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Correlations of *Helicobacter pylori* with liver function, inflammatory factors and serum levels of FoxP3 and RORγt in patients with hepatitis B cirrhosis

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Abstract. – OBJECTIVE: To investigate the correlations of *Helicobacter pylori* (HP) with liver function, inflammatory factors and serum levels of forkhead box P3 (FoxP3) and retinoic acid receptor-related orphan receptor gamma-t (RORγt) in patients with hepatitis B cirrhosis (HBC).

PATIENTS AND METHODS: A total of 60 HBC patients were divided into HBC group (n=30) and HP-infected HBC group (HP&HBC group, n=30). QRT-PCR was conducted to determine the messenger ribonucleic acid (mRNA) levels of FoxP3 and RORγt in serum samples. ELISA was applied to measure the levels of relevant inflammatory factors. Besides, immunohistochemical staining was conducted to detect positive expressions of FoxP3 and RORγt in liver tissues of patients in the two groups.

RESULTS: No significant differences in gender, drinking, smoking, diabetes and age were found between HBC group and HP&HBC group ($p>0.05$). Globulin and albumin levels were comparable between the two groups ($p>0.05$). Liver function indexes, including ALT, AST and TBIL were higher in HP&HBC group than those in HBC group ($p<0.05$). The HBV-DNA level was lower in HBC group in comparison with that in HP&HBC group. The interferon-gamma (IFN-γ) level was remarkably higher in HBC group than that in HP&HBC group ($p<0.01$), and the levels of interleukin (IL)-6, IL-10, IL-17 and transforming growth factor (TGF)-β1 were notably lower in HBC group in comparison with those in HP&HBC group ($p<0.01$). Additionally, the mRNA levels of FoxP3 and RORγt in HBC group were distinctly lower than those in HP&HBC group ($p<0.01$). The mRNA levels of FoxP3 and RORγt were positively related to those of IL-6, IL-10, IL-17, and TGF-β1, and negatively associated with IFN-γ level. Immunohistochemical results indicated that positive expression rates of FoxP3 and RORγt in the liver tissues were approximately 50% in HP&HBC group, and were 15% in HBC group, and the difference was statistically significant ($p<0.05$).

CONCLUSIONS: Expression levels of FoxP3 and RORγt in serum and liver tissues are elevated in HP-infected HBC patients, and inflammatory factors are correlated with their expressions, suggesting the aggravated liver damage.

Key Words:
Helicobacter pylori, Hepatitis B cirrhosis, FoxP3, RORγt, Liver function.

Introduction

Liver cirrhosis is a worldwide public health problem with an increasing mortality rate in recent years[1]. Alcoholic hepatitis, non-alcoholic steatohepatitis and hepatitis C infection are the leading causes of liver cirrhosis in Western countries, while chronic hepatitis B virus (HBV) infection is the main factor for liver cirrhosis in hepatitis B endemic areas[2]. The repeated diffuse degeneration and necrosis of liver cells, fibrous tissue hyperplasia and cellular nodular regeneration are involved in the pathological process, resulting in liver degeneration and cirrhosis[3]. Hepatitis B endangers physical and mental health. It significantly affects liver function, leading to liver metabolism and detoxification disorders and portal hypertension, also complications such as gastrointestinal bleeding[4]. Chronic HBV and hepatitis C virus (HCV) infections account for 58% of cirrhosis cases globally. As one of the most common pathogenic bacteria, more than 50% of global population is affected by *Helicobacter pylori* (HP)[5]. HP is a Gram-negative spiral bacteria with urease, catalase and oxidase activities[6]. Deutsch

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et al[7] revealed that HP infection can enhance the pathological degree of hepatitis C cirrhosis, leading to the imbalance of regulatory T cells (Treg)/T-helper 17 cells (Th17), and thus inflammatory damage of liver tissues[7]. Piotrowski and Boron-Kaczmarska[8] also confirmed that patients infected with HCV are more susceptible to HP infection, and the infection rate rises. Therefore, the functional state of the liver is of great significance in the progression, clinical treatment and prognosis of liver cirrhosis[9]. Thus, exploring the correlations of HP infection with viral hepatitis, liver cirrhosis and malignant liver cancer, and the underlying mechanism facilitate clinical diagnosis and disease treatment[10].

