but free access to water was allowed *ad libitum*. Experimental protocols followed standards and policies of the Animal Care and Use Committee, School of Medicine, Wuhan University.

#### Surgical procedures

Rats were housed for 7 d before experiments. At 6 wk of age, rats were randomly divided into three groups of eight each: sham-operated group, OVX group and OVX+ $E_2+P$  group. Under aseptic conditions, bilateral ovariectomy or sham operation was performed under general anesthesia with ketamine (100 mg/kg, ip). For OVX, two dorsolateral incisions, in the skin and the peritoneum, were made and the ovaries and uterine horn removed. Sham operations consisted of skin and peritoneum incisions. After operation, animals were housed four per cage under previous conditions. On the same day, rats in OVX+ $E_2+P$ group were subcutaneously injected a mixture of estradiol benzoate ( $E_2$ , 5 µg/d) and progesterone (P, 0.2 mg/d). Four weeks after  $E_2$  and P combination treatment, wrap restraint stress experiments were performed in the three groups.

#### Restraint stress-induced bowel dysfunction

The method described by Williams *et al.*<sup>[16]</sup> was used with slight modifications. Rats were acclimated to metal mesh cage placed on a tray for 5 h (9:00-14:00). After the acclimation, they were lightly anesthetized with ether. Then, their forelimbs were restrained with adhesive tapes (12.5 mm in width). The restrained limbs and thoracic trunk were wrapped with other adhesive tapes (50 mm in width). Immediately after those rats were returned to the cages, the number of feces dropped on the tray was counted 1 h after the wrapping. Test compounds were administered 1 h or 30 min before the restraint.

#### Hormone assays

The blood was collected from the heart of anesthetized animals and serum was separated by centrifugation at 1 500 r/min for 20 min and kept at -70 °C until assayed for  $E_2$  and P.The gonadal steroids were measured in duplicate by radioimmunoassay using kits (Tianjin Depu Biotech). Aliquots of serum were added to tubes that had been coated with antibodies to steroid hormone followed by addition of <sup>125</sup>I-hormone. Mixtures were vortexed gently and incubated for 2-3 h at room temperature. Incubation was terminated by aspirating excess of <sup>125</sup>I-hormone and tubes were counted.

#### RNA extraction and RT-PCR

Distal colon tissues were harvested from animals in various treatment groups and frozen in liquid nitrogen. Total RNA from the frozen tissues was extracted using TRIzol as described by the manufacturer's protocol (Invitrogen). RNA was dissolved in RNase-free water and the concentration was determined by measuring the optical density (A) at 260 nm. The purity of the RNA was assessed by the ratio of  $A_{260}/A_{280}$ . Two micrograms of total RNA from each sample was transcribed into cDNA using M-MLV reverse transcriptase (Promega). Briefly, total RNA was mixed with M-MLV reverse transcriptase (200 U/µL), oligo (dT)<sub>18</sub> (50 µmol/L, Sangon, Shanghai), RNase inhibitor (40 U/µL), and dNTPs (1.25 mmol/L each, TaKaRa, Dalian) in a buffer containing 37.5 mmol/L KCl, 25 mmol/L Tris-HCl, and 1.5 mmol/L MgCl<sub>2</sub> (pH 8.3) in a total volume of 25  $\mu$ L. The mixture was incubated at 42 °C for 90 min, heated to 95 °C for 5 min and cooled to 58 °C for 5 min. The resulted cDNA samples were amplified by PCR using Biometra PCR machine (Standard Power Pack P25, Germany) and the following specific primer pairs were used (SBS Genetech, Beijing): rats 5-HT<sub>3</sub>R sense, 5'-GAG ACC ATC TTC ATT GTG CAG CTG GTG CA-3'; antisense, 5'-ACA GCA GCG TGT CCA GCA CAT ATC CCA CC-3', for the 397-bp product<sup>[17]</sup>. Amplification of the rat  $\beta$ -actin gene transcript was used to control the efficiency of RT-PCR among the samples,  $\beta$ -actin sense, 5'-GTC ACC CAC ACT GTG CCC ATC T -3'; antisense, 5'- ACA GAG TAC TTG CGC TCA GGA G-3' for the 542-bp product<sup>[18]</sup>. PCR mixes contained 25 pmoL each of sense

and antisense primers, 2.0 U *Taq* DNA polymerase (Biostar), the buffer supplemented with 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L dNTPs and 1  $\mu$ L cDNA in 25  $\mu$ L. The amplification cycles were carried out under the following conditions: first denaturing at 94 °C for 3 min, then denaturing at 92 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s for a total of 35 cycles, finally a 7 min extension at 72 °C was conducted. The resulted PCR products were analyzed on 20 g/L agarose gels in TBE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8.3) containing 0.05  $\mu$ g/mL ethidium bromide. Appropriate molecular weight markers (100 bp ladder, MBI) were used to verify the required size of the final PCR product. The gels were scanned and the mean density of the products were visualized and photographed under the BIO-PROFIL (VILBER LOURMAT, France).

#### Statistical analysis

Results are expressed as mean $\pm$ SD. The statistical significance of difference was evaluated with one-way analysis of variance (ANOVA), and analyzed by SPSS 10.0. A confidence level of *P*<0.05 was considered significant.

#### RESULTS

#### Fecal pellets output

Table 1 shows the effects of  $E_2$ +P combination and blank control on restraint stress-induced fecal pellet output in rats. The number of fecal pellets counted during observation period was negligible in unrestrained normal rats. In the restraint stress rats, fecal pellet output was measured with pellet counts of  $5.6\pm0.5$  for ovarian hormone-induced sham group. The number of fecal pellets was significantly increased in OVX vehicle-treated group compared with sham and  $E_2$ +P-treated group (vehicle-treated,  $8.6\pm0.6$ ; sham group,  $5.6\pm0.5$ ;  $E_2$ +P-treated,  $4.1\pm0.5$ ; P<0.01). Meanwhile, the time of vitreous pellets output was significantly decreased (P<0.01). The number of fecal pellets was significantly decreased ( $E_2$ +P-treated,  $4.1\pm0.5$ ; sham group,  $5.6\pm0.5$ ; P<0.01) and the time of vitreous pellets output was significantly increased in  $E_2$ +P-treated group (P<0.01).

**Table 1** Effects of ovarian hormone on fecal pellets output in the ovariectomized restraint stress rats (mean $\pm$ SD, *n* = 8)

Fecal pellets (number)	Time of vitreous pellet output (min)
$5.6{\pm}0.5$	$4.8 {\pm} 0.26$
$8.6 \pm 0.6^{\rm d}$	$1.3 {\pm} 0.22^{\rm f}$
$4.1{\pm}0.5^{\rm db}$	$5.8{\pm}0.29^{\rm f}$
	Fecal pellets (number) 5.6±0.5 8.6±0.6 <sup>d</sup> 4.1±0.5 <sup>db</sup>

 $^{b}P = 0.000 vs$  OVX group;  $^{d}P = 0.000 vs$  sham-operated group;  $^{f}P = 0.000 vs$  sham-operated group.

**Table 2** Serum concentration of estrogen and progesterone (mean $\pm$ SD, n = 8)

Group	E <sub>2</sub> (pmol/L)	P (nmol/L)	
Sham-operated	104±6	26±1	_
OVX	$15.7{\pm}0.8^{\rm d}$	$3.2{\pm}0.6^{\mathrm{b}}$	
$OVX + E_2 + P$	$168\pm8^{d}$	$44\pm2^{ m b}$	

 $^{b}P = 0.000 vs$  sham-operated group;  $^{d}P = 0.000 vs$  sham-operated group.

#### Serum concentration of estrogen and progesterone

As expected, serum levels of  $E_2$  were suppressed in OVX rats and restored after treatment with ovarian steroids (shamoperated, 104±6; OVX: vehicle-treated 15.7±0.8;  $E_2$ +P-treated 168±8; P<0.01, Table 2). Serum levels of P were also decreased



**Figure 1** A: 5-HT<sub>3</sub>R mRNA expression in the colon of restraint stress rats. Lane 1: Markers, 100-bp ladder; lane 2: sham-operated group; lane 3: OVX group; lane 4: OVX+E<sub>2</sub>+P group. B: 5-HT<sub>3</sub>R mRNA expression in the colon of restraint stress rats. The bar shows the relative optical density of 5-HT<sub>3</sub>R band obtained from different groups. Values were reported as the ratio of 5-HT<sub>3</sub>R to  $\beta$ -actin signals and normalized by the corresponding RT-PCR products for  $\beta$ -actin, used as the internal control. PCR was carried out for 35 cycles. Amplified cDNA products were separated by 20 g/L agarose gel electrophoresis. <sup>b</sup>*P* = 0.000 *vs* OVX group; <sup>d</sup>*P* = 0.000 *vs* sham-operated group.

in OVX rats and increased following treatment with  $E_2$ +P (shamoperated, 26±1; OVX: vehicle-treated, 3.2±0.6;  $E_2$ +P-treated, 44±2; P<0.01).

#### Expression of 5-HT<sub>3</sub>R in the colon of restraint stressed rats

Effects of ovarian hormone on the expressions of 5-HT<sub>3</sub>R mRNA in colon tissues of restraint stress-induced rats were examined by RT-PCR as shown in Figure 1, which revealed a marked increase in OVX vehicle-treated group (relative optical density:  $1.12\pm0.07$ ). The expression of 5-HT<sub>3</sub>R mRNA in colon was increased to a maximum in OVX vehicle-treated group (sham:  $0.85\pm0.06$ , P<0.01) and significantly decreased after E<sub>2</sub>+Ptreatment ( $0.65\pm0.05$ , P<0.01, Figure 1B). Furthermore, 5-HT<sub>3</sub>R mRNA expression was decreased by 1.3-fold in E<sub>2</sub>+P-treated group (P<0.01 compared with sham group, Figure 1B). However, there was a trend of lower expression of 5-HT<sub>3</sub>R mRNA in colon with the increasing serum levels of E<sub>2</sub> and P.

#### DISCUSSION

IBS is a complex GI disorder with a poorly understood pathophysiology in which three major mechanisms interact: altered gastrointestinal motility, increased sensory function of the intestine and psychosocial factors<sup>[19]</sup>. Various observations suggested that fluctuations in sex hormones in women might have an influential role in IBS: for many women, symptoms became amplified around the time of menses<sup>[8]</sup>. In menstruating women, symptoms are influenced by menstrual cycle and bloating, abdominal pain, and diarrhea tend to be amplified during the late luteal and early menses phases<sup>[20,21]</sup>. GI symptoms increase and intestinal transit decreases during pregnancy (a time of high  $E_2$  and P levels)<sup>[22]</sup>, and rectal sensitivity is greater during menses compared with other menstrual-cycles in women with IBS<sup>[8]</sup>. The precise mechanism responsible for the changes in gastrointestinal motility is still unknown. Earlier research has shown that  $E_2$  can delay gastric emptying and GI transit in rats<sup>[23]</sup>, low dose of P (1 mg/kg, i.p.) enhanced the gastric emptying and high dose of P(5 mg/kg, i.p.) inhibited it. P(1 mg/kg) increased the intestinal transit while higher dose (10-20 mg/kg) had no effect. E<sub>2</sub> suppresses gastric motility response to thyrotropinreleasing hormone (TRH) and restraint stress in conscious rats<sup>[14]</sup>. The precise sex steroid that is responsible for these changes is controversial. It appears that sex steroids play an important role in modulating these effects as postmenopausal women being treated with sex hormone-replacement therapy (HRT) had a decreased rate of gastric emptying of solids compared with men. In contrast, postmenopausal women without hormone replacement had rates of solid emptying similar to those of men<sup>[24]</sup>. Results in pre- and postmenopausal women taking sex HRT showed they had slower gastric emptying than men<sup>[25]</sup>. Whereas

recent studies have also confirmed that HRT is associated with an increased risk of  $\mathrm{IBS}^{[26]}$ .

Stress is known to be an important factor in causing IBS, since it significantly alters bowel functions. Several rodent models of bowel dysfunction caused by restraint stress have been investigated for pharmacological analysis of a stressrelated bowel disorder like IBS<sup>[12,13,27]</sup>. There were similarities between the intestinal effects of wrap restraint stress in rats and IBS in human. Therefore, wrap restraint stress rat is an appropriate animal model to study stress-related intestinal dysfunction. The role of sex hormones in the development of IBS is the subject of ongoing study. Our results indicate that in the restraint stress rats, serum levels of E<sub>2</sub> and P were suppressed in OVX rats and restored following chronic treatment with ovarian steroids. Meanwhile, OVX vehicle-treatment significantly increased the number of fecal pellets, moreover, the time of vitreous pellets output was significantly decreased. E2+P treatment significantly decreased the number of fecal pellets and increased the time of vitreous pellets output. Our study indicated that E<sub>2</sub> and P could relieve the rat colon contractile response to restraint stress, thus decrease the number of fecal pellets and increase the number of fecal pellets output.

5-HT<sub>3</sub>R is expressed by most myenteric neurons, including those that excite gastrointestinal muscle<sup>[28-30]</sup>. In contrast to other serotonin receptor subtypes, these receptors are ligandgated ion channels involved in rapid excitatory responses in peripheral and central nervous system<sup>[31]</sup>. 5-HT<sub>3</sub>R mediates a fast inward current in myenteric neurons<sup>[29,30]</sup>, and their activation is thought to enhance cholinergic transmission via the release of acetylcholine from parasympathetic nerve terminals<sup>[13]</sup>, thus resulting in an increase in gastrointestinal motility, fluid secretion and pain. Clinically, 5-HT<sub>3</sub>R antagonists are important in the treatment of symptoms in IBS<sup>[32]</sup>.

Previous studies demonstrated that restraint stress resulted in an increase in fecal pellet output in rats fed the diet, as well as diarrhea in food-deprived rats, which are equally mediated through the endogenous activation of the 5-HT<sub>3</sub>R<sup>[13,14]</sup>. Clinically, 5-HT<sub>3</sub>R antagonist drugs appeared to more effectively diminish bowel pattern disruption in women with IBS as compared to men<sup>[11]</sup>. Recently, it has been demonstrated that both forskolin and 17-beta-estradiol inhibit the function of 5-HT<sub>3</sub>R in a noncompetitive manner and that this inhibition is independent of cAMP levels<sup>[15]</sup>. Because 5-HT<sub>3</sub>R plays a role in the pathogenesis of IBS, it is possible that the attenuated development of IBS in female rats might be related to the modulation of gonadal hormones on 5-HT<sub>3</sub>R and GI system. This study focused on the effects of gonadal steroid hormones on 5-HT<sub>3</sub>R mRNA expression in colon of wrap restraint stress rats. We observed an increase in 5-HT<sub>3</sub>R gene expression in OVX rats. The increase in 5-HT<sub>3</sub>R gene

expression was prevented by treating the OVX rats with a combination of  $E_2$  and P. These results suggest that 5-HT<sub>3</sub>R mRNA expression is sensitive to the absence of  $E_2$  and P. Their roles in regulation of 5-HT<sub>3</sub>R mRNA expression deserve further study. If the increase in the density of 5-HT<sub>3</sub>R gene expression induced by  $E_2$  and P deficiency reflects overactivity of 5-HT<sub>3</sub>R, then such changes may be relevant to hormone- and age-related GI dysfunction.

In conclusion, we suggest that female gonadal hormones may play an important role in regulation of colon 5-HT<sub>3</sub>R. Exploration of 5-HT<sub>3</sub>R expression by gonadal steroid hormones could contribute to a better understanding of the interactions between ovarian steroids and GI and may have major pathophysiological implications for 5-HT-related bowel disorders, such as IBS.

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• BRIEF REPORTS •

# Meta-analysis of intraperitoneal chemotherapy for gastric cancer

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# Abstract

**AIM:** To assess the efficacy and safety of intraperitoneal chemotherapy in patients undergoing curative resection for gastric cancer through literature review.

**METHODS:** Medline (PubMed) (1980-2003/1), Embase (1980-2003/1), Cancerlit Database (1983-2003/1) and Chinese Biomedicine Database (1990-2003/1) were searched. Language was restricted to Chinese and English. The statistical analysis was performed by RevMan4.2 software provided by the Cochrane Collaboration. The results were expressed with odds ratio for the categorical variables.

**RESULTS:** Eleven trials involving 1 161 cases were included. The pooled odds ratio was 0.51, with a 95% confidence interval (0.40-0.65). Intraperitoneal chemotherapy may benefit the patients after curative resection for locally advanced gastric cancer, and the combination of intraperitoneal chemotherapy with hyperthermia or activated carbon particles may provide more benefits to patients due to the enhanced antitumor activity of drugs. Sensitivity analysis and fail-safe number suggested that the result was comparatively reliable. However, of 11 trials, only 3 studies were of high quality.

**CONCLUSION:** Intraperitoneal chemotherapy after curative resection for locally advanced gastric cancer may be beneficial to patients. Continuous multicenter, randomized, double blind, rigorously designed trials should be conducted to draw definitive conclusions.

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# INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide. Surgery is the main choice of radical treatment, however, even for patients with apparently completely resection, the five-year survival rate is about 30-60%, which has been disappointing. Peritoneal metastasis is the most common type of recurrence after surgery and has the worst prognosis in patients with advanced stomach cancer. To improve the survival of the patients with gastric cancer, intraperitoneal chemotherapy (IPT) as a possible effective treatment for peritoneal dissemination in locally advanced gastric cancer has been investigated clinically, but the results among these studies are still different and disputed. To evaluate whether IPT benefited the patients undergoing radical resection for locally advanced gastric cancer, we summarized all the available randomized trials and combined the results for meta-analysis.

# MATERIALS AND METHODS

#### Materials

Medline (PubMed) (1980-2003/1), Embase (1980-2003/1), Cancerlit Database (1983-2003/1) and Chinese Biomedicine Database (1990-2003/1) were searched. Languages were restricted to Chinese and English.

#### Inclusion and exclusion criteria

To be included, trials had to be randomised and controlled. Trials could be single-blind, double-blind or not blind. Chemotherapy groups were treated intraperitoneally, including intraperitoneal chemotherapy with or without activated carbon particles (CH), intraperitoneal hyperthermic perfusion. No oral or intravenous chemotherapy, chemoimmunotherapy, or radiotherapy were used. Those receiving gastrectomy alone were included in control group. All patients must have had a potential curative surgery for locally advanced gastric cancer.

Data extracted included the baseline data of intraperitoneal chemotherapy group and control group, assessments of eligibility and trial quality, number of survival and death and the statistical consideration.

# Methods

**Data collection** The content terms of stomach neoplasms, intraperitoneal chemotherapy and surgery, and the methodological terms of clinical trial, phase III, randomized trial, double blind method were used. These searches were supplemented by hand searching of the reference lists of identified trials and review articles. Two reviewers assessed independently the outcome data using a predesigned strategy. They also evaluated study quality using Jadad-scale<sup>[1]</sup> plus allocation concealment. Intention-to-treat analyses were also performed. Agreement was achieved through discussion when they have different evaluations.

#### Statistical analysis

The results of eligible trials were pooled using RevMan4.2 software provided by the Cochrane Collaboration. The result was expressed with odds ratio (OR) for the categorical variables. *Q* statistic test was used for gross statistical heterogeneity. Sensitivity analysis was performed by excluding the trials in which Jadad-scale was low. Publication bias was assessed by calculating the Rosenthal s fail-safe number. Subgroup analysis was performed by collecting summaries for subsets of studies with different characteristics.

# RESULTS

# Study selection

There was good agreement between the two reviewers on the eligibility, quality scores and data extraction of analysis. Eighteen

Author	Country	Publication time	Chemotherapy regimens	IPT (number of d	Surgery eath/total)	Follow-up (mo)	Quality score
Rosen HR	Austria	1998	MMC+Carbon	23/46	27/45	36	4
Sautner T	Austria	1994	Cisplatin	29/33	31/34	60	3
Takahashi T	Japan	1995	MMC+Carbon	35/56	46/57	36	3
Yu W	Korea	2001	MMC+5-Fu	55/125	72/123	60	2
Yonemura Y	Japan	2001	MMC+CDDP	44/92	27/47	60	1
Hamazoe R	Japan	1993	MMC	18/42	22/40	60	1
Chen ZX	China	1996	DDP	8/25	13/21	18	1
Zhang WS	China	1998	5-Fu	14/37	15/26	36	1
Fujimoto S	Japan	1998	MMC	27/71	36/70	96	1
Tan CQ	China	2000	MMC	2/22	11/29	36	1
Gao ZA	China	2002	MMC+DDP+HCPT	17/60	31/60	36	2

Table	e 1	Data	from	11	rand	lomized	l control	led	trials	5

Review: Intraperitoneal chemotherapy after curative resection for gastric cancer: meta- analysis of randomized trials Compariso: 01 chemotherapy *vs* surgery Outcome: 01 OR

Study or	Chemotherapy	Surgery	OR (fixed)	Weight	OR (fixed)
sub-category	n/N	n/N	95% Cl	%	95% Cl
Hamazoe R	18/42	22/40		7.14	0.61 [0.26, 1.47]
Sautner T	29/33	31/34		2.05	0.70 [0.14, 3.41]
Takahashi T	35/56	46/57		9.47	0.40 [0.17, 0.93]
Chen ZX	8/25	13/21		5.32	0.29 [0.09, 0.98]
Rosen HR	23/46	27/45		7.56	0.67 [0.29, 1.53]
Zhang WS	14/37	15/26	<b>e</b>	6.07	0.45 [0.16, 1.24]
Fujimoto S	27/71	36/70	<b>B</b>	12.45	0.58 [0.30, 1.13]
Tan CQ	2/22	11/29		4.78	0.16 [0.03, 0.84]
Yu W	55/125	72/123	<b>B</b>	22.52	0.56 [0.34, 0.92]
Yonemura Y	44/92	27/47		10.33	0.68 [0.33, 1.38]
Gao ZA	17/60	31/60	<b>B</b>	12.31	0.37 [0.17, 0.79]
Total (95% Cl)	609	552	•	100.00	0.51 [0.40, 0.65]
Total events: 272 (che	emotherapy), 331 (surg	gery)			
Test for heterogeneity	y: Chi?= 5.38, df = 10 (	P = 0.86), I?= 0%			
Test for overall effect	$z = 5.39 \ (P < 0.00001)$				
			0.1 0.2 0.5 1 2 5	10	

Favours chemotherapy Favours surgery

#### Figure 1 Odds ratio for 11 randomized controlled trial.

Review: Intraperitoneal chemotherapy after curative resection for gastric cancer: meta- analysis of randomized trials Compariso: 01 chemotherapy *vs* surgery Outcome: 01 OR

Study or sub-category	Chemotherapy n/N	Surgery n/N	OR (fixed) 95% Cl	Weight %	OR (fixed) 95% Cl
Sautner T	29/33	31/34		3.08	0.70 [0.14, 3.41]
Takahashi T	35/56	46/57		17.57	0.40 [0.17, 0.93]
Rosen HR	23/46	27/45		14.03	0.67 [0.29, 1.53]
Yu W	55/125	72/123		41.77	0.56 [0.34, 0.92]
Gao ZA	17/60	31/60	<b>_</b> _	22.83	0.37 [0.17, 0.79]
Total (95% Cl)	320	319	•	100.00	0.51 [0.36, 0.71]
Total events: 159 (cl	nemotherapy), 207 (surg	gery)	-		
Test for heterogenei	ty: Chi?= 1.69, df = 4 (P	<b>P</b> = 0.79), I?= 0%			
Test for overall effe	ct: Z = 3.99 ( <i>P</i> <0.0001)				

0.1 0.2 0.5 1 2 5 10

Favours chemotherapy Favours surgery

Review: Intraperitoneal chemotherapy after curative resection for gastric cancer: meta- analysis of randomized trials Compariso: 01 chemotherapy *vs* surgery Outcome: 01 OR

Study or sub-category	Chemotherapy n/N	Surgery n/N		OF 9	R (fixed 5% Cl	l)			Weight %	OR (fixed) 95% Cl
Sautner T	29/33	31/34							10.75	0.70 [0.14, 3.41]
Takahashi T	35/56	46/57				-			49.63	0.40 [0.17, 0.93]
Rosen HR	23/46	27/45		-		+			39.62	0.67 [0.29, 1.53]
Total (95% Cl)	135	136				•			100.00	0.51 [0.31, 0.93]
Total events: 87 (che	emotherapy), 104 (surge	ery)								
Test for heterogenei	ity: Chi?= 0.84, df = 2 (P	<b>P</b> = 0.66), I?= 0%								
Test for overall effe	ct: $Z = 2.20 \ (P = 0.03)$									
			0.1	0.2	0.5	1	2	5	10	

Favours chemotherapy Favours surgery

Figure 3 Sensitivity analysis 2 (by excluding the low quality trials).

random trials including surgery plus intraperitoneal chemotherapy, preliminary surgery alone were analysed, from which 7 reports were excluded for repetitive studies. Table 1 shows the details of the 11 trials<sup>[2-12]</sup> included in the analysis with a total enrollment of 1 161 patients, in which 609 patients were assigned to the treatment group and 552 to the control group. The average sample size was 106 patients. Of 11 trials, three studies were of high quality according to the Jadad-scale (with three scores), one trial mentioned double-blind design and sample-size calculation, and two studies described intention-to-treat analysis. The fail-safe number of 104 suggested that no important publication bias existed in this meta-analysis.

