

Impact of *Helicobacter pylori* on the growth of hepatic orthotopic graft tumors in mice

JUNWEI WANG¹⁻³, XIAOQIAN WANG³, NANHONG TANG³, YANLING CHEN^{1,3} and FEIFEI SHE^{1,2}

¹Key Laboratory of Ministry of Education for Gastrointestinal Cancer, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian; ²Key Laboratory of Tumor Microbiology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian; ³Department of Hepatobiliary Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian 350001, P.R. China

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Abstract. *Helicobacter pylori* is a well-known causative organism of chronic gastric diseases and has been found in many hepatic carcinoma samples. To explore the expression of apoptosis-related proteins and carcinoma development in *H. pylori*-infected livers, we utilized BALB/cAnSlac mice to establish an *H. pylori*-infected model by oral inoculation and orthotopic grafts of hepatic tumors by H22 cells, respectively. We found that *H. pylori* colonies could not be cultured from all liver and tumor samples. However, its 16S rRNA was detectable in 85.3% of livers and 66.7% of tumors in the infected mice. Inflammatory cells were observed and thinly distributed in the lobule portions of the liver, and *H. pylori* mainly existed in the infected hepatic sinusoids and the necrotic areas of the infected tumors. No significant difference was found in liver to body weight ratio between the infected and uninfected. Moreover, the pathological tumor difference was unremarkable between the two. The proliferating cell nuclear antigen (PCNA) and Bcl-2-associated X protein (Bax) expression in the infected tumors was significantly higher and lower, respectively, than those of the uninfected tumors. However, no significant difference in Bcl-2 (B-cell lymphoma 2) expression existed. The results indicate that *H. pylori* found in the livers which were infected by *H. pylori* oral inoculation could contribute to the infiltration of inflammatory cells in livers. Although *H. pylori* has no significant impact on

the liver to body weight ratio or tumor Bcl-2 expression, it may upregulate PCNA expression and downregulate Bax expression, respectively. All our findings show that *H. pylori* may promote proliferation and inhibit apoptosis of tumor cells.

Introduction

As one of the most common pathogens in human bacteria-related chronic infection and carcinomatous diseases, *Helicobacter pylori* infects almost 50% of the world population (1). In 1994, the World Health Organization (WHO) and International Agency for Research on Cancer (IARC) classified it as a type I carcinogen (2). It has been reported that *H. pylori* plays a crucial role in the occurrence and development of chronic active gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma (MALT) and even gastric adenocarcinoma (3). Moreover, *H. pylori* is also closely related to some hematological systemic disorders, for example, hypoferric anemia and idiopathic thrombocytopenic purpura. Recently, it was demonstrated that it is involved in extragastric diseases such as atherosclerosis, ischemic heart disease, immunologic dysfunction, migraines and pediatric growth retardation (4-7). According to abundant clinical studies which had demonstrated morphological and molecular biological evidence, the infection rate of *H. pylori* is higher in patients with chronic hepatic diseases such as chronic hepatitis, liver cirrhosis and liver cancer than the rate of healthy persons (8-14). Xuan *et al* (14) even positively cultured *H. pylori* in 3 (10.7%) of their 28 primary hepatic carcinoma patients.

Primary hepatic cancer is an epidemic malignant tumor worldwide, and ~665,000 new cases are diagnosed every year (15). With an extremely high mortality rate, it ranks second as a cancer-related cause of death and is responsible for 746,000 deaths worldwide in 2012 (16). Its 5-year survival rate in developed countries, such as the United States, is 9% (15), whereas <5% worldwide (17), due to its high postoperative recurrence rate and poor prognosis (18,19). Thus, interference from the source is the most effective measure that might etiologically prevent the occurrence and development of primary hepatic cancer. Hepatitis B virus, hepatitis C virus, aflatoxin, chemical carcinogens and parasitic infections have been identified as pathogenic factors in primary hepatic cancer (20-22).

Correspondence to: Professor Feifei She, Key Laboratory of Tumor Microbiology, School of Basic Medical Sciences, Fujian Medical University, 1 Xueyuan Road, Fuzhou, Fujian 350001, P.R. China

E-mail: shefeifei@yeah.net

Professor Yanling Chen, Department of Hepatobiliary Surgery, Fujian Medical University Union Hospital, 29 Xinquan Road, Fuzhou, Fujian 350001, P.R. China

E-mail: ylchen@medmail.com.cn

Abbreviations: *H. pylori*, *Helicobacter pylori*; PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein

Key words: *H. pylori*, hepatic tumor, PCNA, Bcl-2, Bax

However, basing on the discovery that *H. pylori* was found in the liver of patients who suffered from chronic hepatic diseases, researchers have proposed that it plays a role in hepatitis and hepatic cancer. This novel hypothesis prompted studies concerning the correlation between *H. pylori* and hepatic cancer (23,24). The findings may also be helpful in prophylaxis and treatment of hepatic cancer.

Ito *et al* (25), Zhang *et al* (26) and Chen *et al* (27) co-cultured human hepatoma cells with *H. pylori* *in vitro* and determined the interaction of *H. pylori* with hepatocyte surface molecular receptors. *H. pylori* adhered to and invaded hepatoma cells. As a result, it caused a cytotoxic effect that upregulated tumor-related cyclin D1 and PCNA (proliferating cell nuclear antigen). However, they mainly performed studies *in vitro* that could hardly simulate the microenvironment of liver *in vivo*. Thus, the function of *H. pylori* in liver cancer remains unclear. In the present study, BALB/cAnSlac mice were orally inoculated with an infective dose of *H. pylori*. Then, we developed a tumor-bearing mouse model. Finally, we evaluated the infection status and explored the role of *H. pylori* in the development of hepatic cancer.

Materials and methods

Animals. Six to eight weeks old male and female specific-pathogen-free (SPF) BALB/cAnSlac mice whose fecal DNA were determined by C97/C98 *Helicobacter* spp. primers were obtained from Slaccas Laboratory Animal Co., Ltd., (Shanghai, China; License no. SCXK 2002-0003, Certificate no. 2007000551273) and housed under SPF conditions in Fujian Medical University Laboratory Animal Center (Fuzhou, China; License no. SYXK 2012-0001). All the following pathogens were excluded from SPF BALB/cAnSlac mice [SPF Laboratory Animal Standard of the People's Republic of China (GB 14922.2-2011)]. *Salmonella* spp.; *Yersinia pseudotuberculosis*; *Yersinia enterocolitica*; *Pathogenic dermal fungi*; *Streptobacillus moniliformis*; *Bordetella bronchiseptica*; *Mycoplasma* spp.; *Corynebacterium kutscheri*; *Tyzzer's organism*; *Escherichia coli* O115 a, C, K (B); *Pasteurella pneumotropica*; *Klebsiella pneumonia*; *Staphylococcus aureus*; *Streptococcus pneumoniae*; β -hemolytic *streptococcus*; *Pseudomonas aeruginosa*; lymphocytic choriomeningitis virus (LCMV); hantavirus (HV); ectromelia virus (Ect.); mouse hepatitis virus (MHV); sendai virus (SV); pneumonia virus of mice (PVM); reovirus type III (Reo-3); minute virus of mice (MVM); Theiler's mouse encephalomyelitis virus (TMEV); mouse adenovirus (Mad); polyomavirus (POLY); rat parvovirus (KRV); rat parvovirus (H-1); rat coronavirus (RCV)/sialodacryoadenitis virus (SDAV). All mice were fed a sterilized commercial diet, given water *ad libitum*, and allowed to acclimatize for at least 1 week before the experiments. All animal protocols met the approval of the Institutional Animal Care Committee.