At present, the relation between HP and hepatitis B cirrhosis (HBC) has not been elucidated. Understanding their correlation and the potential mechanism contributes to develop immune interventions to improve the treatment success rate of HBC, which is of great clinical significance. In the present study, the correlations of HP with inflammatory factors and serum levels of forkhead box P3 (FoxP3) and retinoic acid receptor-related orphan receptor gamma-t (RORγt) in patients with HBC were investigated, and the effect of HP on liver function in patients with HBC was explored.

Patients and Methods

Research Subjects

60 HBC patients treated in our hospital from April 2019 and April 2020 were enrolled as the research subjects, including 32 males and 28 females aged 40-60 years (mean age: 50 years). Patients were divided into HBC group (n=30) and HP-infected HBC group (HP&HBC group, n=30). All patients met the diagnostic criteria for HBC in *The Guideline of Prevention and Treatment for Chronic Hepatitis B* in China. The patients in HP&HBC group met the diagnostic criteria for HP infection according to *Fifth Chinese National Consensus Report on the Management of Helicobacter pylori Infection*. Moreover, the influences of disturbing factors from other organs were eliminated. This study was approved by the Ethics Committee of Shengjing Hospital, Affiliated Hospital of China Medical University. Signed written informed consents were obtained from all participants before the study.

Detection of HP in Two Groups

Both groups of patients underwent carbon-14-urea breath test (^{14}C-UBT), and the patients with positive results were diagnosed with HP infection. The exhaled gas of patients was collected using a CO_2 gas collecting bottle, and ^{14}C radioactivity of the collected CO_2 was determined using a liquid scintillator. ^{14}C-UBT = [sample bottle count (dpm) - background bottle count (dpm)] / efficiency of the instrument (dpm/mmol CO_2). HP was defined to be positive when ^{14}C-UBT >100 dpm/mmol CO_2.

Collection of Serum Samples in Two Groups

Before 9 o'clock in the morning, 10 mL of fasting peripheral blood was extracted from each patient. Liver function indexes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL), and globulin (GLB), albumin (ALB) and HBV-deoxyribonucleic acid (DNA) were examined in the Department of Clinical Laboratory in our hospital.

Detection of inflammatory Factors in Serum Via Enzyme-Linked Immunosorbent Assay (ELISA)

Before 9 o'clock in the morning, 3 mL of fasting venous blood was extracted from each patient. The samples were centrifuged, and the supernatant was taken and stored in a refrigerator at -80°C. After that, serum levels of interferon-gamma (IFN-γ), interleukin (IL)-6, IL-10, IL-17 and transforming growth factor (TGF)-β1 were detected by ELISA in a standard protocol.

Determination of the Messenger Ribonucleic Acid (mRNA) Expression Levels of FoxP3 and RORÐt Via Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The samples were incubated with chloroform and centrifuged at 12,000 rpm for 10 min. Next, the supernatant was taken, and fully mixed with an equal volume of isopropanol, followed by standing at room temperature for 10 min and centrifugation at 12,000 rpm for 10 min. After that, the supernatant was discarded, and the precipitate was washed with 75% ethanol twice. Finally, the precipitate was dissolved in DEPC water. RNA concentration and purity were measured, followed by reverse transcription to cDNA. The PCR cycling protocol consisted of pre-denaturation at 95°C for 30 s, 38

Table I. Primer sequences.

Primer	Primer sequence
FoxP3	Forward: 5'-TTCATGCACCAGCTCTCAAC-3'
FoxP3	Reverse: 5'-GACACCGTAGTAGGCTGTTC-3'
RORγt	Forward: 5'-CATCTCCAGCCTCAGCTTTGA-3'
RORγt	Reverse: 5'-TCCCCCAGAAGTCCTTAAATCC-3'
β-actin	Forward: 5'-AGAAACCGATAATCCACTTGTTG-3'
β-actin	Reverse: 5'-GTGCAAGAAAATTGGGTGGCAAAT-3'

cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 31 s, and final extension at 72°C for 7 min. The above cycling process was utilized for FoxP3 and RORγt. With β-actin as an internal reference, the relative mRNA expression levels of FoxP3 and RORγt were evaluated by $2^{-\Delta\Delta Ct}$ method. The primer sequences used for qPCR were shown in Table I.

Measurement of the Associations of FoxP3 and RORγt With Inflammatory Factors Via Spearman's Correlation Analysis

Spearman's correlation analysis was used to test the associations of mRNA expression levels of FoxP3 and RORγt with IFN-γ, IL-6, IL-10, IL-17 and TGF-β1 levels.