#### Meta-analysis

Figure 1 shows the result of the meta-analysis. There was no statistically significant heterogeneity in our analysis, so a fixed effect model was used and the odds radio was 0.51 (95% CI 0.40 to 0.65). The sensitivity analysis was performed by excluding trials with Jadad-scale scores between 1 and 3 and revealed the same difference between intraperitoneal chemotherapy and surgery alone (odds ratio 0.51, 95% CI 0.36 to 0.71: odds ratio 0.54, 95% CI 0.31 to 0.93) (Figures 2, 3).

#### Subgroup analysis

As shown in Table 2, intraperitoneal hyperthermic chemoperfusion (IHCP) or chemotherapy with activated carbon particles (CH) produced more benefits to patients than those without hyperthermia or CH; the group of trials from Asian countries exhibited a trend towards a more significant effect than those from non-Asian countries, which showed no effect with IPT; for trials with more than 5 years of follow-up, the effects were less obvious than those of shorter follow-up, if indicated that IPT might afford long-term survival by delaying relapse and recurrence.

#### Table 2 Subgroup analysis

Characteristic	No. of trials	OR	95% CI
IPT (without CH and IHCP)	2	0.57	(0.35-0.92)
IPT (only with CH)	2	0.52	(0.29-0.94)
IHCP	7	0.48	(0.35-0.67)
Follow-up time (mo) <60	6	0.40	(0.27-0.59)
Follow-up time (mo) $\geq 60$	5	0.60	(0.44-0.82)
Asia	9	0.49	(0.38-0.64)
Non-Asia (Austria)	2	0.67	(0.32-1.41)

IPT: intraperitoneal chemotherapy; CH: carbon particles; IHCP: intraperitoneal hyperthermic chemoperfusion.

#### Side effect analysis

All studies described the side effects of medicine, including anastomotic leakage, leukocytopenia, fever, intestinal obstruction, fistula, intraabdominal bleeding, and prolonged abdominal pain, *etc.* Of 11 trials, 5 had mild complications; 3 had no significant differences in adverse effects between the surgery group and the chemotherapy group; 2 trials produced complications in the chemotherapy group: one was bowel fistula, the other was intraabdominal abscess which turned better after effective treatment. One trial from Austria reported serious side effects by IPT, the complications of the treatment group and control group were respectively 35% and 16%, the death rates, 11% and 2%, which terminated the trial ahead of schedule.

#### DISCUSSION

IPT is applied to kill residual tumor cells left behind during surgery, which can not be achieved by the intravenous approach, especially for stage 3 and 4 gastric carcinomas. It is of much importance to extirpate the free tumor cells in the abdominal cavity and micrometastases on the peritoneal surface to attain longer survival. Animal trials and phase II clinical trials have revealed that IPT could be effective to prevent peritoneal dissemination and liver metastasis<sup>[13]</sup>.

The aim of meta-analysis is to provide a comprehensive, up-to-date summary of average effect of all the relevant randomized controlled trials, to provide reliable guidance for clinical practice and future research<sup>[14]</sup>. Based on our results, IPT may benefit the patient after curative resection for locally advanced gastric cancer, and the combination of IPT with hyperthermia or activated carbon particles may provide more benefit to patients due to the enhanced antitumor activity of drugs<sup>[15]</sup>. Sensitivity analysis and fail-safe number suggested that the results were comparatively reliable.

Two trials from Austria showed that intraperitoneal chemotherapy was not beneficial to patients, one of them terminated ahead of time because of serious adverse effects. Nine Asian studies (from China, Japan, Korea) confirmed a significant survival benefit for patients with tolerable side effects of antitumor drugs. Considering the difference between western studies and Asian studies<sup>[16,17]</sup>, we speculate that they may have different aetiology or biology methods<sup>[18]</sup>.

The adverse effect was an important factor to influence the result of intraperitoneal chemotherapy. However, only 4 trials described the side effects of medicine on the basis of World Health Organization classification, and the different forms of illustration made it difficult to analyze the effect according to evidence based medicine. There fore, we should observe and describe the toxicity of medicine by WHO standard in future clinical research.

Of included 11 trials, only 3 trials were of high quality (Jadadscale with 3 scores) and the other 8 trials were of low quality, which weakened our evidence. On the other hand, reliance on published trials alone might distort the outcome of meta-analysis, because positive studies were more likely to be published than negative ones.

At present, the treatment effect of gastric cancer is still disappointing. Many surgeons hold that the stomach cancer patients cannot benefit from IPT, which is different from our meta-analysis results. To draw definitive conclusions, move effective multicenter, randomized, double blind, rigorously designed trials are needed.

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• BRIEF REPORTS •

# Diallyl disulfide-induced G2/M arrest of human gastric cancer MGC803 cells involves activation of p38 MAP kinase pathways

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# Abstract

**AIM:** To determine the role of p38 MAP kinase signal transduction pathways in diallyl disulfide (DADS)-induced G2/M arrest in human gastric cancer MGC803 cells.

**METHODS:** MGC803 cell growth inhibition was measured by MTT assay. Phase distribution of cell cycle was analyzed by flow cytometry. Expression of Cdc25C, p38, phosphorylation of p38 (pp38) were determined by Western blotting.

**RESULTS:** MTT assay showed that SB203580, a specific p38 MAPK inhibitor blocked DADS-induced growth inhibition. Flow cytometry analysis revealed that treatment of MGC803 cells with 30 mg/L DADS increased the percentage of cells in the G2/M phase from 9.3% to 39.4% (*P*<0.05), whereas inhibition of p38 activity by SB203580 abolished induction of G2/M arrest by DADS. Western blotting showed that phosphorylation of p38 was increased 3.52-fold following treatment of MGC803 cells with 30 mg/L DADS for 20 min (*P*<0.05), whereas Cdc25C was decreased 68% following treatment of MGC803 cells with 30 mg/L DADS for 24 h (*P*<0.05). Decreased Cdc25C protein expression by DADS was attenuated by SB203580 (*P*<0.05).

**CONCLUSION:** DADS-induced G2/M arrest of MGC803 cells involves activation of p38 MAP kinase pathways. Decreased Cdc25C protein expression by p38 MAPK played a crucial role in G2/M arrest after treatment with DADS.

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# INTRODUCTION

Unlimited and uncontrolled cell proliferation is obviously characteristics of tumor cells<sup>[1,2]</sup>. Given that disruption of cell cycle plays a crucial role in cancer progression<sup>[3]</sup>, its modulation

by phytochemicals seems to be a logical approach in controlling carcinogenesis. Thus cell cycle regulation and its modulation by various natural (plant-derived) and synthetic agents are gaining widespread attention in recent years<sup>[4-18]</sup>. Most of the plant products with anticancer activity are strong antioxidants and some of them are effective modulators of protein kinase/ phosphatases that are associated with cell cycle regulation. DADS is a major component of cooked garlic and oil-soluble organosulfur compound in processed garlic, which inhibits the proliferation of human breast, hepatoma, lung, bladder, colon cancer cells and human leukemia HL-60 cells<sup>[19-25]</sup>. Previous studies showed that the ability of DADS to suppress HCT-15, HT-29 cell proliferation was related to its propensity to induce a G2/M arrest<sup>[23,24]</sup>. However, the molecular mechanisms by which DADS exerts its effects on tumor cells leading to inhibition of cell growth and induction of G2/M arrest are largely unknown. p38 MAP kinase (p38) is a member of the mitogen-activated protein (MAP) kinase signaling cascade which has been shown to regulate a variety of cellular events such as cell proliferation, differentiation, and apoptosis<sup>[26-28]</sup>, and may therefore be a potential target of DADS action. Gastric cancer is one of the most common malignant tumors in China<sup>[29-30]</sup>. Our previous studies showed that DADS could inhibit human gastric cancer MGC803 cell growth<sup>[31]</sup>. In this study, signaling pathways of p38 MAPK-induced G2/M arrest in DADS treated MGC803 cells were investigated for their involvement in the mechanisms of DADS-induced growth inhibition.

# MATERIALS AND METHODS

# Materials

Human gastric cancer cell line MGC803 was purchased from Cancer Research Institute of Hunan Medical University. DADS was purchased from Fluka Chemika (Ronkonkoma, NY). Monoclonal anti-p38, anti-pp38 antibodies and SB203580 were purchased from Cell Signaling (Beverly, MA). Polyclonal anti-Cdc25C antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

# Methods

Cell culture and MTT assay MGC803 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 100 mL/L fetal bovine serum (Sijiqing Co.1 Hangzhou) with addition of 100 U/mL penicillin, 100 U/mL streptomycine. Cells in suspension (50  $\mu$ L) were added to each well of a 96-well culture plate and incubated for 24 h at 37 °C in a humidified atmosphere of 50 mL/L CO<sub>2</sub> in air. The 96-well culture plate was divided into 4 sections, with one section being treated by culture media, the others were treated by one of the followings at 50 µL: culture media containing 2×reagents (10 µmol/L SB203580, 30 mg/L DADS, 30 mg/L DADS+10 µmol/L SB203580). In the latter group, SB203580 was added 1 h prior to DADS. The cultures were again incubated as above. After 72 h, 20 µL 5 g/L MTT solution was added to each well, and the cultures were further incubated in 100 µL DMSO solution. A microplate reader was used to measure absorbance at 570 nm for each well. Growth inhibition rate was calculated as follows: growth inhibition rate =  $(1 - A_{570/nm})$ of treated cells/ $A_{570/nm}$  of control cells)×100%.

Cell cycle analysis Cells were incubated in culture media alone or culture media containing reagents (10  $\mu$ mol/L SB203580, 30 mg/L DADS, 30 mg/L DADS+10  $\mu$ mol/L SB203580), at 37 °C for 24 h. In the latter group, SB203580 was added 1 h prior to DADS. Cells were harvested in cold PBS, fixed in 700 mL/L ethanol, and stored at 4 °C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and suspended in 1 mL of PI staining reagents (20 mg/L ribonuclease and 50 mg/L propidium iodide). Samples were incubated in the dark for 30 min before cell cycle analysis. The distribution of cells in the cell cycle was measured by a flow cytometer.

Western blotting MGC803 cells treated with different reagents were harvested, rinsed twice with cold PBS, and incubated in the lysis buffer containing 50 mmol/L Tris (pH7.5), 150 mmol/L NaCl, 10 mL/L Np-40, 1 g/L SDS, 10 g/L sodium deoxycholate, 1 mmol/L DTT, 1 mmol/L PMSF, 2.5 mg/L leupeptin, 25 mg/L aprotinin on ice for 20 min. Following the centrifugation at 12 000 g for 30 min at 4 °C. The amount of protein in the supernatant was determined using BCA protein assay reagent. Equal amount of protein sample was completely vortexed with 2×SDS-gel buffer, and boiled for 5 min at 100 °C to dissolve the bound proteins. The samples were segregated on 100 g/L SDSacrylamide gel, transferred onto a nitrocellulose membrane and blocked with 50 g/L defatted milk, then probed with different primary antibodies. Anti-mouse or anti-rabbit IgG conjugated peroxidase was as a secondary antibody. The filters were then incubated in SuperSignal ECL-HRP detection reagent for 1 min followed by exposure to X-ray film.

#### Statistical analysis

Results were analyzed by SPSS10.0 statistical software. Data were expressed as mean $\pm$ SD. Comparisons between different groups were made by one-way ANOVA (with LSD for post hoc analysis) or  $\chi^2$  test. *P*<0.05 was taken as statistically significant.

#### RESULTS

#### Effect of reagents on growth inhibition of MGC803 cells

As shown in Table 1, 30 mg/L DADS suppressed MGC803 growth by 58.6% (P<0.05). SB203580 10 µmol/L alone slightly reduced cell growth, In contrast, SB203580 blocked DADS-induced growth inhibition (P<0.05). Thus, addition of SB203580 to the cells decreased the inhibitory ability of DADS to MGC803 cells growth.

Table 1 A<sub>570nm</sub> of MGC803 cells exposed to reagents

<i>n</i> = 12	Control	SB203580 (10 μmol/L)	DADS (30 mg/L)	DADS (30 mg/L) +SB203580 (10 µmol/L)
χ	0.78	0.076	0.32	0.53
S	0.038	0.035	0.028	0.024
IR (%)		3.4	58.6ª	31.7ª

<sup>a</sup>*P*<0.05 vs control; IR: inhibition rate.

**Table 2** Distribution of MGC803 cells in cell cycle (%)

	Control	SB203580 (10 μmol/L)	DADS (30 mg/L)	DADS (30 mg/L) +SB203580 (10 µmol/L)
G1	$62.5{\pm}0.9$	62.8±1.3	33.7±1.2	54.0±1.5
S	$28.2{\pm}1.2$	$27.3{\pm}0.8$	$27.9{\pm}0.8$	$24.8{\pm}1.1$
G2	$9.3{\pm}0.8$	$9.9{\pm}1.1$	$39.4{\pm}1.3^{a}$	$21.2{\pm}0.9^{\rm a}$

<sup>a</sup>P<0.05 vs control.

Effect of reagents on cell cycle distribution of MGC803 cells Flow cytometry revealed that the proportion of cells in the G2/M phase after treatment with 30 mg/L DADS for 24 h was 39.4%, three times more than that in untreated cells (9.3%). SB203580 10  $\mu$ mol/L alone had no effect on cell growth (*P*>0.05). But inhibition of p38 activity by SB203580 abrogated induction of G2/M arrest by DADS. MGC803 cells treated with DADS in the presence of SB203580 decreased the G2/M phase to 21.2%, compared to 39.4% by DADS alone (*P*<0.05).

# Expression of p38, pp38, and Cdc25C after reagents treatment of MGC803 cells

Western blotting revealed that phosphorylation of p38 was increased 3.52-fold following treatment of cells with 30 mg/L DADS for 20 min. At the same time, the total p38 amount did not change. Furthermore, DADS-induced-phosphorylation of p38 was completely inhibited by SB203580 (10  $\mu$ mol/L) (Figure 1).



**Figure 1** Expression of p38, phosphorylation of p38 proteins in MGC803 cells following treatment of reagents for 20 min.

DADS treatment for 24 h decreased the level of Cdc25C by 68%, and pretreatment of MGC803 cells with SB203580 partially reversed the down-regulation of Cdc25C level by DADS. In contrast, SB203580 alone had no significant effect on Cdc25C expression (Figure 2).



**Figure 2** Expression of Cdc25C protein in MGC803 cells following treatment of reagents for 24 h.

#### DISCUSSION

Mitogen-activated protein kinase (MAPK) pathway has a central role in transducing extracelluar signals into cellular responses. p38 kinase is a member of the mitogen-activated protein kinase family that is activated by a variety of environmental stress. Rapid initiation of G2 arrest after UV radiation is mediated by p38 kinase<sup>[32]</sup>. Inhibition of p38 blocks the rapid initiation of G2 delay in both human and murine cells after ultraviolet radiation. p38 kinase is responsible for rapid initiation of the G2 delay in IME cells after the hypertonic stress created by addition of NaCl<sup>[33]</sup>. Inhibition of p38 kinase blocks the rapid initiation of this checkpoint both in an immortalized cell line (mIMCDs) and in second-passage IME cells from mouse inner medulla. Genistein-induced G2/M arrest is associated with the activated p38 mitogen-activated protein kinase<sup>[34]</sup>. Thus p38 is a critical event for initiating the G2/M checkpoint and inducing G2/M arrest. Our research showed that DADS-induced MGC803 cells

G2/M arrest and growth inhibition correlated with increased p38 phosphorylation. We used SB203580, a specific inhibitor of p38 to address the potential role of p38 kinase in the regulation of cell-cycle progression. Inhibition of p38 activity by SB203580 abolished induction of G2/M arrest by DADS. Therefore, our research data demonstrated that DADS- induced G2/M arrest of MGC803 cells involved activation of p38 MAP kinase pathways.

p34<sup>cdc2</sup> is the key regulator of cell-cycle progression through G2-M<sup>[35]</sup>. In particular, activation of p34<sup>cdc2</sup> kinase activity is required for progression from G2 to M. phosphorylation of the inhibitory residues Thr14/Tyr15 of p34<sup>cdc2</sup> leads to decreased kinase activity and subsequent arrest at the G2/M phase<sup>[36]</sup>. The Cdc25C protein phosphatase is a key regulator of  $p34^{cdc2}$ phosphorylation status and kinase activity by dephosphorylating Thr14/Tyr15 residues<sup>[37]</sup>. In vitro, p38 binds and phosphorylaties Cdc25C at serine 216<sup>[32]</sup>. Phosphorylation of Cdc25C triggers cell-cycle arrest by the sequestration of Cdc25C by 14-3-3<sup>[38]</sup>. Frey et al. found that p38 was involved in genistein-induced G2/M arrest and down-regulation of Cdc25C expression in immortalized human mammary epithelial cell line MCF-10F<sup>[35]</sup>. Hepatitis B virus X protein (pX) is implicated in hepatocarcinogenesis by an unknown mechanism. Research data<sup>[39]</sup> showed that pX-dependent activation of p38 MAPK inactivated Cdc25C by phosphorylation of Ser216, thus initiating activation of the G2/M checkpoint, resulting in 4pX-1 cell growth retardation. These data suggest that p38 participation in down-regulation of the Cdc25C level may be an important way to impair its actions and an important event in G2/M checkpoint regulation. In the present studies, decreased Cdc25C protein phosphatase by DADS was attenuated by SB203580. Thus it indicates that regulation of Cdc25C protein expression by p38 is a critical event for G2/M arrest after treatment with DADS.

In summary, DADS-induced G2/M arrest and growth inhibition of MGC803 cells involves activation of p38 MAP kinase pathways. Decreased Cdc25C protein expression by p38 is a critical event for G2/M arrest by DADS. However, it should be noted that although p38 inhibitor SB203580 abrogated DADS-induced G2/M arrest, the reversal was not total. SB203580 could not completely abolish induction of G2/M arrest by DADS. This implies that p38 activation is not the sole prerequisite for DADS induced G2/M arrest, other mechanisms may be involved in G2/M arrest Additional studies are needed to clarify these mechanisms. A deeper understanding of the molecular mechanisms involved in the regulation of cell cycle control is very important for the development of novel anticancer strategies.

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BRIEF REPORTS •

# ADAM17 mRNA expression and pathological features of hepatocellular carcinoma

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# Abstract

**AIM:** To study the expression of a disintegrin and metalloproteinase 17 (ADAM17) mRNA in hepatocellular carcinoma (HCC) and to evaluate the relationship between ADAM17 mRNA expression and clinicopathological features of HCC.

**METHODS:** Hepatocellular carcinomas (HCC) from 31 cases were divided into small HCC (SHCC), nodular HCC (NHCC) and solitary large HCC (SLHCC) according to tumor diameter and the number of nodes. ADAM17 mRNA expressions were compared among those groups by means of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The relationship between ADAM17 mRNA expression level and clinicopathological features of HCC was evaluated.

**RESULTS:** NHCC had lower differentiation and was more frequently of microvascular invasion (10/12) than SHCC (3/11) and SLHCC (3/8) (*P*<0.05), but no statistical difference was observed between SHCC and SLHCC comparing their clinicopathological features. ADAM17 mRNA expression was detected in 77.4% (24/31) of HCC tissues and was significantly higher than that in paired non-cancerous liver tissues in which only 35.5% (11/31) of the samples were detected of the expression (P<0.05). The expression of ADAM17 mRNA was much higher in NHCC than in SHCC and SLHCC (P<0.05), while no significant difference was discovered between SHCC and SLHCC. The quantities of ADAM17 mRNA were significantly higher in poorly differentiated HCC than in well or moderately differentiated HCC, but no statistical difference was found concerning liver cirrhosis, tumor capsule formation or microvascular invasion of the cancer.

**CONCLUSION:** The increased expression of ADAM17 may play a key role in the development of HCC. The expression levels of ADAM17 mRNA varied among different pathological types of HCC. Lower mRNA expression of ADAM17 mRNA in SLHCC may be associated with the better molecular pathological features of SLHCC.

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# INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases in the world. Approximately 560 000 new cases of HCC are diagnosed each year, constituting 6% of all new human cancers<sup>[1]</sup>. The HCC mortality rate in China is approximately 20.4/100 000 and is the second leading cause of cancer death among Chinese males<sup>[2]</sup>. The prognosis of large hepatocellular carcinoma (LHCC) is generally considered to be worse than small hepatocellular carcinoma (SHCC), but longterm survival of some LHCC, especially solitary large hepatocellular carcinoma (SLHCC), could be frequently observed after curative resection<sup>[3-5]</sup>. From the clinical observation and our research, we hypothesized that SLHCC was of relatively better biological behavior<sup>[2]</sup>. Furthermore, we have preliminarily proved our hypothesis in a series of researches by showing that SLHCC was of relatively better pathological features and surgical prognosis<sup>[2,3,6]</sup>. Although many systemic studies have been performed, the unique clinical and molecular pathological features of SLHCC are still far from a clear deep understanding and need further investigation.

