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Expression of IL-17 and its gene promoter methylation status are associated with the progression of chronic hepatitis B virus infection

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Abstract

To explore interleukin-17 (IL-17) and its epigenetic regulation during the progression of chronic hepatitis B virus (HBV) infection.

A total of 162 patients with chronic HBV infection, including 75 with chronic hepatitis B (CHB), 54 with hepatitis B-associated liver cirrhosis and 33 with hepatitis B-associated hepatocellular carcinoma (HBV-HCC), were enrolled in this study. Thirty healthy adults of the same ethnicity were enrolled in the control group. Whole venous blood was obtained from the patients and normal controls (n = 30). Clinical and laboratory parameters were assessed, and we performed enzyme-linked immunosorbent assay and quantitative real-time PCR to measure the serum levels and relative mRNA expression of IL-17, respectively. IL-17 promoter methylation in peripheral blood mononuclear cells was assessed by methylation-specific PCR. We analyzed the serum and mRNA levels of IL-17 and IL-17 promoter methylation in the 4 groups as well as the effect of methylation on serum IL-17 levels. Correlations between the IL-17 promoter methylation status and clinical parameters were analyzed by Spearman correlation analysis.

Compared to the normal control group, the patient groups exhibited significantly higher serum and relative mRNA levels of IL-17. The methylation distribution among the patients was significantly lower than that among the normal controls ($P < .05$), with the HBV-HCC group showing the lowest *IL-17* gene methylation frequency. The average IL-17 promoter CG methylation level was negatively correlated with IL-17 mRNA expression ($r = -0.39$, $P = .03$), and negative correlations between IL-17 promoter methylation and prothrombin time activity ($r = -0.585$, $P = .035$), alanine aminotransferase ($r = -0.522$, $P < .01$), aspartate aminotransferase ($r = -0.315$, $P < .05$), and the model for end-stage liver disease score ($r = -0.461$, $P < .05$) were observed. IL-17 serum levels in the methylated-promoter groups were significantly lower than those in the unmethylated-promoter groups.

IL-17 expression and promoter methylation were associated with chronic HBV infection progression, especially in the HBV-HCC group. The IL-17 promoter status may help clinicians initiate the correct treatment strategy at the CHB stage.

Abbreviations: ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CHB = chronic hepatitis B, HBsAg = hepatitis B surface antigen, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls, MELD = model for end-stage liver disease, PTA = prothrombin time activity, TBIL = total bilirubin.

Keywords: chronic, DNA, hepatitis B, interleukin 17, methylation, methylation-specific polymerase chain reaction

1. Introduction

Hepatitis B is a viral-induced infectious disease found worldwide. Once infected with hepatitis B virus (HBV), approximately 10% of people will become chronically ill, and some cases progress to cirrhosis and hepatocellular carcinoma (HCC).[1] In general, the course of chronic HBV infection can be divided into chronic hepatitis B (CHB), hepatitis B-associated liver cirrhosis (HBV-LC) and hepatitis B-associated HCC (HBV-HCC). With the implementation of a universal vaccination policy for the HBV vaccine, the incidence of new chronic HBV infections in the Chinese population has been greatly reduced. However, the number of chronic HBV infections is still large.[2]

Studies to date have shown that HBV infection and prognosis are mainly related to 3 factors: the environment, the virus, and host factors. In particular, the host immune response plays an important role in the prognosis of HBV infection, and cytokines have recently been shown to be involved in the processes of liver injury and regeneration and to play important roles in the development and prognosis of autoimmune diseases and chronic inflammation. Therefore, increasing attention has been paid to the roles of cytokines. Among the many cytokines that influence the pathogenesis of HBV, interleukin-17 (IL-17) may be a key factor associated with chronic hepatocyte injury and viral infection.[3,4]

IL-17, an inflammatory cytokine secreted mainly by $CD4^+$ helper T cell subgroup Th17 cells, primarily participates in inflammatory reactions and immune defense and is closely related to the occurrence of some autoimmune diseases. In the liver, IL-

Editor: Muhammed Mubarak.

The authors declare that they have no funding and competing interests.

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Medicine (2019) 98:23(e15924)

Received: 19 January 2019 / Received in final form: 25 April 2019 / Accepted: 10 May 2019

http://dx.doi.org/10.1097/MD.0000000000015924

17 induces metalloproteinases by activating a variety of cells and recruits inflammatory cells, including neutrophils, causing hepatitis. IL-17 also promotes the secretion of a variety of proinflammatory factors, such as interleukin-6 (IL-6) and granulocyte colony-stimulating factor, to regulate liver inflammation.[5] Furthermore, in addition to viral immune responses, IL-17 has been reported to be involved in alcoholic liver disease and drug-induced liver injury.[6,7] IL-17 is a potential biomarker of acute rejection and plays important roles in the occurrence and development of rejection in liver transplantation and the prognosis of patients with liver failure.[8,9] As an important inflammatory factor, IL-17 may have a crucial role in the clinical prognosis of patients with hepatitis B-related liver failure. Indeed, because the level of IL-17 may be negatively correlated with clinical prognosis, it may be possible to assess the prognosis of hepatitis B-induced liver failure by monitoring the level of IL-17 to guide clinical treatment. Recently, some researchers have confirmed that IL-17 signal transduction is key in liver fibrosis and HCC, with IL-17 acting a mediator of liver fibrosis with different etiologies. Overall, high IL-17 levels are associated with poor prognosis in HCC.[10–13]

DNA methylation is the most common epigenetic modification, and abnormal DNA methylation can cause long-term silencing of gene expression and lead to many diseases.[14–18] Nonetheless, little research has been performed on methylation of the *IL-17* gene at different stages of chronic HBV infection, including CHB, HBV-LC, and HBV-HCC.

