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• REVIEW •

# Hepatitis E: An overview and recent advances in vaccine research

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## Abstract

Hepatitis E virus (HEV) is an unclassified, small, non-enveloped RNA virus, as a causative agent of acute hepatitis E that is transmitted principally via the fecal-oral route. The virus can cause large water-born epidemics of the disease and sporadic cases as well. Hepatitis E occurs predominantly in developing countries, usually affecting young adults, with a high fatality rate up to 15-20% in pregnant women. However, no effective treatment currently exists for hepatitis E, and the only cure is prevention. But so far there are no commercial vaccines for hepatitis E available in the world. Although at least four major genotypes of HEV have been identified to date, only one serotype of HEV is recognized. So there is a possibility to produce a broadly protective vaccine. Several studies for the development of an effective vaccine against hepatitis E are in progress and the best candidate at present for a hepatitis E vaccine is a recombinant HEV capsid antigen expressed in insect cells from a baculovirus vector. In this article, the recent advances of hepatitis E and the development of vaccine research for HEV including recombinant protein vaccine, DNA vaccine and the recombinant hepatitis E virus like particles (rHEV VLPs) are briefly reviewed.

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## INTRODUCTION

Hepatitis E previously known as enterically transmitted non-A, non-B hepatitis, is an infectious viral disease with clinical and epidemiological features of acute hepatitis. It is a water-born disease, transmitted primarily by contaminated water. There is also a possibility of zoonotic spread of the virus, since several non-human primates, pigs, cows, sheep, goats and rodents are susceptible to the infection[1,2]. Hepatitis E virus (HEV) is a principal cause of acute hepatitis in adults throughout much of Asia, Middle East and Northern Africa[3] and transmitted from person-to-person through the fecal-oral route. HEV had provisionally been classified into the *caliciviridae* family from 1988 to 1998, but now it is classified into the separate genus *Hepatitis E-like viruses*[4-6] because the phylogenetic analysis of non-structural regions of the virus did not support the classification of HEV into the *Caliciviridae* family[7]. Although at least four major genotypes have been identified, only one serotype of HEV is recognized[8-10]. HEV infection is endemic in developing countries where sanitary conditions are not well maintained. Over 50 outbreaks have been reported in Southeast and Central Asia, the Middle East, northern and western parts of Africa, and Mexico[11-15]. Most of hepatitis E cases in developed countries have been linked to travel to endemic areas. However, recent studies revealed that hepatitis E also occurred in patients who had never been abroad[16-18]. China is one of the high epidemic areas and there have been 11 hepatitis E epidemics reported to date. The largest one in the world occurred in Xinjiang Uighur Autonomous Region, the Northwest of China, during 1986-1988, with a total number of 119 280 cases and more than 700 deaths[19-20]. Hepatitis E accounts for more than 50% of acute viral hepatitis in young adults of developing countries, although only 1% to 3% of non-pregnant patients progress to fatal fulminant hepatitis, the case-fatality rate can be as high as 20% among pregnant patients[21], constituting a serious public health problem and stressing the need for development of an effective vaccine. The development of an attenuated or killed vaccine is not currently possible because of lacking an efficient cell culture system for replication of HEV[22-27], although some cell lines have been reported for culturing and isolating HEV *in vitro*[28,29]. Therefore, either a nucleic acid-based vaccine or a recombinant protein vaccine is needed. Compared with gene engineering vaccine for hepatitis B, the study of hepatitis E recombinant vaccine was only a recent endeavor, but some progress has been made[30].

## HEV GENOME

HEV genome consists of a linear, single-stranded, positive-sense RNA of approximately 7.5 kb containing a 3' poly (A) tail and 3' noncoding (NC) regions, and contains three overlapping open reading frames (ORFs). All three coding frames are used to express different proteins[31-33] (Figure 1). ORF1 begins at the 5' end of the viral genome after a 27-bp non-coding sequence and extends 5 079 nt to the 3' end. ORF1 encodes a polyprotein of about 1 690 amino acids (aa) consisting of non-structural proteins that are involved in viral genome replication and viral protein processing, as its sequence contains motifs characteristic of viral methyltransferases, papain-like cysteineproteases, helicases and RNA-dependent RNA polymerases. In addition, ORF1 has two regions called Y and X domains of unknown function. The very short 5' NTR at the 5' end of the viral genome of 27-35 nucleotides is consistent with a capped genome. The ability of a monoclonal antibody to recognize 7-methylguanosine in RNA extracted from virions of different HEV genotypes suggests that HEV RNAs are capped[34,35]. ORF3 is 369 nt long, located at the end of ORF1 and overlaps ORF1 at its 5' end by only 1 nt, and overlaps ORF2 by 328 nt. It encodes for a 123-aa protein (pORF3), which is expressed intracellularly. The studies of the biology of HEV replication have shown that pORF3 may be capable of associating with the liver cell cytoskeleton and appears to serve as a cytoskeletal anchor site, where pORF2 and RNA can bind to begin the process of viral nucleocapsid assembly[36].

The ORF2 is located between 5 147 and 7 127 nt, consists of 1 980 nt and encodes 660 aa (71-88 kDa) most likely representing one or more structural or capsid protein(s)[2,29-31].

However, the size of the ORF2 protein in native virons is not known[37]. *In vitro* assays suggest that the -88 kDa of glycoprotein is co-translationally translocated across the endoplastic reticulum and is expressed intracellularly as well as on the cell surface[38]. ORF2 contains important epitopes that can induce neutralizing antibodies and has been the focus of vaccine development[39]. Major epitopes appear to exist near the carboxyl ends of ORF2 and ORF3. Epitopes contained in ORF2 are more conserved (90.5%) than epitopes contained in ORF3 (73.5%) in different strains. Many different ORF2 antigens have been shown to induce antibody (Table 1). There are a number of reports suggesting that truncated ORF2 peptide of shorter length might be more antigenic than the full-length protein[40-44]. However, in the majority of cases, it has not been shown that the resulting antibodies are neutralizing and, therefore, it is not known whether these antigens could serve as vaccine candidates. Only three ORF2 antigens (trpE-C2, Burma 62 kDa, Pakistan 55 kDa) thus far have been shown to induce antibodies that neutralize the virus[33].

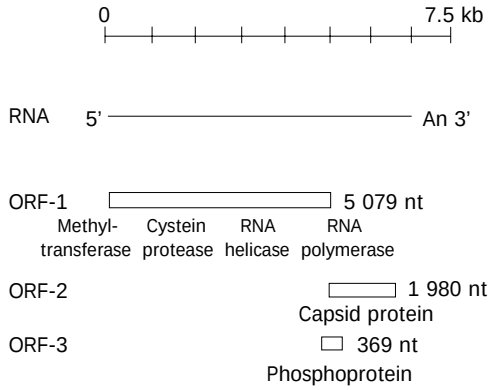


**Figure 1** Genetic map of hepatitis E virus.

**Table 1** HEV ORF2 antigenic peptides

| HEV (origin) | Designation | Amino acid | |
|---|---|---|---|
| | | N' | C' |
| Expressed in *E. coil* | | | |
| China | ORF2 | 1 | 660 |
| Burma | TrpE-C2 | 221 | 660 |
| Burma | SG3 | 328 | 654 |
| China | ORF2.1 | 394 | 660 |
| Mexico, Burma | 3.2 | 612 | 654 |
| Expressed in insect cells | | | |
| Burma | 72 ku | 1 | 660 |
| Pakistan | 63 ku | 112 | 660 |
| Burma | 62 ku | 112 | 636 |
| Pakistan | 55 ku | 112 | 607 |
| Pakistan | 53 ku | 112 | 578 |
| Burma | 50 ku | 112 | 534 |
| DNA vaccine | | | |
| Burma | pJHEV | 1 | 660 |
| China | pSVL-ORF3 | 1 | 123 |

## HEV GENOTYPE

The genome sequence of HEV seems relatively stable[45]. The genome of strains isolated from geographically distinct locations is generally more diverse. At present, no consensus exists on genotype classification. The detected HEV strains are currently genetically characterized in laboratories on the basis of ORFs regions[32]. Recently, some research workers have also started to characterize HEV strains antigenetically using specific antibodies produced by the recombinant expressed capsid proteins[46,47]. On the basis of viruses having nucleotide divergence of not more than 20% of the nucleotides in the ORF2 region[48], the genomes of several HEV strains from different parts of the world can be grouped into at least four major genotypes[8,31,32,49]: Genotype 1 -including the isolates from South-East Asian (Burmese, some Indian strains), North and Central Asian (strains from China, Pakistan, Kyrgyzstan, and India), and North African strains; Genotype 2 -comprising the single North American (Mexico) isolate; Genotype 3 - consisting of the US and swine isolates; and Genotype 4 - including a subset of isolates from China and most isolates from Taiwan. Genetically heterogeneous isolates from several European countries have been designated new genotypes, but probably should be grouped with the US isolates into a large, heterogeneous group[31,50]. Two novel isolates of HEV have recently been described in Argentina. Distinct from all previously described isolates, they represent two diverse subtypes of a new genotype of HEV[51]. Despite the diversity of HEV genotype, no evidence has been found that heterogeneity results from the genetic diversity, thus HEV seems to exist as a single serotype[8-10].

## HEV VACCINE RESEARCHES

At present, no commercially available vaccines exist for the prevention of hepatitis E. However, several studies for the development of an effective vaccine against hepatitis E are in progress[37,52-56]. Several lines of evidence have suggested the feasibility of a HEV vaccine. First, serum antibodies to HEV develop in response to naturally acquired and experimentally induced HEV infections in cynomolgus monkeys[57]. Second, seroepidemiology of hepatitis E suggests that people previously infected with HEV are protected during epidemics of the disease[58]. Finally, successful passive immune prophylaxis in animals indicated that effective vaccination against hepatitis E based on humoral immunity is possible[59]. Although different geographical isolates of HEV have been identified, only one serotype has been recognized[8]. So it may be possible to produce a broadly protective vaccine.

ORF2-encoded protein of HEV is the most promising subunit vaccine candidate because it possesses a good antigenicity. So far, HEV ORF2 gene or its fragments have been expressed in prokaryote cells[56,60-64], insect cells[37,65-68], yeast cells[69-71], animal cells[72], and plants (tomatoes)[30], *etc*., and the expression products possess immunogenicity.

### *Expression in prokaryote cells*

It was reported recently that the smallest fragment of ORF2, which is capable of combining the neutralizing antibody of HEV, is located between 452 and 617 aa. This fragment does not only induce neutralizing antibody, but also cross react to the antibody of other genotype[22]. It was demonstrated that the 2/3 length of C-terminal region of HEV ORF2 contains epitopes, which are recognized by both acute-phase and convalescent-phase antibody to HEV and are likely to be associated with limited immunity to the infection, but these epitopes may be masked when larger portions of ORF2 are expressed as recombinant proteins[64]. So far, many different length of ORF2 fragments have been expressed in *E. coli*[56,63,64], but only a few expression products have been found with significant immunoactivity. The first candidate HEV vaccine was a recombinant fusion protein including 439 amino acids (221-660 aa). It comprised tryptophan synthetase and the carboxy terminal fragment of the ORF2 protein of the Burmese strain (genotype 1) and was expressed in *E.coli*. Two cynomolgus macaques were vaccinated with the fusion protein,

and neither of them developed hepatitis following experimental challenge. The animals challenged with the heterologous HEV, the Mexican strain (genotype 2), did get infected, but did not develop hepatitis[73]. Another study also shows that the immunization with the bacterially expressed ORF2 peptide (pE2) corresponding to 394-607 aa, may prevent HEV infection in primates experimentally transmitted with the homologous strain of HEV[56].

### Expression in insect cells

It is thought that the best candidate at present for a hepatitis E vaccine is a recombinant HEV capsid antigen expressed in insect cells from a baculovirus vector[25]. When ORF2 of genotype 1 strain is expressed from a baculovirus vector in insect cells, the initial 72-ku protein is quickly processed to smaller proteins, possibly via a protease encoded by the baculovirus[66]. The most abundant proteins are 56 ku and 53 ku in size, respectively. However, the only neutralization epitope identified to date was mapped to a region of the ORF2 protein of Sar-55 between amino acids 578 and 607[74]. Therefore, the 56 ku protein (112-607 aa) but not the 53 ku protein (112-578 aa) should contain this epitope. The recombinant 56 ku protein that is more soluble than the full-length protein was an efficient immunogen when adjuvanted with alum[53,57]. Two 400 ng doses of the vaccine were injected to rhesus monkeys intramuscularly and high antibody titers (1:10 000) were achieved. The monkeys were protected against hepatitis E following intravenous challenge with 300 000 monkey infectious doses ($MID_{50}$) of the homologous (Sar-55) or 100 000 $MID_{50}$ of a heterologous HEV (Mexican 14)[57]. To evaluate the immunogenicity and protective efficacy of the 53 kDa protein, the same research group immunized rhesus monkeys with the 53 ku vaccine, which was derived from processing of the ORF2 protein of Sar-55 and purified from the medium of recombinant baculovirus-infected insect cells and precipitated with alum. Two doses of vaccine containing 385 ng of alum-precipitated 53 ku protein were inoculated into the monkeys intramuscularly. The immunized monkeys were challenged with a high (1000 $MID_{50}$) or low (100 $MID_{50}$) dose of homologous virus. The result showed vaccination with the 53 ku protein greatly reduced virus shedding, but did not protect against hepatitis following the high dose challenge. Virus was not detected in the vaccinated animals following the low dose challenge, suggesting that sterilizing immunity might have been achieved. This study indicated that the 53 ku protein did not function as a better vaccine than did the 56 ku protein and actually appeared to have been less effective in preventing disease[37]. The researches have shown that almost complete vaccine-induced protection lasts for at least 6 mo and partial protection persists for at least 1 year following vaccination[75].

### Expression in yeast and other cells

Recently, yeast expression system has been successfully used for production of vaccines, for example, the recombinant hepatitis B surface antigen[76,77]. There are some advantages of using this expression system, such as the expression products similar to the natural protein, maintaining the biological activity of the production, to produce easily in large scale and so on[78]. In recent studies, the ORF2 of HEV (69-660 aa and 112-660 aa) was successfully expressed in *pichia pastoris* and the expression products of recombinant protein (59 ku) was purified and immunized to rhesus monkeys. High titer of anti-HEV (1:8000) was detected in the immunized monkeys[69-71,79]. Research on using plants for expression and delivery of oral vaccine has attracted much academic attention and has become a hot spot of study since 1990 when Curtiss *et al*.[80] first reported the expression of *Streptococcus* mutants surface protein antigen A (SpaA) in tobacco, and great progress has been made since then[81]. In a recent study, the ORF2 partial gene of HEV named E2 (810 bp, 349-604 aa) was constructed into plasmid pCAMBIA1301 and yielded the reconstructed plant binary expression plasmid p1301E2[62]. The p1301E2 was expressed in tomatoes and the recombinant antigen derived from them has normal immunoactivity. The transgenic tomatoes may hold a good promise for producing a new type of low-cost oral vaccine for hepatitis E[30].

### Subunit HEV vaccines

DNA immunization usually induces both cellular and antibody immune response. So it might provide a longer duration of protection. Therefore, it has become another focus of HEV vaccine research. Recombinant DNA vaccine is a recently developed new type of vaccine. Through directly injecting the recombinant plasmid DNA with the target gene into human or animals, the DNA will express the expected protein inside the host cells and therefore induce the immune responses to prevent and fight the disease. Recently, a new technology has been introduced for the development of subunit vaccines involving the direct injection of purified plasmid DNA containing protein coding sequences of interest and appropriate regulatory elements allowing expression in mammalian tissues. This novel technology has several potential advantages over other vaccine approaches[82]. First, the antigens expressed in living cells are in their native form, improving processing and presentation and usually resulting in the activation of both arms of the immune system. Second, DNA can be made inexpensively, in large quantities, at high levels of purity, and is extremely stable. Third, the vector is unlikely to be, or to become, pathogenic, in contrast to live-virus vaccines, and there is little or no immune response to the vector.

In an early study, an HEV cDNA pSVL-ORF3 was constructed by inserting the full length of ORF3 fragment into prokaryotic expression vector pSVL. A total amount of 100 μg of the cDNA was injected to BALB/c mice intramuscularly and anti-HEV IgG was detected in 12 of the 16 immunized mice. However, no antibody was found in the mice injected with the empty vector. The result indicated that the recombinant HEV cDNA could induce the antibody response in mice[83]. Later, another HEV cDNA pJHEV was constructed by inserting full length of ORF2 fragment. The HEV structural protein was expressed in Cos-7 cells under the control of a hCMV promoter. The successful construct was further tested in BALB/c mice for the induction of an ORF2 specific immune response. All the mice immunized with the cDNA were found seroconverted, but no anti-HEV responses induced in the mice of control group. Sera from the mice injected with pJHEV specifically recognized HEV ORF2 structural protein expressed in recombinant baculovirus in an enzyme-linked- immunosorbent assay (ELISA) and Western blot[82]. Furthermore, it was also shown that the antiserum generated by the DNA vaccine could bind specifically to native HEV[84]. Recently, a full-length HEV cDNA clone was constructed in a pSGI vector. The three ORFs were amplified separately and then reconstructed to the full-length clone. The *in vitro* transcribed RNA of the full-length cDNA clone was infective in a HepG2 tissue culture. Viral replication was detected for six passages with strand-specific PCR[85].

### The rHEV VLPs

In spite of the above vaccine candidates, recently the recombinant hepatitis E virus (rHEV) virus-like particles (VLPs) are also the focuses of vaccine research for hepatitis E. With 111 amino acids truncated at the *N*-terminal, when the capsid protein of HEV was expressed in the baculovirus expression system, it was spontaneously assembled into virus-like particles[54]. Electron cryomicroscopy shows that these VLPs are formed with

60 copies of a 54 ku protein arranged in T=1 symmetry[54, 86, 87]. As a mucosal immunogen, the VLPs have several advantages: they are composed of a single protein assembled into particles without nucleic acid, which makes them unable to replicate; they are easy to prepare and purify in large quantities, with a yield of approximately 1 mg /$10^7$ insect cells; rHEV VLPs are antigenically similar to the native virions; they are highly immunogenic in experimental animals when injected parenterally; they are very stable at low pH such as in stomach; and oral delivery of rHEV VLPs could induce the same immune responses as occur in natural infection[86,54].

In a previous study[87], mice were orally inoculated with purified rHEV VLPs without adjuvant. Serum IgM response was obtained within 2 wk after the first administration. Serum IgG and IgA were detected by 4 wk, and the intestinal IgA response was found at 8 wk post-immunization. Therefore, the oral immunization of rHEV VLPs is capable of inducing both systemic and intestinal antibody responses. However, since mice are not susceptible to HEV, the same group recently immunized cynomolgus monkeys orally with 10 mg of purified rHEV VLPs, serum IgM, IgG, and IgA responses were observed. All these antibody responses were obtained without adjuvants. When the monkeys were challenged with native HEV by intravenous injection, they were protected against infection or developing hepatitis. These results suggested that rHEV VLPs could be a candidate for the oral hepatitis E vaccine[88]. Some similar experiments have been described previously[89-91].

## PRE-CLINICAL AND CLINICAL TRIALS

So many vaccine candidates for hepatitis E have been explored as described above. But so far, there is only one HEV vaccine candidate progressed to the stage of clinical trials. That is the 56 ku (expressed in insect cells from a baculovirus vector) recombinant vaccine developed at the NIH, USA[68]. In phase I trial, the vaccine was found to be safe and immunogenic in 88 American volunteers. A further phase I evaluation was performed in Nepal, where hepatitis E is endemic. Three doses of 5 μg and 20 μg, respectively, were injected into 22 Nepalese volunteers each at zero, one and six months. No serious adverse events were observed. By the second month, 43 of 44 volunteers had seroconverted to anti-HEV. By the 7th month, the remaining volunteers also developed antibody to HEV. The study indicated that the HEV vaccine candidate was safe and immunogenic. This same lot of vaccine is currently being used in phase II/III clinical trials in Nepal[25], where as many as 90% of the jaundice cases are caused by HEV.

In recent pre-clinical trails, the vaccine used in the experiment was from the same lot that was prepared for the above clinical trails. The results indicated that two doses of HEV vaccine as small as 1 μg were highly effective in preventing not only hepatitis but also infection following intravenous administration of $10^4$ $MID_{50}$ of virulent virus. However, these doses of vaccine will not be sufficient for long-term protection. Other studies have shown that a third dose of vaccine at 6 or 12 mo following the first dose will enhance immunogenicity and/or efficacy[75,92]. The study has also confirmed that the cross-protection against a Mexican genotype 2 isolate and extended the evidence for cross-protection to a US genotype 3 isolate of HEV. The results of this experiment have shown that the manufacture of a candidate hepatitis E vaccine can be scaled up to produce more clinical quality and that such a vaccine is highly immunogenic and effective for preventing hepatitis E in the pre-clinical trails. It is likely that protection against infection will be more effective following natural oral challenge with relatively small doses of HEV[25].

## CONCLUSIONS

Despite the achievements mentioned above, there are still many questions to be answered in future. For example, there is a study showing that acute hepatitis E can be induced by plasma transfusion from a donor with HEV viremia, which indicates the possibility of transfusion transmitted hepatitis E[93]. Some similar studies also showed the possibility of post-transfusion hepatitis E[94-97]. Therefore, effective measures for preventing post-transfusion hepatitis E must be taken. To achieve effective immunization, how should the HEV vaccine be given by, orally or intramuscularly? Will a monovalent vaccine protect against all HEV strains including HEV from animals and genotypes and provide a long-term immunity to hepatitis E? To facilitate vaccine development and to improve our knowledge about the mechanism of virus replication, an effective practical cell culture system should be established and demonstrated.

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• ESOPHAGEAL CANCER •

# Clinicopathologic analysis of esophageal and cardiac cancers and survey of molecular expression on tissue arrays in Chaoshan littoral of China

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## Abstract

**AIM:** To investigate clinical and pathologic data of esophageal carcinoma (EC) and cardiac carcinoma (CC) among residents in Chaoshan region of China.

**METHODS:** Clinical and pathologic data of 9 650 patients with EC and 4 173 patients with CC in the Chaoshan population were collected and analyzed. Moreover, Chaoshan esophageal carcinoma tissue arrays were made for high-throughput study.

**RESULTS:** Male to female ratio was 3:1 in patients with EC and 4.75:1 in CC. The average age of the occurrence of EC was 54.6 years, and of CC was 58.1 years. For both EC and CC, age at diagnosis was a little younger in Chaoshan region than in most other areas. The most commonly affected site of esophageal carcinoma was the middle third of esophagus (72.0%); the second was the lower third (15.3%). The main gross type of esophageal carcinoma was ulcerative type (41.50%); the medullary type was the second (39.6%). Squamous cell carcinoma accounted for the overwhelming majority of esophageal cancer (96.4%); adenocarcinoma accounted for the overwhelming majority of cardiac carcinoma (94.5%). Chaoshan esophageal carcinoma tissue arrays were easily for high-throughput study, and tissue cores with a diameter of 1.5 mm could better keep more structure for molecular expression study.

**CONCLUSION:** Both EC and CC are common in males. The average occurrence age of EC and CC is younger in Chaoshan than in most other regions of China. The most commonly affected site of esophageal carcinoma was the middle third of esophagus (72.0%). Squamous cell carcinoma accounted for the overwhelming majority of esophageal cancer; adenocarcinoma accounted for the overwhelming majority of cardiac carcinoma. Tissue arrays technology is applicable for rapid molecular profiling of large numbers of cancers in a single experiment.

Su M, Li XY, Tian DP, Wu MY, Wu XY, Lu SM, Huang HH, Li DR, Zheng ZC, Xu XH. Clinicopathologic analysis of esophageal and cardiac cancers and survey of molecular expression on tissue arrays in Chaoshan littoral of China. *World J Gastroenterol* 2004; 10(15): 2163-2167
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## INTRODUCTION

Esophageal cancer (EC) ranks among the 10 most common cancers in the world, and is almost uniformly fatal. Chaoshan area is a unique littoral high-risk area of EC in China, within which Nanao island has the highest risk, the second being Jieyang county. According to the report from the Department of Public Health, Guangdong Province in 1993, the mortalities of EC in Nanao island were: 108.68±7.88/100 000 in standardized Chinese population, 145.44±10.49/100 000 in standardized world population, 261.16±25.01/100 000 in standardized world population between the age of 35-64. The annual average incidence rates in males and females were 132.19/100 000 and 69.20/100 000 in Nanao island from 1987 to 1992[1].

The predominant inhabitants of Chaoshan are offsprings of immigrants who hundreds or thousands of years ago came from the Central Plains of China, now a world well-known high risk region for EC. Chaoshan residents who have a high risk of EC and cardiac carcinoma (CC) are a relatively isolated population who have kept the old Chinese language (Chaoshan dialect) and customs. It is important to see if there is any evidence for the reducing incidence and mortality of EC and CC in Nanao island so far[2] as the incidences of EC and CC present a downward trend in most other high risk regions. This unique society provides us an unparalleled base for the genetic and also environmental study of esophageal carcinoma. In the current study, we explored the clinical and pathologic features of EC and CC.

In addition, scholars have discovered that many genes and signaling pathways are involved in EC and CC development[3-7]. However, genetic tumor markers have not gained in EC and CC diagnostics and prognosis prediction. Identification and evaluation of new molecular parameters are of utmost importance in cancer research. Here we present a high-throughput approach to rapidly identify relevant molecular expression changes in Chaoshan EC tissue arrays.

## MATERIALS AND METHODS

### *Clinical data*

Data about age, gender, and X-ray or pathological diagnoses of 13 823 patients with carcinoma of esophagus (9 650 cases) or

cardia (4 173 cases) were collected from the Tumor Hospital (1978-1998), First Affiliated Hospital (1989-1998), and Second Affiliated Hospital (1983-1998) of Shantou University Medical College, the Central Hospital of Shantou and the Hospital of Jieyang.

### *Sample collecting and EC tissues arrays construction*

Seventy esophageal squamous carcinoma tissue specimens were selected from Department of Pathology, Shantou University Medical College in 2002. The specimens were fixed in 40 g/L neutrally buffered formaldehyde, embedded in paraffin. Sections of 5 μm stained with hematoxylin and eosin were obtained to confirm the diagnosis and to identify different viable, representative areas of the specimen. From these defined areas core biopsies were taken with a precision instrument. Sixty-eight EC and para-cancerous tissue cores with a diameter of 1.5 mm from each specimen were punched and arrayed in 8×9 on a recipient paraffin block. Five-μm sections of the EC tissue array block was cut and placed on adhesive coated slides (cooperated with Cybrdi, Dr. Li Jun).

### *Immunohistochemistry*

The expression of Erk1/Erk2 MAPK signaling protein was analyzed using Erk1/Erk2 Mouse derived anti-activated MAP kinase monoclonal antibody (1:400, Sigma), Histostain™-SP kit and DAB visualization methods according to the manufacture's instruction (China Beijing Zhongshan Biological Technology CO., LTD.) And the expression of epidermal growth factor receptors were primarily analyzed using phosphor-EGFR (Try845) rabbit polyclonal antibodies and HRP-linked anti-rabbit IgG (Cell Signaling Technology, Inc. #2231, #7074). Four conventional normal esophageal epithelium tissues from autopsy were used as normal tissue controls. The human breast cancer tissue was used as positive control. Negative control was designed using phosphate-buffered saline (PBS) instead of primary antiserum. The detailed immunohistochemical process was carried out according to the manufacture's instructions.

### *Assessment of staining*

The positive immunohistochemical staining of Erk1/Erk2 proteins was shown as brown signals in the nuclei; and the immunohistochemical signals of phosphor-EGFR (Tyr845) was in membrane and cytoplasm. The percentage of positive stained cells was evaluated for each tissue sample by counting all cells at 5 high power fields of micrometric rule (5 mm×5 mm). The cases having positive cancer cells or epithelium accounting for more than 75% of all cancer cells or epithelium on the slide were defined as a score of ++++, 50-74% were defined as a score of +++, 25-49% were defined as a score of ++, 6-24% were defined as a score of +, 1-5% were defined as a score of ±, less than 1% were defined as a score of -.

### *Statistical analysis*

Data were stored in a computer data base (FoxPro, version 2.5 b) and analyzed using a computer (Pentium 4) spread sheet (Microsoft Excel 97) and professional statistical computer software (SPSS, version 11.0 and SAS, version 6.08 ). $P<=0.05$ was taken as significant. Immunoreactivity was classified as continuous data (undetectable levels or 0% to homogeneous staining or 100%) for all markers.

## RESULTS

### *Gender and age*

Genders of the patients were recorded in 9 635 cases. The 8 665 EC cases had age records, the youngest and the oldest were 17 years and 91 years respectively, and 3714 CC cases had age records. The male to female ratio and average age of morbidity for EC and CC in Chaoshan region are shown in Table 1. Constituent ratio of EC, CC in every age group is shown in the histogram (Figure 1).

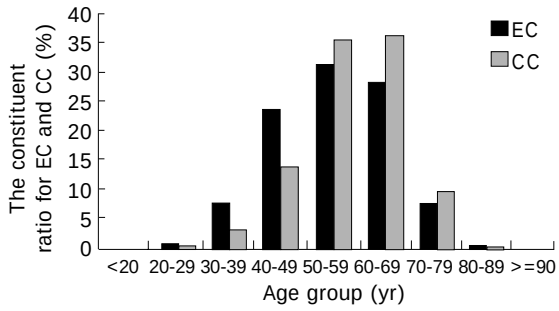


**Figure 1** The histogram of the constituent ratio for EC and CC in Chaoshan region.

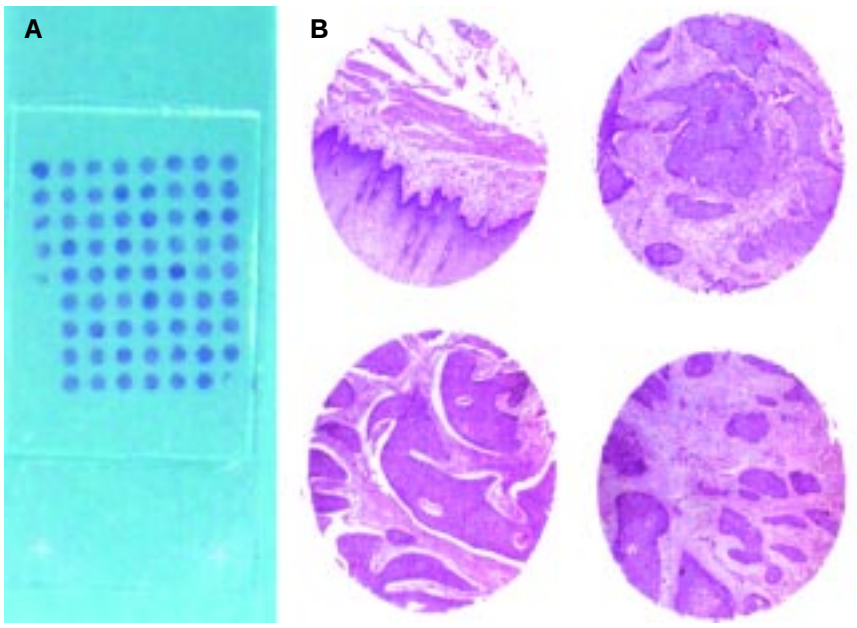


**Figure 2** A: Overview of Chaoshan esophageal carcinoma tissue array (tissue cores with a diameter of 1.5 mm with 68 samples); B: Esophageal para-cancerous tissue and squamous carcinoma tissue (HE staining, original magnification: ×40).

**Table 1** Sex ratio, average age of morbidity for EC and CC in Chaoshan littoral region (mean±SD)

| | *n* | Sex ( male:female) | Age(yr) | *F* | *P* |
|---|---|---|---|---|---|
| EC | 9 635 | 7 228:2 407(3.0:1) | 54.61±10.73 | 103.24 | <0.001 |
| CC | 4 167 | 3 442:725(4.75:1) | 58.14±9.45 | | |

EC:CC=2.31:1.

### *Pathology*

The overwhelming majority of ECs were squamous cell carcinoma (96.4%); whereas CCs were composed mainly of adenocarcinomas (94.5%), and squamous cell carcinoma ranked second (4.4%). The detailed information of pathology is shown in Table 2.

**Table 2** Pathology for EC and CC in Chaoshan littoral region (%)

| | Site | | | Gross type | | | | Histological type | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | U | M | L | UT | MT | ST | FT | SCA | ACA | UCA | O |
| EC | 12.7 | 72.0 | 15.3 | 41.5 | 39.6 | 9.6 | 9.4 | 96.4 | 2.7 | 0.6 | 0.3 |
| CC | | | | | | | | 4.4 | 94.5 | 0.9 | 0.2 |

Site (EC, *n*=6 384); Gross type (EC, *n*=1 193); Histological type (EC, *n*=7 272; CC, *n*=3 086); U: Upper third; M: Middle third; L: Lower third; UT: Ulcerative type; MT: Medullary type; ST: Scirrhous type; FT: Fungating type; SCA: Squamous cell carcinoma; ACA: Adenocarcinoma; UCA: Undifferentiated carcinoma; O: others.

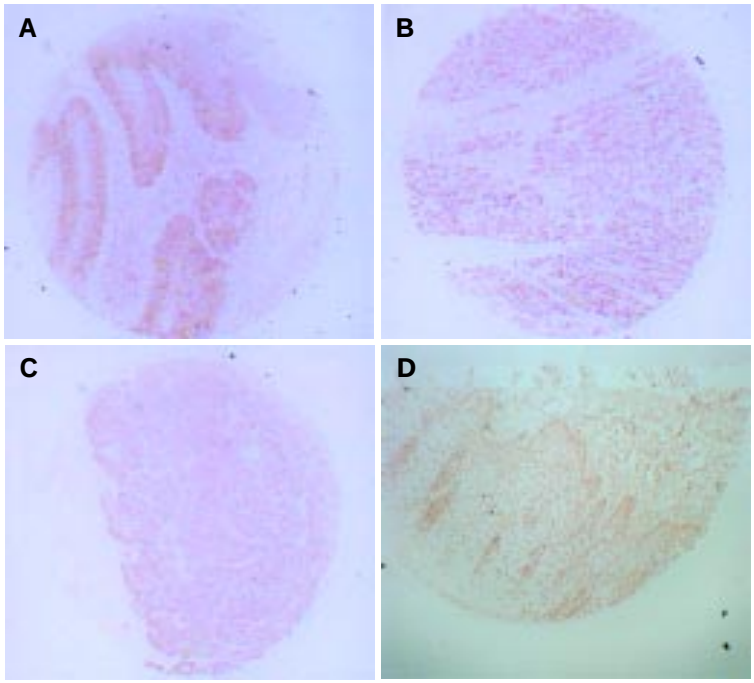


**Figure 3** Phosphor-EGFR (Tyr845) expression on esophageal squamous carcinoma tissue (A, B, C) and esophageal para-cancerous tissue (D) (Cell Signaling Technology kit, original magnification: ×40).

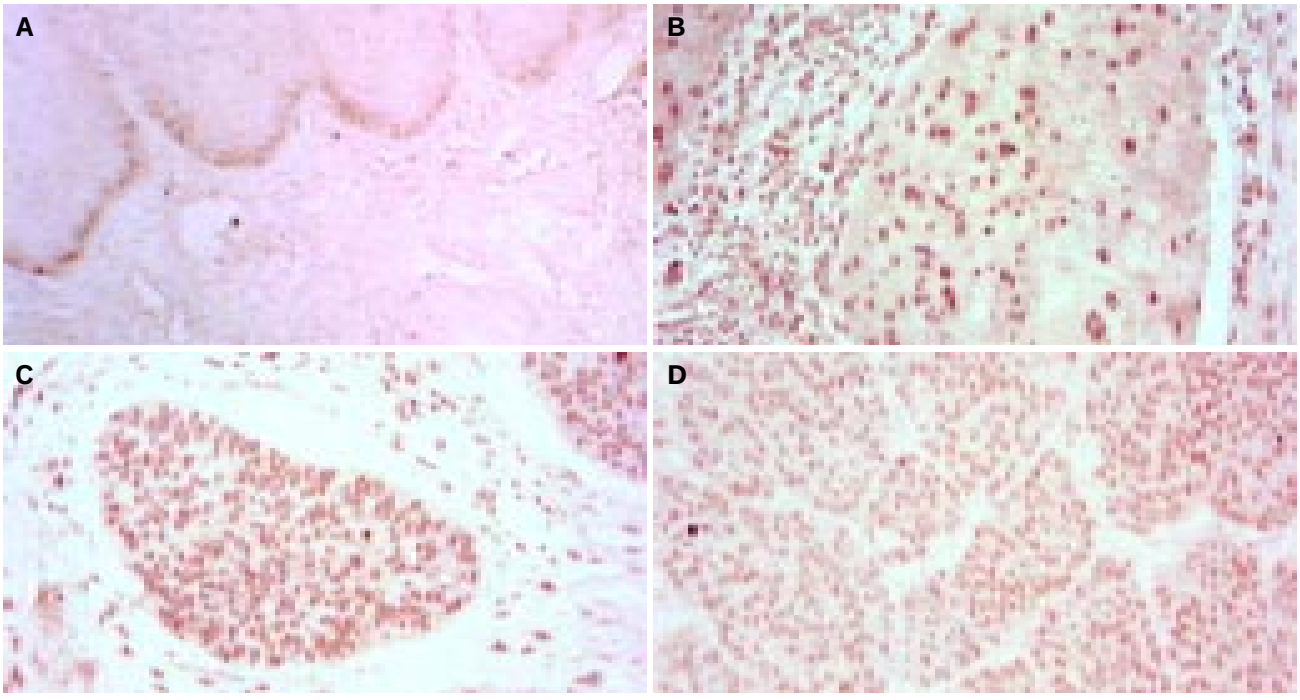


**Figure 4** Erk1/Erk2 expression on normal esophageal squamous tissue (A, conventional tissue section, SP×100) and esophageal squamous carcinoma tissue (B, C, D tissue arrays) (SP×200).

**Table 3** Expression of Erk1/Erk2 in esophageal squamous carcinoma tissue and para-cancerous tissue (%, mean±SD)

| | Normal esophageal squamous cell ($n$=5) | Para-cancerous tissue ($n$=5) | Grade I ($n$=19) | Grade II ($n$=34) | Grade III ($n$=9) | $F$ | $P$ |
|---|---|---|---|---|---|---|---|
| Activated ERK1/ERK2 | 5.10±1.44 | 76.80±0.14 | 76.80±0.09 | 75.80±0.09 | 69.30±0.14 | 2.60 | 0.04 |

There was marked difference between normal esophageal epithelium and cancer tissues or para- cancerous tissue. But there was no marked difference among para–cancerous tissues and esophageal squamous carcinoma tissues ($F$=1.463, $P$=0.236).

### *Chaoshan EC tissues arrays and immunohistochemistry*

Sections from tissue arrays were kept structure well for pathologic and immunohistochemical research (Figure 2). The expression of phosphor-EGFR(Tyr845) in esophageal squamous carcinoma tissue are relative diversity from ± to ++++, but no distinct differences among esophageal squamous carcinoma tissues according to grading (Figure 3). The expression of activated ERK in esophageal squamous carcinoma tissue and para-cancerous tissue are shown in Table 3 (Figure 4).

## DISCUSSION

In data from Yangquan city of Shanxi Province (502 cases)[8], the median age of EC patients was 59.17 years, 4.56 years older than that of Chaoshan EC patients. And also data from Linxian of Henan Province (2 601 cases)[9], the proportion of EC patients from 20 to 30 years was 0.35%, from 30 to 40 was 5.19%; whereas both of the two proportions were lower than those from our data (0.70% and 7.70% respectively). In our data from Chaoshan region, the ulcerative type (41.5%) was the most common gross type of EC, which suggests that most EC patients were in the terminal stages when they arrived at hospitals.

Both preceding evidences indicated the average age of EC occurrence was younger in Chaoshan population than those in central plains of China. Synthetically analysis of two reports from Henan Province (1 045 cases and 1 332 cases respectively)[9,10] showed the proportion of CC patients less than 50 years old was 15.56%, which was slightly lower than that from Chaoshan CC data (17.60%). The similar phenomena were seen in other papers[11,12]. It might indicate that the age of incidence of cardiac cancer is also younger in Chaoshan region than in most other areas. Pathological analysis of 1 572 EC patients from Henan Province indicated histological type for the overwhelming majority of EC was squamous cell carcinoma (SCC), and the proportion of SCC (95.1%) was similar to that in our data (96.44%)[13]. In comparison of the constituent ratios of histological types for cardiac carcinoma with data respectively from the Chinese Academy of Medical Science[14], Erlangen - Nurmberg University in Germany[15] and our data, it homoplastically shows that adenocarcinoma accounts for the overwhelming majority of cardiac carcinoma, while the proportions of other histological types are relatively low.

Both data from Yangquan and Linxian[16] also showed the middle third of esophagus was the most commonly affected site analogously, followed by the lower third and then the upper third, similar to our results.

Tissue array technology is applicable for rapid molecular profiling of large numbers of cancers in a single experiment. But a possible limitation of the tissue array technology is that the minute tissue samples acquired from the original tissues may not always be representative of the entire tumor, in light of the intratumor heterogeneity characteristic to most cancers. The comparisons between similarly acquired specimens from different stages of tumor progression placed on the same tissue microarray should be less problematic. If tumor arrays are used to investigate prevalence or prognostic significance of molecular changes, the critical issue is the extent to which minute tissue samples are representative of their donor tumors. The findings of this study suggest that significant results can be obtained on tumor arrays issue cores with a diameter of 1.5 mm. Well and truly, one should consider the tumor tissue array technology as a rapid, high-throughput survey method to pinpoint the biologically most prevalent or clinically most promising genes and molecular markers for detailed studies combined with conventional tissue specimens.

The epidermal growth factor (EGF) peptide induces cellular proliferation through the epidermal growth factor receptor (EGFR), a $M_r$ 170 000 single-pass transmembrane tyrosine kinase, which is believed to play important roles in the control of cell growth and differentiation. The EGFR activates ras and the MAP kinase pathway, ultimately causing phosphorylation of transcription factors such as c-Fos to create AP-1 and ELK-1 that contribute to proliferation. Gene amplification and overexpression of EGFR have been reported in various human tumors, including head and neck/oral cancer[17-22].

Mitogen-activated protein kinase (MAPK) cascades have been shown to play a key role in transduction extracellular signals to cellular responses. Extracellular signal-regulated kinase (ERK) has been the best characterized MAPK and the Raf-MEK-ERK pathway represents one of the best characterized MAPK signaling pathway. The activated ERKs translocate to the nucleus and transactivate transcription factors, changing gene expression to promote growth, differentiation or mitosis[23].

Our primary immunohistochemical study showed that phosphor-EGFR (Tyr845) and activated ERK were expressed in both para- cancerous and esophageal cancerous cells. The intensity of the expression was much higher in cancer than in normal tissue, suggesting that EGFR, Erk1/Erk2 MAPK signaling pathway might play an important role in the regulation of proliferation of esophageal cancer[24,25].

In summary, Both EC and CC are common in males. The average age of occurrence is younger in Chaoshan than in most other regions of China. It is suggested that either genetic factors might play an important role in the pathogenesis of esophageal and cardiac cancers in Chaoshan or Chaoshan residents exposed themselves to some high risk environmental factors. Tissue microarrays technology is applicable for rapid molecular profiling of many tissue samples in a single experiment.

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• ESOPHAGEAL CANCER •

# Expression of cyclooxygenase-2 in human esophageal squamous cell carcinomas

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## Abstract

**AIM:** To determine whether cyclooxygenase-2 (COX-2) was expressed in human esophageal squamous cell carcinoma.

**METHODS:** Quantitative reverse transcription-polymerase chain reaction (RT-PCR), western blotting, immunohistochemistry and immunofluorescence were used to assess the expression level of COX-2 in esophageal tissue.

**RESULTS:** COX-2 mRNA levels were increased by >80-fold in esophageal squamous cell carcinoma when compared to adjacent noncancerous tissue. COX-2 protein was present in 21 of 30 cases of esophageal squamous cell carcinoma tissues, but was undetectable in noncancerous tissue. Immunohistochemistry was performed to directly show expression of COX-2 in tumor tissue.

**CONCLUSION:** These results suggest that COX-2 may be an important factor for esophageal cancer and inhibition of COX-2 may be helpful for prevention and possibly treatment of this cancer.

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## INTRODUCTION

Esophageal cancer is the fourth most prevalent malignancy in China. Although several factors have been implicated for the recent rise in the frequency of esophageal carcinoma, including diet, activation of *c-myc* oncogenes and inactivation of tumor suppressor genes (*p53*)[1,2], the exact pathogenic mechanisms and promoting factors of this cancer remain to be clarified. There is no effective strategy for treatment of this disease. Recently epidemiological studies suggest intake of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin can reduce the risk of colorectal cancer[3,4]. Based on these findings, subsequent studies have addressed the role of COX enzyme as a target of these compounds.

COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin $H_2$, the precursor of several molecules including prostaglandin (PG), prostacyclin, and thromboxane. Results from recent studies have established the presence of two distinct COX enzymes, a constitutive enzyme (COX-1) and an inducible form (COX-2). They share over 60% identity at the amino acid level and have similar enzymatic activities, but both isoforms are suggested to have different biological functions[5-9]. COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out "housekeeping" functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX-2 mRNA and protein are undetectable in normal tissues, but can be rapidly induced by proinflammatory or mitogenic stimuli including cytokines, endotoxins, interleukins, and phorbol ester[10-12].

Multiple lines of experimental evidence have also suggested that COX-2 is involved in carcinogenesis. For example, COX-2 is up-regulated in transformed cells[13] and in various forms of cancer[14-17], whereas levels of COX-1 are relatively stable. Moreover, COX-2 knockout mice develop 75% fewer chemically-induced skin papillomas than control mice. Recently, it has been shown that selective COX-2 inhibitors may significantly suppress experimentally-induced colon carcinogenesis[18,19]. Epidemiological studies have shown that intake of aspirin was associated with up to a 90% decreased risk of developing esophageal cancer[20,21], and in induced esophageal carcinomas, indomethacin had antitumor activity[22,23].

Here we investigated whether COX-2 was up-regulated in esophageal squamous cell carcinomas from Chinese patients. Our data show that levels of COX-2 are markedly increased in esophageal squamous cell carcinomas, which raises the possibility that selective inhibitors of COX-2 may be useful in the prevention or treatment of this important disease.

## MATERIALS AND METHODS

### *Samples*

We examined 30 cases of esophageal squamous cell carcinoma. Both tumor tissue and surrounding normal tissue were obtained from surgical patients with esophageal squamous cell carcinoma at Tongji Hospital during operations. The age of the patients was 57±9 years (mean±SD; range, 37 to 75 years). Of the patients, 23 were men and 7 were women. A small portion from each tissue sample was immediately frozen in liquid nitrogen and stored at -80 ℃. Tissue samples were fixed in 40 g/L neutral buffered formaldehyde, processed through graded ethanol solutions, and embedded in paraffin and immunostained for COX-2. A subset of paired tumor tissues and surrounding normal tissue was further examined by RT-PCR and Western blotting.

### *RNA extraction*

Protein and total RNA were extracted using TriZol Reagent (Life Technologies. GIBCO BRL). RT-PCR kit was purchased from Takara Bio Co., Ltd. COX-2 affinity-purified polyclonal antibodies were obtained from Santa Cruz Biotechnoloy, Inc. Rabbit anti-goat IgG conjugated with horse-radish peroxidase (HRPO) was obtained from KPL, Inc., USA. ImmunoResearch Lab Enhanced chemiluminescence assay was purchased from PIERCE, Inc. SDS-PAGE standard was obtained from Bio-Rad, Inc.

### *Western blotting for detection of COX-2*

Protein in the various samples were extracted and purified by TriZol Reagent, and the protein concentration was evaluated by Bradford method. A portion of protein (20 μg) extracted from each tissue sample was subjected to electrophoresis in 80 g/L polyacrylamide slab gels. After transfer to PVDF membrane and blocking with fat free milk powder, blots were probed with COX-2 antibodies (1:750), followed by incubation with HRPO-conjugated secondary antibody (rabbit anti-goat 1:800). COX-2 proteins were visualized by enhanced chemiluminescence.

### *Semi-quantitative RT-PCR*

Total RNA was isolated and purified by TriZol Reagent, and the RNA concentration was determined. Semi-quantitative analysis of the expression of COX-2 mRNA was performed using the multiplex RT-PCR technique. In this assay, we used a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal standard. A sample (1 μg) of total RNA was reverse-transcribed using the Takara Bio RT-PCR kit according to the manufacturer's protocol. PCR was performed in a 25 μL reaction mixture containing 5 μL of cDNA template, 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L dNTPs, 100 nmol/L of each primer for COX-2 (sense primer, 5'-CCGAGGTGTATGTATGAGTG-3'; antisense primer, 5'-AGGAGAGGTTAGAGAAGG-3') or 100 nmol/L GAPDH (sense primer, 5'-CCTTGCCTCTCAGACAATGC-3'; antisense primer, 5'-CCACGACATACTCAGCAC-3') and 1 U of *Taq* DNA polymerase. The PCR primers used for detection of COX-2, and GAPDH yielded cDNA products of 314-bp and 340-bp, respectively. The conditions for PCR were one cycle of denaturing at 94 ℃ for 5 min, followed by 35 cycles of at 94 ℃ for 45 s, 56 ℃ for 45 s, 72 ℃ for 1 min with a final extension at 72 ℃ for 7 min. The electrophoresed PCR products were scanned by densitometry and the ratio of COX-2 to GAPDH was calculated in each sample.

### *Immunohistochemistry*

Tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated. Heat antigen retrieval was performed as described previously[24]. Slides were then processed for immunohistochemistry. In the primary antibody reaction step, slides were incubated with the COX-2 antibody (final concentration 5 μg/mL) for 1 h at room temperature. For positive controls, sections of colon cancer expressing the COX-2 protein were included in each staining procedure. For negative controls, nonimmunized rabbit IgG or Tris-buffered saline was substituted for the primary antibody to distinguish false positive responses from nonspecific binding to IgG or the secondary antibody. In addition, preabsorbed antibody with an excess amount of immunogen abolished the staining. Staining was repeated twice to avoid possible technical errors and similar results were obtained in all cases.

### *Evaluation of COX-2 immunohistochemistry*

All immunostained sections were coded and evaluated without prior knowledge of the clinical or pathological parameters. In each section, five high-power fields were selected at random and a total number of at least 700 cells were evaluated. The results were expressed as the intensity of staining. The intensity of staining was judged relative to smooth muscle cells in the sample, and estimated on a scale 0-3 (0 negative; 1 weak; 2 moderate; and 3 strong). All slides were interpreted by two investigators on three different occasions. Their evaluations were similar and <10% disagreement between the investigators was noted. In case of major disagreement, the final evaluation of such sections was determined by consensus using a multihead microscope.

### *Immunofluorescence assay*

Tissue samples were cut into 6 μm crystal-sections, and processed with COX-2 specific antibody and fluorescein labeled secondary antibodies and all the sections were observed immediately under fluorescence microscope.

### *Statistical analysis*

Statistical analysis was performed using SAS software. Results of immunohistochemistry were analyzed by Fisher's exact test. The correlation of RT-PCR and western blotting was analyzed by logistic regression analysis. $P<0.05$ was considered statistically significant.

## RESULTS

### *COX-2 mRNA expression in esophageal squamous cell carcinoma by semi-quantitative RT-PCR*

To examine the level of COX-2 mRNA, RT-PCR was performed to estimate the expression of COX-2 in tumor tissues relative to that in the matched normal tissues, which was expressed as COX-2/GAPDH ratio. GAPDH was used as an internal control. Semiquantitative RT-PCR indicated that COX-2 mRNA was found in 21 of 30 esophageal squamous cell carcinomas. In surrounding normal tissue of esophageal squamous cell carcinoma, COX-2 mRNA could not be detected (Figure 1). In the 21 esophageal squamous cell carcinoma tissues, the COX-2/GAPDH ratios ranged from 0.6 to 1.0, levels of COX-2 mRNA were increased by >80-fold compared to adjacent noncancerous tissue.

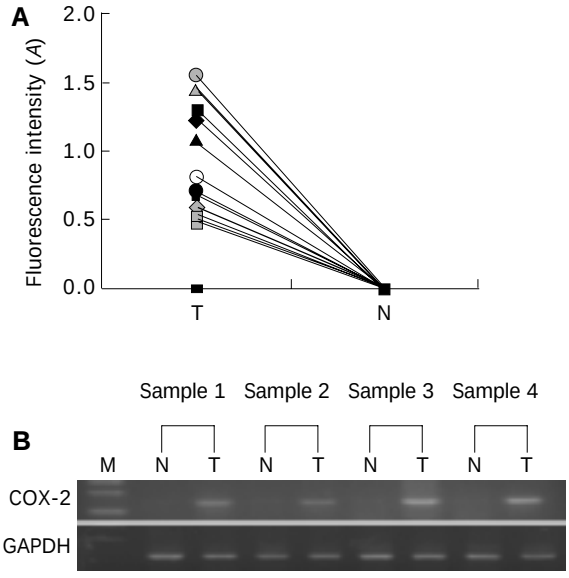


**Figure 1** Semiquantitative result of RT-PCR analysis of COX-2 mRNA expression in squamous carcinoma tissues and matched normal tissues of the esophagus. A: Data are expressed as the fluorescence values of COX-2 band in tumor and nontumorous

tissue samples from 30 patients with esophageal squamous cell carcinoma. B: Representative results of semiquantitative RT-PCR, indicated COX-2 mRNA was found in esophageal squamous cell carcinoma samples (T), while in surrounding normal tissue (N), COX-2 mRNA could not be detected. The coamplified GAPDH gene served as an internal control. PCR product sizes are 314 bp for COX-2 and 340 bp for GAPDH. M indicates DNA marker. The samples in lanes 1N and 1T, 2N and 2T, 3N and 3T, 4N and 4T are paired samples from 4 patients, respectively.

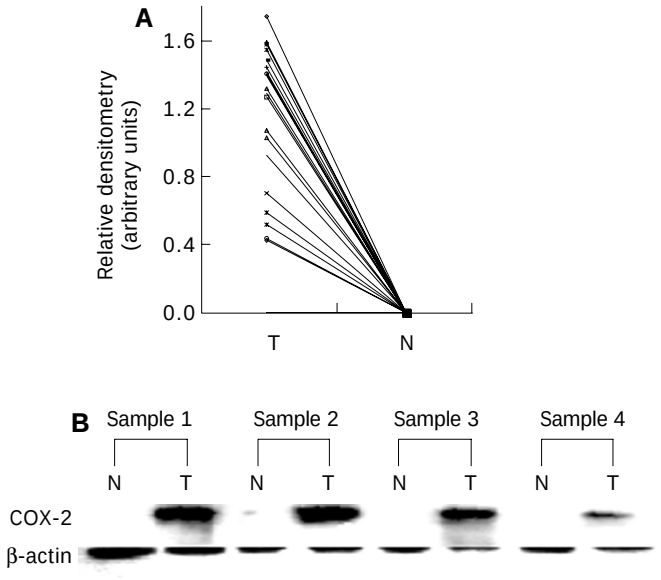


**Figure 2** Western blot analysis of COX-2 in squamous carcinoma tissues and matched normal tissues of the esophagus. COX-2 expressions in representative tumor (T) and nontumorous (N) are shown. A: Data are expressed as the absorbency values of COX-2 band in tumor and nontumorous tissue samples from 30 patients with esophageal squamous cell carcinoma. B: Representative result of Western blot analysis. COX-2 protein was detected in tumor tissue but was undetectable in nontumorous tissue in the same patients. β-actin was used as an internal control . The samples in lanes 1N and 1T, 2N and 2T, 3N and 3T, 4N and 4T are paired samples from 4 patients, respectively.

### *Western blotting*

To determine whether levels of COX-2 protein were also increased in esophageal squamous cell carcinomas, western blot analysis of paired tumorous and nontumorous tissue was performed. COX-2 protein was detected in tumor tissue from 21 of 30 patients but was undetectable in nontumorous tissue in the same patients. β-actin was used as an internal control (Figure 2). Furthermore, the results of western blot analysis paralleled those of mRNA RT-PCR analysis. There was a significant positive correlation between western blot analysis and RT-PCR analysis results ($r$=0.708, $P$<0.01) (Figure 3).

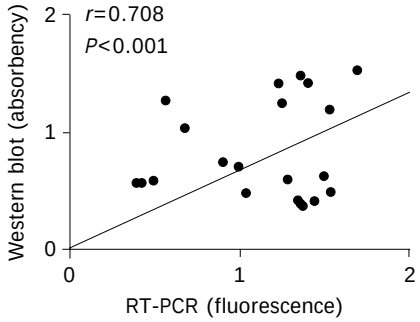


**Figure 3** Correlation between western blot analysis and RT-PCR analysis results. The results showed that the expression of COX-2 in cancer tissues (western blot analysis) was highly correlated with by RT-PCR analysis ($r$ value=0.708, $P$<0.001).

**Table 1** Expression of COX-2 in esophageal squamous cell carcinomas detected by immunohistochemistry

| | Intensity | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | |
| Tumor tissue | 10 | 2 | 12 | 6 | $P$<0.05 |
| Normal tissue | 27 | 3 | 0 | 0 | |

The intensity of staining was estimated on a scale 0–3. (0, negative; 1, weak; 2, moderate; and 3, strong).

### *COX-2 expression in esophageal squamous cell carcinoma by immunohistochemistry*

Immunohistochemistry was performed to directly indicate cell specific COX-2 protein expression. In nontumorous tissue specimens, a weak COX-2 staining or no COX-2 staining was detected. In contrast, a moderate to strong expression of COX-2 was noted in many esophageal squamous cell carcinomas of the same patients. Specifically, a strong expression of COX-2 protein was present in 6 of 30 (20%) esophageal squamous cell carcinomas, moderate expression was present in 12 of 30 (40%), and weak expression was present in 2 of 30 (15%). The level of COX-2 expression in esophageal squamous cell carcinomas was significantly higher than that in surrounding normal tissue (Table 1). Expression of COX-2 was localized to tumor cells, not to surrounding stromal cells or infiltrating inflammatory cells (Figure 4).

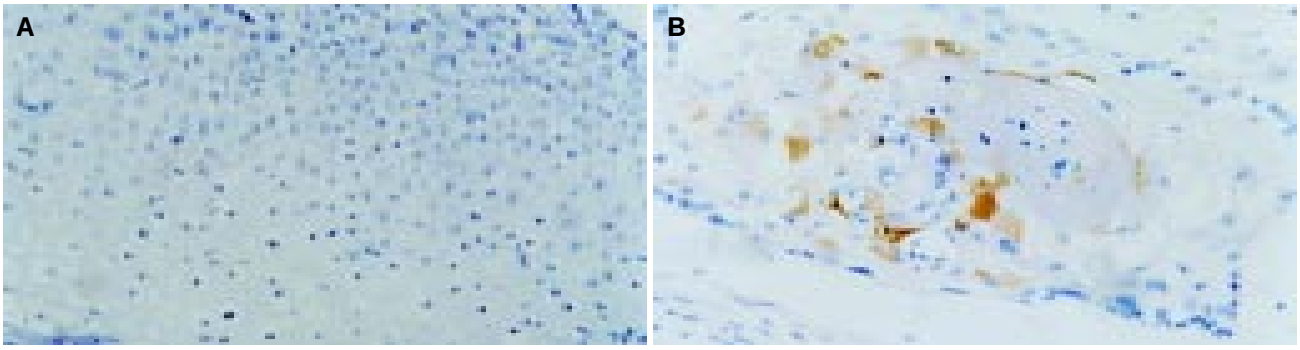


**Figure 4** Representative results of immunohistochemical analysis of COX-2 in squamous carcinoma tissues and matched normal tissues of the esophagus from the same patient (Magnification x200). In normal esophageal tissue there is no positive staining for COX-2 (A) but in esophageal squamous cell carcinoma tissue, carcinoma cells display a strong staining for COX-2, which indicate COX-2 expression specifically in cancer tissues (B).

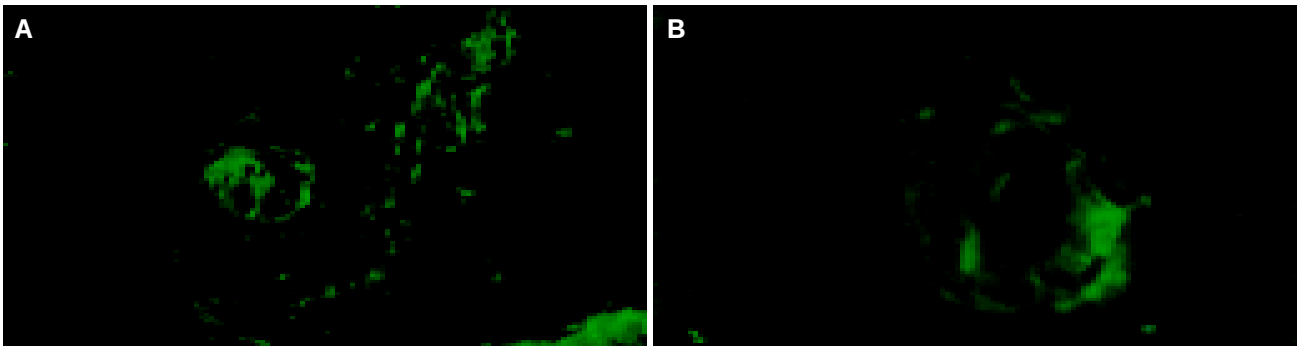


**Figure 5** Immunofluorescence analysis of COX-2 in squamous carcinoma tissues (Magnification, x400). A and B are from 2 different patients with esophageal squamous cell carcinoma, Both display a strong green fluorescence staining for COX-2 in cancer-nests.

### *COX-2 expression in esophageal squamous cell carcinoma by immunofluorescence*

Immunofluorescence was performed to directly indicate whether COX-2 protein was expressed in esophageal cancer. COX-2 expression was detected in esophageal squamous cell carcinomas. Immunofluorescence in esophageal squamous cell carcinomas displayed a cancer-nest structure (Figure 5).

## DISCUSSION

Epidemiological studies indicate that the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) results in a marked reduction in adenoma size and number and, not infrequently, leads to a complete regression of colonic polyps in patients with familial adenomatous polyposis[25-27]. NSAIDs also reduce the risk of sporadic colorectal, breast, and lung cancers[28,29]. These effects of NSAIDs on the inhibition of carcinogenesis have also been demonstrated in animal experiments, including pancreatic tumor model induced by N-nitrosobis (2-oxopropyl) amine in hamsters[30]. Several lines of evidence indicate that the antitumor effects of NSAIDs may be due in part to the inhibition of COX-2 activity[19,31-33]. Indeed, in carcinomas of the colon and lung and also in gastric and esophageal carcinomas, the level of COX-2 expression is significantly increased. These findings suggest that up-regulation of this enzyme might be a common mechanism for carcinogenesis of cells of an epithelial origin. Indeed, in the present large sampled study we found that COX-2 expression was increased at both the RNA and protein levels in a large proportion of esophageal squamous cell carcinoma compared with paired normal surrounding esophageal tissues by RT-PCR and Western blot analyses. RT-PCR analyses indicated that 21 of 30(70%) esophageal squamous cell carcinoma cases displayed increased COX-2 mRNA. Furthermore, the results of mRNA analysis paralleled those of western blot and immunohistochemistry. Thus, approximately 67% of the carcinoma samples exhibited positive staining of COX-2 protein, whereas paired normal tissues showed little or no COX-2 staining. Up to 60% of carcinoma cases showed moderate to strong COX-2 immunostaining (intensity 2 or 3). Also, immunofluorescence demonstrated the expression of COX-2 in esophageal squamous cell cancer-nests. These findings suggest that COX-2 may also be a marker for the malignant potential of esophageal squamous cell carcinoma, although this function remains to be clarified. Taken into consideration of the limited number of specimens, a conservative interpretation of the increased COX-2 mRNA and protein expression is necessary. Examination of a larger number of paired samples of tumor tissue and nonmalignant mucosa from esophageal cancer patients, as well as normal mucosa from persons without esophageal cancer in future studies will be necessary to more clearly determine the status of COX-2 in normal esophageal mucosa and in esophageal cancer. Because COX-2 was up-regulated at the transcriptional level, which can increase COX-2 protein expression, subsequent determination of COX-2 protein synthesis and bioactivity at the protein level is important. It is also important to study the possible link between the known genetic alterations and COX-2 in esophageal cancer. However, the present results indicate COX-2 is elevated in esophageal cancer in a fashion similar to that in intestinal tumors.

COX-2 could potentially cause carcinogenesis via multiple mechanisms, and the role of COX-2 in tumor development and progression *in vivo* is not known. The most obvious possibility is that overexpression of COX-2 leads to high levels of prostaglandins (PGs) in tumor tissue. This hypothesis is supported by the finding of elevated levels of PGs in cancer tissues, compared with corresponding normal tissues[34,35]. PGs produced by COX-2 may subsequently facilitate tumor progression by acting as differentiation and growth factors, immunosuppressors and angiogenic agents[36-38]. Additionally, it has been shown that elevated $PGE_2$ levels in COX-2 overexpression cancer cells correlate with the metastatic potential of the cancer cells, and can be reduced in a dose-dependent manner by COX inhibitors[39]. On the other hand, there is also evidence that NSAIDs might exert their antineoplastic effect by a PG-independent pathway, and the COX-2 enzyme itself may promote cancer development and progression[40]. Thus, NSAIDs suppressed proliferative activity in colon cancer cells devoid of cyclooxygenase and PGs[31]. Moreover, in extrahepatic tissues in which cytochrome P450 content is low, COX may be important for generation of carcinogens. For example, several classes of chemical carcinogens, e.g., dihydrodiol derivatives of polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines, are activated to mutagenic derivatives by COX[41]. The generation of carcinogens by COX-2 may be important, therefore, for understanding the link between cigarette smoking or consumption of grilled or fried meat and esophageal cancer[42]. Additional studies are needed to determine which of these mechanisms are more important in esophageal squamous cell carcinoma.

Selective inhibition of COX-2 in esophageal cancer cells induces apoptotic cell death and reduces proliferative activity and these effects correlate with the inhibition of PG synthesis. Similar results have been reported[43-45]. The significance of COX-2 likely varies between different classes of tumors. We cannot conclude from this and other published data whether the antiproliferative and proapoptotic effects of COX-2 inhibition on esophageal cancer cells are exclusively mediated through the inhibition of PG synthesis or if other mechanisms are also involved. However, our data provide a theoretical basis for evaluation of long-term intake of COX inhibitors on reduction of the incidence of esophageal cancer. Defining actual response of individual tumors to COX-2 inhibitors *in vivo*, however,

awaits further evaluation, especially since the level of COX-2 expression varies substantially between different tumors. Based on our data, a more thorough study on COX-2 expression is needed in patients with esophageal squamous cell carcinoma. Patients with a high COX-2 expression may benefit from specific or nonspecific COX-2 inhibitor medication, such as aspirin, for potential carcinogenic prevention of these cancers. Long-term intake of COX-2 inhibitors may be recommended for family members, especially with a positive family history of esophageal squamous cell carcinoma.

Taken together, our study suggests that COX-2 may play a role in esophageal squamous cell carcinoma development and/or progression and COX-2 inhibitors may be potential agents for the prevention or treatment of human invasive esophageal squamous cell carcinoma. Therefore, long-term intake of NSAIDs, such as aspirin, might reduce the risk of esophageal squamous cell carcinoma. To verify this hypothesis studies to evaluate the efficacy of COX-2 inhibitors in relevant animal models of esophageal squamous cell carcinoma are needed. In addition, studies to define the possible role of COX-2 in esophageal squamous cell carcinoma and the mechanisms by which COX-2 inhibitors exert antitumor activity are essential.

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• GASTRIC CANCER •

# Gastric malignancies in Northern Jordan with special emphasis on descriptive epidemiology

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## Abstract

**AIM:** To study the epidemiology of gastric malignancies in Jordan as a model for Middle East countries where such data is scarce.

**METHODS:** Pertinent epidemiological and clinicopathological data for 201 patients with gastric malignancy in north of Jordan between 1991 and 2001 were analyzed.

**RESULTS:** Male: female ratio was 1.8:1. The mean age was 61.2 years, and 8.5% of the patients were younger than 40 years of age. The overall age-adjusted incidence was 5.82/100 000 population/year. The age specific incidence for males raised from 1.48 in those aged 30-39 years to 72.4 in those aged 70-79 years. Adenocarcinomas, gastric lymphomas, malignant stromal tumors, and carcinoids were found in 87.5%, 8%, 2.5%, and 2% respectively. There was an average of 10.1-month delay between the initial symptoms and the diagnosis. Only 82 patients underwent "curative" gastrectomy. Among adenocarcinoma groups, Lauren intestinal type was the commonest (72.2%) and the distal third was the most common localization (48.9%). The mean follow up for patients with gastric adenocarcinoma was 25.1 mo (range 1-132 mo). The 5-year survival rates for stages I ($n$=15), II ($n$=41), III ($n$=59), and IV ($n$=53) were 67.3%, 41.3%, 5.7%, and 0% respectively ($P$=0.0001). The overall 5 year survival was 21.1%.

**CONCLUSION:** Despite low incidence, some epidemiological features of gastric cancer in Jordan mimic those of high-risk areas. Patients are detected and treated after a relatively long delay. No justification in favor of a possible gastric cancer screening effort in Jordan is supported by our study; rather, the need of an earlier diagnosis and subsequent better care.

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## INTRODUCTION

Gastric cancer (GC) is one of the most prevalent cancers and, today, is the second cause of cancer death worldwide[1,2]. The pattern and incidence of GC vary widely between different parts of the world. Costa Rica and Japan have the first and second highest rates in the world with a death rate of 77.5 and 50.5 /100 000 population, respectively[2,3]. In the United States 12 100 deaths from GC were expected during 2003 with a death rate of 6.8/100 000, and it was estimated that 224 00 (13 400 in men) new cases of GC were diagnosed in the same year[4]. The risk of developing GC was relatively lower in the Middle East and North Africa compared to those of western countries[1,5].

The epidemiology of GC has been widely studied in the Western world as well as in Japan[6-12]. However, only few scattered reports from the developing world have been published[13-17]. There is a lack of good descriptive data on GC from the Middle East countries, where both cancer registration and prevalence of risk factors are relatively unknown. Because of the decreasing trend that took place in the Western world as a result of possibly, socio-economic development and its consequences, it is important to gain an insight into what is happening in other parts of the world such as in the Middle East. This prompted us to report on the epidemiological and clinicopathological features of gastric malignancy in Jordan in comparison to other countries whenever possible. This could assist in the better understanding of the important risk factors, which contribute to the development of GC. This will also give a clue about whether or not screening programs are needed in our region.

## MATERIALS AND METHODS

### *Patients*

This is a retrospective study of gastric malignancy in the north of Jordan over an eleven-year period (Jan 1991 to Dec 2001). The population of Irbid province (north of Jordan) as determined in the last official census conducted in Jordan in 1994 was 745 774 out of which 385 264 (51.66%) were males. Fifty percent of the Jordanian population are below the age of 16 years[18]. A total of 76% of the population live in cities and towns.

Initial data were obtained from the computer database at the Department of Pathology at Jordan University of Science and Technology. This is the only pathology center serving the area. Histologically confirmed primary gastric malignancy was found in 201 patients, including 176 patients with adenocarcinoma, 16 patients with primary gastric lymphoma (PGL), 5 patients with malignant gastric stromal tumors, and 4 patients with gastric carcinoids. Further information was obtained from the medical records of these patients at Princess Basma Teaching Hospital and Prince Rashed Teaching Hospital, the only two tertiary centers in Northern Jordan. All available endoscopy reports were reviewed. Patients and/or family members were contacted whenever possible.

A single pathologist reviewed all the histological slides. Gastric adenocarcinoma was classified into intestinal (IT), diffuse (DT), or mixed types according to the histological criteria

of Lauren[19]. The site of the tumor was assessed pathologically in the resected specimens and endoscopically in the remaining cases, and was defined as that part of the stomach harboring the bulk of the tumor. Tumor staging in each patient was based on clinical information, preoperative radiological investigations, operative findings, and pathological examination. The staging was made in accordance with the TNM system endorsed by the Union Internationale Contre Le Cancer (UICC)[20].

Vital status of patients was ascertained from death certificates or from families who were contacted.

All deaths within 30 d of surgery were considered surgical mortality. The incidence rates were adjusted to the world population. Our hospital based incidence data were matched with the Jordanian National Cancer Registry (JNCR) data only for the period 1996-2001, since the Registry was established in 1996. The survival rate was analyzed for each stage by the Kaplan-Meier method, and the survival curves were compared by the log-rank test using the Statistical Package for the Social Sciences Software Program (SPSS®, Inc., Chicago, Illinois, USA). Differences were considered statistically significant at $P<0.05$.

## RESULTS

During the study period 201 patients with primary gastric malignancy were identified, 128 (63.7%) patients were males with a male: female ratio of 1.8:1.

Sixty-five percent of the patients (131/202) were diagnosed during the second half of the study period. Analyses of the data from the JNCR, which was established in 1996, showed that all the 131 study patients diagnosed with gastric malignancy between 1st January 1996 and 31st December 2001 appeared in the registry and there were no additional patients from northern Jordan in the registry not identified by our study.

The overall age-adjusted incidence (world population) was 5.82/100 000 per year. Table 1 shows the age specific incidence rate (ASIR) per 100 000 population by gender. The peak incidence was in the age group 60-69 years (39.8%), followed by the age group 70-79 (22.9%). Approximately 8.5% of the patients were younger than 40 years of age. The mean age for the whole group was 61.2 years (SD±12.3, range 24-91 years).

Presenting features for our patients are summarized in Table 2. Acute presentation was seen in 59 (29.4%) patients; 37 patients (18.4%) presented with upper gastrointestinal bleeding, 17 (8.5%) with gastric outlet obstruction, and in the remaining 5 (2.5%) patients perforation was the mode of presentation.

**Table 2** Presenting features of patients with gastric malignancy

| | Number of patients | % |
|---|---|---|
| Abdominal pain | 144 | 71.6 |
| Weight loss and/or anemia | 130 | 64.7 |
| Dyspepsia | 98 | 48.8 |
| Nausea, vomiting | 96 | 47.8 |
| Abdominal mass | 62 | 30.8 |
| Anorexia | 58 | 28.9 |
| Dysphagia | 44 | 21.9 |
| Gastrointestinal bleeding | 37 | 18.4 |
| Obstruction | 17 | 8.5 |
| Perforation | 5 | 2.5 |

There was an average of 10.1-month delay (range 2-17 mo) between the initial symptoms and the diagnosis. In 119 patients this was a result of reluctance in seeking medical advice and/or delay in referring patients for endoscopy. However, in five patients the delay was caused by a false negative upper gastrointestinal endoscopy.

Table 3 shows the distribution of the different histological types according to age and sex. The histologic subtypes of the

**Table 1** Age specific incidence rate per 100 000 population/year by age and gender

| Age (yr) group | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Population | No. of cancers[1] | ASIR[2] | Population | No. of cancers[1] | ASIR[2] |
| 0-9 | 113 142 | 0 | 0 | 106 465 | 0 | 0 |
| 10-19 | 092 899 | 0 | 0 | 087 629 | 0 | 0 |
| 20-29 | 076 938 | 0 | 0 | 071 445 | 1 | 0.13 |
| 30-39 | 043 068 | 7 | 1.48 | 038 967 | 9 | 2.1 |
| 40-49 | 024 175 | 11 | 4.14 | 023 480 | 6 | 2.32 |
| 50-59 | 019 763 | 21 | 9.66 | 017 526 | 9 | 4.67 |
| 60-69 | 010 157 | 53 | 47.44 | 009 009 | 27 | 27.25 |
| 70-79 | 003 641 | 29 | 72.4 | 004 264 | 17 | 36.24 |
| ≥80 | 001 481 | 7 | 43 | 001 725 | 4 | 21.08 |
| Total | 385 264 | 128 | 3.02 | 360 510 | 73 | 1.84 |

[1]Number of cancers during the 11-year study. [2]Age specific incidence rate per 100 000 population/year.

**Table 3** Histopathological distribution of gastric malignancies according to age and sex (%)

| Histopathologic diagnosis | Age (yr) | | Sex | | Total |
|---|---|---|---|---|---|
| | Mean | Range | Male | Female | |
| Intestinal type adenocarcinoma | 62.7 | 35-91 | 90(70.9) | 37(29.1) | 127(63.2) |
| Diffuse type adenocarcinoma | 51.9 | 24-75 | 9(31) | 20(69) | 29(14.4) |
| Mixed type adenocarcinoma | 64.3 | 51-72 | 11(57.9) | 8(42.1) | 19(9.5) |
| Adenosquamous carcinoma | 82 | 82 | 1 | - | 1(0.5) |
| Primary gastric lymphoma | 57.9 | 41-82 | 12(75) | 4(25) | 16(8) |
| Carcinoid | 66 | 41-81 | 2(50) | 2(50) | 4(2) |
| Malignant stromal tumors | 69.4 | 43-84 | 3(60) | 2(40) | 5(2.5) |
| Total | 61.2 | 24-91 | 128(63.7) | 73(36.3) | 201(100) |

**Table 4** Distribution of gastric adenocarcinoma according to site (%)

| Histopathological type | Proximal third | Middle third | Distal third | Entire stomach | Total |
|---|---|---|---|---|---|
| Intestinal type adenocarcinoma | 22(17.3) | 30(23.6) | 70(55.1) | 5(3.9) | 127 |
| Diffuse type adenocarcinoma | 6(20.7) | 7(24.1) | 7(24.1) | 9(31) | 29 |
| Mixed type adenocarcinoma | 3(15.8) | 5(26.3) | 8(42.1) | 3(15.8) | 19 |
| Adenosquamous carcinoma | - | - | 1 | | 1 |
| Total | 31(17.6) | 42(23.9) | 86(48.9) | 17(9.7) | 176 |

82 patients who had gastrectomy were correctly diagnosed in pregastrectomy endoscopic biopsies except in 2 cases, which were diagnosed as IT adenocarcinoma and turned out to be of the DT in subsequent examinations of the resected specimens. Pathological examination of the 65 resected IT and mixed adenocarcinomas revealed that 51 of the cancers were moderately differentiated, 10 were poorly differentiated and four were well differentiated.

Table 4 shows the distribution of various histologic types of gastric adenocarcinoma according to the sites affected.

For gastric adenocarcinoma, using the TNM staging, 15 patients had stage I, 41 patients had stage II, 59 patients had stage III, and 53 patients had stage IV disease. In the remaining 8 patients, the stage could not be determined due to insufficient data (stage X).

Gastrectomy was performed for 82 patients (D1 in 63 and D2 in 19 patients). Palliative resection or bypass procedures were done for 95 patients, while 3 patients with PGL had chemotherapy only. The remaining 21 patients were too frail to be treated. No patients had D3 gastrectomy. None of the patients with adenocarcinoma was given adjuvant therapy. Postoperative morbidity occurred in 25.4%(45/177) of the patients. Eight patients died during the postoperative period giving a surgical mortality of 4.5%(8/177).

For the 176 patients with adenocarcinoma, the mean follow up until December 2002 was 25.1 mo (range 1-132 mo). At that time, vital status was available for 163 patients (92.6%). The remaining 13 patients (mean age 59.6 years; 11 males and 2 females) were lost to follow up at a mean of 28.2 mo (range 7-86 mo). The stages of the disease for these 13 patients were I, II, III, IV, and X in 1, 2, 4, 4, and 2 patients respectively.

Survival analysis included the 8 early postoperative deaths and the later non-cancer deaths. The median survival for stages II, III, and both stages IV and X was 59, 19, and 13 mo respectively. Figure 1 demonstrates the survival curves for stages I-IV using Kaplan-Meier method. The 5-year survival rates for stages I, II, III, and IV were 67.3%, 41.3%, 5.7%, and 0% respectively ($P$=0.0001). The overall 5 year survival was 21.1%.

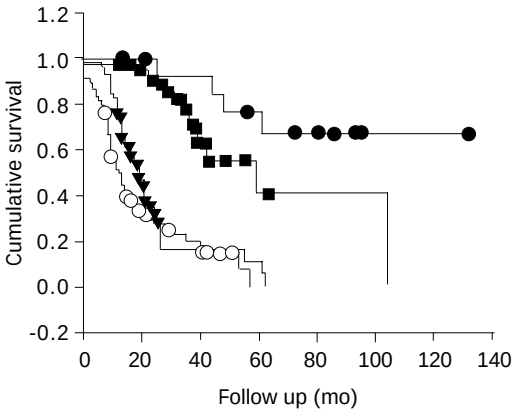


**Figure 1** Survival rate of gastric adenocarcinoma patients according to stage using Kaplan-Meier analysis. (●) Stage I; $n$=15, (■) Stage II; $n$=41, (▼) Stage III; $n$=59, (○) Stage IV and X; $n$=61.

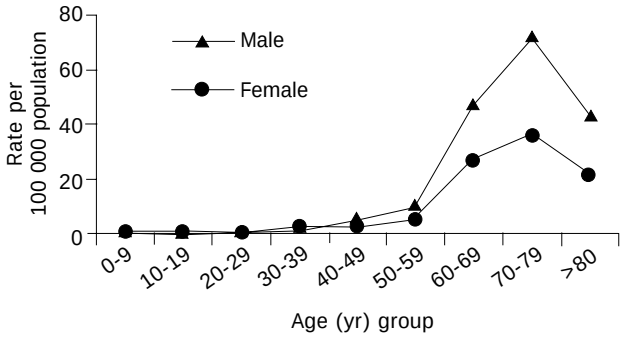


**Figure 2** Age specific incidence rate per 100 000 population / year by gender.

## DISCUSSION

There was consistency between our study and the data of the JNCR. The incidence was calculated and corrected for age in relation to the world population. The age-adjusted incidence of gastric malignancy in this study was 5.82/100 000 per year, which was significantly lower than those of developed countries but rather similar to most other Middle East countries[1,2]. However, this incidence would rise sharply when the ASIR is calculated because of the specific pyramidal age distribution of our population where approximately 50% of our population are below the age of 16 years (1994 census). The ASIR for males per 100 000 population raised from 1.48 in those aged 30-39 years to 72.4 in those aged 70-79 years (Figure 2). Similarly, figures from the JNCR showed that during 1997, the ASIR raised sharply from 3.3 to 108 for the same gender and age groups[18]. During the same year GC was the eighth commonest cancer, representing 4% of all cancer cases, and it accounted for 22% of all primary gastrointestinal tract cancers.

It is well known that the incidence of GC is decreasing globally. The apparent increase in diagnosing GC during the second half of the study period most likely reflects a better diagnostic yield after the relatively recent introduction of endoscopic services throughout the country rather than an actual increase in the incidence. Still the following two factors may play a role in this increasing trend. First, the rapid change in dietary habits Jordan is witnessing might constitute a risk factor. Vitamin C-rich fresh vegetables and fruits, starch, and natural unprocessed wheat products were the major constituents of Jordanian food. However, canned food, hot spices, pickles, and animal proteins are now dominating the Jordanian menu. It is known that the environmental risk factors for GC could be dietary in origin[21,22]. Second, the already high prevalence of *Helicobacter pylori* in Jordan is increasing and the exposure time is also increasing with the improvement in life expectancy among Jordanians[23,24]. It is proposed that Lauren's IT of GC is related to chronic *Helicobacter pylori* infection. Among our study group IT adenocarcinoma was the commonest histological subtype (63.2%).

Most resections were done with palliative intent. The low rate of gastrectomy with "curative" intent could be explained by the high proportion of patients with advanced GC at

presentation. However, we believe that surgical undertreatment might be a contributing factor. Our patients tended to present late as evidenced by the facts that there was a long interval between onset of symptoms and presentation, 29.4% presented acutely, and that only 40.8%(82/201) of them underwent D1 or D2 gastrectomy. This is not only due to insufficiency of current endoscopic services, but also due to the fact that many people in Jordan who have non- specific dyspeptic symptoms will seek the advice of non-physician personnel who will either prescribe herbal medicine for long term treatment or induce patches of second or third degree burns to the epigastric region using hot metals in order to ameliorate the deep seated pain! Subsequently some of these patients in whom the cause of dyspepsia is cancer will present to the hospital with late stage GC or one of its complications. In addition, the elderly people usually fail to make use of the available medical services due to inadequacy of primary health care in Jordan.

Some GC patterns in north of Jordan were similar to those reported from high-risk regions worldwide[3,25]. In particular, the mean age was in the seventh decade, male to female ratio was 1.8:1, IT adenocarcinoma was more common than DT (4.4:1), and distal location was more frequent than proximal one (2.5:1). Since the demographic features among Jordanians are homogenous, we believe that these trends apply to whole Jordan. In contrary, in a low-risk area, such as Kenya, the DT is the commonest subtype[26].

In our study, DT adenocarcinoma occurred in younger patients with a mean age of 51.9 years and was commoner in females with a male to female ratio of 0.45:1 (Table 3). Similar findings of diffuse tumors prevailing among young patients and women were reported from Sweden, Finland, Mexico and Taiwan[6,8,16,17], but not from neighboring Saudi Arabia[14,15].

Our survival rate for stages I and II is consistent with the rate reported in the Western literature[27,28]. However, this rate was much lower for our patients with more advanced stages probably reflecting undertreatment at our institute. After the introduction of a specialized upper gastrointestinal unit at our hospital in January 2001, we have adopted a more radical approach.