A disintegrin and metalloproteinase 17 (ADAM17) is a kind of transmembrane metalloproteinase<sup>[7]</sup>, which can cleave the ectodomain of many transmembrane proteins. The substrates of ADAM17 mediated cleavage include tumor necrosis factor-a (TNF- $\alpha$ ), tumor necrosis factor receptor type I, (TNFR I) and tumor necrosis factor receptor type II (TNFR II), interleukin1 receptor type II (IL1R II), Notch receptor, L-selectin, mucin1 (MUC1), CD30, tumor necrosis factor-related activationinduced cytokine (TRANCE) and many ligands of epidermal growth factor receptor (EGFR), such as transforming growth factor- $\alpha$  (TGF- $\alpha$ ), heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin and so on<sup>[8-19]</sup>. Hassan reported that the oxygen radicals generated by smoke stimulated ADAM17 to cleave transmembrane amphiregulin. The binding of amphiregulin to EGFR then promoted proliferation of lung cancer cells<sup>[20]</sup>. The cleavage function of ADAM17 was required for the activation of EGFR by TGF- $\alpha$  in Hela cells, and the high expression level of ADAM17 in mammary tumors was correlated with high activation rate of EGFR<sup>[21]</sup>. Another experiment showed that single metastatic lung cancer cell in bone marrow presented overexpression of ADAM17<sup>[22]</sup>. All these suggested that ADAM17 played a key role in the development of some cancers. It is well known that the EGFR mediated pathway played an important role in HCC development<sup>[23,24]</sup>. The overexpression of EGFR and its ligands were observed in HCC tissue and were related to the prognosis of HCC patients<sup>[25,26]</sup>. However, much less is known about the mechanisms involved in signal transduction, which activate the EGFR in HCC. It is even not sure whether there is an increased activation rate of EGFR in HCC besides the overexpression of EGFR and its ligands. As ADAM17 played an important role in the activation of EGFR<sup>[27]</sup> and up to now, there is no report about the expression and function of ADAM17 in HCC, so to have a better understanding of the molecular mechanisms involved in HCC development and the unique molecular pathological features of SLHCC, we studied the expression of ADAM17 in HCC and paired non-cancerous liver tissues.

# MATERIALS AND METHODS

# Materials

Thirty-one fresh HCC specimens (26 from males and 5 from females) were obtained by surgical resection in Xiangya Hospital, Central South University between May 2002 and February 2003. Tissues 1 cm away from the edge of the cancer were used as the paired non-cancerous control. The tissues used for RNA extraction were immediately snap-frozen in liquid nitrogen and stored at -70 °C. The specimens used for pathological study were fixed with 10% formalin, dehydrated by conventional methods, embedded in paraffin, cut into slices of 5  $\mu$ m thick and stained by haematoxylin and eosin.

The specimens were divided into SHCC group (largest diameter less than 5 cm for single tumor nodule or the sum of diameters less than 5 cm for two tumor nodules, n = 11), SLHCC group (single tumor nodule with largest diameter more than 5 cm, n = 8) and NHCC group (two or more tumor nodules in the liver, only two tumor nodules and the sum of diameters less than 5 cm were excluded, n = 12).

#### Clinicopathological study

All specimens were examined under a microscope after haematoxylin-eosin staining by two pathologists. Four aspects of clinicopathological features including liver cirrhosis, Edmondson classification, capsule formation and microvascular invasion were studied.

### RT-PCR

The total RNA was extracted from HCC tissue and paired noncancerous liver tissue by using the TRIzol (Invitrogen, USA). The quality of RNA was checked through the ribosomal RNA bands on the gel. Two µg of each intact total RNA sample was reverse-transcribed to complementary DNA (cDNA) by using RT-PCR kit (MBI, USA). The PCR primer sequences used were as follows: sense: 5'-GCACAGGTAATAGCAGTGAGTGC-3' and antisense: 5'-CACACAATGGACAAGAATGCTG-3' for ADAM17; sense: 5'-TTCCAGCCTTCCTTGG-3' and antisense: 5'-TTGCGCTCAGGAGGAGC AAT -3' for  $\beta$ -actin. The sizes of PCR products were 440 bp for ADAM17 and 218 bp for β-actin. The procedure was as follows: denaturation at 94 °C for 5 min, then 40 cycles of denaturation at 94 °C for 50 s; annealing at 52 °C for 1 min and extension at 72 °C for 1 min. The PCR products were electrophoresed in 20 g/L agarose gels, and visualized under ultraviolet light. The expression ratio of ADAM17 mRNA to β-actin was determined by Eagle Eye II photo-analysis system.

# Statistical analysis

The  $\chi^2$  test was used for quantitative enumeration data. The student's *t* test and one-way analysis of variance were used for qualitative data. The statistic analysis was performed by statistical software SPSS11.0. *P* value less than 0.05 was considered significant.

# RESULTS

#### Clinicopathological features of HCC in three groups

According to the results of pathological study (shown in Table 1), the NHCC group had higher incidences of microvascular invasion (10/12) compared with SLHCC (3/8) and SHCC (3/11) (P<0.05). Only 8.3% NHCC (1/12) was classified as Edmondson I-II, while 62.5% SLHCC (5/8) and 63.6% SHCC (7/11) were classified as Edmondson I-II. The differentiation of NHCC was significantly poorer than SLHCC and SHCC (P<0.05). The other two pathological features of SLHCC and SHCC were also better than NHCC but the difference did not reach statistical significance. No statistical difference of the four pathological features was observed between SLHCC and SHCC.

#### **Table 1** Pathological features of HCC in three groups

Pathological features	SHCC ( <i>n</i> = 11)	SLHCC ( <i>n</i> = 8)	NHCC ( <i>n</i> = 12)
Liver cirrhosis			
Present	7	6	9
Absent	4	2	3
Microvascular invasion			
Present	3	3ª	10
Absent	8	$5^{\mathrm{a}}$	2
Capsule formation			
Present	8	6	4
Absent	3	2	8
Edmondson classification			
I-II	7	5	1
III-IV	4	3	11

<sup>a</sup>*P*<0.05 *vs* NHCC.

#### Expression of ADAM17 mRNA in HCC and paired non-cancerous liver tissue

As shown in Figure 1, RT-PCR products of ADAM17 and  $\beta$ -actin presented at the same site as previously designed. The expression of ADAM17 mRNA was detected in 77.4% (24/31) of HCC tissues, much higher than that in paired non-cancerous liver tissues in which only 35.5% (11/31) tissues were detected of the expression (*P*<0.05).



**Figure 1** ADAM17 transcription was detected in HCC but no obvious transcription was observed in paired non-cancerous tissues.

#### Transcription level of ADAM17 in SLHCC, SHCC and NHCC

As shown in Figures 2, 3, the transcription level of ADAM17 was  $0.8\pm0.7$  (mean $\pm$ SD) in SLHCC and  $0.9\pm0.6$  in SHCC. Both were significantly lower than that in NHCC ( $1.6\pm0.5$ ) (P<0.05). No statistical difference in transcription level of ADAM17 was observed between SLHCC and SHCC.



**Figure 2** The expression of ADAM17 mRNA was higher in NHCC compared with SHCC and SLHCC.



**Figure 3** Transcription level of ADAM17 in three groups of HCC.

### Relationship between transcription level of ADAM17 and clinicopathological features of HCC

The transcription level of ADAM17 was compared between different pathological types of HCC. The ADAM17 transcription level was much higher in HCC samples classified as Edmondson III-IV (P<0.05). While no statistical difference in transcription level of ADAM17 was observed when capsule formation, liver cirrhosis, and microvascular invasion were concerned (Table 2).

**Table 2** Relationship between ADAM17 transcription and pathological features of HCC

	Sample (n)	Transcription level of ADAM17 (mean±SD)	Р
Liver cirrhosis			
Present	22	$1.141{\pm}0.743$	>0.05
Absent	9	$0.996{\pm}0.662$	
Microvascular invasion			
Present	16	$1.279{\pm}0.648$	>0.05
Absent	15	$0.893{\pm}0.750$	
Capsule formation			
Present	18	$1.058{\pm}0.157$	>0.05
Absent	13	$1.187{\pm}0.800$	
Edmondson classification			
I-II	13	$0.768{\pm}0.204$	< 0.05
III-IV	18	$1.349{\pm}0.157$	

# DISCUSSION

HCC ranks fifth in frequency worldwide among all malignancies<sup>[1]</sup>. Surgery is the only potential curative treatment of HCC<sup>[28]</sup>. The post-operative survival of SHCC was generally considered to be better than LHCC, but long-term disease-free survival of some LHCC was also frequently observed after curative resection. Furthermore, some clinical investigation showed that the 5-year survival rate of LHCC after curative resection was not statistically different from SHCC<sup>[4,5]</sup>. This kind of LHCC was named SLHCC because it was of some unique characters such as: isolated lesion, expanding growth and relatively integrated fibrous capsule formation et al.<sup>[2]</sup>. Previous studies showed that SLHCC had relative better prognosis after curative resection<sup>[2,3,6]</sup>. Consistent with this, the relatively better pathological features of SHCC were found in this study. The differentiation of SLHCC was much better than that of NHCC, and NHCC was more frequently of microvascular invasion compared with SLHCC. No statistic difference in pathological features was observed between SHCC and SLHCC.

ADAM17 is a sheddase of many transmembrane proteins. The substrates of ADAM17 mediated shedding include many ligands of EGFR such as TGF- $\alpha$ , HB-EGF, amphiregulin *et al.*<sup>[14]</sup>. It has been well known that the activation of EGFR is essential for the carcinogenesis and metastasis of many cancers, while ADAM17 mediated shedding is a key mechanism of sending signals to activate EGFR<sup>[19-21]</sup>. Overexpression of ADAM17 has been observed in gastric tumor, mammary cancer, leukemia cell lines and prostate cancer cell lines<sup>[20,21,29-32]</sup>. In this study the transcription of ADAM17 was detected in 77.4% (24/31) HCC samples and was statistically higher than that in paired non-cancerous samples in which only 35.5% (11/31) were detected (*P*<0.05). This suggests that ADAM17 may play a key role in HCC development.

Previous studies showed that SLHCC had relative better prognosis after curative resection. To understand the underlying molecular mechanism, the transcription levels of ADAM17 in SLHCC, SHCC and NHCC were also studied. As expected, the transcription level of ADAM17 was much higher in NHCC than in SLHCC and SHCC, while no statistical difference was observed between SLHCC and SHCC. The transcription level of ADAM17 mRNA was also detected to be statistically higher in samples classified as Edmondson III-IV compared with those classified as I-II. The higher expression of ADAM17 mRNA in NHCC as well as in poorly differentiated HCC suggested that it might facilitate tumor invasiveness. The lower transcription level of ADAM17 in SLHCC was probably associated with the relatively better molecular pathological features of SLHCC.

The relationship between ADAM17 mRNA expression and the other two tumor features: microvascular invasion and tumor capsule formation was also studied in this study. Although much higher ADAM17 mRNA level was detected in tumors with microvascular invasion and those without integrated capsule formation, the difference did not reach statistical significance. This maybe due to the relatively small sample size in this study. As only 31 samples were used in the study, especially after the samples were divided into three groups, the significance of the research results may be impaired. The role of ADAM17 in HCC invasiveness needs further investigation with a larger sample size.

The increased transcription of ADAM17 may facilitate the growth and invasiveness of HCC in several ways. (1) Overexpressions of EGFR and its ligands were observed in HCC and were related to the prognosis. However, ADAM17 mediated shedding is a key mechanism of sending signals to activate EGFR. ADAM17 is required for the activation of EGFR by TGF-alpha or amphireglin in several cancer cells and the high expression of ADAM17 in mammary tumors was correlated with a high activation rate of EGFR<sup>[20,21]</sup>. As ADAM17 regulates the ligands production and activity of EGFR, the overexpression of ADAM17 is probably as important as the increased expression of EGFR and its ligands for HCC development; (2) ADAM17 can function as an effector of G protein-coupled receptor (GPCR) -mediated signaling. Activation of GPCR specifically results in ADAM17 cleavage and release of amphiregulin, which could activate EGFR and regulate the proliferation and motility of squamous cell carcinoma<sup>[32]</sup>. Overexpression of GPCR was also observed in HCC and was strongly correlated with carcinogenesis of HCC<sup>[33]</sup>. ADAM17 may be a key element of communication between GPCR and EGFR in HCC and facilitates HCC development; (3) TRANCE could activate osteoclast and help cancer cells metastasize to bones. Overexpression of TRANCE was found in bone metastatic lesion of several cancers<sup>[34]</sup>. ADAM17 may play a role in the bone metastasis of HCC by cleaving TRANCE; (4) The shedding of TNFR by ADAM17 may cause disorder of host immune system as the soluble form of TNFR could bind to the TNF and block its attack to cancer cells<sup>[35]</sup>.

Other transmembrane proteins associated with HCC

metastasis such as Fas ligand, CXCL12, E-cadherin, interleukin- $6\alpha^{(36-39)}$  were also the suspected substrates of ADAM17 mediated shedding, so it is of particular significance to study the sheddase role of ADAM17 in HCC development. The ADAM metalloproteinase family includes more than 30 members by now<sup>[40]</sup>. Many of them not only function as metalloproteinases to shed transmembrane proteins<sup>[41-43]</sup> in cancer but also can work as adhesion molecules<sup>[44,45]</sup>. In contrast to the matrix metalloproteinases, relatively few data on expression of ADAM12 and ADAM9 were recently studied in HCC tissue and were of particular importance<sup>[46]</sup>. To study the role of other ADAM family members may help us better understand HCC development.

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BRIEF REPORTS •

# Clinical significance of serum IGF-I, IGF-II and IGFBP-3 in liver cirrhosis

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# Abstract

**AIM:** To investigate the relationship between insulin-like growth factor-I, -II (IGF-I and IGF-II), IGF-binding protein 3 (IGFBP-3) and Child-Pugh score in patients with liver cirrhosis, and to search for potential clinical markers of liver function.

**METHODS:** Forty-four patients with advanced liver cirrhosis of viral origin were divided into 3 groups according to severity of cirrhosis (Child-Pugh score) and 38 healthy subjects served as controls. Serum levels of IGF-I, IGF-II and IGFBP-3 were measured by immunoradiometric assay.

**RESULTS:** Serum IGF-I, IGF-II and IGFBP-3 levels were significantly lower in patients with cirrhosis than in controls, and serum concentrations of IGF-I, IGF-II and IGFBP-3 were associated with the severity of liver dysfunction, and dropped sharply during the progression of liver failure. Among these 3 parameters, serum IGF-II was the most sensitive and effective indicator for liver dysfunction. Concentrations of IGF-I <30 ng/mL, IGF-II <200 ng/mL and IGFBP-3 <6 ng/mL implied a negative prognosis for patients with liver cirrhosis.

**CONCLUSION:** Serum IGF-I, IGF-II and IGFBP-3 may provide a new dimension in the assessment of liver dysfunction. Combined detection of serum IGF-I, IGF-II and IGFBP-3 with Child-Pugh score is more effective in predicting prognosis than Child-Pugh score alone.

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# INTRODUCTION

Insulin-like growth factor-I and II (IGF-I, IGF-II), two major forms of insulin-like growth factors (IGFs) family, are single-chain molecules with three intrachain disulfide bridges consisting of 70 ( $M_r$  7 646) and 67 ( $M_r$  7 471) amino acid residues respectively<sup>[1,2]</sup>. Both of them may be considered as important anabolic hormones which are active throughout one's life, inducing anabolic metabolism and stimulating DNA synthesis, cell proliferation and meiotic division<sup>[3,4]</sup>. Compared with IGF-I, IGF-II is more well-known as a tumor genesis marker<sup>[5]</sup>. As serum IGF-II may be produced and secreted by hepatoma cells, the hormone

could accelerate or magnify its function of continuously stimulating cell growth by directly or indirectly combining with its receptor in neighboring cells or hepatoma cells themselves to form intracellular shortcuts<sup>[6,7]</sup>.

Insulin-like growth factor binding protein-3 (IGFBP-3) consisting of 264 amino acid residues binds nearly 95% circulating IGFs in the human body, forming a stable ternary complex ( $M_r$  150 000) with the acid-labile subunit (ALS). The complex is believed to serve as a reservoir in circulation to prolong half-lives of both IGF-I and IGF-II<sup>[8,9]</sup>. Since most circulating IGF-I, IGF-II and IGFBP-3 are synthesized by hepatocytes, lower levels of the above three parameters should be found in patients with liver diseases<sup>[10]</sup>. This study was designed to clarify the influences of associated liver cirrhosis as assessed by Child-Pugh score (CP score) on these parameters, to determine whether measurement of these three parameters with CP score could be a more reasonable clinical option for evaluating liver function.

#### MATERIALS AND METHODS

#### Patients

From February to September 2001, 44 patients with hepatitis Binduced liver cirrhosis (30 males and 14 females with a mean age of 57.41 years, ranging from 38 to 83) were studied. Patients with liver cirrhosis were diagnosed by liver biopsy and/or by computerized tomography, ultrasonography and clinical biochemical examinations, according to Chinese diagnostic criteria of liver cirrhosis set up in 1995. Patients were divided into 3 groups by CP score: Fifteen people in the group of CP A (scored 5-6), 19 in the group of CP B (scored 7-10) and 10 in the group of CP C (scored 11-15). A total of 38 healthy subjects were served as controls (23 males and 15 females, mean age of 49.97 years, ranging from 35 to 82). After detection of serum IGF-I, IGF-II and IGFBP-3 levels, patients were followed-up in the liver clinic for a period of 6 mo.

#### Study protocols

Serum IGF-I was quantified by immunoradiometric assay kit (Immunotech A BECKMAN Company). Serum levels of IGF-II and IGFBP-3 were detected by DSL-2 600 ACTIVETM immunoradiometric assay kits (Diagnostic System Laboratories Inc, USA).

Sample collection, procession and storage were referred to the following: A 6 mL venipuncture blood was collected into a dry, heparinized tube in the morning from every subject. Then serum or plasma was separated from cells by centrifugation. Samples should be kept at below -30 °C after aliquoting so as to avoid repeated freezing and thawing.

Assay procedures were firmly accorded to instructions of the kits. Briefly, taking protocol of IGF-II for example, first,  $100 \mu$ L of the reconstituted standards, controls and pretreated samples were added to appropriate tubes. Then all the tubes were mixed and incubated at room temperature for 3 h, centrifuged at 180 r/m, and decanted except for total count tubes by simultaneous inversion. The tubes were shaken violently to facilitate complete drainage and allowed to drain on absorbent for 1-2 min. Two

milliliters of deionized water was added to all tubes, except total count tubes, and the tubes were decanted. After  $100 \,\mu$ L of anti-IGF-II [<sup>125</sup>I] reagents was added, all tubes were mixed by shaking and were incubated at room temperature for 1 h on a shaker at 180 r/min, then were decanted except for total count tubes by simultaneous inversion. The tubes were struck sharply on absorbent material for 1-2 min and analyzed, after 2 mL of wash solution being added to each tube except for total count tubes. All tubes were counted in a gamma counter for 1 min after decantation.

#### Statistical analysis

Data were listed as mean $\pm$ SD. Comparison between means was tested by Student's *t* test, Friedman's ANOVA and Duncan test in SAS. *P* value less than 0.05 was considered significant.

# RESULTS

# Serum IGF-I, IGF-II and IGFBP-3 in cirrhotic liver tissues and matched controls

The mean values for IGF-I, IGF-II and IGFBP-3 in 44 cirrhotic patients ( $66\pm58$ ,  $367\pm193$  and  $12.0\pm7.6$  ng/mL, respectively) were significantly lower than those in 38 healthy subjects ( $260\pm74$ , 1 094 $\pm119$  and 39 $\pm7$  ng/mL, respectively, P<0.001, Table 1). Since mean values of the three parameters were negatively correlated to age according to our statistical show (r = -0.646, -0.612, -0.609), data were revised with covariance analysis to obviate age-related effect on three parameters, and similar result was obtained (P<0.001).

# Serum IGF-I, IGF-II and IGFBP-3 levels in patients with different CP scores

The mean values for IGF-I, IGF-II and IGFBP-3 were  $119\pm67$ ,  $507\pm185$  and  $19\pm8$  ng/mL, respectively, in CP A;  $45\pm29$ ,  $343\pm154$  and  $10\pm5$  ng/mL, respectively, in CP B. The mean values for IGF-I, IGF-II and IGFBP-3 in patients classed as Child-Pugh C stage, the worst stage of liver dysfunction with incurable ascites and elongation of APTT (activated partial thromboplastin time), were  $27\pm11$ ,  $201\pm115$  and  $6\pm3$  ng/mL, respectively. Significant difference of the three parameters was found between control group and any stage of cirrhosis (P<0.001). These three parameters gradually diminished, along with disease progression. Statistic analysis also showed that the mean levels for IGF-I and IGFBP-3 in CP A were significantly higher than those in CP B/C (P<0.001), and these values were a little higher in CP B than in CP C, however no significant difference of the two parameters was observed between these 2 categories (P>0.05). Only mean levels of IGF-

II showed clear statistical difference in patients between CP B and CP C (P<0.05), suggesting that IGF-II reduced significantly due to the severity of liver dysfunction (Table 2, Figure 1). Thus serum IGF-II was a more sensitive and effective indicator than IGF-I and IGFBP-3. Furthermore, the depressed IGF-II level was significantly correlated with the IGF-I variation, as well as with CP score (P<0.001).



**Figure 1** Serum IGF-I, IGF-II and IGFBP-3 levels in patients with different Child-Pugh scores.

#### DISCUSSION

Low levels of circulating IGF-I in cirrhosis have been described<sup>[11]</sup>. In recent studies, Assy et al<sup>[12]</sup>. confirmed that basal IGF-I and IGFBP-3 levels were significantly lower in patients with liver cirrhosis in their pilot study of IGF-I generation test, in which the two parameters were observed before and after stimulation with recombinant human growth hormone (rhGH). It was also approved by Donaghy et al<sup>[13]</sup>. that basal IGF-I and IGFBP-3 levels dropped markedly in cirrhosis due to severe GH resistance in these patients, caused by the feedback maladjustment of GH-IGF-I-IGFBP-3 axis. He considered that the fact of impaired IGF-I and IGFBP-3 production and the severity of GH resistance seen in cirrhosis likely reflected the effect of injury to the liver, the central organ of the endocrine GH-IGF-IGFBP-3 axis. GH resistance, an increasingly recognized feature related to the reduction of IGF-I, IGF-II and IGFBP-3 in liver dysfunction, may have been further pathogenically effected by the severity of liver dysfunction, disorder of portosystemic shunting and malnutrition of hepatic storage. In addition, the production/ secretion of GH receptor was also markedly reduced due to severely damaged hepatocytes, thus leading to the disturbance of feedback maladjustment and GH resistance<sup>[14,15]</sup>.