In this study, based on the changes in IL-17 expression in patients with CHB, HBV-LC, and HBV-HCC, we detected the DNA methylation frequency in the same patients. The aim was to explore epigenetic factors leading to changes in IL-17 expression and to find new ideas for studying the pathogenesis of different stages of chronic HBV infection in an effort to identify new targets for regulating IL-17 overexpression and providing new clues for clinical treatment.

2. Materials and methods

2.1. Ethics statement

Our research followed the Helsinki Declaration of the World Medical Association and was approved by the Ethics Committee of Qilu Hospital, Shandong University (No. 2170023). All participants in the study provided informed consent.

2.2. Design and participants

This study was conducted from March 2017 to August 2018. To estimate the group size, a pilot study was conducted using PASS software 11.0 for Windows (NCSS statistical software, Kaysville, UT). According to parameters of alpha = 0.05, a 2-tailed design, a power of 90%, and a group size pattern as 1:2:3:3, we needed at least 15:30:45:45 patients in each group. Initially, 173 hepatitis B surface antigen (HBsAg)-positive patients were selected; 6 were excluded due to severe lesions in other systems, 2 were pregnant, and 3 were severely alcohol dependent. Ultimately, 162 patients, including 75 with CHB (CHB group), 54 with HBV-LC (HBV-LC group), and 33 with HBV-HCC (HBV-HCC group), were enrolled in the case group; 30 healthy adults of the same ethnicity were enrolled in the control group. Of the 162 patients in the case group, 111 were male and 51 female; their ages ranged from 19 to 69 years old, with an average age of 46.67 ± 13.25 years. Of the 30 patients in the normal control group, 22 were male and 8 female; their ages ranged from 23 to 56 years old, with an average age of 43.40 ± 9.69 years. This study conformed to the Guidelines for the Prevention and Treatment of Chronic Hepatitis B formulated by the American Association for the Study of Liver Diseases in 2009.[19]

(1) CHB group: Patients had a greater than 6-month-long positive history of serum HBsAg. Those who were still positive for HBsAg and/or HBV DNA had persistent or repeated elevations of the serum alanine aminotransferase (ALT) level or hepatitis-associated pathological changes detected by histological examination of a liver sample.
(2) HBV-LC group: Cirrhosis is the result of the progression of CHB. The histological manifestations in the liver are diffuse fibrosis and pseudolobule formation, with liver function damage and portal hypertension.
(3) HBV-HCC group: This group was defined according to the 2010 update of the American Association for the Study of Liver Diseases Practice Guidelines for Management of HCC.[20]

The diagnostic criterion for CHB was persistent positive HBsAg for no <6 months before the start of patient enrollment.

No immunomodulator or antiviral therapy was used by any of the patients for half a year.

Exclusion criteria:

(1) HBV infection combined with hepatitis A virus, hepatitis C virus or another hepatophilic virus infection;
(2) alcoholic liver disease, metabolic liver disease, autoimmune liver disease, and others; and
(3) severe diseases in other systems.

2.3. Determination of clinical parameters

HBsAg and hepatitis B e antigen levels were measured using a COBAS system (Roche Diagnostic Products, Shanghai Co, Ltd, Shanghai, China). Serum ALT, aspartate aminotransferase (AST), total bilirubin (TBIL), and albumin (ALB) levels were detected with an automatic biochemical analyzer (Olympus AU5400, Japan). HBV viral load was determined using a PCR system (DA7600, Somerfly Co, Ltd, Shenzhen, China). Routine blood work was performed with a Beckman Coulter automatic blood cell counter. Coagulation was determined with a CA-530 automatic coagulation instrument. All of the above clinical parameters were assessed at the Department of Laboratory Medicine, Qilu Hospital, Shandong University, China.

2.4. Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation

Anticoagulant-treated peripheral venous blood from the patients and healthy controls (HCs) was homogenized with normal saline. The surface of a lymphocyte separation solution with a specific gravity of 1.077 was laid flat with a Pasteur pipette. The volume ratio of diluted blood to lymphocytes was 2:1, and the interface that formed between the layers was clear. The sample was centrifuged for 20 minutes at room temperature at 2000 r/min, after which the mononuclear cells were gently isolated from the white interface and transferred to another 1.5-mL centrifuge tube with a suction tube. RNA and DNA were extracted from these cells.