Bacterial strains and culture conditions. *H. pylori* type strain NCTC 11637 (National Institute For Communicable Disease Control And Prevention, Chinese Center For Disease Control And Prevention, Beijing, China) was used in the present study. Columbia agar base was used (Oxoid Ltd., Hampshire, UK) that was supplemented with 8% sheep

blood (Bio-Kont Technology Co., Ltd., Xiamen, China) and *H. pylori* selective supplements (Oxoid) containing vancomycin (0.01 mg/ml), cefsulodin (0.5 mg/ml), trimethoprim (0.5 mg/ml) and amphotericin B (0.5 mg/ml). The bacteria were incubated in microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂, 37°C, humidity >90%; LEEC Touch 190S; LEEC Ltd., Nottingham, UK) and harvested in sterile phosphate-buffered saline (PBS) after 48 h of growth, centrifuged at 4000 x g for 2 min, and adjusted in sterile PBS to a final concentration of 5x10⁹ colony-forming units per milliliter (CFU/ml). Additionally, *H. pylori* was not only assessed by Gram staining and phase microscopy for purity, morphology and motility, but also tested for urease, catalase and oxidase activity before any animal experiments.

Cell line and cultivation. H22 murine hepatic hepatoma cells were obtained from China Center for Type Culture Collection (Wuhan, China) and syngeneic to BALB/cAnSlac mice. The cells were initially grown in a complete RPMI-1640 medium (HyClone Laboratories Inc., Logan, UT, USA) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO₂ *in vitro* for 2-3 days. Subsequently, the cell suspension was tested for *Helicobacter* and other microorganisms prior to mouse injection or implantation. The cell suspension was spread evenly over Columbia agar base and kept in microaerophilic condition for microaerophilic bacteria detection. So did the Luria-Bertani base in usual condition (37°C) for aerobic bacteria detection. Additionally, after centrifugation the DNA was extracted by Genomic DNA Mini extraction kit (Beyotime Institute of Biotechnology, Haimen, China) for *Helicobacter* genus-specific 16S rRNA (C97/C98) test. Under the circumstance that all detection methods above were negative for microorganisms, we conducted the further protocols. The cell suspension was collected and centrifuged at 2000 x g for 5 min and adjusted with a serum-free RPMI-1640 medium to a final concentration of 1x10⁷/ml. Every BALB/cAnSlac mouse was intraperitoneally injected with a 0.2-ml cell suspension for the first *in vivo* subcultivation for a 7-day period. Finally, the mouse was euthanized by cervical dislocation, and its ascites was collected and centrifuged. Ascitic precipitate cells were washed and adjusted to the same concentration. The cell suspension was intraperitoneally injected into another BALB/cAnSlac mouse for the second *in vivo* subcultivation. Following similar, previously discussed protocols, a third *in vivo* subcultivation was performed. Then, the centrifuged cells were adjusted to 4x10⁷/ml and were used in an orthotopic implantation.

Drug pretreatment. *Lactobacilli* which inhabit the stomachs of the mice would interfere with the growth of *H. pylori* even eradicate it (28). All 70 experimental mice were treated on the 1st to 3rd day with the drugs listed in Table I, according to the methods described by Thalmaier *et al* (29). A mixed solution of ciprofloxacin (Zhejiang Jingxin Pharmaceutical, Co., Ltd., Zhejiang, China), amikacin (Shanghai Harvest Pharmaceutical Co., Ltd., Shanghai, China), imipenem (Merck Sharp & Dohme Corp., Kenilworth, NJ, USA), vancomycin (Zhejiang Hisun Pharmaceutical Co., Ltd., Zhejiang, China), and fluconazole (Yangtze River Pharmaceutical Group, Taizhou, China) were administered once daily.

Table I. Drugs and dose scheme ($\mu\text{g}/\text{mouse}/\text{day}$).

Antibiotics	Oral dose	Intraperitoneal dose
Ciprofloxacin	500	0
Amikacin	375	375
Imipenem	1250	1250
Vancomycin	1000	1000
Fluconazole	150	0

μg , microgram.

Infection protocols. On day 3 (after the last drug treatment), 70 mice were randomly divided into group A ($n=40$, *H. pylori* infected mice) and group B ($n=30$, uninfected mice). From 3rd to 9th day, all mice were fasted for 15-18 h overnight before the treatment on the following day. The next day, every mouse was orally inoculated with a 0.2-ml sterilized alkaline buffer (300 mM NaHCO_3) to neutralize gastric acidity. Then, 15-30 min later, each mouse in group A was administered an oral *H. pylori* suspension (1×10^9 CFU; in 0.2 ml of sterilized PBS) once daily for a continuous 7-day period (4th to 10th day; defined as the first week); group B was administered a 0.2-ml sham dose of sterilized PBS in a same manner and schedule (30-32). All mice underwent hepatic surgery during the 9th week.

Orthotopic hepatic carcinoma model. At the beginning of the 9th week, the mice in group A were randomly divided into group A1 ($n=20$; *H. pylori* infected and tumor positive mice) and group A2 ($n=20$; *H. pylori* infected and tumor negative mice), and group B was divided into group B1 ($n=20$, uninfected and tumor positive mice) and group B2 ($n=10$, uninfected and tumor negative mice). The left liver lobe of the mice was implanted with 50 μl of H22 cell suspension (2×10^6 cells; mice in group A1 and B1) or just 50 μl serum-free RPMI-1640 medium (mice in group A2 and B2) with a subcapsular intrahepatic injection according to Yao *et al* (33) and Aprahamian *et al* (34). Each mouse was anesthetized with 80 mg/kg ketamine (Sunkind Co., Ltd., Shanxi, China) by intraperitoneal injection. The mouse was placed in a supine position on the operating table when it was fully anesthetized. A small median longitudinal incision was made below the xiphoid to expose the left lobe of the liver, and sterile gauze was placed under it to prevent peritoneal sowing. H22 cells in a serum-free RPMI-1640 medium were slowly injected into the parenchyma of the left hepatic lobe at a 30-degree angle with a 100- μl microinjector (Shanghai Bolige Industry & Trade Co., Ltd., Shanghai, China), thus, a transparent bleb of medium could be seen within the hepatic capsule. A sterile cotton swab was gently compressed on the injection site for hemostasis, and the abdomen was closed separately in a two layer method (peritoneum and skin) with a 4-0 silk suture. The mice were kept in SPF warm incubator for observation and finally returned to the SPF animal room when they had fully recovered from the anesthesia.

Tissue collection and bacterial isolation. The mice were fasted for 24 h overnight before necropsy. At the end of the 13th week, all mice were euthanized by cervical dislocation and necropsied for bacterial isolation and histopathology. The hemorrhagic ascites was firstly collected from bulge abdomen of tumor positive mouse by a 2-ml aseptically injector. The ascites was fully mixed and piped (200 μl) onto the Columbia agar base for *H. pylori* culture in microaerophilic environment. The remainder was centrifuged at 4000 \times g for 5 min. The supernatant was tested for AFP (α -fetoprotein) by ELISA kit (Wuhan Boster Biological Technology, Co., Ltd., Wuhan, China) and compared with that in serum of both tumor positive and negative mouse. The DNA of cellular precipitate was extracted by Genomic DNA Mini extraction kit (Beyotime Institute of Biotechnology) and tested for *Helicobacter* genus-specific 16S rRNA (C97/C98). Samples of blood, stomach and liver were aseptically collected and cultured during necropsy. A total of 150 μl of blood samples were collected from inferior vena cava puncture. One hundred microliters were spread evenly over a Columbia agar plate for *H. pylori* cultivation and 50 μl were stored at -80°C until for DNA and AFP analysis. The liver, as a whole, was removed and weighed. Then, the left liver lobe (group A2 and B2) was directly divided into three sections. The first section (50-100 mg) was ground in sterile grinders with 100 μl of sterile PBS and spread evenly over a Columbia agar plate for *H. pylori* cultivation. The second section (15 \times 15 \times 3 mm) used for histological analysis was placed into 10% normal buffered formalin for 24 h, and embedded in paraffin. Sections (3- μm) were cut. The slides were stained with hematoxylin and eosin (H&E; conducted by the Pathology Department of Affiliated Union Hospital of Fujian Medical University, Fuzhou, China) for assessment of histopathological changes and immunohistochemistry (IHC) staining for *H. pylori*, PCNA, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax). The third section was stored at -80°C for DNA and protein analysis. Equally, the tumor in the left liver lobe (A1 and B1 group) was also divided, processed and analyzed as the protocols described above. Finally, the stomach was removed, opened along its greater curvature, and rinsed gently in sterile cold PBS. When the contents had been totally removed, the stomach was placed (mucosal side up) on sterile gauze and longitudinally dissected along the greater curvature into three fragments, so that each one contained the gastric cardia, body and antrum. The three gastric fragments were processed and analyzed. All Columbia agar plates were incubated in a microaerophilic condition (5% O_2 , 10% CO_2 , 85% N_2 , humidity $>90\%$) at 37°C . The plates were evaluated for *H. pylori* growth from 3rd to 10th day after necropsy. The presence of colonies were confirmed using morphology, Gram staining, biochemical tests (urease, catalase and oxidase reactions) and polymerase chain reaction (PCR). The plates were discarded and defined as negative if no positive colony was detected until the 10th day.