Table 1 Serum concentrations of IGF-I, IGF-II and IGFBP-3 in patients with liver cirrhosis and healthy subjects

Category	n	IGF-I (ng/mL)	IGF-II (ng/mL)	IGFBP-3 (ng/mL)
Cirrhosis	44	$66{\pm}58^{ m b}$	$367 \pm 193^{\mathrm{b}}$	12±8 <sup>b</sup>
Control	38	260±74	1 094±119	$39\pm7$

 $^{b}P < 0.001 vs$  control.

Table 2 Serum concentrations of IGF-I, IGF-II and IGFBP-3 in healthy subjects and patients with different CP scores

Category	п	IGF-I (ng/mL)	$P^1$	IGF-II (ng/mL)	$P^1$	IGFBP-3(ng/mL)	$P^1$	
Control	38	260±75	0.001	109±119	0.001	$39{\pm}7$	0.001	
CP A	15	$119 \pm 67$	0.001	507±185	0.001	19±8	0.001	
CP B	19	45±29	0.001	$343 \pm 154$	0.001	$10\pm5$	0.001	
CP C	10	27±11	0.001	201±115	0.001	6±3	0.001	

 $P^{1}$  represents the statistical comparison in columns between healthy subjects and patients with different Child Pugh scores (A: scored 5-6; B: scored 7-10; C: scored 11-15). P<0.001 vs between different groups of the same parameter.

Our data confirmed that serum IGF-I, IGF-II and IGFBP-3 levels were significantly lower in patients with liver cirrhosis than those in control group. Circulating concentrations of IGF-I, IGF-II and IGFBP-3 were decreased in patients with liver cirrhosis and apparently correlated with the degree of liver dysfunction. These lines of evidence indicate that impaired hepatic IGF-I, IGF-II and IGFBP-3 levels may be the real potential indicators for evaluation of liver dysfunction and clinical outcome.

Though no significant difference of serum IGF-I and IGFBP-3 was observed between CP B and CP C in our study, serum IGF-II responded comparably lower in patients with CP C than with CP B, confirming that fluctuation of serum IGF-II level remained statistically significant even in patients with severe liver dysfunction assessed by CP score, and the range of serum IGF-II concentrations was much more clearly delineated from normal to excessively low in patients with severe dysfunction than the case for serum IGF-I and IGFBP-3. With regards to the facts above, serum IGF-II concentration could be an important indicator for hepatic dysfunction and clinical prognosis, and was more sensitive and effective than serum IGF-I and IGFBP-3. The assumption was also supported by  $Nicolic^{[16]}$ , who recently reported that serum IGF-II was lower in patients of cirrhosis than in healthy subjects, and that serum IGF-II was markedly lower in patients with Child-Pugh B/C score (P<0.05). Moreover, he found that serum IGF-II was significantly correlated with IGF-I and CP score (P = 0.007). So Nicolic suggested that single IGF-II determination, a safe, reliable and convenient measurement, may be applicable for the assessment of patients with liver cirrhosis instead of GH stimulated IGF-I generation test and combined measurement of serum IGF-I, IGF-II and IGFBP-3. Other studies showed that both IGF-II and IGF-I were synthesized by hepatocytes, however, specific IGF-II mRNA has been found in hepatocytes, which indicated the impaired serum IGF-II production was the direct effect of decreased liver function, while baseline IGF-I and IGFBP-3 levels were decreased under other circumstances, besides liver dysfunction, such as low serum albumin, malnutrition and glucose metabolic abnormality, most of which are the complications of cirrhosis<sup>[17-19]</sup>.

Our research confirmed that serum concentrations of IGF-I, IGF-II and IGFBP-3 were correlated with CP scores (P<0.001), which is consistent with previous studies<sup>[20,21]</sup>. Thus, we investigated serum levels of IGF-I, IGF-II and IGFBP-3 that had similar effects on evaluation of hepatic dysfunction and proposed that the combined detection of serum IGF-I, IGF-II and IGFBP-3 effectively predicted functional liver reserve, prognostic and clinical states of the patients. More relation has been found between IGF-I and the degree of portal hypertension and portosystemic shunting compared with the degree of liver function impairment<sup>[22,23]</sup>. In agreement with our results, IGFBP-3 has been reported suitable to predict liver synthetic capacity, because IGFBP-3 uniquely reflects GH activity, so it is downregulated during depletion of either GH or its receptor, and diminished even in the presence of rebounded GH concentration<sup>[24,25]</sup>. Biological functions of IGF-I and IGF-II are modulated by specific high-affinity IGFBP-3. IGFBP-3 level might be less age-dependent than IGF-I level<sup>[26-28]</sup>. The significance of IGF-II detection has been described above.

Assy *et al*<sup>[29]</sup>. measured serum levels of IGF-I and IGFBP-3 before and 24 h after a single subcutaneous injection of rhGH (0.14 U/kg bw). IGF-I level below 10 nmol/L was considered indicative of a poor prognosis with 15% survival at one year, whereas above 10 nmol/L indicated a 100% survival rate in 1-2 years. He then concluded that stimulated IGF-I of less than 10 nmol/L might be a true predictor of a negative prognosis in patients with liver cirrhosis. Castilla-Cortazar *et al*<sup>[30-32]</sup>. obtained similar results in their study.

In our research, all patients were followed up in the liver clinic for a period of 6 months after the measurement, among which 6 patients' levels were below the specific levels (IGF-I <30 ng/mL, IGF-II <200 ng/mL and IGFBP-3 <6 ng/mL), and 5 (83%, 4 in CP C, 1 in CP B) of them died of liver failure or bleeding in less than half a year. It is suggested that hepatic cirrhosis patients with low baseline IGF-I, IGF-II and IGFBP-3 levels have a lower survival rate than those with high levels. In agreement with our result, Assy<sup>[12]</sup> also speculated that CP score alone could not be regarded as an ideal predictive method for patients with liver cirrhosis.

So, combined evaluation of baseline IGF-I, IGF-II and IGFBP-3 with CP score gives better prediction than CP score alone of patients' liver function. It appears to be a good predictor of survival and an early indicator of liver dysfunction. However, long-term follow-up with multi-center and large sampled studies are expected.

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BRIEF REPORTS •

# **Expression of TREM-1 mRNA in acute pancreatitis**

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# Abstract

**AIM:** To explore the expression of triggering receptor expressed on myeloid cells (TREM-1) mRNA in acute pancreatitis (AP).

**METHODS:** Using the reverse transcription polymerase chain reaction (RT-PCR), we examined the expression of TREM-1 mRNA in 10 cases of mild acute pancreatitis (MAP), 8 cases of severe acute pancreatitis (SAP), and 10 cases of healthy control subjects. And we also examined the expression of TREM-1 mRNA in 14 cases of AP (including 10 MAP and 4 SAP) before treatment, after successful therapy and clinically cured.

**RESULTS:** The expression of TREM-1 mRNA in the groups of MAP, SAP patients and healthy control subjects was 0.771±0.274, 1.092±0.331 and 0.459±0.175, respectively; there was a significant difference among the three groups (P<0.05). And there was also a significant difference between the AP patients (0.914±0.341) and healthy control subjects (0.459±0.175) (P<0.05). Moreover, in the 14 cases of AP, before treatment, after successful therapy and clinically cured, the expression of TREM-1 mRNA was 0.905±0.226, 0.739±0.169 and 0.633±0.140, respectively, and there was a significant difference among the three stages (P<0.05).

**CONCLUSION:** The expression of TREM-1 mRNA in the patients with AP increases obviously, and correlates with the degree of AP. Furthermore, the expression of TREM-1 mRNA is distinctly different at the different stages of AP. It indicates TREM-1 may play an important role in the occurrence and development of AP.

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# INTRODUCTION

TREM-1 is a new activating receptor of the Ig superfamily expressed on human myeloid cells. And it is selectively expressed on blood neutrophils and a subset of monocytes and upregulated by bacterial lipopolysaccharide (LPS)<sup>[1]</sup>. Researchers have demonstrated that TREM-1 mediates activation of neutrophil and monocytes, and may have a predominant role in inflammatory responses such as septic shock and systemic inflammatory response syndrome (SIRS)<sup>[1,2]</sup>. In this study, we aimed to explore the expression of TREM-1 mRNA in AP and tried to reveal etiology of AP and underlying mechanism.

# MATERIALS AND METHODS

# Patients and specimens

Data obtained from 18 AP patients without any treatment during 48 h of the onset of the disease and 10 healthy control subjects were explored. According to the standards of diagnosis and classification of AP proposed at the Sixth National Conference on Pancreatic Surgery of China in 1996<sup>[3]</sup>, we divided the AP patients into two groups: 10 cases of MAP, and 8 of SAP. Among the AP patients, we examined the expression of TREM-1 in 14 patients at different stages of the disease (before treatment, after successful therapy and clinically cured). We collected 2 mL of blood from the patients and controls before breakfast in the morning and stored them in the EDTA tube. The total leucocytes in the blood were separated by centrifugation at 1 200 r/min for 15 min.

# **Reagents and primers**

Trizol reagents, Oligo (dt) 15, M-MLV reverse transcriptase and DEPC were the products of Promega Co. (USA). Taq DNA polymerase were purchased from Sangon Co. (Shanghai). The RNase inhibitor was from Takara Co. (Japan). According to the human TREM-1 and  $\beta$ -actin cDNA sequence reported in GeneBank, we designed two sets of primers: TREM-1 sense 5'-GTG TGT GAT CTA CCA GCC T-3', antisense 5'-TTC AGA GTC AGG AGT GGA G-3' (amplified fragment length: 232 bp);  $\beta$ -actin sense 5'-GTG CGT GAC ATT AAG GAG-3', antisense 5'-CTA AGT CAT AGT CCG CCT-3' (amplified fragment length: 520 bp).

# Total RNA extraction

The total RNA was extracted from the leucocytes by Trizol reagent. The extracted RNA concentrations and purity were determined at  $A_{260}$  and  $A_{280}$  spectrophotometrically (Beckman DU650, USA). Then 750 mL/L ethanol was used to preserve total RNA at -20 °C.

# Reverse transcription polymerase chain reaction

The total RNA was denatured by incubation for 5 min at 70 °C and rapidly cooled on ice for about 2-3 min. Single-stranded cDNA was synthesized by mixing  $1-2 \mu g$  denatured RNA with  $0.5 \,\mu\text{L}$  (40 u/µL) RNase inhibitor, 1 µL (200 u/µL) M-MLV reverse transcriptase, 4 µL reaction buffer, 1 µL Oligodt (15), 1 µL (10 mmol/L) dNTP, and adding DEPC-treated water up to 20 µL. The RT reaction mixture was incubated at 37 °C for 60 min, followed by 95 °C for 5 min to inactivate M-MLV reverse transcripatase, and stored at 4 °C for use. A 5 µL of the cDNA was used as template in PCR amplification for TREM-1. The mixture (50  $\mu$ L) consisting of 1  $\mu$ L of primers (p1/p2 of TREM-1 and  $\beta$ -actin) each, Taq DNA polymerase (0.5  $\mu$ L (5 U/ $\mu$ L), 10×PCR buffer 5 µL, MgCL<sub>2</sub> 3 µL (25 mmol/L), by addition of deionised water up to 50 µL, was reacted on a programmable thermocycler (Perkinelmer 9 600). The DNA was predenatured at 95 °C for 5 min, followed by 35 cycles of denaturation (45 s at 95 °C), annealing (45 s at 56 °C), and extension (45 s at 72 °C). Then the mixture was finally extended for 10 min at 72 °C, and stored at -20 °C.  $\beta\text{-}$ actin was served as internal control in each experiment under the same conditions.

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#### Electrophoresis identification and quantitative scanning

PCR products (7-9  $\mu$ L) with DL 2 000 marker were electrophoresed through 10 g/L agarose gel in 1×TBE at 70 V for 30 min. The samples were stained with ethidium bromide (0.5 mg/L) and observed by ultraviolet illumination and photographed. Each lane was scanned longitudinally. The *A* values of TREM-1 and  $\beta$ -actin bands were measured by a gel imaging analysis system (GELWORKS 1D advanced v4.01). The *A* ratios of TREM-1/ $\beta$ actin representing the relative levels of the expression of TREM-1 mRNA were calculated for semi-quantitative analysis.

#### Statistical analysis

Results were presented as mean $\pm$ SD. Statistical comparisons were analyzed through the SAS 8.1 software. Analysis of variance was used. *P*<0.05 was considered statistically significant.

#### RESULTS

#### Expression of TREM-1 mRNA and semi-quantitative analysis

The expression of TREM-1 mRNA in the groups of MAP, SAP patients and healthy control subjects were  $0.771\pm0.274$ ,  $1.092\pm0.331$  and  $0.459\pm0.175$ , respectively. There was a significant difference among the three groups (P<0.05). The difference was also significant (P<0.05) (Figures 1, 3) between the AP patients ( $0.914\pm0.341$ ) and healthy control subjects ( $0.459\pm0.175$ ). In AP patients of before treatment, after successful therapy and clinically cured, the expression of TREM-1 mRNA was  $0.905\pm0.226$ ,  $0.739\pm0.169$  and  $0.633\pm0.140$ , respectively. There was a significant difference among the three stages (P<0.01 or P<0.05). And the expression of TREM-1 mRNA was higher in clinically cured group than in healthy control subjects (P<0.05) (Figures 2, 4).



Figure 1 Electrophoresis of the PCR products. M: DL2000 marker; 1: the PCR product of normal subject; 2: the PCR product of MAP; 3: the PCR product of SAP. The amplified fragment lengths of  $\beta$ -actin and TREM-1 are 520 bp and 232 bp respectively.



**Figure 2** Electrophoresis of the PCR products. M: DL2 000 marker; 1: the AP patient before treatment; 2: the same patient after successful therapy; 3: the same AP patient after clinically cured; 4: negative control. The amplified fragment length of  $\beta$ -actin is 520 bp, of TREM-1 is 232 bp. 10 g/L agarose gel.



**Figure 3** The *A* ratios of TREM-1/ $\beta$ -actin in different degree of AP: There was a significant difference among the three groups in the expressions of TREM-1 mRNA (*P*<0.05). And between the AP patients and healthy control subjects, the difference was significant (*P*<0.05).



**Figure 4** The *A* ratios of TREM-1/ $\beta$ -actin in different stage of AP: 1, the group in the stage of before treatment. 2, the group in the stage after successful therapy. 3, the group in the stage of clinically cured. 4, the group of normal subject. There was a significant difference among the three stages in the expression of TREM-1 mRNA (groups 1, 2 and 3. *P*<0.05). And in the group of clinically cured the ratio was higher than in healthy control subjects (*P*<0.05).

#### DISCUSSION

AP is a disease of self-digestive chemical inflammation with unclear etiology. In most cases, they present a self-limited course. Twenty percent of patients with AP have a severe form of the disease. SAP is such a severe disease that it may be associated with both systemic and local complications, such as respiratory insufficiency with adult respiratory distress syndrome (ARDS), renal failure, the development of pancreatic and peripancreatic infection. The mortality of SAP is higher than 40%<sup>[4,5]</sup>. Even now, there are no efficient and sensitive methods to treat AP successfully because the pathogenesis of AP still remains obscure<sup>[6]</sup>. Recent studies<sup>[7]</sup> show that factors like the pancreatic enzymes activated abnormally lead the body to secrete and amplify large quantities of cytokines. Finally, the cytokines increase vascular permeability, activate complement components, resulting in microcirculatory impairment and imbalance of thrombo-fibrinolytic system. Many of these events occur not only in the pancreas itself, but also in the other vital organs and tissues, leading to SAP and complications<sup>[8,9]</sup>. Generally, there are three phases of AP progression: local acinar injury, systemic response, and general sepsis<sup>[7]</sup>. Moreover, some data suggest that the changes in concentration of proinflammatory cytokines can be an early diagnostic criterion for AP<sup>[10,11]</sup>. Most authors have realized that the pathogenesis of SAP is very complicated. It is a multifactorial as well as multifaceted disease. First of all, the etiologic agents initiate the pancreatic acinar injury by release of pancreatic enzymes and over-stimulation of

macrophages and neutrophils, and then the cytokines and inflammatory mediators are delivered<sup>[12,13]</sup>.

In 2000, Bouchon et al. had identified a new activating receptor of the Ig superfamily expressed on human myeloid cells, called triggering receptor expressed on myeloid cells (TREM). TREM-1 is selectively expressed on blood neutrophils and a subset of monocytes and up-regulated by bacterial LPS. Engagement of TREM-1 triggers secretion of IL-8, monocyte chemotactic protein-1, and TNF-alpha and induces neutrophil degranulation. Intracellularly, TREM-1 induces Ca2+ mobilization and tyrosine phosphorylation of extracellular signal-related kinase 1 (ERK1), ERK2 and phospholipase C-gamma. To mediate activation, TREM-1 associates with the transmembrane adapter molecule DAP12<sup>[14]</sup>. Furthermore Bleharski et al.<sup>[15]</sup> suggested that activation of TREM-1 on monocytes participated in the early induced and adaptive immune responses involved in host defense against microbial challenges. Thus, TREM-1 was considered mediating the activation of neutrophil and monocytes, and might have a predominant role in inflammatory responses.

Wilson *et al.*<sup>[16]</sup> discovered that SAP had many similarities to sepsis syndrome and septic shock. The haemodynamic features of cardiovascular instability, reduced ejection fraction and decreased systemic vascular resistance were indistinguishable in each of these conditions. In addition, there are many striking similarities in the cytokine and inflammatory mediator profiles, suggesting that the haemodynamic abnormalities may result from the same pathogenic mechanisms, albeit as a result of different inflammatory stimuli.

In 2001 through their experiments, Bouchon et al.[2] believed that inflammatory responses to microbial products were amplified by a pathway mediated by TREM-1. TREM-1 was an activating receptor expressed at high levels in neutrophils and monocytes that infiltrate human tissues infected with bacteria. Furthermore, it was up-regulated in peritoneal neutrophils of patients with microbial sepsis and mice with experimental LPSinduced shock. Notably, blockade of TREM-1 protected mice against LPS-induced shock, as well as microbial sepsis caused by live Escherichia coli or caecal ligation and puncture. Their results demonstrate a critical function of TREM-1 in acute inflammatory responses to bacteria and implicate TREM-1 as a potential therapeutic target for septic shock. A recent study shows that TREM-1 amplifies Toll-like receptor-initiated responses against microbial challenges and potentiates the secretion of proinflammatory chemokines and cytokines in response to bacterial and fungal infections. Blockade of TREM-1 reduced inflammation and increased survival in animal models of bacterial infections that caused systemic hyper-inflammatory syndromes<sup>[17]</sup>. But nobody knows how TREM-1 expressed in AP as yet. According to the theory mentioned above, we carried out a study of the expression of TREM-1 in AP.

We demonstrated that the expression of TREM-1 in leucocytes of patients with AP was much higher than that in healthy control subjects and correlated with the degree of AP. Furthermore, significant differences in the expression of TREM-1 mRNA were found at the different stages of AP. The expression of TREM-1 decreased gradually with the improvement of severity of the AP patients. This experiment indicates that the expression of TREM-1 is relevant to progression of AP. Because of lack of relevant data about the patients after they were discharged from the hospital, we only compared the expression of TREM-1 between the cured group and healthy control subjects and found that there was still a significant difference between them. Therefore, we suggest that the pathological changes of the pancreas continued even after the patients were cured clinically.

This study may help reveal the etiology of AP and the mechanism of its progression. It may provide a new standard for the diagnosis and classification of AP as well as an efficient method to cure AP by blocking the expression of TREM-1.

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BRIEF REPORTS •

# Development of an ELISA kit using monoclonal antibody to *Clostridium difficile* toxin A

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# Abstract

AIM: To establish an ELISA kit using monoclonal antibodies against *Clostridium difficile* (*C. difficile*) toxin A.

**METHODS:** An indirect sandwich ELISA was described using the purified rabbit monospecific antiserum as capturing antibody. After the polystyrene microtitre plates with 96 flat-bottomed wells were coated with rabbit antiserum, the wells were blocked with 100 g/L BSA in PBS-T. *C. difficile* toxin A or culture filtrates were added to each well and then monoclonal antibodies IgG-horseradish peroxidase conjugate was added as detecting antibody, tetramethylbenzidine was used as substrate and  $A_{450}$  of the stopped reacting product was recorded in an automated plate reader.

**RESULTS:** The tested specimens included culture filtrates of 2 strains of toxigenic *C. difficile*, 2 strains of non-toxigenic *C. difficile*, 26 strains of *E. coli*, 2 strains of *S. dysenteriae*, 1 strain of *Bif. infantis*, 5 strains of *V. cholera*, 2 strains of *S. typhi*, 7 strains of *C. botulinum*, 1 strain of toxigenic *C. sordllii*, and 1 strain of *C. butyricum*. A total of 47 strains of culture filtrates were all negative except for 2 strains of toxigenic *C. difficile*. The detective limitation of toxin A was 0.1 ng/mL.

**CONCLUSION:** An ELISA kit with high specificity and excellent sensitivity for the rapid detection of *C. difficile* toxin A was established. It will be a useful tool for diagnostic test of *C. difficile* toxin A.

Fu SW, Zhang YL, Zhou DY. Development of an ELISA kit using monoclonal antibody to *Clostridium difficile* toxin A. *World J Gastroenterol* 2004; 10(18): 2747-2749 http://www.wjgnet.com/1007-9327/10/2747.asp

# INTRODUCTION

*Clostridium difficile* (*C. difficile*), which has been reported as the major cause of antibiotic-associated colitis (AAC) and pseudomembranous colitis (PMC) in humans and experimental animals, produces at least two toxins, named toxin A and B. Toxin A, a tissue-damaging enterotoxin, can lead to hemorrhagic fluid accumulation in rabbit ileal loops and is cytotoxic for cultured fibroblasts. Toxin B is an extremely potent cytotoxin for many cultured cells<sup>[1,2]</sup>.

Polyclonal antibodies are used in immunoassays and detection for *C. difficile* toxin in many clinical researches. They

are against toxin A and toxin B, and may be against other antigens at the same time, causing false positive results<sup>[3-5]</sup>. The problem may be solved when monoclonal antibodies were used. They can not only improve the specificity of the method, but also decrease the false positive results as well. The present report describes an enzyme-linked immunosorbent assay (ELISA) which used monospecific antibody as a capture antibody, and monoclonal antibody as a detection antibody, for detection of *C. difficile* toxin A in clinical specimens.

# MATERIALS AND METHODS

# Bacterial strains and culture filtrates

The 49 strains of bacteria included 2 strains of toxigenic *C. difficile*, 2 strains of non-toxigenic *C. difficile*; 26 strains of *E. coli*, 2 strains of *S. dysenteriae*; 1 strain of *Bif. infantis*; 5 strains of *V. cholera*; 2 strains of *S. typhi*; 7 strains of *C. botulinum*; 1 strain of *C. sordellii*; and 1 strain of *C. butyricum*. All strains were cultured with proper medium separately and the culture filtrates were used for the assay.