2.5. Measurement of IL-17 levels in the peripheral blood by enzyme-linked immunosorbent assay (ELISA)

In the morning after fasting, 5 mL of venous blood was collected, and the serum was separated. The instructions of the procedure were strictly followed. The IL-17 colorimetric reaction was evaluated at 490 nm using a fluorescent enzyme labeling instrument, and the concentration of IL-17 in each group was calculated according to a standard curve. The minimum detectable dose was 10.24 pg/mL. An ELISA kit was purchased from Bender Med Systems, Germany, and ELISA Light Absorption Enzyme Marker was purchased from Lab Systems, Finland.

2.6. Detection of IL-17 gene expression in PBMCs by reverse transcription PCR (RT-PCR)

RNA was extracted from mononuclear cells by the Trizol method, and RT-PCR was performed as described below. The 20-μL reaction consisted of 4.0 μL 5* buffer, 1 mmol/L dNTPs, 50 pmol random primers, 20 U RNasin, 10 U AMV Reverse Transcriptase, and 1 to 2 μg RNA template. The amplification conditions were 30°C for 10 minutes, followed by 42°C for 60 minutes and 95°C for 5 minutes. Primer Premier 5 software was used to design PCR primers for the *IL-17* gene (Shanghai Boya Biotechnology Corporation, Shanghai, China). The following human IL-17 primers were used: forward, 5′-AGA GAT ATC CCT CTG TG ATC-3′, and reverse, 5′-TAC CCC AAA GTT ATC TCA GG-3′.

The amplified product was 199 bp. The 20-μL PCR reaction for assessing the level of β-actin consisted of 2.0 μL 10* buffer, 2.0 mmol/L $MgCl_2$, 3.5 μL DNA template, and 1 U Taq enzyme. The amplification conditions were 95°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 50°C for 60 seconds, and 72°C for 45 seconds, with an extension at 72°C for 10 minutes. The forward and reverse primers used for human β-actin were 5′-CATGTACGTTGCTATCCAGGC-3′ and 5′-CTCCTTAATGTCACGCACGAT-3′, respectively. The amplified product was 480 bp. The PCR product (15 μL) was examined after electrophoresis through a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and analyzed with a Hema gel image analysis system (Gel-Pro Analyzer Version).

2.7. Preparation of DNA

DNA was extracted from mononuclear cells using the phenol-chloroform method. Approximately 5 μg DNA was diluted to 50 μL with double-distilled water, and 5.5 μL 2 mol/L NaOH (final concentration 0.2 mol/L) was added. The mixture was fully mixed and incubated in a water bath at 37°C for 30 minutes; 30 μL 10 mmol/L hydroquinone was added, and the mixture became yellow. Next, 520 μL 3 mol/L sodium bisulfite (pH 5.0) was added to the mixture, which was gently shaken, covered with 200 μL paraffin oil, and incubated in a 50°C water bath for 18 hours. The upper layer of paraffin oil was absorbed, and the DNA in the lower layer was purified using a resin-based purification kit (Beijing Saibersen Biological Company, Beijing, China) and finally eluted in 50 μL TE buffer. A total of 50 μL 0.6 mol/L NaOH (final concentration 0.3 mol/L) was added to the eluent, mixed well and incubated at room temperature for 15 minutes. Next, 1/10 volume of 3 mol/L sodium acetate (pH 5.2) and 3 volumes of ice-cold absolute ethanol were added to the mixture, followed by overnight precipitation at −20°C. The mixture was centrifuged at 1200 r/min for 15 minutes (4°C), and the precipitate was washed with 70% ethanol, precipitated for 10 minutes, and centrifuged at 10,000 r/min for 5 minutes (4°C). The supernatant was discarded, and the precipitate was dissolved in 30 μL deionized water and stored at −70°C.

Placental DNA was treated with the Sss-1 enzyme and then with sodium bisulfite to produce a positive control DNA template for PCR. For the negative control, deionized water was used instead of template DNA in the PCR reaction.

2.8. Detection of the methylation status of the IL-17 gene promoter in PBMCs by methylation-specific PCR (MSP)

Fresh peripheral venous blood (5 mL) was obtained from each subject, and heparin anticoagulation (25 μL/mL) and density gradient centrifugation were used to isolate PBMCs. DNA was extracted according to the instructions of a DNA extraction kit (Qiagen Company, Germany). DNA transformation was carried out according to the instructions of a DNA methylation modification kit purchased from Shanghai Jemmey Company. The basis of the assay is that sodium bisulfite converts all cytosines except those in methylated CpG regions to uridine; primers for PCR amplification are designed according to the altered sequence with thymidine pairing with the converted cytosine sites. Primers for amplifying methylated and unmethylated sequences were designed with online MethPrimer software; the primers were synthesized, packaged, and dissolved in high-pressure sterilized, triple-distilled water by Shanghai Bioengineering Company. We adjusted the concentrations of the primers to 10 μmol/L and stored them at −20°C.

The sequences for the primers were as follows: methylation reaction primer forward, 5′-TTTTTAATTCGTAACTGGG-CGC-3′, and reverse, 5′-ATAACCGGTGCCGTATCGTC-3′; unmethylated primer forward, 5′-TTTAATTCGTAACTGGGC-GTGG-3′, and reverse, 5′-AATAACCGGTGCCGTATCGT-CATC-3′.