DNA extraction and PCR amplification. DNA of samples (blood, stomachs, livers and tumors) were isolated with Genomic DNA Mini extraction kit (Beyotime Institute of Biotechnology). According to the manufacturer's instruction, ~ 25 mg of tissue was completely triturated with an electric grinder (Tiangen Biotech, Co., Ltd., Beijing, China),

and then, the homogenate (or 50 μ l of blood) was mixed in a vortex with 180 μ l of sample lysis buffer A and 20 μ l of Proteinase K. The mixture was incubated overnight at 55°C, thus, the tissues could be fully cleaved. The next day, 200 μ l of sample lysis buffer B was added to and incubated with them for 10 min at 70°C. The sample was fully mixed with 200 μ l of dehydrated ethanol and then added to a clean spun column. The mixture was washed and centrifuged with a series of washing buffer. Finally, 50 μ l of elution buffer was pipetted into the spun column. The DNA was collected with the last high-speed centrifugation, and its concentration was adjusted to 80 ng/ μ l for the following analysis. The extracted DNA was amplified with *Helicobacter* genus-specific 16S rRNA primers (35): sense primer, 5'-GCT ATG ACG GGT ATC C-3' (C97F) and antisense primer, 5'-GAT TTT ACC CCT ACA CCA-3' (C98R). The sense and antisense primers amplified a 400-bp product. We mixed 2 μ l of 80 ng/ μ l extracted DNA, 1 μ l of 20 pmol from each primer, 12.5 μ l of 2X Power Taq PCR MasterMix (Bioteke Corp., Beijing, China), and 8.5 μ l of sterilized ultrapure water. The mixture was amplified after a PCR cycle: initial denaturation with a Taq polymerase at 95°C for 10 min, denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 75°C for 30 sec (35 cycles), and a final extension step at 75°C for 5 min. The amplified products were loaded onto 1.5% (weight/volume) agarose (Solarbio Technology Co., Ltd., Beijing, China) gels containing 0.04% (volume/volume) GoodView (Beijing SBS Genetech Co., Ltd., Beijing, China), and the production size was compared with a 100-bp DNA marker ladder (Pregene Biotechnology Co., Ltd., Beijing, China). *H. pylori* type strain NCTC 11637 was used as a positive control, while sterilized ultrapure water was used as a negative control for each PCR cycle. Bands were visualized and photographed under UV light. The 16S rRNA positive samples were sequenced by Biosune Biotechnology Co., Ltd., (Beijing, China) and blasted with *H. pylori* type strain NCTC 11637.

Immunohistochemical staining and quantitative analysis. Tissue sections were deparaffinized in xylene and rehydrated through graded alcohols to water. Slides were antigen unmasked by maintaining in a citrate buffer (10 mmol/l; pH 6.0) at a sub-boiling temperature for 20 min. The sections were cooled on a bench top for 30 min and washed with PBS (pH 7.4) three times for 5 min each. Hydrogen peroxide (3%) was incubated with the slides for 10 min and washed away, so endogenous peroxidase activity would be ceased. Sections were then incubated with polyclonal rabbit anti-*H. pylori* antibody (1:50 diluted in PBS, Product no. B047101; Dako, Glostrup, Denmark), monoclonal mouse anti-PCNA antibody (1:200 diluted in PBS, Product no. sc-56; Santa Cruz Biotechnology, Dallas, TX, USA), monoclonal mouse anti-Bcl-2 antibody (1:50 diluted in PBS, Product no. sc-7382; Santa Cruz Biotechnology), or polyclonal rabbit anti-Bax antibody (1:50 diluted in PBS, Product no. sc-526; Santa Cruz Biotechnology) overnight at 4°C. The next day, we removed the primary antibody and washed the sections. A prepared rabbit or mouse SP kit (ZSGB-BIO, Beijing, China) was used according to the manufacturer's instructions, and we incubated the sections with solution A for 30 min and then with solution B for 45 min at 37°C. We removed the reagents and washed them. DAB (ZSGB-BIO) was added to each section

and the staining closely monitored. As soon as the sections had developed, they were immersed in PBS and counterstained in hematoxylin. Finally, the sections were dehydrated with graded alcohols to form xylene before mounting. The tissue sections must not dry out during the whole immunocytochemical staining procedure. Brown granules which were observed in the gastric lumens, hepatic sinusoid, cytoplasm, or nucleus were regarded as positive staining. The slides were observed under a light microscope (Olympus Corp., Tokyo, Japan), and five photographs of each section were randomly obtained at x40 magnification using the same conditions (light source, color saturation, brightness, gain and contrast). Photograph quantification was performed using Image-Pro Plus software, version 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). In each field, the integrated optical density (IOD) for all positive staining was measured.

Protein extraction and western blotting. Twenty milligrams of the liver or tumor was completely triturated with an electric grinder, and the homogenate was mixed with 250 μ l of cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1 mM of phenylmethylsulfonyl fluoride (PMSF). The mixture was centrifuged (14,000 \times g, 4°C for 5 min), and the supernatant was collected. The protein concentrations were detected using a BCA kit (Beyotime Institute of Biotechnology). The proteins (50-100 μ g/lane) were electrophoresed in a 12% SDS-polyacrylamide gel and then transferred onto a PVDF membrane (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% skim milk in TBST at room temperature for 1 h and subsequently incubated overnight at 4°C with a primary antibody (PCNA at 1:500, Product no. sc-56; Bax at 1:500, Product no. sc-526; and Bcl-2 at 1:500, Product no. sc-7382). The next day, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at 1:5,000. Finally, the protein bands on the membranes were detected using an ECL kit (Beyotime Institute of Biotechnology) and scanned with the ImageQuant LAS 4000 digital imaging system (GE Healthcare, Buckinghamshire, UK) or exposed to a medical radiography film (Kodak, Tokyo, Japan). The bands were quantified by Image-Pro Plus software. The IOD of each band was measured and expressed as mean \pm standard deviation (mean \pm SD).

Statistical analysis. Measurement data are presented as mean \pm SD for normal data and median with ranges for non-normal data, while enumeration data are presented as ratios. The data were analyzed using one-way ANOVA if they corresponded with both a normal distribution and homoscedasticity. The SPSS program package (version 11.0; SPSS, Inc., Chicago, IL, USA) was used for our statistical analysis. Differences were considered significant at P-value <0.05.