# Purification of C. difficile toxin A

Toxigenic *C. difficile VPI 10463* was grown anaerobically at 37 °C for 72 h in brain heart infusion (Difco) by the dialysis bag methods. Toxin A was purified by precipitation with 500 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and acid precipitation at pH5.5, then by ion-exchange chromatography on DEAE-Toyopearl 650 mol/L.

# Preparation of monospecific toxin A antiserum

Purified toxin A was inactivated with 4 g/L formaldehyde at 37 °C for 72 h, mixed with SEPPIC adjuvant(5.41:4.6 v/v, Montanide ISA206, France), and then injected in to a New Zealand white male rabbit of 2 kg weight every 2 wk over a period of 4 wk. Booster injections of 0.2 mg of toxoid A were prepared with adjuvant and injected subcutaneously at 4 wk intervals over a period of 32 wk, and antiserum was collected 1 wk after the final booster dose.

# Production of monoclonal antibody

Purified toxin A of *C. difficile* ( $100 \mu g$ ) was inactivated with 4 g/L formaldehyde at 37 °C for 72 h, and mixed with SEPPIC adjuvant, BALB/c mice were injected intraperitoneally with 0.5 mL of toxoid A at 2 wk intervals over a period of 8 wk. Three days before fusion, one mouse was boosted with the same quantity of toxoid A without the adjuvant. The splenocytes from immunized mice were fused with myeloma cells Sp2/0. The hybridoma cells were screened by indirect ELISA and cloned by limiting dilution method.

# Purification of antibodies

Antibodies were purified by precipitation with 400 g/L (NH<sub>4</sub>)  $_2$ SO<sub>4</sub> followed by precipitation with 330 mL/L (NH<sub>4</sub>) $_2$ SO<sub>4</sub> for 3 times, centrifuged at 6 000 r/min for 30 min, and then loaded on Sephacryl-300 chromatography column.

# Conjugate of monoclonal antibody to horseradish peroxidase<sup>[6]</sup>

Sodium periodate solution (0.3 mL, 0.1 mol/L) and 5 mg horseradish peroxidase dissolved in 1 mL of water, the mixture was stirred

for 30 min at 4 °C and dialyzed at 4 °C against 0.01 mol/L sodium acetate buffer (pH4.4) overnight, followed by addition of 0.5 mL of 0.16 mol/L ethylene glycol, stirred for 1 h at 4 °C. Monoclonal IgG of 5 mg in 1 mL of 0.05 mol/L sodium carbonate buffer (pH9.6) was added immediately, stirred and dialyzed at 4 °C with same buffer overnight before any unconjugated enzyme was removed by addition of 0.2 mL sodium borohydride solution (5 mg/mL, 4 °C, 3 h), then precipitated by addition of equal volumes of 100% ammonium sulfate (4 °C, 2.5 h), centrifuged (6 000 r/min, 15 min), dialyzed against 0.01 mol/L PBS (pH7.4) at 4 °C overnight, and stored at 4 °C after diluted with 500 mL/L glycerol (1:1).

#### Indirect sandwich ELISA

Ninety-six-well polystyrene flat-bottomed microtitre plates were coated with 100 µL of purified rabbit monospecific antitoxin  $(8 \,\mu\text{g/mL}, \text{ capture antibody})$  in 0.05 mol/L carbonate buffer (pH9.6) and incubated overnight at 4 °C, the plates were washed once in PBS-T (0.01 mol/L PBS containing 0.5 g/L Tween-20, pH7.4). After 200 µL of 100 g/L BSA in PBS-T was added to the wells and incubated at 37 °C for 2 h, washed 5 times in PBS-T with 3 min incubation at room temperature between each wash, 100 µL of C. difficile toxin A or test samples in PBS-T were added to each well and incubated for 1 h at 37 °C, washed for 5 times. Then 100 µL of 1:1 000 diluted monoclonal antibodies IgG-horseradish peroxidase conjugate (detecting antibody) was added for 1 h at 37 °C, wells were washed 5 times with PBS-T, and 0.1 mL of TMB(3,3',5,5'-tetramethylbenzidine) substrate was added to each well. After 15 min at 37 °C in the dark, the reaction was stopped by the addition of 1 drop of 2 mol/L sulfuric acid and A<sub>450</sub> was measured. ELISA titers of positivity were expressed as  $A_{450}$  >/=0.10. Non. toxigenic C. difficile culture filtrate was used as negative control.

#### Protein determination

Protein concentration was determined by the method of Coomassie brilliant blue G-250, and bovine serum albumin was purchased from Sigma Company of USA.

#### RESULTS

### Properties of the antibodies

Six hybridoma cell lines (2H7, 3E9, 4B5, 5C10, 6G8 and 8A1) secreting mAbs against *C. difficile* toxin A were produced. The Ig subclasses of mAbs 2H7, 3E9 and 6G8 were IgM, mAbs 4B5 and 8A1 were IgG1, mAb 5C10 was IgG2a. All 6 mAbs had no neutralization activity. Epitope recognized by 5 mAbs (2H7, 4B5, 5C10, 6G8 and 8A1) differed from mAb 3E9. Relative affinities of mAbs 8A1 and 4B5 were all above  $10^5$ , and those of the other 4 mAbs were  $10^4$ . mAbs 8A1 were conjugated to horseradish peroxidase for ELISA,  $A_{403}/A_{280}$  was 0.40 and the optimal dilution was 1:1000. The monospecific antiserum could neutralize all activities of *C. difficile* toxin A. The ELISA titer was  $10^6$ /mL.



Figure 1 Detection of *C. difficile* toxin A with ELISA.

#### Sensitivity of ELISA

Purified *C. difficile* toxin A was detected with ELISA and a high sensitivity of 0.1 ng/mL was found.

#### Specificity of ELISA

The tested specimens were from culture filtrates of 49 strains. Forty-seven strains were all negative except that 2 strains were positive. Table 1 illustrates the ELISA results with a high specificity.

**Table 1** Detection of *C. difficile* toxin A in culture filtrates by

 ELISA

Charles in	Number		Culture supernatant			
Strain			sitive (n)	Negative (n)		
C. difficile toxigenic	2		2	0		
C. difficile non-toxigenic	2		0	2		
E. coli	26		0	26		
S. dysenteriae	2		0	2		
Bif. infantis	1		0	1		
V. cholera	5		0	5		
S. typhi	2		0	2		
C. botulinum	7		0	7		
C. sordellii	1		0	1		
C. butyricum	1		0	1		

#### Stability of the ELISA kit

Ninety-six-well polystyrene flat-bottomed microtitre plates were coated with 100  $\mu$ L of purified rabbit monospecific antitoxin (8  $\mu$ g/mL) in carbonate buffer (pH9.6) and incubated overnight at 4 °C, the plates were washed once in PBS containing 0.5 g/L Tween-20, pH 7.4 (PBS-T). After 200  $\mu$ L of 100 g/L BSA in PBS-T was added to the wells and incubated at 37 °C for 3 h, and then vacuumed and kept in plastic bag at 37 °C for 6 d, the titer was detected, indicating that the kit can be kept at 4 °C for at least 1 year.

#### DISCUSSION

A number of rapid (enzyme immunoassay and latex agglutination) and conventional (direct plating and tissue culture) tests have been developed as aids in the diagnosis of *C. difficile* infection: (1) Tissue culture cytotoxicity assay is the best available laboratory test for determination of the role of C. difficile in the pathology of a given patient diarrhea, it has an excellent sensitivity and specificity, but its utility is limited because of its inherent technical complexity, time requirement, specimen-handling requirements, and high costs; (2) Latex agglutination test was found to be nearly as sensitive as the cytotoxicity assay and it did not detect toxin A but a C.difficile cell-associated protein; (3) Organism culture: first, the asymptomatic carriage rate of this organism may be as high as 20% in patients receiving antibiotics making interpretation of positive culture data difficult. Second, organisms that do not produce toxin are thought to be avirulent. Isolates must be proved to produce toxin to be considered pathogenic; (4) ELISA. ELISA has the potential of greatly simplifying and improving the efficiency of the laboratory diagnosis of C. difficile associated diseases. It has the high sensitivity and excellent specificity comparable with tissue culture cytotoxicity assay. Using the commercial enzyme immunoassays for the detection of toxin A or toxin B, the results could be available in 2-4 h. ELISA was a perfect method to detect toxin A in feces<sup>[7,8]</sup>.

The advantage of ELISA for detection is that: first, as a clinical expression of *C. difficile* infection, toxin A was more stable than toxin B. Second, toxin A was easy to be purified and its antibodies were easy to produce. Third, the monoclonal antibodies can be prepared in unlimited quantities and are more reproducible reagents than other antibodies. After comparing

the antibody preparations by indirect ELISA, Lyerly *et al.* reported that ELISA with monoclonal antibodies could detect 4 ng of toxin A, but the polyclonal antibodies purified by affinity chromatography and monospecific antiserum could detect 1 ng of toxin A<sup>[8]</sup>. Laughon *et al.* reported that they could detect 0.1 ng (1.0 ng/mL) of toxin A with a monospecific antiserum<sup>[9]</sup>. In this study we present ELISA kits which used the purified rabbit monospecific antitoxin as capturing antibody and monoclonal antibodies IgG-horseradish peroxidase conjugate as detecting antibody, the detective limitation of toxin A was 0.1 ng/mL.

Antibiotic-associated diarrhea and PMC are the major problems in a variety of health care settings. Requests for the laboratory diagnosis of *C. difficile*-associated diseases are frequently made, but there have been no diagnostic reagents in China until now. Thus, rapid diagnosis of *C. difficile* in patients with PMC and antibiotic-associated diarrhea is very important and guides both the treatment and control of nosocomial spread of infection. The ELISA kits with a high specificity, sensitivity and stability for the rapid detection of *C. difficile* toxin A are presented here. The usefulness of the ELISA kits awaits further studies in clinic.

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• BRIEF REPORTS •

# Relationship between urokinase-type plasminogen activator receptor and vascular endothelial growth factor expression and metastasis of gallbladder cancer

Shu-Qiang Yue, Yan-Ling Yang, Jing-Shi Zhou, Kai-Zong Li, Ke-Feng Dou

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# Abstract

**AIM:** To investigate the relationship of urokinase type plasminogen activator receptor (uPAR) and vascular endothelial growth factor (VEGF) expression with clinical and pathological characteristics of human gallbladder cancer.

**METHODS:** uPAR and VEGF expressions in 68 gallbladder cancer tissues were detected with anti-receptor immunohistochemical stain.

**RESULTS:** Expression rate of uPAR was 57.4% (39/68), and VEGF 51.5% (35/68) in gallbladder cancer tissues. Expression of both uPAR and VEGF was significantly related to metastasis, but not significantly correlated with differentiation stage and size of gallbladder cancer.

**CONCLUSION:** Expression of uPAR and VEGF may be an invasive phenotype of gallbladder cancer and indicator for predicting prognoses, and uPAR expression is significantly correlated with the expression of VEGF.

Yue SQ, Yang YL, Zhou JS, Li KZ, Dou KF. Relationship between urokinase-type plasminogen activator receptor and vascular endothelial growth factor expression and metastasis of gallbladder cancer. *World J Gastroenterol* 2004; 10(18): 2750-2752 http://www.wjgnet.com/1007-9327/10/2750.asp

# INTRODUCTION

The main lethal cause of patients with malignant tumor is the invasion and metastasis of tumor cells. Tumor blood vessels can not only get rich nutrient from hosts, but also spread many malignant cells to hosts. And all these result in unceasing tumor growth and metastasis<sup>[1-4]</sup>. Urokinase-type plasminogen activator (uPA) is a kind of serine protease, which can activate plasminogen to fibrinolysin, the latter can degrade most kinds of extracellular matrix, which then form extracellular local lysis region, thus constructing the path for metastasis. The role of uPA in vivo is dependent on the expression of corresponding receptor (uPAR) in cell membrane. The conjugation of uPA and uPAR can reinforce tumor cells' infiltration ability<sup>[5,6]</sup>. Formation of tumor blood vessels is a very complicated process. Vascular endothelial growth factor (VEGF) has an important role in the formation of tumor blood vessels, as well as in growth and metastasis of tumor, because it can adjust angiogenesis and is specific<sup>[7-9]</sup>. Prognosis

of operation of gallbladder cancer is poor, and its relapse rate is very high. So investigation of metastasis and recurrence of gallbladder cancer has great clinical significance. We made use of immunohistochemistry to detect the expression of uPAR and VEGF in gallbladder cancer, and investigated the relationship between the expression of uPAR and VEGF and pathologic characteristics, invasion and metastasis of gallbladder cancer. Image analyses were also used to quantitatively analyze the relationship between them.

# MATERIALS AND METHODS

# Materials

Sixty-eight specimens of gallbladder cancer resected or biopsied from 1990 to 2001 were pathologically diagnosed. Retrospective analyses were performed on these routine paraffin embedded sections of 4  $\mu$ m thick. According to WHO classification standard of gallbladder cancer differentiation, 21 cases were in grade I, 27 cases grade II, 20 cases grade III. Twenty-six cases had a diameter of tumor </=2 cm, 23 cases 2-4 cm, 19 cases >/=4 cm. Metastasis was found in 31 cases by clinic examination and in surgical operation. Murine anti-uPA, -VEGF and -SABC monoclonal antibodies were purchased from Wuhan Bosted Co, and uPA from Guangdong Tipus Co.

# Methods

Anti-ligand antibodies were used to determine uPAR<sup>[10-13]</sup>: routine de-waxing, trypsin digestion, non-specific antigen blocking. A 1 mg/L of urokinase was used to saturate receptors; anti-uPA monoclonal antibody was added; the rest procedures were according to routine SABC. The procedures of VEGF mAb staining was according to instruction of SABC test kit. There were blank, substitute and normal controls. Positive cells were defined as cytoplasm and/or cell membrane stained clearly buffy or brown. Samples were analyzed by image analyzer. Firstly, strong positive expression regions were selected under low power visual field, then, 10 high power visual fields (400 times) were randomly selected, their grey scales were detected, the average value was used as average expression intensity of the sample.

# **Statistics**

Analyses were performed by  $\chi^2$ -test, *t*-test and correlationtest. *P*<0.05 was considered significant.

# RESULTS

# Results of immunohistochemistry

Positivity rates of uPAR and VEGF expressions in tissue of gallbladder cancer were 57.4% (39/68) and 51.5% (35/68), respectively. uPAR and VEGF were negatively stained in corresponding noncancerous tissues, including relatively normal liver tissue and normal mucosa tissue of gallbladder.

# Expression of uPAR and VEGF and clinical pathological stages

Analysis of the relationship between expression rate and

intensity of uPAR and VEGF and clinical features revealed that expression of uPAR and VEGF was closely correlated to metastasis of gallbladder cancer, but not significantly correlated to the differentiation stage and size of gallbladder cancer (Tables 1, 2).

 Table 1
 Relationship between uPAR expression and clinical pathological stages of gallbladder cancer

Tumor	Patients (n)	uPAR-positive patients ( <i>n</i> )	Positive rate (%)					
Diameter of tumor								
=2 cm</td <td>26</td> <td>15</td> <td>57.7</td>	26	15	57.7					
2-4 cm	23	11	47.8					
>/=4 cm	19	13	68.4					
Pathological stage								
Ι	21	12	57.1					
II	27	16	59.3					
III	20	11	55.0					
Metastasis								
Positive	31	$27^{ m b}$	87.1 <sup>b</sup>					
Negative	37	12	32.4					

 $^{b}P < 0.01$  vs others.

 Table 2
 Relationship between VEGF expression and clinical pathological stage of gallbladder cancer

Tumor	Patients (n)	uPAR-positive patients ( <i>n</i> )	Positive rate (%)
Diameter of tumor			
=2 cm</td <td>26</td> <td>14</td> <td>53.8</td>	26	14	53.8
2-4 cm	23	12	52.2
>/=4 cm	19	9	47.4
Pathological stage			
Ι	21	12	57.1
II	27	15	55.6
III	20	8	40.0
Metastasis			
Positive	31	$24^{\rm b}$	77.4 <sup>b</sup>
Negative	37	11	29.7

<sup>b</sup>P<0.01 vs others.

#### Image analysis of expression of uPAR and VEGF

Grey scales for positively expressed uPAR and VEGF were 238.4 $\pm$ 6.2 and 231.2 $\pm$ 4.1, respectively, that for negative expression were 32.1 $\pm$ 4.3 and 36.2 $\pm$ 3.7, respectively. Correlation analysis showed that the expression intensity of uPAR was significantly positively correlated to that of VEGF ( $\gamma$  = 0.671).

# DISCUSSION

Human uPAR is composed of 313 amino acid residues. The binding site for uPAR and its ligand-uPA is domain I which is close to N-terminal. Amino acid residues involved in the interaction with ligand are mediated by hydrophobic interaction<sup>[14-17]</sup>. uPAR and its ligand-uPA's binding is highly specific. Moreover, this kind of highly effective binding (Kd = 0.1-1.0 nmol/L) makes uPA strongly gather on cell surface, thus activating plasminogen to fibrinolysin locally, leading to extracellular matrix hydrolyzing<sup>[18-21]</sup>. On the other hand, uPAR also has high avidity to pro-uPA. After pro-uPA binding to its membrane receptor, pro-uPA is easily activated into uPA by fibrinolysin around, then pre-fibrinolysin is activated into fibrinolysin by uPA, forming positive feedback enlargement effect. In addition,

fibrinolysin on cell membrane is not easily hydrolyzed to inactive form by its inhibitor- $\alpha 2$  anti- fibrinolysin<sup>[22-25]</sup>. Furthermore, uPAR also activates pre-fibrinolysin by taking part in complex formation of pro-uPA and pre-fibrinolysin on cell surface. Therefore, expression of uPAR in tumor cells has an important localizing role in process of local extracellular matrix hydrolysis, and closely correlates to metastasis<sup>[26-30]</sup>.

VEGF is a kind of specific vascular endothelial cell stimulating factor. It high-effectively and specifically acts on vascular endothelial cells, and intensively promotes splitting and chemotaxis by: (1) increasing microvessel permeability, leading to plasm fibrous protein exosmose, thus providing a fiber network for cell migration during the process of vascularization<sup>[31-34]</sup>; (1) directly stimulating endothelial cell proliferation by acting on two special receptors flt and flk (kdk) of endothelial cell, and producing plasminogen activator (tissue-type and urokinase-type) and collagenase<sup>[35-37]</sup>. It not only promotes endothelial cell movement, which is in favor of vascularization, but also benefits cancer cells shedding and entrance to blood vessel or infiltrating to neighboring fibrous protein and connective tissue matrix. This specificity provides conditions for tumor invasion and metastasis.

Our results display that expression of uPAR and VEGF is closely correlated to invasion and metastasis of gallbladder cancer, but not significantly correlated to the differentiation stage and size of gallbladder cancer. uPAR and VEGF can be regarded as an invasive phenotype of gallbladder cancer and used for predicting the prognoses, and as evaluation marker for therapeutic efficacy as well. The results also revealed the correlation between the incidence of gallbladder cancer and expression of uPAR and VEGF. On the one hand, extracellular matrix hydrolysis by uPAR provides advantages over vascularization; on the other hand, plasminogen activator induced by VEGF stimulates endothelial cell growth and increases microvascular permeability by interacting with uPAR, herein, extracellular matrix hydrolysis is reinforced by uPAR<sup>[38-40]</sup>. The regulatory mechanism between uPAR and VEGF, and effective gene therapy methods need further investigation.

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BRIEF REPORTS •

# Adjustment of lipiodol dose according to tumor blood supply during transcatheter arterial chemoembolization for large hepatocellular carcinoma by multidetector helical CT

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# Abstract

**AIM:** To work out an individualized lipiodol dose in transcatheter arterial chemoembolization (TACE) for large hepatocellular carcinoma (HCC) according to its blood supply evaluated by CT.

**METHODS:** One hundred patients with large HCC (more than 8 cm in diameter) were studied by multidetector helical CT. Patterns of blood supply of HCC were divided into sufficient blood supply, poor blood supply, mixed blood supply and arteriovenous (A–V) shunt. The dose of ultrafluid lipiodol was determined by diameter and blood supply type of HCC. Patients were divided into two groups (50 cases each): lipiodol perfusion group and iodized oil perfusion group according to tumor diameter and the blood supply type of tumor.

**RESULTS:** The confirmation and effective rates were 82%, 84% in the first group and 36%, 46% in the second group (*P*<0.01).

**CONCLUSION:** A relatively individualized lipiodol dose may be determined according to the blood supply pattern and the tumor diameter by CT imaging.

Cheng HY, Shou Y, Wang X, Xu AM, Chen D, Jia YC. Adjustment of lipiodol dose according to tumor blood supply during transcatheter arterial chemoembolization for large hepatocellular carcinoma by multidetector helical CT. *World J Gastroenterol* 2004; 10(18): 2753-2755

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# INTRODUCTION

A critical issue in treating large hepatocellular carcinoma (HCC) is determining the optimum lipiodol and antitumor drug dose before transcatheter arterial chemoembolization (TACE). Overdose will damage hepatic function and inadequate dose will lead to poor treatment effect. Tumor blood supply is the crucial factor for determining lipiodol dose. Lipiodol dose was worked out according to the HCC blood supply check by CT scan, then the actual perfusion dose and curative effect were compared in order to assess the therapeutic efficacy.

# MATERIALS AND METHODS

# Patients

One hundred patients (91 male and 9 female, age range 20-72 years,

mean age 48 years) with large HCC (diameter larger than 8 cm) were prepared for TACE. All HCC cases were confirmed by clinic, laboratory and image examinations.

# Methods

CT scan CT scan patterns were as follows: multi-slice helical CT (Lightspeed QX/I, GE Corporation); 5 mm-thick-section; Pith 3:1; non-enhanced scanning performed along with arterial phase, portal-venous phase and delayed-phase at 20-25 s, 45-50 s and 2-3 min, respectively; at 120 kV; 270-300 mA; high pressure injector as well as nonionic-contrast media injected at a rate of 3 mL/s (standard 1.2-1.5 mL/kg). The images were obtained by picture archiving and communication system (PACS) and relevant diagnostic reports writing workstation (DELL 21 in monitor, resolving power 1920×1920). Radiologists analyzed the CT images by using analysis software on Radworks 5.1 to determine the tumor contrast features and confirm the blood supply patterns and further work out the lipiodol dose. DSA examination DSA (Advantx TC. GE Corporation) was selected and the hepatic artery, coeliac artery and mesenteric artery were detected; the contrast media was injected to reveal the tumor blood supply; the correlation between CT and DSA images was analyzed. Catheter was put into nourishing artery with super-selection skill and anti-carcinoma drug and iodine oil were perfused.

**Perfusion dose** All cases were randomly divided into two groups. In testing group, dose was determined based on maximum tumor diameter along with the pattern of blood supply. When the sufficient blood supply was concerned, lipiodol dose would be 2-3 times tumor's diameter (If tumor diameter of sufficient blood supply was about 10 cm, the lipiodol dose would be about 20-30 mL). As for the poor blood supply, the dose would be equal to its diameter; lipiodol was perfused till tumor volume was filled completely. To mixed blood supply area. When A-V shunt existed, lipiodol amount was determined according to actual condition during TACE. In control group dose was determined based on tumor size (If tumor was about 10 cm, lipiodol dose would be 10 mL accordingly).