The reaction conditions for the PCR were as follows: 95°C for 3 minutes and 35 cycles of 94°C for 45 seconds, 61°C for 1 minutes, and 72°C for 45 seconds, followed by 72°C for 5 minutes and then a hold at 4°C. After PCR amplification, 6 μL of the product was mixed with 1 μL sample buffer and separated by 1.8% agarose gel electrophoresis (constant pressure, 100 V, 30 minutes). The results were imaged with ultraviolet light and analyzed using a gel electrophoresis image analysis system. To ensure the reliability of the results, a blank control (ddH_2O instead of DNA) was included in each run. After electrophoresis, the gel was removed, rinsed with distilled water for 10 seconds, placed in a box, and stained for 20 to 30 minutes. The gel was then removed and rinsed for 10 seconds with distilled water.

Interpretation of the results was performed as described below. Including a negative control, the reactions were considered positive for methylation if bands were produced using the methylation primers; if only the unmethylated primers produced bands, the reactions were considered negative for methylation. The detection limit for methylation of the IL-17 promoter can reach 0.1%.

The above experiments were completed in the Cardiovascular Laboratory of Shandong University.

2.9. Statistical analysis

The SPSS software program version 19.0 (SPSS Inc, Chicago, IL) was used for all statistical analyses. Continuous variables with a

skewed distribution are expressed as the median (centile 25; centile 75). Categorical values are presented as relative frequencies. Quantitative variables were compared by Student *t* test or the Mann–Whitney *U* test. The chi-square test was applied to categorical data. Spearman grade correlation analysis was employed to test correlation between 2 factors. All statistical analyses were 2-sided, and a P value $< .05$ was considered statistically significant.

3. Results

3.1. General characteristics of the subjects in each group

A total of 162 patients were included in the case group of this study. There were 54 patients with HBV-LC, including 36 males, and the average patient age was 56.41 ± 14.58 years. Of the 33 patients with HBV-HCC, 24 were males, and the average patient age was 59.13 ± 10.49 years. Seventy-five patients had CHB, including 51 males; the average patient age was 48.00 ± 11.59 years. Thirty subjects, including 22 males, were enrolled in the HC group; their average age was 43.40 ± 9.69. The 4 groups were comparable in terms of age and sex ($P = .650$ and .512). Clinical parameters, including ALT, AST, TBIL, and ALB, are shown in Table 1.

3.2. Determination of serum IL-17 level and relative expression of IL-17 mRNA in PBMCs from the patients and HCs

The average serum IL-17 levels in the patients (CHB, HBV-LC, and HBV-HCC cases) were 39.28 ± 2.74 pg/mL, 48.28 ± 3.56 pg/mL, and 62.17 ± 2.91 pg/mL, respectively, and the level in the control group was 20.64 ± 1.09 pg/mL. The average relative expression of IL-17A mRNA in PBMCs from the patients (CHB, HBV-LC, and HBV-HCC) was 0.45 ± 0.19, 0.55 ± 0.21, and 0.86 ± 0.23, respectively, and relative expression in the control group was 0.05 ± 0.11. Serum IL-17 levels and the average relative expression of IL-17A mRNA in PBMCs for the patients were all significantly higher than those for the normal controls ($P < .001$), and these differences were statistically significant. Among the case groups, the HBV-HCC group had the highest serum IL-17 level and highest relative expression of IL-17 mRNA in PBMCs; compared with the HBV-LC group and CHB group values, the values in the HBV-HCC group were significantly different ($P < .05$). However, there was no significant difference between the patients with CHB and patients with LC ($P = .857$ and .613) (Figs. 1 and 2).

3.3. IL-17 gene promoter methylation

(1) In the HBV-HCC group, the rate of complete methylation was 15.2% (5/33), the rate of unmethylation was 66.7% (22/33), and the rate of coexistence of methylated and unmethylated DNA was 18.2% (6/33). In the HBV-LC and CHB groups, the complete methylation rates were 35.2% (19/54) and 46.6% (35/75), respectively, the unmethylated rates were 20.4% (11/54) and 34.6% (26/75), respectively, and the coexistence rates were 44.4% (24/54) and 18.7% (14/75), respectively. In the normal control group, the rates of complete methylation, unmethylation and methylation and unmethylation coexistence were 83.3% (25/30), 13.3% (4/30), and 0.03% (1/30).

(2) Through the Chi-square test, we concluded that the distribution of methylation in the case group was significantly different from that in the HC group ($P < .01$). The IL-17 promoter methylation frequency in the HBV-HCC group was significantly lower than that in the CHB group ($\chi^2 = 7.041$, $P < .01$) and the HBV-LC group ($\chi^2 = 6.438$, $P < .01$). There was no significant difference in IL-17 promoter methylation frequency between the CHB and HBV-LC groups ($\chi^2 = 0.975$, $P > .05$) (Fig. 3).

(3) The correlation between the level of DNA methylation in the promoter region of the *IL-17* gene and expression of IL-17 mRNA was also analyzed. In the patients and corresponding normal controls, Spearman grade correlation analysis revealed that the average level of CG methylation in the IL-17 promoter region was negatively correlated with the level of IL-17 mRNA expression ($r = -0.39$, $P = .03$), as shown in Figure 4.