In Western countries, PGL represented only 2% to 5% of gastric malignancies[29], while it represented 8% of our series, which was similar to the 9% figure from neighboring Iraq[30], but different from the 14-22% figure reported from Saudi Arabia[14,15]. During the past three decades the site of PGL in the Middle East has changed[31]. Small intestinal involvement became less common, and gastric involvement more frequent. This varying pattern seemed to be environmental in origin[31]. Although the ratio of PGL to gastric adenocarcinoma among our patients was higher than in western series, a recent study from Jordan demonstrated that other patterns of PGL were mimicking those of the west[32]. The stomach was the commonest site of involvement, accounting for 62% of all cases[32].

Our rates for the gastric malignant stromal tumors and carcinoids are consistent with rates reported elsewhere[33]. The survival rate for these patients was not calculated due to the small number of patients.

At the end of this discussion, we would like to highlight some of the shortcomings in our study. First, the incidence figures were calculated only after histological confirmation by endoscopy or surgery. In Jordan, hospital postmortem is rarely performed because of religious or social grounds. Additionally, only half of deaths are attended by medical workers personals. This means that some patients with GC were certainly not included in the incidence calculation. The limitation to histologically confirmed cases increases specificity but lowers the sensitivity in identifying cases to be included in the incidence figures. Cases admitted to hospitals with a suspicion of GC or deceased with a diagnosis of GC but without histological confirmation were not considered in the estimation of incidence rates. Additionally as one third of our patients presented with an abdominal mass, and there was a 10-mo delay in diagnosis so the estimated incidence was lower than the real incidence because many patients did not reach the hospitals. Second, Lauren classification is known to be affected by a quite low reproducibility[34]. Specifically, mixed cases might be classified as intestinal or as diffuse type, depending on the examined tissue areas endoscopically biopsied or from the surgical specimens.

In conclusion, the incidence of GC in Jordan is low, but increases appreciably with age. This, in addition to the fact that 50% of the Jordanian population are below the age of 16 years, shows that it does not seem justified introducing a screening program. However, general practitioners should be more liberal in referring patients for endoscopy and resist the temptation to treat dyspeptic patients with anti-ulcer therapy without an endoscopy. Open access endoscopy, greater efforts in patient education and improvement of the diagnostic technical skills may raise the chance of detecting GC early. Some patterns of GC in Jordan (age, sex, tumor location, and histological type) are similar to features from high- risk areas. Although this study clearly highlights the pertinent epidemiological and clinicopathological features of gastric malignancy in Jordan, further studies are needed to evaluate the treatment outcomes.

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• GASTRIC CANCER •

# Identification of tumor markers using two-dimensional electrophoresis in gastric carcinoma

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## Abstract

**AIM:** To study the differential expression of proteins in normal and cancerous gastric tissues, and further identify new molecular markers for diagnosis and prognosis of gastric carcinoma, as well as develop new therapeutic targets of the disease.

**METHODS:** Matched pairs of tissues from 6 gastric cancer patients were analyzed for their two-dimensional electrophoresis (2DE) profiles. Soluble fraction proteins from human normal and cancerous gastric tissue were separated in the first dimension by isoelectric focusing on immobilized pH gradient (IPG, pH3-10) strips, and by 125 g/L sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension with silver nitrate staining. Protein differential expression was analyzed by use of image analysis software to find out candidates for gastric cancer-associated proteins.

**RESULTS:** Nine protein spots overexpressed in tumor tissues as compared with noncancerous regions. In the next step, 9 tumor-specific spots were cut off from Coomassie Brilliant Blue staining gels, digested in gel with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin. Protein identification was done by peptide mass fingerprinting with matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). In total, 5 tumor-specific protein spots corresponding to 5 different polypeptide chains were identified, including annexin V, carbonic anhydrase, prohibitin, fibrin beta and fibrinogen fragment D. Among these 5 spots, the potential significance of the differential expressions is discussed.

**CONCLUSION** Differential expression analysis of proteomes may be useful for the development of new molecular markers for diagnosis and prognosis of gastric carcinoma.

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## INTRODUCTION

Gastric cancer is a prevalent tumor worldwide. It is a multistage process involving multiple factors in aetiology and many gene-environment interactions. It is important to emphasize the heterogeneity of the histological background on which the tumor develops. Methods have been developed to identify tumor associated antigens such as molecular cloning in expression system or using a biochemical strategy based on the extraction of antigenic peptides bound to major histocompatibility complex class 1 molecules from tumor cells. These methods have allowed the recognition of certain human tumor antigens[1]. Several tumor markers of gastric cancer have been identified[2-4], including Lewis antigen, sulfomucin, CA50. However, no evidence has been obtained indicating that the detection of these markers precedes clinical diagnosis of gastric cancer. Proteomics studies of clinical tumor samples have led to the identification of cancer-specific protein markers, which provides a basis for developing new methods for early diagnosis and early detection of cancers as well as clues to understanding the molecular mechanism of cancer progression[5]. In order to identify proteins that elicit humoral responses in gastric cancer patients, proteome-based approach was used. A number of proteins from gastric tumor tissues were separated by 2DE and identified by using mass spectrometry. It includes the systematic cataloging of protein expression on a large scale. Such studies could help elucidate the molecular mechanism of cellular events associated with cancer progression, such as cellular signaling[6,7].

## MATERIALS AND METHODS

### *Tissue samples and preparation*[8]

Six pairs of primary, and advanced poorly differentiated gastric adenocarcinoma tissues and corresponding adjacent noncancerous gastric tissues were obtained with informed consent from patients who underwent gastrectomy at the First Affiliated Hospital of Zhengzhou University and Beijing Cancer Hospital. Cancer samples were obtained from the "core" part of the tumor to avoid the adjacent noncancerous tissue. For each of the normal tissues, surface epithelium was selectively procured by dissection with special care for minimal contamination of nonepithelial cells, and samples were immediately snap-frozen in liquid nitrogen. They were classified histologically according to Lauren's classification after H &E staining.

Fragments of normal and malignant tissues were sharp dissected and homogenized with a homogenizer in 2 mL fresh lysis buffer [2 g/L dithiothreilol (Amersham Bioscience, USA), 200 mL/L trichloroacetic acid (Sigma, USA) and 800 g/L acetone], then placed into tubes at 4-8 ℃ for 8-10 h. The mixture was centrifuged at 1 000 r/min for 5 min to remove tissue and cell debris, then centrifuged in a Beckman TL-100 table top ultracentrifuge at 430 000 *g* in a TLA-100.2 rotor for 30 min at 4 ℃. The supernatant was taken as soluble fraction. Protein was lyophilized, resuspended in isoelectric focusing gel rehydration solution {7 mol/L urea, 2 mol/L thiourea, 40 g/L 3-([3-cholamidopropyl] dimethylammonio) - 1-propanesulfonate

(CHAPS), 50 mmol/L dithiothreilol, 20 g/L IPG buffer pH 3-10, L} and stored at -80 ℃ until use. These were used as the 2DE samples for the soluble fraction. Protein concentration of 2-DE samples was estimated according to a commercial Bradford reagent. BSA was used as standard.

### *2DE with IPG strips*

First-dimension bioelectric focusing was carried out on Multiphor II system basically as described by the manufacturer Amersham Bioscience Inc. Samples containing up to 200 μg protein for analytical gels were diluted to up to 450 μL with dehydration solution (8 mol/L urea, 20 g/L CHAPS, 100 mol/L dithiothreilol, 5 g/L IPG buffer). Pre-cast Immobilized pH gradient (IPG) strip (24 cm, pH 3-10, linear gradient) was used for the first-dimensional separation. Strips were applied by overnight rehydration at 50 V, and for 1 h at 1 000 V. Then a gradient was applied from 1 000 to 8 000 V for 8 h to give a total of 180 000 Vh. All IEF steps were carried out at 20 ℃. After the first-dimensional IEF, IPG gel strips were placed in an equilibration solution (6 mol/L urea, 200 g/L SDS, 300 g/L glycerol, 50 mol/L Tris-HCl, pH 8.8) containing 10 g/L dithiothreilol and shaken for 15 min. The gels were then transferred to the equilibration solution containing 25 g/L iodoacetamide to alkylate thiols and shaken for a further 15 min before being placed on a 125 g/L polyacrylamide gel slab. Separation in the second dimension was carried out using Tris-glycine buffer containing 1 g/L SDS, at a current setting of 5 mA/gel for the initial 0.5 h and 18 mA/gel thereafter and a temperature of 20 ℃.

For silver staining, gels were immersed in ethanol: acetic acid: water (35:7:58) for 1.5 h, followed by washed twice in deionized water for 20 min. Gels were pretreated for 1 min in a solution of 0.2 g/L $Na_2S_2O_3$ and followed by3 of 1-min washes in deionized water. Proteins were stained in a solution containing 2 g/L $AgNO_3$ and 0.075% formalin (37 g/L formaldehyde in water) for 20 min, and washed twice in deionized water for 1 min. Subsequently, gels were developed in a solution of 0.6 g/L formaldehyde, 20 g/L $Na_2S_2O_3$ and 0.004 g/L $Na_2S_2O_3$. When the desired intensity was attained, the developer was discarded and reaction stopped by 10 g/L EDTA-$Na_2$. For Coomassie Brilliant Blue staining of gels, gels were equilibrated in a solution containing 500 mL/L methanol, 50 mL/L acetic acid and 25 g/L Coomassie Brilliant Blue R-250. Gels were rinsed in 300 mL/L ethanol containing 70 mL/L acetic acid.

Protein patterns in the gels after silver staining were recorded as digitalized images using a high-resolution scanner. Gel image matching was done with PDQuest software.

### *In-gel protein digestion*

Protein spots on Coomassie blue stained gel was performed essentially as described. After the completion of staining, the gel slab was washed twice with water for 10 min. The spots of interest were excised with a scalpel and put into 1.5 mL micro-tubes. The particles were washed twice with water and then twice with water/acetonitrile (1:1) for 15 min. The solvent volumes were about twice that of the gel. Liquid was removed, acetonitrile was added to the gel particles and the mixture was left for 2 h. After that, liquid was removed and the particles were rehydrated in 25 mmol/L $NH_4HCO_3$ for 5 min. Acetonitrile was added to produce a 1:1 mixture of 25 mmol/L $NH_4HCO_3$ / acetonitrile and the mixture was incubated for 15 min. All liquid was removed. Gel particles were dried in a vacuum centrifuge, reswelled in 10 mmol/L of dithiothreilol and 25 mmol/L of $NH_4HCO_3$, and incubated for 30 min at 56 ℃ to reduce the peptides. After chillness of tubes to room temperature and removal of the liquid, 55 mmol/L iodoacetamide in 25 mmol/L $NH_4HCO_3$ was added. The tubes were incubated for 30 min at room temperature in the dark to S-alkylate the peptides. Then iodoacetamide solution was removed, the particles were washed with 25 mmol/L $NH_4HCO_3$ and acetonitrile, dried in a vacuum centrifuge, rehydrated in digestion buffer containing 50 mmol/L $NH_4HCO_3$ and 12.5 ng/μL trypsin (TPCK-treated, proteomics grade, Sigma, USA), incubated for 8-12 h at 37 ℃. After digestion, 25 mmol/L $NH_4HCO_3$ was added, and the tube was incubated for 15 min. Acetonitrile was added and the tube was incubated for another 15 min. The supernatant was recovered, and the extraction was repeated twice with 50 g/L TFA/acetonitrile (1:1). The three extracts were pooled and dried in a vacuum centrifuge.

### *MALDI-TOF-MS analysis*

One μL sample with 1 μL matrix solution CCA (α-cyano-4-hydroxycinnamic acid) was spotted on the target and dried. Dried spots were analyzed in an REFLEX-III (Bluker) MALDI-TOF mass spectrometer. The spectrometer was run in positive ion mode and in reflector mode with the setting: accelerating voltage, 20 kV; grid voltage, 76%; guide wire voltage, 0.01%; and a delay time of 150 ns. The low mass gate was set at 500 m/z.

Proteins were identified by peptide mass fingerprinting with the search programs Mascot (http:// www.matrixscience.com/ cgi/index.pl?page=.1). The following search parameters were applied: SWISS-PROT and NCBI were used as the protein sequence databases, a mass tolerance of 50 ppm and one incomplete cleavage were allowed; acetylation of the N-terminus, alkylation of lysine by carboxyamidomethylation were considered as possible modifications[9].

## RESULTS

### *Evaluation of the reproducibility*

Mini 2DE gels (7 cm, pH3-10) were used to evaluate the reproducibility of the soluble protein preparations and to quantify the protein extracts for 2DE gel analysis. To ensure quality and reproducibility of results, 2DE maps (24 cm) were established from each sample based on silver staining of at least three independent gels, pairs of gels were run simultaneously with the same power supply and subjected to subsequent image analysis. Protein extracts prepared from tissues were compared in this way and found to be highly reproducible and similar amounts of total soluble protein were yielded when analyzed on the gels.

### *Overview analysis of the protein expression profiles of paired samples*

2DE Gel separation of proteins from 6 pairs of normal and cancerous epithelial cells was procured from the same gastric carcinoma specimen respectively. A series of 2DE maps were constructed for the soluble fraction proteins of human gastric tissue. A representative 2DE gel images following silver staining of cancer tissues (Figure 1A) and normal tissues ( Figure 1B) were produced in a 2DE imageMaster. Comparison of the differential protein expression between cancer and normal tissues shown in 2DE images was carried out using the AutoDetect Spots menu of PDQuest software. Figure 1A (from cancer tissues) contains a total of 356 spots, whereas Figure 1B (from normal tissues) contains a total of 382 spots. A total of 323 spots from cancer tissues could be matched to those from normal tissues. In total, we were able to identify 9 cancer-specific spots in 2DE gels. The positions of the identified proteins are shown in Figure 1A. These differences were observed in other cancerous samples. Because all of the identified spots were detectable with Coomassie Blue staining, they could be considered as abundant proteins.

### *Protein identification*

The resulting spot identification was mapped onto the analytical

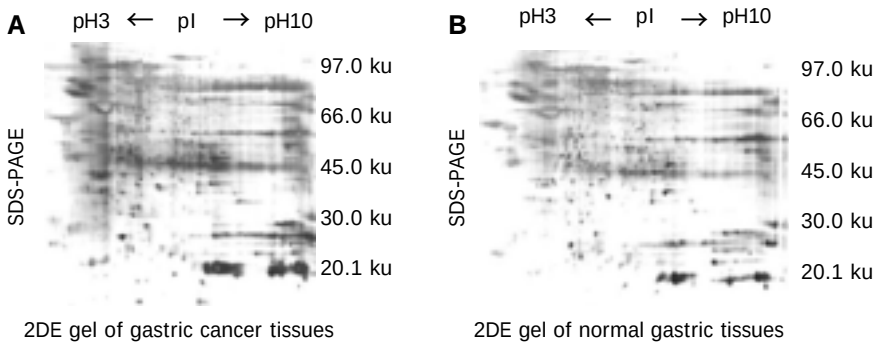


**Figure 1** 2DE maps of human gastric tissue from No.5 patient. A: was from gastric cancer. B: from normal gastric sample of the same patient. Proteins were separated on pH 3-10 linear IPG strip in the first dimension and 125 g/L SDS-PAGE in the second dimension, gels were silver stained. All labeled spots were tumor-specific.

gels stained by Coomassie Brilliant Blue. On the map, 9 cancer-specific spots were excised and subjected to in-gel digestion followed by peptide mass fingerprinting for protein identification. Figure 2 shows the identification of the spot No. 18 as an example . We identified proteins by peptide mass fingerprinting, MS-Fit of UCSF.

The criteria used to accept identifications included the extent of sequence coverage, the number of peptides matched, the probability score, and whether human protein appeared as the top candidates in the first pass search where no restriction was applied to the species of origin.

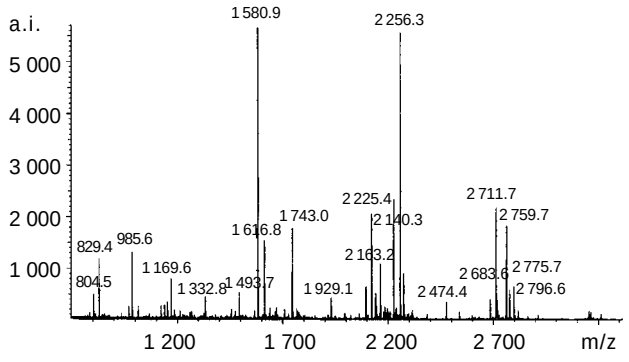


**Figure 2** MALDI-TOF MS of tryptic digests of protein spot No. 18 resolved on 2DE gel, REFLEX III (BLuker). The data were collected on positive ion and reflector mode. Experimentally determined mass values are labeled on peptides. Spot 10 matched peptide 11; amino acid coverage, 60%; the spot was identified as carbonic anhydrase I.

The results of identification are summarized in the Appendix Table 1. For identified protein, probability based score greater than 73 was significant ($P$<0.05). For example, in the case of annexin V, the protein score is 163. Some of these proteins, such as carbonic anhydrase I; chain B, crystal structure of fibrinogen fragment D; fibrin beta and prohibitin, have already been detected in gastric cancer tissues.

## DISCUSSION

Proteome based profiling employs the measurement of protein expression pattern for the identification of individual proteins and clusters of proteins that mediate particular aspects in a physiological and pathophysiological process. The measurement of protein expression patterns of normal and disease tissues or cell populations will lead to the characterization of diagnostic and prognostic markers, and it can be further employed for the analysis of the disease stage which might also have an impact on the therapy. Thus, preferably small clusters of proteins represent the ideal diagnostic markers enabling an easier and more accurate diagnosis of diseases for better therapy[10].

This study was based on an ongoing proteomic analysis of gastric cancer aiming at screening the protein markers in the proteome for diagnosis of gastric cancer. With the availability of high-throughput 2DE gels and initial screening by using automated procedures, identification of changes in the proteome in various tissues will be possible. The approach we described in this study has shown that high-throughput analysis will be a valuable tool. An effort is currently being made using proteome based techniques[11]. 2DE pattern of normal and diseased tissues revealed a number of polypeptides

**Table 1** Protein spots in GC searched by PeptIdent software in the SWISS-PROT database

| Spot ID | SWISS-PROT ID | Peptide matched | Top score | Theoretic pI | Theoretic $M_r$ | Sequence covered rate (%) | Protein name |
|---|---|---|---|---|---|---|---|
| 17 | gi999937 | 17/31 | 163 | 4.98 | 35 839 | 63 | Annexin V |
| 10 | gi515109 | 11/24 | 116 | 6.63 | 28 778 | 60 | Carbonic anhydrase I |
| 5 | gi2781208 | 15/43 | 105 | 5.84 | 38 081 | 55 | Chain B, crystal structure of fibrinogen fragment D |
| 12 | gi223002 | 12/33 | 92 | 7.95 | 51 358 | 42 | Fibrin beta |
| 19 | gi4505773 | 11/46 | 75 | 5.57 | 29 843 | 55 | Prohibitin |
| 4 | gi2781208 | 9/31 | 67 | 5.84 | 38 081 | 37 | Chain B, crystal structure of fibrinogen fragment D |
| 18 | gi15192925 | 8/29 | 63 | 6.90 | 31 032 | 46 | Alcohol dehydrogenase |
| 16 | gi15895617 | 9/31 | 58 | 9.01 | 42 229 | 34 | Sugar transaminase, involved in dTDP-4, 6-dideoxyglucose biosynthesis |
| 11 | gi1586816 | 10/35 | 55 | 9.33 | 42 071 | 27 | Jerky gene |

associated with gastric cancer, which were expressed in gastric cancer tissues, but absent in normal gastric tissues. Some of them might possibly be identified and serve as diagnostic and prognostic markers in gastric cancer.

In the preparation of 2DE maps presented in this study, tissue samples from different individuals were used without pooling of samples. The total homogenate was fractionated by ultracentrifugation into soluble fraction[12]. In order to minimize the influence of the methodology, we attempted, when possible, to make protocols for 2DE PAGE alike. Our initial analyses of six normal tissue samples indicated the overall protein pattern remained very similar across the samples.

We identified nine protein spots which were expressed in cancerous tissues but absent in normal gastric tissues. Of the 9 position identifications, spots 4, and 5 were found as multiple spots on the 2DE gels. Those included the subunits of the proteins, both of which focused in several pI positions but had the same molecular mass. It indicated that these gene products were present as isoforms with post-translation modification[13].

Annexins are $Ca^{2+}$ and phospholipid binding proteins forming an evolutionarily conserved multigene family. For some annexins, it appears that they participate in the regulation of membrane organization and membrane traffic and the regulation of ion ($Ca^{2+}$) currents across membranes or $Ca^{2+}$ concentrations within cells. Some members of the family have been identified extracellularly where they can act as receptors for serum proteases on the endothelium as well as inhibitors of neutrophil migration and blood coagulation[14]. Annexin V is used widely as a marker for apoptotic cells, the annexin V mutants showed defective homotypic cell adhesion and resistance to $Ca^{2+}$-dependent apoptotic agents without exhibiting any changes in the generation of cytosolic $Ca^{2+}$ fluxes[15]. Annexin V also known as calphobindindin I, has been shown to be an endogenous inhibitor of protein kinase C, a key enzyme in cellular signal transduction. The inhibition of protein kinase C by annexin V is presumed to be ultimately related to carcinogenesis and studies have demonstrated that a decrease in the production of annexin V may lead to the dysregulated protein kinase C[16,17]. Annexin V has been found in other cancer tissues or cell lines[18,19], but its presence in gastric cancer has not be documented.

Carbonic anhydrase (CA) is the zinc-containing metalloenzyme that catalyzes the reversible hydration of $CO_2$. The role of the enzyme has been thoroughly investigated. The main functions of the enzyme are to produce $HCO_3^-$ for the intermediate metabolism and to maintain pH, water, and ion equilibrium in the body[20]. CAs show various levels of catalytic activity and binding to inhibitors. They have considerable diversity in tissue distribution and cellular localization, and they perform a variety of biological functions. CAI protein is associated with cell growth. It is likely expressed by rapidly proliferating tumor cells or cells that are about to enter the proliferative state, because the CA domain and other elements of the molecule take part in the regulation of cell growth in certain tumor cell types[21].

The presence of fibrin (ogen) within the tumor stroma likely affects the progression of tumor cell growth and metastasis[22]. The deposition of fibrin (ogen), along with other adhesive glycoproteins, into the extracellular matrix serves as a scaffold to support binding of growth factors and to promote the cellular responses of adhesion, proliferation, and migration during angiogenesis and tumor cell growth. Inappropriate synthesis and deposition of extracellular matrix constituents are linked to altered regulation of cell proliferation, leading to tumor cell growth and malignant transformation[23,24]. Fibrin deposition occurs within the stroma of a majority of tumor types. Fibrin (ogen) content was significantly higher in malignant tumor patients than that in benign disease patients, significant reduction was observed after treatment and became elevated again when there was recurrence or metastasis[25]. Biggerstaff *et al*.[26] suggested that coagulation activation and the subsequent increase in circulating fibrin may enhance platelet adhesion to circulating tumor cells and thereby facilitate metastatic spread. Assessment of fibrin (ogen) not only helps to diagnose cancers but also evaluates the therapautic effect and prognosis[27].

Prohibitin proteins have been implicated in cell proliferation, ageing and the maintenance of mitochondria integrity[28], prohibitins are present in the inner mitochondrial membrane and always bound to each other. They are expressed during development and their expression levels are indicative of a role in mitochondrial metabolism[29]. High level expression of the proteins is consistently seen in primary human tumors. The prohibitin protein has been found having various functions, including cell cycle regulation, apoptosis, assembly of mitochondrial respiratory chain enzymes and ageing. We are currently trying to identify the additional proteins whose expression is significantly altered in cancerous tissue.

This study also confirms that proteomic analysis is a powerful tool for the discovery of such molecular markers. Proteomic analysis allows the characterization of picoquantities of proteins with MS and changes in the levels inherent to the pathophysiology of any cell type, tissue, or whole organism. It is hoped that the identification of protein markers by this approach could discriminate cancerous from normal cells. As demonstrated here, proteomic analysis may be efficiently used to identify new indicators for the diagnosis and prognosis of cancer progression.

In conclusion, the differences of the proteins between normal gastric epithelial cell and malignant cells are complex. Our data only show a few of the highly expressed spots. Further basic and clinical investigation will be needed to demonstrate if these proteins could be markers for GC and to evaluate other non-constant spots in relation to the clinical condition of the patient.

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• LIVER CANCER •

# Therapeutic effects and prognostic factors in three-dimensional conformal radiotherapy combined with transcatheter arterial chemoembolization for hepatocellular carcinoma

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## Abstract

**AIM:** To evaluate the therapeutic efficacy of three-dimensional conformal radiotherapy (3D-CRT) combined with transcatheter arterial chemoembolization (TACE) on the patients with hepatocellular carcinoma (HCC).

**METHODS:** Between 1998 and 2001, 94 patients with HCC received 3D-CRT combined with TACE. A total 63 patients had a Okuda stage I lesion and 31 patients had stage II. The median tumor size was 10.7 cm (range 3.0-18 cm), and liver cirrhosis was present in all the patients. There were 43 cases of class A and 51 class B. TACE was performed using lipiodol, 5-fluorouracil, cisplatin, doxorubicin hydrochloride and mitomycin, followed by gelatin sponge cubes. Fifty-nine patients received TACE only one time, while the others 2 to 3 times. 3D-CRT was started 3-4 wk after TACE. All patients were irradiated with a stereotactic body frame and received 4-8 Gy single high-dose radiation for 8-12 times at the isocenter during a period of 17-26 d (median 22 d).

**RESULTS:** The median follow-up was 37 mo (range 10-48 mo) after diagnosis. The response rate was 90.5%. The overall survival rate at 1-, 2-, and 3- year was 93.6%, 53.8% and 26.0% respectively, with the median survival of 25 mo. On univariate analysis, age ($P$=0.026), Child-Pugh classification for cirrhosis of liver ($P$=0.010), Okuda stage ($P$=0.026), tumor size ($P$=0.000), tumor type ($P$=0.029), albuminemia ($P$=0.035), and radiation dose ($P$=0.000) proved to be significant factors for survival. On multivariate analysis, age ($P$=0.024), radiation dose ($P$=0.001), and tumor size ($P$=0.000) were the significant factors.

**CONCLUSION:** 3D-CRT combined with TACE is an effective and feasible approach for HCC. Age, radiation dose and tumor size were found to be significant prognostic factors for survival of patients with HCC treated by 3D-CRT combined with TACE. Further study for HCC is needed to improve the treatment efficacy.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health threat in Africa and China where rates of hepatitis B infection have always been high[1]. Although early diagnosis and curative surgical resection can achieve the best prognosis, the number of patients who could undergo resection is still limited, even for those with small tumors because of the unique characteristics of this tumor, such as multifocality, early vascular invasion, and concurrent liver cirrhosis[2]. Nonsurgical therapies, such as percutaneous ethanol injection, transcatheter arterial chemoembolization (TACE), have been tried for unresectable HCC[3,4]. TACE has achieved improved survival; however, the antitumor effect of TACE alone has frequently been incomplete, even after repeated treatments[5,6]. Radiotherapy used for HCC has been attempted for more than 4 decades. Early trials adopted whole liver irradiation but the radiation dose was inadequate[7]. Because of the unsatisfactory results obtained with this low-dose whole liver irradiation, doctors have not long applied it in their treatment of HCC. The advanced technique of three-dimensional conformal radiotherapy (3D-CRT), however, has made it possible to deliver a higher dose of radiation to part of the liver accurately without a significant dose increase in the other intra-abdominal critical structures. Several studies reported that 3D-CRT could tolerate higher radiation levels with a substantial tumor response[8-17]. Their findings indicate that 3D-CRT can be an effective component of the treatment strategy for HCC. The principle for 3D-CRT was to escalate the radiation dose in an attempt to elevate the rate of tumor response without damaging the normal liver cells. Stereotactic radiosurgery or hypofractionated radiotherapy has 80-90% local control rate, even for so-called radioresistant tumors, such as renal cell carcinoma and melanoma[18,19]. Because of its success, small-volume radiotherapy has been applied to extracranial lesions, such as lung and liver tumors[20, 21]. Although the role of 3D-CRT in the management of HCC has been increasingly recognized, there are still several questions to be solved, one of which involves the identification of prognostic factors so as to improve the therapeutic effects of the management for HCC after 3D-CRT combined with TACE. Another is how to assess the exact effectiveness and toxicity of 3D-CRT combined with TACE. In our department, 3D-CRT combined with TACE has been actively applied for the treatment of HCC since the 1998. In this retrospective study, we aimed to analyze the effects and prognostic factors affecting survival in 94 HCC patients treated with this therapy.

## MATERIALS AND METHODS

### *Patients*

The diagnosis of HCC was based on histological features or

on radiologic findings (liver tumor CT scan, as well as hypervascular mass in hepatic angiography) and on a serum alpha-fetoprotein (AFP) level exceeding 400 ng /mL. All tumors with an AFP less than 400 ng /mL underwent a biopsy for diagnosis. The exclusion criteria included were as follows: (1) the presence of extrahepatic metastasis; (2) liver cirrhosis of Child-Pugh class C; (3) tumors occupying more than two-thirds of the liver; and (4) a score of Karnofsky performance status of less than 60.

**Table 1** Patient data before initiation of radiotherapy ($n$ =94)

| Data | $n$(%) |
|---|---|
| Age (yr) | |
| <60 | 78(83.0) |
| ≥60 | 16(17.0) |
| Gender | |
| Male | 84(89.4) |
| Female | 10(10.6) |
| Alpha-fetoprotein | |
| >400 ng / mL | 64 (68.1) |
| ≤400 ng / mL | 30(31.9) |
| Child-Pugh classification for cirrhosis of liver | |
| Class A | 43(45.7) |
| Class B | 51(54.3) |
| Karnofsky performance score | |
| >70 | 90(95.7) |
| <70 | 4(4.3) |
| Okuda stage | |
| I | 63(67.0) |
| II | 31(33.0) |
| Tumor size (cm) | |
| <5 | 17(18.1) |
| 5-10 | 40(42.6) |
| >10 | 37(39.3) |
| Tumor type | |
| Massive | 66(70.2) |
| Multinodular | 28(29.8) |
| PVT | |
| Yes | 12(12.8) |
| No | 82(87.2) |
| Albuminemia | |
| <3 g / dL | 15(16.0) |
| >3 g / dL | 79(84.0) |
| Bilirubinemia | |
| <3 mg / dL | 29(30.9) |
| >3 mg / dL | 65(69.1) |
| Chronic hepatitis in serum virology | |
| Type B | 90(95.7) |
| Type C | 4(4.3) |
| Radiation dose | |
| 60 Gy | 34(36.2) |
| 56 Gy | 34(36.2) |
| 48 Gy | 26(27.6) |
| TACE | |
| 1 fraction | 59(62.8) |
| >1 fraction | 35(37.2) |

Ninety-four patients (84 male and female) who received 3D-CRT combined with TACE in our department between August 1998 and August 2001 were enrolled in this study. The patient data are shown in Table 1. Their median age was 51.5 years (range 23–73 years). Sixty-four patients had an serum AFP level >400 ng/mL. Liver cirrhosis was present in all patients, with 43 patients of Child-Pugh class's A. Most patients had good performance status, and 90 patients had a Karnofsky performance score (KPS) of more than 70. The Okuda stage I was in 66 patients and II in 28 patients. The tumor size was calculated according to the mean of three orthogonal diameters on CT. It was <5 cm in 7 patients, 5-10 cm in 30 patients, and >10 cm in 57 patients, with the median tumor size of 10.7 cm (range 3.0-18 cm). The massive tumor was the most frequent type, which was found in 82 patients (87.2%). Portal vein thrombosis (PVT) was present in 12 patients (12.8%). Chronic hepatitis in serum virology was present in 90 patients with type B (95.7%), and in 4 patients with type C (4.3%).

### *TACE procedures*

TACE was performed with the infusion of a mixture of 5–20 mL of iodized oil contrast medium (Lipiodol, Huaihai Pharmaceutical Factory, Shanghai, China), 1.0 g of 5-fluorouracil (5-Fu, Xudong Haipu Pharmaceutical Inc., Shanghai, China) and 40-60 mg of cisplatin (CDDP, Qilu Pharmaceutical Factory, Jinan, China) or 30-50 mg of doxorubicin hydrochloride (Adriamycin, Main Luck Pharmaceutical Inc., Shenzhen, China), and 10 mg of mitomycin (Mytomycin-C C, Kyowa Hakko Kogyo, Tokyo, Japan), followed by 1 mm×1 mm×10 mm of gelatin sponge cubes (Gelfoam. the 3rd Pharmaceutical Factory of Nanjing, Nanjing, China) embolization. To preserve liver function as much as possible, we performed superselective TACE for the feeding arteries of each intrahepatic tumor. When there was an arterial portal shunt or main branch PVT, we performed TACE without lipiodol to prevent severe damage to the normal liver. TACE procedures were performed with a 4-wk interval, and the patients received 1 to 3 times.

### *Radiotherapy*

Radiation treatment was given to patients placed in a supine position, with both arms raised above the head and the head in a natural position. The patients were immobilized in this position using a vacuum pillow (TN-1, TOPSLANE, Shanghai, China) with an oxygen mask (3 000–5 000 cc/min) for respiratory suppression for CT simulator (PQS 2000, Picker, USA). CT data were all transferred to a 3D–radiation treatment planning system (STP 3.0, Leibinger, Freiburg, Germany) by the network. The hepatic tumor, liver, kidneys, spinal cord, and gastroduodenal intestine of each patient were contoured and reconstructed to form a 3D representation. The radiation treatment volumes and treatment angles were designed according to the beam's-eye view technique to minimize critical organ injury. Each beam's shape was designed using a multileaf collimator or custom-made block. Three-dimensional CT-based computerized treatment planning was used to determine the best combination of coplanar and noncoplanar portals. A dose-volume histogram (DVH) was generated from the stereotactic treatment planning system for each patient. Gross tumor volume (GTV) was defined as the hepatic tumor volume, visualized by three-dimensional computation of contrast CT-defined contours. Clinical target volume (CTV) was defined as GTV plus a 0.5 cm margin. Planning target volume (PTV) was defined as CTV plus a 0.5 cm margin at medial/lateral/ventral/dorsal sides, but plus a 1.5-2 cm margin at cranial/caudal sides to account for daily setup error and respiratory organ motion. Dose inhomogeneity of PTV should be within ±7% of isocenter dose. Normal liver was defined as the total liver volume minus the GTV. The average volume of GTV for these 94 patients was 979±623 mL. The average volume of whole liver was 1 790±645 mL. The number of portals used for radiation treatment ranged from 2 to 6, with a median of 5. Ultimately, the radiotherapy volume involved a portion of the liver, and whole liver radiation was always avoided. 3D-CRT was started within 3-4 wk after TACE using a 6-MV linear accelerator (CLINAC 600C/D, Varian Assoc, USA).

A dose of 4-8 Gy was applied each time and 3 times a week to deliver a total dose of 48-60 Gy. The total dose was determined by the fraction of the nontumorous liver receiving >50% of the isocenter dose. The guidelines were as follows: if <25% of the nontumorous liver received >50% of the isocenter dose, the total dose was increased to 60 Gy with 7.5 Gy each time; if 25-50%, the dose was 54 Gy with 6 Gy each time; if 50-75%, the dose was 48 Gy with 4 Gy each time; and if >75%, no treatment was given. This guideline was more strictly followed after the application of three-dimensional planning. The total radiation dose ranged from 48 to 60 Gy, and the median tumor dose was 56 Gy. During treatment, patients were monitored weekly with complete blood counts and liver function tests and AFP test.

### *Evaluation*

The evaluation of tumor response was based on the change in mean tumor size (calculated according to the mean of three orthogonal diameters) on serial CT scans which are first started 4-6 wk after treatment completion and then performed at 1-, 3-mo interval. The evaluation of the change in tumor size was done at 3 mo after the treatment. Complete disappearance of the tumor was considered a complete response (CR), a decrease of >50% in tumor size was defined as a partial response (PR), a decrease of <50% in tumor size or no change was defined as stable disease (SD), and progression was defined as progressive disease (PD). CR and PR were considered to be responsible, whereas SD or PD not to be responsible. Acute toxicity was that occurred during treatment and 1 mo after the treatment. Subacute or chronic toxicity was defined as that occurring from 1 mo after radiotherapy.

### *Statistics*

Statistical analysis was performed using SPSS 10.0. Overall survival was estimated from the date of diagnosis according to the Kaplan-Meier method. Log-rank statistics were used to identify the prognostic factors important for survival. Cox proportional models using enter stepwise regression were applied to all potentially significant variables for the multivariate analysis. $P<0.05$ was considered statistically significant.

## RESULTS

### *Tumor response*

As shown in Table 2, tumor response (CR+PR) was evaluated by the change in mean tumor size on CT 3 mo after treatment completion.

**Table 2** CT response of HCC to 3D-CRT combined with TACE

| Response | No of patients (%) |
|---|---|
| Complete | 12(12.8) |
| Partial | 73(77.7) |
| Stable disease | 6(6.4) |
| Progressive disease | 3(3.1) |

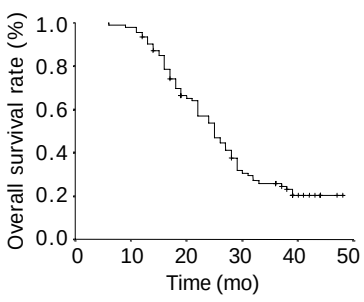


**Figure 1** Actuarial survival of 94 patients treated with 3D-CRT combined with TACE.

### *Overall survival*

The median follow-up was 37 mo (range 10-48 mo) after diagnosis. The overall survival rates of 1-, 2-, and 3-year were 93.6%, 53.8%, and 25.9%, respectively (median survival 25 mo, Figure 1).

### *Factors affecting overall survival*

The results of univariate and multivariate analyses of prognosis factors for overall survival are shown in Table 3. On univariate analysis, age ($P$=0.026, Figure 2), Child-Pugh classification for cirrhosis of liver ($P$=0.01, Figure 3), Okuda stage ($P$=0.008, Figure 4), tumor size ($P$=0.000, Figure 5), tumor type ($P$=0.029, Figure 6), albuminemia ($P$=0.035, Figure 7), and radiation dose ($P$=0.000, Figure 8) were shown as significant factors. Multivariate regression identified the following independent favorable prognostic factors: younger age ($P$=0.024), high radiation dose ($P$=0.001), and small tumor size ($P$=0.000).

**Table 3** Univariate and multivariate analyses of prognosis factors for overall survival

| Univariate analysis | Multivariate analysis | | |
|---|---|---|---|
| | *P* | R (95% *CI*) | *P* |
| Age (yr) | 0.026 | 2.377 | 0.024 |
| Tumor size | 0.000 | 6.183 | 0.000 |
| Radiation dose | 0.000 | 0.491 | 0.001 |
| Child-Pugh classification for cirrhosis of liver | 0.010 | | |
| Karnofsky performance status | 0.913 | | |
| Okuda stage | 0.008 | | |
| Gender | 0.202 | | |
| Tumor type | 0.029 | | |
| PVT | 0.235 | | |
| Albuminemia | 0.035 | | |
| Bilirubinemia | 0.305 | | |
| Chronic hepatitis in serum virology | 0.060 | | |
| Alpha-fetoprotein | 0.861 | | |
| TACE | 0.892 | | |

HR: Hazard ratio; 95% *CI*: Confidence interval.

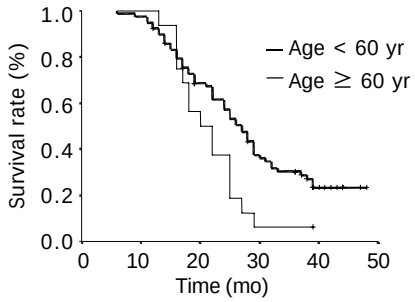


**Figure 2** Univariate analysis of age on survival of patients treated with 3D-CRT combined with TACE.

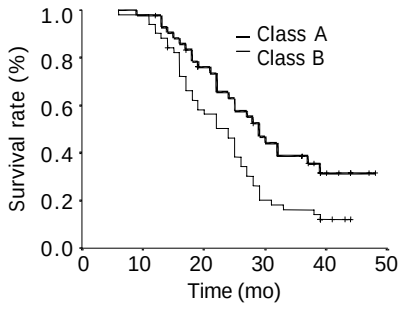


**Figure 3** Univariate analysis of Child-Pugh classification on survival of patients treated with 3D-CRT combined with TACE.

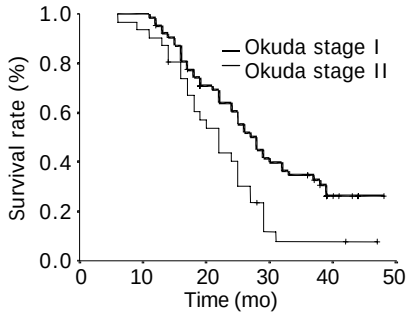


**Figure 4** Univariate analysis of Okuda stage on survival of patients treated with 3D-CRT combined with TACE.

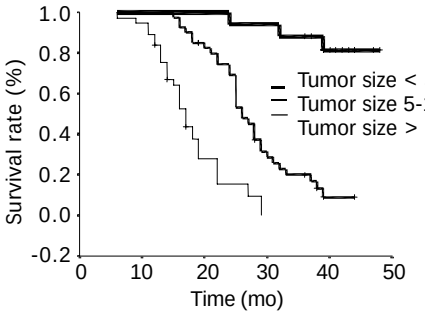


**Figure 5** Univariate analysis of tumor size on survival of patients treated with 3D-CRT combined with TACE.

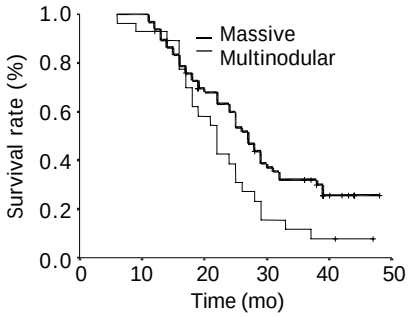


**Figure 6** Univariate analysis of tumor type on survival of patients treated with 3D-CRT combined with TACE.

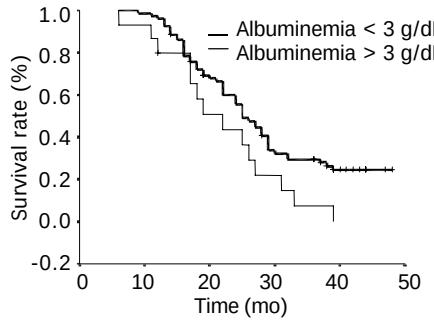


**Figure 7** Univariate analysis of albuminemia on survival of patients treated with 3D-CRT combined with TACE.

### *Toxicity*

In terms of acute toxicity, alterations in the liver function test (23 patients, 24.5%) and fever (51 patients, 54.3%) were frequently found in patients during the early time after TACE. These effects were transient and most patients recovered within 1-2 wk. Hematologic toxicity involved thrombocytopenia (platelets <50 000/mm$^3$) in 13 patients and leucopenia (white blood cells <2 000/mm$^3$) in 2 patients. Subacute and chronic toxicity involved radiation-induced liver disease (RILD) in 12 patients, 4 of whom died from RILD, and gastroduodenal ulcer in 5 patients. The patients other than the 4 victims of RILD were treated conservatively, and no death was found related to the treatment.

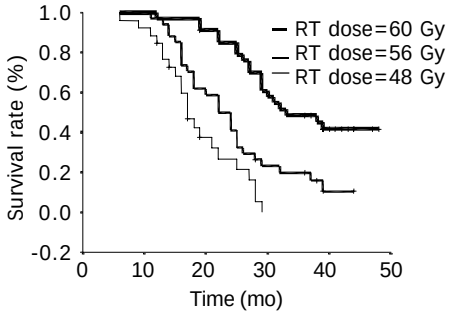


**Figure 8** Univariate analysis of radiation dose on survival of patients treated with 3D-CRT combined with TACE.

## DISCUSSION

Although TACE has been proved to be effective in treating HCC, and selectively and repeatedly used for patients with unresectable HCC, no survival benefits have been observed in at least two randomized trials of TACE[22,23]. TACE is not a curative method and its limitation has also been well documented. After TACE, the tumor cells remain viable, especially in and around the capsule, and tumors may recur by the blood supply from the collateral circulation or portal vein or recanalization of the originally embolized artery[6,24,25]. In advanced HCC, it is almost impossible to achieve a measurable response with TACE[26].

Local radiotherapy can be an effective adjunct to the palliative treatment of HCC, even with portal vein thrombosis. Some studies of local radiotherapy, however, as either combination therapy with TACE or salvage therapy following TACE, have not shown a survival benefit, despite tumor response[4-17]. This is because HCC eradication requires at least 50 Gy of radiation[1,2]; but 33 Gy is a sufficient dose to induce RILD for whole liver radiation. With three-dimensional radiation planning, conformal radiotherapy can minimize scattering, limit unnecessary exposure of normal tissue, and deliver higher doses (40-80 Gy) to tumors[27,28]. Therefore, it can be a feasible approach in the treatment of HCC with high dosage of radiation.

3D-CRT has been reported by a few studies to be effective in treatment of primary and metastatic tumors of lung and liver[20, 21], but it has not been common to be applied in the treatment of HCC. The common therapy for HCC has been reported to be a daily dose of 1.8-2 Gy, 5 fractions per week, to a total delivered dose of 40-60 Gy[4-17]. In our study of the 94 cases of HCC, we suggested that employing 3D-CRT could be beneficially combined with TACE with the following considerations.

Firstly, the combination of 3D-CRT with TACE may remedy the limitation of each alone and has synergistic effects. Secondly, tumor shrinkage after TACE allows the use of smaller irradiation fields, which permits higher tumor doses and improves the normal liver tolerance. Thirdly, combination therapy may also serve the purpose of eliminating residual cancer cells after TACE. Furthermore, the anticancer drug retained in the tumor may have a documented radiosensitizing effect[6,29]. The anticancer drug, when mixed with lipiodol, has been reported to maintain relatively high concentrations in tumors as long as 27 d and decrease to a trace level after 47 d[30]. Guo *et al.*[6] reported that TACE followed by irradiation was a promising approach for large HCC and confirmed that TACE combined with radiotherapy was more effective than TACE alone.

In discussing the factors affecting the prognosis, we are not going to deal with the commonly known ones, which were also found in our study, such as age, Child-Pugh classification for cirrhosis of liver, Okuda stage, tumor size and type, and albuminemia. Only radiation dose is to be discussed, for it is of clinical importance in exploration of a better therapeutic method for HCC. In our present study, the radiation dose started at 60 Gy in 8 fractions within 17 d in cases with <25% of the nontumorous liver receiving >50% of the isocenter dose, at 56 Gy in 9 fractions within 19 d in cases with 25-50%, and 48 Gy in12 fractions within 26 d in cases with 50-75%. Using the linear quadratic model, the biologic effective dose (BED) was here defined to be nd $(1+d/\alpha/\beta)$ in Gray, where n is the fractionation number, d is the daily dose, and $\alpha/\beta$ ratio is assumed to be 10 for tumors. The BEDs for our 3 different dose groups were equivalent respectively to those for 87.5 Gy total doses, 72 Gy total dose and 56 Gy total dose with a daily fraction of 2 Gy. The univariate and multivariate analyses of prognosis factors for overall survival showed that radiation dose is closely related to the prognosis. It seems clear that our virtually high total doses resulted in better response rates and overall survival. In our study, the radiologically documented response rates were 90.5%, our survival rates at 1-, 2-, and 3-year were 93.6%, 53.8%, and 25.9%, respectively, and our median survival rate was 25.0 mo. Besides better survival effect, hypofractionated 3D-CRT offers advantages of a shorter treatment course than a conventional radiotherapy and higher acceptance on the part of the patients without increasing side effects.

It has been reported that if less than 25% of the normal liver is treated with radiotherapy, then there may be no upper limit on dose associated with RILD; if 33%, 67%, or the whole liver is under uniform irradiation of 90 Gy, 47 Gy, or 31 Gy, respectively, there may be 5% risk of RILD associated with the treatment[31]. In analyzing the correlation of RILD with patient-related and treatment-related dose-volume factors, Cheng *et al*.[15] showed that after 3D-CRT, 12 of 68 patients developed RILD which was not found to be associated with their tumor volume. Compared with the documented ones, our results showed no increased toxicity in spite of the increased dose per fraction. This indicated that 3D-CRT played a critical role in the treatment of HCC.

In conclusion, 3D-CRT hypofractionated combined with TACE is a very safe and effective treatment method in higher tumor control and similar normal-tissue toxicity to conventional radiotherapy for HCC.

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• LIVER CANCER •

# Transfection of IL-2 and/or IL-12 genes into spleen in treatment of rat liver cancer

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## Abstract

**AIM:** To test the efficacy of gene therapy in rat liver tumor.

**METHODS:** A retroviral vector GCIL12EIL2PN encoding human IL-2 (hIL-2) and mouse IL-12 (mIL-12) fused gene and its packaging cell were constructed. The packaging cell lines contained of IL-2 and/or IL-12 genes were injected intrasplenically to transfect splenocyte at different time. The therapeutic effect, immune function and toxic effect were evaluated.

**RESULTS:** The average survival times of the 4 groups using IL genes at days 1, 3, 5 and 7 after tumor implantation were 53.3±3.7, 49.3±4.2, 31.0±2.1 and 24.3±1.4 d respectively in IL-2/IL-12 fused gene group, 25.0±2.5, 23.5±2.0, 18.3±2.4 and 12.0±1.8 d respectively in IL-2 gene treatment group, and 39.0±4.8, 32.0±3.9, 23.0±2.5 and 19.4±2.1 d respectively in IL-12 gene treatment group ($P$<0.01, $n$=10). In the IL-12/IL-2 fused gene treatment group, 30% of rats treated at days 1 and 3 survived more than 60 d and serum mIL-12 and hIL-2 levels were still high at day 3 after treatment. Compared with IL alone, NK cell activity was strongly stimulated by IL-2/IL-12 gene. Microscopy showed that livers were infiltrated by a number of lymphocytes.

**CONCLUSION:** IL-2 and/or IL-12 genes injected directly into spleen increase serum IL-2 and IL-12 levels and enhance the NK cell activity, which may inhibit the liver tumor growth. The therapy of fused gene IL-2/IL-12 is of low toxicity and relatively high NK cell activity. Our data suggest that IL-2/IL-12 fused gene may be a safe and efficient gene therapy for liver tumor. The gene therapy should be administrated as early as possible.

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## INTRODUCTION

Gene therapy to liver cancer is limited by both number and duration of died cancer cells being treated[1,2]. Interleukin 2 (IL-2) and interleukin 12 (IL-12) were secreted mainly by mononuclear cell and B cell[3], which play a prominent role in immune response to tumor. These cytokines are stimulated by antigens, for instance virus, bacteria and tumor cells. Others have shown that IL-2 and IL-12 were inhibited by colon cancer[4] and both can up-regulated the T-cell and NK cell to kill tumor cell after administration of exogenous ILs.

The conditions under which the gene therapies of IL-2 and IL-12 are cytotoxic to liver tumor in an animal model have not been clear yet. Understanding the role and the mechanism of IL-2 and IL-12 in the induction of anti-tumor cytotoxic factors is relevant to both the long-term expression of IL and the safety of gene drug in liver tumor gene therapy.

IL-2, a glycoprotein consisted of 133 amino acids, through promotion of growth and proliferation of T cell, can induce either LAK cell or NK cell. IL-2 also induces immunocyte to produce interferon (INF) and tumor necrosis factor (TNF). Immunoreaction mediated by IL-2 increases therapeutic efficacy of colon cancer[4,5]. Secretion of IL-2 and reaction to IL-2 are decreased in tumor patients by as yet unknown reasons. Previous report has demonstrated that metastases are related to the IL-2 level, and the tumor immune mediated by IL is decreased in favor of carcinogenesis, growth, diffusion and metastasis of tumor[6-10].

IL-12 has p35 subunit and p40 subunit localized respectively on chromosome 3 and chromosome 5. The single subunit has no biological activity, and *p35* gene and *p40* gene are expressed simultaneously to have activity. IL-12 activates NK cell and LAK cell, promotes generation and differentiation of T cell and induces NK cell to express a small amount of TNF-β.

Primary liver cancer is common malignant tumors in China. The surgical treatment of the cancer is a main choice, but it is not ideal therapy because of high recurrence. Gene therapy is the new way for some diseases such as diabetes and tumors. Our previous work was to treat liver cancer with IL-12 gene alone, by which tumor growth was inhibited compared with control. Now we constructed the fused gene of IL-2/IL-12. However, little is known on liver cancer treatment with combination of IL-2/IL-12 gene therapy.

We hypothesized that the immunologic regulation to liver cancer following fused gene therapy of IL-2/IL-12 would be dependent upon the production and duration of the cytokines. The toxic effect of IL-2 and IL-12 would be dependent upon approach of drug delivery. In this study, using the model of implanted hepatoma of rats, we have shown that the liver tumors are reduced after IL-2 and/or IL-12 gene are injected into spleen and the cytokines are transferred into liver and blood circulation by porta vein.

## MATERIALS AND METHODS

### *Construction of retrovirus vector of packaging mIL-12 gene and hIL-2 gene*

Aprotinin, leupeptin, pepstatin, fetal calf serum and protein standard mixture were from Sigma. Plasmid pGCp35IRESp40 containing both p35 subunit and p40 subunit of mIL-12 gene was a gift from Professor Xin-Huan Liu. Plasmid pLIL2SN

containing hIL-2 gene was constructed by our laboratory. Cellular strains of hepatoma CBRH3 were provided kindly by Professor Hong Xie (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). Monotropic packaging cell PE501, amphophilic packaging cell PA317 and cell NIH3T3 were provided by our laboratory. All other chemicals were of analytical grade and obtained from Merck or Sigma.

To amplify full length *p35* and *p40* genomes, PCR fragment was generated from downstream primer of mIL-12 p35 subunit and upstream primer of mIL-12 p40 subunit that templated with pGCp35IRESp-40SN. The products derived from PCR were harvested after 1% agarose gel electrophoresis. According to pLIL2PN templation, hIL-2 genome was then amplified and harvested. Fragments of mIL-12 p35 and p40 derived from PCR were templated to amplify with downstream primer of p35 and upstream primer of p40 by PCR, which contain linker sequence. The mIL-12 fused gene was harvested by electrophoresis[11,12]. The mIL-12 and hIL-2 gene fragments were connected respectively with pGEM™ -Teasy vector (Promega, USA) by T4 ligase (Boehringer Mannheim, Germany). The vector was transfected into *E.coli* TG1 competence cell and the positive clone was screened by PCR. Plasmid DNA was extracted from positive clone after partial exonuclease III digestion of PCR product, resulting in constructing GCILEXPN polyclonal sites after endonulease (*Not* I and *Sal* I ) (Promega, USA) that containing GCIL12EXPN of mIL-12 gene, GCXEIL2PN of hIL-2 gene and GCIL12EIL2PN of mIL-12 and hIL-2 fused gene.

### *Retrovirus packaging, identification, titer determination and expression*

Reverse transcript virus was transfected into PE501 cell by electroporation. The clones were screened by G418 after 48 h. PA317 cell was infected by filtered supernatant 10-14 d late. G418 was screen after 3 d. Viral supernatants were harvested after amplifying 6 monclone from every groups for 2-3 wk. Stock cells were frozen at -80 °C.

Recombined reverse transcript viral vector was identified by RT-PCR. Titers of viral supernatants were determined with NIH3T3 cell. The packaging cells with highest viral titer that contain GCIL12EXPN, GCXEIL2PN and GCIL12EIL2PN were named as PA317-GCIL12EXPN, PA317-GCXEIL12PN and PA317-GCIL12EIL2PN, respectively. Protein expression of mIL-12 and hIL-2 was determined by ELISA. ELISA kits (human IL-2 DuoSet and mouse IL-2 DuoSet) were from R&D Systems.

### *Implanted liver cancer in rats*

Male Wister rats (200-250 g bm) were obtained from Animal Center of Chinese Academy of Sciences. Animals were maintained on a standard diet. Hepatoma CBRH3 cells were injected into abdominal cavity of rat. Rats were sacrificed and tumors were removed from abdominal cavity 7-9 d late. Tumors were cut into pieces of 0.05-0.75 cm and then were implanted into rat liver for one or more locations respectively. The tumors were grown up to 0.6-1 cm in diameter after 7-10 d of implantation.

### *Experimental procedure*

Rats were anaesthetized with diethylether. The effects of IL-2 and/or IL-12 on liver cancer were studied in a total of 75 animals for 5 groups that contained 15 rats in every group: 1. Physiological saline control: 0.8 mL 9 g/L NaCl was injected into spleen following implanted liver cancer at day 1; 2. Blank vector control: $1\times10^7$ packaging cells of PA317-GCXEPN was injected into spleen of rat; 3. mIL-12 gene group: $1\times10^7$ packaging cell PA317-GCIL12EXPN was injected into rat spleen after implanted rat liver cancer on days 1, 3, 5 and 7 thereafter; 4. hIL-12 gene group: $1\times10^7$ packing cell PA317-GCxeILPN was injected into rat spleen after implanted rat liver cancer on days 1, 3, 5 and 7 respectively; 5. hIL-2 and mIL-12 fused gene group: $1\times10^7$ packing cell PA317-GCIL12EIL2PN was injected into rat spleen after implanted rat liver cancer on days 1, 3, 5 and 7 thereafter. In addition, when rat survived over 2 mo, rats were named long term survivors and the liver cancer tissues were implanted into liver once more in order to observe cancer growth.

### *CT imaging and pathology*

CT imaging was observed before and after treatment. The survival time and drug toxicity were observed. To observe the tumor cell and lymphocyte infiltration, the pathologic examination was performed following 5 and 7 d of treatment. The serum IL-2 and IL-12, according to the protocol of R&D Systems, were measured on d 1 before treatment and on d 3,7, 30 and 60 thereafter.

### *Analysis of NK cytotoxic activity*

NK target cell YAC-1 was obtained from the American Type Culture Collection (Bethesda, MD). The cytotoxicity of spleen NK cell was analyzed as follows[13]. A single-cell suspension of spleen cells was centrifuged at 400 r/min for 30 min. The lymphocyte layers were harvested. For the preparation of targeted cells, YAC-1 was labeled with [$^{51}$Cr]$Na_2CrO_4$ and mixed with various numbers of spleen cell in a total volume of 200 μL of DMEM. The experimental radioactivity released (ER) in 100 μL samples of cell-free supernatants was determined. The amounts of radioactivity released in wells containing YAC-1 cells alone with and without 0.01% Triton X-100 were designated the total release (TR) and the spontaneous release (SR), respectively. The percentage of specific $^{51}$Cr release was calculated by[(ER-SR)/(TR-SR)]×100.

### *Statistical analysis*

All results were expressed as the mean±SD of at least 10 individual measurements. A one-way analysis of variance (ANOVA) was first carried out to test for any differences in mean values between experimental and respective control group. If differences were established, the values were compared by two-tailed unpaired *t* test. The values were considered to significant difference if $P< 0.05$.

## RESULTS

### *Identification of packing cell strain*

The total RNA of packaging cell strains (PA317-GCIL12EXPN, PA317-GCXEIL2PN and PA317-GCIL12EIL2PN) was extracted. The RNA was amplified by RT-PCR and the product was harvested by electrophoresis. The sequence showed that PA317-GCXEIL2PN and PA317-GCIL12EIL2PN packaging cell strains contained the mIL-12 sequence, and PA317-GCXEIL2PN and PA317-GCIL12EIL2PN packaging cell strains contained the hIL-2 sequence. The target genes were inserted into viral genomes and the packaging cell strains were transfected by IL gene.

### *Determination of titer of virus and protein expression*

The highest virus titer of packaging cell was $2\times10^6$ CFU/mL of PA317-GCIL12EXPN, $2.4\times10^6$CFU/mL of PA317-GCXEIL2PN and $1.4\times10^6$CFU/mL of PA317-GCIL12EIL2PN respectively. Protein expression was measured by ELISA on these 3 packaging cell strains: 1. mIL-12 fused protein: PA317-GCIL12EXPN expressed mIL-12 fused protein 150 ng/$10^6$/48 h and PA317-GCIL12EIL2PN expressed mIL-2 fused protein 45.8 ng/$10^6$/48 h; 2. hIL-2 protein: PA317-GCEXIL2PN expressed hIL-2 protein 7.5 ng/$10^6$/48 h and PA317-GCIL12EIL2PN expressed hIL-2 protein 6.7 ng/$10^6$/48 h.

**Table 1** Average survival time (day) of rat ($n$=0, mean±SD)

| Group | Injection on d 1 | Injection on d 3 | Injection on d 5 | Injection on d 7 |
|---|---|---|---|---|
| Physiologic saline control | 10.7±1.5 | - | - | - |
| Blank vector control | 11.4±1.3 | - | - | |
| IL-2 gene therapy | 25.0±2.5[b] | 23.5±2.0[b] | 18.3±2.4[b] | 12.0±1.8 |
| IL-12 gene therapy | 39.0±4.8[b] | 32.0±3.9[b] | 23.0±2.5[b] | 19.4±2.1[a] |
| IL-12-IL-2 fused gene therapy | 53.3±3.7[c] | 49.3±4.2[c] | 31.0±2.1[c] | 24.3±1.4[c] |

[a]$P$<0.05 and [b]$P$<0.01 *vs* physiologic saline control and blank vector control; [c]$P$<0.05 *vs* IL-2 group or IL-12 group.

### *Average survival time by IL gene therapy*

Table 1 shows the average survival time of rat. All rats were died within 15 d after implanting liver cancer in both physiologic saline control and blank vector control. Compared with control, the survival time of rat was prolonged significantly by treatment with IL-2 or IL-12 ($P$<0.01). The rat survival time of treatment with fused gene of IL-2/IL-12 was lengthened markedly compared with IL treatment alone ($P$<0.05). Moreover, the survival time of early treatment was much longer than that of later treatment with IL ($P$<0.01). In addition, 6 rats treated early with fused gene of IL-2/IL-12 were lived more than 60 d, but there were 3 rats treated with IL-12 that lived more than 60 d.

When the tumor was undetectable by abdominal pathologic biopsy in the rats that lived more than 60 d, the 4 rats were implanted tumor piece again. It was interested that there were no tumor growths in abdominal cavities 7 d late.

### *Imaging features*

The liver tumor was detected by CT on day 7 after implantion in control (Figure 1). The 6 of 10 rats administrated early with IL-2/IL-12 fused gene showed that the liver tumors were reduced after 2 mo (Figure 2).

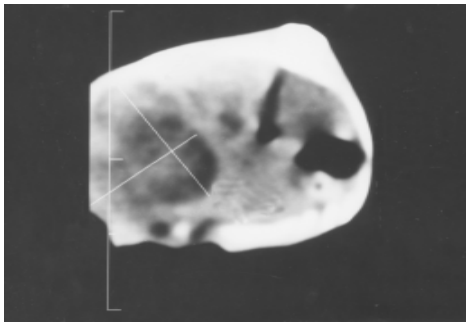

**Figure 1** CT scan of the liver cancer 7 d after implantation.

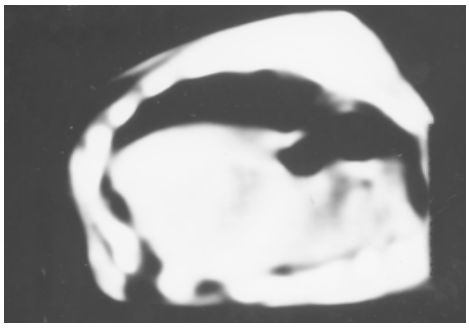

**Figure 2** CT scan of the tumor treated with IL-2/IL-12 fused gene after 2 mo.

### *Pathologic features*

A number of lymphocyte infiltrations in liver tumor were observed significantly after 5 d of treatment with IL-2/IL-12 fused gene and 7 d of treatment with IL-2 gene or IL-12 gene (Figure 3).

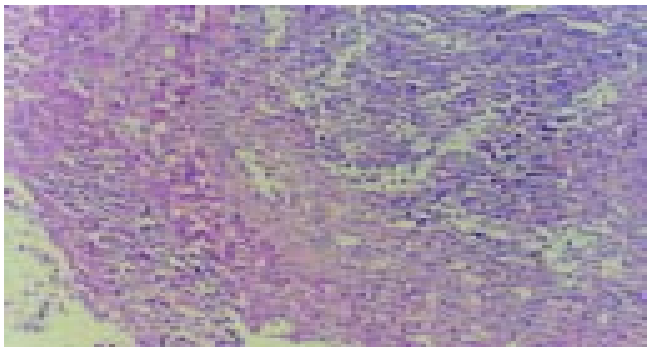

**Figure 3** Pathological changes of implanted liver cancer (HE staining, original magnification: ×200). There are numerous lymphocytes in tumor tissues.

### *Serum mIL-12 and hIL-2 levels*

Compared with control, both IL-2 and IL-12 in serum were increased significantly in rats treated with IL gene on day 1 (Table 2). After the injection of IL-2/IL-12 fused gene, both IL-2 and IL-12 in serum reached the highest level on day 3, then decreased stepwise and maintained at a lower level for 2 mo (Table 3).

**Table 2** Determination of serum hIL-2 or mIL-12 3 d after administration of IL gene **(**ng/mL)

| Group | HIL-2 | MIL-12 |
|---|---|---|
| Physiologic saline control | <0.8 | <0.8 |
| Blank vector control | <0.8 | <0.8 |
| IL-2 gene therapy | 19.4±1.8 | <0.8 |
| IL-12 gene therapy | <0.8 | 22±2.5 |
| IL-12-IL-2fused gene therapy | 18.5±2.4 | 20.5±2.5 |

**Table 3** Determination of serum hIL-2 and mIL-12 on fused gene group (ng/mL)

| Group | HIL-2 | MIL-12 |
|---|---|---|
| Control | <0.8 | <0.8 |
| On d 3 after therapy | 18.5±2.4 | 20.5±2.5 |
| On d 7 after therapy | 10.2±2.5 | 11.5±2.5 |
| One mo after therapy | 5.3±1.2 | 6.2±1.4 |
| Two mo after therapy | <0.8 | <0.8 |

### *IL increases NK cell activity*

IL-2 and IL-12 have been reported to increase NK cell activity. To determine if the IL-2/IL-12 fused gene could induce NK cell activation, rats were treated as described above with IL-2 and/or IL-12. The rats were sacrificed on day 7, and the spleen lymphocytes were assayed for the ability to kill $^{51}$Cr-labeled

YAC-1 target cells. There was a significant increase in NK activity after rats were injected with IL-2 or IL-12 alone ($P<0.01$). Compared with IL alone, treatment with IL-2/IL-12 fused gene markedly enhanced NK cell activity ($P<0.05$).

### *Toxicity of IL*

Three rats (30%) in IL-2 group and 1 (10%) in IL-2/IL-12 group showed anorexia after administration of IL. The symptom was recovered 3-5 d late (Figure 4).

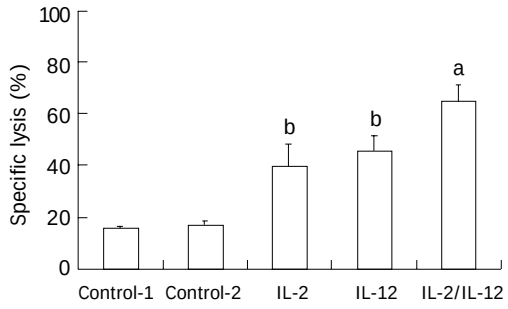


**Figure 4** Activation of NK cell activity after administration of IL-2 and / or IL-12 ($n$=5). $^{b}P<0.01$ *vs* control; $^{a}P<0.05$ *vs* IL-2 group or IL-12 group.

## DISCUSSION

The establishment of DNA recombination and transfection allowed us to utilize gene therapy for killing or inhibiting the tumor cells[14]. The utility of recombinant adenoviral vectors for gene therapy is limited by the low transduction efficiency and lack of specificity for target cells[15,16]. Spread of the virus throughout the cells maximizes the percent of cells within a cell expressing the gene of interest, and should improve the antitumor response. This may be significant for gene delivery expressing cytokines, tumor antigens or enzymes. One limitation of the transduction system is the inefficiency of the combined cytokines expression system. The initial work in this study was to construct the IL-2/IL-12 fused gene vector that was then injected into spleen, in which splenic cells, probably including liver cells, were transducted to express IL-2 and IL-12 simultaneously.

The active IL-12 is a heterogeneous dimmer, which contains p35 subunit and p40 subunit, so that both subunits express simultaneously in cells if used to treat disease. Using PCR technique based on the subunit characterization of different gene sequences, we constructed the active IL-2/IL-12 fused gene and a linker sequence was incorporated into subunits of IL-12, so that the gene could correctly express protein. In present study, the IL-2 and IL-12 levels were not significantly different in serum.

The single gene therapy for the liver cancer is not ideal because it is resulted from multi-factors[17]. Chen *et al.* suggested recently that TRAIL and chemotherapeutic agents or anticancer cytokines combination might be a novel strategy for the treatment of liver cancer. Combination of IL-2/IL-12 results in stable antitumor effect, which induces the cytotoxic T lymphocyte and NK cell[18]. IL-2 and IL-12 have synergetic effect such as immunoregulatory[19,20]. The antitumor immunologic effect from IL-2 and IL-12 depends on available concentration of IL-2 and IL-12. IL-2 and IL-12 also cause a long term antitumor immunologic memory[21]. In present study, retrovirus vector containing IL-2/IL-12 genes was constructed, in which gene products of IL-2 and IL-12 were expressed simultaneously in the liver. We treated rat liver cancer with IL-2 and/or IL-12 gene. The liver cancer rats treated with IL-2 or IL-12 survived longer than those in control ($P<0.01$). Compared with IL alone, combination of IL-2/IL-12 gene showed a longer survival of 14-28 d in early treatment group and 5-12 d in late treatment group.

Spleen is the biggest immune organ that has a lot of immunocytes and produces antibodies and cytokines. Spleen is also the main organ of inducing immunoresponsiveness to heterologous antigen. The retrovirus packaging cell strain was injected into spleen, which expresses the high level of cytokine in order to activate immunocytes. In other hand, the retrovirus packaging cells would be transferred from spleen to liver by splenic veins and the liver cells would be infected by packaging cells resulting in enhanced anti-tumor immunity. In present study, the IL gene was injected into spleen and the blood concentration of IL was the highest on day 3. The IL concentration maintained at a level for 2 mo. Authors from Spain reported that gutless adenoviral vector encoding hIL-2 and mIL-12 was injected into animals and IL was expressed by hepatocytes. The peak concentration of IL-12 was at 10 h and it completely disappeared by 72 h. If the vectors were administrated continually, the serum IL-12 would maintain at least for 48 wk[22,23]. The rats received the splenic gene therapy survived longer than those in control. When combination of IL-2/IL-12 gene was injected into spleen, the high concentration of IL was determined from blood until day 3 and the rats survived a longer time compared with IL therapy alone.

Moreover, early gene treatment is better than late therapy. In this study, 6 rats (30%) with combined gene therapy on early stage survived a long term and the tumor nodes in liver was not detected by CT imaging and pathologic observation, in which the IL-2 and IL-12 kept a high concentration at least for 2 mo. As same as other therapy for cancer, the IL gene therapy should administrate as early as possible. In present study, the liver cancer rats treated with IL-2 or IL-12 on day 1 survived more than 8 d or 13 d respectively compared with that treated on d 7 (Table 1). The combined IL gene therapy has the similar result.

IL-2 or IL-12 produced significant toxic reaction if they were administrated enough dosage to maintain a high blood concentration. When IL-2 or IL-12 was injected into rat liver, some rats showed acute toxic reaction such as anorexia, convulsion and bleeding shock (unpublished data). In present study, when IL genes were injected into spleen, the severe acute toxic reaction was not observed in all groups.

A mechanism of IL gene treatment may be due to enhancement of NK cell activation and production of cytokines including IL[24]. Several investigators have shown that NK cells are a relative smaller cell population in peripheral lymphoid organs but are abundant in the liver. An initial response to tumor cell may involve the innate arm of the immune response resulting in killing of mutant cell strain by NK cell[25,26]. The findings in this study are novel since IL-2/IL-12 fused gene expresses IL-2 and IL-12 simultaneously that result in further stimulation of NK cells. We propose that liver tumor is inhibited because of IL production, such as IL-2 and IL-12, which is stimulated by IL gene therapy.

It is interesting that serum IL-2 and IL-12 levels do not change in IL-2/IL-12 gene therapy compared with IL alone, but NK cell activity is enhanced significantly compared with IL alone. We propose that the NK cell activity is strongly stimulated by both IL-2 and IL-12 at the same time, which was caused by IL-2/IL-12 fused gene expression[27-30].

In summary, treatment with IL-2 and/or IL-12 gene increases serum IL-2 and IL-12 levels and enhances the NK cell activity, which may inhibit the liver tumor growth. The fused gene therapy of IL-2/IL-12 is of low toxicity and relatively high NK cell activity. We suggest that IL-2/IL-12 fused gene therapy may be a safe and efficient method for the treatment of liver cancer. For IL gene therapy, early intervention is better than late one.

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• LIVER CANCER •

# Combined gene therapy of endostatin and interleukin 12 with polyvinylpyrrolidone induces a potent antitumor effect on hepatoma

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## Abstract

**AIM:** To study the antitumor effect of combined gene therapy of endostatin and interleukin 12 (IL-12) with polyvinylpyrrolidone (PVP) on mouse transplanted hepatoma.

**METHODS:** Mouse endostatin eukaryotic plasmid (pSecES) with a mouse Igκ signal sequence inside and mouse IL-12 eukaryotic plasmid (pmIL-12) were transfected into BHK-21 cells respectively. Endostatin and IL-12 were assayed by ELISA from the supernant and used to culture endothelial cells and spleen lymphocytes individually. Proliferation of the latter was evaluated by MTT. H22 cells were inoculated into the leg muscle of mouse, which was injected intratumorally with pSecES/PVP, pmIL-12/PVP or pSecES+pmIL-12/PVP repeatedly. Tumor weight, serum endostatin and serum IL-12 were assayed. Tumor infiltrating lymphocytes, tumor microvessel density and apoptosis of tumor cells were also displayed by HE staining, CD31 staining and TUNEL.

**RESULTS:** Endostatin and IL-12 were secreted after transfection, which could inhibit the proliferation of endothelial cells or promote the proliferation of spleen lymphocytes. Tumor growth was highly inhibited by 91.8% after injection of pSecES+pmIL-12/PVP accompanied by higher serum endostatin and IL-12, more infiltrating lymphocytes, fewer tumor vessels and more apoptosis cells compared with injection of pSecES/PVP, pmIL-12/PVP or vector/PVP.

**CONCLUSION:** Mouse endostatin gene and IL-12 gene can be expressed after intratumoral injection with PVP. Angiogenesis of hepatoma can be inhibited synergisticly, lymphocytes can be activated to infiltrate, and tumor cells are induced to apoptosis. Hepatoma can be highly inhibited or eradiated.

Li PY, Lin JS, Feng ZH, He YF, Zhou HJ, Ma X, Cai XK, Tian DA. Combined gene therapy of endostatin and interleukin 12 with polyvinylpyrrolidone induces a potent antitumor effect on hepatoma. *World J Gastroenterol* 2004; 10(15): 2195-2200
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## INTRODUCTION

Liver cancer is one of the most common neoplasms worldwide. In some parts of Asia and Africa the prevalence is more than 100/100 000 population[1]. Treatments often come to failure because of its high resistance to chemotherapy or radiotherapy and the severe side-effects induced[1]. At present the only curative options are partial hepaectomy or total hepaectomy with liver transplantation. But the chance for surgery is very limited. The effects of some other treatments such as intratumoral injection of ethanol or acetic acid, transarterial catheter embolization, and thermal destruction or microwave coagulation were also very poor due to the high incidence of tumor recurrence and hepatic failure[1-3]. Now combined treatments including biotherapy have been regarded as the most promising methods to cure liver cancer[4,5].

Among the biotherapies, antiangiogenic therapies have recently attracted an intense interest for their broad-spectrum action, low toxicity, and in direct endothelial targeting, the absence of drug resistance[6]. Antiangiogenic therapy induced by endostatin could specifically inhibit endothelial proliferation and potently inhibit angiogenesis and tumor growth accompanied by apoptosis in tumor cells[7-9]. Repeated cycles of antiangiogenic therapy were even followed by prolonged tumor dormancy without further therapy[10].

More and more learners have begun to realize that the occurrence of tumors is a complex course induced by multiple genes and multiple steps. Interference of single factor on tumor often can not get satisfactory results. Although primary tumors were regressed to dormant microscopic lesions after endostatin therapy, tumor cells were not eradicated at all[7,10]. Conventional chemotherapy or radiotherapy can do little on residual tumor cells, accurate recognition and clearance of which depends on the immune system of host[11]. IL-12 is among the most potent cytokines in stimulating antitumor immunity[12-14], which also showed significant inhibitory activity on angiogenesis[15].

So antiangiogenesis therapy by endosatin in combination with antitumor immunotherapy by IL-12 has become an attractive approach. On one hand synergistically antiangiogenic effect may be achieved to induce tumor dormancy. On the other hand, residual tumor cells may be also eradiated by antitumor immunotherapy induced by IL-12. Due to the problems such as bioactive protein production in large quantities, high costs and daily administration of endostatin[10,16], and the severe side-effects of system administration of IL-12[17,18], local gene therapy has proved to be very effective and nontoxic at the same time[19-24]. In this study combined intratumoral gene therapy of endostatin and IL-12 was observed in treatment of mouse hepatoma.

## MATERIALS AND METHODS

### *Plasmid and host*

Mouse endostatin eukaryote expression plasmid pSecES was constructed in our laboratory (data not shown). The expression of endostatin was under the control of *cytomegalovirus* (CMV) promoter. A mouse Igκ signal peptide sequence was located upstream of endostatin sequence to lead endostatin to secrete out. Mouse IL-12 eukaryote expression plasmid (pmIL-12) was

a gift from Professor David M. Mahvi in Wisconsin University, USA. Both $M_r$ 35 000 light chain gene and $M_r$ 40 000 heavy chain gene linked by an internal ribsome entrance site were put downstream of CMV promoter[25]. Empty plasmid pcDNA3.1, pcDNA3.1-lacZ and *E.coli* DH5α were from the collection of our laboratory. DH5α bacteria containing the plasmids were grown in LB medium to mid-log phase. The plasmids were then purified with Qiagen columns (Qiagen). An analytical gel of each plasmid (cut and uncut) was done to ensure that there was no contamination with other nucleic acids. The $A_{260}/A_{280}$ ratios ranged from 1.8 to 2.0. Also each plasmid was confirmed to be free of endotoxin. For gene therapy, concentrated plasmid stock solution was made by lyophilizing and rehydrating with water. Then it was formulated into a solution containing 1 g/L plasmid, 50 g/L polyvinylpyrrolidone (PVP, K30, $M_r$ 40 000, from Sigma), 150 mmol/L NaCl before use.

### Cell culture and animals

BHK-21, human umbilical vein endothelial cell line (HUVEC) ECV304 and mouse hepatoma cell line H22 were purchased from China Center for Type Culture Collection. All the cell lines were maintained in RPMI 1640 medium (GibcoBRL) supplemented with 100 mL/L fetal bovine serum (FBS), $10^5$ U/L penicillin and 100 mg/L streptomycin. Cells were cultured at 37 ℃ with 50 mL/L $CO_2$. Six to 8-wk-old male BALB/c mice of specifical-pathogeny-free (SPF) grade (purchased from Hubei Experimental Animals Center, China) were maintained in a standard SPF animal's room. All animal studies were performed in accordance with acceptable animal use guidelines.

### Secretion and activity of endostatin plasmid and IL-12 plasmid

In 6-well plate, $5\times10^5$ BHK-21 cells per well were transfected with 1 μg pSecES or 1 μg pmIL-12, 3 μL LipoGen (InvivoGen) in accordance with directions of the manufacturer. Forty-eight hours after transfection, 0.1 mL ECV304 cells ($2\times10^7$/L) cultured in RPMI 1640 additionally supplemented with 2 μg/L recombinant human basic fibro growth factor (rhbFGF) (PeproTech, UK) was inoculated to a 96-well plate. 0.1 mL supernant of BHK-21 cells transfected with pSecES was added to ECV304 cells per well. Seventy-two hours later, 20 μL 5 g/L MTT was added per well. After 4 h, the supernant was discarded and 150 μL DMSO was added. Absorbency at 570 nm was assayed. Forty-eight hours after transfection, mouse spleen cells were also produced by routine protocols and cultured in RPMI 1640 medium supplemented with 100 mL/L FBS for 2 h. Then the suspending lymphocytes were adjusted to $1\times10^8$/L and seeded to a 24-well plate (0.5 mL per well). Also 0.5 mL supernant of BHK-21 cells transfected with pmIL-12 was added per well. Forty-eight hours later, the proliferation was evaluated by MTT assay as above. The supernant of BHK-21 transfected with pcDNA3.1-lacZ was used as control. Also transfection rate was evaluated by X-gal staining of BHK-21 transfected with pcDNA3.1-lacZ 48 h later. All the three kinds of supernant was stored at –20 ℃ for further assay.

### Combined gene therapy of endostatin and IL-12 for H22 hepatoma in vivo

In 0.1 mL PBS, $10^5$ H22 cells were inoculated i.m. into the hind limb of BALB/c mice. Then the mice were randomly divided into five groups (6 per group): PVP/NaCl (untreated), pcDNA3.1 (empty vector), pSecES, pmIL-12, pSecES+pmIL-12. Another 6 mice were not inoculated for further assay of basic serum endostatin. From the next day after inoculation, 100 μg pSecES/PVP and/or pmIL-12/PVP, 100 μg pcDNA3.1/PVP or 100 μL PVP/NaCl solution was injected into the inoculation site respectively with a 26 G needle every three days. After inoculation for 21 days, tumors were removed and weighed. Before tumor dissection, mouse blood was collected and stored at 4 ℃ overnight. Then the serum was stored at –20 ℃ for further assay after centrifugation. The data were analyzed statistically by SPSS 10.0 procedure.

### Assay of mouse endostatin and IL-12 (p70) in supernant or serum

The frozen supernant of BHK-21 transfected with pSecES, pmIL-12 or pcDNA3.1-lacZ and the frozen serum were thrawed to room temperature. Then mouse endostatin and IL-12 (p70) were assayed according to the directions of CytElisa mouse endostatin kit (CytImmune, USA) and mouse IL-12 (p70) ELISA kit (Shengzhen Jingmei Biotech, China).

### Detection of MVD, TILs and apoptosis of H22 hepatoma

Paraffin sections (5 μm) of tumor tissues were stained with rat anti-mouse CD31 antibody (eBioscience). Then the primary antibody was detected by biotinylated secondary rabbit anti-rat IgG and SABC immunohistochemistry kit (Wuhan Boster Biotech, China) according to directions of its manufacturer. Paraffin sections were also stained with HE to reveal tumor infiltrating lymphocytes (TILs). Apoptosis of tumors was displayed by TdT-mediated dUTP nick end labeling (TUNEL) staining with an *in situ* cell death detection kit (Boehringer Mannheim, Germany) in accordance with the guardline of its manufacturer.

Tumor microvessel density (MVD) was counted as previously described[26,27]. The CD31 staining sections were scanned in 40× microscope and "hot spots" (area with greatest vascula) were found, from which 3 random fields of 200× microscope were selected. The average vascular number of the three fields was looked as MVD. For HE and TUNEL staining sections, 5 random nonnecrotic tissue fields in 400× microscope was selected. The average number of TILs or apoptotic cells was counted. The data were analyzed statistically by SPSS 10.0 procedure.

## RESULTS

### Secretion and activity of endostatin plasmid and IL-12 plasmid

β-galactosidase expression was assessed by X-gal staining 48 h post-transfection. The tansfection efficiency was about 30%. No endostatin or IL-12 was detected in the supernant of BHK-21 transfected with pcDNA3.1-lacZ. Endostatin secreted by BHK-21 transfected with pSecES was 65.5±10.9 ng/$10^6$ cells. When this supernant was added to ECV304 stimulated by rhbFGF, the proliferation of the latter was inhibited by 29.2% 72 h later (inhibitory efficiency=1-*A* of endostatin group/*A* of control group, $P<0.01$). Also 221.8±44.4 ng/$10^6$ cells IL-12 (p70) was detected in the supernant of BHK-21 transfected with pmIL-12. It could promote mouse spleen lymphocytes to proliferate by 15.2% (proliferation efficiency=*A* of IL-12 group /*A* of control group-1, $P<0.05$). The results are displayed in Figure 1.

### Antitumor effect of combined gene therapy of endostatin and IL-12 for H22 hepatoma in vivo

On the next day after inoculation, the mice received gene therapy. No skin lesions were found in the injection sites. On d 21 after inoculation, tumors were dissected and weighed. Big tumors could be found in all mice of PVP/NS group and pcDNA3.1 group. In pSecES group, all mice formed tumors, some of which were much smaller. Four mice formed tumors in pmIL-12 group and only two mice formed small tumors in pSecES+pmIL-12 group. The average tumor weight of pSecES group was much lower than that of PVP/NS group or pcDNA3.1 group ($P<0.01$) (Figure 2). The inhibitory rate was 56.4% (inhibitory efficiency=1-tumor weight of pSecES group/tumor

weight of pcDNA3.1 group). For pmIL-12 group, the inhibitory rate was 85.7%. When pSecES gene therapy and pmIL-12 gene therapy were used in combination, tumor growth was inhibited by 91.8% ($P$<0.01).