**Reference standard of lipiodol congregation** In CT film, we took the area which lipiodol actually occupied within tumor as a criterion after first TACE: over 75% area as complete filling, area between 50-74% as relatively complete filling, below 49% area as partial filling. Through CT scanning, complete filling and relatively complete filling accorded with pre-operation criterion and partial filling did not.

**Evaluation criterion for curative effect** With short term followup of 6 mo, according to evaluation criterion established by WHO, curative effect could be divided into: complete response (CR), partial response (PR), no change (NC), and progressive deterioration (PD). The three former items were regarded as effective and PD ineffective.

Statistics Confirmation rate and effective rate were assessed by  $\chi^2$  test.

#### RESULTS

#### Four types of blood supply patterns of large HCC

Type I, abundant blood supply, which could be further divided into Ia and Ib subtype. Ia subtype: Tumor displayed obviously homogeneous or non-homogeneous hyper-dense contrast in arterial phase after enhancement in which broadened nourishing vessels were like radiated, piebald, petal or tuberous shape, and were still in enhancement at portal-venous phase and tailed off at delayed-phase. In DSA, tumor's nourishing arteries broadened and small vessel was hyperplasia in radiated and clasp shape. Ib subtype: Compared with surroundings of hepatic parenchyma in CT, tumor presented slight enhancement and were still in relative hypodense in arterial and portal-venous phases where slightly broadened or nodular arteries at arterial phase could be seen. And palisade shape enhancement could be found at portal-venous phase. Many small vessels showed in DSA film.

Type II, poor blood supply, tumor showed no or mild enhancement during arterial phase, portal-venous phase or delayed phase. In DSA, the nourishing arteries presented no or slight widening and small vessels were in absence; during parenchyma phase, the tumor showed slightly staining.

Type III, mixed blood supply, tumor consisted of sufficient blood supply areas and poor blood supply areas, some areas took on apparent contrast while others did slightly. In DSA, sufficient blood supply areas and poor supply areas co-existed in a tumor.

Type IV, A-V shunt portal vein showed at arterial phase in advance while tumor rarely contrasted at arterial or portal venous phase. In DSA, the normal hepatic artery evidently enlarged rather than tumor arteries. A great amount of contrast media could flow into fistula existed in tumor through portal vein or hepatic vein so that involved veins visualized.

From Tables 1, 2, types I-III in CT scan accorded to that in DSA, and the type of A-V shunt in CT scan is very different from that in DSA. The confirmation rate of lipiodol perfusion was 82% in testing group and 36% in control group. In terms of the effective rate, the difference was significant between testing group (84%) and control group (46%).

**Table 1** Blood supply of 100 cases large HCC classified in CT and DSA

Case	Sufficient ase blood supply		Poor blood supply		Mixed blood supply		A-V shunt	
	Case	%	Case	%	Case	%	Case	%
100	75	75	14	14	7	7	4	4
100	72	72	12	12	6	6	10	10
	Case 100 100	Case Suff Case 100 75 100 72	Sufficient           Case         %           100         75         75           100         72         72	Sufficient         Performance           Case         %         Case           100         75         75         14           100         72         72         12	Sufficient         Point           Lase         %         %           100         75         75         14         14           100         72         72         12         12	$\begin{array}{cccc} & Sufficient \\ Sufficient \\ \hline Case \end{array} & & \begin{array}{cccc} & Sufficient \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{cccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{ccccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{ccccc} & Oot \\ & Oot \\ \hline Case \end{array} & & \begin{array}{ccccccccccccccccccccccccccccccccc$	$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$

Tal	ble	2 (	Curative	effects	in	two	grou	ps
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Group	Case	PR	NC	PD	Effective rate (%)
Testing group	50	24	18	8	84.0
Control group	50	7	16	27	46.0

CP: Complete response; PR: Partial response; NC: No change; PD: Progressive deterioration.

#### DISCUSSION

Lipiodol dose is determined by tumor blood supply, patients' general conditions, tolerance to operation, catheter position, *etc.*, of which tumor blood supply plays a crucial role. CT and DSA can display the tumor blood supply well. With sufficient blood supply, tumors in CT scan show apparent enhancement and in DSA display a large amount of inordinately dilated vessels, even "blood lake". With poor blood supply, tumors

reveal little enhancement as well as a small number of vessels. The display rate and staining extent of the vessels were different when the catheter ends reached coeliac trunk, common hepatic artery or proper hepatic artery. Tumor staining extent was significantly higher in latter two because most of blood stream could flow back to splenic artery when catheter was in coeliac trunk. In addition, display rate and staining extent of small vessels were in direct proportion to contrast media amount and injection rate. Two cases revealing no classical trabecula and roundish contrast features in CT were misdiagnosed as poor blood supply type, but the nourishing arteries were presented in arterial phase and a large amount of small vessels appeared in portal venous phase and in DSA lipiodol filled in the tumor completely.

It is of great significance to work out a set of individualized therapy before TACE. Previously, lipiodol dose was determined by tumor's size<sup>[1]</sup>. In another word, lipiodol dose should correspond to tumor size and if lipiodol dose is equal to tumor diameter or 1.5 times the diameter, it will produce good curative effect, and if dose is beyond twice the diameter, it will have fewer effects and even damage the hepatic function, leading to cirrhosis. Matsuo<sup>[2]</sup> believed that in conventional TACE, lipiodol should be over 5 mL if tumor diameter is less than 5 cm, and the maximum dose will be 10 mL when the tumor develops to more than 5 cm in diameter. With the improvement of the catheter quality and the development of the superselective skills, the catheter can be superselected directly into tumor supply arteries with ease, and lipiodol dose can rise up to 20 mL to fill the tumor less than 10 cm in diameter and 30 mL or more to the tumor more than 10 cm. But in poor blood supply cases, the lipiodol dose should not be over its necessity because lipiodol can flow back and disperse into the normal liver parenchyma. As for mixed blood supply, the dose will rely on the sufficient blood supply area in tumor. Tumor has its shape and volume, supposing to make the lipiodol fill in the tumor as much as possible, it is not enough to inject lipiodol dose into tumor just as its maximum diameter, especially in sufficient blood supply tumor. The tumor will survive if lipiodol dose is not sufficient. Theoretically, tumor cell can be killed only when lipiodol has obstructed all supply vessels and drug flows completely into the tumor. Over dose of drug and lipiodol will make hepatic function get worse in the case of the liver cirrhosis. For these reasons above, to make the drug and lipiodol fill as completely in the tumor as possible, lipiodol dose ought to be based not only on tumor diameter but also on individual blood supply.

CT film can reflect tumor's position, shape, size, quantity as well as blood supply, thus providing guidance for therapy. Patterns of tumor blood supply can be divided into 3 types at arterial phase in CT scan: sufficient blood supply, poor blood supply and mixed blood supply. The survival rate of sufficient blood supply is obviously higher than that of poor blood supply<sup>[3,4]</sup>. Vogl<sup>[5]</sup> held that lipiodol filling over 75% area of tumor was complete filling and 50-70% area was comparatively complete filling, and the survival rate was encouraging in these two circumstances.

Chung suggested that over-dose lipiodol injection could lead to pulmonary artery embolism when obvious artery-vein shunt exists, especially in hepatic arterial-venous fistula of large HCC. CT can illustrate obvious A-V shunt and provide guidance for therapy. In such cases, lipiodol dose should be decreased and fistula embolized by gelfoam, then correct dose of lipiodol injected to perfuse tumors.

In the first time of therapy, lipiodol dose should be relatively excessive to completely fill in the tumor, because tumor cells can produce the drug resistance with the increase of TACE operation, the artery will be impaired more or less by lipiodol even produce stenosis or obstruction, and finally, the sufficient blood supply of tumor may turn into poor blood supply, meanwhile, the normal hepatic parenchyma may gradually become cirrhosis, or deteriorated from intrinsic cirrhosis, which would bring side effects and even interrupt the treatment.

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# Simplified purification method for *Clostridium difficile* toxin A

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# Abstract

**AIM:** To establish the purification method for *Clostridium difficile* (*C. difficile*) toxin A.

**METHODS:** *C. difficile VPI 10463* filtrate was cultured anaerobically by the dialysis bag methods. And then the toxin A was purified by precipitation with 500 g/L  $(NH_4)_2SO_4$  and acid precipitation at pH 5.5, followed by ion-exchange chromatography on DEAE-Toyopearl.

**RESULTS:** Purified toxin A exhibited only one band on native polyacrylamide gel electrophoresis (native-PAGE) and Ouchterlony double immunodiffusion. The molecular weight of toxin A was estimated to be 550 000. The purified toxin A had a protein concentration of 0.881 mg/mL. The minimum lethal dose was  $1 \times 10^6$  MLD/mL (i.p.mice). The cytotoxic titer was  $10^7$  CU/mg. The haemagglutinate activity was at a concentration of 1.72 µg/mL. The ratio of fluid volume (mL) accumulated to the length (cm) of the loop was 2.46.

**CONCLUSION:** The modified method for purification of toxin A of *C. difficile* was simple and convenient. It may be even more suitable for purification of toxin A on large scales.

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# INTRODUCTION

*Clostridium difficile* (*C. difficile*) is one major causative agent of pseudomembranous colitis (PMC) and a large number of antibiotic-associated diarrhea in humans and animals. It has been reported that this organism produces at least two toxins, designated A (enterotoxin) and B (cytotoxin). Toxin A, a tissuedamaging enterotoxin, causes hemorrhagic fluid accumulation in rabbit ileal loops and is cytotoxic to cultured fibroblasts. Toxin B is an extremely potent cytotoxin for many cultured cells<sup>[11]</sup>. The importance of toxin A in the pathogenesis of *C. difficile* enteritis has been documented and several investigators have established the purification method of enterotoxin<sup>[21]</sup>. In this report, we presented a simple method for purification of toxin A. The highly purified toxin A was obtained and analyzed for its biological and immunological properties.

# MATERIALS AND METHODS

Bacterial strains and culture filtrates

C. difficile VPI 10463 was grown anaerobically at 37 for 72 h

in Brain Heart Infusion (Difco) by the dialysis bag methods.

# Purification of toxin A

C. difficile VPI 10463 culture filtrates were centrifuged at  $8\,000\,r/min$  for 20 min and filtrated through a 0.45  $\mu m$  membrane filter. The culture supernatant of 200 mL was precipitated by 500 g/L saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> overnight at 4 °C, centrifuged at 8 000 r/min for 15 min. The precipitation was dissolved in 50 mmol/L Tris-HCl buffer (pH7.5) and dialyzed in 10 mmol/L acetic buffer(pH5.5) at 4 °C for 24 h, centrifuged twice, The precipitate was dissolved in 50 mmol/L Tris-HCl (pH7.5) and dialysed against the same buffer, concentrated by PEG 10000 at 4 °C. The sample was then loaded onto anion-exchange DEAE-Toyopearl 650 mol/L chromatography column, the sample was equilibrated by 300 mL of 50 mmol/L Tris-HCl buffer (pH7.5, containing 50 mmol/L NaCl), and then eluted first with 200 mL of a linear NaCl gradient (50-250 mmol/L NaCl), followed by 200 mL of 50 mmol/L Tris-HCl buffer (pH7.5) containing 300 mmol/L NaCl. A second elusion using 200 mL of a linear gradient (300-600 mmol/L NaCl) in the same buffer was performed. The toxins that eluted in the first NaCl gradient were designated toxins A. The toxin was pooled and concentrated, and analyzed for its biological and immunological properties.

# Tissue culture assay

Cytotoxic activity was determined by using African green monkey kidney (Vero) cells as previously described by Kamiya *et al.*<sup>[3]</sup>. For the analysis, tenfold dilutions of test samples were prepared and assayed for activity. The cytotoxic titer, which was expressed as the reciprocal of the highest dilution that caused 100% rounding of the cells by 24 h, was determined by serially diluted 100  $\mu$ L samples transferring to microtiter wells containing Vero cells (10<sup>5</sup>/mL) in 100  $\mu$ L of MEM medium. The specific activity, which was determined from the cytotoxic titer, was defined as the number of cytotoxic units per microgram.

# Haemagglutinate activity

In reference to the methods described by Meng *et al.*<sup>[3]</sup>, rabbit blood cells were washed 3 times with PBS (0.15 mol/L, pH7.2) and then diluted to 25 mL/L suspension. 2×serial dilution of specimens (50  $\mu$ L) was performed with PBS buffer in microplate wells and 50  $\mu$ L of blood cells were added to each well. The HA titer was expressed as the highest dilution that agglutinated rabbit blood cells at 4 °C for 3 h.

# Rabbit ileal loop assay

Enterotoxic activity was determined by the rabbit ileal loop assay as previously described<sup>[3]</sup>. Results were obtained approximately 18 h after injection of 1 mL of test samples and expressed as the ratio of the accumulated fluid (volume) to the length (centimeters) of the loop. Heat-inactivated toxin preparations were used as negative controls.

# Determination of lethal activity

The minimum lethal dose was determined by intraperitoneally injecting mice (14-16 g) with 0.5 mL aliquots of tenfold serial dilutions of test samples and observing the mice over 72 h for toxicity. Two mice were used for each dose of toxin.

#### Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis was performed in 75 g/L and 40-300 g/L gel. Samples were electrophoresed at 120 V for 15 h after initial electrophoreses at 70 V for 30 min. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 and destained. Molecular weights were estimated by comparison with high molecular weight standards (Pharmacia).

#### Immunological methods

Ouchterlony double immunodiffusion was done in 10 g/L agarose. Crossed immunoelectrophoresis (CIP) was performed as previously described<sup>[3]</sup>. Goat antiserum against toxin A used in the analyses was prepared in our laboratory.

#### Protein assay

Protein concentration was determined by the method of Coomassie brilliant blue G-250. Bovine serum albumin was purchased from Sigma Company of USA.

#### RESULTS

High purified toxin A was obtained by precipitation with 500 g/L  $(NH_4)_2SO_4$  and acid precipitation at pH5.5, followed by DEAE-Toyopearl 650 mol/L column chromatography. The elution profile of toxin A by DEAE-Toyopearl 650 mol/L column chromatography is shown in Figure 1. The purified toxin A exhibited only one band on 40-300 g/L native polyacrylamide gel electrophoresis, the molecular mass of toxin A was estimated to be 550 000 (Figure 2). The toxin preparation also gave a single component when analyzed by Ouchterlony double immunodiffusion (Figure 3) and cross immunoelectrophoresis.

The purified toxin A had a protein concentration of 0.881 mg/mL, the cytotoxic titer was  $10^7$  CU units per mg. The HA activity was at a concentration of 1.72 µg/mL, the ratio of fluid accumulated volume (mL) to the length (cm) of the loop was 2.46. The minimum lethal dose was  $1 \times 10^6$  MLD/mL (Table 1). All biological properties of purified toxin A were neutralized by the antiserum of *C. difficile* toxin A.



**Figure 1** Purification of *Clostridium difficile* toxin A by DEAE-Toyopearl 650 mol/L chromatography. Fractions 1-34 were eluted with 50 mmol/L Tris-HCl buffer (pH7.5) containing a linear NaCl gradient (50 mmol/L-250 mmol/L), then fractions 35-50 were eluted with 300 mmol/L NaCl and fractions 51-75 were eluted with linear gradient (300 mmol/L-600 mmol/L NaCl) in the same buffer.

1 2 *M* (x1 000) 660 440 232 140 67

**Figure 2** Analysis of toxin A preparations by native-PAGE (4-30%). Line 1: Purified *C. difficile* toxin A; Line 2: High molecular mass markers, thyroglobulin ( $M_r$  669 000), ferritin ( $M_r$  440 000), catalase ( $M_r$  232 000), lactate dehydrogenase ( $M_r$  140 000), bovine serum albumin ( $M_r$  67 000).



**Figure 3** Analysis of toxin A by Ouchterlony double immunodiffusion. Well 3 contained 20  $\mu$ L of antiserum against strain 10463 culture filtrate, well 1 and well 2 contained 20  $\mu$ L of purified toxin A by acid precipitation and DEAE-Toyopeal 650 mol/L chromatography respectively.

#### DISCUSSION

Affinity chromatography used to purify toxin A in the present study was first described by Krivan *et al*. The methods using bovine thyroglobulin for purification of *C. difficile* toxin A which depends on the temperature was dependent on binding between toxin A and thyroglobulin. A change of the temperature form 4 to 37 toxin A was releases from the glycoprotein molecule<sup>[2]</sup>. In addition to toxin A, there were trace amounts of toxin B and a few other proteins at low level. As thyroglobulin is an expensive biological reagent, this method might not be suitable for purification of toxin A in some laboratories.

Sullivan *et al.* (1982) described a procedure for preparing milligram amounts of homogeneous toxin A from culture filtrate<sup>[4]</sup>. The method consisted of ultrafiltration through an Amicon XM100 membrane filter, anion-exchange chromatography on DEAE-Sepharose CL-6B, and precipitation at pH5.5. We purified toxin A from culture filtrate with the method and found that except for a major protein band, there was a faint band as well on native PAGE. High purified toxin A was obtained by precipitation with 500 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and acid precipitation at pH5.5, followed by DEAE-Toyopearl 650 mol/L column chromatography.

In the studies on the toxins of *C. difficile*, researchers have reported the cytotoxicity of *C. difficile* toxin A for human colonic and pancreatic carcinoma cell lines<sup>[5]</sup>. Although there are a lot

Table 1 Purification of C. difficile toxin A

Purification step	Vol (mL)	Protein(mg/mL)	Lethal dose(Mice, MLD/mL)	Cytotoxicity (CU/mg)
Crude culture supernatant	200	1.86	6×10 <sup>4</sup>	1010
50% (NH <sub>4</sub> ) <sub>2</sub> SO4 precipitation	10	ND	ND	ND
Acid precipitation	10	6.15	$2 \times 10^{6}$	10 <sup>10</sup>
DEAE-Toyopeal 650 mol/L	6	0.881	1×10 <sup>6</sup>	107

of problems to be solved before the toxin being used in clinic, the modified method for purification of toxin A of *C. difficile* was simple and convenient, it may be even more suitable for purification of toxin A on large scale.

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• BRIEF REPORTS •

# **Expression of Survivin in pancreatic cancer and its correlation to expression of Bcl-2**

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# Abstract

**AIM:** To investigate the expression of Survivin in pancreatic cancer and its correlation to the expression of Bcl-2.

**METHODS:** Survivin and Bcl-2 expressions were examined by immunohistochemistry in 42 tissue samples from pancreatic cancer and 10 from normal pancrease.

**RESULTS:** No survivin expression was detected in the tissue samples from normal pancrease, while it was detected in 34 of 42 tissue samples from pancreatic cancer (81.95%). There was a correlation between survivin expression and differentiation and stages of pancreatic cancer. Survivin positive cases were strongly correlated to Bcl-2 expression (28/30 *vs* 6/12, *P*<0.05).

**CONCLUSION:** Overexpression of survivin plays an important role in the development and progression of pancreatic cancer, and correlates to the expression of Bcl-2. Survivin expression can be used as a prognostic factor.

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# INTRODUCTION

The inhibitor of apoptosis protein (IAP) is a member of the widely-expressed gene family of apoptosis inhibitors. It can inhibit apoptosis induced by a variety of stimuli and plays a critical role in the physiologic activities of cells. Survivin is a recently-characterized gene, a member of the IAP family, and has a close relation between anti-apoptosis and tumors. Overexpression of survivin has been reported to play an important role in the development and progression of pancreatic cancer<sup>[1-5]</sup>. In this study, we investigated the relationship between the expression of Survivin in patients with pancreatic cancer and the expression of Bcl-2 in order to provide a theoretical basis for the prevention, diagnosis, and treatment of pancreatic cancer.

# MATERIALS AND METHODS

#### Materials

Ten samples from normal pancreatic tissue and 42 samples from

pancreatic cancer were collected. Normal pancreatic tissues were obtained from autopsy specimens in Department of Anatomy, Medical College, Wuhan University, and their mean age was 49.3 years (range, 33-78). Pancreatic cancer samples were obtained from surgically-resected specimens in Zhongnan Hospital, Wuhan University. All these cancers were diagnosed pathologically.

#### Reagents

Primary antibody for survivin was purchased from Novus Co., Ltd (USA) and Bcl-2 was purchased from Boster Biological Technology Ltd (Wuhan). SP kit was purchased from Zhongshan Biotechnology Co., Ltd (Beijing).

#### Methods

Fixed in routinely-processed formalin and then embedded in paraffin, 4- $\mu$ m thick sections were prepared from the cut surface of blocks at the maximum cross-section. For morphological analysis, the sections were routinely stained with hematoxylin and eosin. Immunohistochemical staining for survivin and Bcl-2 antigen was made by the standard streptavidin/peroxidase (SP) technique. The positive section was used as a positive control. As a negative control, PBS was used instead of the primary antibody for survivin and Bcl-2.

# Scoring criteria

Cytoplasm staining with light yellow or brown was defined as a marker of positive cells. All sections were analyzed by an image analysis system. The mean percentage of positive cells for the expression of survivin and Bcl-2 was determined in at least 10 areas at 400-fold magnification, and the cases with less than 5% positively-stained cells were defined as being negative. The cases with 5% to 10% positively-stained cells were defined as having a positivity rate of "+", 11% to 60% as "++", and more than 60% as "+++".

# Statistical analysis

All statistical analyses were performed with SPSS 11.0 software. Difference and correlation were analyzed by  $\chi^2$  test. *P*<0.05 was considered statistically significant.

# RESULTS

#### Immunohistochemistry

With immunohistochemical staining, we examined the expression of survivin in pancreatic cancer. The results are shown in Figure 1. The expression of survivin was localized in cytoplasm of tumor cells, which are shown as brown granules in Figure 1 (SP), and in Figure 2 (HE). Survivin was expressed in 34 of 42 pancreatic cancer samples, but not expressed in the 10 samples from normal pancreatic tissue. The expression rate was 81.95%.

To study the relationship between the expressions of survivin and Bcl-2, 42 cases were analyzed. The results are shown in Table 2.



**Figure 1** Expression of survivin in poorly and well differentiated pancreatic cancer. A: Expression of survivin in poorly differentiated pancreatic cancer (SP×400). B: Expression of survivin in well differentiated pancreatic cancer (SP×200).



**Figure 2** Poorly and well differentiated pancreatic cancer. A: poorly differentiated pancreatic cancer (HE×400). B: well differentiated pancreatic cancer (HE×400).