Table 1

Demographic characteristics of each group.

Variable	HBV-HCC (n = 33)	HBV-LC (n = 54)	CHB (n = 75)	HC (n = 30)	*P* value
Sex (male/female)	24/9	36/18	51/24	22/8	.512*
Age, yr	59.13 ± 10.49	56.41 ± 14.58	48.00 ± 11.59	43.40 ± 9.69	.650†
$HBeAg^+$ (%)	11 (33.3%)	51 (68%)	39 (72.2%)	NA	<.001*
$HBV\ DNA^+$ (%)	14 (42.4%)	45 (60%)	43 (79.6%)	NA	<.01*
WBC, 10^9/L	6.12 (4.52–8.41)	5.21 (3.41–6.97)	5.35 (4.42–6.12)	NA	.051†
HGB, g/L	134 (114–141)	131 (113–142)	146 (136–154)	NA	<.001†
PLT, 10^9/L	148 (101–243)	102 (83–147)	195 (152–229)	NA	<.001†
ALT, IU/L	56 (38–139)	53 (35–197)	123 (54–469)	NA	<.001†
AST, IU/L	57 (43–99)	57 (31–177)	59 (35–332)	NA	.179†
TBIL, μmol/L	33.2 (16.1–52.6)	28.9 (17–68)	38.4 (16.4–99.7)	NA	.713†
ALB, g/L	33.6 (31.8–39.7)	34.7 (29.1–39.8)	41.6 (35.7–45.7)	NA	<.001†
PTA (%)	74 (60–81)	71 (55–84)	96 (90–106)	NA	<.001†
Lg (HBV DNA)	4.13 ± 1.65	4.37 ± 1.78	4.99 ± 0.95	NA	<.001†
MELD score	6.51 (5.01–9.29)	6.97 (4.31–11.68)	NA	NA	.731‡

Quantitative variables are expressed as the median (centile 25; centile 75) or mean ± SD. Categorical variables are expressed as numbers (%).

*Chi-square test.

†Kruskal–Wallis test.

‡Mann–Whitney U test.

ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CHB = chronic hepatitis B, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls, HGB = hemoglobin, MELD = model for end-stage liver disease, NA = not available, PLT = platelet, PTA = prothrombin time activity, TBIL = total bilirubin, WBC = white blood cell.

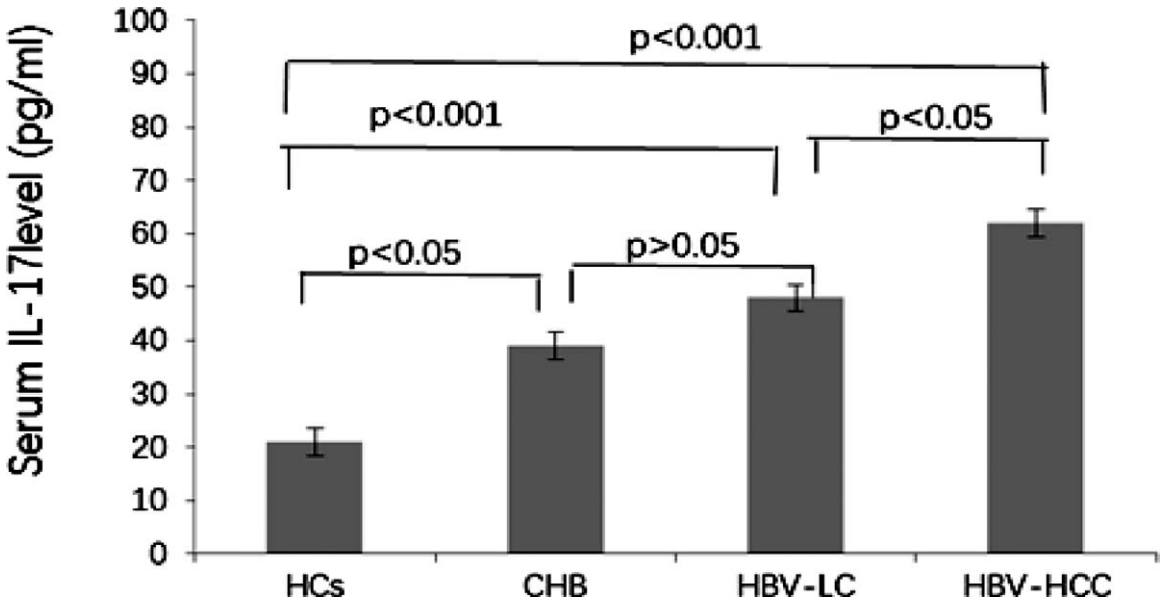

Figure 1. Comparison of serum IL-17 levels among the different hepatitis B virus infection progression groups and healthy controls. Notes: All data are summarized as the mean and standard deviations. $P < .05$ was considered statistically significant. $P > .05$, CHB group versus HBV-LC group; $P < .05$ CHB group, HBV-LC group, HBV-HCC group versus control. CHB = chronic hepatitis B, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls.