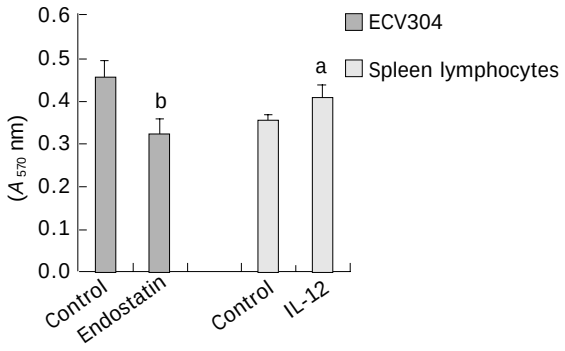


**Figure 1** Proliferation of ECV304 inhibited by the supernant of BHK-21 transfected with pSecES and proliferation of spleen lymphocytes stimulated by the supernant of BHK-21 transfected with pmIL-12. Each bar represents $A$ value of ECV304 or spleen lymphocytes, and mean±SD for 5 wells. [a]$P$<0.05 *vs* control, [b]$P$<0.01 *vs* control.

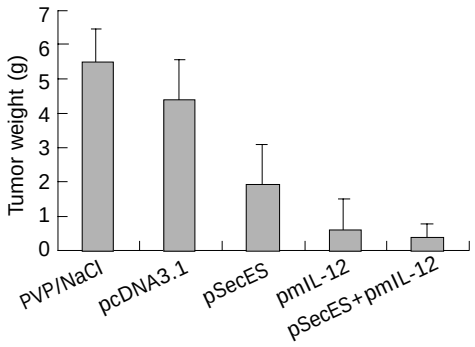


**Figure 2** Hepatoma formation and growth inhibited by gene therapy of endostatin and IL-12. Each bar represents tumor weight of mice treated with PVP/NaCl, pcDNA3.1/PVP, pSecES/PVP, pmIL-12/PVP or pSecES+pmIL-12/PVP, and mean±SD for six mice. $P$<0.01 by ANOVA analysis.

### *Serum endostatin and IL-12 levels after gene therapy*

When gene therapy was finished, the serum endostatin and IL-12 levels were evaluated by ELISA. The serum endostatin level in pSecES group was 117.1±12.8 μg/L, which was much higher than that in pcDNA3.1 group (87.7±6.1 μg/L) or the basic level

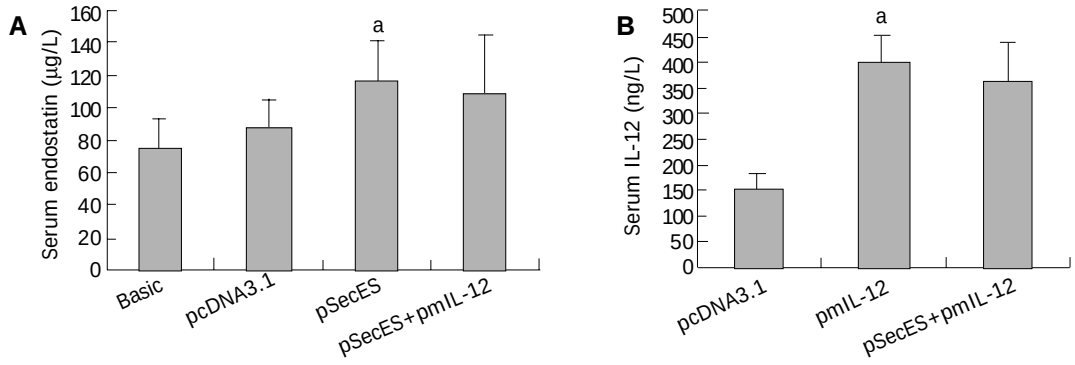


**Figure 3** Increase of mice serum endostatin levels or IL-12 levels after gene therapy. A: Serum endostatin levels of mice treated with pcDNA3.1/PVP, pSecES/PVP, pSecES+pmIL-12/PVP or not inoculated with H22 cells (basic levels). Each bar represents mean ± SD for six mice. [a]$P$<0.05 *vs* pcDNA3.1 group. B: Serum IL-12 levels of mice treated with pcDNA3.1/PVP, pmIL-12/PVP, pSecES+pmIL-12/PVP. Each bar represents mean±SD for six mice. [a]$P$<0.05 *vs* pcDNA3.1 group.

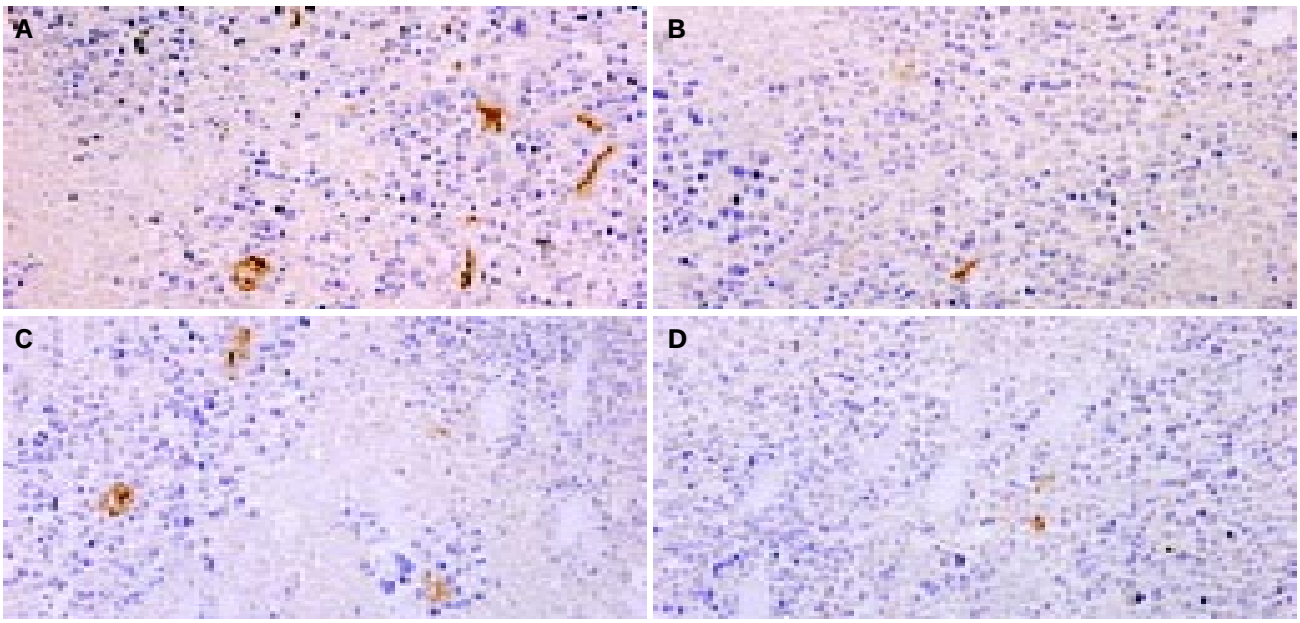


**Figure 4** Hepatoma angiogenesis inhibited by gene therapy of endostatin and IL-12. MVD (CD31staining, 200×) of hepatoma treated with pSecES/PVP (7.3±1.2) (B), pmIL-12/PVP (10.0±1.7) (C) or pSecES+pmIL-12/PVP (3.7±1.2) (D) was less than that treated with pcDNA3.1/PVP (22.7±3.1, $P$<0.05) (A).

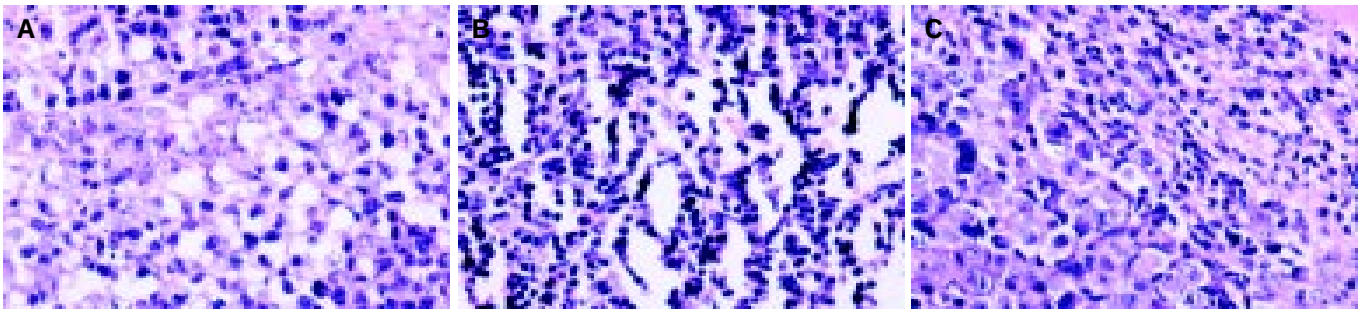


**Figure 5** Lymphocytes infiltration into hepatoma promoted by gene therapy of IL-12. TILs (HE staining, 400×) of hepatoma treated with pmIL-12/PVP (146.2±24.6) (B) and pSecES+pmIL-12/PVP (123.2±21.4) (C) were more than that treated with pcDNA3.1/PVP (45.2±11.8, $P$<0.01) (A).

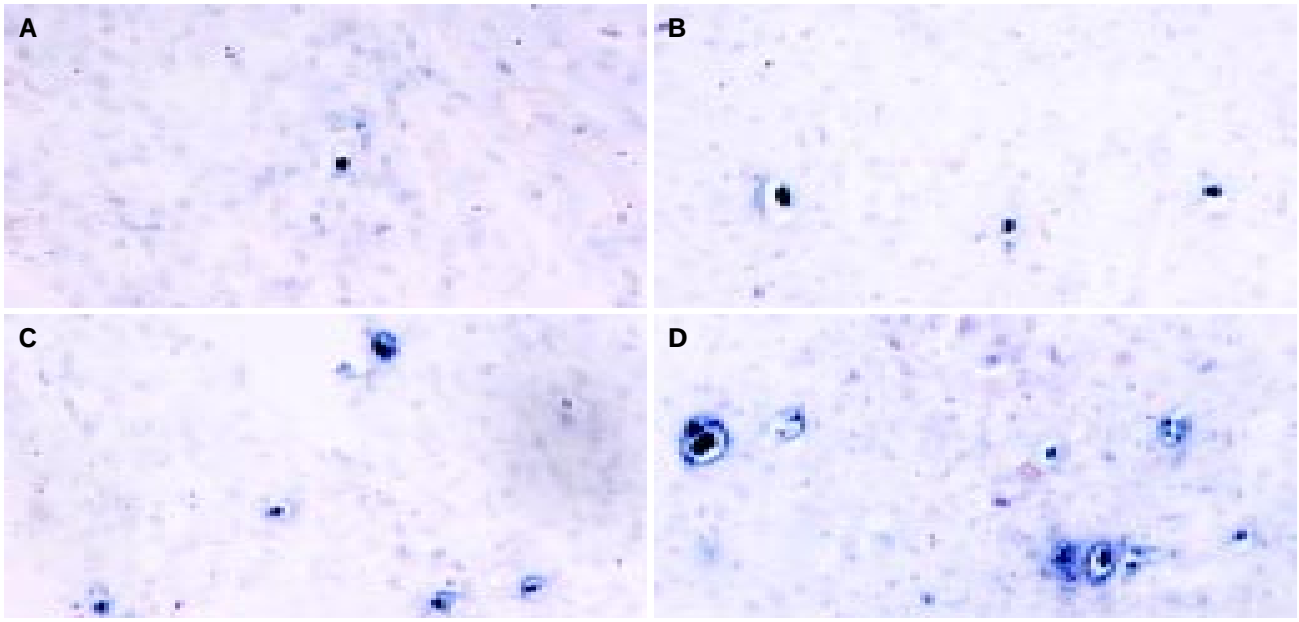


**Figure 6** Apoptosis of hepatoma cells induced by gene therapy of endostatin and IL-12. Apaptotic tumor cells (TUNEL, 400×) treated with pSecES/PVP (11.2±2.3) (B), pmIL-12/PVP (14.4±3.5) (C) or pSecES+pmIL-12/PVP (24.8±4.8) (D) were more than those treated with pcDNA3.1/PVP (1.4±1.7, $P$<0.05) (A).

(75.8±6.6 μg/L) ($P$<0.05). Also, after pmIL-12 gene therapy, the serum IL-12 level was higher in pmIL-12 group (401.0±51.6 ng/L) than in pcDNA3.1 group (154.8±25.8 ng/L) ($P$<0.05) (Figure 3).

### *MVD, TILs and apoptosis of H22 hepatoma after gene therapy*

MVD were counted after CD31 staining in tumor sections. Compared with pcDNA3.1 group, MVD of hepatoma treated with pSecES/PVP or pmIL-12/PVP were much less. And in pSecES+pmIL-12 group, MVD were less than any single gene therapy group ( $P$<0.05) (Figure 4).

Tumor sections were stained by HE, then TILs were evaluated. After pmIL-12/PVP or pSecES+pmIL-12/PVP gene therapy, TILs in hepatoma were more than that treated with pcDNA3.1/PVP ($P$<0.01). The results are displayed in Figure 5.

Also, tumor apoptosis was displayed by TUNEL staining. Figure 6 shows that there were only a few apoptotic tumor cells dispersed in 400× random scopes in pcDNA3.1 group. But in pSecES group or pmIL-12 group, apoptotic tumor cells increased. In pSecES+pmIL-12 group, apoptotic tumor cells were much more than any group ($P$<0.05).

## DISCUSSION

Despite the progress in early diagnosis of liver cancer, its prognosis remains poor. In average, the mean life span was less than a year after diagnosis[1]. How to prevent or cure the recurrence of remained tumor cells after partial hepaectomy and how to inhibit tumor infiltrating in liver cancer patients in advanced stage are urgent challenges for liver cancer therapy. Now antiangiogenic therapy and immunotherapy have been paid more and more attention and many promising conclusions have been drawn[28-31]. But in clinical trails, few satisfactory results were obtained by single biotherapy[17,18,32]. Whether synergistic effects of antiangiogenesis therapy in combination with immunotherapy could be achieved is worthy to be researched.

Studies showed that angiogenesis played a very important role in the course of growth and metastasis of solid tumors including liver cancer. When the tumor size was beyond 2-3 $mm^3$, oxygen and nutrition were not enough by simple diffusion. Then new vessels must be formed for further growth[33]. However, angiogenesis was dependent on the cooperation of pro-angiogenesis factors and anti-angiogenesis factors[34]. Among many inhibitors, endostatin, the C terminal of collagen X VIII, which is a $M_r$ 20 000 protein with 184 amino acids in primary structure, has the strongest antiangiogenesis and antitumor effect. It was identified from the medium of a murine hemangioendothelioma (EOMA) cell line in 1997[7]. Researchers indicated that both natural and recombinant endostatin could markedly inhibit endothelial cell proliferation *in vitro* and angiogenesis in chick chorioallantoic membrane, but had no effect on non-endothelial cells. When endostatin was administrated subcutaneously, primary tumors were regressed to dormant microscopic lesions and metastasis was inhibited. Repeated cycles of endostatin therapy showed prolonged tumor dormancy without further therapy[7,10]. In our previous study, subcutaneous

injection of 10 mg/(kg.d) endostatin produced by gene engineering showed a potent inhibitory effect on the formation and growth of mouse H22 hepatoma[35]. Also the clinical trail of endostatin has been carried out[32]. But to obtain both high quantity and high quality of endostatin was necessary for optimal therapeutic benefit[36]. It was indicated that the activity of endostatin expressed in mammalian cells was 1 000 times than recombinant protein extracted from *E.coli*[37]. So gene therapy of endostatin has become a very good solution and many studies suggested that gene therapy of endostatin showed a powerful inhibition on liver cancer[21,22].

When H22 hepatoma cells, derived from Balb/c mouse liver cancer in 1940 s, were inoculated into the leg muscle of mice, solid tumors formed quickly, which were easy to touch and easy for injection. Compared with viral vectors, non-viral vectors bore some advantages such as easy to handle, high capacity for DNA sequences, low toxicity, and non-immunogenic thus permitting repeated vector administrations[38]. So we constructed endostatin eukarytic plasmid pSecES, inside which a mouse Igκ signal peptide sequence was put upstream endostatin sequence to let endostatin secrete out. *In vitro* high-levels endostatin with inhibitory activity on the proliferation of endothelial cells could be detected from the supernant after transfection. And *in vivo*, for lower degradation of input DNA by endosomes and/or lysosomes and higher transfection efficiency, non-toxic PVP was used to enhance gene expression according to previous reports[39,40]. Plasmid/PVP was injected intratumorally and repeatedly for a higher local concentration. After endostatin gene therapy, mice serum endostatin levels were consistently 2-3-fold higher than basic levels. Compared with controls, tumors formed slowly with less MVD and more apoptosis cells. The average tumor weight inhibitory rate was 56.4%. It was indicated that pro-angiogenic factors such as VEGF were induced when tumor vessels formed gradually, and antiangiogenic factors such as endostatin increased for a negative regulation[41,42]. This opinion was also confirmed by our study that serum endostatin levels in mice burdened with big tumors after vector injection were increased compared with basic levels. When more endostatin were added by gene therapy, the balance would be broken and angiogenensis was inhibited.

Immunocytokines which were important in the regulation of the immune system could overcome immune tolerance against tumor antigens, thus tumor rejection was facilitated. It was benefic for prevention of recurrence of micro tumor cells and advanced tumors which could not accept operation or chemotherapy[11,13]. Different cytokines (IL-2, IL-4, IL-12, INF-γ, TNF-α, GM-CSF) have been used to modulate the host's immune response either *in vitro* or *in vivo*. The activation and proliferation of immunocytes and antitumor immunity were induced more or less[13]. IL-12 is among the most potent cytokines in stimulating antitumor immunity. It is a disulfide-linked heterodimeric cytokine. IL-12 acts by inducting a TH1 type of response, activating natural killer cells and cytotoxic T lymphocytes, enhancing expression of adhesion molecules on endothelial cells, thus facilitating the traffic of lymphocytes to the tumor, and inducting a potent antiangiogenic effect. In previous researches, the antitumor effect of IL-12 was established in various *in vivo* systems[12-14]. However, the latter was reported to cause fatal toxicity in a clinical trial[17], which led investigators to local administration of this cytokine by gene therapy or gene gun, that proved to be very effective and nontoxic at the same time[23,24]. In our study, active IL-12 (p70) was expressed *in vitro* and *in vivo*. Tumors did not formed in 30% mice that received IL-12 gene therapy. The formed tumors were also highly inhibited with more TILs, less MVD and more apoptotic tumor cells. Moreover, the inhibitory effect of IL-12 gene therapy on H22 hepatoma was much better than that of endostatin plasmid ($P<0.05$).

When combined gene therapy of endostatin and IL-12 was used, its antitumor effect was much better than others. Tumor formation was not observed in 66.7% mice. The average tumor weight inhibitory rate was 91.8%. But in mice that received endostatin gene therapy, no tumor regressed. The average tumor weight inhibitory rate was 56.4%. No tumor was formed in 33.3% mice after they received IL-12 gene therapy. The average tumor weight inhibitory rate was 85.7%. It was suggested that synergistic antitumor effect was achieved after combined gene therapy of endostatin and IL-12. The proposed mechanism of the combined gene therapy maybe a synergistic cooperation of an antiangiogenesis effect induced by endostatin and IL-12 in combination with an immune rejection induced by IL-12. On one hand, endostatin and IL-12, which were expressed locally in combination with increased serum levels after gene delivery mediated by plasmid with PVP, could inhibit angiogenesis of hepatoma synergisticly. This was convinced by that MVD in pSecES+pmIL-12 group was much less than any single gene therapy group. But now the antiangiogensis mechanism of endostatin and IL-12 was still not very clear. Through this way, H22 hepatoma cells came to apoptosis or necrosis due to lack of blood supply. On the other hand, more TILs (including activated CTL, NK, TH1 etc.) were observed in tumors that received IL-12 gene therapy or combined gene therapy, which maybe an indication of activated immune response on H22 hepatoma cells. Antitumor immune rejection induced by IL-12 through immunocytes or cytokines also promoted apoptosis of tumor cells. So there were much more apoptotic tumor cells in combined gene therapy group than any single one. Due to these factors, formation and growth of transplanted H22 hepatoma was prevented synergisticly.

Combined gene therapies for liver cancer were also reported previously. Learners have focused on combined genes of two or three immunocytokines, or on suicide gene therapy in combination with genetic immunotherapy[25,30,31,43]. Biotherapy in combination with chemotherapy for lymphoma was also studied[44]. Before our reports, combined treatment with type 5 adenovirus vectors expressing murine angiostatin and IL-12 showed an antitumor effect on a murine breast cancer[45]. Our report is the first to demostrate the potent antitumor effect of combined gene therapy of endostatin and interleukin 12 with polyvinylpyrrolidone on hepatoma.

In conclusion, active target protein can be expressed by repeated local injections of plasmid with PVP. Through this way, a short-term course of combined gene therapy of endostatin and IL-12 can effectively prevent the formation and development of transplanted H22 hepatoma in mice. Antiangiogenic therapy in combination with immunotherapy may be used to regress or to eradicate other solid tumors.

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• LIVER CANCER •

# Analysis of clinical effect of high-intensity focused ultrasound on liver cancer

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## Abstract

**AIM:** To evaluate the clinical effect of high-intensity focused ultrasound (HIFU) in the treatment of patients with liver cancer.

**METHODS:** HIFU treatment was performed in 100 patients with liver cancer under general anesthesia and by a targeted ultrasound. Evaluation of efficacy was made on the basis of clinical symptoms, liver function tests, AFP, MRI or CT before and after the treatment.

**RESULTS:** After HIFU treatment, clinical symptoms were relieved in 86.6%(71/82) of patients. The ascites disappeared in 6 patients. ALT (95±44) U/L and AST (114±58) U/L before HIFU treatment were reduced to normal in 83.3%(30/36) and 72.9%(35/48) patients, respectively, after the treatment. AFP was lowered by more than 50% in 65.3%(32/49) patients. After HIFU treatment, MRI or CT findings indicated coagulation necrosis and blood supply reduction or disappearance of tumor in the target region.

**CONCLUSION:** HIFU can efficiently treat the patients with liver cancer. It will offer a significant noninvasive therapy for local treatment of liver tumor.

Li CX, Xu GL, Jiang ZY, Li JJ, Luo GY, Shan HB, Zhang R, Li Y. Analysis of clinical effect of high-intensity focused ultrasound on liver cancer. *World J Gastroenterol* 2004; 10(15): 2201-2204

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## INTRODUCTION

Since 1942, Lynn *et al.*[1] firstly brought forward the conception of HIFU, i.e. high intensity focused ultrasound. Fry[2] applied HIFU technology to experimentally treat nervous system diseases, and suggested the potential of HIFU in surgical operations. As a result, he found that ultrasound beam could form a preferable focal field at a special depth inside body, and destroy target tissue through focus pointing without damaging neighboring tissues. In 1956, Burov proposed that effect of short-time high-intensity ultrasound irradiation was better than low-intensity ultrasound. With research progress on HIFU technology, this technology has been used in clinic over the years[3-6]. The present study makes an assessment on clinic effect of HIFU in treating liver cancer.

## MATERIALS AND METHODS

### Materials

We performed HIFU treatments in 100 patients (80 male, 20 female, ranging 30-74 years with mean age of 56 years) with liver cancer from July 2001 to July 2003, totally 130 HIFU treatments, 1.30 times per patient. Patients included 62 primary liver cancers and 38 metastatic liver cancers. Sixty-eight patients were with single nodule, 22 with two nodules, 10 with three nodules. Totally 36 tumor nodules with a diameter less than 5 cm, 76 with a diameter between 5-10 cm, 30 with a diameter more than 10 cm were involved. All cases were investigated and verified by pathohistology or an obvious increase of serum AFP and positive imaging, and conformed to diagnostic standard of National Cooperation Conference and Hepatic Carcinoma Prevention and Treatment in 1997. Eighteen patients were in stage I, 48 patients in stage II, and 34 patients in stage III.

### Instrument

JC type focused ultrasound tumor therapeutic system was designed by Chongqing HIFU Technology Co, Ltd. Chongqing, China. It includes two main parts, i.e. ultrasound real-time orientation monitor device and HIFU three-dimensional combination scanning therapeutic device. Under the control of a computer, it can orient to preassigned tumor target zone automatically, determine range of therapy.

The main parameters included therapy frequency 0.8 MHz, mean diameter of focal field 1.1 mm, length of focal field 9.8 mm, focus distance 135 mm, therapy power 140-240 W.

### Methods

Routine examinations and preoperative preparation were conducted according to the principle of surgery. Based on the result of image and ultrasound examination, the therapeutic scheme of HIFU was constituted. HIFU treatment was performed with patient fixed properly. The tumor position and size, therapeutic layers and therapeutic range of every layer were determined by ultrasound diagnostic probe. Then the therapeutic probe treated tumor tissue of every layer from outside body, and in terms of order of layer, from spot to line, and from line to area, leading whole tumor to coagulation necrosis. During the therapeutic process, through changes of graphics of target field and echo of tissue between before and after therapy at every layer, real-time estimate of HIFU therapeutic effect by computer processing image system was carried out, and with feedback, ultrasound therapeutic dosage estimated in the therapy scheme was controlled according to changes of ultrasound photograph. The therapeutic method was divided into complete coverage and local coverage. Twenty-eight cases used complete coverage, including whole tumor focus and normal liver tissue within 2 cm away from edge of tumor, the other cases used local coverage due to reasons, such as large tumor volume, rib overlap or close-by, or involvement of liver tube or cholecyst, *etc*.

### *Observatory parameters*

The following aspects were observed: Improvement of clinic symptoms; changes of liver function 2 d and 2 wk before and after therapy; changes of AFP 2 wk before and after therapy; changes in range and blood supply of coagulation necrosis of tumor focus, also shrink of tumor through re-examination of MRI or CT before and after therapy.

## RESULTS

One hundred liver cancer patients were treated by using HIFU in this group, of which 82 exhibited clinic symptoms. Seventy-one patients were improved obviously after HIFU treatment: appetite increased, weight gained, discomfort or pain on liver region relieved. Remission rate for symptoms was 86.6%(71/82). For 6 patients who had had mild ascites, ascites disappeared after HIFU treatment. For patients with abnormal liver function (ALT 95±44 U/L, AST 114±58 U/L), 2 d after HIFU treatment, liver enzymes rose slightly, but no obvious statistic difference was found compared with pre-treatment. The patients' liver enzymes fell to normal level after 2 wk, respectively, ALT 83.3%(30/36), AST 72.9%(35/48). For the patients whose AFP was increased, AFP was 50% less than original level 2 wk after HIFU treatment, in 65.3%(32/49), only one patient's AFP rose continuously after HIFU treatment, and multi-bone metastases were found by ECT examination. Self-comparison of MRI showed that $T_1$-weighted images and $T_2$-weighted images of tumor therapeutic region were high signal and low signal respectively after HIFU treatment. Enhanced MRI did not show enhanced signal, indicating that coagulation necrosis had occurred in the tumor therapeutic region, blood supply of tumor was diminished or eliminated, tumor of some patients had shrunk obviously after countercheck MRI (Figures 1-3).

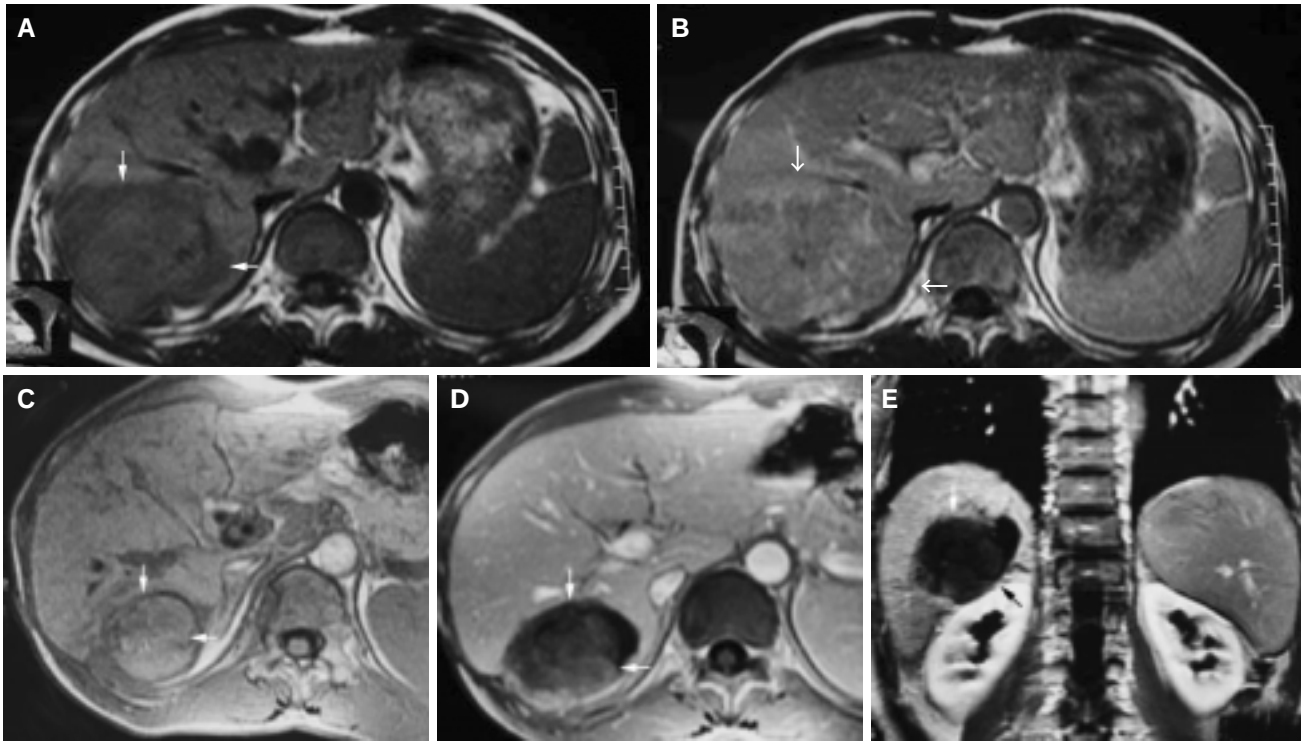


**Figure 1** A 42-year-old male with hepatocellular carcinoma. A: MRI $T_1$-weighted images before HIFU treatment showed that liver tumor of right-posterior lobe was 115 mm×100 mm×66 mm; B: MRI enhanced imaging before HIFU revealed that enhancement of mass in liver right-posterior lobe was higher than that of surrounding normal liver tissues; C: MRI $T_1$-weighted imaging 11 mo after HIFU revealed that liver tumor of right-posterior lobe obviously decreased in size (50 mm×55 mm×60 mm); D, E: MRI enhanced imaging (cross section and coronal section) 11 mo after HIFU revealed that liver tumor had shrunk obviously, blood supply of tumor was eliminated, the tumor therapeutic region had coagulation necrosis.

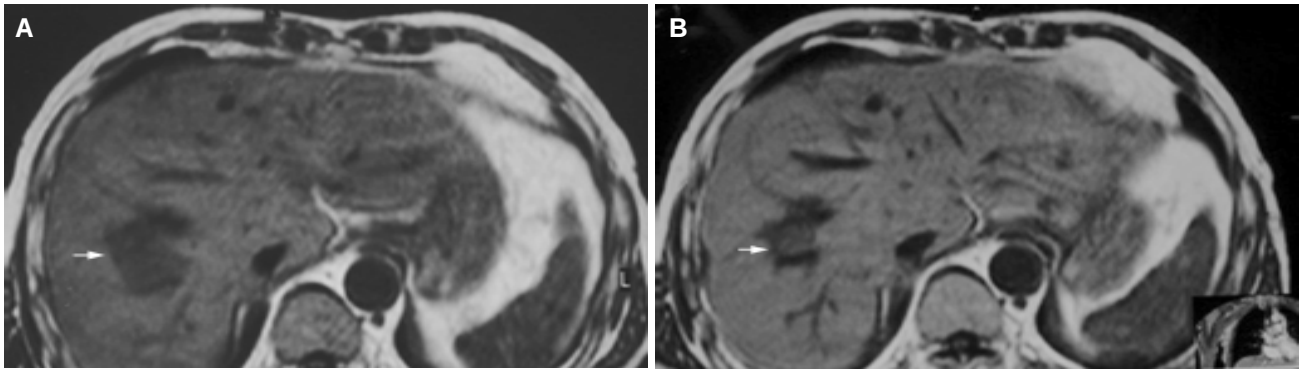


**Figure 2** A 55-year-old man with metastatic hepatocarcinoma in sigmoid cancer postoperation. A: MRI images before HIFU treatment showed that liver tumor of right-posterior lobe was 40 mm×30 mm×30 mm; B: MRI images 2 wk after HIFU revealed that liver tumor of right-posterior lobe obviously decreased in size (20 mm×20 mm×15 mm).

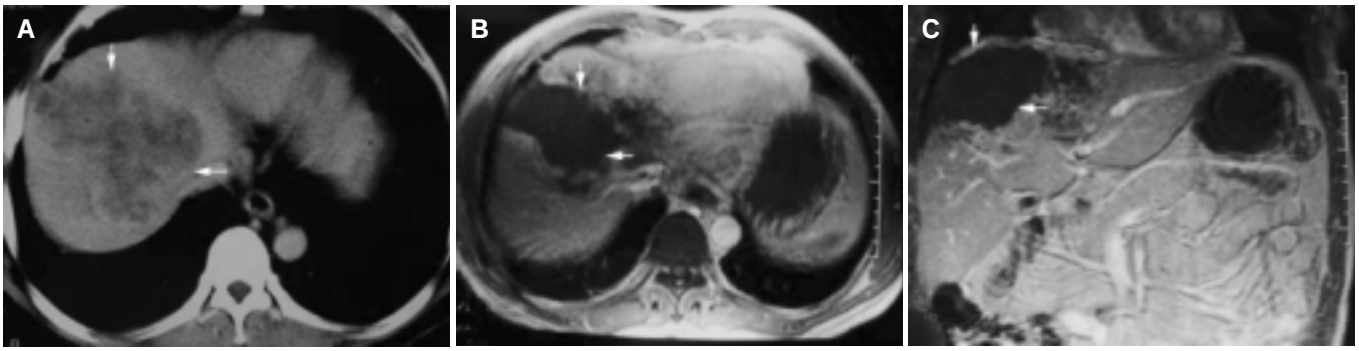


**Figure 3** A 54-year-old man with primary carcinoma of liver in right lobe. A: CT enhanced imaging before HIFU treatment revealed that enhancement of mass in liver right-posterior lobe was higher than that of surrounding normal liver tissues (AFP: 5516 μg/L); B, C: MRI enhanced imaging (cross section and coronal section) 2 mo after HIFU revealed that necrotic tissue in the therapeutic region of the tumor was not enhanced (AFP: 183 μg/L).

## DISCUSSION

HIFU is a high-tech developed successfully in the 1990's, a local way of treating tumor without any damages. It utilizes the physics characteristics of ultrasound beam with assemble and penetration, to focus low energy outside body on inner tumor target field, through instantaneous high temperature effect, cavitate effect, making tumor target tissue of focal zone coagulation necrosis, without destroying surrounding tissues[7-14].

HIFU has the following characteristics in therapy of malignant tumors. Firstly, noninvasiveness. HIFU treats inner tumor without damaging outside body. Previous research on animal angiography indicated that after treating liver tumor of Morris rat using HIFU, nourishing blood vessels which diameter was less than 200 μm of irradiation zone closed, but they were normal for blood vessels which diameter was more than 200 μm[15]. Secondly, accuracy. HIFU can pass through tissues and accurately damage target tissues inside organisms. The boundary between therapy zone and un-therapy zone is clear, tissue beyond target zone is hardly destroyed or without damage[16,17]. Ultrasound Therapy Section of London Emperor Hospital, Britain found that only six cells existed between cells killed completely and cells without damage[18]. Thirdly, real-time therapy. For whole process of therapy, it is a real-time targeting and monitoring process, real-time estimating therapeutic effect and adjusting dosage[19]. Fourthly, suitable therapy. According to the size and shape of tumor, it determines therapy range of tumor target zone, overlays tumor target zone. Therefore, treatment of malignant tumor using HIFU has many advantages such as less pain, no damage, fewer influences on splanchnic function, faster recovery for body and no increase of tumor metastasis chance[20], *etc*.

It is shown that in this research, 100 liver cancer patients had obvious improvement in symptoms and signs after being treated by using HIFU. Short-term effect of HIFU therapy was obvious and affirmative in liver cancer. But for liver cancer patients who had huge block or multifocal big nodules, to make tumor completely occur coagulation necrosis, to gain the purpose of "ablation" tumor integrity, we performed treatment for two or three times. Firstly, we treated deeper tumor parts, to induce coagulation necrosis of the tumor, so as to make it easy to treat remaining superficial parts next time. Conversely, if at first, it makes superficial parts occur coagulation necrosis, and then treats deep tumor tissue, because of change of the impedance dispersion and absorb coefficient of sound, the attenuation of penetrating tissue with ultrasound will aggravate. Meanwhile, focus energy in focal field can not reach ideal degree, as a result it takes more time to make tumor tissue coagulation necrosis.

If tumor locates on the edge of liver with poor blood supply, during therapy of HIFU, We estimate every therapeutic effect by using real-time ultrasound imaging to monitor the change of gray value. If gray value is changed, it indicates that tumor tissue must occur coagulation necrosis. However, tissues with coagulation necrosis do not always exhibit changes of gray value. For tumor in deep part, during real-time monitor, most changes of gray value are not distinct or lightly increased with suffusion. Through quantitative analysis and comparison of gray value of ultrasound imaging before and after therapy, we can find that the difference of gray rank before and after therapy is obvious. This indicates that local tumor tissues have no reversible coagulation necrosis.

For liver cancer patients with abundant blood supply relatively, we should first perform transcatheter arterial chemoembolization before using HIFU, so as to make inner tumor tissue have more iodised oil deposition, which not only is convenient to ultrasound orientation, but also changes the impedance dispersion and absorb coefficient of sound of tumor zone. Accordingly, it is convenient to energy sediment of focal zone, exerts cooperative function of raising temperature, excites high temperature to get to purpose of destruct therapy at local and makes tumor tissue coagulation necrosis[21,22].

Yang *et al*.[15] used HIFU to treat Morris rat hepatoma implanted in the liver. Treatment was administered with a lens-focused 4-MHz transducer that created a focused beam of 550 W/cm$^2$ at peak intensity. One hundred and twelve rats with liver tumors were divided into two groups of 56 each. Group 1 received HIFU therapy while group 2 (the control group) did not. All rats were killed immediately or 1, 3, 7, 14, 21, 28 d after treatment. Eight rats in each group were killed at each interval for pathologic and biochemical studies. Significant inhibition of the tumor growth was seen in the HIFU-treated group, with tumor growth inhibition rates of 65.4-93.1% from on d 3 to 28 after treatment. Ultrasound-treated tumors showed direct thermal cytotoxic necrosis and fibrosis. An additional 56 ACl rats with liver tumors were divided into 4 groups of 14 each. Group 1 received doxorubicin hydrochloride intraperitoneally and HIFU therapy; group 2, HIFU therapy; group 3, doxorubicin hydrochloride; and group 4 (the control group), neither HIFU nor doxorubicin hydrochloride. Significantly improved survival rates were noted in HIFU-treated animals (groups 1 and 2) compared with those of groups 3 and 4.

Research results of home and abroad using animals showed that HIFU can safely and effectively destroy inner liver tissue or liver transplant tumor[23-30]. Clinic results showed that HIFU can also treat liver cancer securely and effectively[11,31,32].

HIFU is a new method with definite therapeutic effect on tumor without damage and poisonous to normal tissues. With improvement of HIFU technology, accumulation of clinic application experience and further research on mechanism of HIFU, HIFU, as a new local therapy, would be applied more extensively to treat liver cancer.

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• COLORECTAL CANCER •

# Honokiol induces apoptosis through p53-independent pathway in human colorectal cell line RKO

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## Abstract

**AIM:** To investigate the signal pathway of honokiol-induced apoptosis on human colorectal carcinoma RKO cells and to evaluate whether p53 and p53-related genes were involved in honokiol-treated RKO cells.

**METHODS:** Cell cycle distribution and subdiploid peak were analyzed with a flow cytometer and DNA fragment with electrophoresis on agarose gels. Transcriptional level of Bax, Bcl-2, Bid and Bcl-xl was accessed by RT-PCR. Western blotting was used to measure p53 protein expression and other factors related to apoptosis. Proliferation inhibition of two cell lines (RKO, SW480) with high expression of p53 and one cell line with p53 negative expression (LS180) was monitored by MTT assay.

**RESULTS:** Honokiol induced RKO cell apoptosis in a dose-dependent manner. The mRNA expression level and protein level of Bid were up-regulated while that of Bcl-xl was down-regulated, but no changes in Bax and Bcl-2 were observed. Western blotting showed p53 expression had no remarkable changes in honokiol-induced RKO cell apoptosis. LS180 cells treated with honokiol exhibited apparent growth inhibition like RKO cells and Sw480 cells.

**CONCLUSION:** Honokiol can induce RKO cells apoptosis through activating caspase cascade by p53-indepenent pathway.

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## INTRODUCTION

Honokiol is a major active constituent extracted from the bark of *Magnolia officianlis* (Chinese name for *Houpu*) (Figure 1). It has a variety of pharmacological effects, such as anti-inflammatory[1], antithrombosis[2], anti-arrhythmic[3], antioxidant[4] and anxiolytic effects[5]. Recently, honokiol has been reported to exhibit a potent cytotoxic activity by inducing cell apoptosis in some cell lines[6-8]. Honokiol-triggered apoptotic process is accompanied with down-modulation of Bcl-XL, release of mitochondrial cytochrome C in CH27 cells[9].

Cells undergoing apoptosis are characterized by distinct biochemical and morphological changes. Many of the biochemical and morphological events of apoptosis are a direct result of caspase-mediated cleavage of specific substrates[10,11]. Together with caspase, Bcl-2 family is involved in inducing cell apoptosis and identified as essential components of the intracellular apoptotic signaling pathways. Cell viability versus death is determined by the relative abundance of various members of the Bcl-2 family, acting in concert with other proteins in the death pathway[12,13].

In many tumor cells, wild-type p53 is considered to participate in apoptosis in response to DNA damage[14]. P53 may transactivate apoptotic regulators, such as Bcl-2[15-17] and Bax[18,19]. Recent studies have shown that p53 plays a role in apoptosis by the mitochondrial-mediated apoptotic pathway[20]. Activation of p53 upregulates Bax[19] and increases the ratio of Bax:Bcl-2, releases cytochrome C and other polypeptides from the intermembrane space of mitochondria into cytoplasm. Cytochrome C activates caspase cascade[12,13].

It is well known that a broad range of agents can induce tumor cell apoptosis through p53-regulated manner by Bax/mitochondria/caspase-9 pathway. But up to now, it is not clear that whether wild-type p53 takes part in honokiol-induced apoptosis. In this present study we examined whether wild-type *p53* and *p53*-related gene were involved in honokiol-treated RKO cells.

## MATERIALS AND METHODS

### Materials

RPMI 1640 medium was obtained from Gibco BRL. Newborn bovine serum was supplied by Sijiqing Biotechnology Co. (Hangzhou, China). Monoclonal antibodies to Bax, Bcl-xl, Bid, Bcl-2, β-actin and p53 were purchased from NeoMarkers, Fremont, CA, USA. Honokiol was got from the National Institute for Pharmaceutical and Biological Products, Beijing, China. The drug was dissolved in DMSO with the stock concentration of 10 mg/mL. It was further diluted in culture medium with the final DMSO concentration <1%. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma Chemical Corporation, USA.

### Cell culture

Human colorectal cell lines RKO, SW480 and LS180 were provided by Cancer Institute of Zhejiang University. Three cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 100 mL/L heat-inactivated fetal bovine serum (Si-Ji-Qing Biotechnology Co, Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in a 50 mL/L $CO_2$ atmosphere.

### Detection of DNA fragmentation

To detect DNA fragments, RKO cells were collected and lysed with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5),

20 mmol/L EDTA, and 10 g/L NP-40. Then 10 g/L SDS and RNase (5 μg/mL) were added to the supernatants, and incubated at 56 ℃ for 2 h, followed by incubation with proteinase K (2.5 μg/mL) at 37 ℃ for 2 h. After DNA was precipitated by addition of both ammonium acetate (3.3 mol/L) and ethanol (995 mL/L) dissolved in a loading buffer, DNA fragmentation was detected by electrophoresis on 15 g/L agarose gels and visualized with ethidium bromide staining.

### *Cell cycle analysis by FCM*

Honokiol-treated RKO cells (5, 10, 15 μg/mL) and vehicle were fixed with 700 mL/L alcohol for 15 min at 4 ℃, then stained with 1.0 μg/mL propidium iodide (PI, Sigma, USA). The red fluorescence of DNA-bound PI in individual cells was measured at 488 nm with a FACSCalibur (Becton Dickinson, USA) and the results were analyzed using ModFit 3.0 software. Ten thousand events were analyzed for each sample.

### *RT-PCR assay*

RKO cells $1\times10^5$ were seeded on 24-well plate. After 24-h culture, cells were treated with 5, 10 μg/mL honokiol and vehicle for 48 h. Total RNA from RKO cells was extracted using Trizol (Invitrogen, USA). In RT-PCR, cDNA synthesis was performed using a RNA PCR kit (TaKaRA Biomedicals, Osaka, Japan) with the supplied oligo dT primer (Table 1). Reverse transcription was performed using a thermal program of 92 ℃ for 2 min, then 38 cycles of annealing for 30 s at 55 ℃, extension for 90 s at 72 ℃. As an internal control, semiquantitative analysis of β-actin was also amplified. This primer pair of β-actin had an optimal annealing temperature of 55 ℃ with 20 cycles and yielded a 350-bp PCR product. Samples were separated on 20 g/L agarose gel and visualized with ethidium bromide staining under UV light.

### *Western blotting*

RKO cells ($5\times10^6$) treated with 5, 10 μg/mL honokiol and vehicle respectively for 24 h were lysed by 4 g/L trypsin containing 0.2 g/L EDTA, then collected after washed twice with phosphate-buffered saline (PBS, pH 7.4). Total protein extract from RKO cells was prepared using cell lysis buffer [150 mmol/L NaCl, 0.5 mol/L Tris-HCl (pH 7.2), 0.25 mol/L EDTA (pH 8.0), 10 g/L Triton X-100, 50 mL/L glycerol, 12.5 g/L SDS]. The extract (30 μg) was electrophoresed on 12 g/L SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane (PVDF, Millipore Corp., Bedford, MA) for 2 h in a buffer containing 25 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine and 200 mL/L methanol. The blots were blocked with 50 g/L nonfat milk in TBST washing buffer for 2 h at room temperature and then incubated at 4 ℃ overnight with antibodies. All antibodies were diluted in TBST according to the manufacturer's instructions. After washed at room temperature with washing buffer, the blots were labeled with peroxidase-conjugated secondary antibodies.

### *Cell proliferation assay*

RKO cells, SW480 and LS180 cells ($1\times10^4$ in 100 μL) were seeded on 96-well plates in triplicate respectively. Following a 24-h culture at 37 ℃, the medium was replaced with fresh medium containing vehicle control or various concentrations of honokiol in a final volume of 200 μL. Cells were incubated at 37 ℃ for 68 h. Then 50 μL of MTT (2 mg/mL in PBS) was added to each well, incubated for an additional 4 h, the plate was centrifuged at 1 000 r/min for 10 min, then the medium was removed. The MTT formazan precipitate was dissolved in 100 μL DMSO, shaken mechanically for 10 min and then read immediately at 570 nm by a plate reader (Opsys MR, Denex Technology, USA).

### *Statistics*

Statistical significance was determined by Student's *t*-test. A *P* value of 0.05 or less was considered significant.

## RESULTS

### *DNA fragmentation detection in RKO cells*

In honokiol-treated RKO cells, a degradation of chromosomal DNA into small internucleosomal fragments was evidenced by the formation of 180-200 bp DNA ladder on agarose gels (Figure 2), hallmark of cells undergoing apoptosis. No DNA ladders were detected in the samples isolated from control cultures. These results indicated that honokiol induced an apoptotic cell death in RKO cells

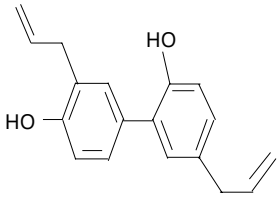


**Figure 1** Chemical structure of honokiol ($C_{18}H_{18}O_2$, $M_r$ 266.33).


**Figure 2** Differences in vehicle and honokiol induced apoptotic DNA laddering of RKO cells. Lane 1: Vehicle; Lane 2: 5 μg / mL; Lane 3: 10 μg / mL.

### *Effect of honokiol on cell cycle phase distribution*

RKO cells were exposed to the increased concentrations of honokiol (5-15 μg/mL) for 48 h, and the cell growth was analyzed using flow cytometry. In the absence of honokiol, the cell populations were at G1, S, and G2/M phases (Figure 3), accompanied with increased concentrations of honokiol by a concomitant increase of the G1 phase (Table 2). From Figure 3, we considered the peak areas of subdiploid were up at the

**Table 1** Expected size of PCR amplification product with each apoptosis modulator primer pair

| Apoptosis modulator | Upstream primer (5'-3') | Downstream primer (5'-3') | Size (bp) |
|---|---|---|---|
| Bax | ACCAAGAAGCTGAGCGAGTGT | ACAAACATGGTCACGGTCTGC | 332 |
| Bcl-xl | GGAGCTGGTGGTTGACTTTCT | CCGGAAGAGTTCATTCACTAC | 379 |
| Bid | GAC CCG GTG CCT CAG GA | ATG GTC ACG GTC TGC CA | 586 |
| Bcl-2 | TTGTGGCCTTCTTTGAGTTCG | TACTGCTTTAGTGAACCTTTT | 332 |
| β-actin | AGGCCAACCGCGAGAAGATGACC | GAAGTCCAGGGCGACGTAGCAC | 350 |

increased concentrations of honokiol. This observation led to a suggestion of G1 arrest. When the cells were exposed to honokiol at 10 μg/mL and above, subdiploid peak was significantly increased and the percentage of apoptotic cells in 10 000 cells was 14.1% and 20.31% respectively (Table 2).

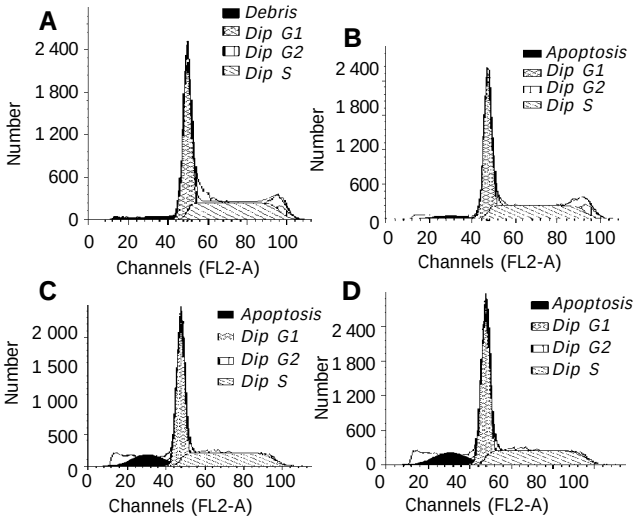


**Figure 3** Apoptosis of RKO cells detected by FCM. A: Vehicle; B: 5 μg / mL honokiol; C: 10 μg / mL honokiol; D: 15 μg / mL honokiol.

**Table 2** Effect of honokiol on cell cycle distribution and apoptosis in RKO cells

| Treatment (μg / mL) | Cell cycle distribution (%) | | | Apoptosis (%) |
|---|---|---|---|---|
| | G0 / G1 | S | G2 / M | |
| Control | 44.76 | 45.09 | 10.14 | 0.21 |
| 5 | 48.03 | 42.37 | 9.60 | 4.51 |
| 10 | 54.04 | 40.30 | 5.66 | 14.10[b] |
| 15 | 58.94 | 39.36 | 1.70 | 20.31[d] |

Cell cycle distribution was determined after 48 h of treatment in each group. The tabulated percentages were an average calculated on the results of three separate experiments. [b]$P<0.01$, [d]$P<0.01$ *vs* corresponding control group.

### *Semi-quantification of Bid, Bax, Bcl-2 and Bcl-xl mRNA expression*

mRNA expression of apoptosis-related genes in response to different levels of honokiol was assessed. The Bcl-xl mRNA expression was significantly reduced in RKO cells exposed to honokiol, while Bid mRNA expression was remarkably up-regulated. No apparent changes of Bax and Bcl-2 were observed (Figure 4).

### *Effect of honokiol on expression of p53-related gene and p53*

*p53* and *p53*-related gene were found to be importantly involved in apoptosis induced by many agents. To study their role in honokiol-treated RKO, we monitored Bax, Bcl-2, Bcl-xl, Bid and p53 protein levels. Results in Figure 5 shows that no significant changes of Bax, Bcl-2 and p53 were found compared with vehicle. The level of Bcl-xl was decreased to vehicle level, while the level of Bid was increased remarkably after treated with 5 and 10 μg/mL honokiol respectively. The protein levels were measured by quantitative Western blot analysis after normalized with β-actin content.

### *Inhibition of proliferation in RKO, Sw480 and LS180 cells*

RKO cells had positively expressed of high wild-type p53 and SW480 cells had positive expression of high mutant p53, while LS180 had negative p53 antigen expression[20-22]. Cells treated with various concentrations of honokiol resulted in a dose-dependent cytotoxicity in three cells. As shown in Figure 6, honokiol-treated LS180 exhibited apparent growth inhibition like RKO cells and Sw480. Honokiol-mediated cytotoxicity occurred at the concentration of 5 μg/mL and above. A significant decrease in cell number was seen at 10 μg/mL. The concentration leading to a 50% decrease in cell number (IC50) was approximate 10.33, 12.98, 11.16 μg/mL in RKO, SW480 and LS180 cells respectively.

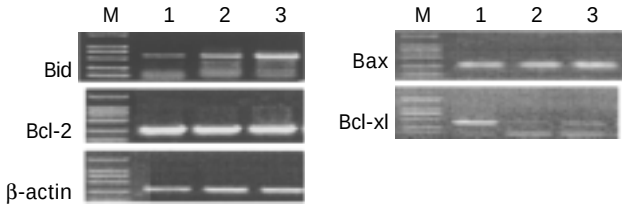


**Figure 4** Analysis of Bcl-2, Bax, Bid and Bcl-xl mRNA by RT-PCR. M: DNA mark; Lane 1: Vehicle; Lane 2: 5 μg / mL honokiol; Lane 3: 10 μg / mL honokiol.

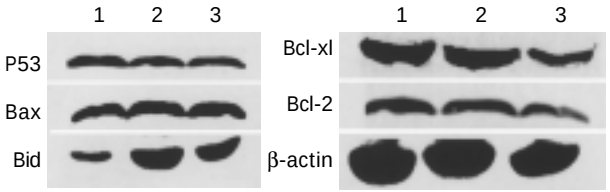


**Figure 5** Western blotting of honokiol- and vehicle-induced Bax, Bcl-2, Bcl-xl, Bid and p53 protein levels in RKO cell line. Lane 1: Vehicle; Lane 2: 5 μg / mL; Lane 3: 10 μg / mL.

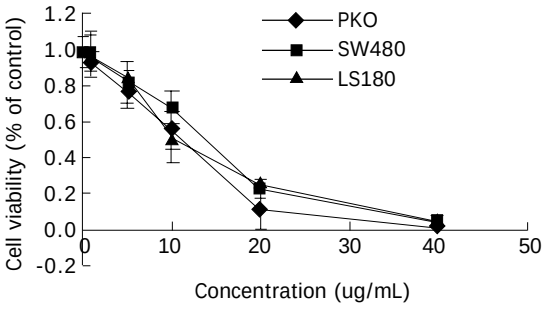


**Figure 6** Concentration-dependent inhibition of RKO, SW480 and LS180 cells exposed to the various concentrations of honokiol shown by MTT assay.

## DISCUSSION

p53 is a crucial protein in cellular stress response. p53-dependent arrest of cells in the G1 phase of the cell cycle was an important component of the cellular response to stress[23]. Wild-type p53 was considered to participate in apoptosis in response to DNA damage in many tumor cells[14,24]. When cells received UV or ionizing radiation and were exposed to anticancer drugs, p53 protein was accumulated[25,26]. The increased p53 could transactivate its downstream target genes to induce cell cycle arrest, DNA repair, and apoptosis[27,28]. p53-dependent apoptosis was also observed *in vivo*[29]. However, p53-independent apoptotic cascades after administration of anticancer drugs or r-irradiation have been described.

In the present study, honokiol-treated RKO cells could induce apoptosis in a concentration- dependent manner, with the ratio of G1 phase increased. The agent also caused an increase of the expression of caspase-3 and caspase-7 in a dose-dependent manner, did not cause the significant increase

of caspase-9 (data not shown). In CH27 cells it promoted to release cytochrome C and activate caspase 3[9].

p53-dependent apoptosis was activated by the Bax/mitochondrial/caspase-9 pathway. Bcl-2 and Bax expression were regulated by p53 both *in vitro* and *in vivo*, and Bax was a direct target of p53 transcriptional activation[30]. Bax, a proapoptotic member of the Bcl-2 family, was located in the outer membrane of mitochondria[25]. Increased in the ratio of Bax/Bcl-2 could cause changes in the membrane potential of mitochondria, consequently, cytochrome C and other polypeptides were released from the intermembrane space of mitochondria into cytoplasm. Once released, cytochrome C could activate procaspase-9 by self-cleavage[12,13] and then other caspases[12].

According to previous data, honokiol led to cell G1 arrest and cytochrome C release like p53. Thus it is necessary to evaluate the mRNA and protein expression of Bax and Bcl-2. Our results showed that honokiol-induced apoptosis of RKO cells was accompanied with up-regulation of Bid and down-modulation of Bcl-xl, but did not involve the regulation of Bcl-2 or Bax protein expression. Since pro-apoptotic Bax is a p53 downstream target, the lack of change of Bax and Bcl-xl supports the view that honokiol probably does not trigger the p53/Bax-mediated apoptosis pathway. Next we examined the expression of p53 in honokiol-treated RKO cells. We found that no significant change of p53 was observed (Figure 5). Finally we analyzed honokiol-induced apoptosis of the three cells with different p53 expression. Our data showed that honokiol-treated LS180 and SW480 cells exhibited apparent growth inhibition like RKO cells.

These results suggest that honokiol-induced caspase activation and cell apoptosis are entirely controlled by the p53-independent pathway.

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• VIRAL HEPATITIS •

# Natural history of chronic hepatitis C in patients on hemodialysis: Case control study with 4-23 years of follow-up

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## Abstract

**AIM:** Hepatitis C virus (HCV) infection is very common among end-stage kidney disease patients on hemodialysis, but its natural history is not known.

**METHODS:** In this study, 189 dialysis patients (case) positive for HCV antibodies who were followed up for more than 4 years were compared with twice as many sex/age matched controls with chronic hepatitis C who were diagnosed in the same month as the case and followed up for comparable periods. The longest follow-up was 23 years in dialysis cases. The disease activities were graded into "asymptomatic" if ALT was less than 40 (35 in cases) IU/L, "low activities" if ALT was 40 (35)-79 IU/L, and "high activities" if ALT was above 80 IU/L during the last or latest 4 year period.

**RESULTS:** All 25 dialysis cases who were followed up for more than 15 years were asymptomatic and 15 of them were negative for HCV RNA. Of the 50 controls followed up for more than 15 years, 34 had high activities, and none cleared HCV RNA. There were 60 controls who were asymptomatic, but they were all positive for HCV RNA, while 22.3% of asymptomatic dialysis cases were RNA negative. No dialysis patients with chronic hepatitis C progressed to cirrhosis, whereas the disease progressed to cirrhosis in more than one quarter of the controls. These differences were highly significant ($P<0.0001$).

**CONCLUSION:** Chronic hepatitic C among hemodialysis patients is mild in disease activity, and is not progressive, perhaps due to immunological abnormalities in these patients. Hepatic C virus is frequently cleared in asymptomatic dialysis patients during a long course. A possible mechanism for viral clearance is viral particle destruction on the surface of the dialyzer membrane.

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## INTRODUCTION

Hepatitis C virus (HCV) infection is very common among patients on hemodialysis for chronic renal failure with global anti-HCV prevalence of up to 91%[1], because of the frequent past blood transfusions and nosocomial infections[2,3]. Although at the clinical setting, dialysis patients with HCV infection seem to have a mild disease, reports on the natural history of hepatitis C in hemodialysis patients vary[4]. Sterling *et al*.[5] compared 50 consecutive patients with end-stage renal disease and HCV infection to HCV-positive controls. Ninety-six percent of patients on dialysis had normal alanine-aminotransferase (ALT) and low necroinflammatory score on biopsy. Despite minimal biochemical evidence of disease, some dialysis patients were more likely to have bridging hepatic fibrosis. Another cohort study[6] showed that the crude relative risk of death comparing HCV positive to negative dialysis patients was not significant. However, adjustment for age, transplantation, and other factors raised the relative risk somewhat. Akpolat *et al*.[7] compared liver histology between 9 dialysis patients and 37 patients with normal renal function, and found less active and progressive chronic hepatitis C in the former. They admitted that the number of patients was too small for conclusion. Thus, more information is required on the natural history of chronic HCV infection in hemodialysis patients. This study was designed to compare end-stage kidney disease patients who were positive for HCV antibodies and control patients infected with HCV but not on dialysis.

## MATERIALS AND METHODS

At the end of 2001, 603 patients with renal failure were dialyzed with 221 consoles at Sanai Amalgamated Dialysis Center, Chiba, which consists of Sanai Memorial Hospital, Sanai Soga Hospital and Ichihara Dialysis Clinic. Of these 603 patients, 142 were tested positive for HCV antibodies. Many of these patients had chronic non-A, non-B hepatitis and were subsequently found to have HCV antibody positive. Dialysis patients commonly had cardiovascular, intracranial and other complications, and they died while on dialysis. At this center, about 55 patients on average died per year. As yet, none of these anti-HCV positive patients turned antibody negative during the observation period after 1991 when C100-3 antibody kit became available at the hospital. The antibody kit was subsequently changed to the 2nd and 3rd generation test. All dialysis patients underwent periodic blood tests that included ALT and aspartate-aminotransferase (AST) every 4 wk. In this study, 189 patients who were anti-HCV positive and followed up for more than 4 years to the end of 2001 constituted the study (case) subjects. Twenty-five patients were followed for more than 15 years (longest 23 years), 94 patients were followed for 10-15 years and 70 for 4-10 years (Table 1). Ten patients were treated with interferon[8] and they were excluded. Currently 3 patients had cirrhosis, they were positive for both HBV and HCV. They were not included. The three patients had cirrhosis when dialysis was started at this hospital.

One patient with renal failure started on dialysis in 1978, 11 in 1983, 5 in 1984, 4 in 1985, 8 in 1986, 13 in 1987, 18 in 1988, 14 in 1989, 21 in 1990, 24 in 1991, 22 in 1992, 14 in 1993, 18 in 1994, 10 in 1995, and 6 in 1996. There were 137 male dialysis patients whose age ranged from 21 to 75 years, averaged 48.6±11.8 years, and 52 females whose age ranged 24 to 83 years, averaged 51.7±11.1 years.

The control group consisted of anti-HCV positive patients with chronic hepatitis who were diagnosed and followed up at the Liver Center of the Chiba University Hospital. The patients who were treated with interferon and had sustained virological response were excluded. At this center, approximately 1 200 HCV positive patients were followed up at an interval of 1 to 3 mo, and about 20-30 new patients were found per month. Out of these patients, two control patients for each dialysis case were chosen who were age (±5 years) and sex matched, and found to be HCV positive in the same month and subsequently followed up for a similar period of time. All these patients were followed up for more than 4 years, and the disease activities were recorded in the last 4 years before death or in the latest 4 year period.

Diagnosis of cirrhosis was made on the basis of liver biopsy, imaging, blood chemistry and physical signs. Imaging findings suggestive of cirrhosis included ascites, liver surface irregularity, splenomegaly, enlarged left gastric vein, enlarged paraumbilical vein, obtuse liver edge, enlarged caudate lobe, excessively large left lobe compared to the right lobe, splenorenal or gastrorenal shunt by ultrasound and CT, and esophago-gastric varicies by endoscopy. The patients with past or currently elevated ALT in the absence of these features were diagnosed as having chronic hepatitis C.

Serum HCV RNA was measured using the branch DNA method and subsequently by Amplicor test (Nippon Roche, Tokyo). Viral load was measured twice at an interval of 2 or more years in dialysis patients to see the change in viral load. Beside the routine liver tests, 7S domain type IV collagen was measured as an indirect indicator of hepatic fibrosis in dialysis cases[9,10].

The disease activities were graded into three categories: "asymptomatic" if ALT levels remained below 40 IU/L for the control group and below 35 IU/L for dialysis cases, the difference in the upper normal limit was due to normally low ALT and AST in dialysis patients[11,12]; "low activities" if ALT fluctuated between 35 (40 in control) and 79 IU/L; and "high activities" if ALT levels exceeded 80 IU/L during the last 4 year period.

Statistical analyses were made by the $\chi^2$ test and ANOVA.

## RESULTS

The disease activities in these dialysis cases who were divided into three groups according to the length of dialysis (more than 15 years, 10-15 years and 4-10 years) are given in Table 1. All the 25 patients on dialysis for more than 15 years were asymptomatic, and only 3 of 50 controls were asymptomatic. The remainder of the controls all had disease activities. Of the 94 patients on dialysis for 10-15 years, 81 (85%) were asymptomatic and 6 (6.4%) had high activities. Whereas in the controls, only 26 (13.8%) of 188 were asymptomatic and 109 (58.0%) had high activities. Of the 70 patients on dialysis for 4-10 years, 58 (83%) were asymptomatic and 5 (7.1%) had high activities. The corresponding figures were 22.1% and 52.1%, respectively, in the controls. These differences were highly significant ($P$<0.001). Table 1 also lists the number of patients in parentheses in whose serum HCV RNA levels were PCR negative or below the quantifiable level by Amplicor. Of the 25 long (more than 15 years) follow-up dialysis cases, RNA was negative or below the quantifiable level in 15 (60%). However, RNA was negative in only one of 14 dialysis cases with low disease activities, the remaining 13 cases were positive. All high activities cases were positive, while none of the control patients was negative for HCV RNA.

The clinical course of these 189 patients was uneventful from the point of view of liver disease. Many of these patients died from liver unrelated diseases, but the liver disease progressed to cirrhosis in none of them. The three cases of HCV positive cirrhosis who were followed up had already had cirrhosis when they came to our hospital. All cases who were followed up for more than 15 years were asymptomatic by the end of the follow-up (Table 1). More dialysis cases who were follow-up for a shorter period of time had disease activities. Thus, chronic hepatitis C among dialysis patients seemed to slowly improve and seldom worsen. In contrast, the disease was progressive in most control patients. In the 50 control patients who were followed up for more than 15 years, the disease progressed to cirrhosis in 18 during the follow-up. In the 228 controls who were followed up for 10-15 years, cirrhosis developed in 62. In the 140 controls who were followed up for 4-10 years, 29 developed cirrhosis.

**Table 1** Clinical evaluation of dialysis (case) and control patients in recent 4 years or last 4 years before liver unrelated death

| Group | | Number | Disease activities | | |
|---|---|---|---|---|---|
| | | | Asymptomatic | Low | High |
| | Follow-up: <15 yr | | | | |
| Case | Male | 14(5) | 14(5) | 0 | 0[b] |
| | Female | 11(10) | 11(10) | 0 | 0[d] |
| Control | Male | 28(0) | 0 | 8(0) | 20[b](0) |
| | Female | 22(0) | 3(0) | 5(0) | 14[d](0) |
| | Follow-up: 10-15 yr | | | | |
| Case | Male | 70(15) | 58(14) | 7(1) | 5[b](0) |
| | Female | 24(0) | 23(10) | 0 | 1[d](0) |
| Control | Male | 140(0) | 16(0) | 29(0) | 95[b](0) |
| | Female | 48(0) | 10(0) | 24(0) | 14[d](0) |
| | Follow-up: 4-10 yr | | | | |
| Case | Male | 53(5) | 41(5) | 7(0) | 5[b](0) |
| | Female | 17(9) | 17(9) | 0 | 0[d] |
| Control | Male | 106(0) | 24(0) | 23(0) | 59[b](0) |
| | Female | 34(0) | 7(0) | 13(0) | 14[d](0) |

( ): HCV RNA, PCR negative or below quantifiable level by Amplicor [b]$P$<0.001, [d]$P$<0.001 by $\chi^2$ test.

**Table 2** Serum HCV RNA in dialysis patients in the study

| Range (KIU/mL) | Group | | |
|---|---|---|---|
| | Asymptomatic | Low activities | High activities |
| <0.5 | 53 | 2 | 0 |
| 0.6-100 | 32 | 2 | 1 |
| 101-400 | 48 | 4 | 1 |
| 401->850[1] | 21 | 6 | 9 |
| Total | 154 | 14 | 11 |

[l]Includes values above 1 000 by Amplicor Version I. Values by branched DNA method were converted to Amplicor values. $P$<0.001 by $\chi^2$ test.

**Table 3** Comparison of the first and second measurements of HCV RNA at intervals longer than 2 years in dialysis patients

| Change | Group | | |
|---|---|---|---|
| | Asymptomatic | Low activities | High activities |
| Unchanged (change <50%) | 44 | 1 | 2 |
| Increased (+ >50%) | 25 | 3 | 2 |
| Decreased (- >50%) | 36 | 5 | 2 |
| Total | 105 | 9 | 6 |

The initial serum levels of HCV RNA in 179 dialysis patients are given in Table 2. Of the 154 asymptomatic cases, the RNA

level was negative or below the quantifiable level in 53 (34.4%) and below 100 KIU/mL in 32 (20.8%), but the remaining 69 patients had RNA levels greater than 101 KIU/mL. All the 11 dialysis patients with high activities had high levels of RNA. Thus, differences among the three activity groups were significant by the $\chi^2$ test ($P$<0.001). Serum RNA could be measured twice at an interval of 2 years or longer in 120 patients. The change was less than 50% of the initial level (unchanged) in 44 (41.9%), increased by more than 50% in 25 (23.8%) and decreased by more than 50% in 36 (41.0%) (Table 3). Serum RNA clearly reflected the absence or presence of disease activities. Serum 7S fragment of type IV collagen was measured in 143 dialysis patients (Table 4). The values ranged from 1.8 to 12.0 μg/mL with a mean of 5.59 ng/mL. The mean±SD for the asymptomatic group was 5.05±1.59 ng/mL, 6.21±1.94 ng/mL for the low activities group and 6.92±2.63 ng/mL for the high activity group. The differences were significant ($P$<0.05) by ANOVA.

**Table 4** Serum 7S fragment of type IV collagen in dialysis patients

| Group | Number of patients | 7S IV collagen (ng/mL) |
|---|---|---|
| Asymptomatic | 118 | 5.05±1.59[a,c] |
| Low activities | 14 | 6.21±1.94[a] |
| High activities | 11 | 6.92±2.63[c] |
| Total | 143 | 5.59 |

[a]$P$<0.05, [c]$P$<0.05 by ANOVA.

## DISCUSSION

In this study, 189 dialysis patients with HCV infection were followed up for more than 4 years in comparison with 378 sex/age matched nondialysis control patients who were diagnosed as chronic hepatitis C. Disease activities were divided into three grades: asymptomatic, low activities (ALT not exceeding 80 IU/L) and high activities (ALT exceeding 80 IU/L) during the last or latest 4 year period. Of the 189 dialysis patients, 25 were followed up for more than 15 years. They all became asymptomatic after a varying period of elevated and fluctuating ALT, and 15 were HCV RNA negative. In a distinct contrast, the majority of controls who were followed up for a comparable period remained to have high activities (Table 1), and none lost HCV RNA, one quarter to one third of them progressed to cirrhosis. Serum type IV collagen 7S domain was measured in dialysis patients, and the results suggested that fibrogenetic activities increased with high disease activities. These results clearly showed that patients on hemodialysis became much better than nondialysis patients with chronic hepatitis C. No dialysis patients progressed to cirrhosis in this series.

The question is why there is such a distinct difference in the natural history of chronic hepatitis C between dialysis and nonuremic patients. It has been suggested that liver injury caused by HCV infection is mainly through immunologic processes[13] rather than the virus that is directly cytopathic[14]. It has been well established that patients with end-stage renal disease and on maintenance hemodialysis have severe immunologic abnormalities with reduced immune responsiveness[15].

Some of our dialysis patients had a high viral load. RNA levels did not show direct correlation with disease activities, but more asymptomatic cases were RNA negative, and RNA levels tended to decrease in more patients when studied at an interval of 2 years or longer. There have been several studies on viral load in hemodialysis patients, but the results were not consistent. According to Umlauft *et al*.[16], HCV viremia fluctuated with the time of undetectable RNA. Whereas in the study of Fabrizi *et al*.[17], HCV load was low and relatively stable. HCV RNA levels decreased in dialysis patients in another report[18].

In 1996, we first described the phenomenon in which the number of HCV viral particles decreased after each dialysis procedure and restored to the previous level at the next dialysis[19]. Subsequently it was found that viral particles were adsorbed onto the dialyzer membrane and destroyed[20]. Our observatrions have since been confirmed. According to Furusyo *et al*.[18], HCV RNA levels were significantly lower in 98 dialysis patients (0.4 mEq/mL) compared with 228 nonuremic patients (2.0 mEq/mL). The dialysis procedure itself might contribute to the reduction of viral load in the long run. Another possible explanation for viral load reduction in dialysis patients was coinfection with another hepatotropic virus[21]. However, our earlier study in this dialysis center, the rates of infection with hepatitis G virus[22] and TT virus[23] were very low. Yokosuka *et al*.[24] followed up 320 patients with chronic type C liver disease and found that no chronic hepatitis patients were seroconverted, seronegative conversion occurred in 2%, only in end stage cirrhotic patients with or without hepatocellular carcinoma. The difference in the temporal virus load between dialysis and non-dialysis patients was so remarkable that it required biological explanation. It is not known whether C-viruses are able to replicate in liver cells of immunologically compromised dialysis patients as in immunologically competent individuals. Hepatocytes of the dialysis patients might not have normal metabolic and synthetic capabilities under the influence of uremic state, as exemplified by very low AST and ALT levels in serum[11,12]. Replication of viral particles within hepatocytes might be reduced for the same reason. It seems that the most plausible explanation would be the negative balance between mechanical destruction of viral particles by membrane dialysis procedure and viral replication.

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• VIRAL HEPATITIS •

# Initial steroid-free immunosuppression after liver transplantation in recipients with hepatitis c virus related cirrhosis

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## Abstract

**AIM:** Steroids can increase hepatitis C virus (HCV) replication. After liver transplantation (LTx), steroids are commonly used for immunosuppression and acute rejection is usually treated by high steroid dosages. Steroids can worsen the outcome of recurrent HCV infection. Therefore, we evaluated the outcome of HCV infected liver recipients receiving initial steroid-free immunosuppression.

**METHODS:** Thirty patients undergoing LTx received initial steroid-free immunosuppression. Indication for LTx included 7 patients with HCV related cirrhosis. Initial immunosuppression consisted of tacrolimus 2×0.05 mg/kg·d po and mycophenolate mofetil (MMF) 2×15 mg/kg·d po. The tacrolimus dosage was adjusted to trough levels in the target range of 10-15 μg/L during the first 3 mo and 5-10 μg/L thereafter. Manifestations of acute rejection were verified histologically.

**RESULTS:** Patient and graft survival of 30 patients receiving initial steroid-free immunosuppression was 86% and 83% at 1 and 2 years. Acute rejection occurred in 8/30 patients, including 1 HCV infected recipient. All HCV-infected patients had HCV genotype II (1b). HCV seropositivity occurred within the first 4 mo after LTx. The virus load was not remarkably increased during the first year after LTx. Histologically, grafts had no severe recurrent hepatitis.

**CONCLUSION:** From our experience, initial steroid-free immunosuppression does not increase the risk of acute rejection in HCV infected liver recipients. Furthermore, none of the HCV infected patients developed serious chronic liver diseases. It suggests that it may be beneficial to avoid steroids in this particular group of patients after LTx.

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## INTRODUCTION

Liver diseases related to chronic viral hepatitis are the leading indication for liver transplantation (LTx) worldwide[1]. Hepatitis B virus and hepatitis C virus (HCV) infection account for the majority of liver diseases related to chronic viral hepatitis. In Europe, 8 422 patients received a liver graft for virus related cirrhosis between January 1988 to December 2000[2]. Posttransplantation HCV infection is a relatively benign condition during the short-term follow-up and liver recipients reach survival rates similar to other indications. However, the natural history of posttransplantation HCV reinfection is variable. Almost all recipients became HCV seropositive during the first year after LTx. At 5 years posttransplantation, up to 30% of the recipients developed cirrhosis[3,12]. The patients' immune status seems to be an important factor regarding the progression of fibrosis. The immunosuppressed liver recipients had a higher rate of fibrosis progression per year compared to patients without immunosuppression[4].

The role of immunosuppressive treatment in the occurrence and severity of HCV reinfection has been discussed controversially[5]. Since the first liver transplantation performed by Starzl in 1963, corticosteroids have been used traditionally for immunosuppression after organ transplantation due to the lack of other available immunosuppressive drugs[6]. Nowadays, a variety of potent and selective immunosuppressive drugs are available, thus allowing avoidance of immunosuppressive drugs with unselective mode of action or unfavorable side effects[7]. Corticosteroids have a well described spectrum of adverse effects including metabolic changes and an increased risk of cardio-vascular diseases. Also, corticosteroids have an unselective immunosuppressive mode of action. Furthermore, corticosteroids increased HCV replication *in vitro* and *in vivo*[8-10]. After liver transplantation, high early HCV-RNA levels could be associated with more severe recurrence of hepatitis[11]. Prieto *et al*. reported positive relationships between the number of rejection episodes, methylprednisolone (MP) boluses, treatment of rejection, cumulative dose of steroids and the development of HCV cirrhosis[12]. These findings implicated that prevention of acute cellular rejection and avoidance of high-dose or maintenance steroids could be beneficial[5], however, the experience with completely steroid-free immunosuppression after LTx is still limited.

Recently, we published a series of 30 patients receiving steroid-free immunosuppression that consisted of tacrolimus and mycophenolate mofetil (MMF)[13]. Since the hypothesis that steroid-free immunosuppression might be particularly beneficial in HCV infected liver recipients, we analyzed this subgroup of liver recipients with regard to the risk of acute rejection, and development of serious HCV related liver disease in the graft.

## MATERIALS AND METHODS

### *Patients*

Thirty adult patients underwent LTx between June 1996 and January 1999. The detailed characteristics have been previously described[13]. The indications for LTx included hepatitis C virus related cirrhosis in seven recipients. The HCV patients included 4 females and 3 males with a median (range) age of 58(43-62) years. HCV-RNA was seropositive prior to transplantation with a

median (range) viral load of $10^4$($10^3$-$10^5$) genome equivalent/mL. The serum HCV-specific RNA was detected by reverse transcription (RT)/polymerase chain reaction (PCR) as previously described by Mihm *et al*.[14]. HCV genotype was classified as II (1b) in all seven patients according to Okamoto *et al*. and Simmonds *et al*.[15,16]. Child-Turcotte-Pugh Score prior to LTx was Child A in 2, Child B in 2 and Child C in 3 patients. Four of the patients had a coexisting hepatocellular carcinoma.

### Methods

Apart from an intravenous (IV) bolus of 500 mg MP before reperfusion of the graft, recipients received no further steroids. Tacrolimus (Prograf, Fujisawa GmbH, München, Germany) was started 6 h after reperfusion at an oral dosage of 2×0.05 mg/kg·d. Thereafter, tacrolimus dosages were adjusted to a target range of 10-15 μg/L during the first three months and 5-10 μg/L after the third month after LTx. Tacrolimus trough levels were measured by a microparticle enzyme immunoassay (Prograf-MEIA II, Abbott Laboratories, Chicago, IL, USA). MMF (Cell Cept, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) was given orally starting from the first postoperative day (pod) at a body mass adjusted dosage of 2×15 mg/kg·d. Liver biopsies were performed routinely on d 7, after 6 mo, every year, and whenever clinically indicated. Acute rejection was defined as abnormal liver function tests, histological changes as graded by the Banff classification, and the necessity for therapy[12]. Treatment of acute rejection consisted of IV-MP boluses of 500 mg over a period of 3-5 d with or without additional prednisolone tapering. Combined antiviral therapy consisting of interferon alfa and "low-dose" ribavirin was used in patients with HCV reinfection accompanied by chronic active hepatitis as described previously by Wietzke *et al*.[17,18].

### Statistical analysis

Actual survival was calculated by the Kaplan-Meier method.

## RESULTS

### Patient and graft survival

Twenty-two of the 30 patients receiving initial steroid-free immunosuppression have been alive after a median (range) follow-up of 1 386(44-2 037) d. Actual patient survival was 86.7% at 2 years and 73.3% at 5 years (Figure 1A). The actual patient survival of HCV-infected recipients was 85.7% at 2 years and 57.1% at 5 years, the difference was not statistically significant (Log-rank test (Cox-Mantel): $P$=0.33) (Figure 1B).

At present, four of the seven HCV infected liver recipients have been alive with a median (range) follow-up of 47(46-60) mo after LTx. None of the deaths in the HCV patients was related to HCV reinfection. The causes of the 3 deaths were chronic graft dysfunction (CDF) on pod 164, heart failure due to preexisting toxic cardiomyopathy on pod 774, and a *de-novo* ovarian carcinoma on pod 950. One of the seven patients with HCV-related cirrhosis underwent successful retransplantation for primary nonfunction (PNF) on day 5 (Table 1).

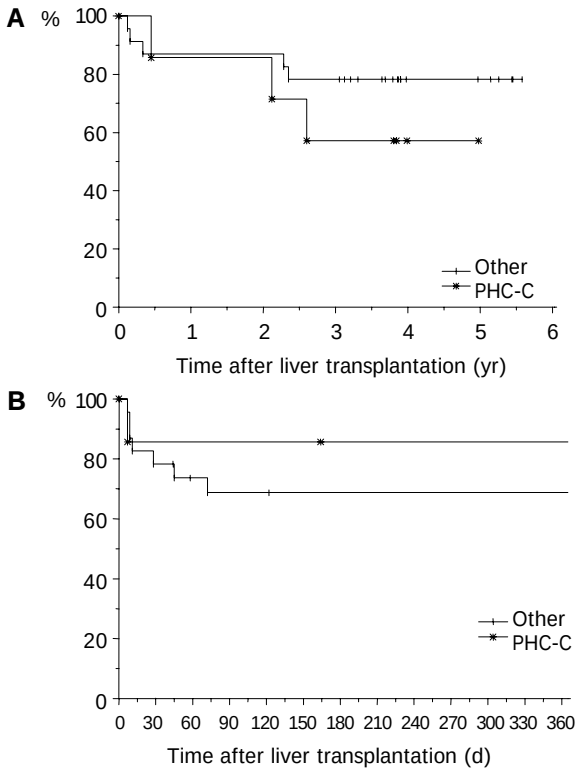


**Figure 1** A: Actual patient survival according to primary indication (HCV related cirrhosis *vs* others) of liver recipients receiving steroid-free immunosuppression. B: Actual rejection free interval according to primary indication (HCV related cirrhosis *vs* others) during the first year after LTx in liver recipients receiving steroid- free initial immunosuppression.

### Acute rejection episodes and immunosuppression

In total, 8 of the 30 liver recipients (26.2%) had an episode of acute rejection. The onset of acute rejection was between pod 7 and 72. The median (range) grade of acute rejection was 5.5(3-8) according to Banff classification. Low tacrolimus trough levels associated with diarrhea were observed in five of the eight recipients with acute rejection. Also, diarrhea was

**Table 1** Characteristics of liver recipients with HCV-infection receiving initial steroid-free immunosuppression

| # | Gender (M/F) | Rge (yr) | Child Class | HCC | HCV genotype | HCV viral load | | | | Liver histology | Outcome (pod) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | prior | ≤1 mo | ≤4 mo | 1 yr | | |
| 5 | f | 60 | C | no | II (1b) | $10^4$ | $10^5$ | $10^4$ | $10^5$ | | died (950), ovarial-ca |
| 9 | f | 62 | B | no | II (1b) | $10^5$ | $10^5$ | $10^6$ | $10^5$ | AR, hepatitis (+/-) | alive (1817), reLTx (5) |
| 12 | m | 57 | A | yes | II (1b) | $10^5$ | $10^5$ | $10^4$ | | | died (774), heart failure |
| 13 | f | 59 | C | yes | II (1b) | $10^5$ | $10^5$ | $10^5$ | | hepatitis (+/-), fibrosis (+) | alive (1456) |
| 19 | m | 58 | B | yes | II (1b) | $10^4$ | $10^3$ | | $10^4$ | hepatitis (+) | alive (1402) |
| 20 | m | 46 | A | yes | II (1b) | $10^3$ | | $10^4$ | $10^4$ | | alive (1389) |
| 25 | f | 43 | C | no | II (1b) | $10^4$ | $10^4$ | $10^5$ | | | died, CDF (164) |

Abbreviations: # (patient number), HCC (hepatocellular carcinoma), HCV (hepatitic C virus), pod (postoperative day), yr (year), mo (mo), prior (prior to LTx), AR (acute rejection), +/++/+++/- (minimal/moderate/severe/reversed), reLTx (liver retransplantation), CDF (chronic graft dysfunction).

the predominant adverse effect when tacrolimus and MMF were given simultaneously. All acute rejection episodes completely resolved after IV-MP boluses and four of the patients with rejection episodes received additional steroid taper. In the further postoperative course, there was no graft loss due to rejection and all of these patients have been alive. Only one of the eight patients with acute rejection had HCV-related cirrhosis. This patient underwent retransplantation on pod 5 for PNF. Until retransplantation, immunosuppression was temporarily discontinued and the patient suffered from diarrhea, thus tacrolimus trough levels were low.