**Table 1** Relationship between survivin expression in pancreatic cancer and clinicopathologic parameters

		Survivin expression			
		(-,+)	(++,+++)	– P	Ι
Sex	Male	11	7		
	Female	13	11	>0.05	
Location	Head	16	14		
	Body,Tail	8	4	>0.05	
Differentiation	High	14	4		
	Low	10	14	< 0.05	0.361
Distant metastasis	Present	8	8		
	Absent	16	10	>0.05	
Stage	I-II	18	8		
	III-IV	6	10	< 0.05	0.311

 Table 2 Correlation between expressions of survivin and Bcl-2

	Survivin expression		л	
	(+)	(-)	P	1
Bcl-2 (+)	28	2		
Bcl-2 (-)	6	6	< 0.01	0.499

# DISCUSSION

Survivin, a recently-characterized member of IAP family, was isolated from the human gene bank by Altieri *et al.* in 1997 using effector cell protease receptor-1(EPR-1) cDNA<sup>[6]</sup>. Recently, great progress has been made in the structure and function of survivin and its correlation to malignant tumors.

Many findings have suggested that survivin may express selectively in different tissues. Survivin was max expressed or poorly expressed in normal terminally-differentiated tissues, whereas it was extensively expressed in many kinds of human tumor tissues<sup>[7-14]</sup>.

Studies have shown that Caspase is responsible for apoptosis, which can activate in cascade and lyze protein, thus determining the pattern of apoptosis. Survivin could directly inhibit the activities of Caspase-3 and 7 and block the process of apoptosis and indirectly inhibit Caspase through P21.Therefore survivin would bind to cell cycle apoptosis factor CDK4 to form survivin-CDK4 complex, and then release of P21 from CDK4 complex.When P21 was bound to mitochondrial Caspase-3,it could inhibit its activity, thus preventing apoptosis<sup>[15-17]</sup>.

The results of our study showed that survivin was highly expressed in pancreatic tumor tissues, but not in normal pancreatic tissues, suggesting that it might play a critical role in the development and progression of pancreatic cancer. Furthermore, there was a positive correlation between survivin expression and tumor TNM staging and differentiation grade. It can be concluded that survivin expression is indicative of higher invasiveness or poor prognosis in pancreatic cancer.

Bcl-2 was the first characterized anti-apoptotic gene with an inhibiting apoptotic pathway different from that of survivin. Bcl-2 could regulate apoptosis by preventing cytochrome C release from mitochondrion to cytoplasm.Whereas, survivin acted by direct inhibition of the terminal effector proteases of apoptosis, i.e., Caspase-3 and Caspase-7.

Our study demonstrated that the expression of survivin was positively correlated with that of Bcl-2. It might be caused by the same transcription and activation mechanism of survivin and Bcl-2, or both might be regulated by GC-rich promoters, and were then transcripted and activated to enhance cell proliferation, acting synergistically to inhibit apoptosis<sup>[18-29]</sup>.

Asanuma *et al.*<sup>[2,5]</sup> investigated whether survivin expression could directly regulate cancer sensitivity to radiotherapy using gene-transducted pancreatic cancer cell strain (MIAPaCa-2). Their results showed that survivin expression could directly down-regulate pancreatic cancer sensitivity to radiotherapy. In addition, Rohayem *et al.*<sup>[12,30,31]</sup> observed that specific

In addition, Rohayem *et al.*<sup>[12,30,31]</sup> observed that specific anti-survivin antibody could be detected in serum of patients with cancers of the lung and colon, suggesting that this antibody could be used as a new diagnostic marker for cancers of the lung and colon. Furthermore, Smith *et al.*<sup>[32]</sup>, Moore *et al.*<sup>[33]</sup>, and Swana *et al.*<sup>[34]</sup> found that survivin levels in urine could be used to diagnose primary and recurrent bladder carcinomas, thus providing a new idea for the diagnosis of pancreatic cancer<sup>[35-39]</sup>.

In conclusion, Survivin can be used as a cancer therapeutic target because of its selective expression in the tissue concerned. Moreover, natural antisense nucleic acid for Survivin-endogenous EPR-1 has become a new hot issue for the tumor gene therapy.

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• BRIEF REPORTS •

# Effects of Antiadhesion preparation on free fibrinogen and fibrin degrading products in abdominal exudates of rabbits postoperatively

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# Abstract

**AIM:** To observe effects of ACOL on fibrinogen (FIB), fibrin degrading products (FDP) and changes of FIB and FDP concentration in rabbits with intro-abdominal exudates during 7 d after major abdominal surgery.

**METHODS:** Sixty New Zealand rabbits were randomly divided into 4 groups: ACOL group, the control group, DCT group and the normal group. After being modeled, except the normal group, the other 3 groups were treated with different ways for a week; the intro-abdominal exudates of rabbits in the 4 groups were drawn for FIB and FDP measurement once daily during 7 d after major abdominal surgery.

**RESULTS:** FIB and FDP in the intro-abdominal exudates altered in a regular way and ACOL could change the concentration of FIB and FDP in the intra-abdominal exudates after major abdominal surgery.

**CONCLUSION:** ACOL can prevent intestinal adhesion by reducing the concentration of FIB and raising that of FDP in the intro-abdominal exudates after major abdominal surgery.

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# INTRODUCTION

With a high incidence of 90%<sup>[1-2]</sup>, intra-abdominal adhesions are the most common complications after major abdominal surgery, and overall 70% patients of intra-abdominal adhesion have the history of previous laparotomies<sup>[3]</sup>. The exact mechanisms about this complication have widely been explored. The damage of peritoneum and its base during laparotomies leads to intra-abdominal traumatic inflammation, and thus the normal intra-abdominal physical condition is destroyed, inflammatory cells and medias are released. Coagulation, kinin

peptide, fibrinolysis, arachidonic acid (AA) participate in this process. The activity of plasminoge activator (t-PA), matrix metalloproteinase (MMP) and their inhibitor changes accordingly<sup>[4-8]</sup>. The related factors of adhesion, such as TNF- $\alpha$ , TGF- $\beta$ , and VEG-F, are involved in regulations of the whole process<sup>[9-11]</sup>, which results in the abnormally higher concentration of free FIB and FDP in the intra-abdominal exudates. The disturbance of the intra-abdominal homeostasis among secreting, dissolving and absorbing together with over-deposition of fibrin in the abdominal cavity finally leads to fibrous adhesion <sup>[12]</sup>. In this research, occurrence and development of intra-abdominal adhesions will be discussed by observing the dynamical changes of FIB and FDP in rabbits' intra-abdominal exudes continuously during 7 d postoperatively. Antiadhesion concentrated oral liquid (ACOL) was used as soon as possible to improve the intra-abdominal physical condition and help to regain the homeostasis of internal environments to prevent the formation of the fibrous jelly in the abdominal cavity. This experiment provided evidence for the punctual and strategic prevention and treatment of the postoperative intra-abdominal adhesion.

# MATERIALS AND METHODS

# Experimental animal

Sixty healthy New Zealand rabbits of both sexes, weighing from 2.5-3.5 kg, were purchased from Laboratory Animal Center, Medical College of Xi'an Jiaotong University.

# Drugs, reagents and instruments

ACOL consisted of Rubarb, Cotex Magnoliae Officinalis, Fructus Auvantii Immature, Caucklandiu Lappal, Folium Raphani Radish Seed, Radix Aucklandiae, Radix Astragali, Cortex Cinnamomi, Fructu Amomi, Radix Angelicae Cinensis, Rhizoma Zingiberis and Radix Salvia Miltiorrhiza. Da-Cheng-Qi Tang (DCT) consisted of Rubarb, Ctex Magnoliae Officinalis, Fructus Aurantii Immature and Natrli Sulfas. Both were provided by Shaanxi College of TCM, and decocted with water and concentrated through reflux with distillatory and water bath to extract the decoction containing 1.0 g crude /mL. FIB test kit was purchased from Changzheng Medical Scientific Limited Company, Shanghai, (batch number f4900704). FDP test kit was purchased from Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, (batch number 900325). Automatic Biochemical Analyzer 7170 type was from Hitachi, Tokyo, Japan.

# Animal models

The experiment was carried out in the clean but not sterile condition under 30 g/L procaine anesthesia (0.5 mL/kg,iv). After shaving and skin-disinfecting, the laparoscopy was performed through a 4 cm vertical midline incision to enable the following performances: (1) appendectomy, (2) perforation of an incision (1 cm in diameters) at 5 cm off pylorus on the lesser curvature with punching device, then repair of the perforation, (3)  $2 \times 2 \text{ cm}^2$  area of the cecal parietal peritoneum being carefully stripped, (4) injection of the solution of Talc powder 1mL/kg and 5 mL distilled water into the abdominal cavity prior to skin closure, (5) splenectomy, and (6) leaving a tube for drainage in the
spleen pit because all the animals were fasted for 8 h after operation. The study protocol was in accordance with the guideline for animal research and was approved by the Research Committee of the hospital.

#### Grouping and medication

The 60 New Zealand rabbits were randomly divided into 4 groups: normal group, ACOL group, control group and DCT group, with 15 rabbits in each group. From the second day postoperation, rabbits in ACOL group were administered ACOL (5 mL/kg) gastrogavage twice daily, the DCT group was administered DCT, the control group was given normal saline (NS) instead, while the normal group had none treatment. The medication was carried out from the second day of modeling and lasted for 1 wk.15 mL NS was injected through the drainage cube into the abdominal cavity of each model animal once daily, and 5 min later the abdominal exudates was drawn for FDP and FIB detection.

#### Statistical analysis

All data were expressed as mean $\pm$ SD, and statistical analysis were performed by Student-Newman-Keuls test, ANOVA analysis, Difference between groups were compared by SNK-*q* test, with *P*<0.05 considered to have statistical significance.

#### RESULTS

# Concentration curves of FIB and FDP in rabbits abdominal exudates during the 7 d postoperatively

The concentration curve of FIB in the rabbits' intra-abdominal exudates showed an increasing trend in the initial 5 d, and reached its peak on the 5th d. After that, it showed a decreasing trend, while on the 7th d still above the preoperative value  $(0.1\pm0.017)$  g/L. (Figure 1). At the same time, the concentration curve of FDP in the exudates showed increase in the first 4 d, arriving at its peak on the 3rd or the 4th d. After that, it showed decrease, while the value of the 7th d was also still above the preoperative value  $(0.3\pm0.012) \mu$ g/mL. (Figure 2). In comparison with the concentration curve of FIB and FDP in the exudates during the 7 d postoperatively, the DCT group was similar to the ACOL group while the normal group similar to the control group. (Figure 1, 2). In comparison with the average concentration

of FIB and FDP in intra-abdominal exudates during 7 d postoperatively, there was remarkable difference among the DCT group, normal group, and control group (P<0.05). The ACOL group showed a remarkable difference (P<0.05), compared with the other three groups. (Tables 1, 2).



**Figure 1** Concentration variety of FIB in healthy rabbits' abdominal cavity during 7 d after operation.



**Figure 2** Concentration variety of FDP in healthy rabbits' abdominal cavity during 7 d after operation.

# Analyses of the average concentration of FIB and FDP in rabbits abdominal exudates

Compared with the control group and the normal group, FIB and FDP in the DCT group showed a significant difference

Table 1 Concentration of FIB and FDP in healthy rabbits' abdominal cavity during 7 d after operation

Group	d 1	d 2	d 3	d 4	d 5	d 6	d 7
FIB (g/L)							
Normal group	$0.236 {\pm} 0.052$	$1.621 \pm 0.083$	$1.927 \pm 0.121$	$3.813 \pm 0.154$	$4.823 {\pm} 0.183$	$3.612 {\pm} 0.076$	$2.721 \pm 0.055$
Control group	$0.27 \pm 0.071$	$1.587 {\pm} 0.012$	$2.471 \pm 0.134$	$3.224 \pm 0.126$	$5.256 {\pm} 0.113$	$3.533 {\pm} 0.054$	$3.081 {\pm} 0.046$
DCT group	$0.145 {\pm} 0.021^{a}$	$0.652{\pm}0.046^{\rm a}$	$1.432 {\pm} 0.061^{a}$	1.961±0.051ª	$2.431 \pm 0.112^{a}$	$1.621 \pm 0.107^{a}$	$1.321 \pm 0.062^{a}$
ACOL group	$0.121 {\pm} 0.032^{a}$	$0.457{\pm}0.054^{a}$	$0.628 {\pm} 0.052^{\rm a}$	$0.927 {\pm} 0.072^{a}$	$1.367 \pm 0.077^{a}$	$1.024{\pm}0.068^{\rm a}$	$1.026 \pm 0.059$
FDP (µg/mL)							
Normal group	$2.328 {\pm} 0.072$	$5.821 \pm 0.176$	$8.368 {\pm} 0.372$	$13.472 \pm 0.961$	$8.462 \pm 0.371$	$7.721 \pm 0.262$	$5.642 {\pm} 0.078$
Control group	$2.452 \pm 0.077$	$5.262 \pm 0.132$	$7.526 {\pm} 0.327$	$15.641 \pm 0.625$	$10.431 \pm 0.362$	$7.651 \pm 0.241$	$4.326 {\pm} 0.062$
DCT group	$8.675 \pm 0.323^{a}$	$12.638 {\pm} 0.672^{a}$	$27.236{\pm}2.331^{a}$	$24.638 {\pm} 2.725^{a}$	$16.369 {\pm} 1.026^{a}$	$12.445 \pm 0.328^{a}$	$9.652 \pm 0.375^{a}$
ACOL group	$13.372 \pm 0.541^{a}$	$19.338 \pm 1.321^{a}$	$17.328 \pm 1.124^{a}$	$34.325{\pm}4.68^{a}$	$22.438 {\pm} 1.372^{a}$	$16.367 {\pm} 0.554^{\mathrm{a}}$	$12.771 \pm 0.366$

<sup>a</sup>*P*<0.05 *vs* other groups.

 Table 2
 Average concentration of FIB and FDP in healthy rabbits' abdominal cavity during 7 d after operation

	Normal group	Control group	DCT group	ACOL group
Concentration of FIB (g/L)	$2.679 {\pm} 0.096$	$2.760 {\pm} 0.079$	$1.36 \pm 60.066^{a}$	$0.793 {\pm} 0.059^{a}$
Concentration of FDB ( $\mu$ g/mL)	$7.442 {\pm} 0.268$	$7.613 {\pm} 0.261$	$15.947 \pm 01.121^{a}$	$19.424 \pm 1.417^{a}$

<sup>a</sup>*P*<0.05 *vs* the normal group and the control group.

(P < 0.05); while in comparison with the other 3 groups, those of the ACOL group showed a significant difference (P < 0.05).

## DISCUSSION

So far, there is no safe and effective prophylaxis available for intra-abdominal adhesions. Scientists try to find the answer from studying the exact mechanism of intra-abdominal adhesions, thus various experiments have been conducted to elaborate the exact mechanism of intra-abdominal adhesions and its prevention. The theory of traumatic inflammation is prevalent in elucidating the mechanism of intra-abdominal adhesions<sup>[14]</sup>. Actually, multiple factors are involved in the development of postoperative intra-abdominal adhesions, such as operative injury, tissue ischemia and foreign materials left during operation<sup>[14-15]</sup>. Any damage to the peritoneum and its base can lead to the release of inflammatory exudates, which contained FIB that causes fibrous adhesion and eventually results in the following two consequences: First, the fibrin is eliminated through the phagocytosis and endogenous fibrinolysis system, and the regeneration of mesothelial cell will cover the wound surface thus to get it repaired physiologically. Second, the wound is not covered by the regenerated mesothelial cells and no physiological repair occurs. Instead, fibrous adheres to other serosa in 12-24 h after operation, and then fibroblasts forms in place of the fibrous matrix, which causes the production of the fibrous collagen and eventually leads to fibrous adhesion. The adhesion can be absorbed completely if mesothelial cells are intact, but when the mesothelial cells are seriously destroyed, its plasminogen activator activity (PAA) is depressed, and with the depression value to that low as 60% or less, the fibrous adhesion will form as a consequence<sup>[16-19]</sup>.

In this experiment, the postoperative concentration levels of FIB and FDP in the rabbits' intra-abdominal exudates were constantly observed during the postoperative period of 7 d. The values of both FIB and FDP in intra-abdominal exudates increased considerably after 24 h postoperatively, arrived at its peak on the 4th or 5th d after operation, and then started to decrease till the 7th d, but still not being able to recover to the its normal preoperative level. The whole process is rather similar to the traumatic inflammation process. Giving the experimental animals some drugs which had been proved effective in promoting the ability of intestinal movement, the incidence of intestinal adhesion decreased remarkably, which indicates that the intestinal adhesion is related to traumatic inflammation, intestinal movement, and intra-abdominal physical conditions.

Under normal condition, there is a little serous fluid in the peritoneal cavity, which, on one hand, is necessary to keep the surface of the viscera moist and reduce the friction among the viscera, and on the other hand, functions to help recover the viscera and defend it against damage. Normally, the omentum, parietal and visceral peritoneum keeps a balance between releasing and absorbing serous fluid in the peritoneal cavity. Maintenance of this balance depends upon the normal sequential movement of intestines and the unobstructed movement of intestinal contents. After abdominal surgery, ileuses paralysis and the retention of the intestinal contents can effect the normal sequential movement of intestines and the smooth movement of intestinal contents. With intestines paralyzed postoperation, the contents are retained in the intestinal cavity. Endotoxin and bacteria in the stool are absorbed from intestines, which results in the abnormally high level endotoxin in the blood, which, in turn, stimulates intestines and worsens their paralysis, thus weakening the intestines' sequential movement and obstructing the smooth movement of their contents. Inflammatory reaction is indispensable to the healing of gastrointestinal and peritoneal damage in operation. Tissue injury, local hemorrhage, cell inactivity and coagulation will call for the inflammation response, which originates from the following vascular reaction: the inflammatory mediums such as histamine, 5-HT, bradykinin, and prostaglandin. cause small blood vessels to first contract transiently, then dilate with congestion, and thus increase the permeability of blood vessels, which enables water, electrolyte, serum proteins, neutrophils and monocytes to enter tissue inter-space, so traumatic inflammatory reaction happens to help heal the damage. The FIB that has permeated into the wound gaps can turn into fibrin and fill the gaps to function as the network for cells to proliferate. However these inflammatory exudes can also enter peritoneal cavity from the wound, and the homeostasis of intraabdominal serous postoperation is destroyed. During the damage, the serous secretion is far beyond the absorbing capability of peritoneal cavity. At the same time, high concentration of FIB in the wound exudes increases the concentration of FIB in peritoneal cavity. With the weakened movement of intestines in the early stage of postoperation and the temporarily destroyed normal physical condition, the exudes among viscera containing higher concentration of FIB easily constitute early fibrous adhesion, which is although still fragile at its early stage. Such traumatic inflammatory response arrives at the climax around 48 to 72 h postoperation, then disappears gradually. The early fragile adhesion can turn into tight one through endometrial stage and mould stage. We can see that the early recovery of the intestinal movement and abdominal physical condition postoperation is vital for prevention of the intestinal adhesion<sup>[20-23]</sup>.

The present study also indicates the traumatic inflammation caused by major abdominal surgery can cause the release of cytokines, the subsequent effects on endothelial cells, inflammatory cells and mesothelini cells, which produce and release plasminogen activator activity inhibitor I or II (PAI1, PAI2). Previous research showed TNF- $\alpha$ , IL-I, TGF $\beta$ , alone or combined, can stimulate the cultured mesothelini cells to produce plasminogen activator activity inhibitor I or II (PAI1and PAI2), which ultimately causes the disturbance of homeostasis between FIB and FDP intra-abdominal exudes. Consequently fibrin depositing on the surface of intra-abdominal viscera constitutes adhesion<sup>[24-27]</sup>.

In essence, intestinal adhesion is that the normal physical condition of peritoneum and the homeostasis between secreting and absorbing sera is destroyed, with higher FIB concentration in the peritoneal cavity, the weakened sequential movement of intestines and the dysfunctional movement of the intestinal contents, so that the wound healing appears among intestine, peritoneal and other viscera. The incidence of intestinal adhesion is related to the degree of the completeness of the peritoneal mesothelial cells in that when mesothelial cells are destroyed seriously, the fibrous adhesion forms. The damage of peritoneal mesothlium cannot be avoided in laparoscopy no matter how gentle the operation is. Since intestinal adhesions postoperation are almost inevitable to some extent<sup>[28]</sup>, recovery of the physical condition in abdominal cavity after operation as soon as possible can reduce the incidence and the adventure of intestinal adhesion<sup>[29]</sup>.

Now we can see, how to shorten the time of traumatic inflammation caused by major abdominal surgery, how to recover the normal physical condition of intra-peritoneum, and how to recover the homeostasis between FIB and FDP, is the vital to prevent the intra-abdominal adhesion postoperatively. The pharmaceutics of laxative remedis represented by Rhubarb start with purgation of intestinal contents, improve the early recovery of intestinal movement as soon as possible. The pharmacologic research of Rhubarb develops quickly, clinic application is wider and wider. The main components of Rhubarb include: Anthraquinones (emodin, rhein, aloe-emdin),

Polysacchavide from Rheum palmatum (DHP<sub>-1</sub>, DHP<sub>-2</sub>) Tannins (hydrolysates type, condensed type), Modern pharmacology finds the effective component in Rhubarb of purgation is Anthraquinone, which effects on colon and increases the tension of the middle and far parts of colon to make them move faster, while no affection on the function of small bowels to absorb the nutritions. experiments reported: if vasectomy the part between colon and small bowels, then inject Anthraquinone into small bowels, but the medicine affects on the colon. Emodin combines with the muscle albumen of affected organ, then behavior cholineroic reaction, which can excite the M receiptors of intestinal smooth muscle, increasing intestinal movement, inhibiting Na<sup>+</sup> K<sup>+</sup> ATP enzyme in intestinal cell membrane. It also can hinder the absorption and transportion of Na<sup>+</sup>, increase osmotic pressure of intra-intestines, keep lots of water, promote intestinal movement to defecate.<sup>[30]</sup>. On one hand through relieving the temporary enterogenic bacterial translocation caused by intestine paralyzed. Rhubarb prevent intestines from ischemia-reperfusion injury, protect gut barrier function, lower the level of endotoxin in blood to demodulate the releasion of cytokine and inflammatory mediums intra-peritoneum. on the other hand, it can lower the ability of endotoxin in blood which stimulates the target cells such as mesothelial cell, platelet et al, Lower the sensitivity of these target cells to the stimulis of endotoxin in blood, make the injurys caused by cytokine, inflammatory mediums easy to be controled. It also can reduce the level of PGE and CAMP in cerebrolate. Lots of experiments prove: Rhubarb remarkably inhibits the functions of cytokine secreted by Macrophage cells which are stimulated by endotoxin. emodin strongly inhibits proliferations of T lymphocyte. Perhaps it is realized through reducing the expression of inflammatory mediums IL-2mRNA and concentration of Ca2+ in cell membrane<sup>[31]</sup>. emodin has excellent Antagonism to early inflammatory exudes, increasing penetration of microvascular, and leucocytes migration<sup>[32]</sup>. In the condition of injures, such as tissue ischemia, endometrial injury, leucocytes Infiltration, and platelet aggregation, lots of free radicals are brought out, which cause pathologic lipidoxidation and induce producation of cytotoxinic lipid free radical and lipid peroxides malondiadehyde (MDA). Rhubarb can inhibit the producation of MDA, relieve the injure of tissue and organ. Rhubarb is a kind of strongly free radical scavengers, and an inhibitor of lipidoxidation, it has function to remove most of free radicals<sup>[33]</sup>. Rhubarb can remove  $O_2$ ,  $H_2O_2$  and other peroxides, inhibit lipid oxidation, Rhubarb themselves on pharmacology have the function of immune regulate two-wayly. emodin improve Indraft of Ca2+ outside of leucocytes, this function have some relations with improving immune of leucocytes; while Polysacchavide from Rheum palmatum inhibits Indraft of Ca<sup>2+</sup> outside of leucocytes and releasion of Ca<sup>2+</sup> inside of leucocytes, and the digree of inhibitation have some relations with its dosage, so for leucocytes it has inhibit function<sup>[34]</sup>, Rhubarb can inhibits the producation of red blood corpuscles antibody, and inhibit the function of T cell, improve phagocytosis of macrophage, it is benefit to regulate immunation. It also can inhibits synthetic of germ's protein and nucleic acid, ACOL adds some medicine such as Radix Astragali, Radix Salvia Miltiorrhiza, Rhizoman Zingiberis to DCT. Those functions lie in invigorating qi and strengthening asthenia, activating blood to resolve stagnation, and improving the whole condition of patiences postoperatively. They also can enhance the resistance of human being, improve the microcirculation. It is benefits to the recovery of the normal physical condition of intra-peritoneum postoperatively. Altogather, ACOL can suppress bacterial and anti-inflammation, protect organs, improve microcirculation through exclude from accumulating. It relieves the accumulation of excrement and bacterial through improving intestinal movement, so it can lower temporary high level toxin in blood, reduce the release and

producation of histamine, bradiykinin, 5-HT, prostaglandin, *et al*, improve the perfusion of blood, relieve the traumitic imflammation intra-peritoneum postoperatively, recover the homeostasis between FIB and FDP as soon as possible, reduce the incidence of adhesion intra-peritoneum.