Liver Biopsy and Immunohistochemistry

Liver biopsy was performed for 10 patients in each group. First, patients and their families were informed of possible adverse reactions and complications and they agreed with the surgery. The procedure was approved by the Ethics Committee before biopsy. Second, under the guidance of B-ultrasound, one liver tissue with a length of 15-20 mm was obtained by quick puncture using a biopsy gun (BARD Magnum, Jersey, NJ, USA). After that, the tissue was sliced into sections, dewaxed, hydrated, and placed in an incubator at 60°C. Then, sections were treated by antigen retrieval heated in a microwave oven, quenching of peroxidase and blocking. After incubating with primary antibody at 4°C overnight, sections were washed in 1× phosphate-buffered saline (PBS) twice (5 min/time), and incubated with the secondary antibody for 40 min. Then, they were washed with 1× PBS for three times (3 min/time). The color was developed with diaminobenzidine (DAB) solution (Solarbio, Beijing, China), and samples were washed with pure water, stained with hematoxylin for 15 min, and washed with pure water for 3 min. The samples were, then, mounted and processed for microscopy to detect positive expressions of FoxP3 and RORγt.

Statistical Analysis

Table II. Comparison of basic clinical indicators between HBC group and HP&HBC group [n (%)].

Indicator	HBC group (n=30)	HP&HBC group (n=30)	*p*	χ^2/t
Gender				
Male	19 (63.3)	13 (43.3)	0.721	0.082
Female	11 (36.7)	17 (56.7)		
Drinking				
Yes	7 (23.3)	10 (33.3)	0.586	0.172
No	23 (76.7)	20 (66.7)		
Smoking				
Yes	14 (46.7)	18 (60.0)	0.762	0.094
No	16 (53.3)	12 (40.0)		
Diabetes				
Yes	24 (80.0)	13 (43.3)	0.637	0.164
No	6 (20.0)	17 (56.7)		
Age (years old)	50.3±7.2	50.3±7.2	0.834	0.274

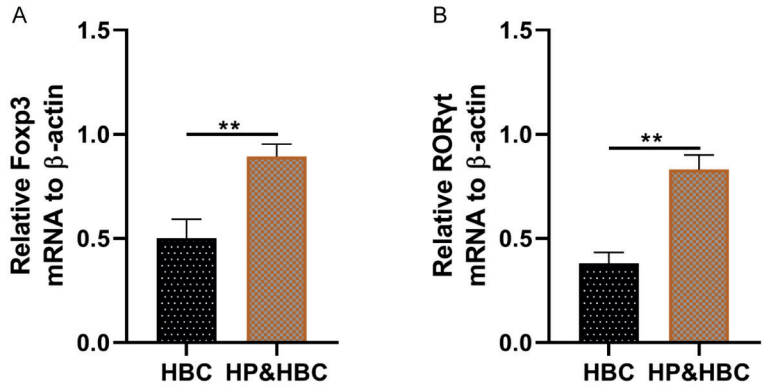

Figure 1. HBV-DNA levels in HBC group and HP&HBC group. *p<0.05, HP&HBC group *vs.* HBC group.

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis of all the experimental data. Measurement data were presented as mean ± standard deviation (SD). Differences between two groups were analyzed by the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). p<0.05 indicated that the difference was statistically significant.

Results

Comparisons of Patients' Clinical Data

There were no significant differences in gender, drinking, smoking, diabetes mellitus and age between HBC group and HP&HBC group (p>0.05) (Table II).

Biochemical Indicators of Patients

No significant differences were observed in ALB and GLB between groups (p>0.05). The levels of liver function indexes (ALT, AST, and TBIL) were significantly higher in HP&HBC group than those in HBC group (p<0.05) (Table III).

Table III. Comparisons of liver function, GLB and ALB between HBC group and HP&HBC group ($\bar{\chi}$ ± s).

Indicator	HBC group	HP&HBC group
ALB	37.5±5.4	38.5±4.9
GLB	33.1±4.8	32.4±5.8
ALT	34.7±39.2	40.5±45.5[a]
AST	30.4±31.8	34.7±41.1[a]
TBIL	19.5±11.3	23.1±9.2[a]

[a]p<0.05, HP&HBC group *vs.* HBC group.

Comparisons of Inflammatory Factors in Serum of Patients

The IFN-γ level was remarkably higher in HBC group than that in HP&HBC group, with a significant difference (p<0.01), and the levels of IL-6, IL-10, IL-17, and TGF-β1 were notably lower in HBC group in comparison with those in HP&HBC group, showing significant differences (p<0.01) (Table IV).