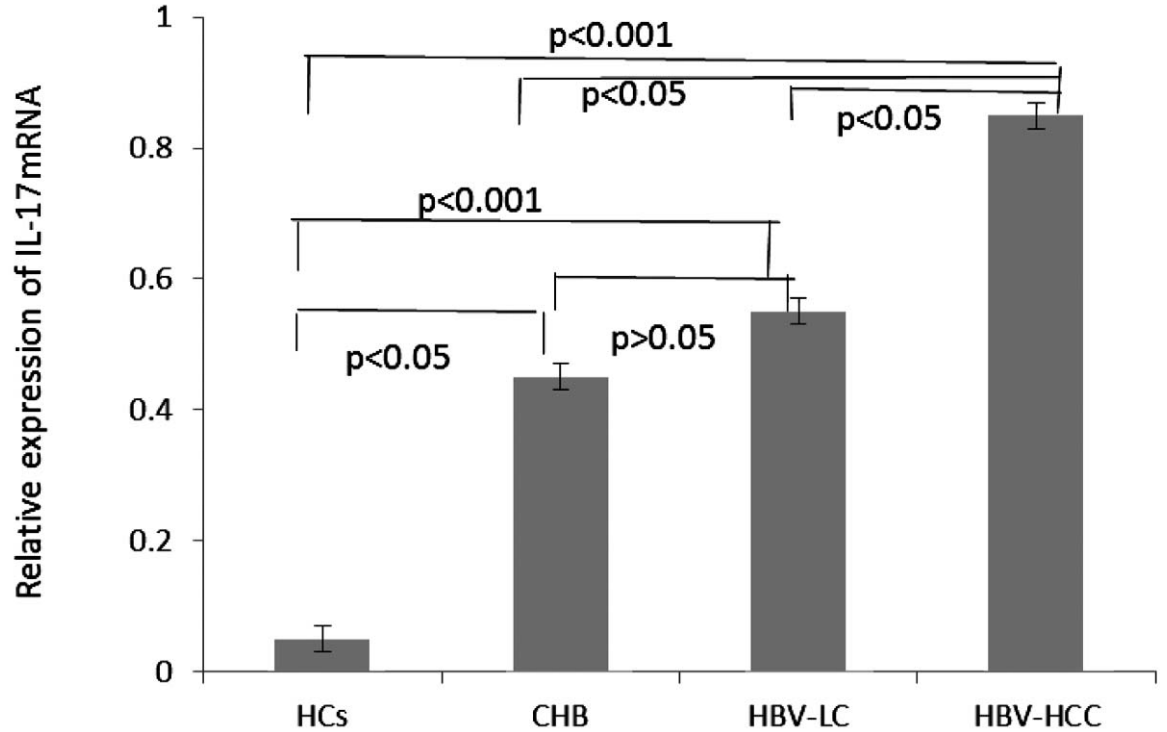

Figure 2. Comparison of IL-17 mRNA levels in PBMCs among different hepatitis B virus infection progression groups and healthy controls. Notes: All data are summarized as the mean and standard deviations. $P < .05$ was considered statistically significant. IL-17 mRNA expression was analyzed by Gel-pro analyzer software. Mean ± s. $P > .05$, CHB group versus HBV-LC group; $P < .05$ CHB group, HBV-LC group, HBV-HCC group versus control. CHB = chronic hepatitis B, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls, PBMCs = peripheral blood mononuclear cells.