Results

Morphological, serologic and ascitic features of mice. The skin ulceration (Fig. 1) was observed in 27.8% (5/18) and 30% (6/20) of mice in group A1 and B1, respectively. However,

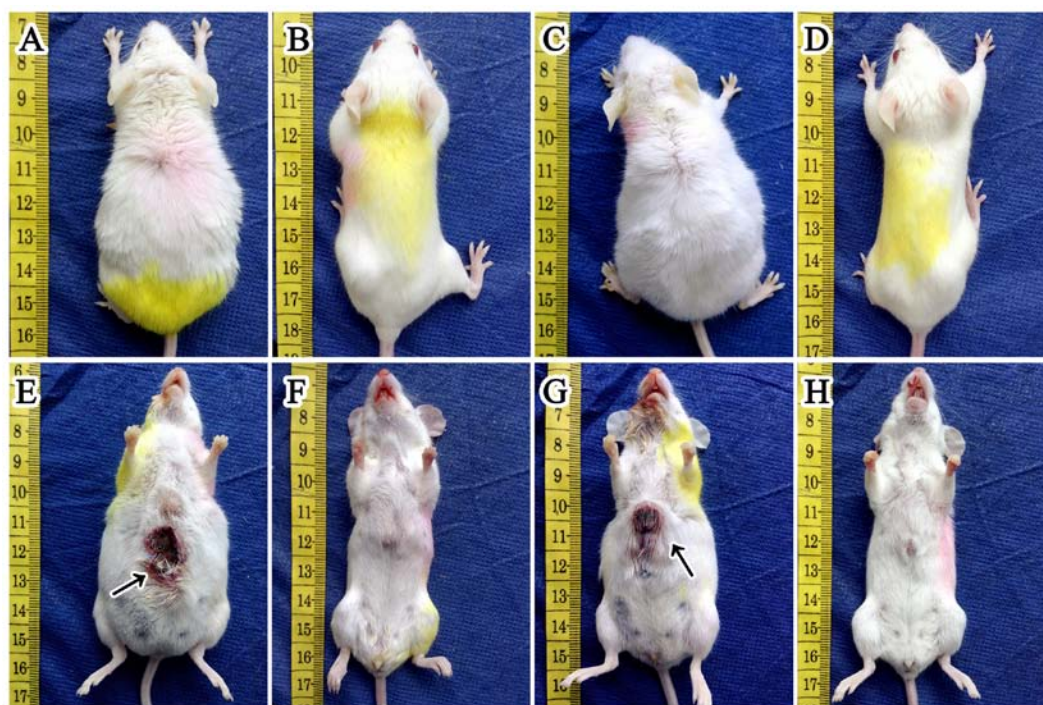


Figure 1. The differences among the mice. (A-D are dorsal views of the mice, and E-H are ventral views of the mice). The weight of the tumor positive mice (group A1 and B1) increased. Their abdomens bulged and representative ulcerations (arrows, 27.8% in A1 and 30% in B1) could be seen on the surface. Moreover, 50% (9/18) and 45% (9/20) of mice in group A1 and B1 had developed a large volume of hemorrhagic ascites. However, the appearance of the tumor negative mice (group A2 and B2) was normal, and no ulceration was visible on their abdomens. (A and E) *H. pylori*-infected and tumor positive mice (group A1); (B and F) *H. pylori*-infected and tumor negative mice (group A2); (C and G) uninfected and tumor positive mice (group B1); (D and H) uninfected and tumor negative mice (group B2). The mouse in each column represents two randomly selected individuals in each group.

there was no significant ($P=0.583$) difference between the two groups. We were unable to collect the skin ulceration for morphological detection. But we analyzed the 'link tissue' which connected hepatic tumor to abdominal wall instead of ulceration by H&E stain. Consequently, we were not surprised at finding that it was full of tumor cells which were characteristic of cellular pleomorphism. Additionally, 50% (9/18) and 45% (9/20) of mice in group A1 and B1 had developed ascites before they were necropsied. Moreover, *H. pylori* could not be cultured from any blood and ascites samples. AFP level (mean \pm SD, ng/ml) of each group was 11.11 ± 0.69 (ascites of A1 group), 10.89 ± 0.70 (serum of A1 group), 10.99 ± 0.67 (serum of A2 group), 10.98 ± 0.72 (ascites of B1 group), 10.88 ± 0.54 (serum of B1 group) and 11.08 ± 0.71 (serum of B2 group), respectively. However, no significant difference ($P=0.962$) could be found between any of the two groups above. Moreover, *Helicobacter* genus-specific 16S rRNA was not detected in blood or ascites samples.

Gastric *H. pylori* determination and histopathological assessment. *H. pylori* was cultured from gastric homogenate from 90% (18 of 20) of the mice in group A1 (*H. pylori* infected and tumor positive mice) and 80% (16 of 20) of the mice in group A2 (*H. pylori* infected and tumor negative mice). *Helicobacter* genus-specific 16S rRNA (Fig. 2) was detected using DNA extracted from the stomach, and a 95% (19 of 20) positive rate was found in both group A1 and A2. Three to five positive PCR products in each group were randomly selected and sequenced. All the sequenced samples were completely homologous to *H. pylori* type strain NCTC 11637.

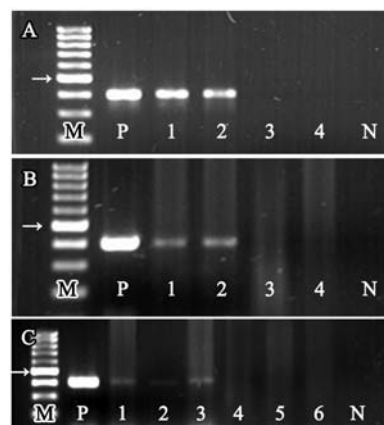


Figure 2. The PCR products of the *Helicobacter* genus-specific 16S rRNA from DNA of different mouse tissue samples, the size of all product was 400 bp. Lane 'M' represents 100 bp DNA marker ladder, and lanes 'P' and 'N' represent positive control and negative control, respectively. The arrows, 500 bp location of the marker. (A) 16S rRNA PCR products detected from bacterial colonies which were cultured from gastric suspensions. Lanes 1 and 2 represent the DNA samples extracted from bacterial colonies cultured from *H. pylori*-infected stomach (group A1 and A2), and lanes 3 and 4 represent the DNA samples extracted from bacterial colonies cultured from uninfected stomach (group B1 and B2). (B) 16S rRNA PCR products detected from gastric samples. Lanes 1 and 2, *H. pylori*-infected gastric DNA samples (group A1 and A2), and lanes 3 and 4, the uninfected gastric DNA samples (group B1 and B2). (C) 16S rRNA PCR products detected from liver and tumor samples. Lanes 1 and 2, liver and tumor DNA samples extracted from *H. pylori*-infected and tumor positive mice (group A1). Lane 3, hepatic DNA samples extracted from *H. pylori*-infected and tumor negative mice (group A2). Lanes 4 and 5, liver and tumor DNA samples extracted from uninfected and tumor positive mice (group B1). Lane 6, hepatic DNA samples extracted from uninfected and tumor negative mice (group B2).