FoxP3 and RORÐt Gene Expression Levels

The mRNA levels of FoxP3 and RORγt in HBC group were distinctly lower than those in HP&HBC group, and the differences were statistically significant (p<0.01) (Figure 1).

Associations of Serum Inflammatory Factors with FoxP3 and RORÐt Gene Expression Levels

Spearman's correlation analysis was conducted to test the associations of expression levels of FoxP3 and RORγt with inflammatory factors in the two groups of patients. The mRNA levels of FoxP3 and RORγt were positively related to those of IL-6, IL-10, IL-17, and TGF-β1, and negatively associated with IFN-γ level (Table V).

Expression Levels of FoxP3 and RORγt Detected by Immunohistochemical Technique

Immunohistochemical results indicated that FoxP3 and RORγt were positively expressed in the liver tissues in both groups, accounting for approximately 50%, and 15% in HP&HBC group

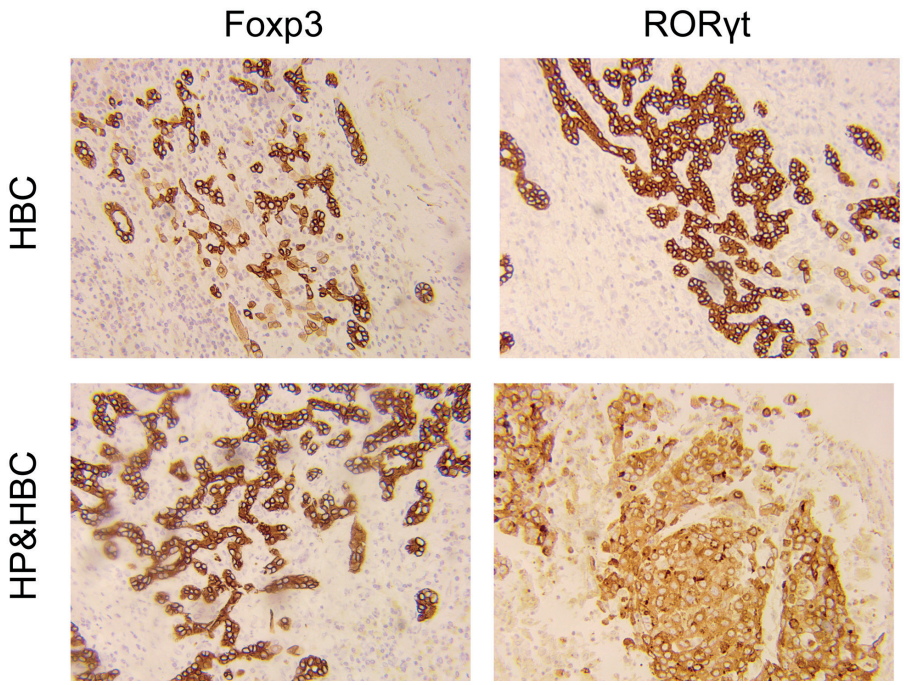

Figure 2. FoxP3 and RORγt gene expression levels in two groups, (magnification: 200×) **p<0.01, HP&HBC group *vs.* HBC group.

and HBC group, respectively, and the difference was statistically significant (p<0.05) (Figure 2).

Comparison of HBV-DNA in the Serum of Patients

The HBV-DNA level was lower in HBC group in comparison with that in HP&HBC group, and the difference was statistically significant (p<0.05) (Figure 3). The result demonstrated that HP can increase HBV-DNA load level in patients with HBV.

Discussion

As one of the most common infectious diseases, infection of hepatitis B mainly attacks the liver[11]. At present, there are about 260 million pa-

Table IV. Comparisons of inflammatory factors between HBC group and HP&HBC group ($\bar{\chi} \pm s$).

Group	IFN-γ	IL-6	IL-10	IL-17	TGF-β1
HBC group	1768.1±348.2[a]	90.5±34.7a	194.2±32.3[a]	65.5±15.3[a]	425.7±68.5[a]
HP&HBC group	1027.2±248.5	107.8±38.4	245.8±37.2	97.6±24.2	604.5±103.5

[a]p<0.05, HP&HBC group *vs.* HBC group.

Table V. Associations of inflammatory factors with FoxP3 and RORγt gene expression levels in HBC group and HP&HBC group ($\bar{\chi} \pm s$).