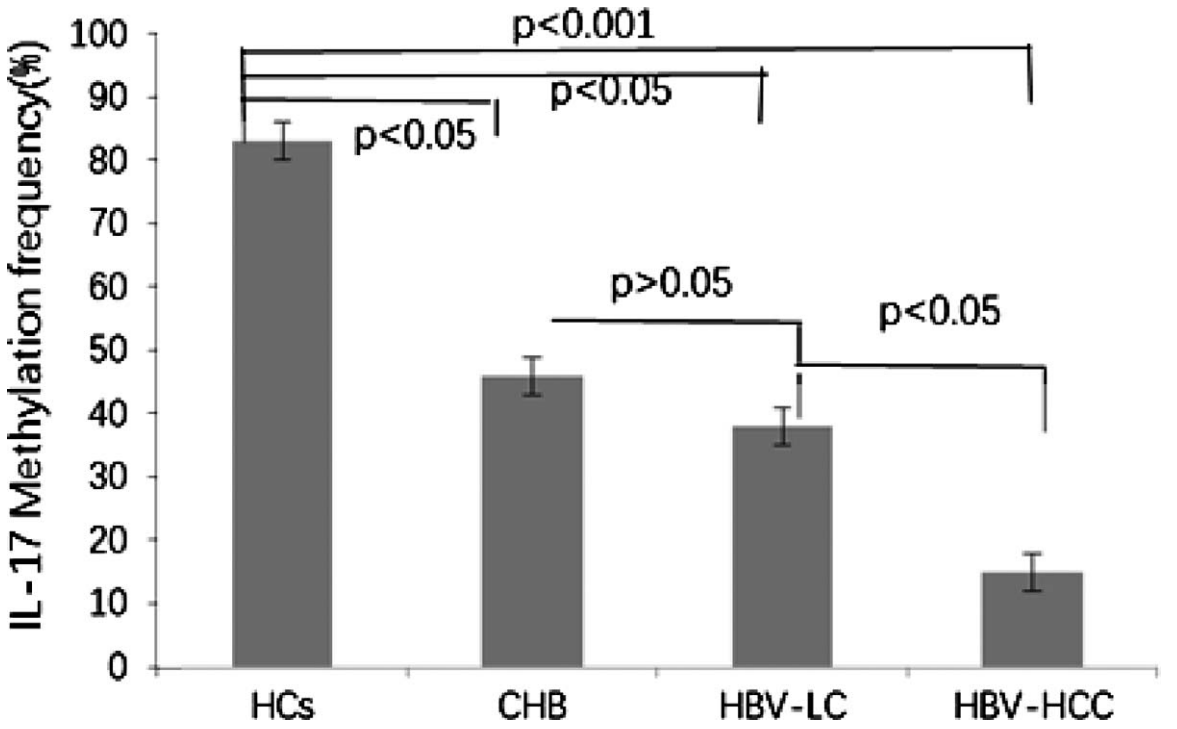

Figure 3. IL-17 promoter methylation frequency among different hepatitis B virus infection progression groups and healthy controls. Notes: All data are summarized as the mean and standard deviations. $P < .05$ was considered statistically significant. $P > .05$, CHB group versus HBV-LC group; $P < .05$ CHB group, HBV-LC group, HBV-HCC group v control. CHB = chronic hepatitis B, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls.

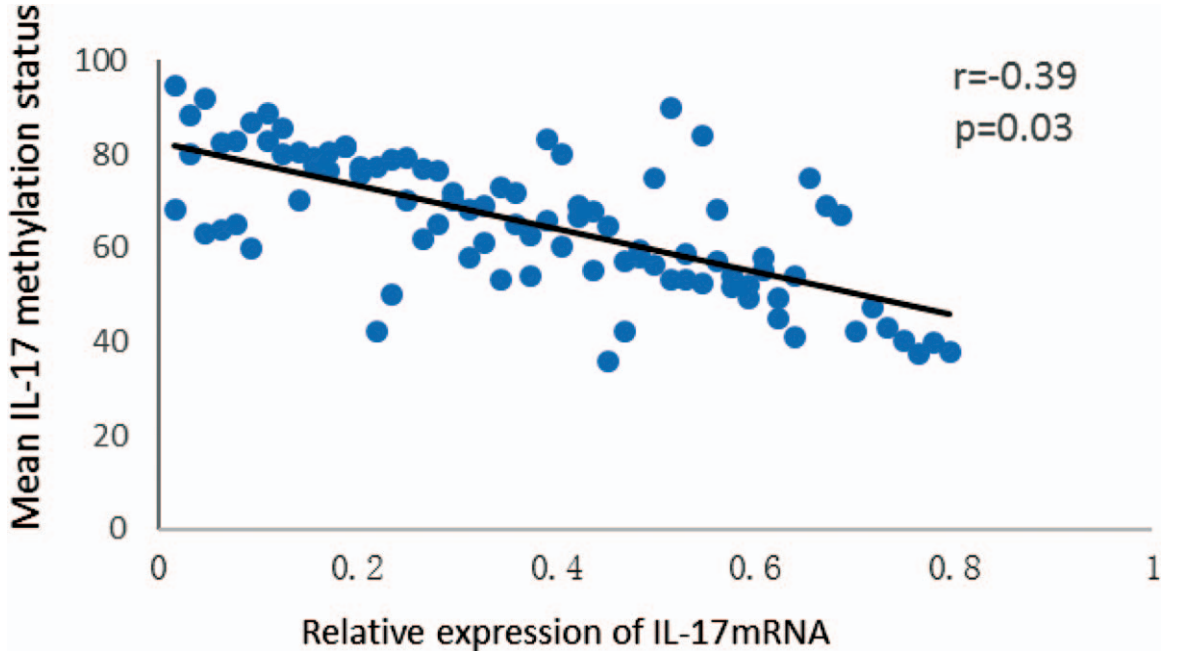

Figure 4. Analysis of the correlation between IL-17 expression level and the level of IL-17 promoter methylation in patients and normal controls. Notes: N = 84, $r = -0.39$, $P = .03$.

Table 2

Relationships between IL-17 promoter methylation and clinical parameters.

Variable	r	P value
Sex (male/female)	0.021	.813
Age, yr	0.149	.391
WBC, 10^9/L	−0.139	.243
HGB, g/L	0.069	.577
PLT, 10^9/L	0.047	.713
ALT, IU/L	−0.522	.001**
AST, IU/L	−0.315	.032*
TBIL, μmol/L	−0.131	.354
ALB, g/L	−0.055	.484
PTA (%)	−0.585	.035
Lg (HBV DNA)	0.216	.09
MELD score	−0.461	.043*

The results of the Spearman correlation analysis showed significant linear correlations between IL-17 promoter methylation and ALT, AST, and MELD score.

* $P < .05$.