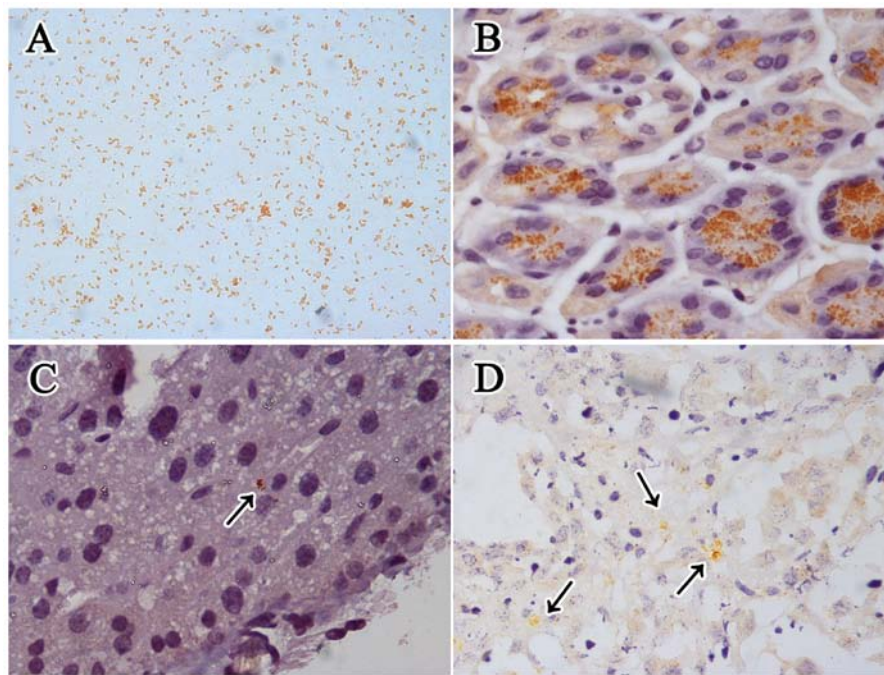


Figure 3. *H. pylori* was detected in the stomach, liver and tumor tissues using immunohistochemistry (IHC) staining (original magnification, x1000). (A) IHC staining of *H. pylori* type strain NCTC 11637. (B) Positive immunoreactivity was observed in the *H. pylori*-infected (group A1 and A2) gastric glands. (C) Positive immunoreactivity (arrows) was observed in some *H. pylori*-infected hepatic sinusoids (group A1 and A2). The hepatic samples were also positive for *Helicobacter* genus-specific 16S rRNA. (D) Immunopositive particles were observed in the necrotic area of tumors in the *H. pylori*-infected mice (group A1). The tumor samples were also positive for *Helicobacter* genus-specific 16S rRNA.

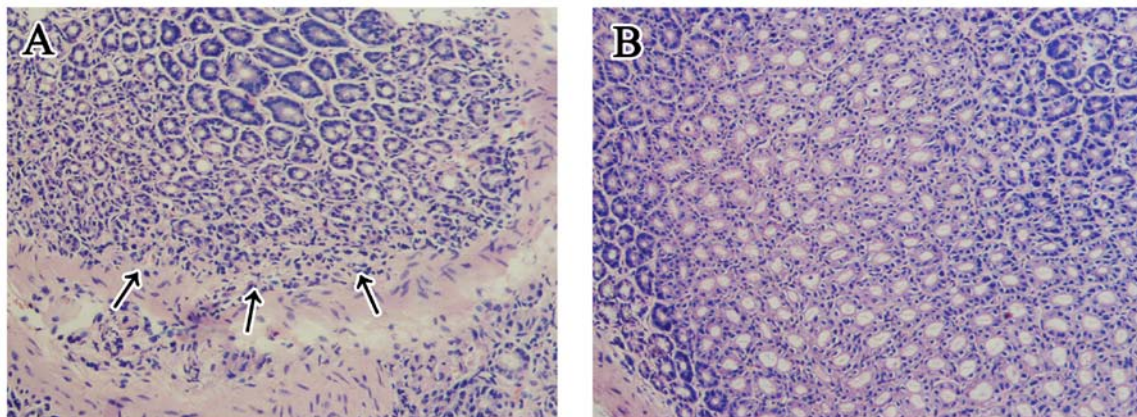


Figure 4. Histopathological changes in the mouse stomachs (both were stained using H&E and magnified x400). (A) Histopathological changes in the *H. pylori*-infected gastric mucosa (group A1 and A2). The gastric glands in the basal layer were obscure, outlined and surrounded by chronic inflammatory cells (arrows); furthermore, the inflammatory changes were mild to moderate. (B) The uninfected gastric mucosa (group B1 and B2). Gastric glands were distinctly and sharply defined and outlined, while chronic inflammatory cells were not apparent.

IHC staining for *H. pylori* in *Helicobacter* genus-specific 16S rRNA positive gastric slides showed that it mainly colonized the gastric glands (Fig. 3). Although there was no apparent ulceration in any *H. pylori*-infected stomach (group A1 and A2), the histopathological changes (Fig. 4) indicated that chronic inflammatory cells infiltrated the pyloric antrum area, and the inflammatory changes were mild to moderate. All the pathogen detections above were negative for *H. pylori* in the uninfected stomachs (group B1 and B2), and chronic inflammatory cells were not apparent.

Detection of *H. pylori* in livers. The mice in group A1 (n=18) and A2 (n=16) whose gastric homogenates were positive for

H. pylori by culture method as well as all individuals in group B1 (n=20) and B2 (n=10) were taken into the following detection and analysis procedures. *H. pylori* could not be cultured in any liver or tumor sample. The positive rate of *Helicobacter* genus-specific 16S rRNA (Fig. 2) for the liver and tumor samples in group A1 was 83.3% (15 of 18) and 66.7% (12 of 18), respectively. The rate for the liver samples in group A2 was 87.5% (14 of 16). Three to five positive PCR products were also sequenced as previously described. All sequenced products were also completely homologous to *H. pylori* type strain NCTC 11637. *H. pylori* was mainly observed in the hepatic sinusoid and necrotic area of the tumors in the infected mice using IHC staining detection (Fig. 3), while none could

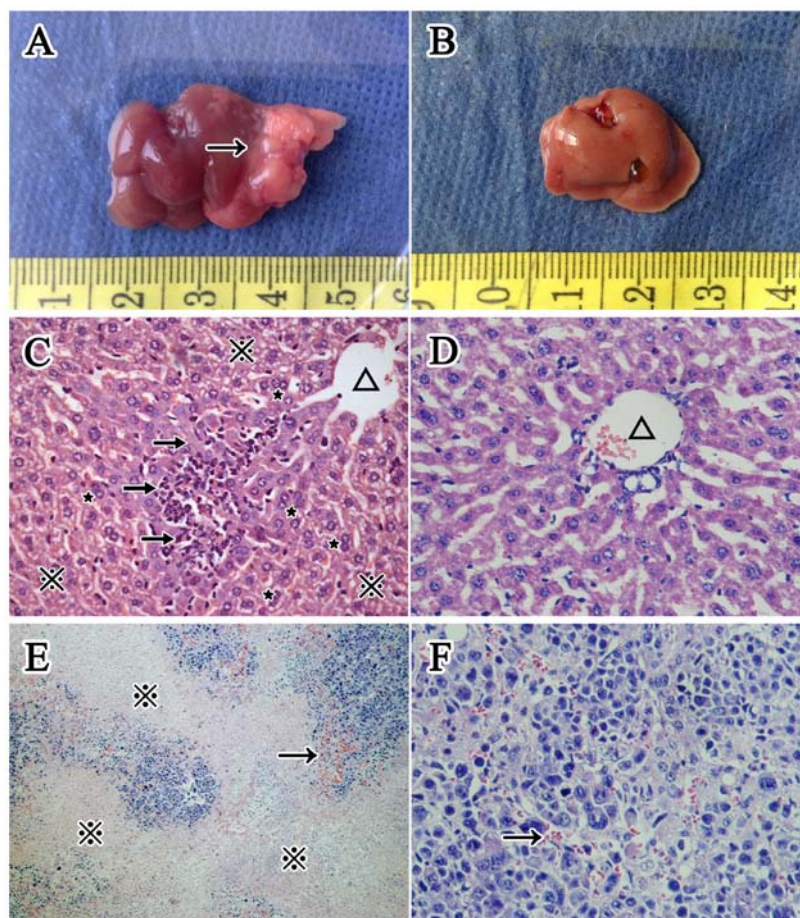


Figure 5. Morphological liver changes. (A) Carcinoma tissue (arrow) can be easily seen in the left liver lobe of the tumor positive mice (group A1 and B1). (B) No aberrant tissue can be observed in the hepatic gross specimen of the tumor negative mice (group A2 and B2). (C) Ballooning degeneration areas (※) and hepatic binucleate cells (asterisk) can be observed in the *H. pylori*-infected liver (group A1 and A2), and the sinusoids were compressed by swollen hepatic cells. Moreover, thinly distributed inflammatory cells (arrows) were also observed in the liver. The triangle represents the central veins. H&E stain (original magnification, x400). (D) Uninfected liver (group B1 and B2). Liver cell cords surround the central vein (triangle) radially, and sinusoids were distinctly and sharply outlined. Chronic inflammatory cells were not present. H&E stain (original magnification, x400). (E) The necrotic areas (※) of the tumors are homogeneously and lightly stained and distributed around the tumor parenchyma. Moreover, vasodilation (arrows) could be observed in the tumor cells. H&E stain (original magnification, x100). (F) Cellular pleomorphism and vasodilation (arrow) can be observed in the tumor parenchyma. H&E stain (original magnification, x400).

be found in the tumor parenchyma. All the *H. pylori* detection methods were negative for the uninfected livers and tumors (group B1 and B2).