Gene		IFN-γ	IL-6	IL-10	IL-17	TGF-β1
FoxP3	Pearson correlation	-0.562	0.503	0.435	0.651	0.523
	Significance (two-tailed)	0.000	0.000	0.000	0.000	0.000
RORγt	Pearson correlation	-0.413	0.342	0.189	0.435	0.298
	Significance (two-tailed)	0.000	0.007	0.018	0.000	0.006
	n	60	60	60	60	60

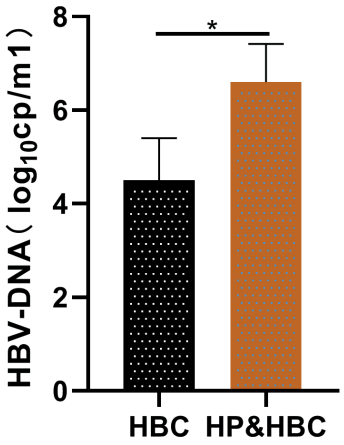

Figure 3. Expression levels of FoxP3 and RORγt detected by immunohistochemical technique.

tients and carriers of HBV worldwide, and about 580,000 people die each year from HBV-related liver cirrhosis or liver cancer[12,13]. The repeated necrosis of liver cells, fibrous tissue hyperplasia and cellular nodular regeneration are typical pathological manifestations of liver cirrhosis. The survival rate of patients with liver cirrhosis caused by HBV infection declines, that is, even minor liver metabolic abnormalities may be dangerous to patients with HBV[14-16]. For untreated patients, up to 40% of chronic HBV infection cases will develop into liver cirrhosis, and decompensated cirrhosis occurs, with complications of liver fibrosis such as jaundice, ascites, variceal bleeding and hepatic encephalopathy[17,18]. Recent studies have indicated a delicate relation between bacterial infection and HBC, of which HP is clearly explored. Some researchers have pointed out that HP can aggravate the pathological degree of hepatitis C cirrhosis, leading to the imbalance of Treg/Th17, and thus the inflammatory damage of liver tissues. If the patient is infected with the HCV, the infection rate of HP also increases[7,8]. HP infection creates an oxidized microenvironment that releases pro-inflammatory, toxic and vasoactive substances and ROS, resulting in inflammation[18,19]. If the excessive production of free radicals cannot be inhibited by the host, reactive oxygen species will destroy cellular components and damage the cells. Currently, pegylated interferon or nucleic acid analogs (lamivudine, adefovir, entecavir and tenofovir alafenamide) used for antiviral therapy can be administered to patients with chronic HBV infection and liver inflammation, so as to alleviate the progression of liver disease[20].

Investigating the correlations of HP infection with HBC and liver cancer, and the underlying mechanism is of great significance, so as to provide theoretical references for clinical treatment of HBC and reduction of mortality. In the present study, the correlations of HP with inflammatory factors and serum levels of FoxP3 and RORγt in patients with HBC were investigated, and the effect of HP on liver function in patients with HBC was explored. The results exhibited that HP could increase HBV-DNA load level in patients with HBV. The higher the HBV-DNA load level is, the stronger the infectivity of hepatitis B will be. As a result, we believed that HP can lead to increased infectivity of hepatitis B. According to the detection resu lts of inflammatory factors in patients with HBC, IFN-γ level was remarkably higher in HBC group than that in HP&HBC group, and the levels of IL-6, IL-10, IL-17 and TGF-β1 were notably lower in HBC group in comparison with those in HP&HBC group. Subsequently, Spearman's correlation analysis revealed that the mRNA levels of FoxP3 and RORγt were positively related to those of IL-6, IL-10, IL-17 and TGF-β1, and negatively associated with IFN-γ level. Besides, immunohistochemical staining uncovered that positive expression rates of FoxP3 and RORγt in the liver tissues of HP&HBC group and HBC group were 50%, and 15%, respectively. Additionally, HBV-DNA load level was positively related to FoxP3 and RORγt expression levels. HP could increase HBV-DNA load level and expression levels of FoxP3 and RORγt, while FoxP3 and RORγt expression levels facilitated the secretion of inflammatory factors, thus aggravating HBC.

Conclusions

These results showed that the mRNA levels of FoxP3 and RORγt in serum and liver tissues are elevated in HP-infected HBC patients, leading to the secretion of a large number of inflammatory factors, and the aggravation of the liver damage. The novelty of this study was that our findings could provide a potential strategy for the treatment and prevention of HBC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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