Only 3 of the 7 HCV patients received steroids after LTx, two in conjugation with tacrolimus first and then cyclosporine (CyA), and one for acute rejection. One patient was reconverted to tacrolimus after suspicion of CDF. MMF was intermittently discontinued in all 7 patients and withdrawn in 5 patients. The cause of MMF withdrawal was diarrhea in 3 patients and conversion to CyA in 2 patients. Maintenance immunosuppression in the HCV infected recipients consisted of tacrolimus monotherapy in 3 patients, tacrolimus/MMF in 2 patients, tacrolimus/prednisolone in 1 patient, and CyA/prednisolone in 1 patient.

### *HCV reinfection*

All 7 HCV infected patients had HCV genotype II (1b). Recurrence of HCV-RNA in serum occurred within 4 mo after LTx in all seven liver recipients with HCV-related cirrhosis. Prior to LTx the median viral load was $10^4$($10^3$-$10^5$) genome equivalent/mL. During the first four months, the viral load ranged from $10^3$ to $6.5\times10^5$ genome equivalent/mL. The virus load was not markedly increased during the first year after LTx (Figure 2). Histologically, only 1 HCV patient had suspicion of minimal portal hepatitis in the most recent biopsy that occurred 15 mo after LTx and transaminases were within normal values (Table 1). Five months after LTx, another recipient had elevated transaminases (AST 55, ALT 109 U/L), suspicion of portal hepatitis and mild fibrosis in the graft. Combination therapy with subcutaneous interferon alfa-2a 3 MU three times per week and oral ribavirin 10 mg/kg body mass in three divided doses per day was given for 6 mo. At the end of treatment, the patient had transaminases within normal values (AST 9, ALT 12 U/L), and histological improvement without fibrosis, but positive serum HCV-RNA. A third recipient developed suspicion of minimal portal hepatitis and fibrosis associated with a reduction of small bile ducts. Liver histology improved after reintroduction of MMF. Initially, this patient underwent retransplantation for PNF and acute rejection was resolved.

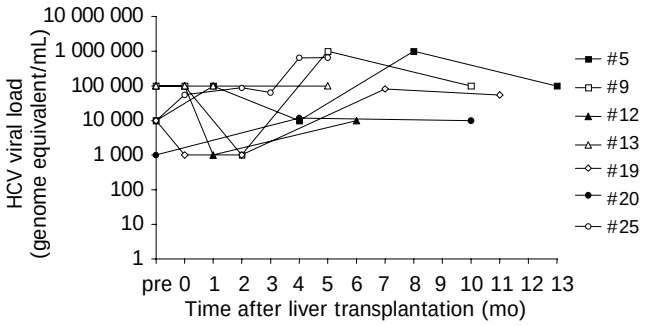


**Figure 2** Serum virus concentration in seven HCV infected recipients during the first year after LTx. All patients received initial steroid-free immunosuppression.

## DISCUSSION

Although steroids are known to increase HCV replication[8], they are given routinely for prophylaxis and treatment of rejection after solid organ transplantation. The potential risk of acute rejection must be faced against the benefit of steroid withdrawal. After LTx, HCV infection has been clearly identified as a risk factor for the development of early acute rejection[19]. On the other hand, the presence, number, and treatment of acute rejections were found to be associated with the histologic recurrence of HCV infection after LTx[20,21]. In 96 liver recipients, histologic recurrence of HCV infection occurred in 18% without acute rejection, 42% with one acute rejection episode, and 70% with more than one episode of acute rejection[21]. In liver recipients, MP treatment for acute rejection was found to increase serum HCV RNA between 4 to 100 fold[8]. OKT3, used for treatment of steroid-resistant rejection after LTx, was associated with more severe recurrences[20]. The mutual relation between treatment of acute rejection and recurrence of HCV-infection after LTx sets the stage for a completely steroid-free immunosuppressive protocol.

Recently, it was clearly demonstrated that steroid-free immunosuppression after LTx was feasible and safe[13,22-24]. The different immunosuppressive regimens consisted of CyA/azathioprine (Aza)[23], CyA or tacrolimus monotherapy[22], CyA/rapamycin, rapamycin monotherapy[24], and tacrolimus/MMF[13]. The reported rates of acute rejection were 65% for CyA and 66% for tacrolimus monotherapy[22]. The combination of CyA/Aza resulted in 80% of acute rejection, but only 9% of acute rejections required treatment. Compared to patients receiving CyA/Aza/prednisone, the incidence and severity of rejection were similar in both groups but the dynamics of virus replication of HCV-RNA was faster among those treated with prednisone. The 2 year survival rate was 70.2% with prednisone and 78.3% without prednisone[23]. The rate of acute rejection using rapamycin/CyA was 28% and with sirolimus monotherapy 75%[24].

We reported that complete avoidance of corticosteroids after LTx could be performed without an increased risk of mortality, morbidity, and severe acute rejection. The rate of acute rejection with the use of tacrolimus in combination with MMF was only 26.2%[13]. This rate of acute rejections might even be reduced, if underimmunosuppression were avoided. Low tacrolimus trough levels were observed in patients with diarrhea when tacrolimus and MMF were given simultaneously. Diarrhea could be avoided after introduction of a two hour dosing interval between tacrolimus and MMF administration[13,25]. Therefore, the rate of acute rejections might be lower if tacrolimus and MMF are administered with a dosing interval.

In our present study, only 1 HCV patient developed acute rejection. This patient had a complicated clinical course with PNF, retransplantation, temporary discontinuation of immunosuppression, acute rejection, and received MP boluses as well as intermittent steroids until pod 255. Reinfection with HCV was noticed in all seven patients. Within 4 mo after LTx, all seven patients were HCV seropositive. Compared to the pretransplant HCV load, there was no significant increase during the first year after LTx. Also, liver histology was completely unsuspicious in 4 HCV patients. Three patients had suspicion of minimal portal hepatitis which was followed up by frequent biopsies in one patient. Another patient with supposed minimal portal hepatitis and additional mild fibrosis received combination therapy which improved transaminases and histology[17]. Also, one patient with previous retransplantation developed portal hepatitis and fibrosis. MMF was reintroduced for reduction of small bile ducts, minimal portal hepatitis and fibrosis and resulted in histological improvement. None of the HCV infected patients developed serious chronic liver disease. However, one patient died of CDF which was related to underimmunosuppression after conversion to CyA for

suspicion of tacrolimus induced neurotoxicity. From our limited experience with the small number of patients, the avoidance of steroids did not alter the posttransplant HCV reinfection in serum and the histological alterations were mild despite all patients had HCV genotype II (1b). Feray *et al*. reported that HCV genotype II (1b) was a risk factor for recurrent hepatitis[26,27]. However, the influence of HCV genotype II (1b) has been controversial. Gordon *et al*. reported a similar frequency of recurrent hepatitis with all HCV genotypes, but HCV genotype II (1b) was associated with a higher rate of cirrhosis[28]. Thus, the avoidance of steroids seems to be beneficial for liver recipients with HCV genotype II (1b).

Hitherto, it remains unclear which immunosuppressive regimen is best for HCV liver recipients. MMF was discussed controversially in liver transplant recipients with HCV[29]. In a recent prospective randomized trial comparing tacrolimus/prednisolone with tacrolimus/MMF/prednisolone in liver recipients with HCV, MMF had no impact on patient and graft survival, rejection or rate of HCV recurrence[30]. However, MMF might reduce the frequency of acute rejection and therefore exposure to steroids[31]. Our intention was to use a safe steroid-free immunosuppressive regimen. Tacrolimus is a potent immunosuppressive drug, but impairment of renal function might limit the use of tacrolimus especially during the early phase after LTx. Thus, we used a combination of tacrolimus and MMF which resulted in a low frequency of acute rejection[13]. However, the combination of tacrolimus and MMF could cause diarrhea or other side effects that often required discontinuation or withdrawal of MMF. Thus, therapeutic drug monitoring of tacrolimus is an essential requirement for the combination of tacrolimus and MMF. Alternatively, anti-IL-2 receptor antibodies might have potential in steroid-free regimens. The use of anti-IL-2 receptor antibodies reduces the frequency of acute rejection after LTx, but there has been limited experience with HCV infection when anti-IL-2 receptor antibodies are given for induction of immunosuppression. Most recently, two immunosuppressive protocols using daclizumab have been reported with controversial results regarding HCV infection. Tacrolimus/MMF was compared with daclizumab/tacrolimus/MMF in a randomized open-label study and corticosteroids were eliminated 24 h after LTx in both arms. Steroids could be avoided safely after pod 1 and HCV recurrence was documented in 2 patients who did not receive daclizumab[32]. In a group of 21 HCV infected liver recipients receiving daclizumab, MMF, and steroids for induction of immunosuppression, patients with HCV infection administered daclizumab were more likely to have an earlier onset of hepatitis, jaundice and greater histological activity compared with a well-matched HCV control group. Also, recurrent hepatitis progressed more rapidly in the daclizumab group. Nelson *et al*. concluded that daclizumab in combination with MMF might be associated with early recurrence of HCV and more rapid histological progression of disease in the early period after LTx[33]. Also, triple and double immunosuppressive regimens had a higher rate of fibrosis and cirrhosis in HCV infected patients compared to monotherapy[34,35]. These findings implicate that strenuous induction protocols should be avoided in HCV infected liver recipients.

As proposed by some centers, early withdrawal or avoidance of steroids in HCV infected patients could be beneficial. From our experience with the limited number of HCV infected liver recipients, initial steroid-free immunosuppression is safe, has a low risk of acute rejection without a need for high-dose steroids and high cumulative steroid dosages, which are likely to in duce less severe HCV reinfection. Furthermore, none of the HCV infected patients developed serious chronic liver diseases. Therefore, it is high time to seriously reconsider the use of any steroids in liver transplantation especially for HCV-infection patients and to prove this approach in a large randomized controlled trial.

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• VIRAL HEPATITIS •

# Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii

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## Abstract

**AIM:** Hepatitis B virus (HBV) genomes in carriers from Hawaii have not been evaluated previously. The aim of the present study was to evaluate the distribution of HBV genotypes and their clinical relevance in Hawaii.

**METHODS:** Genotyping of HBV among 61 multi-ethnic carriers in Hawaii was performed by genetic methods. Three complete genomes and 61 core promoter/precore regions of HBV were sequenced directly.

**RESULTS:** HBV genotype distribution among the 61 carriers was 23.0% for genotype A, 14.7% for genotype B and 62.3% for genotype C. Genotypes A, B and C were obtained from the carriers whose ethnicities were Filipino and Caucasian, Southeast Asian, and various Asian and Micronesian, respectively. All cases of genotype B were composed of recombinant strains with genotype C in the precore plus core region named genotype Ba. HBeAg was detected more frequently in genotype C than in genotype B (68.4% *vs* 33.3%, $P<0.05$) and basal core promoter (BCP) mutation (T1762/A1764) was more frequently found in genotype C than in genotype B. Twelve of the 38 genotype C strains possessed C at nucleotide (nt) position 1858 (C-1858). However there was no significant difference in clinical characteristics between C-1858 and T-1858 variants. Based on complete genome sequences, phylogenetic analysis revealed one patient of Micronesian ethnicity as having C-1858 clustered with two isolates from Polynesia with T-1858. In addition, two strains from Asian ethnicities were clustered with known isolates in carriers from Southeast Asia.

**CONCLUSION:** Genotypes A, B and C are predominant types among multi-ethnic HBV carriers in Hawaii, and distribution of HBV genotypes is dependent on the ethnic background of the carriers in Hawaii.

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## INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of chronic liver diseases including chronic hepatitis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[1]. HBV has been classified into 7 genotypes from A to G based on a sequence divergence of 8% or greater of the entire genome sequences[2-5]. The genotypes of HBV have distinct geographical distributions, which have been associated with anthropologic history[6,7].

Recently, genotypes of HBV have been reported to be an influential factor in the clinical manifestation of chronic liver disease in the host. Genotype A is associated with chronic liver disease more frequently than genotype D in Europe[8]. Genotype C induces more severe liver disease than genotype B found in Asia[9, 10]. Furthermore, it was reported that genotype B had two subgroups[11], and these subgroups influence the clinical manifestations of liver disease in these patients[12].

Hawaii has a large Asian American and Pacific Islander population. Many people have emigrated from various countries in Asia and the Pacific Basin, where HBV infection is endemic. Indeed, the estimated rate of HBV carriers in Hawaii ranges from 1.7% to 3%[13,14], higher than 0.5% of the mainland of United States. However, there has been no information on genotype distribution and sequences of HBV in multi-ethnic carriers in Hawaii.

The aim of this study was to evaluate the HBV genotypes among 61 multi-ethnic carriers in Hawaii by genetic method, and to determine the influences of HBV genotypes on the clinical characteristics.

## MATERIALS AND METHODS

### *Patients*

A total of 61 serum samples with HBsAg-positive were collected from patients with HBV infection who admitted St. Francis Medical Center in Honolulu, Hawaii between 1995 and 2000. All the patients were classified into 4 clinical groups: a symptomatic carriers (ASC) ($n$=14) who had no subjective symptoms and had consistently normal serum alanine aminotransferase (ALT) levels for at least 1 year, patients ($n$=39) with chronic liver disease and persistently elevated serum ALT levels, such as those with chronic hepatitis (CH); patients with liver cirrhosis (LC) ($n$=4), and patients with hepatocellular carcinoma (HCC) ($n$=4). A clinical diagnosis was made on the basis of serum biochemical examination, ultrasonography, computerized tomography and liver biopsy performed as required. Patients coinfected with hepatitis C virus or human immunodeficiency virus were excluded. No patients received antiviral treatment during the follow-up period. The study protocol was approved by the Ethics Committees of the participating institutions in accordance with the *1975* Declaration in Helsinki. Informed consent was obtained from each patient prior to any study related procedures.

### *Genotyping of HBV*

The serum samples were stored at -20 ℃ until assay was performed. Serum DNA was extracted from 100 μL of serum using a DNA extractor kit (Genome Science Laboratory, Fukushima, Japan). The genotypes of HBV were determined by enzyme-linked immunosorbent assay (ELISA) (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd., Tokyo, Japan) with monoclonal antibodies that are type-specific to epitope in the preS2-region product[15]. If the result of ELISA was indeterminate, the genotypes were detected by restriction fragment length polymorphism (RFLP), as previously described[16]. Genotype B was classified into 2 subgroups, "Ba" which has a recombinant sequence of genotype C in the precore/core region, or "Bj" which does not have it, by the method reported previously[12]. Genotype G of HBV was detected by PCR with hemi-nested primers deduced from the unique insertion of 36 nucleotides (nt) in the core gene that is specific to this genotype[17].

### *Sequencing of HBV genome*

Three complete genomes and 61 core promoter/precore regions of HBV were amplified by polymerase chain reaction with several primer sets, as previously described[18]. Amplified HBV DNA fragments were sequenced directly by dideoxy sequencing using a Taq Dye Deoxy Terminator cycle sequencing kit with a fluorescent 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). These nucleotide sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB105172- AB105174.

### *Molecular evolutionary analysis of HBV*

The pairwise nucleotide sequences were aligned using the CLUSTAL W program[19]. The genetic distances were calculated with the 6-parameter method, and the phylogenetic tree was constructed by the neighbor-joining method[20] using ODEN program (version 1.1.1)[21]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1 000 times. Reference sequences of HBV, shown as accession numbers, were obtained from the DDBJ/EMBL/GenBank database.

### *Statistical analyses*

Statistical differences were evaluated using the Mann-Whitney nonparametric test, the Fisher's exact probability test and the Student's *t*-test where appropriate. Differences were considered significant for a *P*-value less than 0.05.

## RESULTS

### *Genotypes of HBV*

The distribution of HBV genotypes among 61 multi-ethnic carriers in Hawaii was 14(23.0%) for genotype A, 9(14.7%) for genotype B and 38(62.3%) for genotype C. All the 9 genotype B strains were found to be Ba that has a recombinant sequence of genotype C in precore/core region. Genotypes D, E, F and G were not found in this study.

### *Comparison with clinical characteristics among HBV genotypes*

Clinical and serological characteristics were compared among the patients infected with genotypes A, B and C (Table 1). Patients with HBV genotype A were of Filipino (*n*=10), Caucasian (*n*=3) and Polynesian (*n*=1) ethnicities. Genotype B was found in patients of Chinese (*n*=4), Taiwanese (*n*=2), Vietnamese (*n*=2), and Hawaiian/Chinese (*n*=1) ethnicities. Genotype C was found in the patients whose ethnic backgrounds were various Asian (*n*=33), Micronesian (*n*=3), and Caucasian (*n*=2). There were no significant differences in terms of the age, gender, serum AST, ALT, ALP, γ-GTP and the clinical stages of liver disease among them. The proportion of HBeAg positive-phenotype in patients with genotypes A, B

**Table 1** Comparison of clinical backgrounds in carriers with HBV genotypes A, B and C

| Features | | | Genotype *n*(%) | | |
|---|---|---|---|---|---|
| | | | A (*n*=14) | B (*n*=9) | C (*n*=38) |
| Age (mean±SD, yr) | | | 49.1±14.5 | 46.9±13.0 | 43.0±11.9 |
| Gender (male:female) | | | 10:4 | 4:5 | 21:17 |
| Race | Asian | Mainland Chinese | 0(0) | 5(33) | 10(67) |
| | | Philippine | 10(83) | 0(0) | 2(17) |
| | | Taiwanese | 0(0) | 2(50) | 2(50) |
| | | Hong Kong | 0(0) | 0(0) | 8(100) |
| | | Vietnamese | 0(0) | 2(40) | 3(60) |
| | | Korean | 0(0) | 0(0) | 7(100) |
| | | Japanese | 0(0) | 0(0) | 1(100) |
| | Micronesian | | 0(0) | 0(0) | 3(100) |
| | Polynesian | | 1(100) | 0(0) | 0(0) |
| | Caucasian | | 3(60) | 0(0) | 2(40) |
| Liver disease | | ASC | 2(14) | 3(33) | 9(24) |
| | | CH | 10(71) | 5(56) | 24(63) |
| | | LC | 2(14) | 1(11) | 1(3) |
| | | HCC | 0(0) | 0(0) | 4(11) |
| | | HBeAg (+) | 8(57) | 3(33)[a] | 26(68)[a] |
| Laboratory finding | | AST (U/L) | 63.3±42.2 | 56.6±58.7 | 54.7±81.9 |
| | | ALT (U/L) | 41.4±31.6 | 34.8±27.0 | 37.3±59.7 |
| | | ALP (U/L) | 359.9±139.4 | 287.3±140.2 | 328.0±166.2 |
| | | γ-GTP (U/L) | 121.0±121.3 | 55.9±37.2 | 66.8±103.0 |

[a]*P*<0.05 between genotypes B and C. ASC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase.

and C was 57.1%, 33.3% and 68.4% respectively, with a significant difference observed between genotypes B and C ($P<0.05$). HCC was found in 10.5% of patients with genotype C.

### Comparison with core promoter/precore sequences

The frequency of mutation in core promoter (nt 1762/1764) and precore (nt 1858 and nt 1896) region was compared among genotypes A, B and C (Table 2). No significant differences in the frequency of the core promoter mutants were found among genotypes A, B and C (21.4%, 44.4%, and 57.9% respectively). Precore stop mutation (A-1896) was detected in genotypes B and C (33.3% and 21.1%) but not detected in genotype A. Sequence analysis of the mutation at nt 1858 in the precore region showed that all genotype B strains and 68% of genotype C strains possessed a T nucleotide (T-1858). In contrast, all genotype A strains had a C nucleotide in this region (C-1858).

### HBV genotype C with C-1858 or T-1858

In order to clarify the significance of nucleotide variety (C or T) at nt 1858 in clinical and virological characteristics, 12 genotype C strains with C-1858 were compared to that of 26 strains of genotype C with T-1858 (Table 3). All strains of both groups were obtained only from carriers whose ethnic background is Asian. HBeAg positive-phenotype was more frequent in genotype C patients with C-1858 than in those with T-1858 (83.3% *vs* 61.5%). The precore stop mutation (A-1896) was found in 30.8%(8/26) of those with genotype C with nucleotide T-1858, but not in those subjects with the C-1858 nucleotide. There were no significant differences in frequencies in terms of the age, gender, serum AST, ALT, ALP, γ-GTP and the clinical stages of liver disease and BCP mutation between them.

### Phylogenetic analysis

To clarify the phylogenetic characterization of genotype C with C-1858, the complete genome of three HBV strains in carriers was sequenced. These subjects were from Micronesia (HI-1), Hong Kong (HI-2) and Vietnam (HI-3). Molecular evolutionary analysis was also conducted (Figure 1). Of them, one strain (HI-1) was clustered into a subgroup of genotype C with T-1858 from Polynesian with significant bootstrap values. The HI-2 and HI-3 strains were clustered into a subgroup of genotype C from Thailand and Vietnam, and separated from

**Table 2** Comparison of core promoter / precore sequences between genotypes A, B and C

| Mutation | | | Genotype *n* (%) | | |
|---|---|---|---|---|---|
| | | | A (*n*=14) | B (*n*=9) | C (*n*=38) |
| CP mutation | | Double mutation | 3(21) | 4(44) | 22(58%) |
| PC mutation | nt 1858 | Cytosine (C) | 14(100) | 0(0) | 12(32) |
| | | Thymine (T) | 0(0) | 9(100) | 26(68) |
| | nt 1896 | Guanine (G) | 14(100) | 6(67) | 30(79) |
| | | Adenine (A) | 0(0) | 3(33) | 8(21) |

CP: Core promoter; PC: Precore; nt: Nucleotide.

**Table 3** Clinical and virological characteristics in carriers of genotype C with C-1858 or T-1858

| | | Mutation *n*(%) | |
|---|---|---|---|
| | | C-1858 (*n*=12) | T-1858 (*n*=26) |
| Age (mean±SD, yr) | | 41.7±13.0 | 43.5±11.6 |
| Gender (male:female) | | 9:3 | 12:14 |
| Race | Asian | | |
| | Mainland Chinese | 3(30) | 7(70) |
| | Filipino | 0(0) | 2(100) |
| | Taiwanese | 0(0) | 2(100) |
| | Hong Kong | 3(38) | 5(63) |
| | Vietnamese | 2(67) | 1(33) |
| | Korean | 1(14) | 6(86) |
| | Japanese | 0(0) | 1(100) |
| | Micronesian | 2(100) | 0(0) |
| | Polynesian | 0(0) | 0(0) |
| | Caucasian | 1(50) | 1(50) |
| Liver disease | ASC | 2(17) | 10(29) |
| | CH | 8(67) | 21(60) |
| | LC | 1(8) | 1(3) |
| | HCC | 1(8) | 3(9) |
| Laboratory finding | HBeAg (+) | 10(83) | 16(62) |
| | AST (U/L) | 34.9±17.0 | 63.8±97.6 |
| | ALT (U/L) | 23.2±11.2 | 43.8±71.3 |
| | ALP (U/L) | 334.6±186.3 | 324.9±157.0 |
| | γ-GTP (U/L) | 87.8±167.3 | 57.2±48.4 |
| CP mutation | Double mutation | 6(50) | 16(62) |
| PC mutation | A-1896 | 0(0) | 8(31) |

ASC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GTP: γ-glutamyl transpeptidase; CP: Core promoter; PC: Precore.

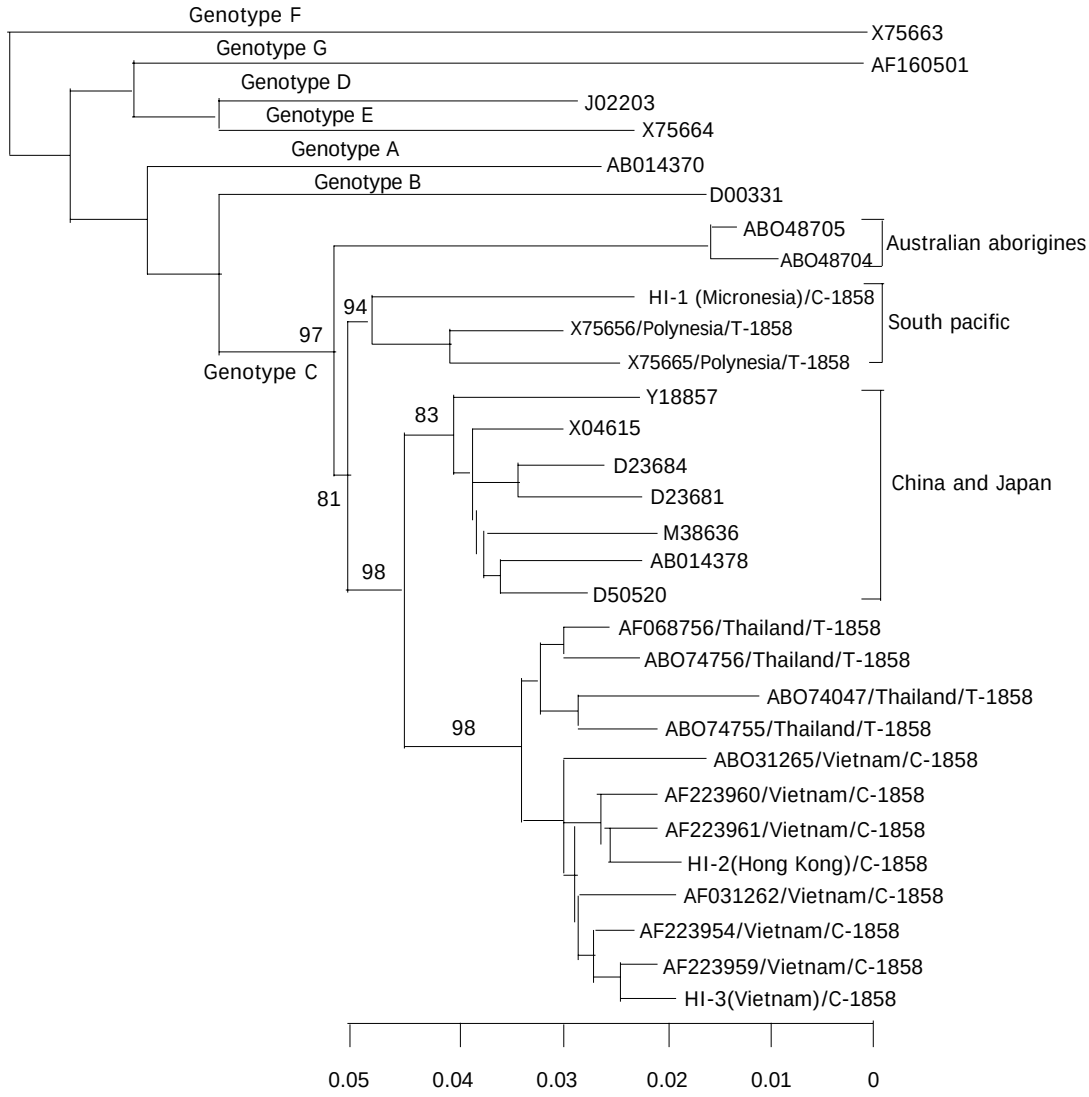


**Figure 1** A phylogenetic tree based on the complete genome sequences of hepatitis B virus with 27 reference strains. Isolate names of HI-1, HI-2 and HI-3 were sequenced in this study. The length of the horizontal bar indicates the number of nucleotide substitutions per site. Numbers beside the main roots are the results of bootstrap analysis.

those strains of China and Japan. Moreover, the strains from Thailand and Vietnam had separate branches, and Hawaiian strains were clustered into the branch with Vietnam strains. Interestingly, all strains from Vietnam had C-1858, and those from Thailand had T-1858.

## DISCUSSION

The findings of the present study indicate that HBV genotypes A, B and C are prevalent in Hawaii, and genotype C is the major genotype. Most cases of genotype A were found in immigrants from the Philippines and countries known to be prevalent regions for genotype A[6]. Genotype B was found only in immigrants from Asian regions where genotype B was endemic. In addition, genotype C was obtained from immigrant who came from various Asian countries, where genotype C was prevalent[22]. These results indicate that the distribution of HBV genotypes in Hawaii is associated with their respective ethnic background.

Recently, it was reported that genotype B could be classified into the Bj (j standing for Japan) and Ba (a standing for Asia) subgroups. Ba shared a genomic sequence with genotype C in the precore/core region, which was prevalent in Asian countries. Bj was restricted to Japan, and did not have this recombination[11]. It was shown that Ba induces more severe liver disease than Bj due to delayed seroconversion of HBeAg[12]. In this study, we found that genotype B, prevalent in Hawaii, was classified as Ba because they were all obtained from carriers with Asian ethnicity (excluding Japanese). In addition, the rate of positive HBeAg (33.3%) and basal core promoter (BCP) mutation (44.4%) in patients of Hawaii infected with genotype B were higher than those in Japanese patients with genotype B[9]. This result is also consistent with a previous report.

The double mutation in the core promoter, A-to-T mutation at nt 1762 and G-to-A mutation at nt 1764, was associated with reduced synthesis of precore mRNA[23,24]. In addition, it has been reported that the BCP mutation was associated with the progression of liver disease[25]. In this study, although it was not significant, BCP mutation was detected more frequently in genotype C than in genotype B. This result supports our previous observation that the BCP mutation was significantly more frequent in genotype C patients than in genotype B patients[9]. In addition, the present study demonstrated that the proportion of HBeAg positivity in genotype C was significantly higher than that in genotype B (68.4% *vs* 33.3%). However, our study could not show the clinical difference between genotypes B and C most likely due to the small number of patients studied. In the future, a case-controlled study in multi-ethnic carriers with larger samples is required to confirm

if genotype C could induce more severe liver disease than genotype B[9,10].

Interestingly, we detected 12 strains of genotype C possessing C-1858 in Hawaii. HBV strain with C-1858 could prevent the A-1896 precore mutation from shutting off the synthesis of HBeAg[26]. This C-1858 variant was frequently found in genotypes A and F[26]. In genotype C, the C-1858 variant was observed in Southeast Asian patients, and the phylogenetic origin of genotype C with C-1858 variant has been reported from Vietnam recently. In this study, the complete genomes of 3 genotype C strains with C-1858 were sequenced. One strain obtained from a Micronesian patient with C-1858 was clustered with previously reported Polynesian strains with T-1858. This indicates that both the C-1858 and T-1858 strains of genotype C are endemic to South Pacific countries. Two other strains obtained from patients with Hong Kong and Vietnamese ethnicities were clustered with the strains of genotype C from Southeast Asian countries. This result is consistent with geographic distribution of HBV genotype[18]. Interestingly, in this subgroup, there were 2 variants of strains, one had C-1858, prevalent in Vietnam, and the other had T-1858, prevalent in Thailand.

The clinical significance of C-1858 or T-1858 among genotype C is not well known. In this study, we compared the clinical and laboratory characteristics between C-1858 and T-1858 variant, but there were no significant differences between them. The number of patients was not enough to clarify the importance of this variation, and its significance for clinical characteristics remains unknown. Further studies would be required using larger numbers of samples.

In conclusion, genotypes A, B and C are the predominant types among multi-ethnic HBV carriers in Hawaii, and the distribution of these genotypes is dependent on the ethnic origin of the carriers in Hawaii. The influence of these genotypes on the clinical manifestations of these HBV carriers in Hawaii is not well defined due to the current small sample size. Case-controlled study with larger cohorts from our unique community is needed.

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• VIRAL HEPATITIS •

# HCV NS5A abrogates p53 protein function by interfering with p53-DNA binding

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## Abstract

**AIM:** To evaluate the inhibition effect of HCV NS5A on p53 transactivation on p21 promoter and explore its possible mechanism for influencing p53 function.

**METHODS:** p53 function of transactivation on p21 promoter was studied with a luciferase reporter system in which the luciferase gene is driven by p21 promoter, and the p53-DNA binding ability was observed with the use of electrophoretic mobility-shift assay (EMSA). Lipofectin mediated p53 or HCV NS5A expression vectors were used to transfect hepatoma cell lines to observe whether HCV NS5A could abrogate the binding ability of p53 to its specific DNA sequence and p53 transactivation on p21 promoter. Western blot experiment was used for detection of HCV NS5A and p53 proteins expression.

**RESULTS:** Relative luciferase activity driven by p21 promoter increased significantly in the presence of endogenous p53 protein. Compared to the control group, exogenous p53 protein also stimulated p21 promoter driven luciferase gene expression in a dose-dependent way. HCV NS5A protein gradually inhibited both endogenous and exogenous p53 transactivation on p21 promoter with increase of the dose of HCV NS5A expression plasmid. By the experiment of EMSA, we could find p53 binding to its specific DNA sequence and, when co-transfected with increased dose of HCV NS5A expression vector, the p53 binding affinity to its DNA gradually decreased and finally disappeared. Between the Huh 7 cells transfected with p53 expression vector alone or co-transfected with HCV NS5A expression vector, there was no difference in the p53 protein expression.

**CONCLUSION:** HCV NS5A inhibits p53 transactivation on p21 promoter through abrogating p53 binding affinity to its specific DNA sequence. It does not affect p53 protein expression.

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## INTRODUCTION

Hepatitis C virus (HCV) is recognized as a major causative agent leading to chronic hepatitis and cirrhosis[1-3], which have a close relationship with the development of hepatocellular carcinoma (HCC)[4,5]. HCV is a positive single-stranded RNA virus belonging to the Flaviridae family, and its genome only contains a single long open reading frame encoding a large polyprotein precursor that is thereafter processed by a combination of cellular and viral proteases into several mature proteins, including three or four structural proteins (core, E1, E2/P7) and at least 6 nonstructural proteins (NS): NS2, NS3, NS4A, NS4B, NS5A, NA5B[6]. HCV NS5A as a nonstructural protein does not assemble into the HCV particles, but still has a lot of functions in the HCV replication and the development of chronic liver disease and hepatocellular carcinoma. Recent studies showed that HCV NS5A could interact with a number of cellular proteins including PKR, p53, Grb2 and Cdk1[7-10], and enhance expression of some genes related to cell proliferation such as PCNA, NF-κB, Stat-3, SRCAP and IL-8[11-14], indicating that HCV NS5A has a critical role in promoting cell proliferation and malignant transformation. HCV NS5A prevents p53 and TNF-a mediated apoptosis[15-17], and possibly perturbs the DNA repair when cells are treated with DNA damage agents including viruses, toxins or physical damage. HCV NS5A is important for the HCV replication. It can form a complex with an SNARE-like protein, hVAP-33 and HCV NS5B[18] and can associate with ER and Golgi complex, and the amphipathic helix (AH) of the HCV NS5A is necessary for this membrane localization and for HCV RNA replication[19,20]. Interaction of HCV NS5A with La protein can also maintain and benefit HCV RNA replication and HCV protein translation[21,22]. Sequence mutation in HCV NS5A might be a reason for the responsiveness in the patient who received IFN treatment[23-25]. The patients with wild type of interferon sensitive-determining region (ISDR) in HCV NS5A usually have a lower responsive rate to the IFN therapy, and the mechanism is that the wild type ISDR can bind PKR, which is induced by IFN and has an anti-viral activity, and can disrupt PKR function[7,26,27]. The tumor suppressor p53 protein has been reported to possess a number of important functions. Activated p53 can transactivate a lot of target genes, maintain normal cell cycle through inducing apoptosis and repairing damaged DNA, and suppressing oncogenic transformation. In this study we explored the interaction between HCV NS5A and p53 and the mechanism by which the HCV NS5A abrogated the p53 transactivation.

## MATERIALS AND METHODS

### *Cell lines and plasmids*

$P^{C53\text{-}NS3}$, $P^{wwp\text{-}luc}$ and $P^{wwp\text{-}mut\text{-}luc}$ were kind gifts from Professor Vogelstein (The Johns Hopkins University). $P^{C53\text{-}NS3}$ is an eukaryotic expression vector that is constructed by cloning normal human p53 cDNA into $p^{CMV}$ plasmid. $P^{wwp\text{-}luc}$ carries luciferase reporter gene driven by p21 promoter that contains the specific consensus DNA sequence binding to p53 protein. $P^{wwp\text{-}mut\text{-}luc}$ is approximately the same vector as $P^{wwp\text{-}luc}$ except for deletion mutation introduced in the p53 binding sequence

of p21 promoter. $P^{CNS5A}$ is made in Dr. Siddiqui's laboratory (University of Colorado, USA) through inserting of HCV NS5A cDNA into $P^{CMV}$ plasmid[12]. The liver carcinoma cell lines Huh7 with *p53* gene mutation and HepG2 with wild-type *p53* gene were from ATCC(USA) and maintained in Dulbecco's Modified Eagle Medium (Life Technologies, USA) complemented with 100 mL/L fetal calf serum. The cells were transfected by the individual plasmids with Lipofectin reagent (Life Technologies, USA) when they became 60-70% confluent. Forty-eight hours after transfection, the cells were harvested for the analysis of relative luciferase activity and electrophoretic mobility shift assay (EMSA).

### *Relative luciferase activity assay*

After 48 h of transfection, cells in the plates were washed two times with PBS, and then 750 μL lysis buffer (0.1 mmol/L $K_2HPO_4$, pH 7.8, 10 mmol/L DTT, 5 g/L NP-40) was added onto plates. The plates were then placed on ice for 15 min and then cells were transferred into a clean 1.5-mL Eppendorf tube. After centrifugation at 15 000 *g* for 10 min, the supernatants were collected for further experiment. For relative luciferase activity assay, 0.4 mL determination buffer (0.1 mmol/L $K_2HPO_4$, pH 7.8, 0.15 mmol/L $MgSO_4$, 10 mmol/L DTT, 0.5 mmol/L ATP) and the different volumes of supernatant containing equivalent protein were put into 5-mL glass tube to observe the times of luminescence within 10 s using a luminometer (Optocomp II, MGM,USA). The experiment was repeated three times and each in triplicate.

### *Electrophoretic mobility-shift assay (EMSA)*

In this experiment, we investigated the ability of p53 binding to the specific consensus DNA sequence, and studied if the binding could be influenced by HCV NS5A. Briefly, this experiment was done as following: (1) Labeling of DNA probe: 50 pmol chemically synthesized oligonucleotide 5' - GGACATGCCCGG GCATGTCC -3' which is the specific consensus DNA sequence to bind p53, 2 μL γ-$P^{32}$ ATP (74 000 GBq/mmoL), 20 U *T4* polynucleotide kinase, 2 μL reaction buffer and incubated for 30 min at 37 ℃, then 60 μL $dH_2O$ was added to the mixture and the labeled oligonucleotides were purified from the free γ-$P^{32}$ ATP by use of Sephadex-G25. The purified $P^{32}$-labeled DNA probe was stored at -20 ℃ until use. (2) Nuclear protein isolation: After transfection with different plasmids for 48 h, cells were washed 3 times with PBS and scrapped down from the plates and then transferred into 1.5-mL Eppendorf tubes and washed 3 times again with PBS. Lysis buffer 30 μL (20 mmol/L Hepes, pH 7.9, 10 mmol/L KCl, 10 mmol/L $Na_3VO_4$, 1 mmol/L ETDA, 100 mL/L glycerol, 1 mmol/L PMSF, 3 g/L aprotinin, 1 g/L leupeptin, 1 mmol/L DTT, 2 g/L NP-40, 20 mmol/L NaF) was added to suspend the cells. After 15 min on ice, and centrifugation at 15 000 ×g for 1 min, the supernatant was discarded and the pellet resuspended in 20 μL nuclear extract buffer (20 mmol/L Hepes, pH7.9, 10 mmol/L KCl, 420 mmol/L NaCl, 10 mmol/L $Na_3VO_4$, 1 mmol/L ETDA, 200 mL/L glycerol, 1 mmol/L PMSF, 3 g/L aprotinin, 1 g/L leupeptin, 1 mmol/L DTT, 20 mmol/L NaF). After incubation at 4 ℃ for 30 min. with mild agitation, and centrifugation at 15 000 *g* for 5 min, aliquot supernatants were collected and stored at -70 ℃ until use. (3) Assessment of p53 binding to DNA probe: For each reaction tube, the followings were added: 2 μg nuclear extraction protein, 2 μL labeled probe containing p53 binding sequence, 1.5 μL EMSA reaction buffer (50 mmol/L NaCl, 0.5 mmol/L ETDA, 40 mL/L glycerol, 1 mmol/L $MgCl_2$, 1 mmol/L DTT, 10 mmol/L Tris-Cl, pH 7.5, 0.05 g/L poly (dI-dC) ) and $dH_2O$ to a total volume of 15 μL. After incubation for 30 min on ice, 20 μL of samples was loaded onto60 g/L undenatured PAGE and run at 160 V for approximately 6 h. The gel was dried and autoradiographed at -70 ℃ for about 16 h.

### *Western blotting to detect p53 and HCV NS5A protein expression*

Cell lysates of Huh7 cells transfected with $P^{C53\text{-}NS3}$ or co-transfected with $P^{CNS5A}$ were made by routine method. The same amount of total protein from the cell lysate was used to load onto 120 g/L PAGE, and then transferred onto PV (polyvinylidene difluoride) membrane. After blocked in block solution (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 3 g/L PVP, 5 g/L Tween 20) for 2 h, the membrane was probed by anti-HCV NS5A or anti-p53, washed and incubated with the second enzyme-labeled antibody, and then observed by using an ECL kit (Boegringer Mannhein Co., Germany).

### *Statistical analysis*

Statistical significance of differences between the 2 groups was determined by applying Student's *t* test or two-sample *t* test. Statistical significance of differences in more than 3 groups was determined by applying one-way ANOVA. A *P* value less than 0.05 was considered significant.

## RESULTS

### *HCV NS5A protein inhibited the transactivation of endogenous p53 on p21 promoter*

Relative luciferase activities in $P^{wwp\text{-}luc}$-transfected HepG2 cells ( $3.95\times10^5$) were significantly higher than that in $P^{wwp\text{-}mut\text{-}luc}$ group ( $0.60\times10^5$, $t$=5.92, $P$<0.01). Compared to $P^{wwp\text{-}luc}$-transfected HepG2 cells, the relative luciferase activities in the HepG2 cells co-transfected with $P^{wwp\text{-}luc}$ and $P^{CNS5A}$ decreased in a $P^{CNS5A}$ dose- dependent manner ($F$=20.71, $P$<0.01), suggesting that HCV NS5A protein can inhibit the transactivation of endogenous p53 on p21 promoter. In contrast, the relative luciferase activities of the HepG2 cells co-transfected with $P^{wwp\text{-}mut\text{-}luc}$ and $P^{CNS5A}$ had little change ($F$=0.76, $P$>0.05) in comparison with $P^{wwp\text{-}mut\text{-}luc}$-transfected HepG2 cells. Those results indicated that endogenous p53 has a transactivation on p21 promoter, and HCV NS5A down-regulated p21 promoter activity through inhibiting endogenous p53 activity (Table 1).

**Table 1** HCV NS5A inhibits the transactivation of endogenous p53 on p21 promoter

| Group | Relative luciferase activity | *F* | *P* |
|---|---|---|---|
| $p^{wwp\text{-}luc}$ (1 μg) | | | |
| + $p^{CNS5A}$ (0 μg) | $3.49\times10^{5b}$ | 20.71 | <0.01 |
| + $p^{CNS5A}$ (0.5 μg) | $2.69\times10^5$ | | |
| + $p^{CNS5A}$ (1.0 μg) | $1.62\times10^5$ | | |
| + $p^{CNS5A}$ (2.0 μg) | $0.58\times10^5$ | | |
| $p^{wwp\text{-}mut\text{-}luc}$ (1.0 μg) | | | |
| + $p^{CNS5A}$ (0 μg) | $0.60\times10^5$ | 0.76 | >0.05 |
| + $p^{CNS5A}$ (0.5 μg) | $0.65\times10^5$ | | |
| + $p^{CNS5A}$ (1.0 μg) | $0.62\times10^5$ | | |
| + $p^{CNS5A}$ (2.0 μg) | $0.64\times10^5$ | | |

[b]$t$=5.92, $P$<0.01.

### *HCV NS5A protein abrogated exogenous p53 transactivation on p21 promoter*

The mean of relative luciferase activity in the Huh7 cells individually transfected with $P^{wwp\text{-}luc}$ and $P^{wwp\text{-}mut\text{-}lu}$ was $0.47\times10^5$ and $0.45\times10^5$ respectively and there were no statistically differences between these two groups ($t$ =1.16, $P$>0.05). This might be because *p53* gene in Huh7 cells has been mutated and p53 protein has lost its normal transactivation function, therefore, no matter what happened to p21 promoter (wild type or mutated), the expression of p21 promoter driven luciferase gene was not affected. When $P^{wwp\text{-}luc}$ or $P^{wwp\text{-}mut\text{-}luc}$ was co-

transfected with $P^{C53\text{-}NS3}$ into Huh7 cells, respectively, the relative luciferase activities in the $P^{wwp\text{-}luc}$ + $P^{C53\text{-}NS3}$ group greatly increased ($\chi=5.63\times10^5$), but the relative luciferase activities in the $P^{wwp\text{-}mut\text{-}luc}$ + $P^{C53\text{-}NS3}$ group only mild increased ($\chi=0.50\times10^5$), and a remarkable variance between the two groups existed ($t$=10.12, $P$<0.01). These results indicated that exogenous p53 should have a strong transactivation on p21 promoter. In order to see if HCV NS5A protein has any effect on the exogenous p53 transactivation, we used $P^{CNS5A}$, $P^{C53\text{-}NS3}$and $P^{wwp\text{-}luc}$ to co-transfect Huh7 cells, and the result showed that the relative luciferase activities in the $P^{CNS5A}$+$P^{C53\text{-}NS3}$+$P^{wwp\text{-}luc}$ group cells decreased in a $P^{CNS5A}$ dose-dependent way ($F$=24.23, $P$<0.01). $P^{CNS5A}$ and $P^{wwp\text{-}luc}$ were also used to transfect Huh7 cells to find out if HCV NS5A had a direct effect on p21 promoter. The result showed that HCV NS5A did not increase or decrease the relative luciferase activity. From those experiments we proposed that HCV NS5A down-regulated p21 promoter activity by inhibiting exogenous p53 activity, and not by HCV NS5A' s direct effect on p21 promoter (Tables 2, 3).

**Table 2** HCV NS5A inhibits exogenous p53 transactivation on p21 promoter

| Group | Relative luciferase activity | *F* | *P* |
|---|---|---|---|
| $p^{wwp\text{-}luc}$ (1 μg) | $0.47\times10^{5ab}$ | 24.23 | <0.01 |
| +$p^{C53\text{-}NS3}$ (1 μg) | $5.63\times10^{5b}$ | | |
| +$p^{CNS5A}$ (0.5 μg) | $1.61\times10^5$ | | |
| +$p^{CNS5A}$ (1.0 μg) | $0.69\times10^5$ | | |
| $p^{wwp\text{-}mut\text{-}luc}$ (1 μg) | $0.45\times10^{5a}$ | 0.69 | >0.05 |
| +$p^{C53\text{-}NS3}$ (1 μg) | $0.50\times10^5$ | | |
| +$p^{CNS5A}$ (0.5 μg) | $0.43\times10^5$ | | |
| +$p^{CNS5A}$ (1.0 μg) | $0.48\times10^5$ | | |

[a]$t$=1.16, $P$>0.05; [b]$t$=10.12, $P$<0.01.

**Table 3** HCV NS5A and p21 promoter activity

| Group | Relative luciferase activity | *F* | *P* |
|---|---|---|---|
| $p^{wwp\text{-}luc}$ (1.0 μg) | $0.44\times10^5$ | 0.52 | >0.05 |
| + $p^{CNS5A}$ (0.5 μg) | $0.46\times10^5$ | | |
| + $p^{CNS5A}$ (1.0 μg) | $0.43\times10^5$ | | |
| $p^{wwp\text{-}luc}$ (1.0 μg) | $0.56\times10^5$ | 28.16 | <0.01 |
| + $p^{C53\text{-}NS}$ (0.5 μg) | $2.68\times10^5$ | | |
| + $p^{C53\text{-}NS}$ (1.0 μg) | $4.84\times10^5$ | | |

### *HCV NS5A inhibited p53 protein transactivation through abrogation of p53 binding to the specific DNA*

Through EMSA, the band of specific p53 and DNA probe complex was found in the nuclear lysates prepared from Huh7 cells transfected with $P^{C53\text{-}NS3}$, and verified by the appearance of the supershift after addition of the monoclonal anti-p53 (a generous gift of Dr. Harris, USA) or polyclonal anti-HCV NS5A antibody (a generous gift of Professor Rice, USA) in the EMSA mixtures. This p53-probe complex band did not occur in the nuclear lysates prepared from Huh7 cells untransfected with plasmids or only transfected with $P^{CNS5A}$, suggesting there is wild-type p53 protein expressed in these cells. In the next experiment, we used different doses of $P^{CNS5A}$ and constant amount (1 μg) of $P^{C53\text{-}NS3}$ to co-transfect Huh7 cells. The p53-probe band became gradually weakened and finally disappeared with increasing doses of $P^{CNS5A}$, suggesting that HCV NS5A could inhibit p53 transactivation through disruption of the binding of p53 to its specific consensus DNA (Figure 1).

### *HCV NS5A had no effect on p53 protein expression*

p53 protein was expressed at about the same level between the Huh7 cells transfected with p53 expression vector and the Huh7 cells co-transfected with $P^{CNS5A}$ and p53 expression vectors (Figure 2). This result indicates that HCV NS5A does not affect p53 protein expression.

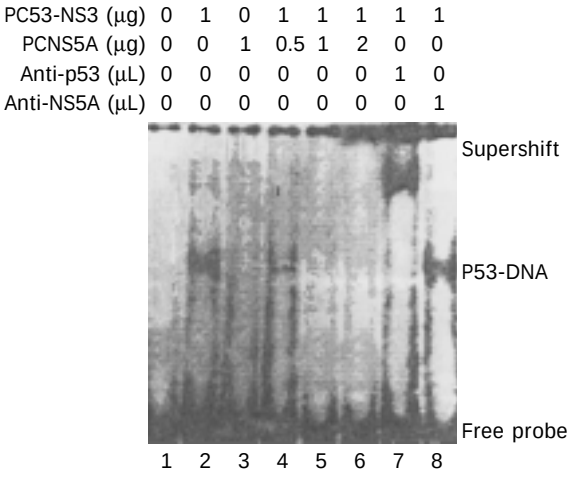


**Figure 1** HCVNS5A inhibits p53 binding to its specific DNA probe. Lane 1: Negative control; Lane 2: Transfected with pC53-NS3 only; Lane 3: Transfected with pCNS5A only; Lanes 4-6: Co-transfected with pC53-NS3 (1 μg) and different dose of pCNS5A; Lanes 7-8: Transfected with pC53-NS3, and antip53, antiNS5A anti-bodies were added to the reaction mixture individually.

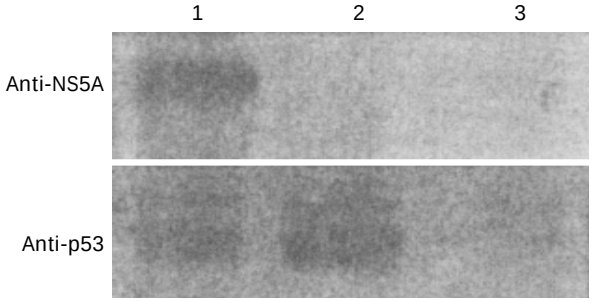


**Figure 2** Western blotting for the expression of NCV NS5A and p53 proteins. Lane 1: Huh 7 cells co-transfected with $P^{C53\text{-}NS3}$ and $P^{CNS5A}$; Lane 2: Huh 7 cells transfected with $P^{C53\text{-}NS3}$; Lane 3: Huh 7 cells untransfected.

## DISCUSSION

HCV NS5A is a phosphoprotein that exists in two different forms of $M_r$ 56 000 and 58 000 with modifications of serine residues, and $M_r$ 58 000 form is produced by additional phosphorylation of the $M_r$ 56 000 form[28-30]. Initial studies found that HCV NS5A contains acidic and proline-rich amino acids in its carboxyl-terminal, and this structure feature resembles eukaryotic transcription activator[31,32]. Later studies showed that the HCV NS5A with amino-terminal deleted (1-146) mutant did have a strong transcriptional activation, but the whole length HCV NS5A showed no apparent transcriptional activity[33]. The potential tansactivation of HCV NS5A has attracted great attention and become the research focus of the nonstructural proteins of HCV due to its important role in the pathogenesis of chronic liver disease and even hepatocellular carcinoma. Clinical observations suggested the responsiveness of patients to IFN treatment might be related to HCV NS5A ISDR sequence mutations[23-25]. Several experiments from Katze´s laboratory and others confirmed that HCV NS5A could repress RNA dependent protein kinase (PKR) function, by which HCV can escape the effect of IFN, and also inhibit apoptosis and promote cell proliferation[7,26,27]. When PKR is dysfunctioned, sustained

expression of eIF-2α in cells results in cell growth and differentiation, and induces malignant transformation and the development of tumors in nude mice. ISDR of HCV NS5A can bind the domain of PKR and prevent the PKR dimerizing which is the critical process to activate PKR function. Mapping studies found that ISDR and the adjacent C-terminal 26 amino acids can form a heterodimer with PKR. The effect of HCV NS5A on the protection of apoptosis induced by TNF-α and p53 was further identified[16,17]. In the present study, we explored the possible effect of HCV NS5A on the transactivation of p53. Luciferase reporter gene driven by p21 promoter was used in this experiment, which showed that both endogenous and exogenous p53 protein could transactivate p21 promoter as expected. But, when HCV NS5A protein was introduced, by transfection or co-transfection with HCV NS5A expressing vector, the p21 promoter function activated by p53 was greatly depressed. This finding is in agreement with previous reports[8,11]. To further understand the mechanism of interaction between HCV NS5A and p53, we did an EMSA experiment for p53 binding to its consensus DNA probe, to see if HCV NS5A could disturb the binding reaction. The result showed that HCV NS5A disrupted p53 binding to its DNA probe, and with the increase of dosage of HCV NS5A expressing vector transfected, inhibition of the binding seemed more remarkable. All the above results suggested HCV NS5A depressed the p53 transactivation by interrupting the binding between p53 and its specific DNA. Recent studies also evidenced that HCV NS5A could physically associate with p53 and form a complex, which might be the reason why p53 binding to its DNA was disrupted[8,34]. Further, we wanted to know whether p53 protein expression was affected by the presence of HCV NS5A. Through western blot, we found that after co-transfection of HCV NS5A expressing vector, the level of p53 protein expression in the cells did not show any change compared to empty vector transfected cells. This result was also similarly found in another report[11]. In summary, according to our experiments and previous reports by others, HCV NS5A represses p53 transactivation function by disrupting p53 binding to its specific DNA through HCV NS5A and p53 complex formation, not by the disturbance of the p53 protein expression. HCV NS5A can interact with many cellular factors in the host cells such as PKR, p53, Bcl2 and disrupt their functions[7,8,26,27,34,35], which are related to the processes of apoptosis. HCV NS5A can also interact with unknown cellular factors and induce the expression of genes such as SRCAP, NF-κB and PCNA[11-13], which are related to the promotion of cell proliferation. HCV NS5A seems to be a very active protein interacting with host cellular proteins and taking a major part in modulating cell growth and death, as well as in the development of hepatocellular carcinoma.

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• VIRAL HEPATITIS •

# Early diagnosis of bacterial and fungal infection in chronic cholestatic hepatitis B

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## Abstract

**AIM:** To investigate the early diagnostic methods of bacterial and fungal infection in patients with chronic cholestatic hepatitis B.

**METHODS:** One hundred and one adult in-patients with chronic hepatitis B were studied and divided into 3 groups: direct bilirubin (DBil)/total bilirubin (TBil) 0.5, without bacterial and fungal infection (group A, *n*=38); DBil/TBil <0.5, without bacterial and fungal infection (group B, *n*=23); DBil/TBil 0.5, with bacterial or fungal infection (group C, *n*=40). The serum biochemical index and pulse rate were analyzed.

**RESULTS:** Level of TBil, DBil, alkaline phosphatase (ALP) and DBil/ALP in group A increased compared with that in group B. The level of ALP in group C decreased compared with that in group A, whereas the level of TBil, DBil and DBil/ALP increased (ALP: 156±43, 199±68, respectively, *P*<0.05; TBil: 370±227, 220±206, respectively, *P*<0.01; DBil: 214±143, 146±136, respectively, *P*<0.01; DBil/ALP: 1.65±1.05, 0.78±0.70, respectively, *P*<0.001). The level of DBil and infection affected DBil/ALP. Independent of the effect of DBil, infection caused DBil/ALP to rise (*P*<0.05). The pulse rate in group A decreased compared with that in group B (63.7±6.4, 77.7±11.4, respectively, *P*<0.001), and the pulse rate in group C increased compared with that in group A (81.2±12.2, 63.7±6.4, respectively, *P*<0.001). The equation (infection=0.218 pusle rate +1.064 DBil/ALP -16.361), with total accuracy of 85.5%, was obtained from stepwise logistic regression. Pulse rate ( 80/min) and DBil/ALP ( 1.0) were used to screen infection. The sensitivity was 62.5% and 64.7% respectively, and the specificity was 100% and 82.8% respectively.

**CONCLUSION:** Bacterial and fungal infection deteriorate jaundice and increase pulse rate, decrease serum ALP and increase DBil/ALP. Pulse rate, DBil/ALP and the equation (infection=0.218 pusle rate+1.064 DBil/ALP-16.361) are helpful to early diagnosis of bacterial and fungal infection in patients with chronic cholestatic hepatitis B.

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## INTRODUCTION

Patients with chronic hepatitis are known to be abnormally susceptible to infection as a result of multiple immunologic defects[1-6]. The incidence of bacteriemia in chronic liver disease is 5- to 7- fold higher than that found in other hospitalized patients[7]. Infection is often associated with systemic inflammatory response syndrome and multiple organ dysfunction syndrome that is easy to develop into liver failure[8,9]. Infection can prove fatal either directly or by precipitation of encephalopathy, gastrointestinal hemorrhage, hepatic hemodynamic derangement, portal hypertension, or renal failure[9-17]. About 33% mortality in bacteriemic patients with chronic liver disease has been noted[18]. Considering the low positivity rate and lengthy bacterial culture, severe chronic liver disease patients with clinical diagnosis of infection should get the antibiotic treatment immediately to improve the survival opportunity before pathogen diagnosis[19-24]. However, clinical diagnosis is made more difficult by the absence of the typical clinical feature of infection - that is, fever, leucocytosis, and localized symptoms[9, 21]. Clinically apparent focus of infection could not be found in 20% to 60% of bacteriemic patients with chronic liver disease[9, 25, 26]. No specific site is identified in one third to one half of the infections, in which case the only clue may be deterioration of hepatic precoma or coma or renal function. The preventive antibiotic treatment is often applied to serious patients who have the liability to infection (such as the protein in ascites <10 g/L, gastrointestinal hemorrhage, and previous spontaneous bacterial peritonitis), and in the meantime it might increase the risk of drug resistance[19, 27-30]. The early diagnostic methods of secondary bacterial and fungal infection in patients with chronic hepatitis B have not been studied. To reduce infection related mortality, the development of new methods for early diagnosis is of great significance.

## MATERIALS AND METHODS

### *Patients*

One hundred and one adult in-patients with chronic icteric hepatitis B in West China Hospital from October 2002 to March 2003 were studied and divided into three groups: direct bilirubin (DBil)/total bilirubin (TBil) 0.5, without bacterial or fungal infection (group A, *n*=38); DBil/TBil <0.5, without bacterial or fungal infection (group B, *n*=23); DBil/TBil 0.5, with bacterial and fungal infection (group C, *n*=40).

### *Inclusion criteria*

All patients in the study met with the following criteria: positive HBsAg or HBVDNA; TBil >17.1 mmol/L; age between 14 to 65 years. In addition, the patients in group C had the following features: leukocytes $10\times10^9$/L or neutrophils $7\times10^9$/L or the ratio of neutrophil 70 % in blood; leukocytes in urine 5 per visual field under high power microscope; leukocytes $0.5\times10^9$/L or neutrophils $0.25\times10^9$/L in ascites; bacterial culture of blood, urine, phlegm or ascites was positive.

### *Exclusion criteria*

The patient who had one of the followings was excluded from

group A and group B: infected with other pathogens rather than HBV; complicated with other diseases which had not relation with hepatitis B; recently occurring gastrointestinal hemorrhage. The patient who had one of the followings was excluded from group C: infected with other virus; complicated with other liver diseases which had not relation with hepatitis B; recently occurring gastrointestinal hemorrhage; leukocytes in urine 5 per visual field under high power microscope but normal in routine blood test in female patients who were in menstruation or in patient who had non-inflammatory nephrosis.

### *Investigation indexes*

Blind tests for TBil, DBil, indirect bilirubin (IBil), DBil/IBil, alanine aminotransferase (ALT), aspartate aminotransferase (AST), AST/ALT, serum total protein (TP), albumin (ALB), globulin (GLOB), ALB/GLOB, γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), DBil/ALP, lactate dehydrogenase (LDH), cholinesterase (ChE), serum total bile acid (TBA) and pulse rate were performed. The first liver biochemical test of group A and B after hospitalization and the first liver biochemical test of group C after confirmation of infection were done, and pulse rate of all patients were measured after 15-min bed rest.

### *Statistical analysis*

All data were presented as mean±SE. Analysis of variance and covariance was used to determine whether there were significant differences among the three groups. Data with significant difference were entered into a stepwise logistic regression analysis. Statistical calculations were performed by SPSS (Version: 11.0, Chicago, USA).

## RESULTS

### *Clinical features of infection*

Fifteen percent of patients in group C showed a rise in leukocyte or neutrophil in blood, 55% of patients showed a rise in the ratio of neutrophil in blood lightly, and 12.5% of patients showed an increase in leukocyte or neutrophil in ascites. Eleven patients showed a leukocyte rise in urine (in these cases, 5 patients showed an increase in leukocyte or neutrophil in blood or in the ratio of neutrophil, 5 patients had a history of fever, 2 patients showed spontaneous bacterial peritonitis). Fifteen patients presented with the localized symptoms of infection. Sixteen patients had a history of fever (the fever was light and persisted shortly in most patients). Positivity rate of bacterial culture was 25% (3/12).

**Table 1** Comparison of serum bilirubin

| Group | Case | Tbil (μmol/L) | DBil (μmol/L) | IBil (μmol/L) | DBil/TBil |
|---|---|---|---|---|---|
| A | 38 | 220±206 | 146±136 | 73±72 | 0.660±0.078 |
| B | 23 | 59±33[b] | 22±12[d] | 38±23 | 0.361±0.105[d] |
| C | 40 | 370±227[bf] | 214±143[bf] | 136±92[df] | 0.631±0.840[f] |

[b]$P$<0.01 *vs* group A; [d]$P$<0.001 *vs* group A; [f]$P$<0.001 *vs* group B.

### *Analysis of variance*

Level of TBil, DBil, ALP and DBil/ALP in group A increased significantly compared with that in group B ($P$<0.01, $P$<0.01, $P$<0.05, $P$<0.01, respectively). The level of ALP in group C decreased compared with that in group A (156±43, 199±68, respectively, $P$<0.05), whereas the level of TBil, DBil and DBil/ALP increased significantly (TBil: 370±227, 220±206, respectively, $P$<0.01; DBil: 214±143, 146±136, respectively, $P$<0.01; DBil/ALP: 1.65±1.05, 0.78±0.70, respectively, $P$<0.001). The level of IBil in group C decreased compared with that in group A ($P$<0.001) (Tables 1, 2).

The pulse rate in group A decreased significantly compared with that in group B (63.7±6.4, 77.7±11.4, $P$<0.001). The pulse rate in group C increased compared with that in group A and normal adults (81.2±12.2, 63.7±6.4, 72, $P$<0.001), but decreased compared with that in infectious patients without hepatitis (81.2±12.2, 90, $P$<0.001) (Table 2, Figure 1).

The level of ALT, AST, AST/ALT, TP, ALB, GLOB, ALB/GLOB, GGT, LDH, and ChE showed no significant difference among the three groups (data not shown).

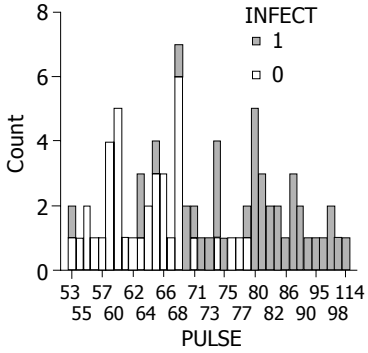


**Figure 1** Pulse rate in cholestatic patients. 1: With bacterial or fungal infection; 0: Without bacterial and fungal infection.

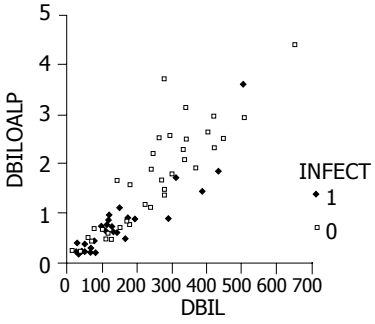


**Figure 2** DBil/ALP and DBil in cholestatic patients. 1: With bacterial or fungal infection, 0: Without bacterial and fungal infection.

### *Analysis of covariance*

There was linear correlation between DBil and DBil/ALP in groups A and C, and the distribution of DBil between the two groups was closely similar (Figure 2). The level of DBil and infection affected DBil/ALP ($P$<0.05, $P$<0.001, respectively)

**Table 2** Comparison of TBA, ALP, DBil/ ALP and pulse rate (mean±SE)

| Group | *n* | TBA(μmol/L) | *n* | ALP(U/L) | *n* | DBil/ALP | *n* | Pulse rate(times/min) |
|---|---|---|---|---|---|---|---|---|
| A | 22 | 148±89 | 29 | 199±68 | 29 | 0.78±0.70 | 38 | 63.7±6.4 |
| B | 16 | 31±17[d] | 18 | 149±50[a] | 18 | 0.14±0.06[b] | 23 | 78±11[d] |
| C | 22 | 140±60[f] | 34 | 156±43[ac] | 34 | 1.65±1.05[df] | 40 | 81±12[d] |

[a]$P$<0.05 *vs* group A; [c]$P$<0.05 *vs* group B; [b]$P$<0.01 *vs* group A; [d]$P$<0.001 *vs* group A; [f]$P$<0.001 *vs* group B.

(Table 3). Independent of the effect of DBil, infection caused DBil/ALP to rise ($P$<0.05) (Table 4).

**Table 3** Tests of between-subjects effects (dependent variable: DBil/ALP)

| Source | Type III sum of squares | Df | Mean square | $F$ | $P$ |
|---|---|---|---|---|---|
| Corrected model | 50.025 | 2 | 25.012 | 129.672 | <0.001 |
| Infection | 1.172 | 1 | 1.172 | 6.074 | 0.017 |
| DBil | 38.709 | 1 | 38.709 | 200.682 | <0.001 |

**Table 4** Pairwise comparisons (dependent variable: DBil/ALP)

| Infection I | Infection J | Mean difference(I-J) | $SE$ | $P$ | 95% | CI |
|---|---|---|---|---|---|---|
| 1 | 2 | -0.293[1] | 0.119 | 0.017 | -0.531 | -0.055 |
| 2 | 1 | 0.293[1] | 0.119 | 0.017 | 0.0551 | 0.531 |

[1]The mean difference is significant at the 0.05 level.

### *Logistic regression*

The stepwise logistic regression was used to establish the best statistical model to early diagnose infection, and the equation (infection=0.218 pulse rate+1.064 DBil/ALP -16.361, $P$<0.001) was obtained. Its overall accuracy was 85.5%, and accuracy for diagnosing infection and noninfection were 85.7% and 85.3%, respectively (Table 5).

**Table 5** Logistic analysis of bacterial and fungal infection

| | B | $SE$ | Wald | Df | $P$ | Exp (B) |
|---|---|---|---|---|---|---|
| Pulse rate | 0.218 | 0.059 | 13.468 | 1 | <0.001 | 1.244 |
| DBil/ALP | 1.064 | 0.447 | 5.657 | 1 | 0.017 | 2.899 |
| Constant | -16.361 | 4.251 | 14.815 | 1 | <0.001 | <0.001 |

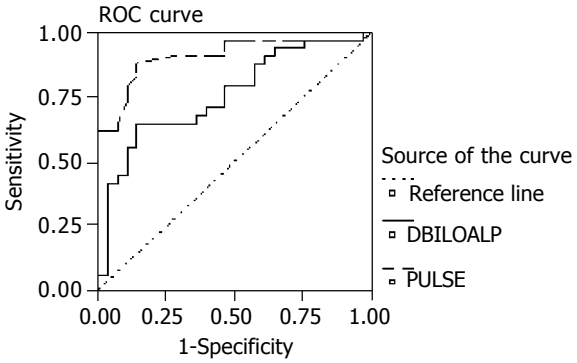


**Figure 3** Receiver operator characteristic curve of pulse rate and DBil/ALP.

### *Screening test*

Because the diagnostic index of pulse rate at 80/min to screen infection was the greatest, the cut off point was selected at 80/min (Figure 3). The sensitivity (Sen), specificity (Spe), diagnostic index (DI), false positive rate ($\alpha$), false negative rate ($\beta$), Youden's index ($\gamma$), crude accuracy (CA), adjusted agreement (AA), positive predictive value (PV+) and negative predictive value (PV-) were 62.5%, 100%, 1.625, 100%, 71.7%, 62.5%, 80.8%, 83.6%, 0% and 37.5%, respectively. Area under the curve (Az)=0.889 ($P$<0.001) (Table 6). Because the diagnostic index of DBil/ALP at 1.0 was the greatest, the cut off point was selected at 1.0 (Figure 3). Sen, Spe, DI, $\alpha$, $\beta$, $\gamma$, CA, AA, PV+ and PV- were 64.7%, 82.8%, 1.475, 81.5%, 66.7%, 47.5%, 73.0%, 73.9%, 17.8% and 35.3%, respectively. Az=0.7373 ($P$<0.001) (Table 6). Pulse rate was a better index than DBil/ALP ($P$<0.001).

## DISCUSSION

Bacterial and fungal infections are the common complications of chronic hepatitis, frequently aggravating hepatitis. Since the bacterium culture in chronic hepatitis patients is often negative, clinical diagnosis plays a major role. However, a large number of patients do not often present with the typical clinical features of infection. In a prospective study, fever was initially absent in 45% of cases, and leukocytosis was absent in 25% of cases[10]. Urinary tract infection is the common type of infection and the source of bacteriemia in up to 50% of patients with cirrhosis, and 20% of episodes of spontaneous bacterial peritonitis[10-30]. However, patients with urinary tract infection often do not have localized symptoms or physical findings[10,1]. Careful urinalysis is important to exclude infection in patients with chronic liver disease[10]. In our study, the leukocyte or neutrophil in blood rose in 15% of infected patients, and the ratio of neutrophil rose lightly in 55% of patients. Leukocyte or neutrophil in ascites increased in 5 patients, and leukocyte in urine rose in 11 patients. Sixteen patients had a history of fever (the fever was mild and short-lasting in most patients). Only 37.5% of patients presented localized symptoms of infection. The positivity rate of bacterial culture was 25%(3/12). Therefore, early diagnosis of bacterial and fungal infection is difficult in chronic hepatitis.

The level of ALP frequently rises in cholestatic patients. However, in serious liver disease, along with the increase of serum TBil and DBil, sometimes ALP decreased. Hadjis discovered that the synthesis of ALP decreased in the case of widespread hepatocyte necrosis. As well as ALT and AST, it is a marker of disease aggravation. In our study, the level of TBil and DBil in cholestatic patients with infection increased compared with that without infection, but ALP in infection patients decreased compared with that without infection.

In order to characterize the contrary alteration of DBil and ALP in infection patients, we introduced a new index -that is the ratio of DBil to ALP. The level of DBil/ALP showed significant difference between the three groups (0.7829±0.7002, 0.1377±0.0652, 1.6468±1.0500, respectively, $P$<0.01). Analysis of covariance showed that the level of DBil and infection affected DBil/ALP, and independent of the effect of DBil, infection led to DBil/ALP increase ($P$<0.05).

To test whether DBil/ALP could be used to screen infection, a screening test was performed. Its sensitivity, specificity, positive predictive value and negative predictive value were 64.7%, 82.8%, 81.5% and 66.7%, respectively (the cut off point at 1.0). Therefore, we hypothesize that DBil/ALP can be used as an "infection index" to diagnose infection early in chronic cholestatic hepatitis B. If DBil/ALP is greater than 1.0, the probability of infection is 82.8%.

The mean pulse rate in normal adults is 72/min, but it frequently increases in infection, often above 90/min. Because the vagus's excitement increased in cholestatic patient, the

**Table 6** Screening test of bacterial and fungal infection

| Diagnosis | | Sensitivity | Specificity | Diagnostic Index | $A_Z$ | Z | $P$ |
|---|---|---|---|---|---|---|---|
| Pulse rat | 80/min | 0.625 | 1.000 | 1.625 | 0.889 | 20.372 | <0.001 |
| DBil/ALP | 1.0 | 0.647 | 0.828 | 1.475 | 0.7373 | 4.022 | <0.001 |

pulse rate decreased compared with that in normal adults (63.7±6.4, 72, $P<0.001$). Although the pulse rate in cholestatic patient increased after infection (81.2±12.2, 63.7±6.4, $P<0.001$), it was slower compared with that in infection patients without cholestasis (81.2±12.2, 90, $P<0.001$). That is why alteration of pulse rate in those patients is likely to be neglected in clinic.

To test whether pulse rate could be used to screen infection, a screening test was done. Its sensitivity, specificity, positive predictive value and negative predictive value were 62.5%, 100%, 100% and 71.7%, respectively (the cut off point at 80/min). Pulse rate was better for screening infection than DBil/ALP ($P<0.001$).

In order to establish the best statistical model to diagnose infection early, the equation (infection=0.218 pulse rate+1.064 DBil/ALP - 16.361, $P<0.001$) was obtained from the stepwise logistic regression. Its total accuracy, the accuracy of diagnosing infection and noninfection was 85.5%, 85.7% and 85.3%, respectively.

It is interesting that there is notable relationship between the logistic equation and screening test. Among the 18 indexes that we studied, only pulse rate and DBil/ALP could affect the logistic equation. The equation can be described approximately as "infection 0.2 pulse rate + DBil/ALP – 16". As 0.2 multiplied by 80 is 16, if pulse rate 80/min, the equation 0 obviously. But if pulse rate <80/min, DBil/ ALP significantly affects the equation. For severe chronic cholestatic hepatitis B, if pulse rate 80/min, perhaps the antibiotic treatment should be applied immediately before pathogen diagnosis to improve the survival opportunity. If DBil/ALP 1.0 but pulse rate <80/min, the equation is helpful to estimate the probability of infection. If DBil/ALP < 1.0 and pulse rate <80/min, the equation is useful to exclude infection. It is a sensitive, quick, simple and economical method to diagnose infection early and possibly improve the survival opportunity of severe chronic cholestatic hepatitis B.

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# Increased oxidative DNA damage, inducible nitric oxide synthase, nuclear factor κB expression and enhanced antiapoptosis-related proteins in *Helicobacter pylori*-infected non-cardiac gastric adenocarcinoma

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## Abstract

**AIM:** Several epidemiological studies have demonstrated a close association between *Helicobacter pylori* (*H Pylori*) infection and non-cardiac carcinoma of the stomach. *H pylori* infection induces active inflammation with neutrophilic infiltrations as well as production of oxygen free radicals that can cause DNA damage. The DNA damage induced by oxygen free radicals could have very harmful consequences, leading to gene modifications that are potentially mutagenic and/or carcinogenic. The aims of the present study were to assess the effect of *H pylori* infection on the expression of inducible nitric oxidative synthase (iNOS) and the production of 8-hydroxy-deoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA injury in human gastric mucosa with and without tumor lesions, and to assess the possible factors affecting cell death signaling due to oxidative DNA damage.

**METHODS:** In this study, 40 gastric carcinoma specimens and adjacent specimens were obtained from surgical resection. We determined the level of 8-OHdG formation by HPLC-ECD, and the expression of iNOS and mechanism of cell death signaling [including nuclear factor-κB(NFκB), MEKK-1, Caspase 3, B Cell lymphomal leukemia-2 (Bcl-2), inhibitor of apoptosis protein (IAP ) and myeloid cell leukemia-1 (Mcl-1)] by Western-blot assay.

**RESULTS:** The concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were significantly higher in cancer tissues than in adjacent non-cancer tissues. In addition, significantly higher concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were detected in patients infected with *H pylori* compared with patients who were not infected with *H pylori*. Furthermore, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

**CONCLUSION:** Chronic *H pylori* infection induces iNOS expression and subsequent DNA damage as well as enhances anti-apoptosis signal transduction. This sequence of events supports the hypothesis that oxygen-free radical-mediated damage due to *H pylori* plays a pivotal role in the development of gastric carcinoma in patients with chronic gastritis.

Chang CS, Chen WN, Lin HH, Wu CC, Wang CJ. Increased oxidative DNA damage, inducible nitric oxide synthase, nuclear factor κB expression and enhanced antiapoptosis-related proteins in *Helicobacter pylori*-infected non-cardiac gastric adenocarcinoma. *World J Gastroenterol* 2004; 10 (15): 2232-2240
http://www.wjgnet.com/1007-9327/10/2232.asp

## INTRODUCTION

*Helicobacter pylori* (*H pylori*) infection is associated with an increased risk of both peptic ulcer and gastric cancer[1] Chronic gastritis induced by *H pylori* significantly increases the risk for non-cardiac gastric cancer[2,3], and host responses that may affect the threshold for carcinogenesis include alteration of epithelial cell proliferation and apoptosis. Although the linkage between *H pylori* infection and gastric cancer is convincing, the molecular mechanism or mechanisms responsible are unclear, and both bacterial and host factors are implicated. One possible factor is that increased inflammation generates reactive oxygen and nitrogen intermediates that have been shown to damage DNA directly[4].

Mucosal hyperproliferation has been reproducibly demonstrated in *H pylori*-infected human[5-7] and rodent gastric tissue[8-10], and proliferation scores normalize following successful eradication in humans[5-7]. However, maintenance of tissue integrity requires that enhanced cell production is accompanied with increased rates of cell loss consequently, studies have also examined the effect of *H pylori* on apoptosis. In contrast to hyperproliferation, *H pylori* has been associated with increased[11-14], unchanged[15], or even decreased[16] levels of apoptosis *in vivo*, and within a particular population, substantial heterogeneity exists among apoptosis scores[11-14] . These observations suggest that increases in proliferation that are not balanced by concordant increases in apoptosis over years of colonization may heighten the retention of mutated cells, ultimately enhancing the risk for gastric malignancy in certain populations. Alteration of epithelial cell proliferation and apoptosis is a manifestation of *H pylori*-induced gastritis. However, the precise mechanisms underlying these effects remain incompletely clarified. In this study, we aimed to assess the effect *H pylori* infection on the expression of iNOS and the production of 8-OHdG, a sensitive marker of oxidative DNA injury in human gastric mucosa with and without tumor lesions, as well as the possible factors affecting cell death signaling due to oxidative DNA damage.

## MATERIALS AND METHODS

### *Patients*

In this study, 40 patients (aged 68.3±11.4 years, range 39-84 years, M/F: 26/14) with non-cardiac gastric adenocarcinoma were included. Specimens from tumor site and adjacent non-tumor site were obtained from surgical resection. By TNM staging according to the criteria described by the International Union Against Cancer[17], there were 2 cases in stage 1, 6 cases in stage 2, 8 cases in stage 3 and 24 cases in stage 4 (Table 1). *H pylori* infection was assessed by the following three methods: histology (haematoxylin and eosin staining or Giemsa staining, rapid urease test (CLO test, Delta West Pty Ltd, Perth, Australia) and serum *H pylori* ELISA IgG assays (Trinity Biotech USA, Jamestown, NY). A patient was considered *H pylori* positive if one or more of the diagnostic methods applied were positive, and *H pylori* negative if all methods were negative. There were 29 patients (72.5%) with *H pylori* infection and 11 patients (27.5%) without *H pylori* infection. We determined the level of 8-OHdG formation by HPLC-ECD, and the expression of iNOS and cell death signaling, including NFκB, MEKK1, Caspase 3, Bcl-2, Mcl-1 and IAP by Western-blot assay.

**Table 1** Characteristics of all patients

| Case No | Stage | *H pylori* infection | Case No | Stage | *H pylori* infection |
|---|---|---|---|---|---|
| 1 | II | + | 21 | III | + |
| 2 | IV | + | 22 | IV | + |
| 3 | IV | + | 23 | IV | + |
| 4 | III | + | 24 | IV | + |
| 5 | IV | + | 25 | IV | - |
| 6 | IV | + | 26 | IV | + |
| 7 | IV | + | 27 | I | + |
| 8 | III | - | 28 | I | - |
| 9 | III | + | 29 | IV | - |
| 10 | III | + | 30 | II | + |
| 11 | IV | + | 31 | IV | + |
| 12 | IV | - | 32 | IV | + |
| 13 | IV | - | 33 | IV | + |
| 14 | IV | + | 34 | III | + |
| 15 | IV | + | 35 | II | + |
| 16 | II | + | 36 | III | + |
| 17 | III | - | 37 | IV | - |
| 18 | IV | + | 38 | IV | + |
| 19 | IV | + | 39 | IV | + |
| 20 | II | - | 40 | II | - |

### *DNA extraction and digestion*

DNA was extracted with Dneasy tissue kit (QIAGEN) according to the protocol of Dahlhaus and Apple[18] with minor modifications as described previously[19]. Briefly, one volume of nuclear fraction obtained from surgical tissue was mixed with ten volumes of extraction buffer (1 mol/L NaCl, 10 mmol/L Tris–HCl, 1 mmol/L EDTA, 5 g/L SDS, pH 7.4) and one volume of chloroform:isoamylalcohol (12:1 v/v). After vigorous shaking, the aqueous phase was separated by centrifugation. This step was repeated three times before DNA was precipitated with absolute ethanol (-70 ℃). DNA pellet was dissolved in TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 7.4), and then incubated with RNase A to remove RNA. After incubation, DNA was extracted with chloroform:isoamylalcohol, and precipitated with absolute ethanol again. The DNA pellet was dissolved in 10 mmol/L Tris–HCl (pH 7.0) and hydrolyzed according to the previous procedure[20]. Briefly, 200 μg DNA in 200 μL 10 mmol/L Tris-HCl (pH 7.0) was first denatured at 95 ℃ for 3 min. The digestion was carried out at 37 ℃ for 1 h with 100 units DNase I (from bovine pancreas, Sigma) in a buffer containing 10 mmol/L $MgCl_2$, followed by incubation with 5 units nuclease $P_1$ (from penicillium citrinum, Sigma) in 100 μmol/L $ZnCl_2$ for an additional hour. The mixture was incubated finally with 3 units alkaline phosphatase (from *Escherichia coli*, Sigoma) at 37 ℃ for 1 h. The incubation was terminated with acetone (HPLC grade, Merck, Germany) to precipitate proteins. After 12 000 *g* centrifugation for 15 min, the supernatant was dried under vacuum in a rotary evaporator (Eyela, Tokyo, Japan) and DNA was dissolved in distilled $H_2O$.

### *8-OHdG assay*

8-OHdG levels were determined by HPLC/ECD consisting of a BAS PM-80 pump (Bioanalytical Systems, West Lafayette, IN, USA), a CMA-200 microautosampler (CAM/Microdialysis, Stockholm, Sweden), a BAS-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), a microbore reversed-phase column (Inertsil-2, 5-μm ODS, 1.0×150 mm I.D. G.L. Sciences, Tokyo, Japan) Beckman I/O 406 interface, and Beckman system gold data analysis software (Beckman Instruments). A potential of +0.6 V was selected for the glassy carbon working electrode which corresponded to a silver/silver chloride reference electrode. The mobile phase consisted of 12.5 mmol/L citric acid, 30 mmol/L NaOH, 25 mmol/L sodium acetate, 10 mmol/L acetic acid glacial and 50 mL/L methanol (pH 5.3). The flow rate was set at 1 mL/min. Under these conditions, the retention time of 8-OHdG was 8.49 min with a significantly higher signal than background noise.

### *Immunoblot analysis*

Cancerous and non-cancerous tissue samples were homogenized with ten volumes of homogenization buffer (50 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L sodium chloride, 10 g/L Nonidet P-401 g/L SDS, 5 g/L deoxycholic acid, 1 mmol/L sodium orthovanadate, 170 μg/mL leupeptin, 100 μg/mL phenylsulfonyl fluoride; pH 7.5). After 30 min of mixing at 4 ℃, the mixture was centrifuged at 800 r/min for 10 min, and the supernatant was collected. The protein content of the samples was determined with the Bio-Rad protein assay reagent using BSA as a standard. For Western blotting analysis, 50 μg protein from both cancerous and non-cancerous tissue samples was resolved on 100 g/L SDS-PAGE gels along with prestained protein molecular weight standards (Bio-Rad). The separated proteins were then blotted onto NC membrane (0.45 μm, Bio-Rad) and reacted with primary antibodies (against iNOS, NFκB, MEKK1, Caspase 3, Bcl-2, Mcl-1 and IAP). After washed, the blots were incubated with peroxidase-conjugated goat anti-mouse antibody. Immunodetection was carried out using the enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Corp., UK). Relative protein expression levels were quantified by densitometric measurement of ECL reaction bands and normalized with values of tubulin. Finally, enhanced chemiluminescence (ECL) was used to detect the membrane, and then the membrane was exposed to X-ray film to observe results. All the immunoblot results were determined by Alpha Imager 2000 documentation & analysis system (Alpha Innotech Corporation).