Morphological changes in the livers. We determined liver to body weight ratio for each group and show the average ratio (mean \pm SD) of the mice in groups A1, A2, B1 and B2 was 0.072261 ± 0.024696 , 0.045331 ± 0.003885 , 0.062900 ± 0.020324 , and 0.042018 ± 0.004516 , respectively. The ratio of tumor positive mice (group A1 and B1) was significantly higher ($P < 0.001$) than that of tumor negative mice (group A2 and B2), while there was no significant difference between *H. pylori* infected and uninfected mice, namely group A1 and B1 ($P = 0.178$) or group A2 and B2 ($P = 0.635$). Cytoplasmic vacuolation (ballooning degeneration) and hepatic binucleate cells could be observed in the *H. pylori* infected livers, and the sinusoids were compressed by swollen hepatic cells. In addition, the infiltrated inflammatory cells were thinly distributed in the hepatic lobules and also observed in the *H. pylori* infected livers. However, no visible microscopic difference existed between group A1 and B1 tumors. Both had characterized

structural disorders and cellular pleomorphism such as atypia, increased nuclear-cytoplasmic ratio, hyperchromatic nuclei and pathological mitosis. Additionally, inflammatory cells and vasodilation could be observed among the tumor cells. The necrotic areas, which were homogeneous and lightly stained, were distributed around the tumor parenchyma (Figs. 1 and 5).

Protein expression in livers and tumors. We detected the expression of specific proteins in the livers and tumors by performing IHC staining and western blot analysis. Similar trends with IHC staining and western blot analysis were noted regarding PCNA and Bcl-2 expression. The PCNA expression (Figs. 6 and 7 and Table II) was significantly ($P \leq 0.01$) increased in the *H. pylori* infected tumors (group A1) as being compared with the uninfected tumors (group B1). Similarly, it was also significantly ($P < 0.05$) increased in the *H. pylori* infected livers (group A1 and A2) as being compared with the uninfected livers (group B1 and B2). Additionally, the tumor PCNA expression (group A1 and B1) was dramatically increased ($P < 0.001$) as compared with the liver (group A1, A2, B1 and

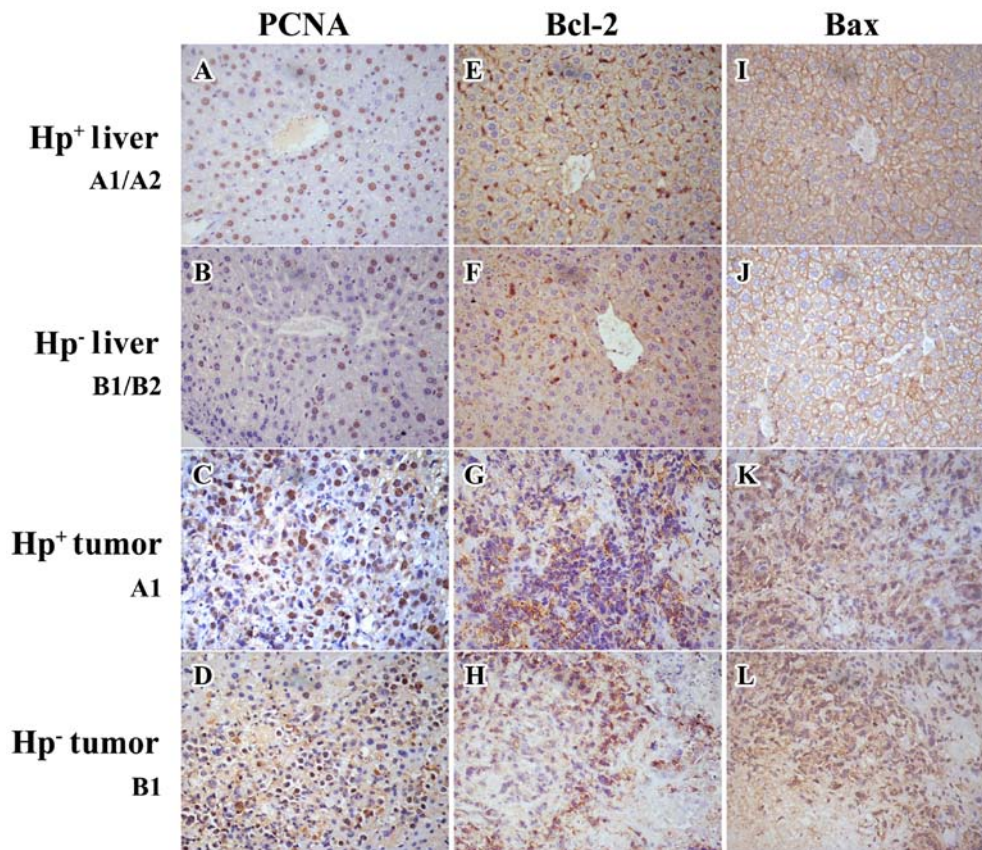


Figure 6. The (A-D) PCNA, (E-H) Bcl-2 and (I-L) Bax expression in the livers and tumors of representative sections. The integrated optical density (IOD) of the positive cells was assessed and expressed as mean \pm SD (Table II). IHC stain (original magnification, x400). (A, E and I) Liver tissues of *H. pylori*-infected mice (group A1 and A2). (B, F and J) Liver tissues of uninfected mice (group B1 and B2). (C, G and K) Tumor tissues of *H. pylori*-infected mice (group A1). (D, H and L) Tumor tissues of uninfected mice (group B1).

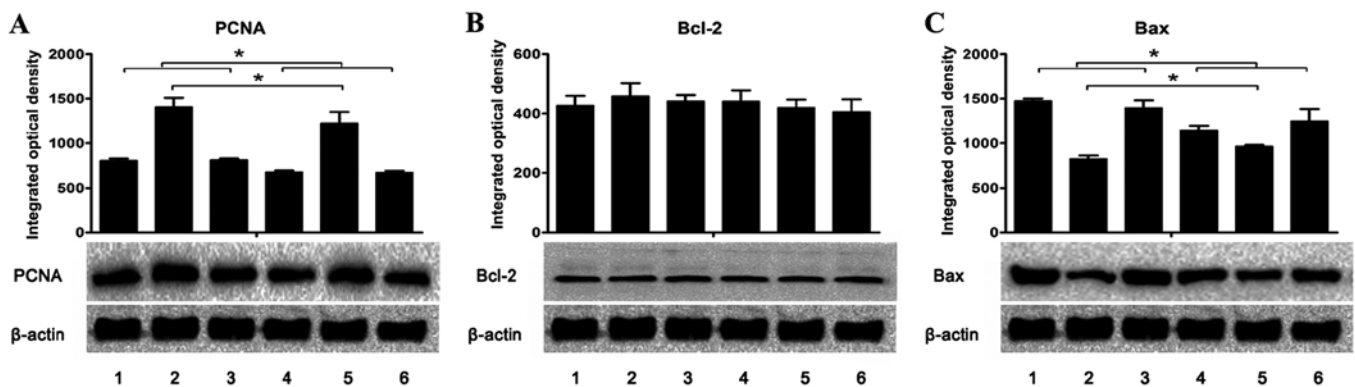


Figure 7. Expression of (A) PCNA, (B) Bcl-2 and (C) Bax in the livers and tumors was assessed using western blot analysis. The integrated optical density (IOD) of each antigen band was measured. The results are expressed as mean \pm SD in the histogram. Lanes 1 and 2, the protein expression in the livers and tumors, respectively, of the *H. pylori*-infected and tumor positive mice (group A1). Lane 3, the protein expression in the livers of *H. pylori*-infected and tumor negative mice (group A2). Lanes 4 and 5, the protein expression in the livers and tumors, respectively, of the uninfected and tumor positive mice (group B1). Lane 6, the protein expression in the livers of uninfected and tumor negative mice (group B2). * $P < 0.05$ as lane 1 compared with lanes 4 or 6, lane 3 compared with lanes 4 or 6, and lane 2 compared with lane 5.