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• CASE REPORT •

# Endoscopic identification of Peyer's patches of the terminal ileum in a patient with Crohn's disease

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# Abstract

We presented a 20-year-old patient with Crohn's disease (CD). Colonoscopy revealed longitudinal ulceration in the terminal ileum and rectal aphtoid ulcers. After treatment with mesalamine and total parenteral nutrition, repeat colonoscopy revealed a granular elevated area in the terminal ileum, which appeared as an irregular dome-like elevation with irregularly arranged villi on magnifying endoscopy. Biopsy specimens taken from the region showed microgranulomas and lymphoid hyperplasia. Scanning electron microscopy revealed the presence of M cells, confirming that the area corresponded to Peyer's patches. Peyer's patches by magnifying endoscopy and electron microscopy may provide insights into the pathogenesis of CD.

Ishimoto H, Isomoto H, Shikuwa S, Wen CY, Suematu T, Ito M, Murata I, Ishibashi H, Kohno S. Endoscopic identification of Peyer's patches of the terminal ileum in a patient with Crohn's disease. *World J Gastroenterol* 2004; 10(18): 2767-2768 http://www.wjgnet.com/1007-9327/10/2767.asp

# INTRODUCTION

The gut-associated lymphoid tissue (GALT) is composed of Peyer's patches, peripheral lymphoid tissues and appendix and plays an important role in the immune system of the gastrointestinal tract<sup>[1]</sup>. The importance of Peyer's patches in the development of Crohn's disease (CD) is generally accepted<sup>[1-8]</sup>, but there have been only a few reports focusing on the endoscopic appearance of Peyer's patches of the terminal ileum in CD<sup>[3-5]</sup>.

We presented a patient with CD in whom specific CD lesions were found in the Peyer's patches of the terminal ileum by magnifying endoscopy, histopathology and scanning electron microscopy. We also discussed the importance of endoscopic observation of the Peyer's patches of the terminal ileum in the diagnosis and assessment of the pathogenesis of CD.

# **CASE REPORT**

A 20-year-old man was admitted to our hospital with right lower abdominal pain, diarrhea and anal fistula for 4 wk. Physical examination revealed localized guarding with tenderness in the right lower quadrant of the abdomen as well as anal discharge. Laboratory analysis showed leukocytosis (10 700/µL), elevated C-reactive protein (27 mg/L) and slight hypoproteinemia, while other biochemical and serological data were within normal limits. Repeated stool cultures yielded no enteric pathogens including *Yersinia* and *tubercle* bacilli. Colonoscopy showed longitudinal ulcers in the terminal ileum (Figure 1A), ileocecal valve, hepatic flexure, and anal canal. A subsequent radiological examination of the small bowel disclosed three discrete longitudinal ulcers with cobblestone appearance in the ileum. After treatment with a total of 2250 mg of mesalamine for 2 wk under total parenteral nutrition, repeat colonoscopy revealed improvement of the above lesions, leaving deformity of the ileocecal valve and anal stenosis. In addition to multiple aphthoid ulcers in the rectum, a granular elevated area was noted in the terminal ileum, which corresponded to the lymph follicle type of the Peyer's patches according to a classification proposed by Fujikura (Figure 1B)<sup>[9]</sup>. On magnifying endoscopy, the surface of the Peyer's patches appeared as an irregular dome-like elevation with irregularly arranged villi (Figure 1C). Histopathological examination of the biopsy specimens taken from the region and rectal aphthoid ulcers disclosed the presence



**Figure 1** Endoscopic findings. A: Endoscopic view showing an ulceration of the terminal ileum. B: Endoscopic view showing granularly elevated mucosa consistent with the lymphoid follicle type of the Payer's patches. C: Magnifying endoscopy showing the irregular villi and the dome.

of microgranulomas and lymphoid hyperplasia (Figure 2). Scanning electron microscopy of the specimen taken from the Peyer's patch revealed the presence of M cells (Figure 3).



**Figure 2** Histological finding of a biopsy specimen obtained from the Peyer's patches in the terminal ileum disclosing the presence of a microgranuloma and lymphoid hyperplasia.



Figure 3 Scanning electron microscopy of the Peyer's patches demonstrating M cells with microfolds.

#### DISCUSSION

Major advances have been made in every aspect of CD and ulcerative colitis (UC), but several problems in the etiology and pathogenesis of inflammatory bowel diseases (IBD) remain unresolved<sup>[1-8]</sup>. It has been recognized that the disorder of GALT plays a crucial role in the development of IBD<sup>[1-8]</sup>. Peyer's patches and related lymphoid follicles have specialized follicle-associated epithelia (FAE) which serve as sites for the induction of mucosal immune responses<sup>[4,5]</sup>. In particular, it has been found that M cells serve as an important first step in this process<sup>[1,4,5]</sup>, and Peyer's patches are abundantly distributed in the terminal ileum<sup>[7,9]</sup>, an area predisposed to CD. However, little attention has been paid to endoscopic observation of Peyer's patches, and therefore, only a few studies<sup>[3-5]</sup> have described endoscopic appearance of Peyer's patches in the terminal ileum. In this regard, Fujikura<sup>[9]</sup> examined the terminal ileum by colonoscopy in 110 normal cases and grossly in 48 autopsy cases, and classified Peyer's patches into three categories as follows; lymph follicle, lymph border and lymphocyte aggregation types<sup>[9]</sup>. Macroscopically, the lymph follicle type appeared as granular or convolute elevation and was associated with lymphoid hyperplasia. The lymphocyte aggregation type appeared as a flat area associated with mild lymphocytic infiltration and few lymphoid follicles<sup>[9]</sup>. Recently, Van Kruiningen et al.<sup>[7]</sup> examined 55 adults without intestinal disease and reported that Peyer's patches were concentrated in the distal 25 cm of ileum but extended proximally to 200 cm from the ileac end.

In the present case, magnifying endoscopy identified the irregular surface of Peyer's patches, which harbored microgranulomas and lymphoid hyperplasia when examined histopathologically. Scanning electron microscopy identified the presence of M cells, confirming that the specimens were taken from the Peyer's patches.

Our findings pose an important question: does the CD lesion

selectively originate from the Peyer's patch? In this regard, Lockhart-Mummery and Morson<sup>[2]</sup> reported in 1960 that the earliest microscopic change in CD was ulceration of the lymphoid follicles and Peyer's patches in the terminal ileum. Since then, several investigators have reported that CD initially occurs as tiny aphthoid lesions at the sites of mucosal lymphoid follicles in the gastrointestinal tract<sup>[3-5]</sup>. Recently, Fujishima et al.<sup>[3-5]</sup> investigated ultrastructurally the epithelium covering solitary lymphoid nodules using biopsy samples obtained from the colorectum during colonoscopy, and indicated that the red halo appearance of such epithelia seemed to precede visible aphthoid ulcers. They suggested that ulcerations in CD might originate from the follicle-associated epithelium (FAE), possibly related to its role as a portal entry for potentially pathogenic agents. These studies have led to the concept that CD could originate from GALT including Peyer's patches and lymphoid follicles in the terminal ileum<sup>[3-8]</sup>. With this concept<sup>[3-7]</sup>, one can explain the reason for the occurrence of the skip lesions and the frequent involvement of the terminal ileum in CD.

Fujikura<sup>[9]</sup> showed that M cells of the Peyer's patches were more frequently seen in the dome area of the lymph follicle and lymph border types than in that of the lymphocyte aggregation type, emphasizing its pathogenetic functions. Further studies of the ultrastructure of M cells should enhance our understanding of their role in the development of IBD. However, it is difficult to obtain M cells by conventional endoscopic biopsy because they are located in the dome area (FAE), but not in the villi of Peyer's patches<sup>[9]</sup>. Our experience indicates that biopsy using magnifying endoscopy is beneficial for accurate biopsy sampling to obtain tissue segments containing M cells, because this tool allows us to clearly recognize the dome area.

In conclusion, we identified CD-specific lesions in the Peyer's patch of the terminal ileum by endoscopy and histopathological examination. Although the pathogenic role of M cells in the development of IBD is still unknown, examination of the Peyer's patches by magnifying endoscopy and electron microscopy may provide insights into the pathogenesis of CD.

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• CASE REPORT •

# Intestinal perforation after combined liver-kidney transplantation for a case of congenital polycystic disease

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# Abstract

**AIM:** To highlight the intestinal perforation (IP), an uncommon and catastrophic complication after combined liver-kidney transplantation.

**METHODS:** Combined liver-kidney transplantation (LKTx) with left kidney excision and a cyst fenestration procedure on the right kidney were performed on a case of 46-year-old female with congenital polycystic disease (CPCD).

**RESULTS:** Two sites of IP were noted 40-50 cm proximal to ileocecal area during emergent laparotomy 10 d postoperatively. Despite aggressive surgical and medical management, disease progressed toward a fatal outcome due to sepsis and multiple organ failure 11 d later.

**CONCLUSION:** Long duration of operation without venovenous bypass, overdose of steroid together with postoperative volume excess may all contribute to the risk of idiopathic multiple IPs. Microbiology and pathology inspections suggested that the infected cyst of the fenestrated kidney might be one reason for the fatal intra-peritoneal infection. Thus for the CPCD patients who seem to be very susceptible to infectious complications, any sign of suspected renal-infection found before or during LKTx is indication for the excision of original kidney. And the intensity of immunosuppression therapy should be controlled cautiously.

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## INTRODUCTION

Intestinal perforation (IP) is a catastrophic complication after

liver or kidney transplantation and well documented in literatures separately<sup>[1,2]</sup>. Herein we report a case of IP after combined liver-kidney transplantation (LKTx).

#### **CASE REPORT**

The recipient was a 46-year-old female who had a history of 6-year aggravating abdominal girth and pain, with symptoms of gross hematuria, intermittent fever and anorexia in the recent one and a half year. The above symptoms severely affected her daily life as well as her profession. She had obvious abdominal protuberance with a liver palpable 5 cm below the right costal margin, the lower edge of left kidney aligned with the navel level and the right kidney 3 cm lower. Percussion tenderness was found in bilateral areas of kidney. Her liver function was Child B. Her serum creatinine was 132 µmol/L and the glomerular filtration rate was 19.3 mL/min. In urine sediment Gram-negative bacillus was found (1/hpf). ABO typing was "O". HLA typing results were: A3, 26 (10); B39 (16), 54 (22); Bw6; DR4, 8; DRw53; DQw7(3), 8(3). Preoperative diagnosis was congenital polycystic disease (CPCD) with chronic renal failure and urinary tract infection.

Allografts came from an "O" type, 23-year-old male cadaver. HLA typing results were: A203 (2), 1102 (11); B27, 38 (16); Bw4; DR15 (2), 16 (2); DRw51; DQw5 (1), 6 (1); DTT-PRA negative; lymphocytotoxic crossmatch negative. Liver and kidney were harvested and preserved routinely.

Bacillus in urine sediment and culture turned negative after therapy whereas WBC counting was  $4.1 \times 10^{9}$ /L (neutrophils 80.5%) before operation. Left kidney excision + right kidney fenestration + combined liver-kidney transplantation were performed on June 23, 2000. About 1 000 mL ascites was found in laparotomy. The fluid in the cysts of right kidney and liver was clear, in contrast to those muddy yellow-white liquids in the cysts of left kidney. Culture of the fluid from left cysts showed Gram-negative bacilli. The recipient hepatectomy and graft liver implant (Piggyback) were done routinely with modification (the hepatic artery and portal vein anastomosis was performed subsequently and revascularized simultaneously). No venovenous bypass was done. The left kidney resection, right kidney depression and graft kidney implantation (in right iliac fossa) were performed in the usual manner. Totally 650 mL urine was collected from recipient's kidneys but no urine drainage was found from the implanted kidney. The whole operation lasted 18 h and 47 min (anhepatic period 2 h 7 min; warm ischemic 0 min, cold ischemic 794 min for liver and 1 143 min for kidney). Bleeding during operation (about 7 000 mL) mainly came from the right kidney. The haemodynamic was stable.

Immunosuppression regimen was initially based on cyclosporine. Oral cyclosporine 500 mg was given the night before operation and 1 000 mg methylprednisolone was given intravenously before revascularization. However, due to the delayed recovery of function of the implanted kidney, postoperative immunosuppression protocol shifted to methylprednisolone + mycophenolate mofetil (MMF, Roche Corp.). The dosage of methylprednisolone during the 8-d post transplantation were 200, 160, 260, 220, 160, 100, 40, 20 mg separately and, maintained at a dosage of prednisolone 20 mg/d. MMF 2 g/d was given orally until the 16th d, when bone marrow suppression was manifested. Sandostatin (somatostatin, Sandoz Corp.) and Losec (Astra) were given daily after operation. Beta-lactam antibiotics, ganciclovir (Cymevene, Roche Corp.) and injection fluconazole (Diflucan, Pfizer Corp.) were used for prophylaxis of bacterial, cytomegalovirus (CMV) and fungus infections separately.

The patient woke up 2 h after returning to ICU. Due to transfusion overload (6000 mL positive balance in the first 24 h) and retention of sodium and water, acute left heart failure, pulmonary edema and ARDS presented in turn. All were reversed 6 d later. The liver function recovered well. Drainage from left side subdiaphragmatic location increased continuously. Its creatinine concentration was 2-fold higher than that of blood  $(1 114 \,\mu\text{mol/L} vs 586 \,\mu\text{mol/L})$ , which indicated urine leakage from the right kidney. Since d 5 postoperatively (POD), daily urine decreased from 900 mL to 40 mL, and serum creatinine peaked to 626 mmol/L (glomerular rate 22.6 mL/min). Haemodialysis was performed daily since d 8 postoperatively. Passage of flatus and green stools began from d 6 postoperatively, and changed to water like, with frequent discharge and urgency to defecate. Temperature mounted since d 7 postoperatively. Obvious abdominal pain and distention with lower abdominal tenderness presented on d 10 postoperatively. White blood cell count rose to 17.35×10<sup>9</sup>/L. No vascular problem of implanted liver and kidney was found in real-time ultrasound examination. In emergent exploration on d 10, two sites of bowel perforation at a diameter of 1.2 cm and 0.6 cm were noted 40-50 cm proximal to ileocecal area; and multiple ulcerative lesions with purulence were scattered on the serosa of terminal ileum. Postoperative pathology found no sign of rejection in the implanted liver and kidney except that, acute tubular necrosis in the graft kidney; acute purulence with multiple acute perforations on the ileum wall; and especially, the inflammation on the serosa side was more severe than that of the mucosa side (Figure 1). Ileum of 60 cm (30 cm proximal to the ileocecal) long was resected and an endto-end anastomosis was done. Distention and abdominal pain were not relieved after laparotomy. And multiple ulcers appeared on the buccal mucosa, with skin rash on the forehead and chest. The temperature rose to 39.5 °C on d 15 postoperatively, accompanied with decreased counting of WBC and PLT  $(1.3 \times 10^9/L)$ ,  $1.8 \times 10^{9}$ /L separately). A bone marrow aspiration showed severe marrow suppression that was not ultimately reversed by granulocyte macrophage colony stimulating factors. Culture findings included: Pseudomonas cepacia (blood, urine, secretion from trachea, and abdominal drainage), Staphylococcus haemolyticus (abdominal drainage and wound secretion), Klebsiella pneumonia, Acinetobacter junii (secretion from trachea), Candida parapsilosis, Candida sake (urine). Serology supervision of CMV, herpes simplex virus and EB virus were always negative. No reperforation was found except multiple superficial ulcers scattered on the whole bowel wall and mesentery in reexploration on d 20 postoperatively. Biopsy of graft liver/kidney and chest rash indicated no evidence of rejection and GVHD. The case progressed to death on d 21 for multiple organ failure (Data shown in Figure 2).



**Figure 2** Alterations of body temperature, white blood cell count, liver and renal function after combined liver-kidney transplantation. Temperature (°C) = daily maximum of body temperature; WBC = white blood cell count (×10<sup>9</sup>/L); TBIL = total bilirubin (µmol/L); GPT = glutamic pyruvic transaminase (IU/L); SCr = serum creatinine (µmol/L); DATE = date posttransplant; Point A: the first emergent laparotomy on the 10th d posttransplantation; Point B: the second laparotomy on the 20th d; Point C: inception of haemodialysis on the 8th d.



Figure 1 A: Lesions of bowel perforation and multiple ulcerative lesions 40-50 cm proximal to ileocecal area; attached with purulent secretion scattered on the serosa of terminal ileum. B: Photomicrograph of mucosal ulceration in ileum wall (HE staining, original magnification:  $\times 100$ ).

#### DISCUSSION

For liver transplantation, postoperative IP mostly happens among pediatric recipients (6.4-20%)<sup>[1,3]</sup>, especially in those who have had previous abdominal surgery but rarely seen in adults. The median delay between transplantation and perforation was 13 d, according to Soubrane O.<sup>[3]</sup>. The sites tended to locate in small intestine<sup>[4,5]</sup> and, according to Marujo, the typical intraoperative findings in whom developed the syndrome of multiple bowel perforations were pinpoint perforations in areas of normal bowel<sup>[6]</sup>. The cause remains obscure and is probably of multifactorial origin. Risk factors have been implicated as following: a previous history of abdominal operation (especially those who received Kasai operation for congenital biliary atresia), steroid therapy, CMV infection, long time duration of operation, relaparotomy for postoperative bleeding, early portal vein embolism and venovenous bypass<sup>[3,6,7]</sup>. Incidence of reperforation was as high as 31-53%<sup>[7,8]</sup>. Even though there was report of 100% success rescue<sup>[6]</sup> and 70% 3-year survival<sup>[3]</sup>, we noticed Shaked's report of 50% (12/24) mortality in primary perforation and 78% (7/9) in reperforation group<sup>[4]</sup>.

The incidence of IP in renal transplant recipients ranged from 0.62% to 3.4%<sup>[9,10]</sup> or, in reports of larger sample, differed from 1.1% to 2.1%<sup>[11,12]</sup>. Most perforations occurred within a few weeks or months after engraftment, the period of most intense immunosuppression<sup>[9,12]</sup>. The pathogenesis was related to a high incidence of diverticular disease in patients with polycystic kidneys and/or chronic renal failure<sup>[11]</sup>. Other risk factors included overimmunosuppression<sup>[9,12]</sup>, CMV infection<sup>[13]</sup>, and the transplant procedure<sup>[11]</sup>. The average mortality rate was 56.5%<sup>[14]</sup>. This high mortality appeared to be related to the effects of immunosuppression and associated response to sepsis<sup>[9]</sup>. Clinical findings in these patients, such as fever or leucocytosis, might be masked by the immunosuppressive agents<sup>[12]</sup>. And pneumoperitoneum on abdominal roentgenograms was not necessarily positive<sup>[1,4]</sup>. Therefore, prompt diagnosis, aggressive surgical care consisting of resectional therapy, broad-spectrum antibiotics, and a reduced immunosuppressive protocol are crucial to outcome<sup>[2]</sup>.

In our case, without a previous history of abdominal surgery and consequent adhesion, resections of the original liver and left kidney as well as implantation of graft liver and kidney were smooth, although the whole duration of operation lasted 18 h due to the limitation of personnel in the renal transplantation group. Diverticulosis and CMV infection were also cautiously excluded by inspection during operation and postoperative supervision separately. Furthermore, no pathologic evidence of GVHD was found in the examinations of either the perforated ileum or biopsy skin rash in this case. In our case, it is hard to blame just the absence of venovenous bypass during the anhepatic phase as the only risk factor in inducing gastrointestinal congestion thus responsible for the perforation<sup>[6]</sup>. But the postoperative sodium and water retention aggravated the bowel edema and stasis authentically. In addition, an obvious risk factor herein, is the relatively high dose of steroid that has not tapered down routinely because of the withdrawal of cyclosporine. Overimmunosuppression may increase risk of IP through inducing damage to the barrier of intestinal mucosa<sup>[9,12]</sup>. Thus we propose that, the long duration of operation without venovenous bypass, overdose of steroid together with postoperative volume excess may all contribute to the risk of idiopathic multiple IPs in the present case.

Also, we suggest that intraperitoneal infection in this case might originate first from the infected cyst of the fenestrated kidney and might be prior to the occurrence of IP, based on the following evidences: (1) Neutrophils counting was above normal before operation. (2) G<sup>-</sup> bacillus was found in the fluid from left cysts during operation. (3) Evidence of urine leakage from the right kidney was present. (4) The inflammation on the serosa side was more severe than that of the mucosa side under pathologic inspection. This supports Jeyarajah's observation in 6 cases of CPCD who received liver and kidney transplantation and that, possibly due to overimmunosuppression or the presence of infectious foci in residual cysts, these patients seemed to be very susceptible to infectious complications after transplantation<sup>[15]</sup>. Unfortunately, the intraperitoneal infection of this case was masked and deteriorated by the post-transplant leukopenia. The cause of marrow suppression in this case might be multifactorial but most likely to be drug-induced. Leukopenia and/or thrombocytopenia, the frequent side effects of MMF, ganciclovir and beta-lactam antibiotics that had been applied in this case, might be accentuated by her renal dysfunction.

In summary, the lesson we learn from this particular case is, for the CPCD, any sign of suspect renal infection found before or during LKTx is indication for the excision of original kidney and, the intensity of immunosuppression therapy should be controlled with caution.

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