### *Statistical analysis*

Each parameter expressed by fold of increase of the data from cancer tissue was compared to the adjacent non-cancer tissue. The results were reported as mean±SE from individual magnitudes. Data were analyzed with Student's *t*-test. Statistical differences were considered significant when $P<0.05$.

## RESULTS

To assess the effect of *H pylori* infection on each determined parameter, the results were expressed by fold of increase comparing the data of cancer tissue with those of the adjacent non-cancer tissue. The concentrations of 8-OHdG were analyzed by HPLC/ECD. There were significantly higher concentrations

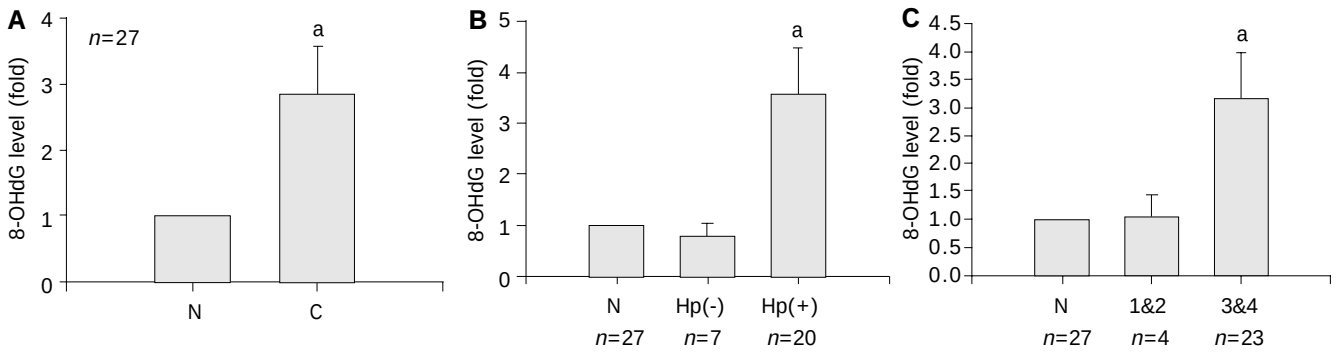



**Figure 1** Concentrations of 8-OHdG. A: Higher concentration of 8-OhdG in cancer tissues compared with adjacent non-cancer tissues. B: Higher concentration of 8-OhdG in cancer tissues of patients with and without *H pylori* infection. C: Significantly higher F8-OHdG concentrations in stage 3 and 4 patients $^{a}P<0.05$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4.

of 8-OHdG in cancer tissues than in adjacent non-cancer tissues (Figure 1). In addition, significantly higher concentrations of 8-OHdG were detected in patients infected with *H pylori* than in those without *H pylori* infection. 8-OHdG levels were also significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

Immunoblot analysis of several factors affecting cell death signaling related to oxidative DNA damage showed increased concentrations of iNOS and NFκB in patients infected with *H pylori* compared to patients who were tested negative for *H pylori* (Figure 2A-B). Furthermore, iNOS and NFκB concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

Although there were some increases in the concentrations of MEKK1 and Caspase-3 in the cancer tissue (Figure 2C-D), these changes were not statistically significant. In addition, no significant increases of MEKK1 and Caspase-3 in patients infected with *H pylori* were noted. The concentrations of Caspase-3 in stage 1 and 2 patients were significantly lower than those in stage 3 and 4 patients.

Among the anti-apoptosis-related proteins, there were some increases in the concentrations of Bcl-2 in the cancer tissue (Figure 2E). However, the changes were not statistically significant. In addition, no significant increase of Bcl-2 in patients infected with *H pylori* was noted. The concentrations of Bcl-2 in stage 1 and 2 patients were significantly lower than those in stage 3 and 4 patients. Mcl-1 and IAP concentrations were significantly higher in the cancer tissues than in the adjacent non-cancer tissues (Figure 2F-G). In addition, Mcl-1 and IAP concentrations were significantly higher in *H pylori*-positive patients than in *H pylori*-negative patients. Stage 3 and 4 patients had significantly higher concentrations of Mcl-1 and IAP than stage 1 and 2 patients.

**Table 2** 8-oHdG, iNOS, NFκB, Mel-1 and IAP Concentrations in cancer and adjacent non-cancer tissues

| | Cancer tissue | *H pylori* (+) | Stages I & II | Stages III & IV |
|---|---|---|---|---|
| 8-OHdG | ↑ a | ↑ a | - | ↑ a |
| iNOS | ↑ b | ↑ b | - | ↑ b |
| NF-κB | ↑ a | ↑ a | - | ↑ a |
| MEKK1 | - | - | - | - |
| Caspase 3 | - | - | ↓ b | - |
| Bcl-2 | - | - | ↓ b | - |
| Mcl-1 | ↑ a | ↑ a | - | ↑ a |
| IAP | ↑ a | ↑ a | - | ↑ a |

↑: increase, ↓: decrease, -: no significant change, $^{a}P<0.05$, $^{b}P<0.01$ compared with control, by one sample *t*-test and independent *t*-test, respectively.

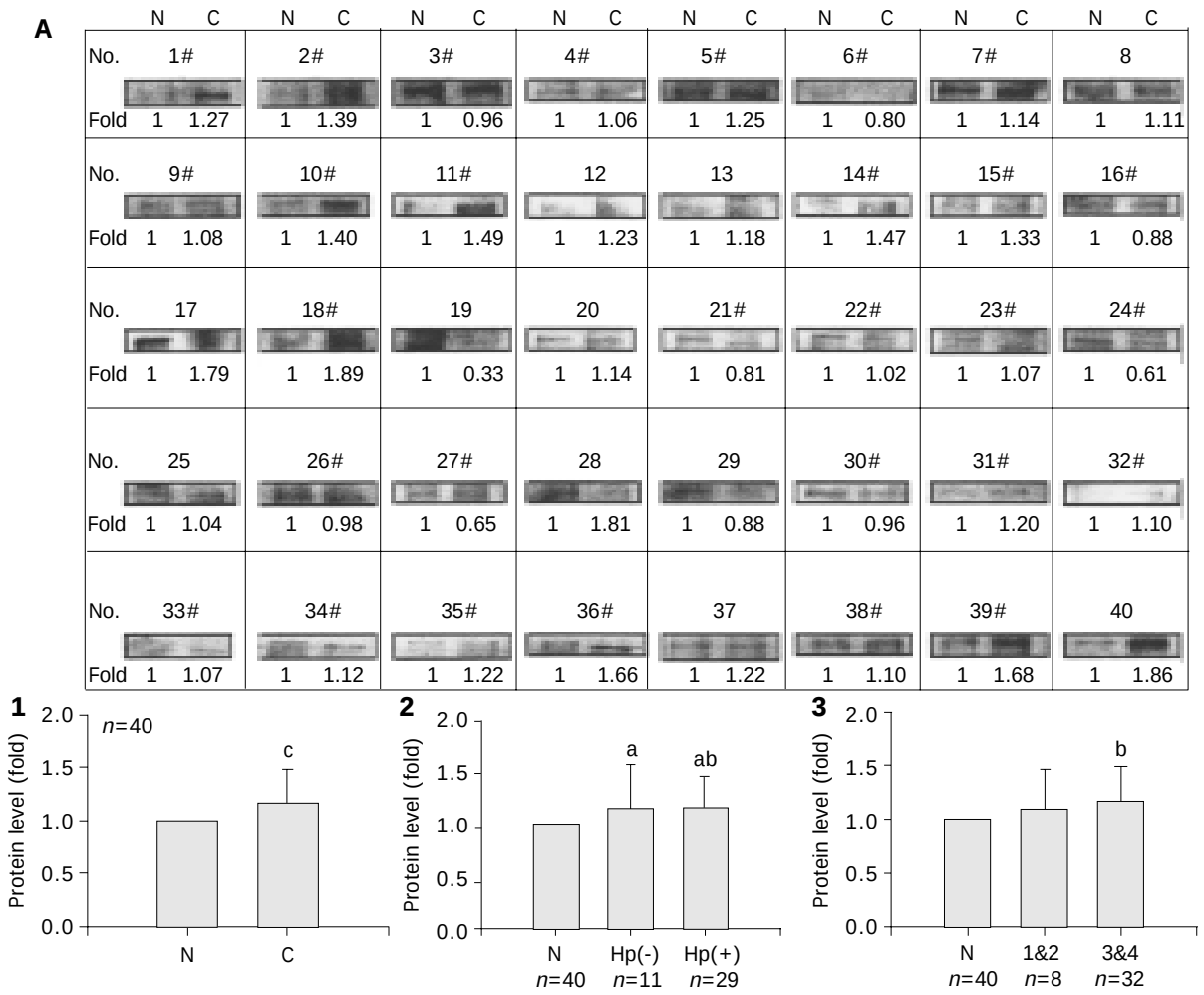
In summary, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in cancer tissues than in adjacent non-caner tissues (Table 2). In addition, significantly higher concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were detected in patients infected with *H pylori* compared to patients who were not infected with *H pylori*.

Furthermore, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

## DISCUSSION

*H pylori* infection may influence the progression from chronic gastritis to adenocarcinoma of the distal stomach by a multifactorial, multistep process, eas which chronic inflammation plays a major role. Free radical mediated oxidative DNA damage was involved in this process[21-23]. The DNA damage provoked by oxygen free radicals can have very harmful consequences, leading to gene modifications that are potentially mutagenic and/or carcinogenic. Recently, 8-OHdG has been accepted as a sensitive marker for oxidative DNA damage in affected organs. Chronic gastritis due to *H pylori* infection is characterized by the accumulation of oxidative DNA damage measured by concentrations of 8-OHdG, indicating mutagenic and carcinogenic potential[24]. In this study, we demonstrated an increase in the concentrations of 8-OHdG in cancer tissues. Furthermore, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer. The increased level of oxidative DNA damage suggested a mechanistic link between *H pylori* infection and gastric carcinoma[25,26].

*H pylori*-associated inflammation related to DNA damage is indicated by increased levels of oxidative DNA damage, increased occurrence of apoptosis, as well as increased expression of iNOS, which seemed to provide the mechanistic links between *H pylori* infection and gastric carcinogenesis[27,28]. Upregulation of iNOS expression might contribute to the oxidative DNA damage observed during *H pylori* infection[25]. Recent studies have shown that nitric oxide (production of iNOS) reacts rapidly with superoxide anions produced by *H pylori*-infected gastric tissues to form peroxynitrate, which could induce oxidative damage[29]. This may increase the mutation rate in infected hyperproliferative gastric mucosa. In addition, the expressions of iNOS are closely related to tumor angiogenesis, and involved in progression of the disease as well as lymph node metastasis. Thus the expressions of iNOS might serve as indexes for evaluating staging of gastric carcinoma. In this study, we observed significantly higher increases in the concentrations of iNOS in patients infected with *H pylori* and in patients with advanced gastric cancer, compared with *H pylori*-negative patients and low stage gastric cancer patients, respectively.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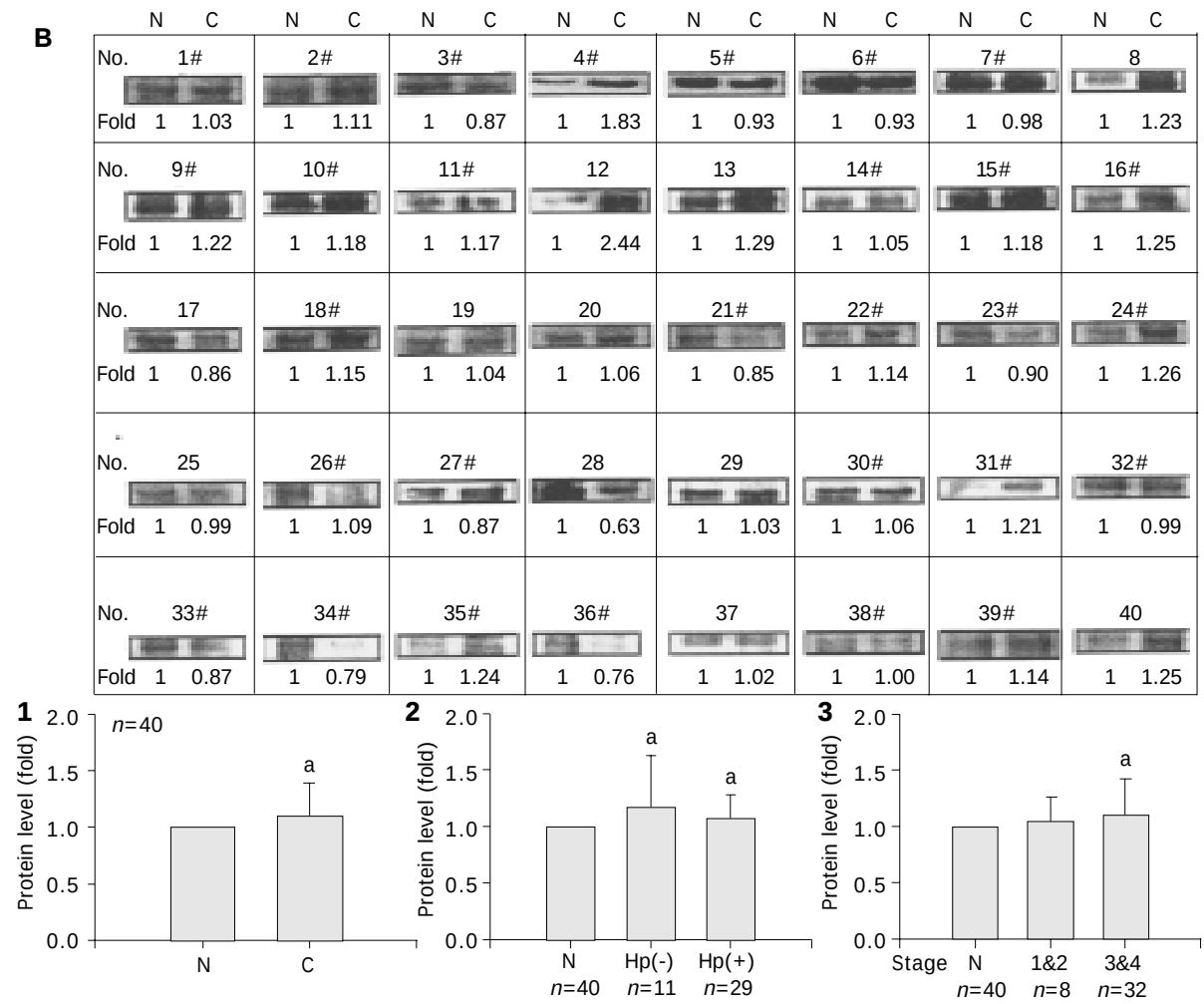
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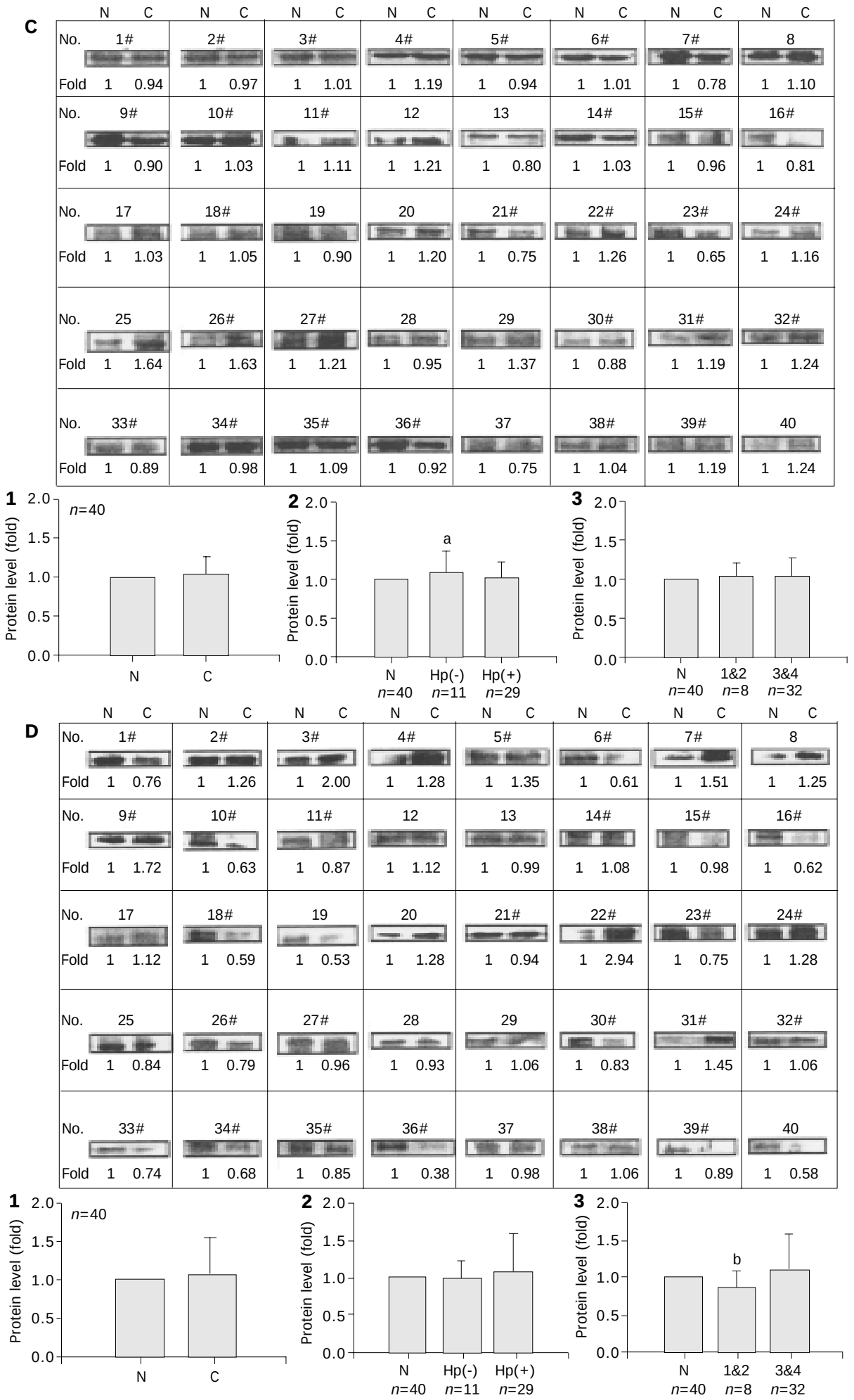
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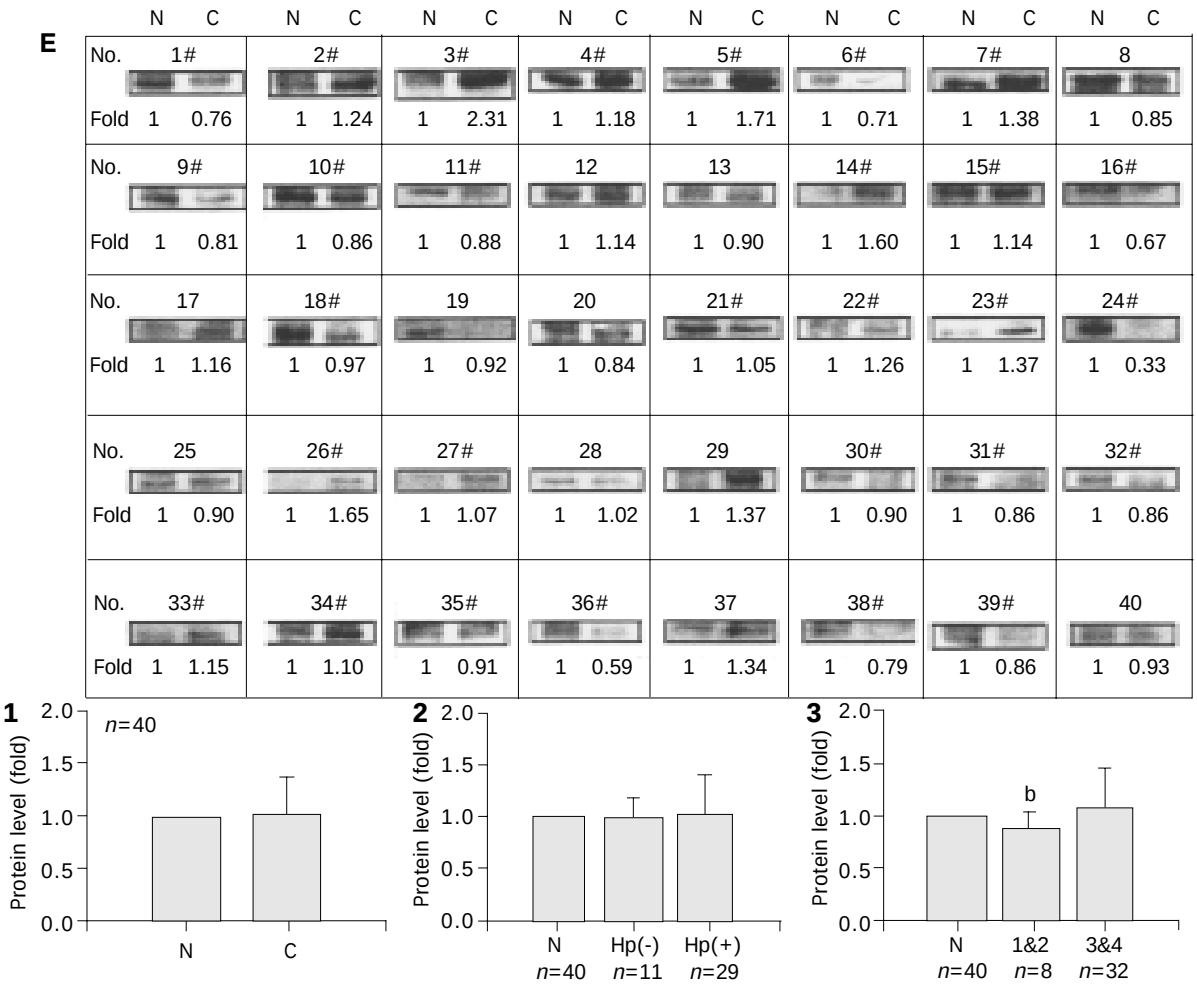
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No. 17 18# 19 20 21# 22# 23# 24#
Fold 1 1.16 1 0.97 1 0.92 1 0.84 1 1.05 1 1.26 1 1.37 1 0.33
No. 25 26# 27# 28 29 30# 31# 32#
Fold 1 0.90 1 1.65 1 1.07 1 1.02 1 1.37 1 0.90 1 0.86 1 0.86
No. 33# 34# 35# 36# 37 38# 39# 40
Fold 1 1.15 1 1.10 1 0.91 1 0.59 1 1.34 1 0.79 1 0.86 1 0.93
1
n=40
Protein level (fold)
2.0
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N
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2
Protein level (fold)
N
n=40
Hp(-)
n=11
Hp(+)
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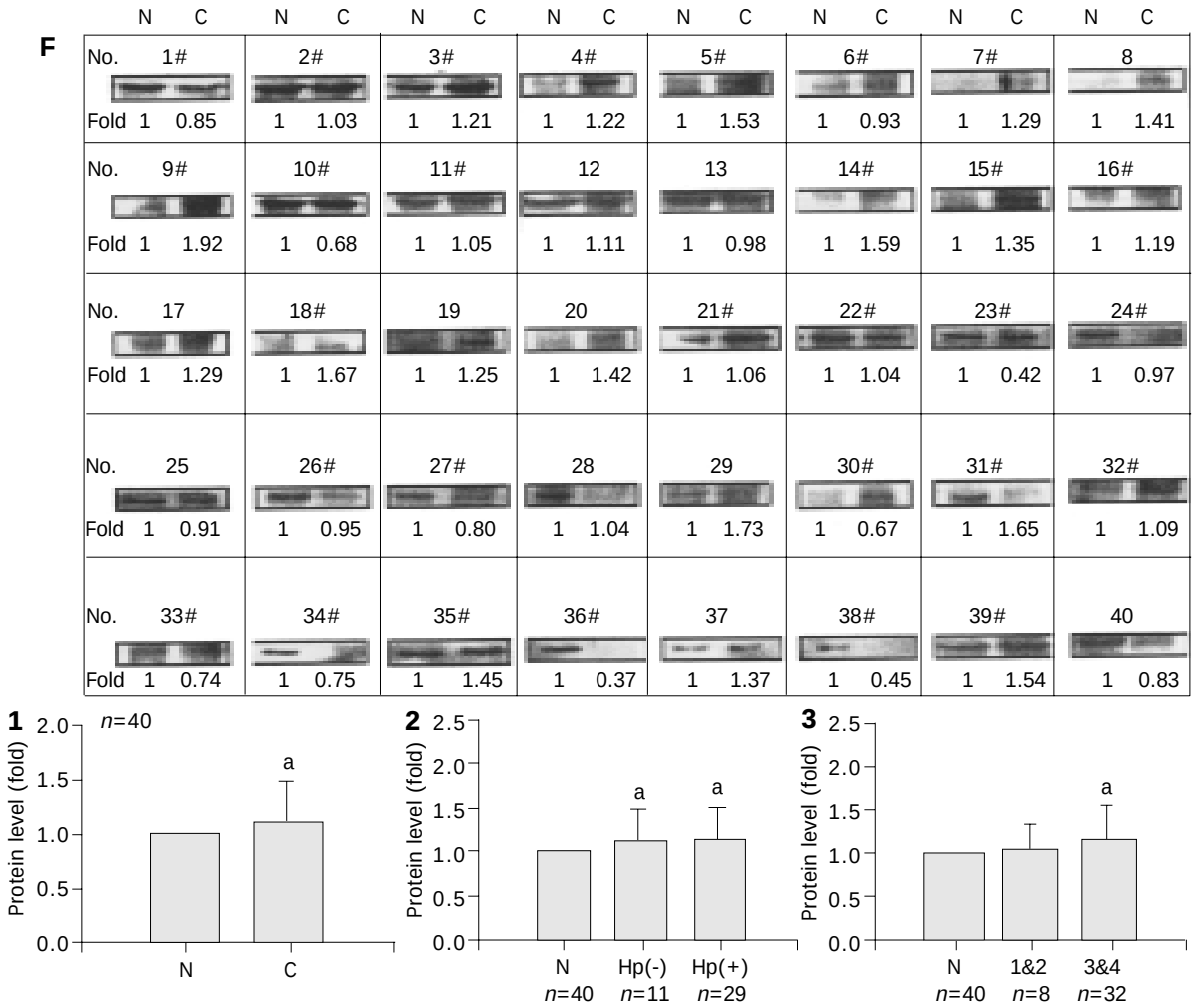
b
Protein level (fold)
N
n=40
1&2
n=8
3&4
n=32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Fold 1 1.29 1 1.67 1 1.25 1 1.42 1 1.06 1 1.04 1 0.42 1 0.97
No. 25 26# 27# 28 29 30# 31# 32#
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No. 33# 34# 35# 36# 37 38# 39# 40
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2.0
1.5
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N
n=40
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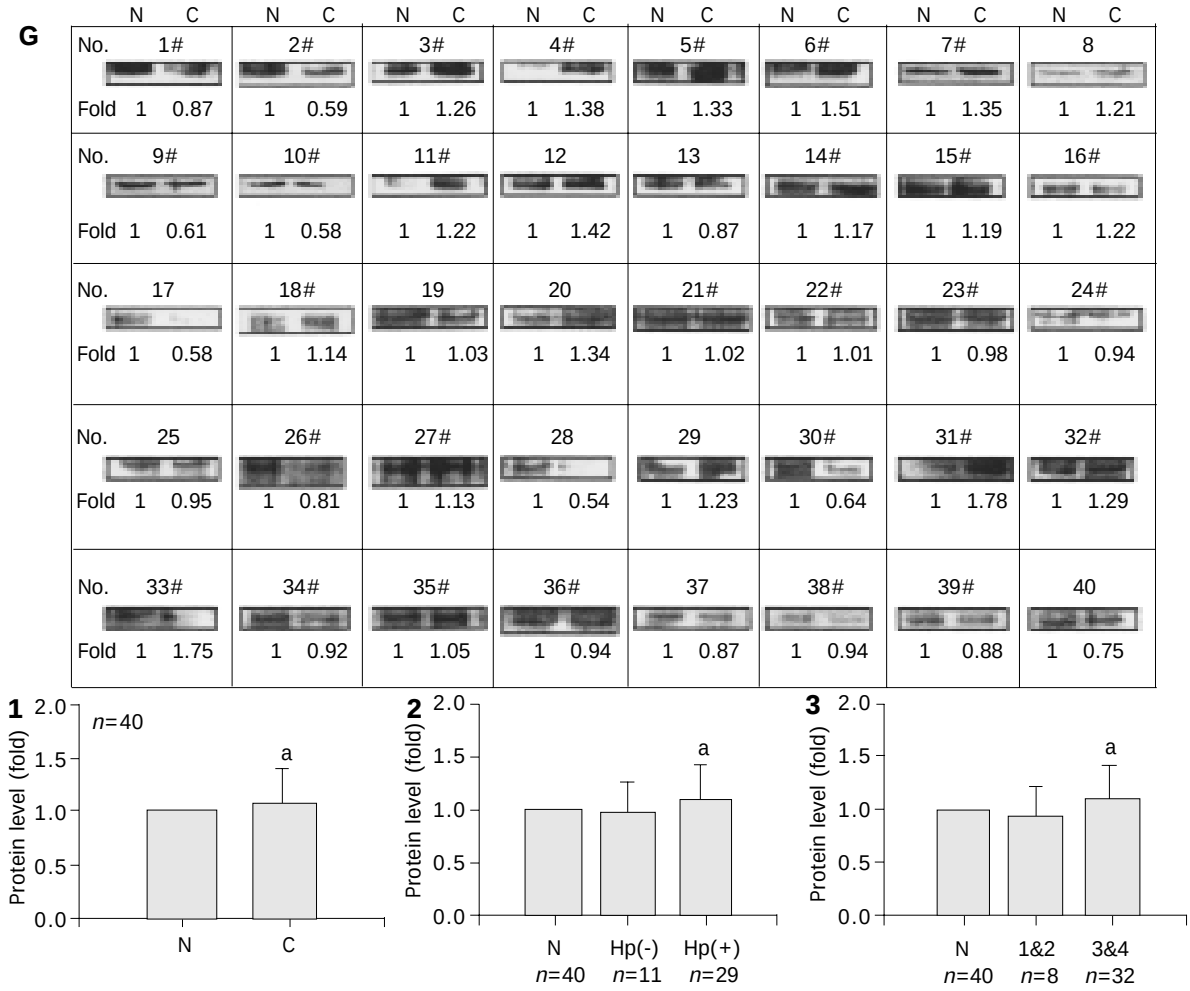


**Figure 2** Immunoblot analysis of factors affecting cell death. A: Immunoblot analysis of iNOS. 1: Significantly higher concentrations of iNOS in cancer tissues compared with adjacent non-cancer tissues. 2: Significantly higher concentrations of iNOS in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher iNOS concentrations in stage 3 and 4 patients. #=*H pylori* infected, $^{a}P<0.05$, $^{b}P<0.01$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. B: Immunoblot analysis of NFκB. 1: Significantly higher concentrations of NFκB in cancer tissues in comparison with adjacent non-cancer tissues. 2: Significantly higher concentrations of NFκB in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher NFκB concentrations in stage 3 and 4 patients #=*H pylori* infected, $P<0.05$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. C: Immunoblot analysis of MEKK1. 1: Concentrations of MEKK1 in cancer tissues and adjacent non-cancer tissues. 2: Significantly higher concentrations of MEKK1 in cancer tissues of patients without *H pylori* infection. 3. No differences in MEKK1 concentrations in cancer tissues with different stages. #= *H pylori* infected, $^{a}P<0.05$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. D: Immunoblot analysis of Caspase 3. 1: Similar concentrations of Caspase 3 in cancer tissues and adjacent non-cancer tissues. 2: Similar concentrations of Caspase 3 in cancer tissues of patients with and without *H pylori* infection. 3: Significantly lower Caspase 3 concentrations in stage 1 and 2 patients. #=*H pylori* infected, $^{b}P<0.01$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. E: Immunoblot analysis of Bcl-2. 1: Similar concentrations of Bcl-2 in cancer tissues and adjacent non-caner tissues. 2: No differences in concentrations of Bcl-2 in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher Bcl-2 concentrations in stage 1 and 2 patients. #=*H pylori* infected, $^{b}P<0.01$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. F: Immunoblot analysis of Mcl-1. 1: Significantly higher concentrations of Mcl-1 in cancer tissues in comparison with adjacent non-cancer tissues. 2: Significantly higher concentration of Mcl-1 in cancer tissues of patients with and without *H pylori* infection. 3: Significant higher Mcl-1 concentrations in stage 3 and 4 patients than #=*H pylori* infected, $^{a}P<0.05$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. G: Immunoblot analysis of IAP. 1: Significantly higher concentrations of IAP in cancer tissues compared with adjacent non-cancer tissues, 2: Significantly higher concentrations of IAP in cancer tissues with *H pylori* infection, 3: Significantly higher IAP concentrations in stage 3 and 4 patients #=*H pylori* infected, $^{a}P<0.05$, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4.

NFκB has been found to be a critical regulator of genes involved in inflammation, cell proliferation, and apoptosis[30]. Recent studies suggested that NFκB might play a critical role in protecting cells against apoptosis[31,32]. The magnitude of the stimulus and the cell type involved may determine whether NFκB leads to cell survival or cell death. Maeda *et al*. reported cag PAI positive *H pylori* was capable of inducing apoptotic effects mainly through the mitochondrial pathway. Antiapoptotic effects mediated by NFκB activation were also observed by Maeda *et al*[33]. In this study, we revealed an

increase in the concentrations of NFκB in cancer tissues. In addition, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer compared with *H pylori*-negative patients and low stage gastric cancer patients, respectively.

Recent investigations implicated mitogen-activated protein kinases (MAPK) as additional mediators of *H pylori*-dependent NFκB activation and IL-8 expression. Studies have demonstrated the presence of cross-talk between the MAPK and NFκB pathways[21-34]. MAPK cascades are signal transduction networks that target transcription factors and thus participate in a diverse array of cellular functions including cytokine production. In this study, there was an increase in the concentrations of MEKK1, a member of the MAPK signaling cascade in cancer tissues. However, the increase was not significant in all the studied patient groups.

Kanai *et al*. noted that transforming growth factor-α played an anti-apoptotic role in gastric mucosal cells by enhancing the expression of Bcl-2 family proteins via an NFκB-dependent pathway[35] NFκB is now known to upregulate transcription of several anti-apoptotic genes, including a cellular inhibitor of apoptosis, a Bcl-2 homolog, and cyclooxygenase-2, as well as pro-apoptotic genes, such as bax and p53. Tumor cells can have abnormal expression levels of factors such as Bcl-2 family proteins that slow the progression of apoptosis and elevate NFκB-regulated transcription, which can inhibit TNF-α-induced apoptosis. However, in this study, no significant changes in the concentrations of Bcl-2 in cancer tissues or patients infected with *H pylori* were noted. Furthermore, upregulation of anti-apoptotic gene mcl-1 might play a role in interleukin-6-mediated protection of gastric cancer cells from the apoptosis induced by hydrogen peroxide[36]. The anti-apoptotic role played by NFκB also involves the ability of this transcriptional factor to induce expression of genes that promote cell survival, such as the genes coding for TRAF1, TRAF2, and the cellular inhibitors of apoptosis 1 and 2 (c-IAP1, c-IAP2)[37]. In this study, we demonstrated an increase in the concentrations of IAP and Mcl-1, anti-apoptosis related proteins in cancer tissues. Furthermore, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer, compared with *H pylori*-negative and non-advanced gastric patients, respectively. Therefore, enhanced antiapoptosis-related protein expression may contribute to disease progression.

Caspase3 is a member of the cell death effector (CED)-3 family, which is involved in the induction of apoptosis. Hoshi *et al*. reported Caspase 3 was involved in the development or regulation of apoptotic cell death in cell turnover of normal and neoplastic mucosa of the human stomach[38]. These results indicate that gastric adenocarcinoma is associated with an inhibition of apoptosis and the augmentation of proliferative activity of tumor cells when compared to non-neoplastic gastric mucosa. However, in this study, there was no difference of increase in Caspase 3 between patients with or without *H pylori* infection or patients with or without advanced gastric cancer.

In conclusion, chronic *H pylori*-induced iNOS expression and subsequent DNA damage as well as signal transduction appear to make an important contribution to *H pylori*-related gastric carcinogenesis. This sequence of events found in this study supports the hypothesis that oxygen-free radical-mediated damage induced by *H pylori* plays a vital role in the development of gastric carcinoma in patients with chronic gastritis.

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• BASIC RESEARCH •

# Japanese herbal medicine, *Saiko-keishi-to*, prevents gut ischemia/reperfusion-induced liver injury in rats via nitric oxide

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## Abstract

**AIM:** To determine whether *Saiko-keishi-to* (TJ-10), a Japanese herbal medicine, could protect liver injury induced by gut ischemia/reperfusion (I/R), and to investigate the role of NO.

**METHODS:** Male Wistar rats were exposed to 30-min gut ischemia followed by 60 min of reperfusion. Intravital microscopy was used to monitor leukocyte recruitment. Plasma tumor necrosis factor (TNF) levels and alanine aminotransferase (ALT) activities were measured. TJ-10 1 g/(kg·d) was intragastrically administered to rats for 7 d. A NO synthase inhibitor was administered.

**RESULTS:** In control rats, gut I/R elicited increases in the number of stationary leukocytes, and plasma TNF levels and ALT activities were mitigated by pretreatment with TJ-10. Pretreatment with the NO synthase inhibitor diminished the protective effects of TJ-10 on leukostasis in the liver, and the increase of plasma TNF levels and ALT activities. Pretreatment with TJ-10 increased plasma nitrite/nitrate levels.

**CONCLUSION:** TJ-10 attenuates the gut I/R-induced hepatic microvascular dysfunction and sequential hepatocellular injury via enhancement of NO production.

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## INTRODUCTION

Herbal medicines that have been used in China for thousands of years are now being manufactured in Japan as drugs containing ingredients of standardized quality and quantity. The clinical efficacy of these medicines has been utilized by Japanese Western-medicine practitioners for more than 20 years and is well recognized. One of the herbal medicines, *Saiko-keisi-to* (TJ-10) (Chinese name; *Chai-Hu-Gui-Zhi-Tang*), is a common drug to treat duodenal ulcer, pancreatitis, and chronic liver disease in Japan. It is an oral medicine and consists of 9 herb components: *Bupleurum* root, *Pinellia tuber, Scutellaria* root, *Jujube* fruit, *Ginger rhizome*, ginseng root, cinnamon bark, peony root and *Glycyrrhiza* root. Another common herbal medicine, Sho-saiko-to (TJ-9), consists of 7 components of them: *Bupleurum* root, *Pinellia tuber*, *Scutellaria* root, jujube fruit, ginger rhizome, ginseng root, and *Glycyrrhiza* root. In a double-blind multicenter clinical trial, TJ-9 was reported to reduce the elevated serum activities of aspartate transaminase, alanine transaminase (ALT), and glutamyl transpeptidase in chronic active hepatitis patients[1]. TJ-9 has been shown to improve liver function as well as the symptoms associated with chronic liver disease including digestive discomfort[2]. Although TJ-10 has been often administered to patients with chronic liver disease as well as TJ-9, there are few epidemiological reports[3], and little is known about mechanisms of its cytoprotective effects on liver damage. Recently, because of its major pharmaceutical effects, TJ-10 is presumed to gradually improve biological defense mechanisms. One of the components of TJ-10, *Saikosaponin*, has been reported to inhibit hepatocyte necrosis induced by galactosamine[4], and *Saikosaponin*-d has been shown to reduce microsomal lipid peroxidation induced by NADPH and $CCl_4$[5]. However, its mode of action has not been fully elucidated.

Nitric oxide (NO) has been found to be a modulator of the adhesive interactions between leukocytes, platelets, and endothelial cells[6-9] as well as an important modulator of tissue blood flow, arterial pressure, and neurotransmission[8], and NO-dependent cell-cell interactions have been demonstrated in tissues exposed to ischemia and reperfusion (I/R), an injury process in which leukocyte-endothelial cell adhesion plays a critical role. A role of NO in the pathobiology of I/R injury has been supported by observations that inhibition of NO biosynthesis could elicit most of the microvascular alterations observed in tissues exposed to I/R[7,10], and NO-donating compounds have been shown to provide significant protection against the microvascular dysfunction that is normally associated with I/R[7]. We developed a murine model of leukocyte-dependent hepatocellular dysfunction that was elicited by gut I/R[11-13]. The model allows *in vivo* assessment of the effects of I/R on leukocyte sequestration in sinusoids of different regions of the liver lobule, leukocyte adherence in postsinusoidal venules, and the number of perfused sinusoids. Using this model, we have recently demonstrated that inhibition of both NO synthase and supplementation with exogenous NO could affect the leukocyte rolling, leukocyte adhesion, and sinusoidal perfusion elicited in the liver by gut I/R[14-16].

Some herbal medicines have been reported to have an inducing effect on NO production by non-stimulated macrophages[17]. We have recently reported that TJ-9 could attenuate the gut I/R-induced hepatic microvascular dysfunction and sequential hepatocellular injury via NO[18]. However, little is known about the effect of TJ-10 on plasma NO levels and I/R injury *in vivo*. TJ-9 is contraindicated for liver cirrhosis, because of its side-effect, causing lung fibrosis. Since TJ-10 has 30% less *Saikosaponin* compared to TJ-9, it seems to be safer. Therefore, frequency and importance of TJ-10 are expected to increase in Japan. In the present study, we investigated whether TJ-10 modulated the gut I/R-induced microvascular dysfunction in the liver, and the role of NO in the responses by inhibiting NO synthase.

## MATERIALS AND METHODS

### Animals and surgical procedure

Male Wistar rats (200-250 g) were fed a standard rat chow for 2 wk, and TJ-10 (1 g/kg in saline) or saline alone was then administered for 7 d intragastrically through a tube. Experiments below were performed 18 h after the final dose of TJ-10 or saline. The rats were fasted for 18 h prior to each experiment, and then intraperitoneally anesthetized with pentobarbital sodium (35 mg/kg). The left carotid artery was cannulated, and a catheter was positioned in aortic arch to monitor blood pressure. The left jugular vein was cannulated for drug administration. All experiments were performed according to the criteria outlined in the National Research Council Guide.

### Intravital microscopy

After laparotomy, one lobe of the liver was examined through an inverted intravital microscope (TMD-2S, Diaphoto, Nikon, Tokyo, Japan) and images were recorded with a silicon intensified target (SIT) camera (C-2400-08, Hamamatsu photonicus, Shizuoka, Japan). The liver was placed on an adjustable Plexiglas microscope stage and covered with a nonfluorescent coverslip that allowed observation of a 2 $cm^2$ segment of tissue. The liver was carefully positioned to minimize the influence of respiratory movements, and its surface was moistened and covered with cotton gauze soaked with saline. Images of microcirculation at the surface of the liver were observed through consecutive microfluorographs of hepatic microcirculation, that is, those of rhodamine-6G-labeled leukocytes in the sinusoids, were observed at 90 min after the onset of SMA occlusion and recorded on a digital video recorder. The number of stationary leukocytes was determined off-line during playback of videotape images. A leukocyte was considered to be stationary within the microcirculation (sinusoids) if it remained stationary for more than 10 s. The lobule, which had the fewest stationary leukocytes, was selected for observation at the basal condition. Stationary leukocytes were quantified in both the midzonal and pericentral regions of the liver lobule and expressed as the number per field of view ($2.1\times10^5$ $\mu m^2$). The percentage of non-perfused sinusoids was calculated as the ratio of the number of non-perfused sinusoids to the total number of sinusiods per microscopic field.

### Experimental protocols

We observed the surface of liver for 10 min before ligating the superior mesenteric artery to ensure that all parameters measured on-line were in a steady state. The superior mesenteric artery was then ligated for 0 (sham) or 30 min with a snare created from a polyethylene tube. At the end of the ischemic period, the ligation was gently removed. Leukocyte accumulation and the number of non-perfused sinusoids (NPS) were measured before ischemia, immediately following reperfusion, and every 15 min for 1 h thereafter. In one set of experiments, 7 untreated animals, and 5 TJ-10-treated animals in the control groups (sham gut I/R) and gut I/R groups were used. In another set of experiments in which TJ-10 was administered, the rats were given a NO synthase (NOS) inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA: Sigma, St. Louis, MO) (0.5 mg/kg, i.v.) 30 min before the onset of ischemia. These experiments were performed with 5 animals in each group. In some experiments, the rats were given dexamethasone (2 mg/kg, Sigma, St. Louis, MO) with or without L-NMMA (0.5 mg/kg, i.v.) 30 min before the onset of ischemia. These experiments were performed with 5 animals in each group.

### Tumor necrosis factor and endotoxin assay

Sixty min after the onset of reperfusion, blood plasma samples for tumor necrosis factor (TNF) detection were collected from the inferior vena cava at a point proximal to the hepatic vein. Plasma TNF concentration was determined in a microtiter plate using a TNF immunoassay kit (BioSource International, Camarillo, CA) based on enzyme-linked immunosorbent assay (ELISA) as described in our previous study[19]. Plasma endotoxin levels were measured by endospecy (an endotoxin-specific chromogen. Seikagaku Co.,Tokyo, Japan) according to our previous report[20].

### Enzyme and nitrite/nitrate assay

Blood samples for enzyme activities were collected from the carotid artery 6 h after the onset of reperfusion. Serum ALT activity was determined by conventional UV methods as previously described[21]. Blood samples for nitrite/nitrate assay were collected from the inferior vena cava 16 h after the last administration of TJ-10 (saline as control). The combined levels of nitrite and nitrate in plasma were determined by a previously reported method[22]. Five separate experiments were performed.

### Statistical analysis

The data were analyzed by standard statistical methods, i.e., ANOVA and Scheffe's (post hoc) test. All values were reported as mean±SD. $P<0.05$ was considered statistically significant.

## RESULTS

Figure 1A shows the effects of TJ-10 and/or L-NMMA on the gut I/R induced leukostasis in sinusoids of the midzonal and pericentral (including the terminal hepatic venule [THV]) regions of the liver lobule (panel A), and the entire liver lobule (sinusoids + THV, panel B). In control rats, gut I/R elicited significant increases in the number of stationary leukocytes compared to basal values. Pretreatment with TJ-10 blunted the gut I/R-induced leukostasis in the midzonal (untreated+I/R: 9.6±0.6, TJ-10+I/R: 3.6±0.6, per field) and the pericentral regions (untreated+I/R: 6.0±0.7, TJ-10+I/R: 3.6±0.5, per field). L-NMMA diminished the protective effect of TJ-10 in the pericentral region (5.6±0.2, per field), but did not significantly affect the gut I/R-induced leukostasis in the midzonal region or the entire liver lobule.

Figure 1B shows the effect of TJ-10 and/or L-NMMA on the gut I/R-induced elevation of plasma TNF levels. In the control rats, gut I/R elevated the plasma TNF-α levels. Pretreatment with TJ-10, however, blunted the gut I/R-induced elevation of plasma TNF-α levels (untreated+I/R: 140.8±12.6, TJ-10+I/R: 61.7±22.5 ng/L). L-NMMA diminished the protective effects of TJ-10 (113.2±13.7 ng/L).

Figure 1C shows the effect of TJ-10 and/or L-NMMA on the gut I/R-induced elevation of serum endotoxin levels. In the control rats, gut I/R elevated the serum endotoxin levels. Pretreatment with TJ-10, however, blunted the gut I/R-induced elevation of serum endotoxin levels (untreated+I/R: 39.2±8.1, TJ-10+I/R: 11.1±3.3 ng/L). L-NMMA diminished the protective effects of TJ-10 (34.7±5.3 ng/L).

Figure 1D illustrates the effect of TJ-10 and/or L-NMMA on the gut I/R-induced elevation of plasma ALT activities. In the control rats, gut I/R elevated the plasma ALT activities. Pretreatment with TJ-10, however, blunted the gut I/R-induced elevation of plasma ALT levels (untreated+I/R: 146.6±13.0, TJ-9+I/R: 76.0±7.0 IU/L). L-NMMA diminished the protective effects of TJ-10 (123.2±11.9 IU/L).

Table 1 shows the effects of TJ-9 and TJ-10 on plasma nitrite/nitrate levels. Pretreatment with TJ-10 increased the plasma nitrite/nitrate levels as well as TJ-9.

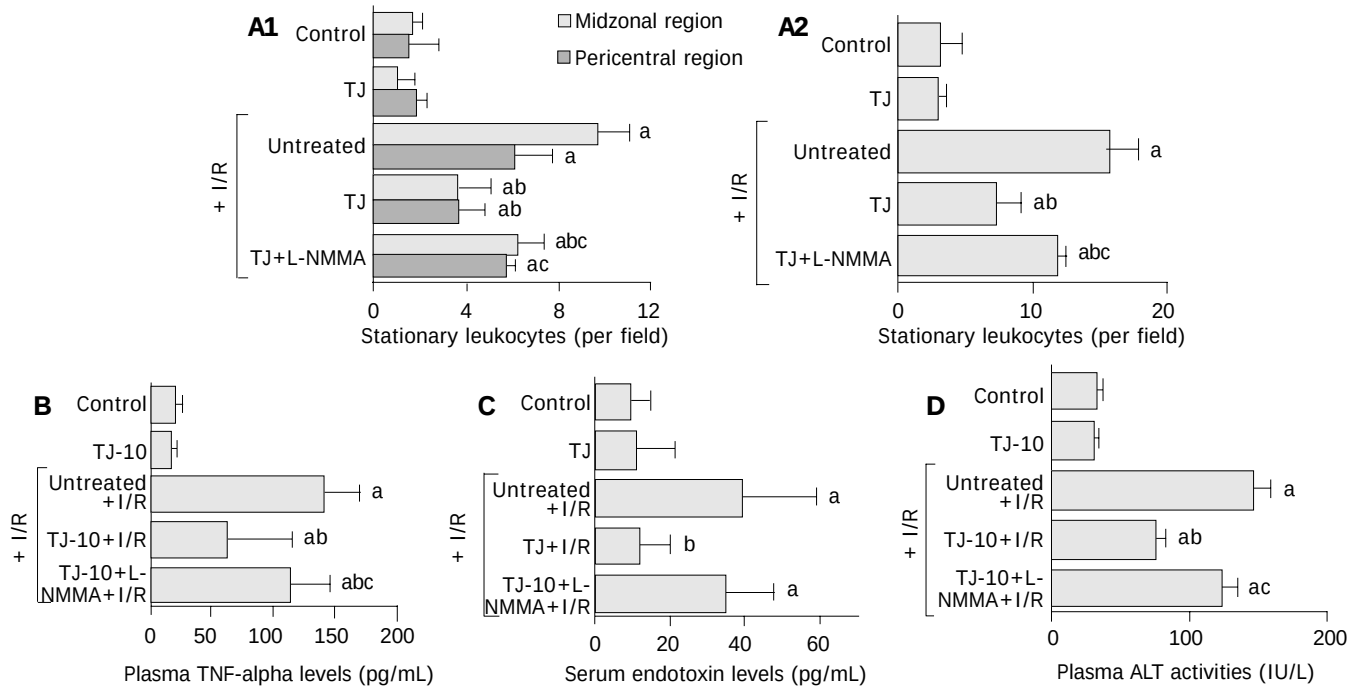


**Figure 1** Effects of Saiko-keishi-to (TJ-10) and/or an NO synthase inhibitor ($N^G$-monomethyl-L-arginine, L-NMMA) on the number of stationary leukocytes, plasma TNF-α levels, serum endotoxin levels, and plasma ALT activities. A: In each region (the midzonal and pericentral regions) (panel A) and the entire (combined) liver lobule (panel B) after 30 min of gut ischemia and 60 min of reperfusion; B: Plasma TNF-α levels at 60 min after gut I/R; C: Serum endotoxin levels at 60 min after gut I/R; D: Plasma ALT activities at 6 h after gut I/R.

**Table 1** Effects of Sho-saiko-to (TJ-9) and Saiko-keishi-to (TJ-10) on plasma nitrite/nitrate levels ($n$ = 5)

| Group | Plasma nitrite/nitrate levels |
|---|---|
| Control | 17.1±2.9 |
| TJ-9 | 35.8±4.3[a] |
| TJ-10 | 39.0±4.0[a] |

[a]$P$<0.05 *vs* control.

## DISCUSSION

Herbal medicines are widely used in Japan. The clinical efficacy of these medicines has been utilized by Japanese Western-medicine practitioners for more than 20 years and is well recognized. In cases of gastroenterological and hepatological diseases, they are often used for patients with chronic liver disease as well as gastrointestinal functional disorder. Although TJ-9 has been the most famous herbal medicine for chronic liver disease in Japan, TJ-10 is another common drug to treat duodenal ulcer, pancreatitis, and chronic liver disease. TJ-10 has effectiveness for more kinds of diseases rather than TJ-9. Indeed, the components of TJ-10 include all of the 7 components of TJ-9. Although TJ-10 has been often administered to patients with chronic liver disease as well as TJ-9, there were few epidemiological reports[3], and little is known about mechanisms of the cytoprotective effects on liver damage.

Previously published work has demonstrated that reperfusion of the ischemic small intestine could elicit an acute inflammatory response both in the intestine and in distant organs, such as the liver[11,12,23] and lung[23,24]. In the liver, the response was characterized by leukocyte plugging of sinusoids, leukocyte adherence in postcapillary venules, a reduction in the number of perfused sinusoids, hepatocellular hypoxia, and leakage of enzymes (ALT) from hepatocytes[11,12,23,25]. Recently, we have reported that TJ-9 could attenuate the gut I/R-induced responses in the liver[18]. In the present study, it was demonstrated that TJ-10 had a similar effect to TJ-9 on the gut I/R-induced responses in the liver. NO has been found to be a modulator of the adhesive interactions between leukocytes, platelets, and endothelial cells[6-9], and NO-dependent cell-cell interactions have been demonstrated in tissues exposed to ischemia and reperfusion (I/R), an injury process in which leukocyte-endothelial cell adhesion plays a critical role. Depletion and/or inactivation of NO has been implicated as a key event in the recruitment of leukocytes in tissues exposed to I/R[26-28]. We previously demonstrated that treatment with L-NMMA resulted in exaggerated leukostasis and cellular injury in the murine liver after gut I/R and that increased delivery/generation of NO in the liver via a NO donor attenuated the inflammatory responses and microvascular dysfunction elicited in the liver by gut I/R[13]. These results suggest a protective effect of NO on the gut I/R-induced responses in the rat liver. The finding in the present study that L-NMMA diminished the protective effect of TJ-10 on the increase in plasma TNF-α and serum endotoxin levels, leukostasis in the liver, and plasma ALT activities, suggested that TJ-10 could prevent the gut I/R-induced cytokine production and microvascular dysfunction in the liver by elevating sinusoidal NO level. NO could modulate leukocyte- and /or platelet-endothelial cell interactions[6,9,13,14]. Since pretreatment with TJ-10 actually increased plasma nitrite/nitrate levels in the present study, the increase in NO production by hepatocytes and macrophages after treatment with TJ-10 appeared to be involved in the cytoprotective effects of TJ-10.

In the present study, L-NMMA diminished the protective effect of TJ-10 on the increase in plasma TNF-α and serum endotoxin levels, leukostasis in the liver, and plasma ALT activities. While, in the previous study[18], L-NMMA did not affect the protective effect of TJ-9 on the increase in leukostasis in the midzonal region, the total number of stationary leukocytes, or the plasma ALT activity. One likely interpretation is that a mechanism other than the increase in NO production mediates the protective effects of TJ-9. Steroids have been known to prevent reperfusion injury[29]. The evidence in our previous study[18] that L-NMMA did not affect either TJ-9- or dexamethasone-induced decrease in the gut I/R-elevated plasma

ALT activities, raises a possibility that TJ-9-increased blood corticosterone level may prevent the gut I/R-induced microvascular and hepatocellular injury. Since L-NMMA diminished the protective effect of TJ-10 on the gut I/R-induced increase in plasma ALT activities, corticosteroid effect was not involved in the prevention of gut I/R-induced hepatocellular injury by TJ-10. *Kampo* (herbal medicines) prescriptions affect as the complex. For example, though one of the components in TJ-10, cinnamon bark, which is not included in TJ-9, had a very strong anti-oxidant effect, there was no significant difference between TJ-9 and 10 in the potential for anti-oxidation[30]. Thus, our results suggest that TJ-10 has different characteristics from TJ-9, even including all components of TJ-9.

Recently , the importance of the way of traditional diagnosis "Sho" in the use of *Kampo* prescriptions has come to be widely described even in package insert drug information pamphlets of *Kampo* prescriptions. "Sho" is judged comprehensively by a complex of subjective and objective symptoms at a certain point of illness. The process is generally complicated, but we sometimes diagnose by tonus felt on the abdomen. Namely, hypertonus is "Jitsu Sho", and hypotonus is "Kyo Sho". General fatigue, sleepiness, sleepless, appetite loss, tiredness of eyes, easily catching cold, dizziness, looking pale, and so on are symptoms of "Kyo Sho". TJ-10 suits for "Kyo Sho" rather than TJ-9. Moreover, most patients with chronic hepatitis have "Kyo Sho". TJ-10 prevents microvascular dysfunction in the liver increase in NO production. Symptoms of "Kyo Sho" described above seem to be caused by microvascular dysfunction. Taken together, TJ-10 is recommended for chronic hepatitis patients with "Kyo Sho".

Although further studies are required to clarify the mechanisms of the protective effects of TJ-10 on reperfusion injury, this study has demonstrated the protective effect of TJ-10 on reperfusion injury via NO.

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• BASIC RESEARCH •

# *KAI1* gene expression in colonic carcinoma and its clinical significances

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## Abstract

**AIM:** To investigate *KAI1* gene expression in the progression of human colonic carcinoma and its clinical significances.

**METHODS:** KAI1 expression was detected by *in situ* hybridization and immunohistochemistry in the 4 established cell lines of colorectal carcinoma with different metastatic potentials, and in 80 specimens of colonic carcinoma, 21 colonic carcinoma specimens with lymphatic metastasis and 20 controls of normal colonic mucosa.

**RESULTS:** The expressions of KAI1 in HT29 and SW480 cell lines were higher than those in LoVo and SW620. The expression of *KAI1* gene was significantly higher in colorectal carcinoma compared with normal colonic mucosa and lymphatic metastasis ($\chi^2$=46.838, $P$<0.01). The expression of *KAI1* gene had no relationship with histological grade. The KAI1 expressions in Dukes A and B carcinoma were higher at both mRNA and protein levels compared to Dukes C carcinoma ($\chi^2$=16.061, $P$<0.05). The expression of KAI1 in colonic carcinoma specimens with lymphatic metastasis was almost lost. The results of *in situ* hybridization were in concordance with immunohistochemistry.

**CONCLUSION:** KAI1 is highly related to the metastasis of colonic carcinoma and may be a useful indicator of metastasis in colonic carcinoma.

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## INTRODUCTION

Colorectal carcinoma is one of the most common forms of malignancy, and metastasis is the major cause of mortality in human population. Timely and precise identification of the occurrence of metastasis and its contributive factors are very important in the enhancement of prognosis and effects of treatment. *KAI1* gene was first identified in human prostate carcinoma and mapped to human chromosome 11p11.2[1]. It encodes 267 amino acids belonging to plasma membrane glycoprotein, which consists of 4 transmembrane domains and 1 large and 1 small extracellular domains. The extracellular domains have 3 potential N-glycosylation sites. According to its special structure, it may be predicted that the function of *KAI1* gene may come down to cell-cell adherence and cell-matrix connection[2]. The role of KAI1 in tumor progression may not be limited to prostatic cancer. It was reported to be important in preventing the development of metastases in a wide variety of human tumor types, including cervical[3], breast[4], pancreatic[5], esophageal[6], bladder[7], and ovarian cancers[8]. In the present study, the expression of KAI1 was detected by *in situ* hybridization in 4 cell lines of colorectal carcinoma, and in paraffin-embedded normal colonic epithelium, carcinoma, and lymphatic metastasis. The purpose was to explore the relationship between KAI1 expression and colonic carcinoma metastasis and its clinical significances.

## MATERIALS AND METHODS

### *Cell lines and culture conditions*

LoVo and HT29 cell lines were cultured in RPMI 1640 supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. SW480 and SW620 were cultured in DMEM medium supplemented with the 100 mL/L FBS. All cells were grown in 50 mL/L $CO_2$ humidfied atmosphere at 37 ℃.

### *Tissue specimens*

A total of 80 colonic carcinoma specimens, 20 normal colonic mucosa samples and 20 lymphatic metastasis samples were obtained from Nanfang Hospital. All the samples were routinely fixed in 40 g/L formaldehyde solution, embedded in paraffin, and cut into 4 μm thick sections. Samples were selected according to the pathological diagnosis and reviewed by a pathologist to confirm the diagnosis.

### *In situ hybridization (ISH) assays*

The digoxigenin-labeled KAI1-cDNA probe for *in situ* hybridization was prepared as described in one of our previous papers[9]. The ISH was performed according to the instruction of the enhanced sensitive ISH detection kit (purchased from Boster Biotechnology Company). Briefly, the sections were deparaffinized with xylene, dehydrated with graded ethanol and incubated in 30 mL/L $H_2O_2$ to block endogenous peroxidases for 30 min at room temperature. After being treated for 10 min with Triton X-100 and for 40 s with 3% pepsin, the sections were prehybridized for 3 h and hybridized overnight at 37 ℃. The final concentrations of the labeled probes were 0.15 ng/μL. After hybridization, excess probes were removed through rinsing in 2×SSC (1×SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate pH 7.0), 0.5×SSC, 0.2×SSC, respectively. The sections were incubated with an antidigoxigenin antibody conjugated biotin for 120 min at room temperature and then added strept-avidin biotin complex for 30 min. For color reaction, diaminobenzidine (DAB) was used. If the ISH signals were present, the cytoplasm would be full of brown granules. For ISH of the four colorectal carcinoma cell lines, the cells were incubated and grown on cover slip for 24 h, fixed with 950 mL/L

ethanol and washed with PBS (phosphate-buffered saline pH 7.2) 3 times, and then carried out ISH as described above. For negative controls, the digoxigenin-labeled KAI1-cDNA probe was replaced by prehybridized solution.

### *Immunohistochemical assays*

To confirm the results of *KAI1* gene expression on *in situ* hybridization, immunohistochemical studies were performed as described previously[9,10]. Briefly, the fixed cells and sections were subjected to immunostaining by using an ultrasensitive streptavidin-peroxidase technique (Maixin Biotechnology Company). Endogenous peroxidases were blocked by incubating with 5 mL/L $H_2O_2$ for 30 min at room temperature. The cells and sections were subsequently treated for 10 min with Triton X-100 and for 40 s with 30 g/L pepsin. The cells and sections were incubated for 30 min at 37 ℃ with normal nonimmune serum before overnight incubation at 4 ℃ with specific monoclonal KAI1-antibody (BD pharmingen technical company) at a dilution of 1:100. The cells and sections were then treated with biotin-conjugated second antibody before adding streptavidin-peroxidase. For color reaction, diaminobenzidine was used. If the positive signals were present, the cytoplasm and membrane were stained brown. For negative controls, the monoclonal KAI1 antibody was replaced by PBS.

### *Review and scoring of the section*

The stained sections were reviewed and scored indepentdently by two pathologists under Olympus microscope. The different degrees of intensity of staining were graded on a scale of 0 to 3 as follows: 0 indicated that the number of positive cells was less than 10%; 1 indicated weak positive and the number of positive cells more than 10%, but less than 30%; 2 indicated positive and the number of positive cells more than 30% but less than 50%; 3 indicated strong positive and the number of positive cells more than 50%.

### *Statistical analysis*

Fisher's exact probability test was adopted to examine the relationship between the variables. A $P$ value <0.05 was considered statistically significant.

## RESULTS

### *KAI1 gene expression in colorectal carcinoma cell lines*

HT29 and SW480 cell lines were derived from primary colorectal adenocarcinoma. SW620 and LoVo cell lines were established from metastatic colorectal carcinoma. Especially, SW620 cell line was isolated from the same case as was SW480 cell line. The expressions of KAI1 mRNA and protein in HT29 and SW480 cells were positive, whereas those in SW620 and LoVo cells were negative (Figures 1, 2).

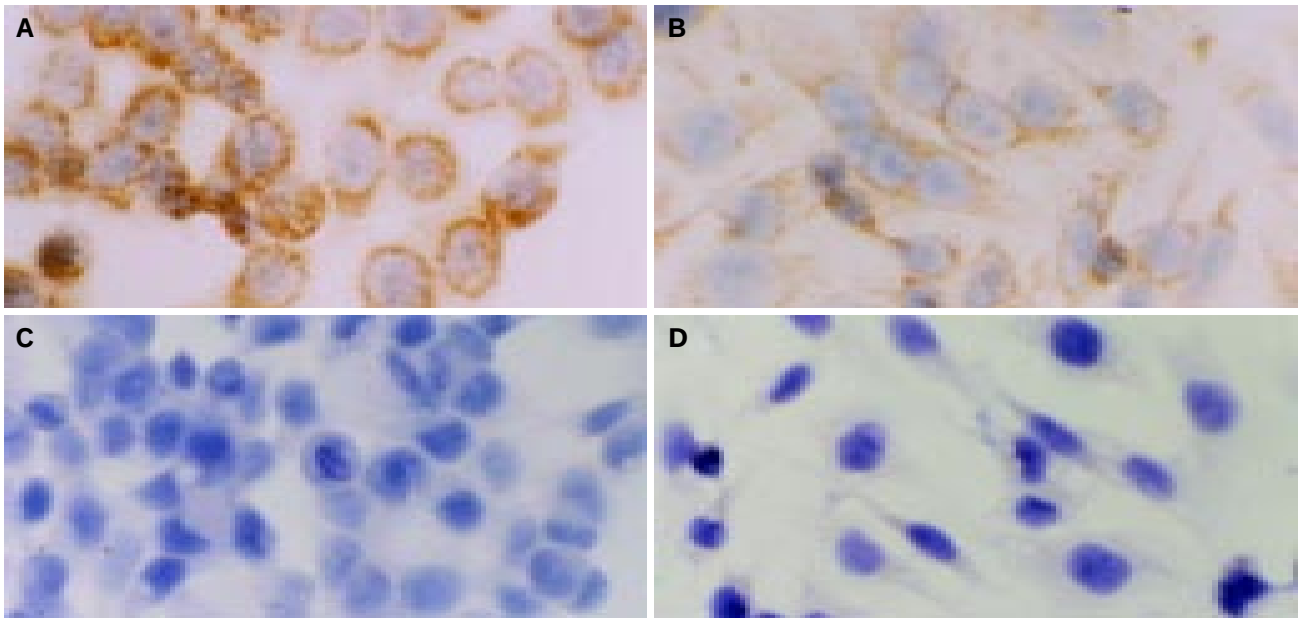


**Figure 1** *In situ* hybridization detection of expression of KAI1 mRNA in colorectal carcinoma cell lines (Original magnification: ×400). A: In HT29 cells, the positive expression (brown granule) was located in cytoplasm; B: In SW480 cells, the positive expression was located in cytoplasm; C: In SW620 cells, the expression of KAI1 mRNA was negative; D: In LoVo cells, the expression of KAI1 mRNA was negative.

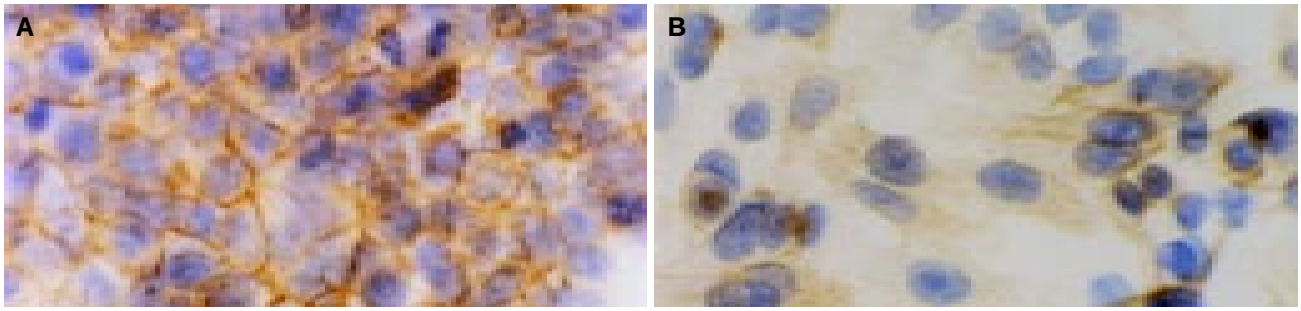


**Figure 2** Expression of KAI1 protein detected by immunohistochemistry in colorectal carcinoma cell lines (Original magnification: ×400). A: In HT29 cells, the positive expression located in cytoplasm and membrane; B: In SW480 cells, the positive expression located in cytoplasm and membrane.

### *Heterogeneous expression of KAI1 mRNA in colonic carcinoma tissue*

Of the 80 colonic carcinoma specimens, 56(70%) were classified as KAI1 positive (Figure 3). There were 4 cases (20%) with positive KAI1 mRNA expression in normal colonic mucosa and 2 cases (10%) in lymphatic metastasis. The results are shown in Table 1. The positivity ratio was significantly higher in colonic carcinoma than that in nomal colonic mucosa and in lymphatic metastasis ($\chi^2$=46.838, $P$<0.01). The expression in lymphatic metastasis was almost lost.

To further investigate the relationship between the expression of KAI1 and the clinical pathology, we sorted the colonic carcinomas based on histological grades and Dukes stages. Histological grade I means well differentiated adenocarcinoma, II means moderatedly differentiated and III means poorly differentiated. The results indicated that KAI1 expression had no relationship with histological grades ($\chi^2$=3.887, $P$>0.05). The expression in Dukes A and B carcinoma was markedly higher in comparison with Dukes C carcinoma ($\chi^2$=16.061, $P$<0.01).

### *KAI1 protein expression analyzed by immunohistochemistry*

There were 52(65%) cases with positive KAI1 protein expressions in the colonic carcinomas (Figure 3). The positive ratios of nomal colonic mucosa and lymphatic metastasis were 25% and 10%, respectively. The KAI1 expressions in 3 groups were significantly different ($\chi^2$=28.298, $P$<0.01). Overall, the immunohistochemical results agreed well with those from the *in situ* hybridization assays.

## DISCUSSION

Loss of the function of metastasis suppressor genes is an important step in the progression of a tumor type. Several candidate antimetastasis or anti-invasion genes have been studied in colorectal carcinoma, including nm23[11], E-cadherin[12], and CD44[13], but inconsistent findings have been reported. For example, in separate studies, nm23 expression has been found to be directly correlated, not to be correlated, or inversely correlated with metastatic potential in colorectal cancer[14-16]. KAI1 has been thought to be one of such metastasis suppressor genes, because it was shown to suppress the ability of human prostatic cancer cells to metastasize when the tumor was transplanted into nude mice[1] and because KAI1 mRNA expression was reduced in advanced pancreatic cancer[17] so that the pancreatic cancer cells spread to lymph nodes and distant organs. Furthermore, the transfer of the KAI1 gene into mammary cancer cells has been shown to lead to suppression of their metastatic potential, whereas their primary tumor growth has not been affected[18-20].

KAI1 is a member of the transmembrane-4 superfamily (TM4SF), many of which, including KAI1, are CD antigens present on the surface of leukocytes[21,22]. At least three TM4SF members are implicated in metastasis, including CD9/MRP-1, CD63/ME491, and CD82/KAI1[23]. KAI1 and other TM4SF members, such as integrins and E-cadherin, have been demonstrated to bind to each other and relay extracellular signals to signal transduction pathways that are important in cellular adhesion, invasive motility, and metastasis[24-27].

In our previous studies, we transfected the KAI1 cDNA

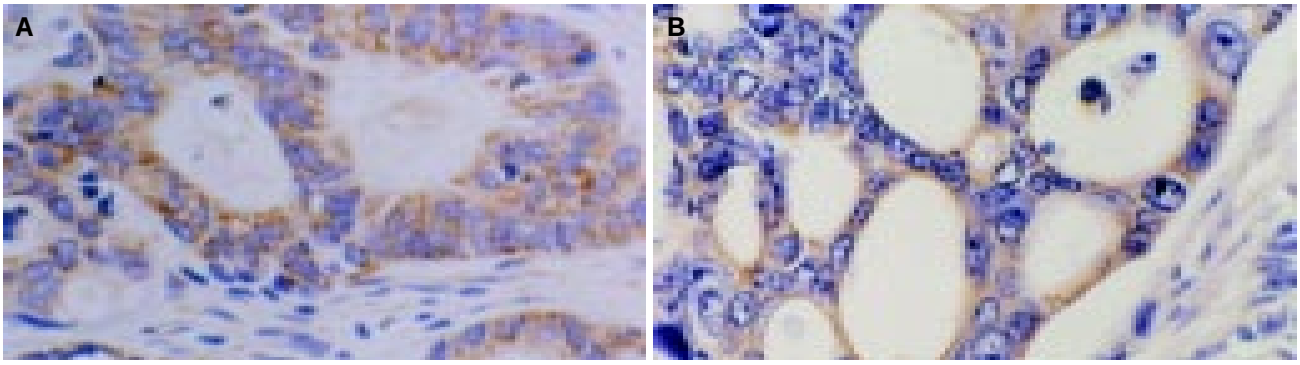


**Figure 3** *In situ* hybridization and immunohistochemical detection of KAI1 mRNA and protein in colonic carcinoma (Original magnification: ×400). A: *In situ* hybridization detection found that strong KAI1 mRNA staining was located in cytoplasm (brown granule); B: Detection by immunohistochemistry showed strong KAI1 protein staining was in cytoplasm and membrane.

**Table 1** *KAI1* gene expression in normal colonic mucosa, colonic carcinoma and lymphatic metastasis *n* (%)

| Tissue | KAI1 / CD82 expression | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | Total |
| Normal colonic mucosa | 16(80.0) | 3(15.0) | 1(5.0) | 0 | 20 |
| Colonic carcinoma | | | | | |
| Histological grade | | | | | |
| I | 6(23.1) | 2(7.7) | 12(46.2) | 6(23.1) | 26 |
| II | 10(26.3) | 12(31.6) | 14(36.8) | 2(5.3) | 38 |
| III | 8(50.0) | 6(37.5) | 2(12.5) | 0 | 16 |
| Dukes stage | | | | | |
| Dukes A | 1(4.3) | 6(26.1) | 9(39.1) | 7(30.4) | 23 |
| Dukes B | 3(17.6) | 6(35.3) | 5(29.4) | 3(17.6) | 17 |
| Dukes C | 20(50.0) | 10(25.0) | 8(20.0) | 2(5.0) | 40 |
| Lymphatic metastasis | 18(90.0) | 2(10.0) | 0 | 0 | 20 |

into a colorectal carcinoma cell line, LoVo, and found that the KAI1 transfectant exhibited significantly increased homotypic cell adhesion and suppressed invasion and metastasis[9]. Our findings were consistent with those in the studies of prostatic cancer[2] and breast cancer[18] cells.

In this study, we first investigated KAI1 mRNA expression in 4 colorectal carcinoma cell lines and found *KAI1* gene expression was much higher in HT29 and SW480 cell lines than in SW620 and LoVo cell lines. SW480 was isolated from a high-grade primary colonic tumor, and SW620 was isolated from a metastatic lymph node from the same patient 1 year later at the time of clinical relapse. KAI1 protein expression was high in SW480 but reduced in SW620. The colonic cancer cell line derived from metastatic lesions, LoVo, hardly exhibited any expression. In our observation we found that *KAI1* gene expressions both at mRNA and protein level, were inversely correlated with the metastatic potential of some established colorectal carcinoma cell lines.

We next examined KAI1 expression in normal colonic mucosa, carcinoma and lymphatic metastasis by *in situ* hybridization. The results indicated that KAI1 gene was highly expressed in colonic carcinoma compared with normal colonic epithelium and lymphatic metastasis. We found that although the expression had no relationship with histological grades, it was significantly correlated with Dukes stages. This indicates that the KAI1 mRNA expression may be positively related with the Dukes stages. The data shown in Table 1 demonstrate that KAI1 expression increases at an earlier tumor stage of colonic carcinoma, while decreases at advanced stages, and is possibly lost in metastases. That is to say, the expression of KAI1 has a reverse correlation with metastasis in colorectal carcinoma.

How does reduced TM4SF expression cause changes of invasive ability of tumor cells? It was hypothesized that tetraspanins might be implicated in the assembly of integrin-contained signaling complexes, thus modulating the function of integrin receptors in cell migration[24]. Some results indicated that integrin-tetraspanin protein complexes played an important role in regulating protrusive activity of the tumor cells and contributed to extracellular matrix-induced production of matrix metalloproteinase 2 (MMP-2), and as a consequence, the invasive ability of cells[28].

KAI1 has been extensively studied for its involvement in the progression of different human cancers. The mechanism of down-regulation of KAI1 has also been analyzed, however, much debate still exists. Recent study found a putative p53 consensus-binding site within the promoter region of KAI1 and demonstrated that the loss of p53 function, which was commonly observed in many types of cancer, led to the down-regulation of the *KAI1* gene, which might result in the progression of metastasis[29]. But other data suggested that the down-regulation of KAI1 was not associated with either mutation, allelic loss, methylation of the promoter, or p53 regulation[30]. Our previous study also demonstrated that mutation of the KAI1 gene, methylation of CpG islands and the abnormality of p53 were not related to low expression of KAI1.

In conclusion, KAI1 expression increases in an earlier tumor stage of colonic carcinoma, decreases in advanced stages, and is possibly lost in lymphatic metastases. The loss of KAI1 expression is correlated with higher stage, a surrogate marker for metastatic potential. The down-regulation of KAI1 in Dukes C and loss in metastasis demonstrate that loss of KAI1 expression occurs in cancer progression. The selection of cells that have the ability to spread from the primary tumor to the metastasis may favor those cells that have lost KAI1 expression. Those cells would be expected to be less adhesive, more invasive, and more motile[31], and these characteristics are necessary for metastasis.

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• BASIC RESEARCH •

# Influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells

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## Abstract

**AIM:** Recent studies in both rodents and humans indicated that bone marrow (BM)-derived stem cells were able to home to the liver after they were damaged and demonstrated plasticity in becoming hepatocytes. However, the question remains as to how these stem cells are activated and led to the liver and where the signals initiating the mechanisms of activation and differentiation of stem cells originate. The aim of this study was to investigate the influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells.

**METHODS:** Serum samples were collected from rats treated with a 2-acetylaminofluorene (2-AAF) /carbon tetrachloride ($CCl_4$) program for varying time points and then used as stimulators of cultured BM stem cells. Expression of $M_2$- and L-type isozymes of rat pyruvate kinase, albumin as well as integrin-β1 were then examined by reverse transcription polymerase chain reaction (RT-PCR) to estimate the differentiation state of BM stem cells.

**RESULTS:** Expression of $M_2$-type isozyme of pyruvate kinase ($M_2$-PK), a marker of immature hepatocytes, was detected in each group stimulated with experimental serum, but not in controls including mature hepatocytes, BM stem cells without serum stimulation, and BM stem cells stimulated with normal control serum. As a marker expressed in the development of liver, the expression signal of integrin-β1 was also detectable in each group stimulated with experimental serum. However, expression of L-type isozyme of pyruvate kinase (L-PK) and albumin, marker molecules of mature hepatocytes, was not detected in groups stimulated with experimental serum.

**CONCLUSION:** Under the influence of serum from rats with liver failure, BM stem cells begin to differentiate along a direction to hepatocyte lineage and to possess some features of immature hepatocytes.

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## INTRODUCTION

Recent studies in both rodents and humans indicated that bone marrow (BM) stem cells were able to home to the liver after they were damaged, and demonstrated plasticity in becoming hepatocytes[1-4]. Questions remain as to how BM stem cells are activated and led to the liver and where the signals initiating the mechanisms of activation and differentiation of stem cells originate. Transfused oval cells (hepatic stem cells) that had a selective tropism for the liver in an animal model of liver-damage suggested that "signal molecules" were present in serum of this animal model and played an important role in mediating both hepatic and non-hepatic stem cell activation[5]. However, the influence of these putative signal molecules in serum on the differentiation state of bone marrow-derived stem cells is yet unclear. The purposes of the present work were to confirm the existence of signaling molecules in serum of liver-damaged rats and to observe its effects on the differentiation of BM stem cells into hepatocytes.

## MATERIALS AND METHODS

### *Establishment of animal model of liver-damage*

Male Sprague–Dawley (SD) rats, 6-week-old, were used for the establishment of an animal model of liver-damage. The model was made by means of a 2-AAF/$CCl_4$ program according to Petersen[1]. In experimental group, 2-acetylaminofluorene (2-AAF, Sigma), 2.5 g/L in earthnut oil, was administered to stomach of rats everyday for 7 d. On the 7th d of 2-AAF administration, an Ld50 dose of $CCl_4$ was given by intraperitoneal injection. Animal blood was taken at the time points of 2, 4, 8, 12, and 24 h after $CCl_4$ injection. Experimental serum was prepared on standby. The serum from normal animals was used as control.

### *Isolation and culture of bone marrow stromal cells*

The SD rats were sacrificed by means of ether asphyxia. Bone marrow was collected from tibiae and BM cells were suspended in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum. After centrifugation and re-suspension, the cells were seeded in a culture flask and cultured under a routine condition (37 ℃, 50 mL/L $CO_2$). The solution of medium was changed every 4 d while the cells floating on the medium were discarded. The cells adhering to bottom of the flask (so-called BM stromal cells) were cultured sequentially for 12-14 d. After 3 population doublings, the purified cells were harvested and used in the following stimulating culture with experimental serum.

### *Stimulating culture of BM stromal cells with experimental serum*

BM stromal cells were cultured sequentially in a specialized medium (DMEM-F12) containing 3 ml/L experimental rat serum. In the control group, the culture medium contained 3 mL/L normal rat serum instead of experimental rat serum. Cultures were grown and submerged for 12 d. The cells were harvested on the 13th culture day for ribonucleic acid (RNA) isolation.

**Table 1** Primer pairs for RT-PCR

| Marker genes | Primer pairs | PCR fragments |
|---|---|---|
| $M_2$-PK | 5'ccatctaccacttgcagttattcga3' / 5'tcatggtacaggcactacacgc3' | 431 bp |
| L-PK | 5'acctctgccttctggatactgact3' / 5'tgcaagactccggttcgtatct3' | 322 bp |
| Albumin | 5'gagcccgaaagaaacgagtgtt3' / 5'ggggaatctctggctcatacg3' | 389 bp |
| INT-β1 | 5'tacttcagacttccgcattgg3' / 5'cagtgactgcaaaaatcgtctg3' | 488 bp |
| GAPDH | 5'ccatggagaaggctggg3' / 5'caaagttgtcatggatgacc3' | 180 bp |

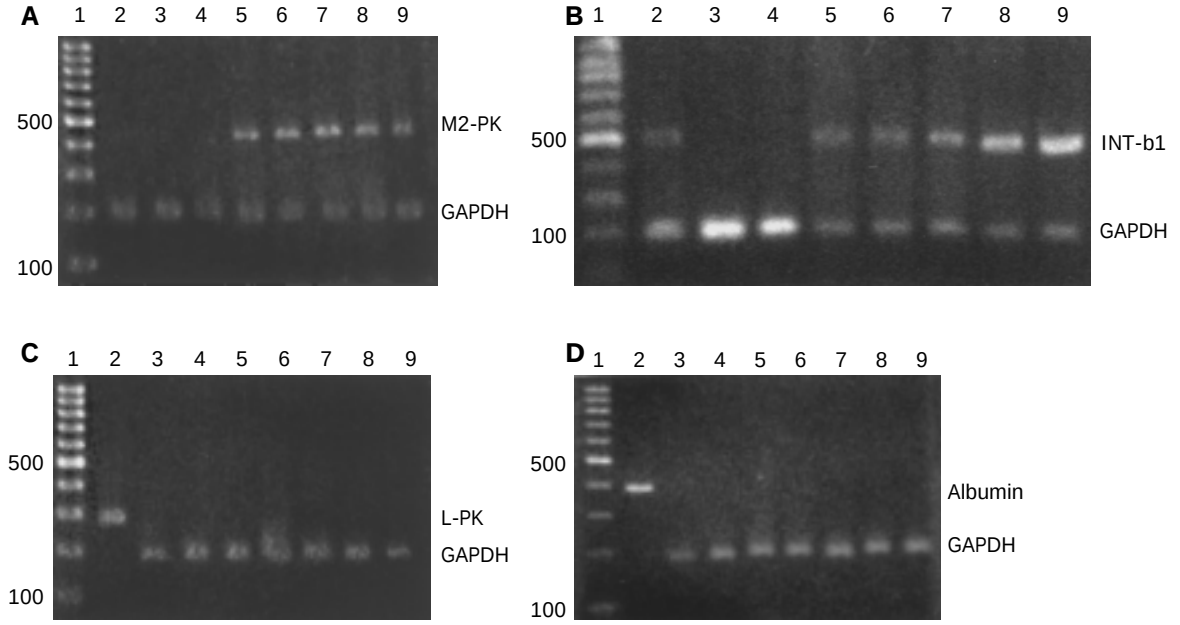


**Figure 1** Expressions of $M_2$-PK, Integrin-β1, L-PK and albumin in BM stem cells. A: Expression of $M_2$-PK in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. B: Expression of Integrin-β1 in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. C: Expression of L-PK in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. D: Expression of albumin in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h.