B2). There was no difference ($P > 0.1$) in the Bcl-2 expression (Figs. 6 and 7 and Table II) in any tumor or liver. With western blot analysis, a significant reduction ($P = 0.035$) in the Bax expression (Fig. 7 and Table II) of the *H. pylori*-infected tumors was demonstrated (group A1) as compared with the uninfected tumors (group B1); there was no difference ($P = 0.176$) in Bax expression (Fig. 6 and Table II) between these two groups with

IHC staining. However, both western blot analysis and IHC staining demonstrated a significant upregulation ($P < 0.05$) of Bax expression in the *H. pylori*-infected livers (group A1 and A2), as compared with the uninfected livers (group B1 and B2). Moreover, the Bax expression in the tumors (group A1 and B1) was significantly decreased ($P < 0.001$), as compared with the livers (group A1, A2, B1 and B2).

Table II. Integrated optical density of the proteins expressed in representative liver or tumor samples detected by immunohistochemical stain and western blot analysis (mean \pm SD).

Group	Sample	PCNA		Bcl-2		Bax	
		IHC	WB	IHC	WB	IHC	WB
A1	Liver	660.384 \pm 24.196 ^{a,b}	804.811 \pm 21.937 ^{a,b}	257.009 \pm 25.241	426.883 \pm 31.775	568.872 \pm 35.024 ^{a,b}	1473.967 \pm 25.356 ^{a,b}
A1	Tumor	769.849 \pm 16.381 ^c	1406.576 \pm 100.647 ^c	284.988 \pm 12.188	458.303 \pm 43.060	309.306 \pm 20.036	822.853 \pm 38.193 ^c
A2	Liver	657.051 \pm 13.166 ^{a,b}	812.288 \pm 15.339 ^{a,b}	243.345 \pm 25.150	441.563 \pm 20.182	533.919 \pm 15.386 ^{a,b}	1395.167 \pm 85.053 ^{a,b}
B1	Liver	452.964 \pm 15.665	677.803 \pm 17.419	283.233 \pm 38.032	440.803 \pm 36.415	449.545 \pm 30.011	1145.367 \pm 50.639
B1	Tumor	725.417 \pm 17.978	1226.722 \pm 124.089	275.016 \pm 21.806	419.970 \pm 26.492	336.763 \pm 15.308	961.160 \pm 18.509
B2	Liver	442.964 \pm 18.235	672.141 \pm 18.418	262.746 \pm 40.646	405.830 \pm 41.563	481.272 \pm 16.762	1248.333 \pm 134.814

IHC, immunohistochemical stain. WB, western blotting. Results are shown with the mean \pm SD. ^aP<0.05 (vs. B1 liver); ^bP<0.05 (vs. B2 liver); ^cP<0.05 (vs. B1 tumor).

Discussion

H. pylori has been classified as a type I carcinogen (2), that is a major causative factor in many gastric diseases. Furthermore, some extragastric diseases are also correlated with its pre-infection during childhood (4-7). Recent studies on the correlation between *H. pylori* infection and chronic hepatic disease have found that the anti-*H. pylori* antibody level in the serum of patients with chronic liver disease is significantly higher than that of healthy patients (8-12,36-40). In addition, *H. pylori* was also detected and confirmed in many liver samples from chronic hepatic disease patients using morphological and genetic detection (8,13,14,24,41-43). Moreover, researchers have successfully cultured *H. pylori* from the liver samples of some clinical hepatic disease cases (14,44). All the above mentioned details suggest that *H. pylori* could infect the human liver and may play a role in the development or progression of hepatic disease.

However, when talking about the role of *H. pylori* in the progression of hepatic diseases, there is no consistent opinion among researchers. Some investigator, such as Fox *et al* (35), Matsukura *et al* (45) and García *et al* (46) claimed that it could not colonize livers and was barely able to promote hepatic diseases. They rather regard it as contaminant instead of infection. However, others, such as Goo *et al* (23), Ito *et al* (24) and Rocha *et al* (47), reported that *H. pylori* not only colonize the livers, but also contribute to the hepatic disease evolution. Nowadays, investigators are gradually discovering a growing kinds of *Helicobacter spp.*, such as *H. bilis*, *H. pullorum* and *H. pylori*, which has been found in the liver of hepatic disease patients. However, attention is mainly focused on *H. bilis* or *H. pullorum* (35,45,46) rather than *H. pylori* for the following reasons. It has been confirmed by *in vitro* study that *H. pylori* was unable to survive in bile products (48). Moreover, cholecystectomy would increase the gastric *Helicobacter* infection risk (49). Relying on the crucial findings that *H. pylori* was unable to survive in hepatobiliary condition, the presence of *H. pylori* was considered a result of contamination rather than colonization, even though it had ever been found in gallbladder and bile of patients (50). In humans, bile is produced continuously by the hepatocytes

(liver bile), but stored and concentrated in the gallbladder (gallbladder bile) (51). Ito *et al* (25), Zhang *et al* (26) and Chen *et al* (27) found *H. pylori* exhibited hepatotoxicity while it was co-cultured with hepatocytes which were capable of producing bile *in vitro*. Hence, they insisted *H. pylori* might play a potential role in hepatocarcinogenesis. Additionally, Goo *et al* (23), Ito *et al* (24) and Rocha *et al* (47) found all liver samples were negative for other gut organisms, such as *E. coli*, which mainly inhabited gut and more likely present in liver than *H. pylori* as a contaminant. Moreover, the successful *H. pylori*-cultivation from livers of patients (14,44) also suggest its colonization rather than contamination. Finally, basing on the finding that *H. pylori* could be recovered from feces, Kelly *et al* (52) and Thomas *et al* (53) agreed with the opinion that it would survived even after exposure to bile. We came to the same conclusion as that summarized by Ito *et al* (25), Zhang *et al* (26) and Chen *et al* (27). However, bile products might transform *H. pylori* from virulent helical form to insufficient coccoid form (54). The colonization and pathology ability of *H. pylori* in liver may depend on its virulence factors, such as CagA or VacA (25,55-57) and forms. Therefore, we thought the reasons for low *H. pylori* frequency in the study of Fox *et al* (35) may due to the insufficient strain or form of *H. pylori* which the researchers had adopted.

By using morphological (IHC staining) and genetic detection (PCR), the present study confirmed that *H. pylori* or only its structural components were both detected in stomachs and livers. It was mainly detected in the hepatic sinusoids and necrotic areas of the tumors. Currently, the following possible perspectives or hypotheses are widely accepted regarding the presence of *H. pylori* in the liver. Firstly, because its genetic components had been detected in cholecystic samples or the bile of gallbladder disease patients, some researchers consider the findings related to duodenal reflux (58-62). Gastrointestinal motility will transport *H. pylori* from the stomach to the descending duodenum. It would be gradually transmitted in a retrograde manner through the major duodenal papilla, sphincter of Oddi, common bile duct, common hepatic duct, left and right hepatic ducts, and cholangiole to its final destination in the liver or gallbladder.

Secondly, the genetic components of *H. pylori* were detectable in the endarterium samples of atherosclerotic patients using PCR detection by Danesh *et al* (7) and others who indicated that *H. pylori* might enter into the circulatory system (5,6,63). A long-term chronic *H. pylori* infection would result in chronic gastritis and a chronic gastric ulcer. Consequently, a small amount of *H. pylori* would constantly leak into the blood through impaired vessels that are in or around gastric ulcers and then wander aimlessly along the portal or lymph circulation. Tian *et al* (66) found that inflammatory cells were mostly thinly distributed along the central veins, hepatic sinusoids, arteriolar, and venules in the periportal area of *H. pylori*-infected livers. Although similar morphological changes were found in *H. pylori*-infected mouse livers of the present study, we could not detect *H. pylori* genus-specific 16S rRNA in any of the blood samples. The low bacteria concentration ($<10^3$ CFU/ μ l) in the blood may be responsible for the negative results (60). Thus, even though it was undetectable in the blood of hepatic positive individuals, we cannot deny the abovementioned secondary hypothesis. Thirdly, inflammatory cells would phagocytize *H. pylori* and arrive at liver when the hepatic homeostatic equilibrium had been broken, for example, an increasing of bile pH value.

With a growing concern regarding the correlativity of *H. pylori* infection and hepatic disease, the positivity rate of *H. pylori* genetic components detected in hepatic samples is increasing. Although it is not uncommon for *H. pylori* to be cultured from the gastric samples of infected patients and animals, a positive culture from hepatic samples is still rarely reported. It has been reported that *H. pylori* could be cultured from clinical hepatic samples (14,44). However, no positive culture result could be found in hepatic or blood samples of *H. pylori*-infected animal models which were developed by various researchers, including ourselves. By referring to the findings of researchers in the field, we analyzed the results and summarized the possible reasons as follows. First, by analyzing the results of *H. pylori*-specific IHC staining, we found that the densities of a positive reaction in *H. pylori*-infected livers were significantly lower than those of the stomachs. Silva *et al* (60) indicated the reasons why they were unable to visualize the *H. pylori* colonies stating that the bacteria were cultured in the conventional conditions and methods. Hence, a low detection sensitivity of bacterial culture may produce a false-negative result. *H. pylori* prefers gaseous conditions (2-5% of oxygen and 5-10% of carbon dioxide), certain temperatures (34 to 40°C; 37°C is its optimum), humidity (>90%), and acidity (pH 5.5 to 8.0; neutral is the optimum). *H. pylori* transforms from its normal form to a coccoid form if its growing environment changes (54), such as cholestasis in which hepatic cells excrete abnormal acidic bile. The coccoid form of *H. pylori* is widely known as a degenerative dead form or a nonculturable form (64,65). In addition to its alkalinity, bile may also contain some other unfavorable ingredients that are unsuitable for the growth of *H. pylori* and would play a crucial role in restraining or killing it. Because the genetic components or structural proteins would not be eliminated completely by inflammatory or hepatic cells. Thus, we were able to realize the existence of *H. pylori* in samples by a more sensitive method, such as morphological IHC stain and PCR. Narikawa *et al* (54) also hypothesized that the coccoid transformation of *H. pylori*

would contribute to the inability of *H. pylori* culture in hepatic samples despite a positive genetic detection.

Although, the liver to body weight ratio in *H. pylori*-infected livers was not significant as compared with uninfected livers, cytoplasmic vacuolation and hepatic binucleate cells were observed by Goo *et al* (23), and the present study. In addition, infiltrated inflammatory cells that were thinly distributed in the *H. pylori*-infected hepatic lobules were also found by Tian *et al* (66). No macroscopic or microscopic differences existed in the *H. pylori*-infected and uninfected tumors, except for the pathogenic detection of *H. pylori*. In the present study, the skin ulceration bearing mouse usually bore an extremely gross exophytic growing hepatic tumor which stuck to the abdominal wall. We did not have the resources for skin ulceration detection, we analyzed the 'link tissue' instead and found that it was full of pleomorphic tumor cells. On that account, we insist that the skin ulceration was a part of the necrotic tumor, which had invaded into the abdominal wall. In order to explore the impact of *H. pylori* in the development and progression of liver and hepatic orthotopic graft tumors, we introduced three tumor markers, namely PCNA, Bax and Bcl-2, which are cellular growth, apoptosis-promotion and apoptosis-inhibition proteins, respectively. In the present study, *H. pylori* may have upregulated the expression of PCNA both in tumor and liver, whereas may have downregulated the Bax expression in the tumor and upregulated it in the liver. No significant impact of *H. pylori* on the Bcl-2 expression could be seen in the tumor or the liver.

PCNA was a 36-kDa antigen which was associated with cellular growth activity (67-70). It plays an important role in cellular proliferation and DNA synthesis, and its expression is usually proportional to DNA synthesis. Hence, it is widely thought to be an indicator and is used to estimate the occurrence, development, metastasis, progression, classification, neoplasm staging, treatment evaluation and prognosis of many tumors (71-74). In actively proliferative cells, such as tumor cells, the expression of PCNA is significantly upregulated (75-80). In the present study, besides the significantly unregulated expression in tumors, PCNA in *H. pylori*-infected livers and tumors was also markedly higher than that in the uninfected ones. Our results corresponded to the findings of Goo *et al* (23) and Tian *et al* (81). We noted that the swollen cells in the *H. pylori*-infected livers compressed the hepatic sinusoids, and subsequently, might lead to the blood and oxygen deficits. The impaired hepatic cells might induce the expression of PCNA indirectly as the deficits in nutrition, oxygen and blood supply. Moreover, inflammatory cells and their infection factors might contribute to PCNA upregulation in hepatic cells. This is our hypothesis for the reasons for PCNA upregulation that was detected in *H. pylori*-infected livers. Moreover, Tian *et al* (81) performed an *in vitro* experiment involving the human liver HepG2 cells and *H. pylori* co-culture indicating an increasing PCNA expression. However, the liver cells in their study were in an abundant nutritious, oxygen and blood condition (82). Thus, we infer from the results that *H. pylori* may promote cellular proliferation in some direct but unknown pathways.

The Bcl-2 family is divided into two categories. One is a type of regulator protein that induces cell apoptosis, such as Bax. The other, especially Bcl-2, is considered an impor-

tant anti-apoptosis protein. The Bcl-2 family antigen would combine with each other and become a protein dimer which act as a molecular switch in the progression of cell death (83-88). The occurrence of carcinoma is an imbalance between cellular growth or proliferation and cellular death or apoptosis. The upregulation of proto-oncogenes or downregulation of anti-oncogenes would finally promote tumor progression. When the Bax expression were higher than that of Bcl-2, they would combine as Bax/Bax homologous dimers and promote cellular apoptosis. Alternatively, the Bcl-2 and Bax antigens would combine as Bcl-2/Bax heterodimers and Bcl-2/Bcl-2 homodimers and play a role in inhibiting cellular apoptosis (86,89-92). Deficits in nutrients, oxygen, and blood supply might be a crucial reason for cellular apoptosis in the present study (93). Additionally, inflammatory factors or mediators excreted by infiltrating inflammatory cells were also responsible for cellular apoptosis. All the above mentioned reasons enabled the cellular apoptosis in *H. pylori*-infected livers and led them to have a higher Bax expression. Hemangiectasis was noted in the tumors, thus, their blood and oxygen supply were abundant. Moreover, the percentage of death and apoptosis was significantly lower in the tumor than the normal liver tissue. Thus, we were able to explain the downregulation of the Bax expression demonstrated in the present study. We conclude from the results that inflammation might be the primary cause of apoptosis in the *H. pylori*-infected livers, but *H. pylori* might act as a crucial factor to inhibit or reduce tumorous apoptosis.

The present study has some shortcomings. We could not establish the *H. pylori* forms and the exact reasons for hepatic inflammation. Additionally, it is unknown whether the difference among groups will be more significantly visible when the model-build period is prolonged. Therefore, further research is essential for the worldwide unsolved issues that were found in exploring the relationship between *H. pylori* and hepatic diseases.

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