### *Isolation of RNA*

RNA was extracted from the cells collected from the cultures described above, according to the protocol of QIAGEN RNA easy mini kit. RNA samples were then stored at -80 ℃.

### *Primers selection*

Genes of $M_2$-type and L-type isozymes of rat pyruvate kinase ($M_2$-PK, L-PK), albumin and integrin-β1 (INT-β1) were selected as the markers representing different differentiation stages of hepatocyte lineage. The gene of glyceraldehyde-3-phosphate-dyhydrogenase (GAPDH) was used as an internal control for RT-PCR reactions. Primer pairs used for RT-PCR are shown in Table 1.

### *RT-PCR reactions*

RNA samples were first reversely transcribed into cDNA, and then used as templates in the following PCR reactions. The reaction cocktails (containing 1 μg template cDNA, 50 μmol/L dNTPs, 400 μmol/L primers, 1×PCR buffer, 2.5 mmol/L $MgCl_2$, 1 U Taq-polymerase, add $H_2O$ to 50 μL of total volume) were run on GeneAmp® PCR System 9600 (AB) with a combined program of program 1 (at 94 ℃ for 5 min), program 2 (at 95 ℃ for 1 min, at 60 ℃ for 1 min, at 72 ℃ for 1 min; 30 cycles), and program 3 (at 95 ℃ for 1 min, at 60 ℃ for 1 min, at 72 ℃ for 5 min). The PCR products were electrophoresed in 12 g/L agarose gel, stained with ethidium bromide, and photographed.

## RESULTS

$M_2$-PK, as a marker of immature hepatocytes, was used to estimate the differentiation state of BM stem cells stimulated by the experimental serum. The results showed that the expression signals of $M_2$-PK were detected in each group

stimulated with experimental serum, but not in the control group of normal BM stem cells that had no stimulation, BM stem cells stimulated with control serum, and normal mature hepatocytes (Figure 1A). Integrin-β1 is a marker expressed during the development of liver. In this study, its expression signals were also detectable in each group stimulated with experimental serum as those observed in the positive control of hepatocytes (Figure 1B). L-PK and albumin, as marker molecules of mature hepatocytes, were used to estimate the terminal differentiation state of BM stem cells under the influence of experimental serum. However, no signals were detected in the experimental groups, except for the positive control of hepatocytes (Figure 1C, D).

## DISCUSSION

The existence of liver stem cells had been widely proved in both rodents and humans[1-4,6-9]. By extension, liver stem cells could be divided into three groups: (1) mature hepatocytes that proliferate during normal liver tissue renewal and after less severe liver damage, (2) oval cells that are activated to proliferate when the liver damage is extensive and chronic, and (3) exogenous liver stem cells that may derived from bone marrow cells and respond to severe liver damage[10]. However, in a narrow sense, the concept of liver stem cells is usually limited to hepatic oval cells and non-hepatic bone marrow stem cells.

A phenomenon that was often observed both in experimental animal models and in clinics was that the proliferation of liver stem cells occurred most often in conditions of severe liver damages or chronic liver diseases[11-15]. Maybe it is the reason that stem cells were seldom detected in healthy livers. Thus it can be understood that liver-damage is an important prerequisite for activation of liver stem cells. This suggests that the signals initiating activation of hepatic or non-hepatic stem cells might originate from damaged livers. This hypothesis had been partially proved by our previous experimental work[5]. In our previous experiments, oval cells isolated from male SD rats were transfused, through caudal vein into the circulatory system of a female rat with liver damage. Sex-determining gene *sry* that was located on Y chromosome was then examined respectively by PCR and *in situ* hybridization technique in the liver, kidney and spleen of experimental animals. The results of cell-transplant experiments showed that *sry* gene was detectable only in the liver but not in the spleen and kidney of rats with liver damages and that no signals could be detected in control animals, neither in the liver, spleen nor in the kidney. It could be also morphologically observed that some exogenous cells with *sry* marker migrated into the parenchyma of liver and settled there, suggesting that transfused oval cells had a selective tropism for damaged liver. These results also suggested that signaling molecules existed in the serum of animals with liver damage and might play a role in mediating stem cell activation.

In the present study, an animal model of liver-damage was established with a 2-AAF/$CCl_4$ program. In this model, the capacity of hepatocyte self-regeneration was first impaired by 2-AAF and then the liver was damaged severely by $CCl_4$. In this status, the damaged liver would likely produce a signal to initiate the activation of stem cells in the bone marrow. The results of the present study showed that the expression of $M_2$-PK, a marker of immature hepatocyte[16-22], could be detected in each group stimulated with experimental serum, but not in any of the control groups. Integrin-β1 is a marker expressed during the development of liver. Its expression could be detected in fetal hepatocyte as early as at 8$^{th}$ wk of gestation[30]. In the present results, the expression signal of integrin-β1 was also detectable in each group stimulated with experimental serum. Thus, the functional state of BM-derived stem cells was changed under the influence of experimental serum, thus differentiating toward the direction of a hepatocyte lineage. Although the markers of a mature hepatocyte, L-PK and albumin were not detectable in stimulated BM stem cells, the leap from an undetermined state to a determined state was a marker of entry into the process of programmed differentiation. A variety of possibilities could account for the lack of detectable signals for L-PK and albumin in stimulated BM stem cells. Among the possibilities, one could be the deficiency in intensity and time of stimulation, while another could, by reasoning, be that the postulated "signal molecules" existing in the experimental serum were involved only in the early activation and determination of BM stem cells, while the terminal differentiation of the cells into hepatocytes might still need other signals.

The results in the present study indicated that the driving force promoting differentiation of BM stem cells to hepatocytes was certainly generated from the serum of rats treated by 2-AAF/$CCl_4$. It has been further testified that some "signal molecules" were existed in the circulation of rats treated by 2-AAF/$CCl_4$ and that they might play an important role in the initiation of activation of stem cell. It would be helpful for understanding the mechanisms of stem cell differentiation if the "signal molecules" could be further identified and isolated.

Recent studies have convincingly demonstrated that adult bone marrow contains cells capable of differentiating into hepatocyte-like cells. Nevertheless, what type of cell population are the ancestor cells for hepatocytes still remains a question. In the vast majority of reports, hematopoietic cells were considered to be capable of "transdifferentiating" into hepatocytes[2,4,23-28]. However, Wagers (2002) deemed that there was little evidence for transdifferentiation of adult hematopoietic stem cells[29]. The present study showed that bone marrow stromal cells demonstrated the plasticity in changing into hepatocytes. By this token, the debate about origin of liver stem cells will keep on.

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• BASIC RESEARCH •

# Pathophysiological significance of a reaction in mouse gastrointestinal tract associated with delayed-type hypersensitivity

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## Abstract

**AIM:** To explore the pathophysiological significance of delayed type hypersensitivity (DTH) reaction in mouse gastrointestinal tract induced by an allergen 2,4-dinitrochlorobenzene (DNCB).

**METHODS:** BALB/c mice were randomly divided into control and $DTH_{1-6}$ groups. After sensitized by DNCB smeared on the abdominal skin, the mice were challenged with DNCB by gavage or enema. The weight, stool viscosity and hematochezia were observed and accumulated as disease active index (DAI) score; the gastrointestinal motility was represented by active charcoal propulsion rate; the colon pathological score was achieved by macropathology and HE staining of section prepared for microscopy; and the leukocyte migration inhibitory factor (LMIF) activity was determined by indirect capillary assay of the absorbance (*A*) of migrated leukocytes.

**RESULTS:** Active charcoal propulsion rates of small intestine in the DNCB gavages groups were significantly higher than that in the control group ($P<0.01$). The DAI scores and pathological score in DNCB enema groups were also higher than that in the control group ($P<0.05$), and there were significant rises in LMIF activity in DNCB enema groups as compared with control groups ($P<0.01$).

**CONCLUSION:** Mouse gastrointestinal DTH reaction could be induced by DNCB, which might facilitate the mechanism underlying the ulcerative colitis.

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## INTRODUCTION

The gastrointestinal tract (GI) is not only an important organ in digestion and endocrine, but also the largest peripheral immunological organ in the body[1]. The secretory immunoglobulin A (s-IgA) and T cell are responsible for developing mucosa vaccines and producing oral tolerance[2,3]. Furthermore, the manifestation of T cell mediated reaction in GI tract was most often in the way of delayed-type hypersensitivity (DTH), thereby resulting in ulcerative colitis[4,5]. 2,4-dinitrochlorobenzene (DNCB), a chemical compound of low molecular weight, could combine with tissue protein to function as a full antigen in activating T cell mediated DTH reaction, such as skin DTH[6] and colon inflammation/ulcer[5,7,8]. Gastrointestinal DTH (or colitis) induced by DNCB in rabbit, guinea pig and rat has been reported[5,7,8], but that in mice has not been reported. In this study, DNCB-induced DTH in mice GI tract were found, and its pathophysiological significance was discussed.

## MATERIALS AND METHODS

### *Animals*

Healthy male BALB/c mice weighing 18-21 g (supplied by the Department of Experimental Animals, Peking University Health Science Center) were used. Animals were fed with a standard diet and allowed free access to water.

### *Preparation of major chemicals*

**DNCB solution** For DNCB sensitization, 330 mg DNCB (Beijing Chemical Reagent Company, Beijing, P.R. China) was dissolved in 10 mL of acetone-olive oil (1:1) vehicle. For DNCB gavage, 300 mg DNCB was first mixed with a minimum volume of polysorbate 80, and a minimum volume of ethanol was added until the mixture was completely dissolved, followed by the addition of olive oil to achieve a final DNCB concentration of 6.6 g/L. The ratios of polysorbate 80-ethanol-olive oil in this vehicle were 6.6%, 8.8% and 84.6%, respectively. Then the 6.6% solution was diluted to 1.3 g/L and 0.3 g/L DNCB solution. For DNCB enema, DNCB was dissolved in 600 mL/L ethanol to achieve 4 g/L, 2 g/L, and 1 g/L DNCB solution, separately. All these DNCB solutions were stored at 4 ℃.

**Activated charcoal suspension** The suspension was made according to Qi *et al*.[9] with a little modification. A total of 6 g activated charcoal (Tianjin 6th Chemical Reagent Company, Tianjin, China) and 2 g astragalin gum (Beijing Chemical Reagent Company, China) were dissolved in 50 mL of normal saline (NS) just before intragastric use.

**RPMI 1640** One milliliter of RPMI 1640 culture medium contained 100 IU penicillin and 100 μg streptomycin, and then the pH was adjusted to 6.8-7.2.

**Phytohemagglutinin (PHA)** A 100 mg PHA was dissolved in 10 mL incomplete RPMI1640, and then sterilized and preserved at -20 ℃.

**Ammonium chloride solution (erythrocyte lytic fluid)** A total of 1.03 g Tris and 3.735 g $NH_4Cl$ were dissolved in 500 mL distilled water.

### *Induction of DTH response in GI tract*

**Grouping** Ninety-six BALB/c mice were randomly divided into 10 groups, including $DTH_{1-6}$, DTH negative ($DTH^-$) group and control group.

**Sensitization** On the first day of experiment, mouse fur on abdomen (2.5 cm diameter) was shaved. A total of 50 μL of 33g/L DNCB was smeared on the shaved skin once a day for 1 or 4 d. Mice in the $DTH_{(-)}$ and control groups were smeared with acetone-olive oil vehicle.

**Gavage** All the mice were fasted for 6 h before gavage. On the second day of experiment the mice in $DTH_1$, $DTH_2$ and $DTH_3$ groups were gavaged with 0.8 mL of 0.3 g/L, 1.3/L and 6.6 g/L DNCB, respectively. The $DTH_{(-)}$ group was gavaged 1.3 g/L DNCB and the control group was gavaged polysorbate 80-ethanol-olive oil vehicle. On the third day, 0.8 mL of activated charcoal was gavaged to each mouse. Twenty minutes later, the mice were killed by decapitation for determination of gastrointestinal motility.

**Enema** On the 5 th day, a silica-gel tube with 10 mm diameter was inserted into the colons of the mice, its tip being 3-3.5 cm far from the anus. The mice in $DTH_4$, $DTH_5$ and $DTH_6$ groups were intracolonically administered 1 g/L, 2 g/L and 4 g/L DNCB (2 μL/g) once a day for 4 d. $DTH_{(-)}$ group was administered 2 g/L DNCB and the control group was administered 600 mL/L ethanol vehicle.

### *Evaluation of the animals*

**Gastrointestinal motility** After each mouse was killed, the whole small intestine was taken out, rinsed with NS quickly, and the intestinal wall was cut open longitudinally, laid and unfolded on a flat plate for estimation of the active charcoal migration distance (cm). The gastrointestinal motility was expressed by charcoal propulsion rate by using the formula: charcoal propulsion rate=migration distance of active charcoal / the distance from pylorus-duodenum junction to ileocecum×100%.

**Body mass and stool** By using disease activity index (DAI) score[10], the body mass and stool were scored as follows: Body mass: score 0-normal; score 1-1-5% lower than normal; score 2-6-10% lower than normal; score 3-11-15% lower than normal; and score 4-above 15% lower than normal. Stool viscosity: score 0-normal; score 2-fluffy; and score 4-diarrhea. Stool hemorrhage: score 0-normal; and score 2-apparent hemorrhage.

**Pathological score** On the 9 th d, all the mice received enema were killed by cervical dislocation. The colon was cut open longitudinally along the attachments of mesenteries and was first macropathologically observed. Then specimens were taken from inflammatory/ulcerative colon and were fixed in 40 g/L formaldehyde. Paraffin-embedded 5-μm thick section was made for HE staining. The macropathological score and microscopy score were obtained by adopting Dr. Murano's method[10] with slight modification as follows: Macropathological score: score 0-no cementation (colon was easily detached from other tissues) and no inflammation in colon; score 1-moderate cementation or local congestion; score 2-severe cementation with one ulcer (<1 cm); score 3- more than one ulcer (<1 cm) with inflammation; and score 4-more than one ulcer (>1 cm) with inflammation.

Microscopy: score 0-normal colon mucosa or slight congestion; score 1-mucosa hyperemia, infiltration of chronic inflammatory cells and decrease of the number of goblet cell in colon mucosa; score 2-mucosa hyperemia, infiltration of chronic inflammatory cells and local superficial erosion; and score 3-atrophic change with ulcer and serious infiltration of chronic inflammatory cells in colon mucosa.

### *Measurement of leukocyte migration inhibitory factor (LMIF) activity*

**LMIF preparation** By following our previous method[11], the spleen lymphocytes ($2\times10^6$/mL) of mice received enema were cultured in PHA (60 μg/mL) for 72 h. Then the supernatant containing LMIF component was collected by centrifugation (1 500 r/min, 15 min), followed by lyophilization. The lyophilized powder was stored at -20 ℃. Working solution (LMIF solution) was prepared by dissolving the lyophilized powder in RPMI-1640 to one third of its original volume before using.

**Preparation of peripheral leukocytes** A suspension of indicator cells (migrated leukocytes) was prepared from the anti-coagulated whole blood of guinea pigs. Three percent gelatin of 1/3 volume of the blood was added to the blood before culturing the sample at 37 ℃ for 30 min. The upper layer rich in leukocytes was taken out and centrifuged (1 000 r/min) for 10 min. Then the sediment was washed twice with Hanks' solution free of $Mg^{2+}$ and $Ca^{2+}$, and again centrifuged (1 000 r/min) for 10 min. Finally, the sediment was used as the indicator cell and was adjusted to a concentration of $(1.6\text{-}1.8)\times10^7$ cells/mL RPMI 1640.

**Detection of leukocyte absorbance** By following our previous work[11] with a little improvement, the leukocyte was injected into a capillary tube of 80 μL volume. After heat-sealing one end of the tube and spinning at 1 000 r/min for 10 min, the tubes were cut at the liquid-cell interface. The portion containing the cells was placed inside each chamber of a 24-well plate filled with 210 μL LMIF solution for culture. Twelve hours later, 200 μL solution was taken out from each chamber and was put in an well of a 96-well plate, followed by an addition of 20 μL methylthiazoletetrazolium, MTT) was added into each well before the plate was incubated at 37 ℃ for 4 h. Then the supernatant was removed, 200 μL of DMSO was added into each well and the plate was shaken for 10 min. The absorbance (*A* value) of the migration cells was determined in Micro plate Reader at a wavelength of 570 nm. The average of *A* values of 3 wells was regarded as a mean *A* value. The above processes were repeated for 9 times.

### *Statistical analysis*

Data were expressed as mean±SE. Analysis of variance (ANOVA) and student's *t* test were used for comparison among the groups and between paired data. $P<0.05$ was considered to be statistically significant.

## RESULTS

### *Gastrointestinal motility*

Active charcoal propulsion rates of small intestine in the $DTH_1$ (0.3 g/L DNCB) and $DTH_2$ (1.3 g/L DNCB) groups were significantly higher than that in the control group ($P<0.01$) and $DTH_{(-)}$ groups ($P<0.05$ or $P<0.01$), whereas there was no significant difference between the control group and $DTH_{(-)}$ group (Figure 1). All the mice in $DTH_3$ group died after the 0.66% DNCB gavage.

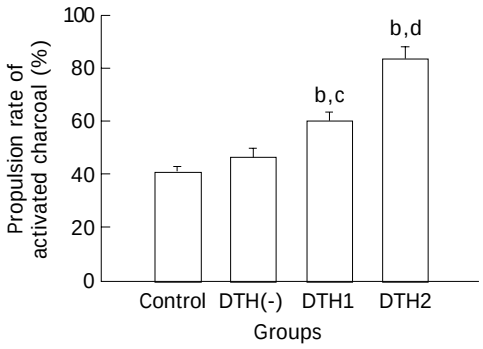


**Figure 1** Effect of DNCB gavage on gastrointestinal motility in sensitized mice. [b]$P<0.01$ *vs* control, [c]$P<0.05$ *vs* $DTH_{(-)}$, [d]$P<0.01$ *vs* $DTH_{(-)}$ and $DTH_1$ $n=8$ in control; $n=7$ in $DTH_{(-)}$, $DTH_1$ and $DTH_2$ groups.

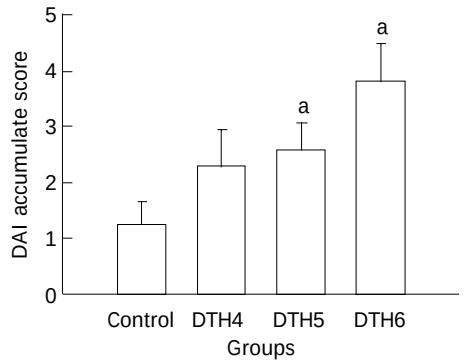


**Figure 2** Effect of DNCB enema on DAI score in sensitized mice. $^{a}P<0.05$ *vs* control. $n=16$ in control group and $DTH_4$ group; $n=11$ in $DTH_5$ group; $n=17$ in $DTH_6$ group.

### *DAI score and pathological score*

Diarrhea was first found in the mice 24 h after DNCB enema, and weight loss was found 3 d later. Serious weight loss and obvious diarrhea were seen in the 4 g/L DNCB group, 24% of whom died. DAI scores are shown in Figure 2.

Pathologically, the control and $DTH_{(-)}$ groups had normal histological structures and glands, no ulcer was found except for occasional slight mucosa congestion (Figure 3A). In $DTH_4$ (1 g/L DNCB) group, there was slight colic edema, mucosa congestion, infiltration of lymphocytes and a decrease in the number of glands and goblet cells (Figure 3B). In $DTH_5$ (2 g/L DNCB) group, intestinal adhesion and flatulence were found. A more disturbing array of glands, local erosion, dramatic decrease in goblet cells and diffuse inflammatory cellular infiltration were found (Figure 3C). In $DTH_6$ (4 g/L DNCB) group, more extensive colic cementation, expansion of the proximal intestinal cavity, some white exudates, mucosa congestion, necrosis and multiple ulcers were found. Under the microscope, mucosa atrophy, decrease of glands and disturbance of tissue structure were observed (Figure 3D), moreover, erosion, hemorrhage, necrosis as well as deeper/extensive ulcers were easily seen (Figure 3E). The pathological score in each group is shown in Figure 4.

### *The LMIF activities and its relationship with colitis*

With the increased DNCB doses in enema, which exacerbated colonic tissue damage, we could see an increase of the LMIF activity (*A* value decreased). The LMIF activity was significantly increased in the $DTH_5$ and $DTH_6$ groups as compared with the control group ($P<0.01$, student's *t*-test) (Table 1). There were also significant differences in LMIF activity ($P<0.01$) among $DTH_4$, $DTH_5$ and $DTH_6$ groups (ANOVA).

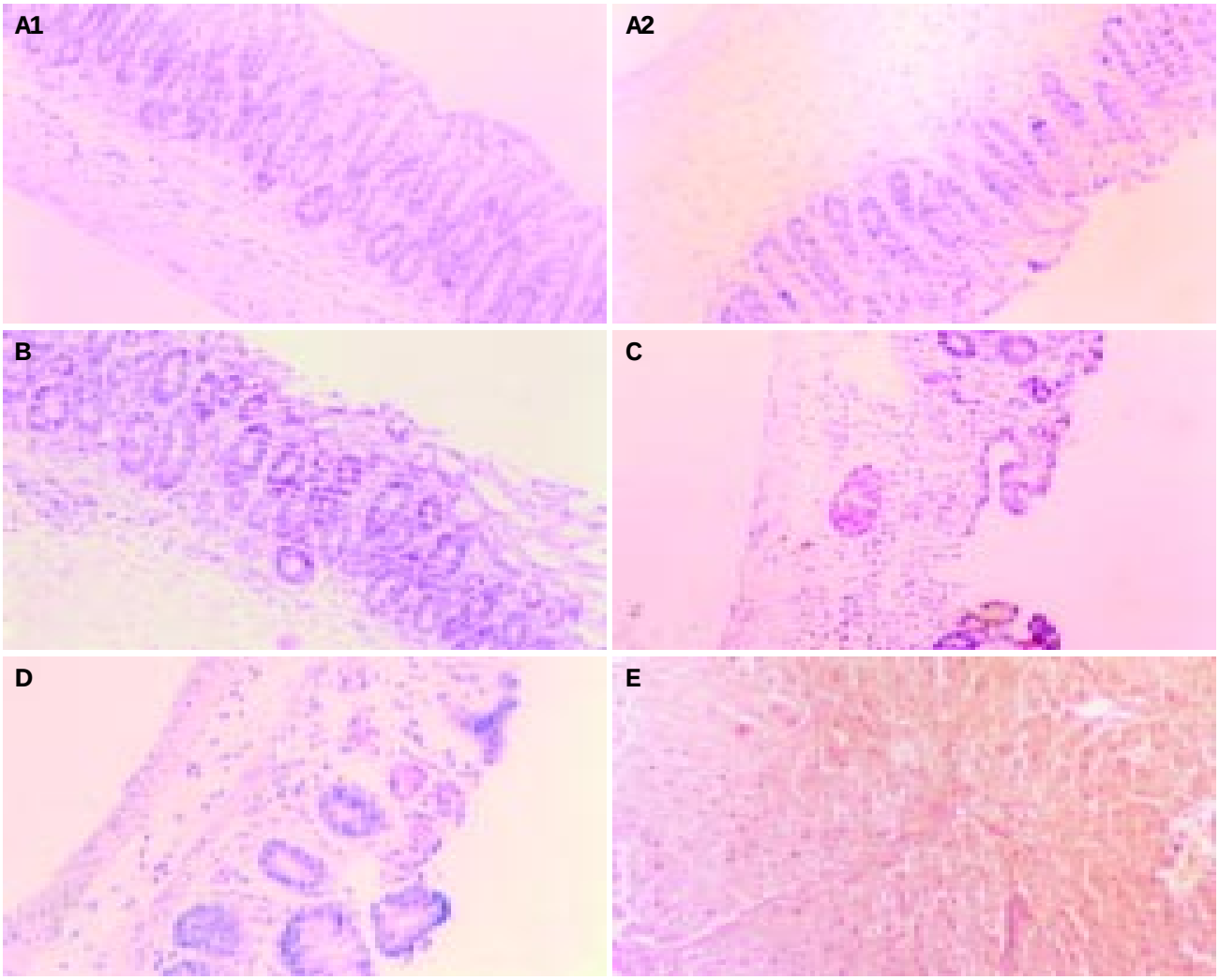


**Figure 3** The pathological change of colon by DNCB enema in sensitized mice. A1: control 600 mL/L ethanol, normal histological structures. A2: $DTH_{(-)}$, only slight mucosa congestion. B: 1 g/L DNCB, hyperemia, infiltration of chronic inflammatory. cells, decrease of the number of goblet cell in colon mucosa. C: 2 g/L DNCB, superficial erosion, chronic inflammation of colon mucosa. D: 4 g/L DNCB, atrophic changes of colon mucosa with chronic inflammation. E: 4 g/L DNCB, ulcer and hemorrhage in colon mucosa (HE, ×200).

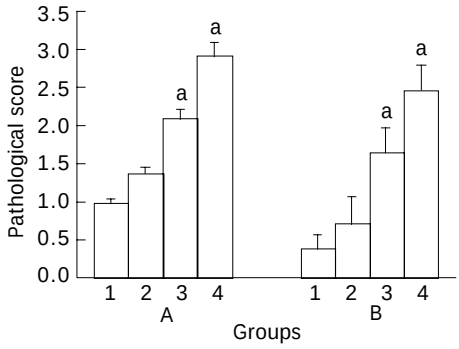


**Figure 4** Pathological score in the mice received DNCB enema after sensitization. 4A: macropathology; 4B: microscopically observation. group 1: control (600 mL/L ethanol, $n$=16); group 2: $DTH_4$ (1 g/L DNCB, $n$=17); group 3: $DTH_5$ (2 g/L DNCB, $n$=17); and group 4: $DTH_6$ (4 g/L DNCB, $n$=17) $^aP<0.05$ *vs* group 4A1 and 4B1, respectively.

**Table 1** Effect of DNCB enema on LMIF activity in sensitized mice

| Group | Skin smearing (50 μL/mouse) | Enema (2 μL/g bm) | *A* value (mean±SE) |
|---|---|---|---|
| Control | Acetone-olive oil | 600 mL/L ethanol | 0.426±0.009 |
| $DTH_{(-)}$ | Acetone-olive oil | 2 g/L DNCB | 0.401±0.007 |
| $DTH_4$ | 33 g/L DNCB | 1 g/L DNCB | 0.383±0.038 |
| $DTH_5$ | 33 g/L DNCB | 2 g/L DNCB | 0.220±0.012$^b$ |
| $DTH_6$ | 33 g/L DNCB | 4 g/L DNCB | 0.139±0.019$^d$ |

$^bP<0.01$ when compared with the control and $DTH_{(-)}$ (student's *t*-test). $^dP<0.01$ between $DTH_4$, $DTH_5$ and $DTH_6$ groups (ANOVA); $n$=9 in each group. *A*, the absorbance of migration leukocytes. The skin smearing and enema were completed daily for 4 d.

## DISCUSSION

For the first time, here, we reported DTH response of mice GI tract by intra-gastrointestinal administration of DNCB. The evidence of gastrointestinal DTH response were as follows: DNCB-sensitized mouse showed increased GI motility, diarrhea, hematochezia and colitis after DNCB challenge; and the activity of LMIF released from sensitized T-lymphocyte was significantly increased after DNCB enema. The mechanism of the DTH phenomenon might be the same as that of the ear skin DTH reaction in our previous study[6].

What is the pathophysiological significance of GI DTH reaction? Early in the 1960's, Bicks *et al.* found that inflammation and ulcer could be induced by DNCB enema in guinea pigs[4], which he thought as being a cellular immune induced by DNCB that resulted in a DTH reaction in GI tract. Later, several other researchers explored the relations between DNCB-activited cellular immune and GI disorder, but they either indirectly inferred the possibility of GI being influenced by DTH skin tests[12] or only focused on the pathological relation of DNCB-colitis with human ulcerative colitis (UC)[5,7,8]. Comparing with previous DNCB-colitis in rat, guinea pig and rabbit by other researchers, our work here not only successfully created DNCB-colitis model in mouse, but also found a more convenient and more sensitive animal model for DTH study[13]. In our study, we proved, using the specific DTH index of LMIF[14,15], that DNCB could induce DTH in mouse GI tract, and this kind of DTH might be the essence of UC.

MIF is one of the cytokines released from sensitized T cell after subjecting the allergen again[14,15]. According to the target cell, LMIF can be classified as LMIF and MMIF[16]. There was a dramatic positive correlation between LMIF activity and the gravity of DTH response[17]. We found that the ConA-stimulated lymphocytes from mesenteric lymph node of DNCB-treated mouse showed higher LMIF activity than the control, and LMIF activity increased as DNCB concentration increased (Table 1), whereas in $DTH_{(-)}$ group, neither higher LMIF activity nor colon inflammation/ulcer was found, indicating a possible relations among GI DTH response, LMIF activity and UC. Secondly, we know that the main clinical manifestations of UC are diarrhea and pus hematochezia, which result from an irritated intestinal peristaltic and inflectional ulcer[18,19]. Our results were in accordance with the clinical manifestations of UC (Figures 1-4), indicating that colitis in mouse caused by DNCB was basically similar to human UC in pathology. Finally, two representatives but separate works by Bartnik *et al.*[20] and Murakami *et al.*[21] support the hypothesis that the essence of UC is gastrointestinal DTH response. Bartnik *et al.* reported that patients with severe or moderate ulcerative colitis showed LMIF release, which was significantly greater than that observed in patients with other large bowel diseases[20]; and Murakami *et al.* reported that the number of MIF expressing cells increased at the colonic mucosa in patients with ulcerative colitis, and MIF induced significant levels of IL-1 and IL-8 in monocytes and dendrite cells in UC patients, indicating a role of MIF in the induction and/or perpetuation of the inflammatory environment in UC[21]. Comparing with all the other cytokines reported in UC[22], we think that the distinctive (unique) significance of LMIF in UC and its pivotal role in connecting DTH with UC have been showed by Bartnik and Murakami.

To sum up, we have enough reasons to conclude that the GI DTH in mouse may provide not only experimental models of human UC, but also insight into pathogenic mechanisms of the UC, an inflammatory bowel disease (IBD) of unknown etiology.

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• BASIC RESEARCH •

# Hepatitis B virus X gene induces human telomerase reverse transcriptase mRNA expression in cultured normal human cholangiocytes

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## Abstract

**AIM:** To study the transcriptional regulation of human telomerase reverse transcriptase (hTERT) mRNA in normal human cholangiocytes (HBECs) after hepatitis B virus X (HBx) gene transfection and to elucidate the possible mechanism of HBV infection underlying cholangiocarcinoma.

**METHODS:** HBECs were cultured *in vitro* and co-transfected with a eukaryotic expression vector containing the HBx coding region and a cloning vector containing coding sequences of enhanced green fluorescent protein (EGFP) using lipid-mediated gene transfer. The transfection efficiency was determined by the expression of EGFP. The expressions of hTERT mRNA and HBx protein in HBECs were detected by RT-PCR and immunocytochemical stain, respectively.

**RESULTS:** The transfection efficiencies were about 15% for both HBx gene expression plasmid and empty vector. No hTERT mRNA was expressed in HBECs when transfected with OPTI-MEM medium and empty vector, but a dramatic increase was observed for hTERT mRNA expression in HBECs when transfected with HBx expression vector. HBx protein was only expressed in HBECs when transfected with HBx expression vector.

**CONCLUSION:** HBx transfection can activate the transcriptional expression of hTERT mRNA. Cis-activation of hTERT mRNA by HBx gene is the primary mechanism underlying the proliferation, differentiation and tumorigenesis of biliary epithelia.

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## INTRODUCTION

Telomeres make up the ends of chromosomes of eukaryote and progressively shorten with each cell cycle. Critically short telomeres induce cellular senescence and death[1]. Telomere lengths become stabilized by the activation of telomerase in most tumor cells, highly proliferative cells and human somatic cells. The activation of telomerase is a crucial step in tumorigenesis and cellular senescence[2]. The most important catalytic protein subunit of telomerase ribonucleoprotein is hTERT whose expression parallels telomerase activity[3]. It is known that hTERT expression is regulated mainly at the transcriptional level and that the core promoter of hTERT encompasses numerous transcription factor binding sites. All these factors, which regulate hTERT promoter region individually or coordinately, comprise a complex regulation system[4]. A recent study has shown that HBV DNA integration locates upstream to the hTERT promoter and that HBV enhancer can cis-activate the transcriptional expression of hTERT gene in hepatocarcinoma cell lines[5]. HBx gene also activates the expression of telomerase[6]. All these findings provide a new mechanism of HBV in liver carcinogenesis. There is a prominent expression of HBx protein in tissues of both intrahepatic[7,8] and extrahapatic cholangiocarcinomas[9]. So far, it is not clear whether HBV infection involves in the tumorigenesis of cholangiocarcinoma and if HBx gene can regulate the expression of telomerase gene. In order to determine the possible correlation of HBV infection and cholangiocarcinogenesis, we transfered HBx gene into human normal cholangiocytes (HBECs) and assayed the expression of hTERT mRNA by RT-PCR.

## MATERIALS AND METHODS

### *Cell and culture*

HBECs, isolated from normal human bile ducts[10], were kindly provided by Dr. Ludwik K. Trejdosiewicz (ICRF Cancer Medical Research Unit, St James's University Hospital, Leeds, UK). HBECs were maintained as adherent monolayers in "HBEC medium" comprising a 1:1 mixture of Ham's F12 and DMEM (Gibco BRL®), supplemented with 50 g/L fetal bovine serum (Gibco BRL®), 5 ng/mL epidermal growth factor (Intergen Company), 0.4 μg/mL hydrocortisone hemisuccinate, 2 nmol/L triiodothyronine and 5 μg/mL insulin (all from Sigma) and 10 ng/mL human recombinant hepatocyte growth factor (R&D Systems,). Cells were seeded in 25 $cm^2$ tissue culture flasks and propagated at 37 ℃ in a humidified atmosphere of 550 mL/L $CO_2$ in air and monolayers passaged approximately once a week at or before confluence by incubation in trypsin-versene for approximately 5 min until the cells were shrunken. Trypsin activity was quenched by addition of fresh medium containing FBS and cells were seeded at 1/2 split ratio.

### *Plasmids and transfection*

The plasmids pcDNA3, pCMV-X and pEGFP were the gifts from Professor Xiao-Dong Zhang (Institute for Molecular Biology, Nankai University, China). The empty pcDNA3 vector, a eukaryotic expression vector, was used as negative control. pCMV-X was constructed by inserting the entire HBx

coding region (HBV nucleotides 1 372-1 833 465 bp) into the *Eco*RI/*Eco*RV sites of the pCDNA3 vector[11]. Cloning vector pEGFP carried an enhanced green fluorescent protein (EGFP) gene that was cloned between the two MCS of the pPD16.43. EGFP encoded by pEGFP could emit bright green fluorescence in eukaryotic cells. All the plasmids contained ampicillin resistance genes for propagation and selection in *E.coli*.

Transient transfection of plasmids into HBECs was performed using Lipofectamine (Gibco BRL®) according to the protocol recommended by the manufacturer. The day before transfection, cells were trypsinized and seeded in 6-well plates. On the day of transfection, cells reached 60% confluence. 2.9 μg pcDNA3 DNA or pCMV-X DNA and 0.1 μg pEGFP DNA were diluted with OPTI-MEM medium (Invitrogen) and then mixed with Lipofectamine. A total of 1 mL transfection medium was added to the cells after the cells were washed one time by OPTI-MEM medium. The cells were incubated at 37 ℃ in a humidified atmosphere of 50 mL/L$CO_2$ in air for 3 h. After 3 h incubation, the transfection medium was replaced with fresh complete medium containing serum and the cells were incubated for another 36 h. Then the cells were harvested and extracted. The expression of transfected gene was examined by immunocytochemistry. The cells transfected with OPTI-MEM medium were used as blank control and transfected with pcDNA3 vector as empty vector control, co-transfected pEGFP as a marker for transfection efficiency[12].

### RT-PCR of hTERT mRNA

Total cellular RNAs were extracted from different groups by TRIzol reagent (Gibco BRL®). A 2 μg of extracted RNA was reverse transcribed into cDNA first-strand with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) and 1 μg of oligo $(dT)_{15}$ primer (Promega) in a final volume of 25 μL of enzyme buffer for 60 min at 42 ℃. hTERT cDNA analysis was performed by PCR amplification of a 145 bp fragment using primer pairs 5'-CGGAAGAGTGTCTG GA GCAA-3' (sense) and 5'-GGATGAAGCGGAGTCTGGA-3' (antisense) as described previously[13]. A 320 bp fragment of GAPDH gene was amplified as an internal control. The primers for GAPDH were 5'GGAAGCTTGTCATCAATGG 3' (sense) and 5'CTGTGGTCATGAGTCCTTC 3'(antisense). PCR was performed with 5 μL of cDNA first-strand in a 50 μL reaction mixture containing 2 mmol/L $MgCl_2$, 1 mmol/L dNTPs, 0.4 μmol/L of each primer, and 2.5 units of *Taq* DNA polymerase (Promega). The reaction mixture was heated at 94 ℃ for 5 min, then 33 cycles of PCR were performed. Each cycle included denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 40 s and extension at 72 ℃ for 90 s. A 10 μL PCR products was assessed by 15 g/L agarose eletrophoresis and ethidium bromide staining (0.5 μg/mL), visualized under ultraviolet light and analyzed by NIH Image software.

### Immunocytochemistry

Cells in different groups were cultured on coverslips and fixed with acetone and methanol. Detection of HBx protein expression in the transfected cells was performed by the ultrasensitive immunochemistry kit (Maixin Company, Fuzhou, China) according to the manufacturer's instructions. Rabbit anti-human HBx polyclonal antibody (1:800) was provided by Dr. Wen-Liang Wang (The 4th Military Medical University, Xi'an, China).

## RESULTS

### Transfection efficiency

Under fluorescence microscope, EGFP can only be observed in the HBx gene transfected cell cultures and the cells of empty vector control (Figure 1). There was no such green fluorescence in the cells of the blank control. Transfection efficiency was estimated by counting the percentage of EGFP-expressing cells in at least 3 fields of vision under fluorescence microscope. For the HBx gene transfected cell cultures and empty vector control, the transfection efficiency was about 15%.

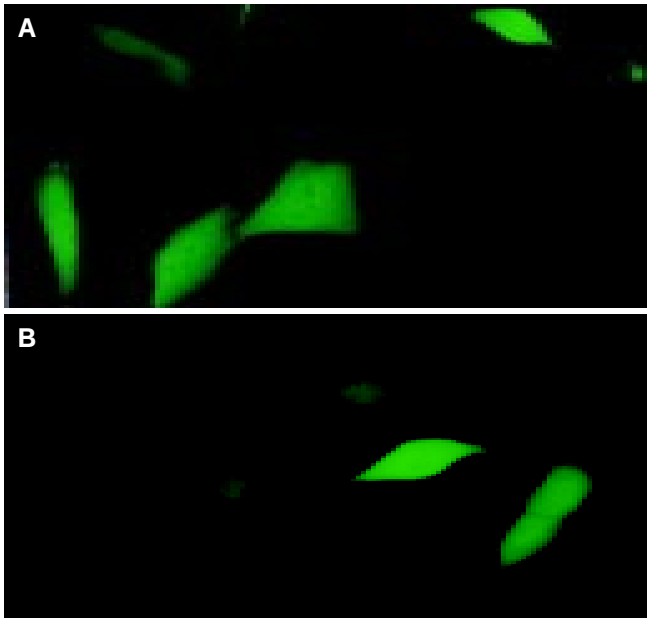


**Figure 1** Transfection efficiency evaluated by EGFP-expressing cells in the HBx gene transfected cell cultures and empty vector control. pEGFP 0.1 μg and pCMV-X (or pcDNA3) 2.9 μg were co-transfected into HBECs by Lipofectamine. Thirty-eight hours after transfection. EGFP-expressing cells were visible under fluorescence microscope in the HBx gene transfected cell cultures (A) and empty vector control (B), but not in the blank control (Under fluorescence microscope ×200).

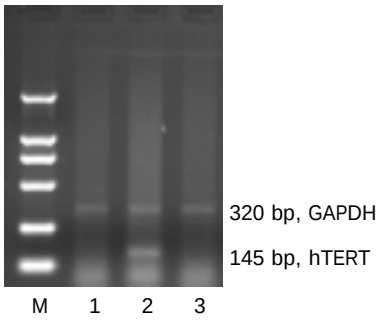


**Figure 2** Analysis of hTERT mRNA expression by RT-PCR. RT-PCR was performed on total RNA extracted from HBECs transfected with OPTI-MEN medium (1), pCMV-X (2) and pcDNA3 (3), respectively. M: DL2000 Marker.

### hTERT mRNA expression

To examine the effects of HBx gene on hTERT transcription, pCMV-X expression vector was co-transfected with pEGFP into HBECs. The expression of human GAPDH RNA in all of the samples was quantitatively measured and used as an internal control. After RT-PCR analysis, a prominent level of hTERT transcript was detected in the HBx gene transfected cell cultures. In contrast, as shown in Figure 2, hTERT mRNA of HBECs in the blank control and empty vector control were undetectable. The relative expression level of hTERT mRNA was determined by measuring band intensities of both hTERT transcript and GAPDH transcript and calculating the ratio of hTERT to GAPDH. As shown in Figure 3, after transferred with HBx

gene, the HBECs exhibit more distinct hTERT mRNA expression in contrast to those transfected with blank control and empty vector.

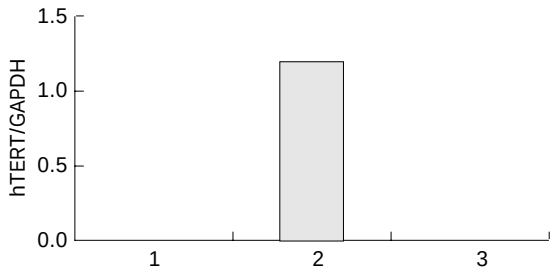


**Figure 3** Quantitative and relative changes of hTERT mRNA expression analyzed by NIH Image software. Dramatic expression of hTERT mRNA was observed in HBECs when transferred with pCMV-X vector (lane 2), but there was no hTERT mRNA expression in HBECs when transferred with OPTI-MEM medium (lane 1) and empty vector (lane 3).

### *HBx protein expression in HBECs*

HBx protein expression in transferred HBECs was identified by immunocytochemistry. Positive signals could be observed sporadically in the HBx gene transfected cell cultures (Figure 4B). As for the HBECs in the blank control and empty vector control, there are no such observable positive signals (Figures 4A, C).

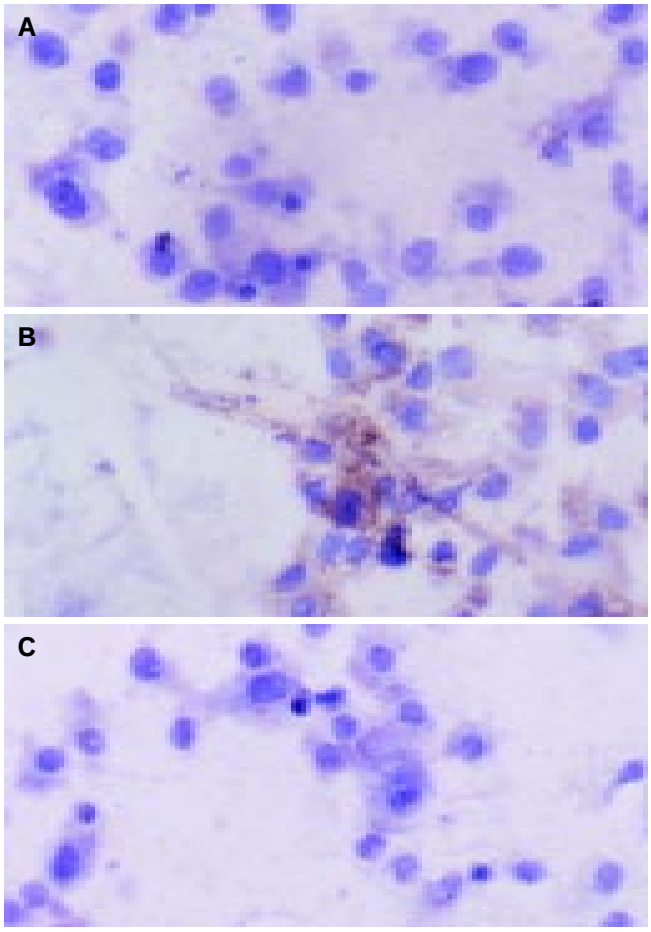


**Figure 4** HBx protein expression in transferred HBECs assayed by ultrasensitive immunocytochemistry. The blank vector and OPTI-MEN transfected HBECs showed no expression of HBx protein (A, C), but pale brown positive signals scattered in pCMV-X vector transferred HBECs (B). Immunocytochemistry (S-P methods, ×200).

## DISCUSSION

HBECs were isolated from normal human bile duct epithelia and cultured *in vitro*. As no retroviral transduction with SV-40 large T antigen cDNA was performed, the cells were not immortalized. They were really primary culture cells and would only grow for a few passages before becoming senescent, the cells simply stopped dividing and died eventually. Though there was expression of telomerase endogenous genes (such as the telomerase RNA component gene) in this kind of finite cell lines, telomerase was inactivated as the expression of hTERT was repressed. The inhibition of hTERT expression originated from the presence of numerous transcription factors in the core promoter of hTERT. Transcriptional repressors such as p53[14,15], Mad1[16,17] and myeloid-specific zinc finger protein 2[18], could specifically inhibit the transcriptional expression of hTERT mRNA in normal human somatic cells. Our results confirmed this hypothesis. When we transferred the empty vector and OPTI-MEN medium into the HBECs, we could not assay the expression of hTERT mRNA. Dramatic hTERT mRNA expression in HBECs transfected with HBx gene showed that HBx gene could cis-activate transcriptional expression of hTERT gene. An early study also showed that normal human cells could restore the telomerase activity in the presence of other oncogenic viruses[19]. It has been reported that HBV genome was integrated into the promoter region of hTERT both in HuH-4 human hepatocellular carcinoma-derived cell line[5] and in liver tumor tissues[20]. The integration of HBV enhancer upstream of the hTERT promoter cis-activated hTERT gene transcription in HuH-4 cells[5]. It is known that the up-regulation of telomerase activity could be observed in hepatocellular carcinoma cell line HepG2 after transferred with X gene[6]. Together with our results, this was a most important demonstration of transcriptional regulation of telomerase gene through HBx gene in carcinogenesis of both human hepatocarcinomas and cholangiocarcinomas. The precise mechanism of such an action is still unknown.

It has been well known that HBx protein encoded by HBx gene, is a potential oncogenic factor and mainly acts as a transcriptional co-activator involving in multiple gene regulation and signaling pathway[21]. Up-regulation of telomerase gene expression may be another major role of HBx at the stage of carcinogenesis[22]. Our early study found that higher expression levels of HBx protein and mRNA could be assayed in the tissues of cholangiocarcinomas[9,23]. Based on our present finding that HBx protein expression could be detected in HBECs transfected with HBx gene, we could suggest that HBV infection and its genome integration might involve in the pathogenesis of cholangiocarcinomas[24]. HBx protein, which has long beed studied as the major causative factor for hepatocarcinogenesis[25,26], might still play more important role in the carcinogenesis of cholangiocarcinomas than other proteins encoded by other genes of HBV[27]. As for the mechanism for HBx protein' carcinogenic action, we deduced there should be a binding site for HBx protein in the core promoter region of hTERT gene although this motif has not been identified. Another notion derived from our study is that HBx protein may recruit some cis-action elements (such as c-Myc[28], AP-2[29]) or repress other factors (like p53[30], E2F1[31]) in mediating the transcriptional regulation of hTERT gene. When exogenous HBx gene was transferred into HBECs, HBx protein translation was achieved in some of HBECs. It is the expression of HBx protein in the transfected cells that be responsible for cis-activation of hTERT mRNA directly or indirectly.

In summery, HBECs do not show the expression of hTERT mRNA and a dramatic high expression of hTERT mRNA can be observed in HBECs transfected with HBx gene. The cis-activation of hTERT gene by HBx is the primary mechanism

underlying proliferation, differentiation and tumorigenesis of biliary epithelia.

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• BASIC RESEARCH •

# *In vitro* anti-coxsackievirus $B_3$ effect of ethyl acetate extract of Tian-hua-fen

Zhen-Hong Li, Bao-Ming Nie, Hong Chen, Shu-Yun Chen, Ping He, Yang Lu, Xiao-Kui Guo, Jing-Xing Liu

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## Abstract

**AIM:** To investigation the anti-coxsackievirus $B_3$ ($CVB_{3m}$) effect of the ethyl acetate extract of Tian-hua-fen on HeLa cells infected with $CVB_{3m}$.

**METHODS:** HeLa cells were infected with $CVB_{3m}$ and the cytopathic effects (CPE) were observed through light microscope and crystal violet staining on 96-well plate and $A_{600}$ was detected using spectrophotometer. The protective effect of the extract to HeLa cells and the mechanism of the effect were also evaluated through the change of CPE and value of $A_{600}$.

**RESULTS:** The extract had some toxicity to HeLa cells at a higher concentration while had a marked inhibitory effect on cell pathological changes at a lower concentration. Consistent results were got through these two methods. We also investigated the mechanism of its anti-$CVB_{3m}$ effect and the results indicated that the extract represented an inhibitory effect through all the processes of $CVB_{3m}$ attachment, entry, biosynthesis and assemble in cells.

**CONCLUSION:** The results demonstrate that the ethyl acetate extract of Tian-hua-fen has a significant protective effect on HeLa cells infected with $CVB_{3m}$ in a dose-dependent manner and this effect exists through the process of $CVB_{3m}$ attachment, entry, biosynthesis and assemble in cells, suggesting that the ethyl acetate extract of Tian-hua-fen can be developed as an anti-virus agent.

Li ZH, Nie BM, Chen H, Chen SY, He P, Lu Y, Guo XK, Liu JX. *In vitro* anti-coxsackievirus $B_3$ effect of ethyl acetate extract of Tian-hua-fen. *World J Gastroenterol* 2004; 10(15): 2263-2266
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## INTRODUCTION

Tian-hua-fen is the dried root of *Trichosanthes kirilowii Maxim* or *Trichosanthes japonica Regel*. The major component of it is mass starch, various amino acids, phytohemagglutinin, saccharide, saponin and some other things[1]. Tian-hua-fen was mentioned in Compendium of Materia Medica written by Li Shizhen in the late 14th Century as a drug to reset menstruation and facilitating the expulsion of retained placenta. For a long time, Tian-hua-fen had been used in the powdered form in conjunction with other Chinese herbal medicines to induce abortion[2]. Clinical applications over the years proved that Tian-hua-fen had multiple pharmacological effects, such as termination of pregnancy, anti-tumor, anti-inflammation, anti-virus and immunoregulation and so on[3-5]. The anti-virus, especially anti-HIV-1, effect of trichosanthin (TCS) has been known to us. But to the author's knowledge research about the non-protein parts of Tian-hua-fen is rare. Coxsackievirus B is the major pathogen of viral myocarditis and now there is no effective therapeutic drug. In the process of screening anti-virus agents from Chinese medicinal herb we found that the nonprotein parts of Tian-hua-fen had a notable anti-virus effect *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### *Preparation of the ethyl acetate extract of Tian-hua-fen*

A total amount of 200 g Tian-hua-fen powder (Shanghai drug store) was macerated with 2 L 750 mL/L ethanol overnight, then was boiled in water under reflux for 3 h and the boiled fluid was filtered. The filtrate was evaporated under reduced pressure to gain a residue. The residue was suspended in water and partitioned with petroleum ether, ethyl acetate and n-BuOH (Analytical pure, Shanghai chemical company Ltd) successively. The four fractions were evaporated under reduced pressure to give petroleum ether fraction (0.56 g), ethyl acetate fraction (0.82 g), n-BuOH fraction (0.85 g) and aqueous fraction (12.7 g), respectively.

### *Preparation of HeLa cells and titration of virus titer*

HeLa cells were stored in liquid nitrogen with 100 g/L dimethyl sulphoxide (DMSO) and 900 mL/L fatal calf serum (FCS) and maintained in culture flasks in complete RPMI 1640 medium (Gibcol, BRL America). Subculture was carried out every 2-3 d after it had formed a confluent monolayer. $CVB_{3m}$ (Stored by our laboratory) was serially diluted to $10^{-10}$ with non-FCS RPMI1640 culture medium. On 40-well plate, 0.025 mL $CVB_{3m}$ with variable dilution and 0.025 mL non-FCS RPMI1640 culture medium were added to each well. Finally, 0.05 mL viable HeLa cells ($3\times10^5$/mL) were added. Each dilution was quadrupled and normal HeLa cells co-cultured only with RPMI1640 containing 10 mL/L FCS were prepared as negative control at the same time. Then the cells were incubated at 37 ℃ with 50 mL/L $CO_2$ for 72 h. The cytopathic effects (CPE) were observed under light microscope. The titer at which cells appeared 50% CPE was designated 1 $TCID_{50}$ (50% tissue culture infectious doses). A 100 $TCID_{50}$ was used as the infectious titer in the following experiment.

### *Assay of the toxicity of the extract to HeLa cells*

A total of 5 mg extract was dissolved in 5 μL DMSO, then 2.5 mL deionized water was added and the liquid was sterilized at 115 ℃ for 20 min. After cooling to 55 ℃, 2.5 mL 2×RPMI 1640 culture medium (without FCS) was added to make the end concentration of the extract (1 mg/mL). The original liquid

was diluted with non-FCS RPMI 1640 culture medium serially from 1:2 to 1:1 024. A total of the 0.025 mL sample with various concentration and 0.025 mL of the same culture medium were added to each well on 96-well plate. Finally 0.05 mL viable HeLa cells ($3\times10^5$/mL) were added. Each concentration of the sample was quadrupled. Two controls, HeLa cells co-cultured only with RPMI1640 (containing 100 mL/L FCS) and 1 g/L DMSO respectively were prepared synchronously. Cells were incubated at 37 ℃ with 50 mL/L $CO_2$ for 72 h. CPE was observed under light microscope and the concentration at which cells appeared <50% CPE (compared with that of extract-free cultures) was regarded as the lowest toxic concentration. In addition, cells were stained with 5 g/L crystal violet (Ameresco) and $A_{600}$ was detected using spectrophotometer.

### *Assay of the anti-$CVB_{3m}$ effect of the extract*

The extract was diluted serially from 1:256 at which it had no toxicity to HeLa cells to 1:8 192 with non-FCS RPMI 1640 culture medium. Then 0.025 mL extract with variable concentration was added to each well of 96-well plate. Then 0.025 mL 100 $TCID_{50}CVB_{3m}$ was overlaid. After incubation at 37 ℃ with 50 mL/L $CO_2$ for 1 h, 0.05 mL viable HeLa cells ($3\times10^5$/mL) were added. Each concentration was quadrupled and three controls, normal HeLa cells co-cultured only with RPMI1640 (containing 100 mL/L FCS), 100 $TCID_{50}CVB_{3m}$ and the extract (1:256 mg/mL), respectively were prepared synchronously. Cells were grown for 3 days and then CPE was observed under light microscope. Later, the cells were stained with 5 g/L crystal violet and $A_{600}$ was measured using spectrophotometer.

### *Primary study on the mechanism of anti- $CVB_{3m}$ effect of the extract*[6]

The extract was diluted with non-FCS RPMI1640 culture medium from 1:256 to 1:8 192. HeLa cells ($1.5\times10^4$/well) were seeded onto three 96-well plates and allowed to attach to the well bottom. When the cells were confluent the culture medium was discarded and the cells were rinsed twice with the same culture medium. The cells on three plates were treated respectively as follows: The first plate: 0.025 mL 100$TCID_{50}CVB_{3m}$ and 0.025 mL extract of variable concentration were added to each well. After incubation at 37 ℃ with 50 mL/L $CO_2$ for 1 h, the mixture was substituted with 0.1 mL non-FCS RPMI1640 culture medium; the second plate: 0.025 mL 100$TCID_{50}$ $CVB_{3m}$ and 0.025 mL extract of variable concentration were added. The plate was incubated at 37 ℃ with 50 mL/L $CO_2$ for 1 h. Then the mixture was substituted with 0.025 mL extract of variable concentration and 0.075 mL non-FCS RPMI1640 culture medium; the third plate: 0.025 mL 100$TCID_{50}CVB_{3m}$ and 0.025 mL non-FCS RPMI1640 culture medium were added first. After incubation at 37 ℃ with 50 mL/L $CO_2$ for 1 h the mixture was discarded and 0.025 mL extract of variable concentration and 0.075 mL non-FCS RPMI1640 culture medium were overlaid. Three controls, HeLa cells co-cultured only with RPMI1640 containing 100 mL/L FCS, 100$TCID_{50}$ $CVB_3$m and extract (1:256 mg/mL) respectively were prepared synchronously and each extract concentration was quadrupled. At last all plates were incubated at 37 ℃ with 50 mL/L $CO_2$. When the cells treated with $CVB_{3m}$ appeared 100% CPE, the cells were stained with 5 g/L crystal violet and $A_{600}$ was detected using spectrophotometer.

## RESULTS

### *Titration of CVB3m titers*

The incubation was terminated after 72 h and CPE was observed under light microscope. The results showed the cells in all quadrupled wells treated with $CVB_{3m}$ at titer of $10^{-1}$-$10^{-6}$ appeared 100% CPE. About the cells treated with $CVB_{3m}$ at titer of $10^{-7}$, the cells in two wells appeared 100% CPE while the others appeared 50% CPE. In regards to the cells co-cultured with $CVB_{3m}$ at titer of $10^{-8}$-$10^{-10}$, the cells in four wells appeared no CPE completely. Then $TCID_{50}$ was calculated as $10^{-7}$ according to Reed-Muench method[7].

### *Assay of toxicity of ethyl acetate extract to HeLa cells*

After incubation for 72 h, CPE induced by the extract was observed under light microscope. The results showed the cells co-cultured with extract at concentration from 1:2-1:128 mg/mL appeared CPE of different degrees, while the cells co-cultured with extract at concentration from 1:256 to 1:8 192 mg/mL appeared no CPE. The 50% toxic concentration was 1:128 mg/mL according to Reed-Muench method. The value of $A_{600}$ also indicated that the extract had no toxicity to HeLa cells from 1:256 mg/mL. The cells co-cultured with 1 g/L DMSO appeared no CPE, which showed that DMSO at this concentration had no toxicity to HeLa cells (Table 1).

### *Assay of the anti-$CVB_{3m}$ effect of the extract*

From 1:256 to 1:8 192 mg/mL the extract showed various protective effects to HeLa cells and the effect was decreased with the increased dilution. The protective effect was best at concentration from 1:256 to 1:1 024 mg/mL. The minimal effective inhibitory concentration (EIC) was 1:8 192. The value

**Table 1** Cytotoxity of ethyl acetate extract to HeLa cells shown in the value of $A_{600}$

| Dilution | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1 024 |
|---|---|---|---|---|---|---|---|---|---|---|
| Value of $A_{600}$ | 0.20±0.029 | 0.22±0.030 | 0.32±0.022 | 0.29±0.069 | 0.38±0.043 | 0.59±0.035 | 0.69±0.075 | 1.30±0.069 | 1.23±0.061 | 1.29±0.056 |

The $A_{600}$ value of the cells co-cultured with the extract at concentration from 1:2 to 1:128 mg/mL was lower than that of normal HeLa cells (1.32±0.034), which showed that the extract had some toxicity to HeLa cells at these concentrations. From 1:256 mg/mL the $A_{600}$ value became close to that of normal HeLa cells, which indicated that from 1:256 mg/mL the extract had no toxicity to HeLa cells again.

**Table 2** Protective effect of ethyl acetate extract on HeLa cells shown in the value of $A_{600}$

| Dilution | 1:256 | 1:512 | 1:1 024 | 1:2 048 | 1:4 096 | 1:8 192 |
|---|---|---|---|---|---|---|
| Value of $A_{600}$ | 1.09±0.017 | 1.02±0.122 | 0.89±0.060 | 0.91±0.039 | 0.83±0.048 | 0.77±0.028 |

The $A_{600}$ value of the cells co-cultured only with RPMI1640 was 1.32±0.02; the $A_{600}$ value of the cells co-cultured only with $10^{-5}$ $CVB_{3m}$ was 0.22±0.026; and the $A_{600}$ value of the cells co-cultured only with extract at concentration of 1:256 mg/mL was 1.21±0.042. As shown in the table the value of $A_{600}$ of the cells treated with extract is higher than that of the cells infected with $CVB_{3m}$ while not treated with the extract ($^bP<0.01$), indicating the protective effect of the extract on' HeLa cells from $CVB_{3m}$ infection.

**Table 3** Protective effect of ethyl acetate extract on HeLa cells shown in percentage ($A_{600}$ value of cells protected with the extract / $A_{600}$ value of normal HeLa cells

| Dilution | 1:256 | 1:512 | 1:1 024 | 1:2 048 | 1:4 096 | 1:8 192 | $10^{-5}$ $CVB_{3m}$ |
|---|---|---|---|---|---|---|---|
| The ratio of $A_{600}$ | 88.56±0.032 | 79.46±0.008 | 73.07±0.057 | 65.57±0.023 | 61.83±0.038 | 56.1±0.015 | 16.7±0.027 |

The percentage of vital cells protected with the extract accounted for compared with normal HeLa cells decreased with the increased extract dilution, and it was preferable from concentration 1:256 mg / mL to 1:1 024 mg / mL. $^bP<0.01$ *vs* group of $10^{-5}$ $CVB_{3m}$.

**Table 4** The value of $A_{600}$ obtained from various infective phase

| Dilution / $A_{600}$ | 1:256 | 1:512 | 1:1 024 | 1:2 048 | 1:4 096 | 1:8 192 |
|---|---|---|---|---|---|---|
| First group | 0.45±0.041 | 0.56±0.011 | 0.63±0.036 | 0.65±0.043 | 0.37±0.076 | 0.38±0.029 |
| Second group | 0.72±0.022 | 0.96±0.013 | 0.69±0.009 | 65.00±0.025 | 0.78±0.056 | 0.73±0.054 |
| Third group | 0.53±0.030 | 0.48±0.036 | 0.40±0.049 | 0.39±0.066 | 0.39±0.077 | 0.32±0.015 |

The $A_{600}$ value of the cells co-cultured only with RPMI1640, cells co-cultured only with $10^{-5}$ $CVB_{3m}$ and cells co-cultured only with extract (1:256 mg / mL) was 1.29±0.011, 0.24±0.023 and 1.20±0.029, respectively. The value of $A_{600}$ of the cells treated with extract of variable concentration at different time of $CVB_{3m}$ infection was higher than that of the cells infected with $CVB_{3m}$ and not treated with the extract, which indicated the protective effect of the extract existed through all the processes of $CVB_{3m}$ attachment, entry, biosynthesis and assemble in cells.

of $A_{600}$ obtained using crystal violet staining showed that starting from 1:256 mg/mL, the value of $A_{600}$ decreased with the increased dilution. There was a negative correlation between them ($r$=0.8525, $P$<0.01, Tables 2-3, Figure 1).

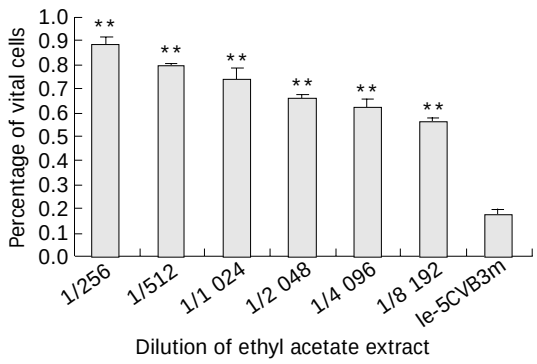


**Figure 1** The protective effect of the extract on HeLa cells expressed in column figure. The value of $A_{600}$ obtained using crystal violet staining decreased with the increased dilution of the extract. From 1:256 to 1:8 192 mg / mL the extract showed various protective effects on HeLa cells and the effect was decreased with the increased dilution of the extract. The protective effect was best at concentration of 1:256 to 1:1 024 mg / mL.

### *Primary study on the mechanism of anti-$CVB_{3m}$ effect of the extract in vitro*

In the first group, the extract was added in the process of virus adsorption. In second group, the extract was added in the process of virus adsorption and also after adsorption while in the third one, the extract was added after virus adsorption. The value of $A_{600}$ of the three groups were obtained. Among all three groups, $A_{600}$ obtained from the cells treated with extract from 1:256 to 1:8 192 mg/mL was higher than that treated with 100$TCID_{50}$ $CVB_{3m}$, and that of the second group was the highest. This suggested the extract exerted anti-$CVB_{3m}$ effect through all the processes of the virus infection (Table 4).

## DISCUSSION

Proliferation of virus primarily depends on the biosynthesis system of host cells because virus deficit the enzymes needed for their proliferation. Virus proliferation was similar to nucleotide replication of host cells and there is also great similarity between its products such as nucleotide and proteins and that of host cells. So the demand for killing virus while not infecting the normal physiological function of host cells brings us great difficulty to design and synthesize effective anti-virus drug. The development of anti-virus drugs is very slow compared with that of anti-bacteria drugs in the last one hundred years since the discovery of virus. Though it is an important way to synthesize anti-virus drugs using chemical methods, it is very slow and time-consuming. In recent years, many countries are paying more and more attention to look for anti-virus agents from Chinese medicinal herbs. Tian-hua-fen is a traditional Chinese medicinal herb. It has been used in Chinese for centuries to induce mid-term abortion. And there were many records about its biological efficacy. In the 70 s, the active ingredient of Tian-hua-fen, TCS, a protein from the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii Maxim* was found and purified. It is a monomeric protein with a pI (isoelectric point) of 9.4 and an apparent molecular weight of 24 kDa. There are no cysteine residues in the molecule[8]. TCS is a member of type 1 ribosome-inactivating protein (RIP) family[7-9]. RIP is a group of cytotoxic proteins acting on eukaryotic ribosomes. They can inactivate 60S ribosomal subunits by only hydrolyzing a single phosphodiester bond between the guanosine residue at position 4 325(G4 325) and the adenosine residue at position 4 326(A4 326) in 28S rRNA. TCS can inactivate eukaryotic ribosomes through its N-glycosidase activity by hydrolyzing the N-C glycosidic bond of adenylic acid at 4 324 site in 28S rRNA of rat liver[10]. Thus cell protein synthesis was inhibited. Clinical application over the years showed TCS had multiple pharmacological effects. The research about TCS was once popular since McGrath *et al.*[11,12] reported it could inhibit HIV-1 for the first time. But the sever side effect prevented its more extensive clinical application[13]. There are some other components such as polysaccharide, phytohemagglutinin, sterol and palmitic acid and so on in Tian-hua-fen besides TCS[14]. Polysaccharide in Tian-hua-fen had marked immunoregulation effect[1]. The galactose-binding lectin from Tian-hua-fen stimulated the incorporation of D-[3-3H]glucose into lipids in isolated rat epididymal adipocytes[15]. In the process of screening anti-virus agents, we found the ethyl acetate extract of Tian-hua-fen had obvious preventive effect on HeLa cells from $CVB_{3m}$ infection. This showed the potential anti-virus effect of the ethyl acetate extract of Tian-hua-fen. In this study, Tian-hua-fen was treated with chemical reagents and then was extracted with organic reagents. Finally, four kinds of extract were obtained. The anti-$CVB_{3m}$ effect of the four fractions was tested and the results showed

ethyl acetate fraction had the best preventive effect on HeLa cells from $CVB_{3m}$ infection. The preventive effect decreased as the extract dilution increased. There was an obvious correlation between them. The ethyl acetate fraction was further chromatographed on silica gel column and their anti-virus effect was also tested. The results suggested that toxicity of the further extract to HeLa cells was decreased compared with the former one and there also existed a negative correlation between the preventive effect and the dilution.

Many methods have been developed for determining the antiviral activities of compounds in cell culture. For viruses that cause discernable cytopathic effects (CPE) microscopically in cells, visual scoring of CPE inhibition is performed most frequently because it is rapid, and allows a number of compounds to be evaluated using 96-well microplates[16]. Since solely relying on visual scoring was inaccurate for assessing the cytotoxicities, the use of a dye or stain is very important. The results indicated that methods using bisbenzimide, crystal violet, fluorescein diacetate, MTT, neutral red, or rhodamine 6G were similar to visual scoring for determining anti-influenza virus activity in cell culture. Rapid staining (15 min) methods could be done with crystal violet and rhodamine 6G, and rhodamine 6G gave a high background in microwells containing only water (no cells or virus) which had to be subtracted[17], whereas other methods for determining antiviral activity of test substances may be much more tedious, requiring more microplates, compound, and/or time than the above methods. These include the plaque reduction assay[18], virus yield reduction assay[19], determining drug effect by counting the number of infected cells stained by fluorescent antibody[20] and [$^3$H]TdR incorpration[21]. For this reason, we preferred cystal violet staining in our study in order to quantitatively screen anti-virus agents.

We also did some primary study on the anti-$CVB_{3m}$ mechanism of the extract. The extract was added before virus adsorption, during adsorption and after adsorption and then the cells were stained with crystal violet and $A_{600}$ was measured. The value of $A_{600}$ of the three groups was higher than that of the cells infected with virus. This indicated the three kinds of treatment had preventive effect on HeLa cells from $CVB_{3m}$ infection at different levels and suggested that the extract could act through virus adsorption, penetration and synthesis in cells.

Our results showed that the ethyl acetate extract of Tian-hua-fen had marked anti-$CVB_{3m}$ effect *in vitro*. Some questions still remained to be answered. Which component play key role in the process of anti-virus? Which has the better anti-virus effect between the crude extract and the further extract? What is the anti-virus mechanism of it? To answer these questions needs more and further research. Anyway it is sure that the ethyl acetate extract of Tian-hua-fen has marked anti-virus effect. The answers to the above questions will help to wide the range of virus that Tian-hua-fen resists and also help to make Tian-hua-fen a clinical-used drug.

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• BASIC RESEARCH •

# FR167653 attenuates murine immunological liver injury

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## Abstract

**AIM:** To study the effect of FR167653 on immunological liver injury (ILI) in mice.

**METHODS:** ILI was established by tail vein injection of 2.5 mg *Bacillus Calmette-Guerin* (BCG), and 10 d later with 10 mg lipopolysaccharide (LPS) in 0.2 mL saline (BCG plus LPS). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) in sera and malondialdehyde (MDA), glutathione peroxidase (GSHpx) contents in liver homogenates were assayed by spectrophotometry. The levels of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) levels in sera were determined using ELISA. Interleukin-1 (IL-1) produced by peritoneal macrophages was determined by the method of $^3$H-infiltrated cell proliferation. The nuclear factor-kappa B (NF-κB) p65 in liver tissue was analyzed with reverse transcription polymerase chain reaction (RT-PCR). Liver samples collected were stained with hematoxylin and eosin.

**RESULTS:** FR167653 (50, 100, 150 mg/kg) could significantly decrease the serum transaminase (ALT, AST) activity and MDA content in liver homogenate, and improve reduced GSHpx level of liver homogenate. Liver histopathological examination showed FR167653 (100, 150 mg/kg) significantly reduced inflammatory cells infiltration and liver cells necrosis. FR167653 (50, 100, 150 mg/kg) significantly lowered TNF-α and NO levels in serum, and IL-1 produced by peritoneal macrophages. Moreover, expression of NF-κB mRNA in liver tissue of ILI induced by BCG plus LPS was significantly reduced by FR167653.

**CONCLUSION:** All results showed that FR167653 had significant inhibitory action on ILI in mice.

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## INTRODUCTION

It has been demonstrated that tissue inflammation plays a critical role in liver pathology via induction of cellular injury. The infiltration of mononuclear phagocytes into the liver has been shown to correlate with the severity of liver injury. Kupffer cells (KCs) are among the first cells that respond to endotoxins, including lipopolysaccharides (LPS), and are considered to be the primary macrophages involved in the clearance of gut-derived bacteria or bacterial toxins. High portal level of LPS can lead to a pronounced secretion of proinflammatory mediators by KCs and ultimately to endotoxin-induced liver injury[1,2]. Moreover, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) β have been linked to liver injury[3-5], and shown to be early and important mediators of liver injury[6-8]. Thus, inhibition of KCs activity and proinflammatory cytokines production would be beneficial to alleviate liver injury.

FR167653,1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo [5,1-*c*][1,2,4] triazin-2-yl]-2-phenylethanedione sulfate monohydrate, was first discovered to be a potent dual inhibitor of IL-1 and TNF-α production in LPS-stimulated human monocytes and phytohemagglutinin-stimulated human lymphocytes[9]. Takahashi *et al*.[10] and Kawano *et al*.[11] also confirmed the inhibitory effect of FR167653 on cytokine production. FR167653 ameliorated endotoxin shock in rabbits and intravascular coagulation in rats[9,12], and also ameliorated cardiac dysfunction caused by chronic infusion of LPS in rats[13]. Furthermore, FR167653 protected lung, liver, and heart against ischemia-reperfusion injury in dogs[14-17], and the protection against liver ischemia-reperfusion injury was associated with inhibition of proinflammatory cytokines from KCs[18, 19]. Recent studies suggest that FR167653 inhibits IL-1β and TNF-α production via specific inhibition of p38 MAPK activity[12,17,20-22]. Because effective therapy has not been established for liver injury, development of an anti-inflammatory compound is urgently needed. From this viewpoint, it is worthwhile to assess the anti-inflammatory and immunomodulatory effect of FR167653. In the current study, we administered FR167653 to a murine model of *Bacillus Calmette Guerin* (BCG) and LPS-induced liver injury and assessed histopathological changes and its effects on cytokine production.

## MATERIALS AND METHODS

### *Animals and reagents*

Male Kunming strain mice weighing 18-22 g were purchased from Animal Center of Anhui Medical University. Mice were allowed to access to food and tap water *ad libitum*, and acclimated to facilities for at least 1 wk before any treatments. 1, 1, 3, 1-tetraethoxypropane (TEP) and 5, 5'-dithibis-(2-nitrobenzicacid) (DTNB) were purchased from FLUKA Co., Switzerland. BCG was purchased from Institute of Shanghai Biological Products. ConA, LPS from *Escherichia coli*, and TNF-α ELISA kit were purchased from Sigma Co., St. Louis, USA. The primers of NF-κB and β-actin were synthesized by BIOASIA Biotech Co. Shanghai. FR167653 supplied by the Fujisawa Pharmaceutical Co. Ltd. (Tokyo, Japan), was dissolved to 20 mL/L with 5 g/L methylcellulose solution. To obtain the dose of 50, 100, or 150 mg/kg body mass (BM), 2.5, 5, or 7.5 μL/g·bm. of 20 mL/L FR167653 solution was injected subcutaneously.

### *Model of BCG and LPS-induced immunological liver injury (ILI) and treatment* [23]

Each mouse was injected with 2.5 mg BCG (viable bacilli) in 0.2 mL saline via tail vein, and 10 d later with 10 mg LPS in 0.2 mL

saline. At 4, 8, and 12 h post-injection of LPS, animals received FR167653 at 50, 100, and 150 mg/kg, s.c., respectively. The control group was subcutaneously administered the same volume of 5 g/L methylcellulose solution. The mice were anesthetized with ether, then sacrificed by cervical dislocation 16 h after LPS injection and blood was collected, and centrifuged at 1 500 r/min for 10 min at room temperature. Serum was aspirated and stored at -70 ℃ until assayed as described below. The liver was also removed and stored at -70 ℃ until required.

### Measurement of ALT, AST

Serum ALT, and AST were determined using commercial kits purchased from Institute of Shanghai Biological Products affiliated to the Ministry of Health.

### Measurement of MDA and GSHpx in liver homogenate

Livers were thawed, weighed and homogenized with Tris-HCl buffer (5 mmol/L, containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged at 1 000 r/min for 10 min at room temperature and the supernatant was used immediately for the assays of MDA and GSHpx. MDA was measured by the thiobarbituric acid method according to standard techniques. The content of MDA was expressed as nmol per gram liver tissue. GSHpx was measured by the DTNB method, and its content was expressed as U per milligram protein.

### Measurement of TNF-α, IL-1, and nitric oxide (NO)

Serum TNF-α and NO were measured using commercial kits produced by Sigma Co. and Beijing Biotinge-Tech Co. Ltd, and their levels were expressed as pg/mL and μmol/L respectively. IL-1 produced by peritoneal macrophages was measured according to the method of $^3$H-infiltrated cell proliferation.

### RT-PCR assay for NF-κB p65 mRNA in liver tissue

Liver tissue RNA was extracted using RNA easy kit (Invitrogen, USA). To test the efficacy of reverse transcriptase, RT-PCR was performed for β-actin mRNA. Briefly, the first strand of cDNA was synthesized by reverse transcriptase and pooled. The resulting cDNA samples were adjusted to PCR buffer conditions and run for PCR simultaneously. The primers for NF-κB p65 were 5'- GCG GCC AAG CTT AAG ATC TGC CGA GTA AAC-3', and 5'-CGC TGC TCT AGA GAA CAC AAT GGC CAC TTG CCG-3'. The primers for β-actin were 5'-TGG AAT CCT GTG GCA TCC ATG AA-3', and 5'-TAA AAC GCA GCT CAG TAA CAG TC-3'. The amplifications of NF-κB p65 and β-actin genes are expected to generate 198 bp and 348 bp fragments, respectively. Amplification was performed for 30 cycles, each of which consisted of 1 min of denaturation at 94 ℃, 1 min of annealing at 58 ℃, and 3 min of primer extension at 72 ℃. Ten μL of reaction mixture was loaded to 10 g/L agarose gel containing 0.5 μg/mL ethidium bromide for electrophoresis, the gel was then placed under ultraviolet light for semi-quantitative detection.

### Histological examination of liver specimens

Formalin-fixed liver specimens were embedded in paraffin and stained with hematoxylin and eosin for conventional morphological evaluation.

### Statistical analysis

All values were expressed as mean±SD. The significance of differences between groups was determined by ANOVA followed by Student's *t* test. *P* value of less than 0.05 was considered significant.

## RESULTS

### Effects of FR167653 on ILI induced by BCG plus LPS in mice

The levels of ALT and AST in plasma and MDA content in liver homogenate were significantly increased after the sequential injection of BCG and LPS. Meanwhile, the GSHpx level in liver homogenate was sharply decreased (Figures 1, 2). FR167653 (50, 100, 150 mg/kg, s.c.) could not only significantly decrease ALT, AST, and MDA levels, but also evidently increase GSHpx level in mice with ILI (Figures 1, 2). Liver histopathologic examination showed extensive inflammatory cells infiltration and liver cells necrosis. FR167653 (100, 150 mg/kg) significantly reduced inflammatory cells infiltration and liver cells necrosis (Figure 3).

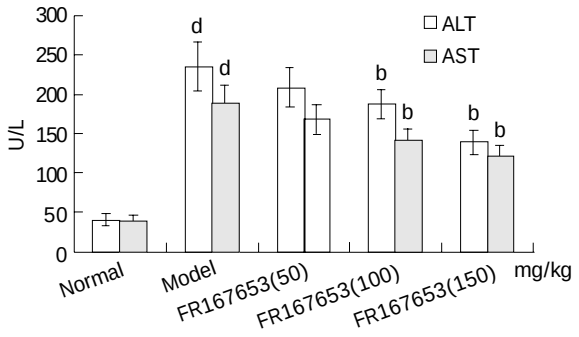


**Figure 1** Effect of FR167653 on serum ALT and AST activities in ILI mice. *n*=8, mean±SD, $^bP$<0.01 *vs* model group; $^dP$<0.01 *vs* normal group.

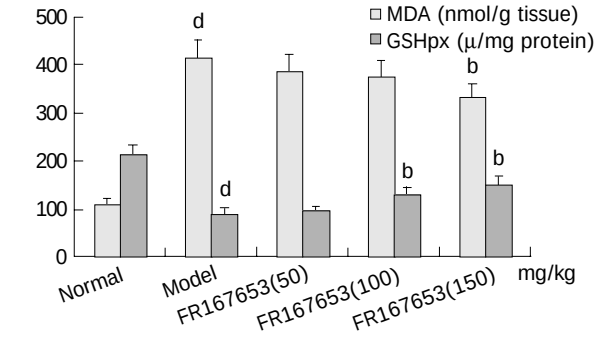


**Figure 2** Effect of FR167653 on MDA and GSHpx contents in liver homogenates of ILI mice. *n*=8, mean±SD, $^bP$<0.01 *vs* model group; $^dP$<0.01 *vs* normal group.

**Table 1** Effect of FR167653 on serum TNF-α, NO and IL-1 from peritoneal macrophages in ILI mice

| Group | Dose (mg/kg) | TNF-α (pg/mL) | NO (μmol/L) | IL-1 ($10^3$/min) |
|---|---|---|---|---|
| Normal | - | Under detection limit | 15.7±4.3 | 6.4±1.42 |
| Model | - | 523.1±28.6$^d$ | 117.8±10.7$^d$ | 21.5±5.04$^d$ |
| FR167653 | 50 | 491.8±20.5$^a$ | 109.3±10.5 | 17.4±3.81 |
| | 100 | 404.9±18.5$^b$ | 95.4±8.9$^b$ | 14.7±3.05$^b$ |
| | 150 | 341.8±19.1$^b$ | 84.3±8.7$^b$ | 11.9±2.85$^b$ |

*n*=8, mean±SD, $^aP$<0.05, $^bP$<0.01 *vs* model group; $^dP$<0.01 *vs* normal group.

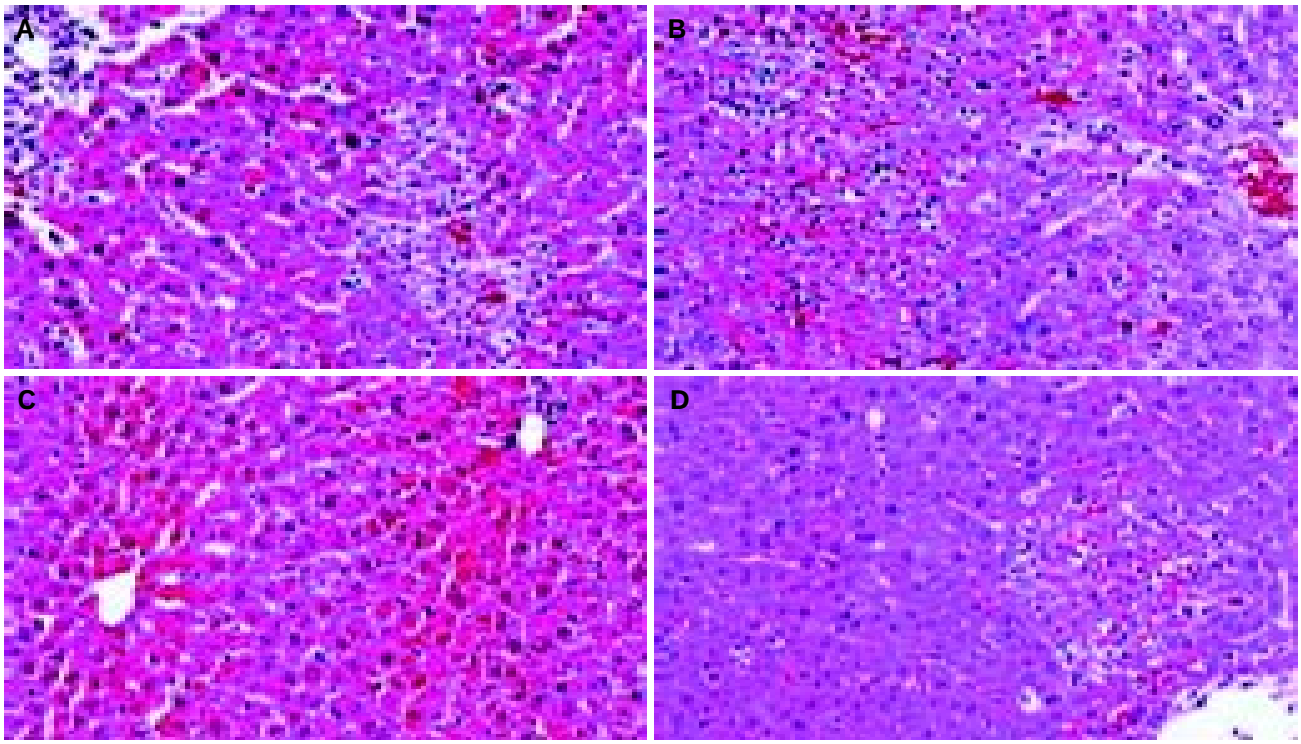


**Figure 3** Effect of FR167653 on liver histopathology of ILI mice. A: Normal group; B: Model group; C: Model plus FR167653 (100 mg/kg); D: Model plus FR167653 (150 mg/kg). All sections were stained with hematoxlin and eosin (200×).

### *Effects of FR167653 on TNF-α, IL-1, and NO*

The levels of TNF-α and NO were elevated significantly in mice injected with BCG and then challenged with LPS. Likewise, IL-1 excreted by peritoneal macrophages was also significantly increased in the model group (Table 1). FR167653 (50, 100, 150 mg/kg) obviously counteracted the increase of TNF-α and NO levels in sera. IL-1 produced by peritoneal macrophages was also significantly inhibited by FR167653 (Table 1).

### *Effects of FR167653 on NF-κB p65 expression in liver tissue*

Gel electrophoresis and semi-quantitative analysis showed that FR167653 significantly reduced expression of NF-κB mRNA in liver tissue of ILI induced by BCG plus LPS compared with model group (Figure 4).

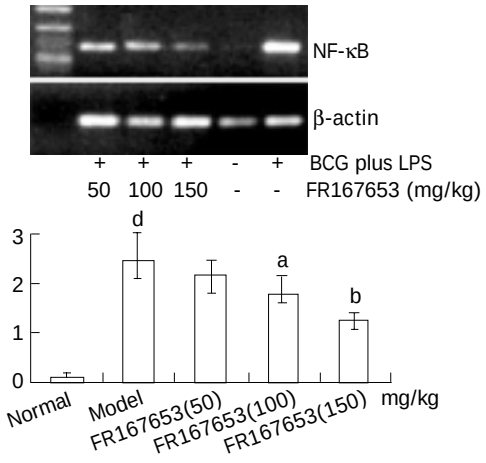


**Figure 4** Effect of FR167653 on NF-kB mRNA in liver tissue of ILI mice. $n$=3, mean±SD. $^{a}P<0.05$. $^{b}P<0.01$ *vs* model group; $^{d}P<0.01$ *vs* normal grorp.

## DISCUSSION

It has been demonstrated that severe hepatitis could be induced by injecting a small dose of bacterial LPS into BCG-pretreated mice[23]. In the present study, ILI was successfully induced by BCG plus LPS. On this basis, FR167653 (50, 100, 150 mg/kg) could significantly lower the increased plasma transaminase levels and MDA contents in liver homogenate. Meanwhile, GSHpx levels were increased significantly. Histopathologic examination showed FR167653 significantly reduced inflammatory cells infiltration and liver cells necrosis.

Although several parameters of the inflammatory response contribute to liver injury[24], one well-studied pathway is the production of TNF-α. In a number of liver injury models, elevated TNF-α levels were present and correlated with liver injury[23,25,26]. Inhibition of TNF-α activity can decrease liver injury. The addition of soluble TNF receptors that diminished the biological effect of TNF-α could significantly decrease liver enzyme levels, improve liver histology, and reduce mortality immediately after acute carbon tetrachloride administration. Similar results were seen in chronic alcohol-induced liver disease in rats. In humans, elevated levels of TNF-α are seen in hepatitis and are associated with increased mortality[27]. Furthermore, TNF-α might act as the first mediator of liver injury, its elevation would result in release of a number of proinflammatory mediators including IL-1, NO, IL-6, IL-8[3,28], further deteriorating the liver injury. In the present study, TNF-α in the sera and IL-1 produced by peritoneal macrophages were significantly increased in mice with ILI compared with control. FR167653, a potent inhibitor of TNF-α and IL-1, suppressed elevated TNF-α and IL-1 levels, which was consistent with previous observations[29]. As potent producers of inflammatory cytokines such as TNF-α and IL-1, KCs have been implicated in the pathway leading to liver injury[30]. Whether inhibition of FR167653 on TNF-α production was associated with diminished KCs proliferation, and decreased inflammatory cells infiltration needs to be further clarified.

A growing body of evidence suggests that sustained production of NO resulting from up-regulation of inducible NOS (iNOS) after LPS challenge may cause hepatocellular injury, either directly[31,32], or indirectly, by forming reactive nitrogen

intermediates[33]. Menezes *et al*.[34,35]demonstrated that a NO scavenger, NOX, decreased hepatocellular injury and improved survival after hemorrhagic shock. As reported previously, the synthesis of NO was regulated by various factors including TNF-α, IL-1, and NF-κB. The inhibition of NF-κB led to a decrease in the inducible isoform of NO and the transcription of TNF-α in KCs[36-38]. Moreover, synthetic double-stranded oligodeoxynucleotides (ODN) transfer with a high affinity to NF-κB (NF-κB/decoy/ODN) *in vivo* effectively suppressed endotoxin-induced fatal liver injury in mice[39]. In our study, we observed significant suppression of NF-κB p65 mRNA in liver tissue and NO in sera of BCG plus LPS - induced ILI by FR167653. It indicated that inhibition of FR167653 on liver injury might be related with decreased NO production through inhibiting NF-κB transcription. Recent studies suggested that p38 MAPK inhibitor, SB203580, diminished phosphorylation of the transactivation domain of the p65 subunit of NF-κB[40]. Moreover, the pharmacological characteristics and chemical structure of FR167653 resemble SB203580[41,42]. Therefore, inhibition of FR167653 on liver injury was associated with reduced expression of NF-κB via p38 MAPK, and led to down-regulation of TNF-α, IL-1, and NO.

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• CLINICAL RESEARCH •

# Therapeutic plasma exchange in patients with hyperlipidemic pancreatitis

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## Abstract

**AIM:** To clarify the effectiveness of plasma exchange by comparing the mortality and morbidity before and after the intervention of plasma exchange.

**METHODS:** Plasma exchange has been available as an optional therapy for hyperlipidemic pancreatitis since August 1999 in our hospital. The patients were assorted into 2 groups (group I: before August 1999 and group II: after August 1999). Group I consisted of 34 patients (before the availability of plasma exchange). Group II consisted of 60 patients (after the availability of plasma exchange). Twenty patients in group II received plasma exchange after giving their consent. The mortality and morbidity were compared between group I and group II. Furthermore, the patients with severe hyperlipidemic pancreatitis (Ranson's score≥3) were analyzed separately. The mortality and morbidity were also compared between those receiving plasma exchange (group A) and those who did not receive plasma exchange (group B).

**RESULTS:** There was no statistical difference in the mortality, systemic and local complications between group I and group II. When the patients with severe hyperlipidemic pancreatitis were analyzed separately, there was no statistical difference between group A and group B.

**CONCLUSION:** Plasma exchange can not ameliorate the overall mortality or morbidity of hyperlipidemic pancreatitis. The time of plasma exchange might be the critical point. If patients with hyperlipidemic pancreatitis can receive plasma exchange as soon as possible, better result may be predicted. Further study with more cases is needed to clarify the role of plasma exchange in the treatment of hyperlipidemic pancreatitis.

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## INTRODUCTION

Hypertriglyceridemia (HTG) is a rare cause of pancreatitis. Hyperlipidemic pancreatitis (HLP) secondary to HTG presents typically as an episode of acute pancreatitis or recurrent acute pancreatitis or rarely as chronic pancreatitis[1]. The typical clinical profile of HLP is a patient with preexisting lipid abnormality along with the presence of secondary factors (such as poorly controlled diabetes mellitus, alcohol abuse, pregnancy, or a medication) that can induce HTG[1]. It is generally accepted that a TG level more than 1 000 mg/dL is needed to precipitate an episode of acute pancreatitis[2]. It is postulated that hydrolysis of TG by pancreatic lipase into free fatty acid is toxic to pancreatic endothelium and acinar cells[3]. In an animal study, hyperlipidemia could intensify the course of acute edematous pancreatitis and necrotizing pancreatitis[3].

Plasmapheresis has been claimed to reduce triglyceride level rapidly in HLP[4-11] and is believed to halt the progression of HLP[8-10]. Actually, experiences of plamapheresis in HLP are limited and only sporadic cases were reported[5-11]. There was no control study in the past concerning whether plasmapheresis could improve the mortality or morbidity of HLP. Our aim was to analyze the benefits of plasma exchange by comparing the mortality and morbidity of HLP patients with those without receiving such an intervention.

## MATERIALS AND METHODS

### *Patient characteristics*

From September 1992 to June 2003, a total of 862 patients with acute pancreatitis were reviewed and 94 patients were consistent with hyperlipidemic pancreatitis (HLP). As plasma exchange has been available as an optional therapy of HLP since August 1999 in our hospital, the patients were assorted into 2 groups (group I: before August 1999 and group II: after August 1999). Group I consisted of 34 patients (before the availability of plasmapheresis) and group II consisted of 60 patients (after the availability of plasmapheresis). Twenty of 60 patients in group II received plasma exchange after giving their consents. The Ranson's score was used for assessment of the severity of pancreatitis. Half of the patients receiving plasma exchange were severe cases (Ranson's score≥3). The anatomical change of acute pancreatitis was assessed according to the Balthazar's grading. The enrolled criteria of plasma exchange in HLP were as followings: (1) overt symptoms of acute pancreatitis, (2) pancreatitis proved by CT, ultrasound or elevation of pancreatic enzymes, (3) triglyceride (TG) >1 000 mg/dL and lactescent serum, (4) exclusion of other causative conditions, such as gall stone, trauma or neoplasm, (5) patient's agreement. The mortality and morbidity between group I and group II were compared.

Furthermore, the patients with severe HLP (Ranson's score≥3) were analyzed separately. We divided the patients with severe HLP (a total of 29 patients) into 2 groups. Group A received plasma exchange, while group B did not receive the intervention. The mortality and morbidity between group A and group B were also compared.

The secondary factors inducing HTG in our patients included diabetes mellitus (46 patients), alcoholic consumption (32 patients) and oral contraceptive (one patient). The median time for starting plasma exchange was 3 d after symptom onset (range, 2-6 d).

### *Apheresis*

Plasma exchange was carried out using membrane filtration in a KM 8800 membrane plasmapheresis monitor (Kuraray, Osaka, Japan) with a Plasmacure plasma separator (Kuraray, Osaka, Japan) to separate plasma from blood. One calculated plasma volume was processed during each session of plasma exchange. One course of plasma exchange treatment consisted of one or two daily sessions based on the doctor 's decision, single session in 13 patients and two sessions in 7. Heparin was used as the anticoagulant. Either a double lumen catheter in a central vein (fifteen patients) or a dialysis catheter in an antecubital vein (five patients) was used for vascular access. Replacement fluid was given with fresh frozen plasma (FFP) in 8 patients and isovolumetric 5 % albumin solution in 12 patients.

### *Statistical analysis*

*t*-test and chi-square test were used for statistical analysis. $P<0.05$ was considered statistically significant.

## RESULTS

The demographic characteristics of all the patients are summarized in Table 1. The mean age and sex distribution were similar in both groups. The initial mean TG level was around 1 900 in both groups. The severity of pancreatitis was predicted by the Ranson's criteria. Severe pancreatitis (Ranson's scroe$\geq$3) was 20.6% in group I and 36.7% in group II ($P$=0.105). The anatomical change of pancreatitis was assessed according to the Balthazar's grading system and 54.2% of group I and 41.3% of group II were belonged to Balthazar grade D or E ($P$=0.305).

The mortality rate, systemic and local complications of both groups are demonstrated in the Table 2. The systemic complication was defined by the Atlanta definition[12]: (1) pulmonary insufficiency, $PaO_2 < 8$ kPa, (2) renal insufficiency, Cr > 2 mg/dL, (3) shock, SBP<12 kPa, (4) UGI bleeding >500 mL/24 h. The local complications included abscess and pseudocyst formation. There was no significant difference between group I and group II in mortality and complications. Further comparison of individual items of systemic and local complications between the two groups revealed no statistical differences (Tables 3, 4).

**Table 1** Demographic characteristics

| | Group I ($n$=34) | Group II ($n$=60) | $P$ value |
|---|---|---|---|
| Age (yr) | 40.8±6.8 | 42.3±8.9 | 0.394 |
| Initial TG | 1 922±1 287 | 1 913±612 | 0.966 |
| DM (%) | 38(13/34) | 55(33/60) | 0.118 |
| Alcohol (%) | 44(15/34) | 28.3 (17/60) | 0.121 |
| Ranson >3(%) | 20.6( 7/34) | 36.7(22/60) | 0.105 |
| Balthazar D, E (%) | 54.2(13/24) | 41.3(19/46) | 0.305 |

**Table 2** Comparison of mortality and morbidity between patients before and after availability of plasma exchange

| | Group I (%, $n$=34) | Group II (%, $n$=60) | $P$ value |
|---|---|---|---|
| Mortality (%) | 5.9(2/34) | 6.7(4/60) | 0.881 |
| Systemic complications (%) | 17.6(6/34) | 18.3(11/60) | 0.934 |
| Local complications (%) | 11.8(4/34) | 6.7(4/60) | 0.395 |

When severe hyperlipidemic pancreatitis (Ranson's score$\geq$3) was analyzed separately (Table 5), the mortality rate, systemic and local complications of group A (with plasmapheresis) and group B (without plasmapheresis) were not statistically different ($P$=0.369, 0.153, 0.454, respectively).

The mean serum concentration of TG and lipase fell significantly after plasma exchange. The serum TG level declined from 2 019±780 mg/dL to 691±331 mg/dL (65.8% reduction) and the serum lipase level declined from 4 007±355 U/L to 447±35 U/L (88.8% reduction).

**Table 3** Comparison of systemic complications between patients before and after the availability of plasma exchange

| | Group I (%, $n$=34) | Group II (%, $n$=60) | $P$ value |
|---|---|---|---|
| ARF | 17.6(6/34) | 10(6/60) | 0.286 |
| UGI bleeding | 0(0/34) | 8.3(5/60) | 0.084 |
| Shock | 8.8(3/34) | 10(6/60) | 0.852 |
| ARDS | 11.8(4/34) | 10(6/60) | 0.790 |

ARF: Acute renal failure; ARDS: Acute respiratory distress syndrome.

**Table 4** Comparison of local complications between patients before and after availability of plasma exchange

| | Group I (%, $n$=34) | Group II (%, $n$=60) | $P$ value |
|---|---|---|---|
| Abscess | 17.6(6/34) | 10(6/60) | 0.286 |
| Pseudocyst | 0(0/34) | 8.3(5/60) | 0.084 |

**Table 5** Comparison of patients with severe hyperlipidemic pancreatitis receiving plasma exchange and not receiving plasma exchange

| Ranson >3 | Group A: PE(+) (%, $n$=10) | Group B: PE(-) (%, $n$=19) | $P$ value |
|---|---|---|---|
| Mortality | 30(3) | 15.8(3) | 0.369 |
| Systemic complications | 70(7) | 42.1(8) | 0.153 |
| Local complications | 10(1) | 21.1(4) | 0.454 |

PE (+): With plasma exchange; PE (-): Without plasma exchange.

## DISCUSSION

The association between hyperlipidemia and acute pancreatitis was first described by Speck in 1865[1]. Studies on patients with familial HTG and their longterm follow-up have shown that extreme elevation of TG occurred during episode of acute pancreatitis[13]. It has been generally believed that a TG level of more than 1 000 mg/dL was needed to precipitate an acute pancreatitis[2]. The hypothesis of hyperlipidemic pancreatitis is that pancreatic damage was resulted from toxic injury to the capillary endothelium and the damage of pancreatic acinar cells was caused by free fatty acids liberated by pancreatic lipase[3]. Conservative treatment (fasting, lipid lowering drugs, insulin or fluid restoration) might decrease TG level slowly in a time span of days to weeks[14]. In contrast, plasmapheresis might remove excessive lipid from serum in about 2 h[4,6]. Sporadic reports about plasmapheresis used in hyperlipidemic pancratits were seen in the past[4-10]. They all concluded that plasmapheresis was helpful for treating or preventing acute hyperlipidemic pancreatitis. However, no control study to assess the value of plasmapheresis in the treatment of hyperlipidemic pancreatitis is available.

Different methods have been used in plasmapheresis. Plasma exchange is superior to double filtration in the removal of excessive TG because the membrane of plasma separator was usually blocked by the larger particles of chylomicron[6]. We used plasma exchange with replacement of albumin or fresh frozen plasma (FFP) in the treatment of HLP in this study. FFP could supply lipoprotein lipase and apolipoprotein from the healthy donor[13]. Lipoprotein lipase and apolipoprotein were essential for the catabolism of TG[13].

In our study, plasma exchange could remove TG effectively

from turbid plasma in a short time (about 2 h). TG declined from 2 019±780 mg/dL to 691±331 mg/dL (65.8% reduction). It was also postulated that plasmapheresis could remove circulating activated enzymes and inflammatory mediators[15], but its beneficial effects in pancreatitis has not been proved[10]. The serum lipase level declined from 4 007±355 U/L to 447±35 U/L (88.8% reduction) after plasma exchange in our patients.

Despite the marked reduction in TG and lipase after plasma exchange, we could not achieve statistically significant improvement in the mortality and morbidity after the intervention of plasma exchange. The mortality was 5.9% before the intervention (group I) and 6.7% after the intervention (group II). The rate of systemic complications (acute renal failure, UGI bleeding, shock, or pulmonary insufficiency) was 17.6% in group I and 18.3% in group II. The rate of local complications (abscess or pseudocyst) was 11.8% in group I and 6.7% in group II. While individual items of complications were considered, there were still no statistical differences between the two groups.

When the patients with severe HLP (Ranson's score$\geq$3) were analyzed separately, the mortality rate was 30% in group A (with plasma exchange) and 15.8% in group B (without plasma exchange). The mortality in severe HLP was not decreased by plasma exchange. The rate of systemic complication was 70% in group A and 42.1% in group B ($P$=0.153). The rate of local complication was 10% in group A and 21.1% in group B ($P$=0.454). Again, plasma exchange was not able to alter the complication rate significantly.

Why could plasma exchange not improve the mortality and morbidity in HLP? We proposed that the time of plasmapheresis might be the critical point. If patients with HLP could receive plasma exchange as soon as possible, better result might be expected[5].

In conclusion, plasma exchange fails to improve the overall mortality and morbidity of HLP in our study. Further study with more cases is needed to clarify the role of plasmapheresis in the treatment of HLP.

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• BRIEF REPORTS •

# Insulin is necessary for the hypertrophic effect of cholecystokinin-octapeptide following acute necrotizing experimental pancreatitis

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## Abstract

**AIM:** In previous experiments we have demonstrated that by administering low doses of cholecystokinin-octapeptide (CCK-8), the process of regeneration following L-arginine (Arg)-induced pancreatitis is accelerated. In rats that were also diabetic (induced by streptozotocin, STZ), pancreatic regeneration was not observed. The aim of this study was to deduce whether the administration of exogenous insulin could in fact restore the hypertrophic effect of CCK-8 in diabetic-pancreatitic rats.

**METHODS:** Male Wistar rats were used for the experiments. Diabetes mellitus was induced by administering 60 mg/kg body mass of STZ intraperitoneally (i.p.), then, on d 8, pancreatitis was induced by 200 mg/100 g body mass Arg i.p. twice at an interval of 1 h. The animals were injected subcutaneously twice daily (at 7 a.m. and 7 p.m.) with 1 μg/kg of CCK-8 and/or 2 IU mixed insulin (300 g/L short-action and 700 g/L intermediate-action insulin) for 14 d after pancreatitis induction. Following this the animals were killed and the serum amylase, glucose and insulin levels as well as the plasma glucagon levels, the pancreatic mass/body mass ratio (pm/bm), the pancreatic contents of DNA, protein, amylase, lipase and trypsinogen were measured. Pancreatic tissue samples were examined by light microscopy on paraffin-embedded sections.

**RESULTS:** In the diabetic-pancreatitic rats treatment with insulin and CCK-8 significantly elevated pw/bm and the pancreatic contents of protein, amylase and lipase *vs* the rats receiving only CCK-8 treatment. CCK-8 administered in combination with insulin also elevated the number of acinar cells with mitotic activities, whereas CCK-8 alone had no effect on laboratory parameters or the mitotic activities in diabetic-pancreatitic rats.

**CONCLUSION:** Despite the hypertrophic effect of CCK-8 being absent following acute pancreatitis in diabetic-rats, the simultaneous administration of exogenous insulin restored this effect. Our results clearly demonstrate that insulin is necessary for the hypertrophic effect of low-doses of CCK-8 following acute pancreatitis.

Hegyi P, Rakonczay Jr Z, Sári R, Czakó L, Farkas N, Góg C, Németh J, Lonovics J, Takács T. Insulin is necessary for the hypertrophic effect of cholecystokinin-octapeptide following acute necrotizing experimental pancreatitis. *World J Gastroenterol* 2004; 10(15): 2275-2277
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## INTRODUCTION

We have previously demonstrated that the administration of low doses of cholecystokinin-octapeptide (CCK-8) accelerated the processes of regeneration following L-arginine (Arg)-induced pancreatitis[1] and this was not observed in rats that were also diabetic[2]. The most significant difference in the regeneration was observed after two wk of CCK-8 treatment[1]. In addition, the histologic examination revealed that the majority of hypertrophized pancreatic acinar cells were found surrounding the enlarged islets of Langerhans following CCK-8 administration. It appears that the close proximity of the islets of Langerhans functions to protect the acinar cells as well as accelerate the regenerative process during Arg-evoked pancreatic tissue damage. A reason for this may be due to the interaction of acinar and islet cells. The exocrine and endocrine pancreas possesses a multitude of complex anatomical and functional interrelations[3]. It is well documented that intact islets of Langerhans are necessary for normal pancreatic exocrine function[4], and so we set out to investigate whether the administration of exogenous insulin could restore the hypertrophic effect of CCK in diabetic-pancreatic rats.

## MATERIALS AND METHODS

Male Wistar rats weighing 250-300 g were divided into five groups. The animals were kept at a constant room temperature of 25 °C with a 12-h light-dark cycle, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). Rats in group D (diabetic -control group) were injected with 60 mg/kg body mass of streptozotocin (Zanosar®, The Upjohn Company, Kalamazoo, MI) intraperitoneally (i.p.). In group DP (diabetic and pancreatitic) the rats received STZ as in group D, and on d 8, pancreatitis was induced by 200 mg/100 g body mass Arg (Sigma, St. Louis, MO) i.p. twice at an interval of 1h. In group DPC, apart from being given Arg and STZ, the rats were also administered 1 μg/kg of CCK-8 (synthesized by Botond Penke, Department of Medical Chemistry University of Szeged) subcutaneously (s.c.) twice daily (at 7 a.m. and 7 p.m.). In group DPI, besides being administered Arg and STZ, the rats received 2 IU mixed insulin (300 g/L short-action and 700 g/L intermediate-action insulin, HUMULIN M3®, Lilly Hungária Kft, Hungary) s.c. twice

**Table 1** Laboratory parameters changes in diabetic-pancreatitic rats due to a 14 d administration of insulin and / or CCK-8

| | Serum glucose | Serum insulin | pm / bm | Pancreatic DNA | Pancreatic protein | Pancreatic amylase | Pancreatic trypsinogen | pancreatic lipase |
|---|---|---|---|---|---|---|---|---|
| Insulin treatment | ↓ | ↑ | ↑ | - | - | ↑ | - | - |
| CCK-8 treatment | - | - | - | - | - | - | - | - |
| Insulin and CCK-8 treatment | ↓ | ↑ | ↑ | - | ↑ | ↑↑ | ↑ | - |

CCK-8: cholecystokinin-octapeptide, pm / bm: pancreatic weight / body weight ratio. ↑ increased or ↓ decreased activity / level, – no change, ↑↑ significantly larger increase of activity compared with the ↑ sign. All changes are given in comparison to the untreated diabetic-pancreatitic rats (group DP).

daily (at 7 a.m. and 7 p.m.). In group DPCI, diabetes and pancreatitis were induced as in group DP and the rats were administered CCK-8 and mixed insulin as mentioned before. The animals were killed in the morning by exsanguination through the abdominal aorta 14 d after pancreatitis induction. The serum amylase, glucose, insulin and plasma glucagon levels, pancreatic mass/body mass ratio (pm/bm), the pancreatic contents of DNA, protein, amylase, lipase and trypsinogen were measured[2]. Pancreatic tissue samples were examined by light microscopy on paraffin-embedded sections. Results were expressed as mean±SE. Statistical analysis was performed by using ANOVA. *P* values <0.05 were accepted as significant.

## RESULTS

In diabetic groups D, DP, and DPC, the serum glucose levels (22.5±1.5 mmol/L , 29.5±0.8 mmol/L and 29.0±0.7 mmol/L respectively) were significantly elevated and the insulin levels (0.70±0.30 U/l, 0.35±0.09 U/l and 0.20±0.05 U/l, respectively) were significantly lower *vs* those in insulin-treated groups DPI and DPCI (14.5±2.3 mmol/L and 12.9±2.8 mmol/L ; 401±38 U/L and 138±35 U/L, respectively). These results clearly indicated the presence of diabetes mellitus in the animals studied and the efficacy of insulin treatment. There were no significant differences in plasma glucagon and serum amylase levels in group D as compared with any of the other groups. In groups DPI and DPCI, pm/bm (2.98±0.21 mg/g, 3.58±0.11 mg/g, respectively) was significantly elevated *vs* group DP (2.09±0.26 mg/g). However, no significant differences were observed in pm/bm between group DPC and group DP (2.62±0.26 mg/g and 2.09±0.26 mg/g, respectively). In group DPCI (insulin- and CCK-8-treated diabetic-pancreatitic rats), the pancreatic protein content (250±35 mg/p) was significantly elevated *vs* groups DP and DPC (147±10 mg/p and 148±10 mg/p, respectively). In groups DPI and DPCI (insulin-treated rats), the pancreatic amylase content (3 948±1 288 U/p, 10 502±2 935 U/p, respectively) was significantly elevated *vs* groups DP and DPC (158±5 U/p and 182±25 U/p, respectively). In group DPCI, pancreatic amylase content (10 502±2 935 U/p) was significantly elevated *vs* group DPI (3 948±1 288 U/p). No significant differences were found in the pancreatic DNA and lipase contents between group D and any other groups. In group DPCI, the pancreatic trypsinogen content was significantly elevated *vs* group DPI (976±132 IU/p and 592±63 IU/p, respectively). However, no significant differences were observed in the pancreatic trypsinogen content between group DPC (655±36 IU/p) and DP (776±48 IU/p). Histological examination revealed signs of chronic inflammation in diabetic-pancreatitic rats, where acute inflammatory cells had been replaced by interstitial tissues, mononuclear cells and fibroblasts. Histological examination did not show any differences between groups DPC and DP. In group DPCI, a more intense mitotic activity was observed *vs* group DPC due to the effect of insulin (Table 1).

## DISCUSSION

The intraperitoneal administration of high doses of Arg induces selective pancreatic acinar cell damage without any morphological change to the islets of Langerhans[5]. STZ has been reported to be specifically toxic to the β-cells of the islets of Langerhans and to induce a dose-dependent and irreversible diabetes in rats without any morphological change to the exocrine pancreas[6]. These models seemed to be suitable for studying the correlation between diabetes mellitus and pancreatitis. The role of insulin in the process of spontaneous and CCK-8-promoted pancreatic regeneration following acute pancreatitis has not yet been characterised in detail. The interesting finding which showed that periinsular acini remained intact during Arg-induced-pancreatitis as well as a lack of the hypertrophic effect of CCK in diabetic rats prompted us to continue studies on the effects of insulin in the process of pancreatic remodelling. Our hypothesis stated that insulin was necessary for the regenerative effect of CCK-8, therefore, we evoked diabetes and pancreatitis in rats as described earlier[2] and then, in the diabetic rats insulin was administered by s.c. injections. The elevation of serum insulin level and the diminution of serum glucose level, clearly showed the efficacy of the insulin treatment. CCK-8 could only elevate pw/bw, the pancreatic contents of protein, amylase and lipase in the presence of insulin. Moreover, CCK-8 could also increase the number of acinar cells with mitotic activity when insulin was administered, but CCK-8 alone had no effect on the laboratory parameters or the mitotic activity in diabetic-pancreatitic rats. Lines of evidence demonstrate that both pancreatic secretory and growth processes are (at least partially) under the control of pancreatic islet hormones. Hypoinsulinemia was known to cause pancreatic atrophy and fat infiltration of the exocrine pancreas in guinea pigs[7]. In contrast to this, endogenous and exogenous insulin evoked an increase in pancreatic enzyme synthesis and growth[8]. These direct (via acinar insulin receptors) and indirect (influence on CCK receptors) effects of insulin are well understood. It was also demonstrated that insulin binding to its receptors on the pancreatic acini could be correlated with the subsequent stimulation of protein synthesis[9]. Another indirect observation was that anti-insulin serum completely blocked the CCK-8-stimulated pancreatic secretion in rats[10]. It further suggests that endogenous insulin is necessary for the stimulatory action of CCK-8 on pancreatic exocrine secretion and growth[10]. The present study proves our hypothesis that insulin is indeed necessary for the hypertrophic effect of CCK-8 following acute necrotizing experimental pancreatitis.

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• BRIEF REPORTS •

# Effects of terlipressin on systolic pulmonary artery pressure of patients with liver cirrhosis: An echocardiographic assessment

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## Abstract

**AIM:** Portopulmonary hypertension is a serious complication of chronic liver disease. Our aim was to search into the effect of terlipressin on systolic pulmonary artery pressure among cirrhotic patients.

**METHODS:** Twelve patients (6 males and 6 females) with liver cirrhosis were recruited in the study. Arterial blood gas samples were obtained in sitting position at rest. Contrast enhanced echocardiography and measurements of systolic pulmonary artery pressure were performed before and after the intravenous injection of 2 mg terlipressin.

**RESULTS:** Of 12 patients studied, the contrast enhanced echocardiography was positive in 5, and the positive findings in contrast enhanced echocardiography were reversed to normal in two after terlipressin injection. The mean systolic pulmonary artery pressure was 25.5±3.6 mmHg before terlipressin injection, and was 22.5±2.5 mmHg after terlipressin ($P$=0.003). The systolic pulmonary artery pressure was above 25 mmHg in seven of these 12 patients. After the terlipressin injection, systolic pulmonary artery pressure was <25 mmHg in four of these cases (58.3% *vs* 25%, $P$=0.04).

**CONCLUSION:** Terlipressin can decrease the systolic pulmonary artery pressure in patients with liver cirrhosis.

Altintas E, Akkus N, Gen R, Helvaci MR, Sezgin O, Oguz D. Effects of terlipressin on systolic pulmonary artery pressure of patients with liver cirrhosis: An echocardiographic assessment. *World J Gastroenterol* 2004; 10(15): 2278-2280
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## INTRODUCTION

The spectrum of pulmonary vascular disorders in liver disease and portal hypertension ranges from hepatopulmonary syndrome characterized by intrapulmonary vascular dilatations (IPVD) to pulmonary hypertension (portopulmonary hypertension), in which pulmonary vascular resistance is elevated[1].

Hepatopulmonary syndrome (HPS) is incurable but resolves over time after liver transplantation[2]. In patients with cirrhosis, 1% pulmonary hypertension is developed[2,3]. Portopulmonary hypertension (PPH) is irreversible and there is no effective treatment[4]. Mortality of liver transplantation in patients with PPH ranges from 50% to 100%[4]. The common causes of HPS and PPH are portal hypertension and portosystemic shunting, indicating that vasoactive and angiogenetic factors originating from the liver can control pulmonary circulation[4].

So far, observational studies have examined several drugs[5-8]. No clearly effective medical therapies for PPH have been found. In the present study, we investigated the effects of terlipressin that was used in the treatment of variceal hemorrhage on systolic pulmonary artery pressure (SPAP) among cirrhotic patients.

## MATERIALS AND METHODS

### *Patients*

Twelve cirrhotic patients without any malignancy, heart failure, renal failure (serum creatinin >20 mg/L), chronic obstructive lung disease, pneumonia, and anemia (hemoglobin level <100 mg/L) were studied. All patients had portal hypertension. The presence of hepatic dysfunction or portal hypertension was assessed by the following: (1) clinical history of complications related to liver disease and portal hypertension (ascites, hepatic encephalopathy, esophagogastric varices, variceal bleeding and spontaneous bacterial peritonitis); (2) liver function tests (aspartate and alanine aminotransferase, alkaline phosphatase, total bilirubin, prothrombin time, and albumin); and (3) abdominal ultrasound evidences of cirrhosis and portal hypertension (small, nodular liver, hepatofugal portal venous flow, portosystemic collateral circulation, splenomegaly, and ascites). The severity of hepatic dysfunction was stratified according to the Pugh-Child's criteria.

### *Methods*

Chest X-ray and electrocardiogram were performed for all patients. Blood samples were obtained to calculate the score of Pugh-Child in the morning of study day. Arterial blood gas samples were obtained from a single radial artery puncture while the patient was breathing room air in sitting position at rest.

Transthoracic contrast-enhanced echocardiography (CEE) was performed to investigate the intrapulmonary vascular dilations. CEE was performed after the administration of 10 mL of hand-agitated normal saline solution in the supine position via an upper extremity peripheral vein. Positive results were qualitatively defined as any visual opacification of the left heart chamber more than three cardiac cycles after appearance of microbubbles in the right ventricle[9]. These findings suggested intrapulmonary passage of microbubbles through either dilated precapillary and capillary vessels or direct arteriovenous communications. Echocardiographic assessments were carried out with Wingmed system five 1.7 MHz electronic probe by an experienced cardiologist. Left lateral position was used during measurements. Left atrium, left ventricular diastolic and systolic dimensions were calculated with standard M-mode echocardiographic pictures in parasternal long axis view. Wall motions and wall thickness were also evaluated. Fractional

shortening was studied at level of chordae tendinae (left ventricular end diastolic dimension-left ventricular end systolic dimension/left ventricular end diastolic dimension ×100). Each parameter represented the mean value of three successive measurements. Maximum flow rates with continuous wave Doppler (CW) at level of tricuspid valves were used to calculate SPAP (modified Bernoulli equation: $\triangle p=4V^2$). SPAPs were computed by adding estimated right atrial pressure (5 mmHg) to pressure gradient calculated by modified Bernoulli equation.

CEE and measurements of SPAP were performed before and after the intravenous injection of 2 mg terlipressin (Glypressin ® 1 mg flacon, ERKIM Ilac AS, Istanbul, Turkey).

All statistical calculations were done using the SPSS 11.0 software. The measurements were given as the mean values. The paired sample test, Cochran's Q test, and Friedman's test were used to determine the difference of SPAP, the frequency of CEE, and the frequency of patients whose SPAP $\geq$ 25 mmHg respectively before and after terliprssin injection. $P< 0.05$ was accepted as statistically significant.

## RESULTS

The clinical and demographical characteristics of patients are shown in Table 1. A total of 12 patients, six males and six females were studied, with a mean age of 52 years. The mean score of Pugh-Child was 5.08, and the mean value of arterial $pO_2$ was 77.83 mmHg. Ascites was detected in two patients. Two patients suffered from dyspnea. In two patients, chest X ray films showed bilaterally reticolonodular densities at the basal areas.

Before the injection of terlipressin intravenously, a positive CEE was detected in 5 patients, and two of them were disappeared after the drug was injected. Three of the patients who had evident hypoxemia suffered dyspnea, and CEE was positive in these patients. After terlipressin injection, CEE was negative in two of the three patients. Arterial $pO_2$ was high in the rest two patients with CEE (+), after terlipressin injection CEE positivity was continued. There was not any difference before and after the drug injection according to the frequency of the positivity of CEE (5/12 *vs* 3/12, $P$=0.15 according to Cochran's Q test). This meaningless result might be due to the small number of patients. The SPAP value was $\geq$25 mmHg in four of five patients with positive CEE. Two patients with a negative CEE after the drug injection had the lowest SPAP value in this group, 24 mmHg and 27 mmHg respectively.

Although the value of SPAP was 25.5±3.6 mmHg before the drug injection, it was decreased to 22.5±2.5 mmHg after terlipressin injection ($P$=0.003). The value of SPAP was above the level of 25 mmHg as the limit of pulmonary hypertension in seven patients. After the injection of terlipressin, it continued to be higher above this level in three patients [7/12(58.3%) patients versus 3/12(25%) patients]. This difference was statistically significant ($P$=0.04). CEE was positive in one of the three patients whose SPAP was >25 mmHg after terlipressin injection. The positivity of CEE disappeared after terlipressin injection in one patient who had a SPAP value lower than 25 mmHg.

## DISCUSSION

The association of pulmonary hypertension with portal hypertension, also known as portopulmonary hypertension (PPH), is a complication of chronic liver disease that has been associated with high morbidity and mortality at the time of liver transplantation[2-4]. PPH has been defined as mean pulmonary artery pressure >25 mmHg in the presence of a normal pulmonary capillary wedge pressure and portal hypertension[2-4].

The presence of intrapulmonary vascular dilatations can be confirmed using one of the three imaging modalities: contrast-enhanced echocardiography, perfusion lung scan - technetium 99 m-labeled macroaggregated albumin scanning, and pulmonary arteriography[9,10]. A Doppler echocardiogram is a highly sensitive and noninvasive diagnostic modality for both measuring PAP and determining IPVD. Therefore, it should be considered as the first screening method of choice[11].

Heretofore, therapy for chronic management of PPH is lacking. Recently, continuous intravenous infusion of epoprostenol has been demonstrated to improve symptomatology and survival in the general population of patients with PPH[5]. Anecdotal reports suggested that long-term epoprostenol therapy given by continuous infusion might be effective for patients with portopulmonary hypertension, but the efficacy of epoprostenol has not been rigorously studied in this subgroup of patients[5-7]. Kuo *et al*.[5] reported the use of epoprostenol in the more specific instance of PPH. Over a period of 6-14 mo, epoprostenol (10-28 ng/kg.min) therapy was associated with a 29-46% decrease in mean pulmonary artery pressure, a 22-71% decrease in pulmonary vascular resistance, and a 25-75% increase in cardiac output in a group of four patients[5]. These results suggest that effective chronic therapy for PPH is available. In combination with inhaled nitric oxide as acute intraoperative therapy, epoprostenol infusion represented an additional therapeutic option for treatment of PPH in the liver transplant candidate[5].

**Table 1** Characteristics of patients. PBS: primary biliary cirrhosis, HBV: hepatitis B virus, HCV: hepatitis C virus, N: Normal, BB-Nod: bilateral basillary nodularity, (-): absent, (+): present, PAP: pulmonary artery pressure and, CEE: contrast enhanced echocardiography

| Sex | Age (yr) | Etiology | Pugh-Child Score | pO2 (mmHg) | Ascites | Dyspnea | Chest X-ray | PAP-0 (mmHg) | PAP-1 (mmHg) | CEE-0 | CEE-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 60 | PBS | 5 | 89 | (-) | (-) | N | 28 | 26 | (+) | (+) |
| F | 55 | HBV | 4 | 85 | (-) | (-) | N | 24 | 24 | (-) | (-) |
| M | 54 | Alcohol | 4 | 65 | (-) | (+) | BB-Nod | 27 | 21 | (+) | (-) |
| F | 48 | Cryptogenic | 5 | 60 | (-) | (+) | N | 24 | 20 | (+) | (-) |
| M | 58 | HCV | 4 | 63 | (-) | (+) | N | 28 | 24 | (+) | (+) |
| M | 56 | HBV | 4 | 73 | (-) | (-) | N | 25 | 25 | (-) | (-) |
| M | 52 | HBV | 5 | 88 | (-) | (-) | N | 30 | 22 | (+) | (+) |
| F | 50 | HBV | 10 | 90 | (+) | (+) | BB-Nod | 31 | 26 | (-) | (-) |
| M | 48 | HCV | 5 | 85 | (-) | (-) | N | 21 | 22 | (-) | (-) |
| M | 60 | HBV | 7 | 80 | (+) | (-) | N | 20 | 19 | (-) | (-) |
| F | 55 | HCV | 4 | 71 | (-) | (-) | N | 27 | 22 | (-) | (-) |
| F | 39 | HBV | 4 | 85 | (-) | (-) | N | 21 | 19 | (-) | (-) |

There are no long-term studies or guidelines on the use of pharmacotherapy in PPH. In view of the rarity of this disease, much of the traditional treatment of this disease has been empirical. Inhaled NO could decreased the pulmonary artery pressures in some patients with PPH and might have some promise for long-term treatment of this disease[12]. Other medications have been reported to cause amelioration of pulmonary hypertension in patients with portal hypertension including beta-blockers, nitrates, calcium-channel blockers, prostacyclin and prostacyclin analogs, phosphodiesterase inhibitors (sildenafil), L-arginine, and, endothelin antagonists[3]. In view of the decreased incidence of variceal bleeding in patients taking beta-blockers and nitrates, we encourage the use of these medications in patients with PPH. Beta-blocking agents, which are used in the treatment for portal hypertension may have deleterious effects on the setting of pulmonary hypertension because they decrease cardiac output and increase pulmonary vascular resistance. Vasodilators are usually ineffective and poorly tolerated because these patients usually have a decreased systemic vascular resistance. Most patients with porto-pulmonary hypertension do not receive anticoagulants because the risk of bleeding is deemed to be elevated, especially when esophageal varices are present.

In patients with hepatopulmonary syndrome, supplemental oxygen and liver transplantation were the usual treatments of choice[2]. Pharmacologic approaches were limited in improving hypoxemia[2]. Outcome following liver transplantation was variable, increased cardiopulmonary mortality occurred in patients with moderate to severe pulmonary hypertension. Although a few reports have demonstrated improvement of pulmonary hypertension after liver transplantation, this procedure was a very risky one in patients with markedly increased pulmonary artery pressures[13]. Report about combined liver-lung transplantation might open a perspective for selected patients with porto-pulmonary hypertension[14].

Terlipressin is a long-acting vasopressin analogue that has been proved useful in the treatment of variceal hemorrhage. Terlipressin could reduce portal pressure in cirrhotic patients mainly through intense splanchnic vasoconstriction that decreases portal venous inflow[15]. Hepatic blood flow might also be reduced by terlipressin[15]. The systemic haemodynamic response to terlipressin was moreover associated with the decrease in portal pressure[16]. After administration of terlipressin, the azygos blood flow decreased significantly[17]. In patients with cirrhosis, a single injection of 2 mg terlipressin significantly and markedly reduced portal pressure and azygos blood flow for up to 4 h[17]. The azygos blood flow (superior porto-systemic collateral circulation) correlated strongly with portal venous flow in patients with portal hypertension[17]. It could be expected that terlipressin could reverse the vasodilatation of dilated intrapulmonary arteries with HPS and PPH. It can also decrease SPAP by reducing the increased blood flow that may facilitate pulmonary arterial hypertension. In our study, it reduced SPAP from 25.5±3.6 mmHg to 22.5±2.5 mmHg and, this result was statistically meaningful. CEE that showed intrapulmonary vascular dilatation was positive in 5 patients, and it was reversed to normal in 2 patients after terlipressin injection. However, more studies are needed to decide whether this result is meaningful or not.

Chronic terlipressin therapy in combination with a multidisciplinary, well-planned evaluation and treatment plan, may be the answer to a heretofore untreatable disease. This one is a prestudy, because more and detailed studies are required to show its efficiency. In the context of persisting uncertainty about the cause and treatment of PPH, future studies must focus on the pathophysiology of PPH, predicting reversibility after liver transplantation, and identifying other treatment options.

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• BRIEF REPORTS •

# Empirical antibiotic treatment with piperacillin-tazobactam in patients with microbiologically-documented biliary tract infections

Gabrio Bassotti, Fabio Chistolini, Francis Sietchiping-Nzepa, Giuseppe de Roberto, Antonio Morelli

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## Abstract

**AIM:** To report our experience with empiric antimicrobial monotherapy (piperacillin/tazobactam, of which no data are available in such specific circumstances) in microbiologically-documented infections in patients with benign and malignant conditions of the biliary tract.

**METHODS:** Twenty-three patients, 10 with benign and 13 with malignant conditions affecting the biliary tree and microbiologically-documented infections were recruited and the efficacy of empirical antibiotic therapy was assessed.

**RESULTS:** The two groups featured similar demographic and clinical data. Overall, the infective episodes were most due to Gram negative agents, more than 60% of such episodes (mostly in malignant conditions) were preceded by invasive instrumental maneuvers. Empirical antibiotic therapy with a single agent (piperacillin/tazobactam) was effective in more than 80% of cases. No deaths were reported following infections.

**CONCLUSION:** An empiric therapeutic approach with piperacillin/tazobactam is highly effective in biliary tract infections due to benign or malignant conditions.

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## INTRODUCTION

The common causes of intra-abdominal infections are those related to the biliary tract[1]. However, to obtain a microbiological diagnosis in biliary tract infections (BTI) is not easy, due to the difficulty of sampling bile and the low incidence of positive blood cultures. Therefore, antimicrobial therapy is often empirical[2], and the choice of an appropriate regimen depends on the knowledge of the most common causative bacteria and the reported efficacy of antimicrobial drugs in BTI. Moreover, the paucity of randomized clinical trials for BTI treatment probably justifies the fact that there is no standardized approach to these infections[2].

The present study was to report our experience with empirical single antibiotic treatment with piperacillin-tazobactam of BTI in patients with benign and malignant diseases of the biliary tract, since there are no specific data on this compound in the treatment of such infections.

## MATERIALS AND METHODS

Twenty-three consecutive patients (15 men, 8 women, age range 22-88 years) with microbiologically documented BTI entered the study. Underlying disease and causative organisms were assessed. Empirical treatment (4.5 g t.i.d) was started immediately after obtaining samples (blood and/or bile) for microbiological cultures, and concordance with antibiogram and its efficacy were also evaluated. The treatment was judged effective when fever and clinical symptoms of infection resolved within 72 h, whereas the persistence of fever beyond 72 h from the start of treatment, the deterioration of clinical conditions or the death as a result of the primary infection was considered as failure.

## RESULTS

Overall, records were obtained from 10 patients with benign and 13 patients with malignant conditions affecting the biliary tree. Table 1 shows the clinical characteristics of the two groups. In more than 60% of patients, BTI were preceded by an invasive procedure on the biliary tree, and this was less frequent in benign than in malignant conditions (50% *vs* 77%).

**Table 1** Demographic and clinical variables of 23 patients with BTI

| | Benign conditions | Malignant conditions |
|---|---|---|
| No (%) | 10 / 23(43.5) | 13 / 23(56.5) |
| Average duration of treatment (d) | 7±1 | 10±1 |
| Underlying condition (*No.*) | Choledocholitiasis (7) | Cholangiocarcinoma (6) |
| | Acute cholecystitis (2) | Pancreatic carcinoma (4) |
| | Iatrogenic stenosis (1) | Gallbladder carcinoma (2) |
| | | Infiltrating hepatoma (1) |
| Previous instrumental invasive maneuvers (*No*) | None (5) | None (3) |
| | PTD (3) | PTD (10) |
| | ERCP (2) | |

Abbreviations: BTI=biliary tract infections; ERCP=endoscopic retrograde cholangio-pancreatography; PTD=percutaneous transhepatic drainage.

**Table 2** Microbiological variables in 23 patients with BTI

| | Benign conditions (10 patients) | Malignant conditions (13 patients) |
|---|---|---|
| Insulation medium (No.) | Blood (7) | Blood (6) |
| | Bile (3) | Bile (7) |
| Polymicrobial infections | 1 (4%) | 7 (30%) |
| Isolated pathogens (No. cases) | *E.coli* (4) | Enterococcus spp (6) |
| | Enterococcus spp (3) | Staphylococcus spp (6) |
| | Pseudomonas spp (3) | Candida spp (5) |
| | Enterobacter spp (2) | *E.coli* (2) |
| | Streptococcus spp (2) | Pseudomonas spp (2) |
| | Klebsiella spp (1) | Proteus spp (1) |
| | Candida spp (1) | Enterobacter spp (1) |
| | | Salmonella spp (1) |

Table 2 shows the microbiological characteristics of the pathogens isolated in both groups. As expected, most infections were caused by Gram negative agents, and 30% of them (almost exclusively found in malignant conditions) were polymicrobial. *Candida spp* were always isolated from bile in polymicrobial infections. In 19(82.6%) patients there was no need of modifying the empiric therapeutic schedule, whereas in the remaining 4 the antibiotic regimen was modified according to the antibiogram showing resistance or insensitivity to piperacillin/tazobactam. In all patients with BTI due to benign conditions, decrease of fever and improvement of clinical conditions were observed within 3-18 h. A slower trend was observed in patients with BTI due to malignant conditions (improvement within 8-24 h), probably due to more polymicrobial infections and resistances to the empiric regimen. After the results from the antibiogram were obtained, these latter patients treated with more targeted antibiotic regimens, had the disappearance of fever and improvement of the clinical conditions. No deaths were reported attributable to BTI.

## DISCUSSION

In this study, we reported our experience with an empiric antibiotic regimen in BTI, and showed that a monotherapy with piperacillin/tazobactam (that, to the best of our knowledge has still not been assessed in such circumstances) might be effective in more than 80% of cases. The organisms more commonly cultured in our patients, in both benign and malignant conditions, were Gram negative bacteria, the pathogens were more frequently associated with obstructive conditions of the biliary tree[3,4]. Several infective episodes followed invasive instrumental procedures, especially percutaneous drainage (that also gave a discrete yield for bile culture, as previously shown for this procedure[5]), and were mostly represented by polymicrobial infections.

A preferred therapeutic schedule for BTI, until recently, was usually a combination of a penicillin (usually ampicillin) and an aminoglycoside[6-9]. This combination had limited anaerobic coverage, frequent resistance (to ampicillin) of Gram negative bacteria, and the risks of renal damage (aminoglycoside, significantly increased in patients with cholestasis)[10]. However, other antibiotics (such as the ureidopenicillins) exhibited a broad spectrum of activity, that included many anaerobes, *enterococci* and *P.aeruginosa*, in addition to Gram negative bacilli[11], so that they may result in appealing for use as single agents. Actually, it has been shown that monotherapy with a ureidopenicillin (mezlocillin, piperacillin) is equally or more effective than the traditional approach with ampicillin/aminoglycoside for treatment of BTI[12-14], although in patients undergoing nonsurgical invasive procedure of the biliary tree and/or with suspected increased risk of *P.aeruginosa* the association of ureidopenicillin/aminoglycoside has been still justifiable[15,16]. On the other hand, the combination of piperacillin with the beta-lactamase inhibitor tazobactam (that displays a substantial elimination in bile[17,18]) might be a reasonable alternative when the local resistance pattern featured a high incidence of ureidopenicillin-resistant *E.coli* or *Klebsiella spp*[2,19], as also shown by its effectiveness as single empiric agent in high-risk, febrile neutropenic patients with cancer[20].

Experience with quinolones for treatment of BTI was still limited[2,21]. However, there is good evidence that monotherapy with these compounds might be as effective as combination therapy for treatment of BTI[22-24].

To date, the combination of piperacillin/tazobactam has been demonstrated clinically- and cost-effective in both uncomplicated and complicated intraabdominal infections[25-27], although no specific data on BTI are available. Therefore, we feel that our experience might be a useful adjunct to the therapeutic armamentarium.

In conclusion, empiric antibiotic treatment with piperacillin/tazobactam is frequently effective in BTI due to benign and malignant conditions. Of course, in such circumstances an early operative drainage of the biliary tree is always mandatory, regardless of the presence or absence of suppuration in the common bile duct[28], to prevent relapses and septic complications.

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• BRIEF REPORTS •

# Determination of glycated hemoglobin in patients with advanced liver disease

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## Abstract

**AIM:** To evaluate the glycated hemoglobin ($HbA_{1c}$) determination methods and to determine fructosamine in patients with chronic hepatitis, compensated cirrhosis and in patients with chronic hepatitis treated with ribavirin.

**METHODS:** $HbA_{1c}$ values were determined in 15 patients with compensated liver cirrhosis and in 20 patients with chronic hepatitis using the ion-exchange high performance liquid chromatography and the immunoassay methods. Fructosamine was determined using nitroblue tetrazolium.

**RESULTS:** Forty percent of patients with liver cirrhosis had $HbA_{1c}$ results below the non-diabetic reference range by at least one $HbA_{1c}$ method, while fructosamine results were either within the reference range or elevated. Twenty percent of patients with chronic hepatitis (hepatic fibrosis) had $HbA_{1c}$ results below the non-diabetic reference range by at least one $HbA_{1c}$ method. In patients with chronic hepatitis treated with ribavirin, 50% of $HbA_{1c}$ results were below the non-diabetic reference using at least one of the $HbA_{1c}$ methods.

**CONCLUSION:** Only evaluated in context with all liver function parameters as well as a red blood count including reticulocytes, $HbA_{1c}$ results should be used in patients with advanced liver disease. $HbA_{1c}$ and fructosamine measurements should be used with caution when evaluating long-term glucose control in patients with hepatic cirrhosis or in patients with chronic hepatitis and ribavirin treatment.

Lahousen T, Hegenbarth K, Ille R, Lipp RW, Krause R, Little RR, Schnedl WJ. Determination of glycated hemoglobin in patients with advanced liver disease. *World J Gastroenterol* 2004; 10(15): 2284-2286
http://www.wjgnet.com/1007-9327/10/2284.asp

## INTRODUCTION

Measurement of glycated hemoglobin ($HbA_{1c}$) is used for routine evaluation and management of patients with diabetes mellitus. Concentrations of $HbA_{1c}$ provide a means of assessing long-term glycemic status and correlate well with development of complications related to diabetes mellitus[1,2]. The liver plays a major role in regulating glucose metabolism because it is the main source of endogenous glucose and a major site involved in insulin metabolism. Because liver disease is associated with an increased prevalence of impaired glucose tolerance and diabetes mellitus, there is a need for tools to measure its long-term glycemic control[3]. Previous studies indicated that both $HbA_{1c}$ and fructosamine measurement should not be used in patients with liver cirrhosis, although the reason for this was unclear[4-6]. Shortened erythrocyte life span as in hemolytic anemia is known to cause clinically and analytically low $HbA_{1c}$ values independent of glycemia[7], but measurement of fructosamine, which has been used to document glycemic status over a period of 2-4 wk, should not be affected by erythrocyte life span. This study described the determination of $HbA_{1c}$ and fructosamine as well as parameters of liver disease and anemia in patients with advanced liver disease.

## MATERIALS AND METHODS

Blood samples were collected, with and without EDTA, from 15 consecutive patients with compensated liver cirrhosis and 20 patients with chronic hepatitis and fibrosis of the liver. Diagnostic liver biopsies were performed routinely in all patients during the course of treatment in the Division of Gastroenterology and Hepatology, Department of Internal Medicine, Medical University in Graz. Liver cirrhosis was histologically defined as a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules[8,9]. Of 15 patients with compensated liver cirrhosis Child-Pugh class A (total bilirubin <2 mg/dL, serum albumin >3.5 g/dL, prothrombine time 1-4 s prolonged, no hepatic encephalopathy and no ascites), 6 were tested positive for hepatitis C, 8 had alcoholic liver disease and 1 had primary biliary cirrhosis. Of the 20 patients with chronic hepatitis and fibrosis, 19 were tested positive for hepatitis C and 1 suffered from alcoholic liver disease. Ten of these patients with chronic hepatitis C were treated with interferon-α plus the antiviral drug ribavirin that can cause reversible hemolytic anemia[10]. None of the patients included in the study had a history of impaired glucose tolerance or diabetes mellitus.

$HbA_{1c}$ was measured within 3 d of collection using the Hi-Auto $A_{1c}$ HA-8140 HPLC (Menarini Diagnostics, Florence, Italy), the DCA 2000 immunoassay method (Bayer, Vienna, Austria) and the Roche Cobas Integra immunoassay method (Roche, Vienna, Austria). Each of these $HbA_{1c}$ methods was certified by the National Glycohemoglobin Standardization Program (NGSP)[11]. Routine hematological data were determined with a Coulter counter (Beckman, Vienna, Austria). Blood glucose was determined with a hexokinase/glucose-6-phosphate dehydrogenase colorimetric method (Gluco-Quant; Roche, Vienna, Austria) and used as mean of 4-6 measurements on separate days during the preceding 1 mo. The relationship of blood glucose and $HbA_{1c}$ was calculated according to MBG (mmol/L)=(1.98·$HbA_{1c}$) -4.29[12]. Fructosamine was determined with a colorimetric test that uses nitroblue tetrazolium in alkaline solution (Unimate FRA; Roche, Vienna, Austria). Reference

ranges were provided by each manufacturer and in most cases represented the mean±2SD of a population without known diabetes. All determinations were analyzed blindly and the procedures were in accordance with the declaration of Helsinki and the local ethics committee recommendations.

## RESULTS

Forty percent (6/15) of the patients with liver cirrhosis had $HbA_{1c}$ levels below the non-diabetic reference range with at least one $HbA_{1c}$ method, while fructosamine concentrations were either within the reference range ($n$=10) or elevated ($n$=5) (Figure 1). Twenty percent (2/10) of the patients with chronic hepatitis had $HbA_{1c}$ levels below the non-diabetic reference range with at least one $HbA_{1c}$ method. Fructosamine concentrations of all the 10 patients with chronic hepatitis were below the non-diabetic reference range. In patients with chronic hepatitis treated with ribavirin, 50%(5/10) of $HbA_{1c}$ levels were below the non-diabetic reference range detected by at least one of the HbA1c methods (Figure 1). One patient in this group demonstrated a fructosamine concentration within the diabetic range.

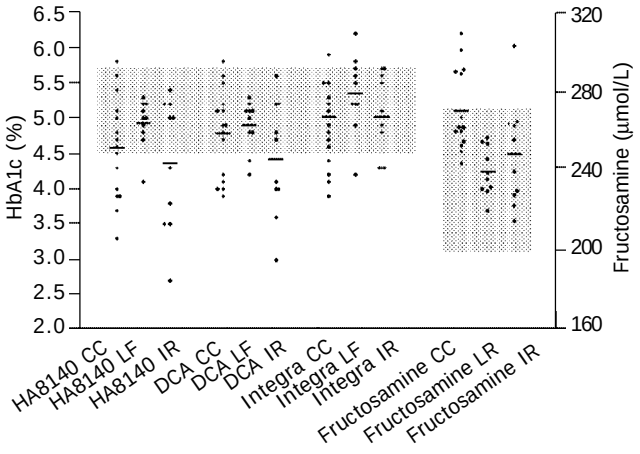


**Figure 1** $HbA_{1c}$ level and fructosamine concentration in patients with liver disease. CC: Compensated cirrhosis; LF: Chronic hepatitis (liver fibrosis); IR: Chronic hepatitis with interferon and ribavirin treatment. Shaded areas represent the mean±2SD reference range for each test ($HbA_{1c}$: 4.5-5.7%; fructosamine: 200-272 µmol/L).

Table 1 shows the percentage of patients in each group (cirrhosis, chronic hepatitis, chronic hepatitis with interferon and ribavirin treatment) that the levels of erythrocyte, hematocrit and hemoglobin were below the normal range, and reticulocyte counts above the normal range. Although 30-53% of the patients with cirrhosis and chronic hepatitis demonstrated moderate anemia, none had a reticulocyte count within normal. All of those with low $HbA_{1c}$ also demonstrated anemia but some patients with anemia did not have low $HbA_{1c}$. Seventy to eighty percent of the patients with chronic hepatitis treated with ribavirin demonstrated moderate anemia and 30% also had high reticulocyte counts (Table 1). All of those with high reticulocyte counts, as well as some of those with anemia and normal reticulocyte counts, had below-normal $HbA_{1c}$. This study showed elevated reticulocytes, which might be a sign of shortened erythrocyte life span, in only 3 patients with chronic hepatitis and ribavirin treatment. In these patients $HbA_{1c}$ was below the non-diabetic reference range on all methods. We also found $HbA_{1c}$ values below the non-diabetic reference range in up to 40% of the patients with liver cirrhosis and in 50% of the patients with chronic hepatitis treated with ribavirin as measured by at least one of the $HbA_{1c}$ methods. In these groups of patients the $HbA_{1c}$ levels were negatively correlated to the percentage of reticulocytes (Pearson correlation, $r$=-0.55 to -0.79 depending on method, $P$<0,05 for all methods). There was no significant relationship between $HbA_{1c}$ and reticulocyte count in the patients with chronic hepatitis and no ribavirin therapy.

We performed an one-sample $t$-test comparing mean blood glucose calculated of $HbA_{1c}$ results (MBG (mmol/L) = (1.98·$HbA_{1c}$)-4.29) and measured blood glucose as the actual value. $HbA_{1c}$ results of the HPLC Menarini HA-8140 and the immunoassay method DCA 2000 were used to calculate a desired blood glucose value because in Pearson correlation they did not correlate with blood glucose. In patients with chronic hepatitis treated with ribavirin the one sample $t$-test for measured blood glucose and calculated blood glucose resulted in a significant difference ($t_{9\,Menarini}$=7.68, $P$<0.05; $t_{9\,DCA}$=6.67, $P$<0.05). In patients with liver cirrhosis calculated blood glucose was up to 1 mmol/L lower than measured blood glucose but a high standard deviation (Table 2) caused no statistical difference.

No correlation was found for all 3 groups between $HbA_{1c}$ results and hepatic serum parameters as -glutamyl transferase (GGT), glutamate-oxalate transaminase (GOT) and glutamyl-pyruvic transaminase (GPT). In all 3 patient groups total protein measured in serum was within normal and albumin was normal in all patients with chronic hepatitis. Three patients with compensated cirrhosis had serum albumin below normal. There was no correlation found in all 3 patient groups between

**Table 1** Percentage of patients outside the reference range for parameters of anemia

| Group | Patients below normal (%) (Reference range) | | | Patients above normal (%) |
|---|---|---|---|---|
| | Erythrocytes (4.5-5.9 T/L) | Hct (40-50%) | Hb (13-17 g/dL) | Reticulocytes (5-20%) |
| Cirrhosis ($n$=15) | 46 | 53 | 40 | 0 |
| Chronic hepatitis ($n$=10) | 30 | 30 | 30 | 0 |
| Chronic hepatitis /ribavirin ($n$=10) | 80 | 80 | 70 | 30 |

Hct: Hematocrit; Hb: Hemoglobin.

**Table 2** Values of measured blood glucose (mean±SD) and calculated mean blood glucose values [MBG (mmol/L)=(1.98·$HbA_{1c}$)-4.29]

| | Measured MBG (mmol/L) | Menarini HA-8140 Calculated MBG (mmol/L) | DCA 2000 Bayer Calculated MBG (mmol/L) |
|---|---|---|---|
| Cirrhosis ($n$=15) | 5.8±1.9 | 4.8 | 5.1 |
| Chronic hepatitis ($n$=10) | 5.1±0.3 | 5.4 | 5.4 |
| Chronic hepatitis /Ribavirin ($n$=10) | 5.2±0.4 | 4.3 | 4.5 |

MBG: Mean blood glucose.

fructosamine results and total protein or albumin. In patients with hepatic cirrhosis, mean fructosamine was within the high non-diabetic reference range. In patients with chronic hepatitis, fructosamine was close to the middle of the non-diabetic reference range. Five patients with cirrhosis and one patient with chronic hepatitis treated with ribavirin had high fructosamine levels even though they had normal blood glucose values.

## DISCUSSION

The liver plays a major role in regulating glucose metabolism because it is the main source of endogenous glucose and a major site involved in insulin metabolism. The most common pathogenic agents in liver disease are alcohol abuse and infectious hepatitis that may cause disturbed erythropoiesis and decreased red cell survival. Macrocytic anemia is a common feature in liver disease but is still incompletely understood[13]. The antiviral drug ribavirin has been widely used in combination with interferon in the treatment of chronic hepatitis C and a major side effect of ribavirin is a reversible hemolytic anemia[10].

Glycated hemoglobin (GHb) measured as $HbA_{1c}$ in diabetic patients, is used for evaluating long-term control of diabetes mellitus. GHb is the result of irreversible non-enzymatic glycation at one or both $NH_2$-terminal valines of the hemoglobin's -chain. The extent of glycation and the relative involvement of the hemoglobin's -and -chains still remain unclear. Depending on the determination method used the concentration of $HbA_{1c}$ is approximately 4-6% in healthy non-diabetic patients. Glycated hemoglobin most accurately reflects the previous 2-3 mo of glycemic control. Diabetic patients could present with abnormal liver chemistries, representing findings from benign hepatic steatosis to severe cirrhosis of the liver. Some medications to treat diabetes mellitus have an effect on liver metabolism or could even cause hepatotoxic reactions. Liver cirrhosis promotes glucose intolerance and diabetes through various mechanisms including insulin resistance and impaired insulin secretion. Sixty to 80% of patients with liver disease have glucose intolerance and 10-15% eventually develop overt diabetes.

In this study we demonstrated $HbA_{1c}$ values below the non-diabetic reference range in up to 40% of the patients with liver cirrhosis while fructosamine results were either within the reference range or elevated in the diabetic range. However, protein metabolism was normal in our patients and although fructosamine results depend on glycation of serum proteins the results might be altered by reduced hepatic protein synthesis due to impairment of liver function. In 50% of the patients with chronic hepatitis treated with ribavirin, $HbA_{1c}$ values were below the non-diabetic reference range as measured by at least one of the $HbA_{1c}$ methods. In these groups of patients the $HbA_{1c}$ results were negatively correlated to the percentage of reticulocytes that might be caused by disturbed erythropoiesis and decreased red cell survival. In patients with liver cirrhosis and chronic hepatitis treated with ribavirin, the $HbA_{1c}$ calculated value of mean blood glucose was up to 1 mmol/L (18 mg/dL) lower than measured mean blood glucose. This underlines that impairment of liver function has influence on results of $HbA_{1c}$ determination. Fructosamine may be a more reasonable marker for long term glucose control in patients with liver disease, but based on our findings we recommend frequent blood glucose monitoring as a measure for glucose control in patients with advanced liver disease.

We conclude that only evaluated in context with all liver function parameters as well as a red blood count including reticulocytes, $HbA_{1c}$ should be used in patients with liver disease. Although the pathophysiologic reasons have still not been confirmed, our data demonstrate that $HbA_{1c}$ and fructosamine measurements should be used with caution when evaluating long-term glucose control in patients with hepatic cirrhosis or in patients with chronic hepatitis with ribavirin treatment. This interference may be due to alterations in erythrocyte lifespan and altered protein metabolism, but further investigations are needed to elucidate the exact cause of the interference in patients with liver disease.

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• BRIEF REPORTS •

# Expression and localization of c-Fos and NOS in the central nerve system following esophageal acid stimulation in rats

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## Abstract

**AIM:** To determine the distribution of neurons expressing c-Fos and nitric oxide synthase (NOS) in the central nerve system (CNS) following esophageal acid exposure, and to investigate the relationship between c-Fos and NOS.

**METHODS:** Twelve Wistar rats were randomly divided into two equal groups. Hydrochloric acid with pepsin was perfused in the lower part of the esophagus for 60 min. As a control, normal saline was used. Thirty minutes after the perfusion, the rats were killed and brains were removed and processed for c-Fos immunohistochemistry and NADPH-d histochemistry. Blood pressure (BP), heart rate (HR), and respiratory rate (RR) during the experimental procedures were recorded every 10 min.

**RESULTS:** There were no significant differences in BP, HR and RR between the two groups. c-Fos immunoreactivity was significantly increased in rats receiving acid plus pepsin perfusion in amygdala (AM), paraventricular nucleus (PVN), parabrachial nucleus (PBN), nucleus tractus solitarius and dorsal motor nucleus of vagus (NTS/DMV), nucleus ambiguous (NA), reticular nucleus of medulla (RNM) and area postrema (AP). NOS reactivity in this group was significantly increased in PVN, PBN, NTS/DMV, RNM and AP. c-Fos and NOS had significant correlation between PVN, PBN, NTS/DMV, RNM and AP.

**CONCLUSION:** Acid plus pepsin perfusion of the esophagus results in neural activation in areas of CNS, and NO is likely one of the neurotransmitters in some of these areas.

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## INTRODUCTION

Reflux esophagitis (RE) is a common gastrointestinal motility disorder. Esophageal reflux occurs when gastric contents move in a retrograde direction into the esophagus, and esophagitis develops by prolonged exposure to gastric contents. This happens when the lower esophageal sphincter fails to provide an adequate mechanical barrier, when the esophageal peristaltic contractions fail to provide adequate clearance of the gastric contents, and/or when gastric contents exist for a prolonged time due to gastroparesis[1]. Esophageal motility is controlled by a variety of factors of which the nerve system is the most important one. Locally, motility disorder caused by esophagitis is usually due to the decreased release of acetylcholine[2,3], signal transduction failure[4], and/or decreased intracellular $Ca^{2+}$[5]. In CNS, little has been known about the distribution of activated neurons after esophageal acid exposure[6].

It is reported that *c-fos* is the most well characterized IEGs (immediate early genes) in neurons; the *c-fos* message is induced within minutes of stimuli and the protein is expressed within 1-3 h[7-10]. The expression of *c-fos* in CNS is considered to be a marker of neuronal activity following an appropriate stimulus, and the site of central expression of c-Fos in response to a stimulus is used as a means of elucidating the course of the response[11-16]. Nitric oxide (NO) acts as an intercellular messenger in CNS. As a highly diffusible and short-lived gas, NO is always studied by means of nitric oxide synthase (NOS)[17]. Studies have shown that NOS-containing neurons are identical to those selectively stained for NADPH diaphorase[18]. The present study was designed to determine the distribution of neurons expressing c-Fos and NOS in CNS following esophageal acid exposure, and to investigate the relationship between c-Fos and NOS.

## MATERIALS AND METHODS

### Animals

Twelve male Wistar rats weighing 220-260 g were housed in standard home cages under conditions of controlled illumination (12:12 h light/dark cycle), humidity, and temperature (18-26 ℃) for at least 7 d prior to the experimental procedure. They were fed a standard rat diet and tap water. The animals were deprived of food but not water 12-16 h before each experiment. They were randomly divided into two equal groups. All procedures were approved by the Committee for Animal Care and Usage for Research and Education of the Peking University.

### Methods

Rats were anaesthetized with an intraperitoneal injection of urethane (1.0 g/kg). After a rat reached a complete state of anesthesia, the abdominal wall and gastric wall were incised, and a drainage cannula was inserted in the gastric cardia to collect run-off solution from the esophagus. The anesthetized rat, strapped supine to an animal board, was then positioned with its head elevated at a slight angle (20-30°). A single lumen clear vinyl tube (ID 0.05 mm, *A* 0.8 mm) was passed by mouth into the esophagus. The tip of the cannula was located 3 cm above the esophagogastric junction. The cannula was then positioned and connected to a continuous perfusion pump (Medical Equipment Ltd. Zhejiang University, Hangzhou, China). A solution containing hydrochloric acid (HCl 0.1 mol/L) and pepsin (2 000-4 000 U/mL) (pH 1.5) was perfused continuously at a rate of 10 mL/h for 60 min. As a control, normal saline was used. Blood pressure (BP), heart rate (HR) and respiratory rate

(RR) during the experimental procedures were recorded every 10 min. After perfusion, the rat was left undisturbed for another 30 min before being deeply anesthetized with urethane (1.5 g/kg i.p.). The animal then was transcardially perfused with 9 g/L saline followed by 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH 7.3). The brain was removed and postfixed in the same fixative overnight and cryoprotected by immersion in 200 g/L sucrose for 72 h. Coronal sections (40 μm) of the brain were cut in a cryostat. Every fourth section was used to reveal c-Fos immunoreactivity and NADPH-diaphorase (NADPH-d) staining, and the second set of sections was used as a control for the immunohistochemical reaction.

The sections were collected and rinsed in 0.01 mol/L PBS containing 3 g/L Triton X-100 (PBST). Then they were incubated at 37 ℃ for 2 h in a solution containing 1 mmol/L NADPH (Biomol, London, UK), 0.5 mmol/L nitroblue tetrazolium (Biomol), Tris-HCl 50 mmol/L, and Triton X-100 2 g/L. After a rinse in PBST, sections were placed into a 50 g/L goat serum for 30 min at room temperature (RT), and incubated overnight at RT in primary antibody c-Fos (1:200, Santa Cruz Biotechnology, California, USA). After washing for 15 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG (Zymed, South San Francisco, Canada) diluted 1:300 in PBST at RT for 2 h, and then incubated in peroxidase-conjugated streptavidin (1:300 dilution, Zymed) for 2 h at RT. The immunoreactivity was visualized by incubating with 0.05 mol/L Tris-HCl buffer containing 0.1 g/L 3,3'-diaminobenzidine, and 0.3 mL/L $H_2O_2$ for 10-20 min at RT. The stained sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

### *Statistical analysis*

BP, HR and RR recorded every 10 min during the 90-min experimental procedures were averaged per animal and then per experimental group, respectively. The distribution of c-Fos and NADPH-d positive cells was detected under a microscope (Olympus, Tokyo, Japan), and the cells were counted on LEICA Q550CW system (Leica Microsystems Imaging Solutions Ltd, Wetzlar, Germany). The numbers of cells containing c-Fos immunoreactivity and NADPH-d were counted unilaterally in specific nuclei in several sections; 5 sections for amygdala (AM), nucleus tractus solitarius and dorsal motor nucleus of vagus (NTS/DMV), nucleus ambiguous (NA) and reticular nucleus of medulla (RNM) and 4 sections for paraventricular nucleus (PVN), supraoptic nucleus (SON), parabrachial nucleus (PBN) and area postrema (AP). The average number of c-Fos or NADPH-d positive neurons per section for each rat was calculated, respectively, by dividing the total number of c-Fos or NADPH-d positive cells obtained from all sections by the number of sections taken for each brain nucleus. Data were expressed as mean±SD of the respective brain areas. Statistical analyses were performed by SPSS 12.0 using the *t*-test, and a *P* value of less than 0.05 was considered statistically significant. The relationship between c-Fos and NADPH-d positive cells was performed by the correlation analysis.

## RESULTS

### *BP, HR and RR to acid-pepsin perfusion*

Esophageal acid perfusion did not change BP (18.03±1.07 *vs* 17.26±0.62 kPa, $F$=2.663, $P$=0.134), HR (275.30±14.43 *vs* 265.00±22.12 beats/min, $F$=1.343, $P$=0.273), and RR (92.00±10.41 *vs* 94.56±9.46 breathes/min, $F$=0.078, $P$=0.785) compared with control group.

### *c-Fos and NADPH-d staining in CNS*

The c-Fos positive cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB. In both groups of rats, c-Fos expression was observed in several brain regions. In telencephalon and diencephalon, c-Fos positive cells were mainly located in AM (Figure 1A, B), PVN (Figure 1C, D), SON and the numbers of the former two areas increased significantly in the acid-pepsin perfusion group (Table 1). Esophageal exposure to acid and pepsin also stimulated a significantly greater number of c-Fos-labeled neurons in areas of brain stem including PBN, NTS/DMV, NA (Figure 2A, D), RNM and AP (Table 1). NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites and axons. Acid-pepsin perfusion significantly increased the numbers of NADPH-d stained cells in PVN (Figure 1C, D), PBN, NTS/DMV, RNM and AP (Table 1). There were some coexistence of Fos

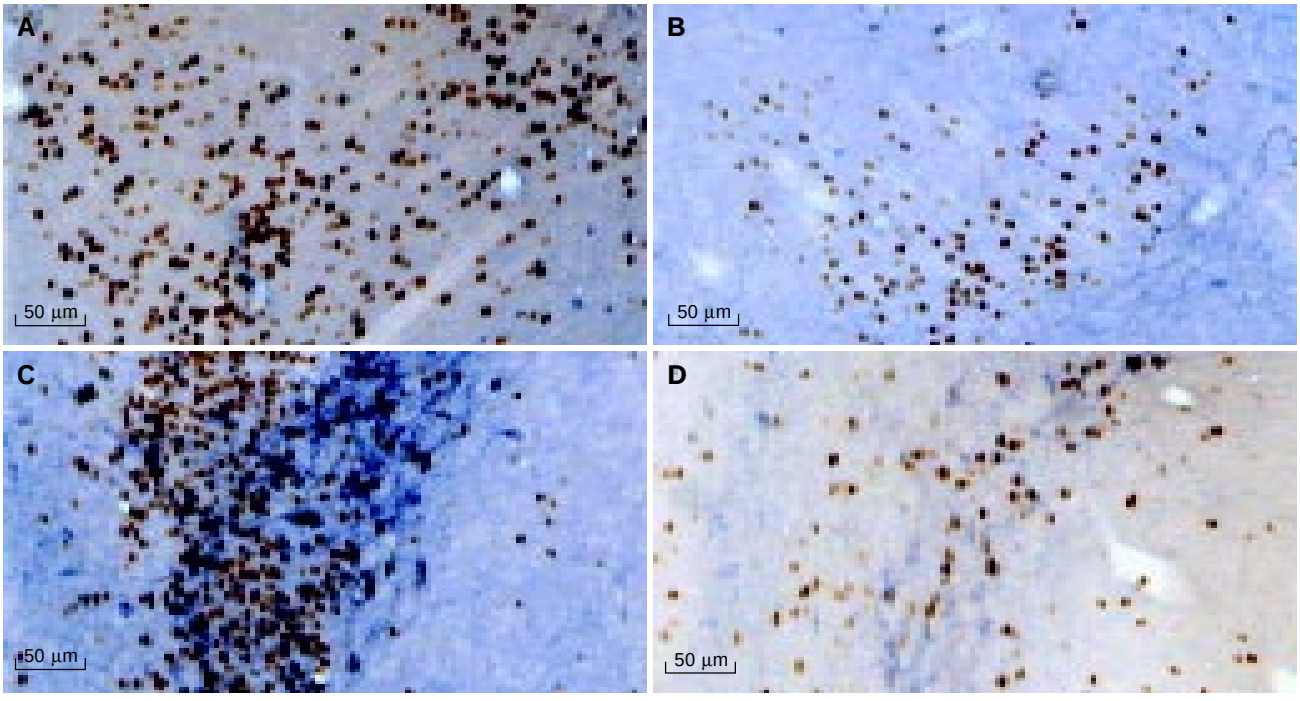


**Figure 1** Photomicrographs showing c-Fos and NOS positive neurons in amygdale (A and B), paraventricular nucleus (C and D). A and C were taken from rats with acid-pepsin perfusion, while B and D were taken from rats with saline perfusion. (3V: the third ventricle).

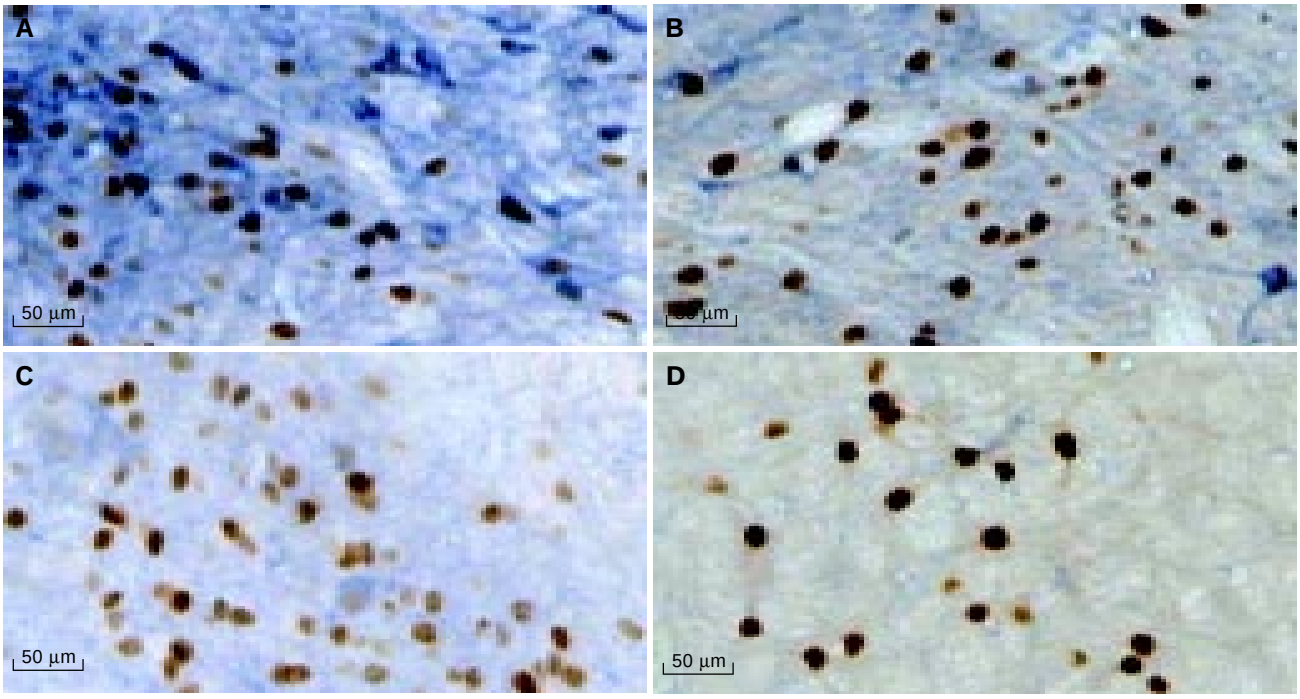


**Figure 2** Photomicrographs showing c-Fos and NOS positive neurons in nucleus tractus solitarius (A and B), nucleus ambiguous (C and D). A and C were taken from rats with acid-pepsin perfusion, while B and D were taken from rats with saline perfusion.

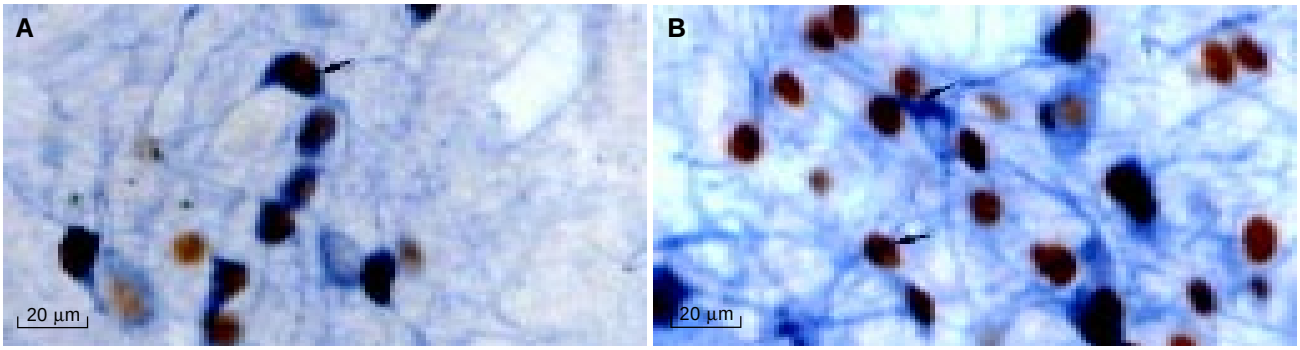


**Figure 3** Photomicrographs showing the coexistence of c-Fos and NADPH-d positive staining, i.e. colocalization (left) and close proximity (right).

**Table 1** Effects of esophageal acid-pepsin perfusion on c-Fos and NOS expression in brain nuclei, as determined by the average of number of c-Fos or NADPH-d positive neurons / section

| Nuclei | Acid-pepsin perfusion | | | Saline perfusion | | |
|---|---|---|---|---|---|---|
| | c-Fos | NOS | c-Fos&NOS | c-Fos | NOS | c-Fos&NOS |
| AM | 341.3±13.7[b] | 8.0±2.0 | 1.7±0.7 | 166.2±2.7 | 6.5±0.5 | 1.9±0.4 |
| PVN | 551.1±11.6[b] | 151.8±48.5[b] | 127.6±34.1[b] | 232.2±12.9 | 66.9±1.5 | 64.1±4.4 |
| SON | 181.0±3.5 | 96.2±2.4 | 66.0±7.0 | 183.3±5.8 | 95.3±4.2 | 64.9±2.1 |
| PBN | 103.0±4.1[b] | 17.1±1.8[b] | 2.9±1.0[b] | 79.7±2.6 | 3.4±0.6 | 1.1±0.5 |
| NTS / DMV | 161.1±6.9[b] | 48.8±6.8[b] | 32.3±4.7[b] | 75.0±0.8 | 23.7±0.7 | 8.4±1.5 |
| NA | 42.7±0.8[b] | 2.1±0.4 | 1.0±0.2 | 25.0±1.5 | 2.0±0.6 | 1.0±0.2 |
| RNM | 77.4±7.6[b] | 15.1±1.5[b] | 7.6±1.1[b] | 32.9±0.4 | 5.1±0.5 | 1.9±0.3 |
| AP | 190.1±11.1[b] | 6.0±2.3[b] | 2.3±1.1[a] | 107.2±2.1 | 1.9±0.6 | 0.9±0.3 |

Data are expressed as mean±SD. [a]$P<0.05$, acid-pepsin perfusion *vs* saline perfusion. [b]$P<0.01$, acid-pepsin perfusion *vs* saline perfusion. c-Fos, c-Fos positive neurons; NOS, NADPH-d positive neurons.

and NADPH-d positive staining (Figure 3). The coexistence included colocalization that was visualized as blue-stained perikarya (NADPH-d activity) containing a clearly visible dark brown nucleus (c-Fos protein), and close proximity that was visualized as c-Fos positive nucleus being within neuronal processes of NADPH-d, and that was the presence of NADPH-d positive staining within 3 μm from c-Fos-positive nucleus. Both of them have been adopted as a criterion of close proximity[19,20]. The coexisting cells were mainly observed in PVN, SON and NTS/DMV.

### *Correlation between c-Fos and NADPH-d positive cells*

There was a high correlation between c-Fos and NADPH-d positive cells in PVN, PBN, NTS/DMV, RNM and AP. The correlation coefficient (r) was 0.805, 0.943, 0.923, 0.947, 0.869 (all $P<0.01$) respectively. There was no correlation between c-Fos and NADPH-d expression in AM, SON, and NA.

## DISCUSSION

Acid, in combination with pepsin, was chosen to be the stimulant in this rat model of gastroesophageal reflux. This combination has been shown to cause esophagitis in experimental models[21,22].

The nerve supply to esophagus is composed of extrinsic and intrinsic components. The extrinsic innervation is mainly through the autonomic nervous system, which is divided into sympathetic and parasympathetic components. The parasympathetic innervation of esophagus is supplied by the vagus nerves. Three types of vagal afferent fibers are classified on the basis of their sensitivity to mechanical stimulation: those responding to mucosal stroking (mucosal receptors), those responding to circular tension (tension receptors) and those responding to mucosal stroking and circular tension (tension/mucosal receptors)[23]. Sensory afferents from the esophagus usually travel to NTS. DMV, which contains preganglionic motor neurons, has efferent fibers. The dorsal vagal complex (DVC) comprising NTS and DMV is the center of the integration of vagal control of esophagus[24,25]. Exposing the subdiaphragmatic vagus nerves (SDV) to horseradish peroxidase (HRP), Norgren *et al.* found that retrogradely labeled neurons occurred within NA and the reticular formation caudal to NA, and DMV whereas anterograde HRP reaction product occurred in NTS and AP[26]. Besides, connections of NTS with the medullary reticular formation and AP existed[27]. They were reported to take part in some visceral reflexes. In the present study, c-Fos positive neurons were seen in NTS, DMV, NA, RNM, and AP. In comparison with the controls, the number was greater in the acid-pepsin group. In this context, the present results confirm those reports mentioned above. During the esophageal exposure to acid, a cascade of chemoreceptors that lie along the passage is stimulated. Some of these signals are carried by vagal afferents to NTS in brainstem. From there, visceral information is disseminated to various brain sites, where it affects regulatory functions by engaging endocrine, autonomic, and some other effector mechanisms. But how all these different pathways interconnect within subnuclei is still unknown. It has been reported that PBN is related to noxious information from the visceral organs[14]. Esophageal acid exposure also induces high density of c-Fos expression in PBN.

A significantly increased number of c-Fos positive nuclei was observed in AM and PVN. Although many of c-Fos staining cells were seen in SON, there was no significant increase in this area in response to acid-pepsin perfusion. PVN is immediate beneath the ependyma of the third ventricle. The afferent connections of PVN are from hippocampal formation, septal nuclei, locus ceruleus, AM, and NTS. The efferent connections appear, in part, to be reciprocal to the afferent systems. The AM has reciprocal connections with locus ceruleus, substantia nigra, NTS, DMV, PBN, reticular formation, and nuclei of the hypothalamus. The present study showed that only some of those areas expressed c-Fos immunoreactivity, which suggests that those activated neurons are related to esophageal innervation. In order to exclude the potential contribution of the pressor response to the induction of c-Fos in NTS and other nuclei, BP, HR, and RR were recorded during the experimental procedures. There were no significant changes in BP, HR, and RR between the two groups.

It has been reported that NOS exists in neurons of DVC. The premotoneurons in NTS express NOS, and NO acting in the NA takes part in the esophageal peristalsis[28]. The present study showed that many NADPH-d positive neurons were seen in PBN, NTS/DMV, RNM, and that some were seen in NA and AP. This suggests that NO release may modulate characteristics of the activated neurons in these nuclei that are evoked by esophageal acid stimulus. It has been reported that NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), reduces the spontaneous discharge rate of the NTS neurons *in vivo* and *in vitro*, which confirms that NO has the excitatory effect on NTS[29]. L-NAME also reduces the c-Fos expression in DVC, suggesting that c-Fos expression is, in part, related to NO release in DVC. Little has been known about the neurotransmitters in telencephalon and diencephalon. In the present study, many NADPH-d positive cells were observed in PVN and SON, but only few were found in AM.

The present study observed the coexistence of c-Fos and NADPH-d positive staining. It is possible that the neuronal cells containing NOS are activated during esophageal acid exposure, which may cause NO release to themselves or to other brain regions in modulating the esophageal reflux.

In conclusion, acid-pepsin exposure to lower part of the esophagus stimulates the mucosal receptors, which in turn activates the neurons of NTS through vagal afferent fibers, and finally the neurons in DMV and NA to modulate the esophageal peristalsis. The possible nuclei involved in these procedures are AM, PVN, PBN, RNM, and AP. Double labeled staining of c-Fos and NADPH-d suggests that NO is one of the neurotransmitters in PVN, PBN, NTS/DMV, RNM and AP.

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• BRIEF REPORTS •

# Effect of octreotide on human pancreatic cancer cells after transfected with somatostatin receptor type 2 gene

Zheng-Ren Liu, Ren-Yi Qin, Gao-Song Wu, Qing Chang, Da-Yu Wang, Sheng-Quan Zou, Fa-Zu Qiu

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## Abstract

**AIM:** To observe the effect of octreotide on apoptosis rate of human pancreatic cancer cells PC-3 after transfected with somatostatin receptor type 2 (SST2) gene.

**METHODS:** SST2 plasmid was transfected into PC-3 cells by liposome. Result of transfection was detected by immunocytochemical staining and Western blotting. Apoptosis rates of PC-3 cells under different dosages of octreotide were measured by MTT assay and flow cytometry (FCM).

**RESULTS:** Apoptosis rate caused by octreotide of transfected PC-3 cells was 7.56±1.06% at the dosage of 0.20 μg/mL, 9.25±1.73% at the dosage of 0.40 μg/mL and 14.18±2.71% at the dosage of 0.80 μg/mL. Apoptosis rate caused by octreotide of non-transfected PC-3 cells was 5.76±0.75% at the dosage of 0.20 μg/mL, 6.69±0.80% at the dosage of 0.40 μg/mL and 7.26±1.28% at the dosage of 0.80 μg/mL. Transfected PC-3 cells growth inhibition rate caused by octreotide was 9.36±1.34% at the dosage of 0.20 μg/mL, 12.03±1.44% at the dosage of 0.40 μg/mL and 20.23±4.21% at the dosage of 0.80 μg/mL. Non-transfected PC-3 cells growth inhibition rate caused by octreotide was 6.44±0.66% at the dosage of 0.20 μg/mL, 7.65±0.88% at the dosage of 0.40 μg/mL and 9.29±1.32% at the dosage of 0.80 μg/mL. We found that octreotide caused higher apoptosis rate and inhibition rate in transfected groups than in non-transfected groups ($P<0.05$) at the tested dosages (0.20, 0.40 and 0.80 μg/mL).

**CONCLUSION:** Deficiency of SST2 was probably the major reason why octreotide had little effect on PC-3 cells. Transfecting SST2 gene could strengthen the ability of octreotide of killing PC-3 cells. It provided an experimental evidence for using both octreotide and transfection with SST2 gene on clinical treatment of pancreatic cancer.

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## INTRODUCTION

Octreotide, the artificial synthetic somatostatin analogue, which possesses the advantage of relative specificity, long lasting and small ill effect, has already been applied to clinical therapy extensively. Its anti-tumor mechanism mainly depends on the expression of somatostatin receptor, especially SST2[1-3]. Most tumors highly express SST2, but some do not express or low express. For example, 90% human pancreatic cancer cells have lost the ability of expressing SST2, which leads to the unsatisfactory treatment effect in pancreatic cancer[4]. We did the experiment to explore octreotide's effect on the apoptosis rate of pancreatic cancer (PC-3) cells after transfected with SST2 gene.

## MATERIALS AND METHODS

### *Materials*

PC-3 cells were a gift from China Medical University. RPMI 1640, fetal calf serum, Liposome 2000 Reagent were purchased from Gibco Co. USA. Octreotide was kindly presented by Novartis Co. Swiss. MTT, DMSO and G418 were purchased from Sigma Co. USA. Goat-anti-human SST2 monoclonal antibody was purchased from Santa Cruz Co. USA. Rabbit-anti-goat and DAB were purchased from Zhongshan Biotechnology Co. Beijing, China. ECL kit was purchased from Amersham Pharmacia Biotechnology Inc. ABC test kit was purchased from Huamei Co. China. Human SST2 plasmid was kindly presented by Junbo Hu of Maryland University, USA.

### *Methods*

**SST2 plasmid amplification and transfection** We adopted colon bacillus (DH-5α) to amplify and extract the human SST2 plasmid, which was identified by endonuclease. We cultured the PC-3 cells with RPMI 1640 supplemented with 100 mL/L fetal calf serum. When PC-3 cells were in exponential growth phase, the human SST2 plasmid was transfected into PC-3 cells mediated by Liposome 2000 reagent and screened with culture medium with 600 mg/L G418.

**Immunocytochemical staining** Transfective effect was detected by immunocytochemical staining. ABC test kit was adopted to stain the cells crawling on the slides with immunocytochemical staining. Goat-anti-human SST2 monoclonal antibody was diluted to 1:100. Blank groups used PBS instead of goat-anti-human antibody.

**Western blotting** Expression of SST2 was detected by western-blot. Transfected cells were collected and lysed with the cell-lysis to extract protein. Goat-anti-human SST2 monoclonal antibody (1:1 000) was used. The signals were developed with the ECL kit.

**FCM** Apoptosis rate was detected by flow cytometry. When the PC-3 cells were in exponential growth phase, octreotide at different dosages (0.05, 0.10, 0.20, 0.40, 0.80 μg/mL) was added. After 24 h, culture medium was renewed and octreotide added according to the above dosages. Cells in the culture medium were collected and preserved in 700 mL/L alcohol at -20 ℃. Cells were collected after 48 h; the apoptosis rate was detected by flow cytometry.

**MTT assay** MTT assay was adopted to detect the sensitivity of PC-3 cells *in vitro* after using different dosages of octreotide. We divided them into three groups: blank groups (no cells), control groups (no drug) and octreotide groups. Octreotide treated cells were divided into five groups according to the dosages (0.05, 0.10, 0.20, 0.40, 0.80 μg/mL). Then the transfected PC-3 cells and non-transfected cells were inoculated into the 96-well culture panel, about $3\times10^6$/L in each well. Each panel had 10 replicate wells. After 24-h culture, octreotide was added and then cultivated for another 48 h. The sensitivity of PC-3 cells was detected with MTT assay and inhibition rate was measured by detecting the value of the *A* (λ=550 nm). Inhibition rate of PC-3 cells growth=[(*A*control group-*A*blank group)-(*A*experiment group-*A*blank group)]/(*A*control group-*A*blank group)×100%.

### *Statistical analysis*

*t*-test and SPSS software were employed to analyze data. $P<0.05$ indicates significant difference.

## RESULTS

### *Expression of SST2 in transfected and non-transfected PC-3 cells*

Cells crawling on the slides were stained by immunocytochemical staining. SST2 gene expressing PC-3 cells would present brown particles spreading mainly in the cytoplasm and cell membrane (Figure 1). PC-3 cells non-transfected were negative-expression (Figure 2). We proved that PC-3 cells did not express SST2. In contrast, they could express SST2 after transfected with SST2 gene. And it also proved that the transfection was effective and accorded with the requirement of our experiments.

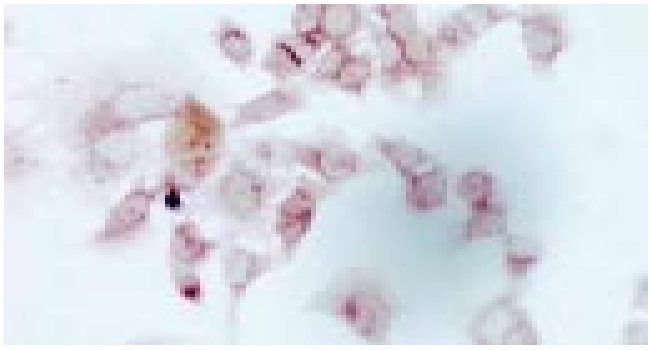

**Figure 1** SST2 expressed in the transfected PC-3 cells (10×40).

**Figure 2** SST2 did not express in non-transfected PC-3 cells (10×40).

### *Western blotting of SST2 expression*

Protein of transfected PC-3 cells was extracted and the expression of SST2 was detected by Western blotting. SST2 in transfected PC-3 cells was detected (Figure 3), the $M_r$ is about 40 000. It also proved that the transfection was effective at protein level. This was the base of our next experiments.

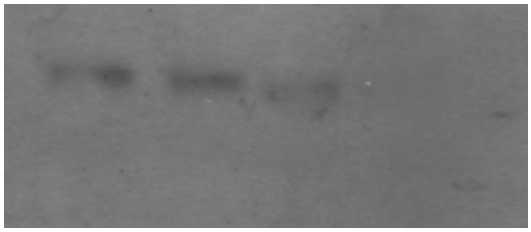


**Figure 3** SST2 could be detected in the transfected PC-3 cells.

### *Analysis of PC-3 cell inhibition rate by MTT assay*

Inhibition rate was detected by MTT assay. Growth inhibition rate of transfected PC-3 cells caused by octreotide was 3.45±0.91% at the dosage of 0.05 μg/mL, 5.18±1.21% at the dosage of 0.10 μg/mL, 9.36±1.34% at the dosage of 0.20 μg/mL, 12.03±1.44% at the dosage of 0.40 μg/mL and 20.23±4.21% at the dosage of 0.80 μg/mL. The inhibition rate caused by octreotide for non-transfected PC-3 cells was 3.28±0.54% at the dosage of 0.05 μg/mL, 4.08±0.45 % at the dosage of 0.10 μg/mL, 6.44±0.66% at the dosage of 0.20 μg/mL, 7.65±0.88% at the dosage of 0.40 μg/mL and 9.29±1.32% at the dosage of 0.80 μg/mL. After using octreotide (0.05-0.80 μg/mL) for 48 h, we found that it caused a higher inhibition rate in transfected groups than in non-transfected groups $P<0.05$) at different dosages (0.20, 0.40 and 0.80 μg/mL). It proved that the expression of SST2 could enhance octreotide's effect on the inhibition of PC-3 cells (Figure 4).

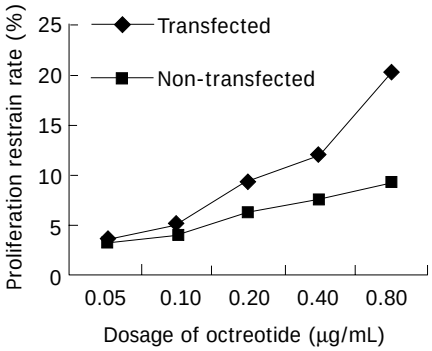


**Figure 4** Transfected PC-3 cells growth inhibition rate under octreotide.

### *Flow cytometric analysis of PC-3 cell apoptosis rate*

When 0.05-0.80 μg/mL octreotide was added and incubated for 48 h, the PC-3 cells would show the typical apoptosis peak measured by flow cytometry. Moreover, the higher the drug dosage was, the higher the apoptosis rate was. Higher dosages (0.20, 0.40 and 0.80 μg/mL) of octreotide caused a greater increase of inhibition in PC-3 cells transfected groups than in non-transfected groups ($P<0.05$) (Table 1).

**Table 1** Apoptosis rate of PC-3 cells transfected and non-transfected with SST2 gene under different dosages of octreotide (mean±SD)

| Dosage of octreotide (μg / mL) | Apoptosis rate of transfected PC-3 cells (%) | Apoptosis rate of non-transfected PC-3 cells (%) | *P* |
|---|---|---|---|
| 0.05 | 3.65±0.66 | 2.77±0.33 | >0.05 |
| 0.10 | 4.45±0.78 | 3.23±0.57 | >0.05 |
| 0.20 | 7.56±1.06 | 5.76±0.75 | <0.05 |
| 0.40 | 9.25±1.73 | 6.69±0.80 | <0.05 |
| 0.80 | 14.18±2.71 | 7.26±1.28 | <0.05 |

## DISCUSSION

At present, somatostatin has been widely used in therapy of various tumors especially endocrine tumors. In a majority of cases, favorable effect has been noted. In combined treatment with classical chemotherapeutic drugs, such as 5-fluorouracil, somatostatin has synergistic action of killing tumor cells[5]. The mechanism of somatostatin's anti-tumor effect *in vitro* lies in combining with its receptor. Somatostatin receptor belongs to a protein G coupled receptor family[6]. According to the similarity of structure and difference in affinity to somatostatin, somatostatin receptors are classified into two categories: (1) having strong affinity to somatostatin, including SST2A, SST2B, SST3 and SST5; (2) having weak affinity to somatostatin, including SST1 and SST4[7,8]. It is known that somatostatin receptors are extensively distributed in human central nervous system and other tissues. The category and amount of somatostatin receptor expression is different in various tumors. Somatostatin receptor, especially SST2 is usually highly expressed in many tumor tissues. SST2 may play a role in anti-tumor effect of somatostatin (It is considered as a tumor suppressing gene)[9-11]. The mechanisms of somatostatin's anti-tumor action are as follows: (1) direct negative effect on tumor growth; (2) inhibiting the growth of tumors by interfering with synthesis of growth factors, which are produced via paracrine and autocrine of tumor cells; (3) anti-tumor effects by inhibiting excretion of somatotropin, insulin, gastrin and other hormones or depressing insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and other growth factors; (4) interference with DNA synthesis of tumor cells; (5) suppression of angiogenesis of tumors[12].

Substantial evidence has shown that loss or low expression of SST2 was observed in primary pancreatic cancer[9,13-15]. Our previous research results have also shown that expression of SST2 was detected in 12.5 % of primary pancreatic cancers. These results suggest that expression of SST2 was deficient in a majority of pancreatic cancer tissues, which may be the main reason why somatostatin and somatostatin analogues were nearly inefficacious in the treatment of pancreatic cancer. But it is interesting that expression of SST2 was observed in the majority of adjacent histologically noncancerous pancreas, which was beneficial for somatostatin and somatostatin analogues to suppress fast invasion and growth of tumor cells. These results also suggest that if expression of SST2 in pancreatic cancer and adjacent histologically noncancerous pancreas was reduced; somatostatin and somatostatin analogues were nearly inefficacious in the treatment of pancreatic cancer. But if expression of SST2 in both pancreatic cancer and adjacent histologically noncancerous pancreas were observed, somatostatin and somatostatin analogues were efficacious[11,1]. Therefore, different expression of SST2 in pancreatic cancer and adjacent histologically noncancerous pancreas is bound to result in different effects of treatment, which may be the main reason why reports of effects of somatostatin and somatostatin analogues treatment on pancreatic cancer are inconsistent at the present time. We believe that it is very important to make clear whether SST2 is expressed in pancreatic cancer and adjacent histologically noncancerous pancreas before somatostatin and somatostatin analogues are used in treatment of pancreatic cancer. We have demonstrated that transfecting SST2 gene into human pancreatic cancer cells which lack SST2 could markedly up-regulate SST2 expression, increase apoptosis rate of human pancreatic cancer cells and enhance somatostatin analogue octreotide to inhibit growth of human pancreatic cancer cells and induce apoptosis. These results suggest that it may be an exciting molecular biological therapy for human pancreatic cancer deficiency of SST2 gene via transfection with SST2 gene and combination with somatostatin analogue.

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• BRIEF REPORTS •

# Inhibitory effect of *Huangqi Zhechong* decoction on liver fibrosis in rat

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## Abstract

**AIM:** To assess the inhibitory effect of *Huangqi Zhechong* decoction on hepatic fibrosis in rats induced by $CCl_4$ plus alcohol and high fat low protein diet.

**METHODS:** Male SD rats were randomly divided into hepatic fibrosis model group, control group and 3 treatment groups consisting of 12 rats in each group. Except for the normal control group, all the rats were subcutaneously injected with $CCl_4$ at a dosage of 3 mL/kg. In 3 treated groups, either high-dose group (9 mL/kg), or medium-dose group (6 mL/kg), or low-dose group (3 mL/kg) was daily gavaged with *Huangqi Zhechong* decoction, and saline vehicle was given to model and normal control rats. Enzyme-linked immunosorbent assay (ELISA) and biochemical examinations were used to determine the changes of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), laminin (LN), type-III-procollagen-N-peptide (PIIIP), and type IV collagen content in serum, and hydroxyproline (Hyp) content in liver after sacrificing the rats. Pathologic changes, particularly fibrosis were examined by hematoxylin and eosin (HE) and Van Gieson staining.

**RESULTS:** Compared with the model control group, serum ALT, AST, HA, LN, PIIIP and type IV collagen levels dropped markedly in Huangqi Zhechong decoction groups, especially in the medium-dose Huangqi Zhechong decoction group (1 954±576 U/L *vs* 759±380 U/L, 2 735±786 U/L *vs* 1 259±829 U/L, 42.74±7.04 ng/mL *vs* 20.68±5.85 ng/mL, 31.62±5.84 ng/mL *vs* 14.87±1.45 ng/mL, 3.26±0.69 ng/mL *vs* 1.47±0.46 ng/mL, 77.68±20.23 ng/mL *vs* 25.64±4.68 ng/mL, respectively) ($P<0.05$). The Hyp content in liver tissue was also markedly decreased (26.47±11.24 mg/mgprot *vs* 9.89±3.74 mg/mgprot) ($P<0.01$). Moreover, the stage of the rat liver fibrosis in Huangqi Zhechong decoction groups was lower than that in model group, and more dramatic drop was observed in medium-dose Huangqi Zhechong decoction group ($P<0.01$).

**CONCLUSION:** *Huangqi Zhechong* decoction can inhibit hepatic fibrosis resulted from chronic liver injure, retard the development of cirrhosis, and notably ameliorate the liver function. It may be a safe and effective therapeutic drug for patients with fibrosis.

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## INTRODUCTION

Liver fibrosis is common in most chronic liver diseases regardless of the etiology[1-8]. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis[9]. *Huangqi Zhechong* decoction is a traditional Chinese medicine. The aim of the present study was to investigate the protective effects of *Huangqi Zhechong* decoction on liver fibrosis in rats of $CCl_4$-induced cirrhosis.

## MATERIALS AND METHODS

### *Reagents*

$CCl_4$ (Xi'an Chemical Factory) was diluted into 400 g/L in olive oil (Xi'an Chemical Factory). *Huangqi Zhechong* decoction was self-made by the Pharmaceutical Department of the Second Hospital, Xi'an Jiaotong University. The kit for Hyp was bought from Nianjing Jiancheng Biological Institute. Kits for HA, LN, PIIIP and type IV collagen were bought from Senxiong Company, Shanghai.

### *Animals*

Sixty adult male SD rats weighing 150-200 g were provided by the Laboratory Animal Center of the College of Medicine, Xi'an Jiaotong University. The rats were randomly divided into 5 groups of 12 each: control group; model group; and 3 treatment groups. Except for the control rats, all rats were subcutaneously injected with 400 g/L $CCl_4$ ($CCl_4$:Olive oil 2:3), 3 mL/kg·b.w., at every 3 d for 6 wk, and fed with high fat low protein diet (75% pure maize plus 20% lard and 0.5% cholesterol) and 300 mL/L alcohol as drinks. In the 3 treatment groups, *Huangqi Zhechong* decoction was administered daily via gastric tube to high-dose, medium-dose and low-dose groups at a dosage of 9 mL/kg, 6 mL/kg and 3 mL/kg for 6 wk, respectively. After 6 wk, except the dead, all the rats were anesthetized with 200 g/L urethane (5 mL/kg, abdominal injection). Blood was taken from abdominal aorta, centrifuged at 4 ℃, and plasma were kept at -20 ℃ for assays.

### *Pathological observations*

Hepatic tissues were fixed in 40 g/L solutions of formaldehyde in 0.1% mol/L phosphate-buffered saline (pH 7.4), and embedded in paraffin. Five-micrometer thick section slides were prepared. All the sections stained with HE and standard van Gieson staining (VG) were coded and scored by blind reading. Van Gieson's method was used to detect collagen fibers[10]. Liver condition was classified according to the standard formulated by China Medical Association in 1995[11],

and fibrosis was graded from 0 to 4 (0: no fibrosis; 1: portal area fibrosis; 2: fibrotic septa between portal tracts; 3: fibrosis septa and structure disturbance of hepatic lobule; and 4: cirrhosis).

### Statistical analysis

Results were expressed as mean±SD. Quantitative data were analyzed by using ANOVA in statistical software SPSS 11.0. *A* value of $P<0.05$ was considered statistically significant. Ridit test was used for statistical analysis of the qualitative data.

## RESULTS

### Hyp content in liver tissues

Liver Hyp level was significantly lower in rats treated with $CCl_4$ and *Huangqi Zhechong* decoction compared to the rats treated with $CCl_4$ alone ($P<0.01$). And the liver Hyp level of rat in 3 *Huangqi Zhechong* decoction treatment groups has no significant difference from control group (Table 1).

### Plasma levels of ALT and AST

Plasma levels of ALT and AST in model group were higher than those in the controls ($P<0.01$), while the *Huangqi Zhechong* decoction treatment groups showed significant lower ALT and AST levels than the model group. Furthermore, among the 3 treatment groups the medium-dose group showed the best effect and the levels of ALT and AST in serum showed no difference compared with the normal group (Table 1).

**Table 1** Level of serum ALT, AST and liver Hyp

| Group | *n* | ALT (U/L) | AST (U/L) | Liver Hyp (μg/mgprot) |
|---|---|---|---|---|
| Control | 12 | 86.0±17.7 | 329±40 | 10.02±1.05 |
| Model | 11 | 1 954±576[d] | 2 735±786[d] | 26.47±11.24[d] |
| High-dose group | 9 | 989±576[ad] | 1 584±988[c] | 15.01±7.59[b] |
| Medium-dose group | 10 | 759±380[bc] | 1 259±829[a] | 9.89±3.74[b] |
| Low-dose group | 10 | 1 003±530[ad] | 1 650±928[c] | 10.06±2.58[b] |

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; [c]$P<0.05$, [d]$P<0.01$ *vs* control group.

### HA, LN, PIIIP and type IV collagen levels in serum

Serum levels of HA, LN, PIIIP and type IV collagen in model group, compared with the controls, were all markedly increased ($P<0.01$). And compared with the model group, the serum levels of HA, LN, PIIIP and type IVcollagen were significant decreased in the 3 treatment groups ($P<0.01$) (Table 2).

**Table 2** Serum levels of HA, LN, PIIIP and type IV collagen

| Group | *n* | PIIIP (ng/mL) | Type IV collagen (ng/mL) | LN (ng/mL) | HA (ng/mL) |
|---|---|---|---|---|---|
| Control | 12 | 0.34±0.67 | 18.47±3.43 | 10.07±1.74 | 17.96±5.86 |
| Model | 11 | 3.26±0.69[d] | 77.68±20.23[d] | 31.62±5.84[d] | 42.74±7.04[d] |
| High-dose group | 9 | 2.01±0.40[bd] | 39.14±4.97[bd] | 16.32±2.73[bd] | 21.71±5.69[b] |
| Medium-dose group | 10 | 1.47±0.46[bd] | 25.64±4.68[b] | 14.87±1.45[bd] | 20.68±5.85[b] |
| Low-dose group | 10 | 1.84±0.27[bd] | 29.09±2.78[b] | 17.02±2.74[bd] | 24.18±7.89[b] |

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; [c]$P<0.05$, [d]$P<0.01$ *vs* control group.

**Table 3** Pathological observation of liver condition

| Group | *n* | Liver condition | | | | | *U* |
|---|---|---|---|---|---|---|---|
| | | 0 | I | II | III | IV | |
| Model | 11 | 0 | 0 | 0 | 3 | 8 | |
| High-dose group | 9 | 0 | 2 | 3 | 3 | 1 | 2.98[a] |
| Medium-dose group | 10 | 0 | 4 | 4 | 2 | 0 | 4.01[b] |
| Low-dose group | 10 | 0 | 2 | 4 | 3 | 1 | 3.75[b] |

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; The value of *U* represents the Ridit value of the two groups, $U>1.96$ means $P<0.05$, $U>2.58$ means $P<0.01$.

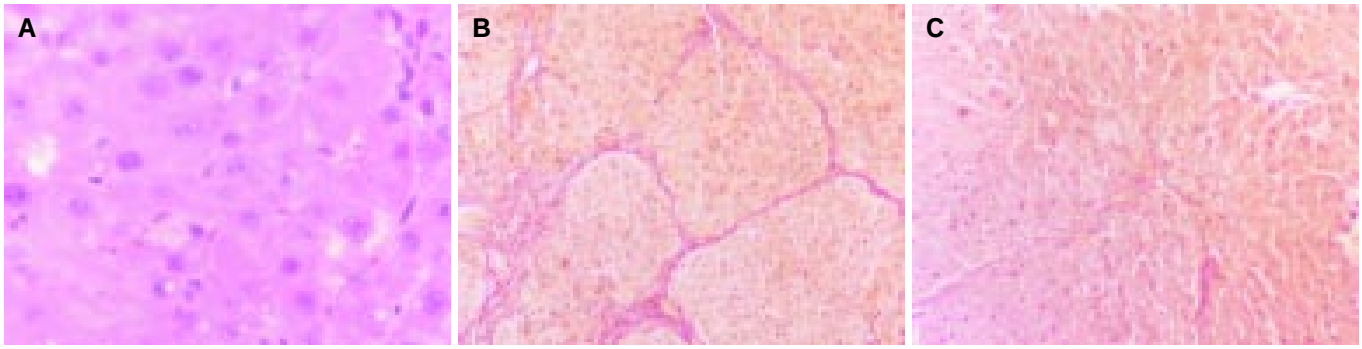


**Figure 1** Liver tissue under light microscope. A: Normal liver tissue in control group (HE staining, original magnification: ×400); B: Liver fibrosis tissue in model group, more fibrous tissue was formed in liver. A large amount of inflammatory cells soaked into the intralobular and the interlobular (van Gieson staining, original magnification: ×200); C: Liver fibrosis tissue in *Huangqi Zhechong* decoction group. The pathological change of liver was rather lighter compared with the model (van Gieson staining, original magnification: ×200).

### *Pathological assay*

At the end of the study, the liver of control rats had no appreciable alterations (Figure 1A). In the model group, the margin of liver was uneven; more fibrous tissues formed and extended into the hepatic lobules to separate them incompletely; a large amount of inflammatory cells infiltrated in the intralobular and the interlobular regions; the liver structure was disordered with some displacement of central veins, and there were more necrotic and degenerated liver cells compared with the control (Figure 1B). While in the 3 treatment groups, especially in the medium-dose group, the pathological changes of liver was rather milder, showing less fibrous tissue proliferation and inflammatory cell infiltration in the interlobular space; the hepatic cell cords arranged radially with less displacement of central veins and less degenerated or necrosis hepatic cells,without any pseudolobule observed (Figure 1C). Compared with the model group, the liver condition of the rats was significantly improved in *Huangqi Zhechong* decoction groups (Table 3).

## DISCUSSION

The incidence rate of chronic hepatopathy in China is high, which afflicts the patients by progressively developing irreversible cirrhosis[12,13]. Hepatic fibrosis is the intermediate and crucial stage of this process, characterized by reversibility. If treated properly in this stage, cirrhosis could be successfully prevented[14]. But it remains a problem to prevent cirrhosis or to control its progression in patients with a chronic liver disease[15]. Great efforts have been made to find safe and effective drugs. Recent clinical and experimental observations have demonstrated that Chinese medicines might be of some preventive and therapeutic values against fibrosis[16-18].

Of *Huangqi Zhechong* decoction, the Chinese traditional medicine, the *Astragalus* has the effects of activating blood circulation to relieve stasis, strengthening "spleen", supplementing and smoothening "qi" to eliminate fullness, reinforcement body's immunological function. It also could preserve the integrity of hepatocytes, eliminate toxic free radicals, inhibit lipid peroxidation of cytomembrane, relieve necrosis of hepatocytes, and obviously antifibrosis[19-23]. *Thoroughfare* is mainly used to activate blood circulation, remove stasis, and dredge the liver[24].

*Huangqi Zhechong* decoction has been used in clinic for many years to prevent liver fibrosis and shown good effect. However, its effect and the associated mechanisms need further experimental evidence. $CCl_4$ is a super-hepatotoxin, with which the $CCl_3$ free radical is produced during metabolic processes and acts on liver cells to covalently conjugate with the membranous unsaturated lipid to cause lipid peroxidation and necrosis of hepatocytes[25-27]. For this reason, $CCl_4$ was used to induce liver fibrosis.

We started to treat the rats with *Huangqi Zhechong* decoction at the same time of subcutaneous injection with $CCl_4$. After 6 wk, the histological features showed that there were no appreciable alterations in control group. But pathological evaluation showed that the rats in model group almost had integrity fibrosis septum, and pseudolobular could be seen in nearly every section. While the rats received *Huangqi Zhechong* decoction had less fibrosis, the reticular fibrosis in the interlobular septum was limited remarkably, and no pseudolobular could be seen. In addition, *Huangqi Zhechong* decoction, especially the medium-dose administration could decrease the scores of hepatic fibrosis grading.

ALT and AST are indexes to describe liver functions. Most part of ALT is presented in the cytoplasm of liver cell, discharged in blood when degeneration, hyper permeability and necrosis of liver cells occur. So the increase of ALT level in serum reflects the degree of liver cell injury. Our study showed that the *Huangqi Zhechong* decoction could decrease serum levels of ALT and AST in rats with hepatic injury caused by $CCl_4$. It indicates that *Huangqi Zhechong* decoction may work through protecting the liver cells.

HA, LN, PIIIP and type IV collagen are good serum markers of hepatic fibrosis. In this study, the serum contents of these 4 markers in the model group were much higher than those of the controls ($P<0.01$). And the *Huangqi Zhechong* decoction groups had significantly low HA, LN, PIIIP and type IV collagen levels in serum than those in the controls, which indicated that *Huangqi Zhechong* decoction could successfully prevent hepatic fibrosis.

Hyp content in liver is another important index to react the hepatic fibrosis. In fibrotic liver, collagen fibers increase, which induced the rise of Hyp content in liver[28]. So Hyp level could provide the information about the degree and variant process of cirrhosis. In this study, we observed that the liver Hyp level in the model group was much higher than that of the controls and *Huangqi Zhechong* decoction groups.

In summary, *Huangqi Zhechong* decoction may play a role in antifibrotic therapy. It can protect the liver cells and inhibit the deposition of collagen fibers in liver. It may provide a safe and effective strategy for inhibition of cirrhosis in clinic use.

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# Transanal approach in repairing acquired rectovestibular fistula in females

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## Abstract

**AIM:** To summarize the operative experience of the transanal approach in acquired rectovestibular fistula repair.

**METHODS:** Ninety-six cases of acquired rectovestibular fistula in young females were analyzed retrospectively. The etiology and operative procedure were discussed. Operative essential points were, the patient was laid in prone frog position, with the knees and hips flexed at 90°; the perineum was elevated; and the anal opening was exposed. Four stay sutures were applied to the margin of the fistular orifice in the anal opening at points 3, 6, 9 and 12 o'clock. A circular incision of mucosa surrounding the stay sutures was made. The fistula was dissected from its anal opening to its vestibular opening. The wound of vestibule was sutured, and the rectoanal wound was then sutured transversely.

**RESULTS:** All the 96 patients recovered uneventfully from operation with a successful rate of 93.75%.

**CONCLUSION:** The transanal approach in the treatment of the acquired rectovestibular fistula is a simple and feasible technique.

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## INTRODUCTION

Acquired rectovestibular fistula in small girls is a common anorectal disease. Its etiology is still indefinite. Some of pediatric surgeons described it as a kind of congenital malformation. In China, most thought it as an acquired disease caused by the infection of crypts of *Morgagni*[1-7]. There are two main surgical procedures that are commonly adopted, transanal and perineal approach[8-13].

## MATERIALS AND METHODS

### *Patients*

Ninety-six patients with an average age of four years and two mo, ranging from 6 mo to 14 years, were admitted and operated. Most patients came with the complaint of passing stool or flatus through vagina. Half of them had a history of perineal inflammation at the early life of 2-3 mo. An external opening was usually found in the vestibule, a little bit right or left to the vaginal opening, while the internal opening was always found at the mid-point of the dentate line on the anterior anorectal wall. The diameter of fistula orifice ranged from 2 to 8 mm.

### *Preoperative preparation*

Metronidazole tablet (30 mg/ kg per day, divided t.i.d) and oral garamycina (15 mg/kg per day, divided t.i.d) were given before operation for 3 d. Nothing but water by mouth was allowed for 1 d before operation day. Enema was performed at the night before operation day and on the morning of the operation day.

### *Operative methods*

Under general anesthesia, patient was laid in prone frog position, with knees and hips flexed at 90° fixed at the caudal end of the operation table, with a soft pillow placed under the pubis. Almost half roll of sterile bandage was inserted into rectum to prevent rectal discharge. The tail of bandage might be soaked with iodophor before being sent deeply into the rectum, and it was tied by a long heavy silk, which was left outside the anal orifice in order to make the removal of the bandage easily after operation. The anal opening was widely stretched bilaterally to expose the inner opening of fistula, four stay sutures were applied to four points at 3, 6, 9 and 12 o'clock respectively. The stay sutures were pulled, and a circular incision of mucosa around the inner opening of fistula was made by Bovie. The fistula was dissected toward the outer opening in the vestibule and resected. The wound of vestibule was sutured interruptedly with 5-0 Dexon. Usually fistula was about 0.8 cm long. The levator ani muscle was approximated longitudinally to abolish the dead space anterior to the rectum. The anorectal wall was freed proximally for about 1.0 cm to make the wound closed without tension. The packing bandage in rectum was pulled out after operation.

### *Postoperative treatment*

Nothing but water by mouth was provided for 3 d after operation, and intravenous antibiotics were given 3 to 5 d. Perianal area was kept clean and dry by warm ventilation 3 to 5 times every day for some 5 d.

## RESULTS

Six cases, about 6.25% of all patients, had recurrence of fistula. However, three of them healed spontaneously 2 to 3 wk later without any specific treatment except sitz bath with 30 g/L boric acid.

## DISCUSSION

Bryndorf and Madsen reported rectovestibular fistula, a fistulous tract between the bowel and the low female genital tract, in 1960[14]. The disease is more common in Asian than in Europeans. There were two controversial opinions about its etiology, the congenital or the acquired. Chatterjee *et al*.[1-5] described rectovestibular fistula with normal anus in females as a kind of congenital malformation, double terminal of the alimentary tract. The family history, coexisting anomalies as

anal stenosis or sacral vertebrae abnormality and the stratified squamous epithelium lining of fistular wall were the basis favouring the congenital consideration. The inflammatory changes of fistula might be secondary.

Most doctors in China believe that the development of rectovestibular fistula with normal anus in small girls was similar to the inflammatory fistula-in-ano in males[6-8]. The inflammation started by the infection of crypts of Morgagni, perianal abscess formation followed, and finally it ruptured through the vestibule. On the other hand, some deep crypts or anal glands cystoid dilatation as well as infantile hypoergic immunity of the rectoanal mucosa make the rectum mucosa barrier imperfect[15-17]. All these might play a role in anal fistula formation. Certainly it was not the congenital double termination of the alimentary tract. None of patients had a definite history of fistula at birth, and neither did a surgical specimen show normal histology of mucosa, submucosa and continuous smooth muscle in the fistula tract.

Ninety-six females with acquired rectovestibular fistula were treated by transanal approach in the last 10 years with a satisfactory result. The main experience may be concluded as: (1) Rectal bandage packing may prevent stool contamination during operation. (2) Fistula should be dissected completely by Bovie to make a bloodless operation field. (3) Rectum and vagina should be separated clearly. (4) Anterior rectal wall should be sutured perfectly without tension. (5) Dead space anterior to rectum should be eliminated by suturing the levator ani muscles and central body.

The favorite age for operation ought to be 3 to 5 years in order to wait for the improvement of the symptoms and also for better development of perianal structures of the child.

Recurrence of fistula, if happened, often occurred about 7 d after operation, and its symptoms included perineal inflammation, followed by passing of stool or flatus through the sutured incision. By sitz bath in 30 g/L boric acid and waiting for inflammation subsiding, half of the patients with early recurrent fistula healed spontaneously.

Diverting colostomy provides a clean operation area for anal fistula repair[11,12], but it needs at least three operations. We believe that a careful preoperative, operative and postoperative treatment offers a satisfactory result without a diverting colostomy.

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• CASE REPORT •

# Hepatitis B virus reactivation in a patient undergoing steroid-free chemotherapy

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## Abstract

A 62-year-old Japanese man who was positive for hepatitis B surface antigen (HBsAg) and anti-HBe antibody, underwent chemotherapy for non-Hodgkin's lymphoma (NHL). Mutations were detected in the precore region (nt1896) of HBV. Because steroid-containing regimen may cause reactivation of hepatitis B virus (HBV) and hepatitis may progress to be fulminant after its withdrawal, we administered CHO (CPA, DOX and VCR) therapy and the patient obtained complete response. However, he developed acute exacerbation of hepatitis due to HBV reactivation. Recovery was achieved with lamivudine (100 mg/d) and plasma exchange. The present case suggests that acute exacerbation of hepatitis can occur with steroid-free regimen. Because the efficacy of the prophylactic use of lamivudine has been reported and the steroid enhances curability of malignant lymphoma, the steroid containing regimen with prophylaxis of lamivudine should be evaluated further.

Shimizu D, Nomura K, Matsumoto Y, Ueda K, Yamaguchi K, Minami M, Itoh Y, Horiike S, Morita M, Taniwaki M, Okanoue T. Hepatitis B virus reactivation in a patient undergoing steroid-free chemotherapy. *World J Gastroenterol* 2004; 10(15): 2301-2302
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## INTRODUCTION

CHOP regimen consisting of cyclophosphamide (CPA), doxorubicin (DOX), vincristine (VCR), and prednisolone (PSL) is performed as the first line therapy for non-Hodgkin's lymphoma (NHL) because of its high response rate and less incidence of side effect. However, CHOP regimen for hepatitis B virus (HBV) carrier may cause reactivation of HBV due to prednisolone and hepatitis may progress to be fulminant after its withdrawal. On the other hand, it has already been proven that steroid containing-regimens show higher complete response and survival rate than steroid free regimens[1]. Thus, it is difficult to choose which chemotherapeutic regimen for NHL carrying HBV.

We described an asymptomatic HBV carrier patient with NHL who received CHO therapy. Although he achieved complete response, hepatitis acutely exacerbated. Lamivudine and plasma exchange improved hepatic failure.

## CASE REPORT

A 62-year-old man complained of tonsil swelling, redness and sore throat. He was referred to our hospital in August 2003. Needle biopsy from tonsil revealed him as having NHL (diffuse large B-cell lymphoma). He was admitted to our hospital on August 21 because tonsil swelling developed rapidly. Two enlarged cervical lymph nodes were palpable and right tonsil swelled. Hematological studies showed WBC 5 600/mL, Hb 15.4 g/dL and platelet $7.2\times10^4$/mm$^3$, LDH 167 IU/L, GOT 25 IU/L, GPT 15 IU/L, ALP 392 IU/L, Ch-E 163 IU/L, T-bil 1.82 mg/dL, prothrombin time (PT) 14.7 s, IL-2 receptor 564 U/mL, indocyanine green test (ICG) 25%. Serological tests showed HBsAg (+), HBeAg (-), HBeAb (+). HBV-DNA level was 4.7 LGE/mL (TMA method). Mutant virus having mutation at precore and core promoter region was detected by enzyme-linked mini-sequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) and enzyme-linked specific probe assay (Smitest HBV core promoter mutation detection kit, Genome Science Laboratory, Tokyo, Japan). Ultrasonography revealed cirrhotic pattern of liver and mild splenomegaly. Neck magnetic resonance imaging revealed swelling of right tonsil and two cervical lymph nodes. Computed tomography scanning revealed no lymph node swelling in chest and abdomen. Bone marrow aspiration revealed involvement of abnormal lymphocytes. Thus, we diagnosed he was at stage IV.

He received first course of CHO regimen on August 22. Although tonsil swelling improved, liver dysfunction gradually developed after the third course. After the forth course, liver dysfunction got severe, as serum LDH level was 499 IU/L, GOT 650 IU/L, GPT 422 IU/L, T-bil 1.49 mg/dL, PT 25.9 s and HBV-DNA level increased 6.2 LGE/mL. We diagnosed it as acute exacerbation of HBV and started both lamivudine (100 mg/d) and plasma exchange. Liver dysfunction improved gradually. In January 2004, hematological data improved as LDH 328 IU/L, GOT 98 IU/L, GPT 54 IU/L, T-bil 3.66 mg/dL, PT 19.6 s, HBV DNA 2.8 LGE/mL. He was following good clinical course and remained complete response for NHL.

## DISCUSSION

It has been reported that steroid may induce exacerbation of hepatitis after cessation of steroid therapy or during tapering of steroid. Cheng *et al*. reported that 50 NHL patients carrying HBV were randomized to receive either ACE (epirubicin, CPA and etoposide) or PACE (predonisolone+ACE). The cumulative incidence of HBV reactivation was 38% and 73%, respectively. On the other hand, it is clear that PACE chemotherapy was

more effective than ACE arm in the treatment of NHL, because CR rate was 46% and 35%, overall survival rate at 46 mo was 68% and 36%, respectively[1]. Thus, there is no consensus regarding the best therapy for NHL with HBV.

Regarding to HBV-DNA, cases with a point mutation from G to A at nucleotide 1896 of the precore region of HBV tended to develop fulminant hepatitis with steroid containing treatment[2-5]. Because we detected the mutant HBV in peripheral blood before chemotherapy, we performed CHO regimen and obtained CR for NHL. However, hepatitis acutely exacerbated.

Recently, several reports have shown promising results for the prophylactic use of lamivudine in cancer patients before chemotherapy[6-8]. However, we did not administer prophylaxis of lamivudine in the present patient, because (1) he was an asymptomatic HBV-carrier, (2) lamivudine-resistant virus might appear after lamivudine treatment or acute exacerbation after discontinuation of lamivudine, and (3) in Japan, adefovir dipivoxil was not available. Theoretically, the steroid containing regimen with lamivudine prophylaxis for NHL patients carrying HBV may be the best therapy. To prove the efficacy of this therapy, further studies are required.

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• CASE REPORT •

# Alverine citrate induced acute hepatitis

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## Abstract

Alverine citrate is a commonly used smooth muscle relaxant agent. A MEDLINE search on January 2004 revealed only 1 report implicating the hepatotoxicity of this agent. A 34-year-old woman was investigated because of the finding of elevated liver function tests on biochemical screening. Other etiologies of hepatitis were appropriately ruled out and elevated enzymes were ascribed to alverine citrate treatment. Although alverine citrate hepatotoxicity was related to an immune mechanism in the first case, several features such as absence of predictable dose-dependent toxicity of alverine citrate in a previous study and absence of hypersensitivity manifestations in our patient are suggestive of a metabolic type of idiosyncratic toxicity.

Arhan M, Köklü S, Köksal AS, Yolcu ÖF, Koruk S, Koruk I, Kayaçetin E. Alverine citrate induced acute hepatitis. *World J Gastroenterol* 2004; 10(15): 2303-2304
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## INTRODUCTION

Toxic hepatitis is a liver injury caused by drugs and chemicals. The severity varies from nonspecific changes in liver functions such as abnormalities in liver enzymes to fulminant hepatic failure and cirrhosis. Its incidence has been increased over the past several decades. Drugs have been estimated to be responsible for 15-20% of all cases of fulminant and subfulminant hepatitis in Western countries and 18% of acute liver failure in the United States[1,2].

Alverine citrate is a smooth muscle relaxant agent, commonly used in patients with irritable bowel syndrome in association with simethicone. Hepatotoxicity of alverine citrate is extremely rare and has been reported only in 1 patient before[3]. Herein we report a new case of alverine citrate-induced hepatotoxicity occurred in a middle-aged woman that resolved completely after discontinuation of the drug.

## CASE REPORT

On July 24, 2003, a 34-year-old woman was admitted to the Outpatient Clinic of Gastroenterology Department of Yuksek Ihtisas Hospital with the chief complaint of dyspepsia lasting for 2 years. She described bloating after meals and sometimes had gastroesophageal reflux symptoms. She did not have any chronic disease states. Physical examination was completely normal. Her biochemical tests including liver enzymes (AST, ALT, ALP and GGT) were all within normal limits. Upper gastrointestinal endoscopy was performed showing grade I esophagitis and pangastritis. On July 29, omeprazole PO 20 mg bid, liquid alginic acid (Gaviscon) PO 10 mL qid (30 min after meal and at bedtime) and meteospasmyl (a tablet containing 60 mg alverine citrate and 300 mg simethicone) PO tid (before meals) were prescribed. Till the 14th d after prescription she told that she used all of the drugs as recommended. At that time omeprazole was cessated and she continued to medical treatment with Gaviscon and meteospasmyl.

She was admitted to another hospital because of arthralgia lasting for years on August 18, 2003, but therapy was managed on an outpatient basis. Amitryptyline 25 mg PO bid was added to Gaviscon and Meteospasmyl and a gastroenterology consultation was requested because of the finding of abnormal liver function test values on biochemical screening. On August 18, serum liver enzymes were as follows: ALT: 319 IU/L, AST: 211 IU/L (N: 40 IU/L for both); ALP: 184 IU/L (N: 35-129), GGT: 116 IU/L (N: 5-40) and normal direct and indirect bilirubin levels. She began to take amitryptyline on August 23 and readmitted to our hospital on August 26. Her ALT and AST levels were 1 119 IU/L and 853 IU/L, respectively. Other biochemical tests such as, hemoglobin level, white blood cell count, platelet count, and prothrombin time were within normal limits. Hepatobiliary imaging with ultrasonography was normal. Viral markers for hepatitis including hepatitis A, B and C viruses, cytomegalovirus, Epstein-Barr virus, and herpes simplex virus were all negative. Autoantibodies (antinuclear, antimitochondrial, anti-smooth-muscle, anti-liver-kidney microsomal enzymes and anti-soluble liver antigen) were also negative. She denied taking any other medications and using alcohol or any herbal and folk remedies anytime. There were no known environmental issues that could be contributing.

Liver enzyme elevations were thought to be drug induced. Amitryptyline, Gaviscon and Meteospasmyl were all immediately discontinued on August 26. The ALT and AST values decreased to 597 IU/L and 237 IU/L 8 d after withdrawal of all drugs and became completely normal 18 d after cessation. The time course of liver function tests is presented in Table 1. Liver biopsy was not performed, and rechallenge with Meteospasmyl was not attempted because of ethical reasons. Since she had continuing mild symptoms related to gastroesophageal reflux, she continued to take Gaviscon and omeprazole without any increase in biochemical parameters.

**Table 1** Time course of liver function tests

| Date | AST (IU/L) | ALT (IU/L) | GGT (IU/L) | ALP (IU/L) |
|---|---|---|---|---|
| 7/24/2003 | 30 | 32 | 21 | 162 |
| 8/18 | 211 | 319 | 116 | 184 |
| 8/26 | 853 | 1 119 | - | - |
| 9/3 | 237 | 597 | - | 229 |
| 10/2 | 22 | 21 | 35 | 75 |

Omeprazole, alginic acid, and alverine citrate were started on 7/30/2003. Omeprazole was cessated on 8/10/2003. Amitryptyline was added to alginic acid and alverine citrate on 8/23/2003 and all medications were discontinued on 8/26/2003. ALP: Alkaline phosphotase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transferase.

## DISCUSSION

Meteospasmyl is a most widely used drug for irritable bowel syndrome. It contains 60 mg alverine citrate and 300 mg simethicone. Simethicone has been used as an antifoaming agent and considered as an inert one[4]. It is excreted via feces without any metabolizing in the gastrointestinal system. It has been widely used in patients with gastrointestinal gas and for the improvent of visibility during radiologic examination[5]. To our knowledge, no systemic side effect regarding simethicone was reported in the literature.

Alverine is one of the papaverine congeners. Its antispasmodic effects are mediated by processes involving smooth muscle cells, extrinsic nervous system, and calcium channels[6]. In humans, the drug is completely absorbed by the gastrointestinal tract and mainly metabolised in the liver, with neglicible amounts excreted in the urine[3]. Side effects such as flushing on the face and neck, nausea, headache, dizziness, and allergic skin eruptions may be observed. To our knowledge, hepatotoxicity associated with alverine use was reported once in the literature. Hepatotoxicity was not reported for the other papaverine derivatives other than trimebutine[7].

In our patient increase in liver enzymes was ascribed to alverine treatment due to several reasons. There was a temporal relationship between alverine treatment and hepatitis. Liver function tests were normal before treatment and increases appeared three weeks after the start of alverine treatment. The timeline of exposure to alverine and initial observation of increase in liver function tests were consistent with drug-induced liver disease[8]. Liver function tests completely normalized three weeks after withdrawal of the drug. Furthermore other etiologies of hepatitis (alcohol, steatohepatitis, autoimmune hepatitis and viral hepatitis) were appropriately ruled out. Omeprazole was not thought to be responsible for our patient's hepatitis in view of her prior uses of this drug without adverse effects. Neither chemical nor clinical side effects were seen in her consequent follow-up after alginic acid and omeprazol were started again. We therefore classified our case as probable alverine-induced hepatotoxicity according to the Naranjo probability scale[9].

Drugs cause liver injury either via intrinsic toxicity (dose-dependent or predictable) or host idiosyncrasy (dose-independent or unpredictable). Idiosyncratic hepatotoxicity may be in metabolic or immunoallergic form. Metabolic-type idiosyncratic injury develops as a result of the susceptibility of rare indivuduals to hepatotoxicity from a drug that is usually safe at convential doses. In this pattern, reactions would occur among suspectible individuals who possess an isolated genetic enzymatic alteration not expressed under normal conditions, which would become clinically apparent following the administration of certain drugs[10]. The mechanism of alverine induced liver disease is unknown. In the first case described in the literature alverine hepatotoxicity was related to immune mechanisms due to the presence of transient hypereosinophilia, eosinophil polymorhphonuclear cells in the liver inflammatory infiltrates, and antinuclear autoantibodies. In our patient autoantibodies were negative and there was no hypereosinophilia in the peripheral blood smear. Several features such as the absence of predictable dose-dependent toxicity of alverine and fever, rash, and arthralgia (hypersensitivity manifestations) in our patient were consistent with a metabolic type of idiosyncratic toxicity.

In conclusion, alverine is a smooth muscle relaxant drug. This report presents the second case of alverine-associated possible liver toxicity. Alverine citrate should be included in the list of drugs causing toxic hepatitis.

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• CASE REPORT •

# Pregnant woman with fulminant hepatic failure caused by hepatitis B virus infection: A case report

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## Abstract

**AIM:** To report the experience in successfully treating pregnant women with severe hepatitis.

**METHODS:** Comprehensive medical treatments were performed under strict monitoring.

**RESULTS:** Pregnant woman with severe hepatitis was successfully rescued.

**CONCLUSION:** Vital measures taken in the treatment of pregnant women with severe hepatitis include termination of the pregnancy at a proper time and control of various complications, such as disseminated intravascular coagulation (DIC), hepatorenal syndrome, hepatic encephalopathy and infection.

Yang YB, Li XM, Shi ZJ, Ma L. Pregnant woman with fulminant hepatic failure caused by hepatitis B virus infection: A case report. *World J Gastroenterol* 2004; 10(15): 2305-2306
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## INTRODUCTION

Hepatitis B virus (HBV) infection is common in China[1], and it is one of the most common causes of severe hepatitis in pregnancy, with a high mortality rate of 43-80%[2]. In this paper, we reported a pregnant woman with severe hepatitis B infection complicated by postpartum massive hemorrhage, hepatorenal syndrome, hepatic encephalopathy, spontaneous bacterial peritonitis and infection of biliary tract. Under strict monitoring, we applied comprehensive medical treatments. Both the mother and the child were discharged in healthy condition.

## CASE REPORT

A 33-year-old pregnant woman was admitted in our hospital on April 15, 2003, presenting with a 38-week pregnancy, 10-d puffiness and yellow urine, and 3-d deep jaundice. She had a history of HBsAg(+), HBeAg(+) and HBcAb(+) for about 10 years, but her liver function had been normal until she got pregnant. The last menstrual period (LMP) was 2002-7-22. The expected date of confinement (EDC) was 2003-4-29. Physical examination showed she had normal vital signs, an urgent and painful looking, clear mind, icteric sclera and xanthochromia, bulbar conjunctiva edema, normal heart and lung on ausculation, absence of abdominal tenderness or rebound tenderness, no detection of liver or spleen and no shifting dullness on palpation, moderate edema of both legs. Obstetrical conditions were as follows: she had left occipitoanterior position (LOP) of fetus, the height of fundus was 36 cm, the abdomen circumference was 99 cm, fetal heart rate (FHR) was 120 beats/min. No dilatation of cervix was found. Laboratory findings were as follows: aspartate transaminase (AST) was 125.0 U/L, alanine transaminase (ALT) was 138.0 U/L, albumin (ALB) was 32.5 g/L; total bilirubin (TB) was 383.9 μmol/L, blood urea nitrogen (BUN) was 36.03 mmol/L, creatinine (CREAT) was 402.6 μmol/L, white blood cell count (WBC) was $21.60\times10^9$/L, hemoglobin (Hb) was 82 g/L, prothrombin time (PT) was 24 s, uric protein was (+++). β-ultrasonography showed her liver was in a chronic hepatitis state, and moderate ascites and a little liquid in both sides of thoracic cavity were also found. Markers of series of hepatitis virus were negative for hepatitis A, C, D and E, but positive for HBsAg (+), HBeAg (+) and HBcAb (+). Therefore she was diagnosed as pregnancy associated with hepatitis B infection and fulminant hepatic failure of pregnancy (FHFP).

After admission, the pregnant woman received supportive treatments. Later, because of "fetal distress", she received a cesarean section plus hysterectomy under general anesthesia. During the operation, we found yellow ascites of about 1 500 mL, and the same color of amniotic fluid was also found. The newborn was a mature male baby with normal vital signs, and transmitted to pediatrics department and given hepatitis B virus (HBV) specific immunoglobin (HBIG) and HBV vaccine. After the delivery of placenta and membrane, there was no sign of uterine contraction, and massive hemorrhage occurred (about 2 000 mL). So hysterectomy was performed. The liver was obviously small, which was about the palm size and a little harder than normal.

After operation, the patient developed a clinical course of exacerbation. She developed hepatic encephalopathy of degree III, hepatorenal syndrome, spontaneous bacterial peritonitis, infection of biliary tract, secondary fungal infection, septicemia and prolonged healing of the wound. The comprehensive and well-designed rescue measures were taken and her condition was under control on June 25. After a hospitalization of 104 d, the patient and her newborn were discharged in generally good conditions.

## DISCUSSION

Generally, a pregnant woman with fulminant hepatic failure refers to failure of liver function caused by viral hepatitis. Often the complications included dis- seminated intravascular coagulation (DIC) that presents a hemorrhage trend [3,4], hepatic encephalopathy, hepatorenal syndrome, toxic intestinal tympanites, cerebral edema and infection of biliary tract, and so on. A pregnant woman with severe hepatitis often had hypodynamia, deep jaundice, hypocoagulability, hypoproteinemia, coma and acute renal failure. It was reported that many viruses including hepatitis virus (HAV, HBV, HCV, HDV, HEV, HGV, and so forth[5,6]), cytomegalovirus (CMV), TTV[7] and herpes virus[8], could cause severe hepatitis. But HEV and HBV

infections were most frequent[9-12]. Clinically, about 0.2-0.5% of total patients with hepatitis would develop into severe hepatitis. The prognosis of severe hepatitis during late pregnancy was so poor that it could be listed among the causes of maternity and parity mortality. In this report, the patient with a 10-year history of hepatitis had a 38-wk pregnancy. She developed severe hepatic dysfunction with hypocoagulability, hepatic encephalopathy and hepatorenal syndrome. She was positive for HBsAg, HBeAg, and HBcAb. Therefore the diagnosis of pregnancy associated with severe hepatitis and HBV infection was confirmed[13,14]. Under strict monitoring, we took comprehensive therapeutic measures and got a successful result. The key points of rescuing the patient were as follows.

The general treatment included: (1) The patient was asked to have an absolute rest in bed with a diet low in lipid and protein[15] and rich in fiber and vitamins. The total energy should be controlled at 1 500 kcal. Intra-gastrointestinal food could not only neutralize gastric acid, promote gastro- intestinal movement, but also reduce the incidence of toxic intestinal tympanites, endotoxemia, infection of biliary tract, peritonitis or even septicemia. (2) Protecting liver function, gastric membrane and preventing gastrointestinal hemorrhage. (3) Using antibiotics to prevent and treat infections, gamma globulin to promote immune state and neutralize endotoxins. HBIG, which can neutralize HBV and is relatively safe for gestational period, should also be used. (4) Maintaining the balance of body fluid and electrolytes and using dexamethasone for a short period of time ( 3-5 d) to improve the toxic symptoms and the maturity of fetal lung, thus getting ready for planned delivery.

To terminate pregnancy at a proper time, internal carotid artery centesis was performed and a tube placed to detect the central vein pressure and determine the amount of transfusion, thus preventing the incidence of cerebral edema or pulmonary edema. Before cesarean section, coloclysis and atropine were used. But sedatives such as luminal were not taken into consideration. General anesthesia instead of epidural anesthesia was to avoid epidural hematoma[16], and during cesarean section[17], we chose longitudinal incision. After cesarean section, we performed hysterectomy and placed an absorbable hemostatic gauze on the cervical stump to reduce hemorrhage during and after operation. We also detected the size of liver during operation to make a general evaluation of its condition of direct prognosis. We perfused the abdomen cavity with iodophors to reduce abdominal infection. We remained a gastric tube during operation to prevent intestinal tympanites and to put in medicines that could improve the movement of intestine. After operation, we remained a drainage tube in abdomen to facilitate the expulsion of ascites and to inspect whether there was continuous hemorrhage.

To prevent and cure complications, measures were taken to reduce possible motivations such as using none or as few as sedatives and narcotics, to avoid too much or too fast diuretics or relieving of ascites, to prevent constipation or mass hemorrhage or infection, to adjust intestinal environment by lactulose towards pH<7 to reduce absorption of $NH_3$ and endotoxin which would exacerbate the condition of hepatic encephalopathy, to take metronidazole and norfloxacin to compress intestinal bacteria which could produce $NH_3$, to use coloclysis to accelerate the expulsion of intestinal bacteria and endotoxin and $NH_3$-removing medicines, to keep normal neurotransmitter by applying L-dopa or aceglutamide, to inject hepat amine intravenously to keep amino-acid balance, to use Chinese formulated medicines such as *Angong niuhuang wan*. Heparin in low dose was used to prevent dissipation of large amount of plot and thrombin factors and to improve clotting mechanism. Thrombin factors such as fresh frozen plasma, plot, cryoprecipitate, pro- thrombin complex, and fibrinogen were used in a great quantity. Hepatorenal syndrome was treated by restricting the infusion of fluid, correcting hypoproteinemia and hypovolemia in time. Diuretics such as furosemide, diuretics complex (furosemide + dopamine + phentolamine) or mannitol + furosemide were used.

To promote regeneration of hepatocytes, glucagon and insulin were used to trigger the synthesis of hepatocyte DNA, human albumin was used to neutralize indirect bilirubin, stimulate regeneration of hepatocytes and prevent further necrosis of hepatocytes.

Blood routine, clotting function, liver function and biochemical indices, especially ALT, AST, alkaline phosphatase (AKP), acetylcholine esterase (CHE), alpha fetoprotein (AFP) and glucose (GLU) were monitored. Intensive nursing and sterile operation were performed, regular mouth care and clean of incision and perineum were maintained. 50% glucose + insulin was used to promote wound healing.

Before labor, HBIG or anti-HBV medicines (safe for both maternity and fetus) were given to interrupt intrauterine HBV infection[1]. The newborn was also given HBIG and HB vaccine[18] to build up active and passive immunity.

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# ISI journal citation reports
# 2003 – GASTROENTEROLOGY AND HEPATOLOGY

| Rank | Abbreviated journal title | ISSN | 2003 Total cites | Impact factor | Immediacy index | 2003 Articles | Cited Half-life |
|---|---|---|---|---|---|---|---|
| 1 | GASTROENTEROLOGY | 0016-5085 | 46 174 | 12.718 | 2.810 | 316 | 6.9 |
| 2 | HEPATOLOGY | 0270-9139 | 30 844 | 9.503 | 1.574 | 296 | 5.5 |
| 3 | SEMIN LIVER DIS | 0272-8087 | 2 524 | 6.524 | 0.810 | 42 | 4.7 |
| 4 | GUT | 0017-5749 | 20 612 | 5.883 | 1.147 | 307 | 6.5 |
| 5 | J HEPATOL | 0168-8278 | 11 111 | 5.283 | 0.879 | 281 | 5.0 |
| 6 | LIVER TRANSPLANT | 1527-6465 | 2 944 | 4.242 | 0.621 | 211 | 2.9 |
| 7 | AM J GASTROENTEROL | 0002-9270 | 17 923 | 4.172 | 0.678 | 391 | 5.1 |
| 8 | ALIMENT PHARM THERAP | 0269-2813 | 60 32 | 3.529 | 0.573 | 372 | 3.9 |
| 9 | AM J PHYSIOL-GASTR L | 0193-1857 | 11 047 | 3.421 | 0.634 | 268 | 5.9 |
| 10 | GASTROINTEST ENDOSC | 0016-5107 | 10 047 | 3.328 | 1.798 | 331 | 4.8 |
| **11** | **WORLD J GASTROENTERO** | **1007-9327** | **2 387** | **3.318** | **0.345** | **632** | **2.4** |
| 12 | J VIRAL HEPATITIS | 1352-0504 | 1 320 | 3.258 | 0.386 | 70 | 4.0 |
| 13 | ENDOSCOPY | 0013-726X | 4 238 | 3.227 | 0.349 | 152 | 5.5 |
| 14 | INFLAMM BOWEL DIS | 1078-0998 | 1 278 | 3.023 | 0.625 | 40 | 3.8 |
| 15 | HELICOBACTER | 1083-4389 | 798 | 2.624 | 0.426 | 68 | 3.6 |
| 16 | NEUROGASTROENT MOTIL | 1350-1925 | 848 | 2.500 | 0.333 | 66 | 3.7 |
| 17 | DIS COLON RECTUM | 0012-3706 | 8 130 | 2.343 | 0.191 | 256 | 7.6 |
| 18 | SCAND J GASTROENTERO | 0036-5521 | 7 271 | 2.140 | 0.118 | 238 | 7.6 |
| 19 | LIVER | 0106-9543 | 1 337 | 2.076 | | 0 | 5.4 |
| 20 | J GASTROINTEST SURG | 1091-255X | 1 236 | 1.881 | 0.430 | 142 | 3.6 |
| 21 | PANCREAS | 0885-3177 | 2 271 | 1.855 | 0.364 | 140 | 5.8 |
| 22 | INT J COLORECTAL DIS | 0179-1958 | 1 167 | 1.848 | 0.373 | 83 | 6.4 |
| 23 | GASTROENTEROL CLIN N | 0889-8553 | 1 517 | 1.684 | 0.019 | 54 | 6.7 |
| 24 | PANCREATOLOGY | 1424-3903 | 240 | 1.596 | 0.115 | 52 | 2.2 |
| 25 | EUR J GASTROEN HEPAT | 0954-691X | 3 163 | 1.578 | 0.367 | 177 | 4.6 |
| 26 | J CLIN GASTROENTEROL | 0192-0790 | 3 215 | 1.564 | 0.484 | 155 | 7.2 |
| 27 | J GASTROEN HEPATOL | 0815-9319 | 3 171 | 1.530 | 0.254 | 193 | 4.5 |
| 28 | DIGEST LIVER DIS | 1590-8658 | 637 | 1.463 | 0.151 | 159 | 2.5 |
| 29 | J PEDIATR GASTR NUTR | 0277-2116 | 3 963 | 1.402 | 0.257 | 183 | 6.1 |
| 30 | DIGESTION | 0012-2823 | 2 193 | 1.399 | 0.091 | 55 | 7.7 |
| 31 | DIGEST DIS SCI | 0163-2116 | 9 149 | 1.387 | 0.144 | 347 | 7.9 |
| 32 | CAN J GASTROENTEROL | 0835-7900 | 813 | 1.265 | 0.134 | 82 | 3.9 |
| 33 | J GASTROENTEROL | 0944-1174 | 1 625 | 1.179 | 0.319 | 191 | 4.5 |
| 34 | DIGEST DIS | 0257-2753 | 534 | 1.151 | 0.031 | 32 | 5.7 |
| 35 | Z GASTROENTEROL | 0044-2771 | 918 | 1.076 | 0.112 | 116 | 5.4 |
| 36 | ABDOM IMAGING | 0942-8925 | 1 190 | 0.996 | 0.194 | 124 | 5.0 |
| 37 | BEST PRACT RES CL GA | 1521-6918 | 342 | 0.992 | 0.000 | 66 | 3.4 |
| 38 | HEPATOL RES | 1386-6346 | 406 | 0.991 | 0.168 | 155 | 2.6 |
| 39 | GASTROEN CLIN BIOL | 0399-8320 | 1 497 | 0.884 | 0.235 | 153 | 6.3 |
| 40 | HEPATO-GASTROENTEROL | 0172-6390 | 3 886 | 0.837 | 0.044 | 544 | 5.0 |
| 41 | DIS ESOPHAGUS | 1120-8694 | 354 | 0.809 | 0.027 | 75 | 4.7 |
| 42 | ACTA GASTRO-ENT BELG | 0001-5644 | 345 | 0.670 | 0.065 | 31 | 5.2 |
| 43 | DIGEST SURG | 0253-4886 | 548 | 0.619 | 0.114 | 79 | 4.2 |
| 44 | CURR OPIN GASTROEN | 0267-1379 | 300 | 0.598 | 0.050 | 60 | 3.9 |
| 45 | REV ESP ENFERM DIG | 1130-0108 | 255 | 0.348 | 0.211 | 57 | 6.0 |
| 46 | CHIR GASTROENTEROL | 0177-9990 | 74 | 0.157 | 0.039 | 76 | |
| 47 | LIVER INT | 1478-3231 | 4 | | 0.053 | 75 | |

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• NEWS •

# Citation indexes of Chinese scientific journals in 2003 (ISI JCR 2004)

| Rank | Abbreviated journal title | 2003 total cites | Impact factor | Index immediacy | 2003 articles | Cited half-life |
|---|---|---|---|---|---|---|
| **1** | **WORLD J GASTROENTERO** | **2 387** | **3.318** | **0.345** | **632** | **2.4** |
| 2 | CHINESE J ASTRON AST | 229 | 1.768 | 0.065 | 62 | 2.2 |
| 3 | CELL RES | 354 | 1.729 | 0.192 | 52 | 3.2 |
| 4 | FUNGAL DIVERS | 207 | 1.437 | 0.189 | 53 | 2.5 |
| 5 | CHINESE PHYS | 855 | 1.347 | 0.193 | 233 | 2.3 |
| 6 | ACTA PHYS SIN[1] | 2 410 | 1.130 | 0.172 | 586 | 2.9 |
| 7 | CHINESE PHYS LETT | 1 955 | 1.095 | 0.173 | 664 | 2.4 |
| 8 | ACTA PETROL SIN[1] | 444 | 1.078 | 0.067 | 89 | 4.0 |
| 9 | ASIAN J ANDROL | 188 | 1.064 | 0.140 | 57 | 2.6 |
| 10 | ACTA GEOL SIN | 467 | 1.040 | 0.457 | 46 | 4.4 |
| 11 | EPISODES | 411 | 1.020 | 0.056 | 36 | 7.0 |
| 12 | ACTA PHARMACOL SIN | 1 586 | 0.884 | 0.120 | 208 | 4.8 |
| 13 | SCI CHINA SER D | 737 | 0.801 | 0.272 | 151 | 3.8 |
| 14 | CHEM J CHINESE U[1] | 2 391 | 0.796 | 0.093 | 569 | 3.7 |
| 15 | COMMUN THEOR PHYS | 727 | 0.666 | 0.242 | 310 | 2.3 |
| 16 | ACTA CHIM SINICA | 1 375 | 0.643 | 0.105 | 381 | 3.9 |
| 17 | BIOMED ENVIRON SCI | 387 | 0.609 | 0.043 | 47 | 6.7 |
| 18 | CHINESE SCI BULL | 2 302 | 0.593 | 0.136 | 544 | 4.7 |
| 19 | CHINESE J CHEM | 578 | 0.592 | 0.118 | 255 | 2.7 |
| 20 | ACTA MECH SINICA | 270 | 0.587 | 0.043 | 70 | 6.2 |
| 21 | CHINESE J STRUC CHEM[1] | 319 | 0.548 | 0.039 | 152 | 3.3 |
| 22 | CHINESE J CATAL | 396 | 0.542 | 0.083 | 180 | 3.6 |
| 23 | SCI CHINA SER B | 838 | 0.541 | 0.069 | 72 | 9.2 |
| 24 | CHINESE J INORG CHEM[1] | 481 | 0.535 | 0.075 | 281 | 3.0 |
| 25 | ACTA BIOCH BIOPH SIN[1] | 386 | 0.524 | 0.053 | 190 | 3.7 |
| 26 | CHINESE J ORG CHEM[1] | 430 | 0.497 | 0.069 | 246 | 3.3 |
| 27 | J RARE EARTH | 263 | 0.486 | 0.049 | 144 | 3.6 |
| 28 | ACTA PHYS-CHIM SIN[1] | 516 | 0.468 | 0.093 | 257 | 3.2 |
| 29 | ADV ATMOS SCI | 231 | 0.449 | 0.069 | 101 | 4.3 |
| 30 | SCI CHINA SER C | 228 | 0.440 | 0.029 | 68 | 4.3 |
| 31 | J UNIV SCI TECHNOL B | 145 | 0.437 | 0.087 | 104 | 2.7 |
| 32 | CHINA OCEAN ENG | 95 | 0.413 | 0.119 | 59 | |
| 33 | CHINESE MED J | 1 245 | 0.393 | 0.081 | 431 | 6.0 |
| 34 | ACTA MECH SOLIDA SIN | 115 | 0.389 | 0.021 | 47 | 4.0 |
| 35 | CHINESE J GEOPHYS[1] | 278 | 0.375 | 0.038 | 133 | 4.4 |
| 36 | CHEM RES CHINESE U | 175 | 0.370 | 0.054 | 112 | 3.6 |
| 37 | CHINESE J CHEM | 172 | 0.357 | 0.062 | 145 | 3.0 |
| 38 | J WUHAN UNIV TECHNOL | 139 | 0.356 | 0.037 | 81 | 3.1 |
| 39 | SCI CHINA SER E | 210 | 0.355 | 0.028 | 72 | 3.8 |
| 40 | ACTA POLYM SIN[1] | 410 | 0.351 | 0.055 | 146 | 4.4 |
| 41 | CHINESE ANN MATH B | 169 | 0.343 | 0.019 | 52 | 5.4 |
| 42 | CHINESE CHEM LETT | 717 | 0.342 | 0.028 | 394 | 3.8 |
| 43 | PROG NAT SCI | 360 | 0.335 | 0.136 | 154 | 3.6 |
| 44 | RARE METAL MAT[1] | 435 | 0.329 | 0.149 | 262 | 3.1 |
| 45 | T NONFERR METAL SOC | 413 | 0.322 | 0.026 | 348 | 3.6 |
| 46 | ACTA BOT SIN | 712 | 0.321 | 0.063 | 222 | 5.3 |
| 47 | PROG CHEM | 121 | 0.319 | 0.045 | 66 | 3.9 |
| 48 | J CENT SOUTH UNIV T | 80 | 0.299 | 0.000 | 75 | |
| 49 | SPECTROSC SPECT ANAL[1] | 427 | 0.298 | 0.020 | 352 | 3.4 |
| 50 | ACTA MATH SIN | 327 | 0.297 | 0.041 | 49 | 6.6 |
| 51 | HIGH ENERG PHYS NUC[1] | 293 | 0.285 | 0.105 | 238 | 3.0 |
| 52 | J MATER SCI TECHNOL | 318 | 0.275 | 0.026 | 190 | 3.7 |
| 53 | ALGEBR COLLOQ | 106 | 0.274 | 0.038 | 52 | 5.9 |
| 53 | RARE METALS | 98 | 0.274 | 0.033 | 61 | |
| 55 | J ENVIRON SCI-CHINA | 137 | 0.255 | 0.014 | 140 | 4.3 |
| 56 | APPL MATH MECH | 422 | 0.251 | 0.086 | 152 | 5.1 |
| 57 | SCI CHINA SER A | 722 | 0.247 | 0.024 | 85 | 7.3 |
| 57 | ACTA METALL SIN | 569 | 0.247 | 0.097 | 258 | 4.8 |
| 57 | J INORG MATER[1] | 346 | 0.247 | 0.030 | 234 | 4.0 |
| 60 | J IRON STEEL RES INT | 40 | 0.245 | 0.000 | 43 | |
| 61 | PROG BIOCHEM BIOPHYS[1] | 206 | 0.241 | 0.048 | 145 | 3.0 |
| 62 | CHINESE J ANAL CHEM[1] | 971 | 0.224 | 0.036 | 389 | 5.2 |
| 63 | J INFRARED MILLIM W | 107 | 0.160 | 0.019 | 105 | 3.5 |
| 64 | J COMPUT MATH | 188 | 0.153 | 0.013 | 79 | >10.0 |
| 65 | CHINESE ASTRON ASTR | 57 | 0.149 | 0.000 | 57 | |
| 66 | J COMPUT SCI TECHNOL | 50 | 0.140 | 0.040 | 99 | |
| 67 | ACTA MATH SCI | 119 | 0.127 | 0.000 | 50 | >10.0 |

[1]Chinese.

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• NEWS •

# Impact factors and citation times of Chinese scientific journals covered by ISI JCR (2000-2003)

| Abbreviated journal title | Impact factor | | | | Citation time | | | |
|---|---|---|---|---|---|---|---|---|
| | 2000 | 2001 | 2002 | 2003 | 2000 | 2001 | 2002 | 2003 |
| ACTA BIOCH BIOPH SIN[1] | 0.289 | 0.399 | 0.596 | 0.524 | 150 | 217 | 377 | 386 |
| ACTA BOT SIN | 0.434 | 0.284 | 0.376 | 0.321 | 698 | 516 | 1 336 | 712 |
| ACTA CHIM SINICA[1] | 0.435 | 0.530 | 0.536 | 0.643 | 844 | 990 | 1 298 | 1 375 |
| ACTA GEOL SIN | 1.000 | 0.271 | 0.531 | 1.040 | 323 | 234 | 316 | 467 |
| ACTA MATH SCI | | | 0.104 | 0.127 | | | 108 | 119 |
| ACTA MATH SIN | 0.234 | 0.324 | 0.407 | 0.297 | 212 | 275 | 299 | 327 |
| ACTA MECH SINICA | 0.575 | 0.734 | 0.726 | 0.587 | 180 | 242 | 265 | 270 |
| ACTA MECH SOLIDA SIN | 0.215 | 0.191 | 0.226 | 0.389 | 68 | 70 | 78 | 115 |
| ACTA METALL SIN | | | | 0.247 | | | | 569 |
| ACTA PETROL SINICA[1] | | | 0.534 | 1.078 | | | 289 | 444 |
| ACTA PHARMACOL SIN | 0.485 | 0.631 | 0.688 | 0.884 | 854 | 958 | 1 575 | 1 586 |
| ACTA PHYS SIN[1] | | 0.657 | 1.182 | 1.130 | | 1 227 | 2 277 | 2 410 |
| ACTA PHYS SIN | 0.210 | 0.369 | | | 100 | 176 | | |
| ACTA PHYS-CHIM SIN[1] | 0.192 | 0.269 | 0.361 | 0.468 | 119 | 283 | 350 | 516 |
| ACTA POLYM SIN | | 0.377 | 0.288 | 0.351 | | 314 | 358 | 410 |
| ADV ATMOS SCI | | 0.327 | 0.288 | 0.449 | | 146 | 172 | 231 |
| ALGEBR COLLOQ | 0.089 | 0.188 | 0.150 | 0.274 | 25 | 84 | 94 | 106 |
| APPL MATH MECH | 0.166 | 0.155 | 0.199 | 0.251 | 250 | 267 | 354 | 422 |
| ASIAN J ANDROL | | | 0.827 | 1.064 | | | 99 | 188 |
| BIOMED ENVIRON SCI | 0.400 | 0.437 | 0.500 | 0.609 | 359 | 332 | 390 | 387 |
| CELL RES | | 2.102 | 1.958 | 1.729 | | 244 | 308 | 354 |
| CHEM J CHINESE U[1] | 0.590 | 0.904 | 0.830 | 0.796 | 1 421 | 1 959 | 2 189 | 2 391 |
| CHEM RES CHINESE U | 0.260 | 0.223 | 0.229 | 0.370 | 125 | 106 | 131 | 175 |
| CHINA OCEAN ENG | 0.137 | 0.206 | 0.196 | 0.413 | 24 | 46 | 41 | 95 |
| CHINESE ANN MATH B | 0.153 | | 0.296 | 0.343 | 116 | | 146 | 169 |
| CHINESE ASTRON ASTR | 0.164 | 0.144 | 0.181 | 0.149 | 49 | 47 | 60 | 57 |
| CHINESE CHEM LETT | 0.229 | 0.289 | 0.347 | 0.342 | 536 | 603 | 733 | 717 |
| CHINESE J ANAL CHEM[1] | | 0.288 | 0.240 | 0.224 | | 992 | 949 | 971 |
| CHINESE J ASTRON AST | | | 0.879 | 1.768 | | 1 | 65 | 229 |
| CHINESE J CATAL | | | | 0.542 | | | | 396 |
| CHINESE J CHEM | 0.707 | 0.663 | 0.558 | 0.592 | 318 | 369 | 413 | 578 |
| CHINESE J CHEM | 0.124 | 0.223 | 0.222 | 0.357 | 55 | 101 | 117 | 172 |
| CHINESE J GEOPHYS[1] | 0.097 | 0.155 | 0.122 | 0.375 | 13 | 34 | 45 | 278 |
| CHINESE J INORG CHEM[1] | | 0.301 | 0.494 | 0.535 | | 141 | 280 | 481 |
| CHINESE J ORG CHEM[1] | | 0.294 | 0.377 | 0.497 | | 271 | 338 | 430 |
| CHINESE J STRUCT CHEM[1] | | 0.184 | 0.324 | 0.548 | | 168 | 260 | 319 |
| CHINESE MED J[1] | 0.107 | 0.108 | 0.182 | 0.393 | 752 | 807 | 909 | 1 245 |
| CHINESE PHYS | | 0.828 | 1.185 | 1.347 | 39 | 200 | 542 | 855 |
| CHINESE PHYS LETT | 0.638 | 0.813 | 1.036 | 1.095 | 761 | 1 215 | 1 644 | 1 955 |
| CHINESE SCI BULL | 0.414 | 0.511 | 0.570 | 0.593 | 1 321 | 1 628 | 2 030 | 2 302 |
| COMMUN THEOR PHYS | 0.302 | 0.397 | 0.453 | 0.666 | 350 | 479 | 577 | 727 |
| EPISODES | | | | 1.020 | | | | 411 |
| FUNGAL DIVERS | | | | 1.437 | | | | 207 |
| HIGH ENERG PHYS NUC[1] | 0.264 | 0.324 | 0.248 | 0.285 | 225 | 271 | 248 | 293 |
| J ASIAN NAT PROD RES | 0.294 | 0.508 | | | 16 | 54 | | |
| J CENT SOUTH UNIV T | | | 0.052 | 0.299 | | | 6 | 80 |
| J COMPUT MATH | 0.250 | 0.168 | 0.135 | 0.153 | 175 | 155 | 173 | 188 |
| J COMPUT SCI TECHNOL | | | 0.154 | 0.140 | | | 53 | 50 |
| J ENVIRON SCI-CHINA | | | | 0.255 | | | | 137 |
| J INFRARED MILLIM W[1] | 0.146 | 0.198 | 0.121 | 0.160 | 39 | 68 | 85 | 107 |
| J INORG MATER[1] | | 0.131 | 0.222 | 0.247 | | 77 | 260 | 346 |
| J IRON STEEL RES INT | 0.195 | 0.171 | 0.083 | 0.245 | 18 | 21 | 31 | 40 |
| J MATER SCI TECHNOL | 0.241 | 0.269 | 0.239 | 0.275 | 164 | 220 | 271 | 318 |
| J RARE EARTH | 0.125 | 0.236 | 0.287 | 0.486 | 57 | 117 | 147 | 263 |
| J UNIV SCI TECHNOL B | 0.118 | 0.099 | 0.397 | 0.437 | 38 | 37 | 100 | 145 |
| J WUHAN UNIV TECHNOL | 0.185 | 0.140 | 0.376 | 0.356 | 41 | 56 | 119 | 139 |
| PROG BIOCHEM BIOPHYS[1] | 0.077 | 0.112 | 0.160 | 0.241 | 95 | 109 | 140 | 206 |
| PROG CHEM | | | | 0.319 | | | | 121 |
| PROG NAT SCI | 0.249 | 0.288 | 0.264 | 0.335 | 167 | 279 | 309 | 360 |
| RARE METAL MAT ENG[1] | 0.242 | 0.319 | 0.225 | 0.329 | 103 | 151 | 223 | 435 |
| RARE METALS | | 0.142 | 0.284 | 0.274 | | 60 | 69 | 98 |
| SCI CHINA SER A | 0.309 | 0.340 | 0.295 | 0.247 | 528 | 627 | 682 | 722 |
| SCI CHINA SER B | 0.751 | 0.840 | 0.702 | 0.541 | 799 | 925 | 895 | 838 |
| SCI CHINA SER C | 0.291 | 0.396 | 0.358 | 0.440 | 155 | 171 | 180 | 228 |
| SCI CHINA SER D | 0.475 | 0.610 | 0.688 | 0.801 | 236 | 369 | 580 | 737 |
| SCI CHINA SER E | 0.309 | 0.376 | 0.412 | 0.355 | 91 | 140 | 197 | 210 |
| SPECTROSC SPECT ANAL[1] | | 0.250 | 0.293 | 0.298 | | 280 | 377 | 427 |
| T NONFERR METAL SOC | 0.294 | 0.340 | 0.293 | 0.322 | 221 | 306 | 374 | 413 |
| **WORLD J GASTROENTERO** | **0.993** | **1.445** | **2.532** | **3.318** | **327** | **722** | **1 535** | **2 387** |
| TOTAL | | | | | 13 557 | 20 957 | 28 866 | 35 842 |

[1]Chinese.