** $P < .01$.

ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HGB = hemoglobin, MELD = model for end-stage liver disease, PLT = platelet, PTA = prothrombin time activity, TBIL = total bilirubin, WBC = white blood cell.

3.4. Correlations between IL-17 promoter methylation and clinical parameters

In addition, we examined correlations between methylation and clinical parameters in the 4 groups using the Spearman method. We found no associations between IL-17 promoter methylation and sex ($r = 0.021$, $P = .813$), age ($r = 0.149$, $P = .391$), $\log_{10}$ HBV DNA level ($r = 0.216$, $P = .09$), ALB ($r = -0.055$, $P = .484$), TBIL ($r = -0.131$, $P = .354$), platelet count ($r = 0.047$, $P = .713$), white blood cell count ($r = -0.139$, $P = .243$) or hemoglobin ($r = 0.069$, $P = .577$) in the case groups. In contrast, negative correlations between IL-17 promoter methylation and prothrombin time activity (PTA) ($r = -0.585$, $P = .035$), ALT ($r = -0.522$, $P < .01$), AST ($r = -0.315$, $P < .05$) and the model for end-stage liver disease (MELD) score ($r = -0.461$, $P < .05$) were observed, as shown in Table 2.

3.5. Effect of IL-17 promoter methylation on the serum IL-17 level

The HBV-HCC, HBV-LC, CHB, and HC groups were each divided into a methylated DNA group and an unmethylated DNA group. Based on this stratification, a significant difference in serum IL-17 levels between each methylated group and unmethylated group was found, with serum IL-17 levels in each methylated DNA group being significantly lower than those in the unmethylated DNA group ($P < .05$), as shown in Figure 5.

4. Discussion

CHB is a worldwide infectious disease. Once a patient becomes chronically infected, the covalently closed circular DNA of HBV is difficult to eradicate from the nucleus of hepatocytes, which increases the risk of developing hepatic decompensation, cirrhosis, and HCC.[21,22]

CHB is related to immunity, whereby an abnormal cellular immune response is closely associated with the occurrence and progression of chronic HBV infection and liver damage, and IL-17 plays an important regulatory role in the complex immune network of CHB. In this study, we confirmed that serum IL-17 levels and relative expression of IL-17 mRNA in PBMCs from CHB, HBV-LC, and HBV-HCC groups were significantly higher than those in PBMCs from HCs; moreover, the differences between the HCC group and the control group were statistically significant. Previous studies have shown that higher IL-17 levels in the liver are accompanied by higher serum IL-17 and IL-17 mRNA levels in PBMCs.[23,24] Regardless, due to the lack of liver biopsy, the relationship between IL-17 levels in the liver and serum IL-17 levels or IL-17 mRNA levels in PBMCs needs to be further confirmed.

In the present study, we also analyzed correlations between IL-17 promoter methylation and clinical parameters. ALT and AST are the main markers of liver inflammation, and we found negative correlations between IL-17 promoter methylation and PTA ($r = -0.585$, $P = .035$), ALT ($r = -0.522$, $P < .01$), and AST ($r = -0.315$, $P < .05$). MELD is a new systematic model for evaluating liver reserve function. The TBIL level, PTA, serum creatinine level and etiology of liver pathogens are used to evaluate the severity and prognosis of disease, and a higher score indicates a greater risk of death in the short term.[25] The results of our study also showed that IL-17 promoter methylation was significantly associated with MELD score in patients. Overall, the lower the level of IL-17 promoter methylation, the more serious is the patient's condition. This observation also proves that the IL-17 promoter methylation status can reflect the severity of a patient's condition, which suggests that methylation is related to disease activity. Nonetheless, we found no associations between IL-17 promoter methylation and HBV DNA, and further research is needed.

Epigenetic inheritance is a heritable change in gene expression without DNA sequence changes and includes DNA methylation and histone modification.[26,27] DNA methylation involves the addition of a methyl group to DNA, and aberrant promoter demethylation is usually associated with overexpression of genes that might participate in the pathogenesis of many diseases.[28] A large number of studies have confirmed that epigenetics is an important mechanism regulating the differentiation of $CD4^+$ T cells into different subsets,[29] and an imbalance in Th1, Th2, Th17, and other related cytokines has been observed in the peripheral blood of patients with liver diseases.[30,31] In general, the DNA methylation status is usually stable, rendering this measure suitable for use as a biomarker for disease detection and prognosis prediction,[32] and the most commonly used MSP method was used in this study. Previous studies have shown that chronic hepatitis-cirrhosis-HCC is a process of sustained progression. Our results showed that the degree of *IL-17* gene promoter methylation was significantly different between the case group and the HC group. The degree of IL-17 promoter methylation was lowest in the patients with HBV-HCC, and the degree of IL-17 promoter methylation in the CHB patients was lower than that in the HCs and higher than that in the HBV-LC patients. This trend may have some predictive value for disease progression. We also observed a significant difference in serum IL-17 levels between the methylated DNA group and the unmethylated DNA group among the CHB, HBV-LC, or HBV-HCC patients and HCs. Serum IL-17 levels in the group with methylated DNA were significantly lower than those in the group with unmethylated DNA. Furthermore, the serum IL-17 level was negatively correlated with methylation status, and

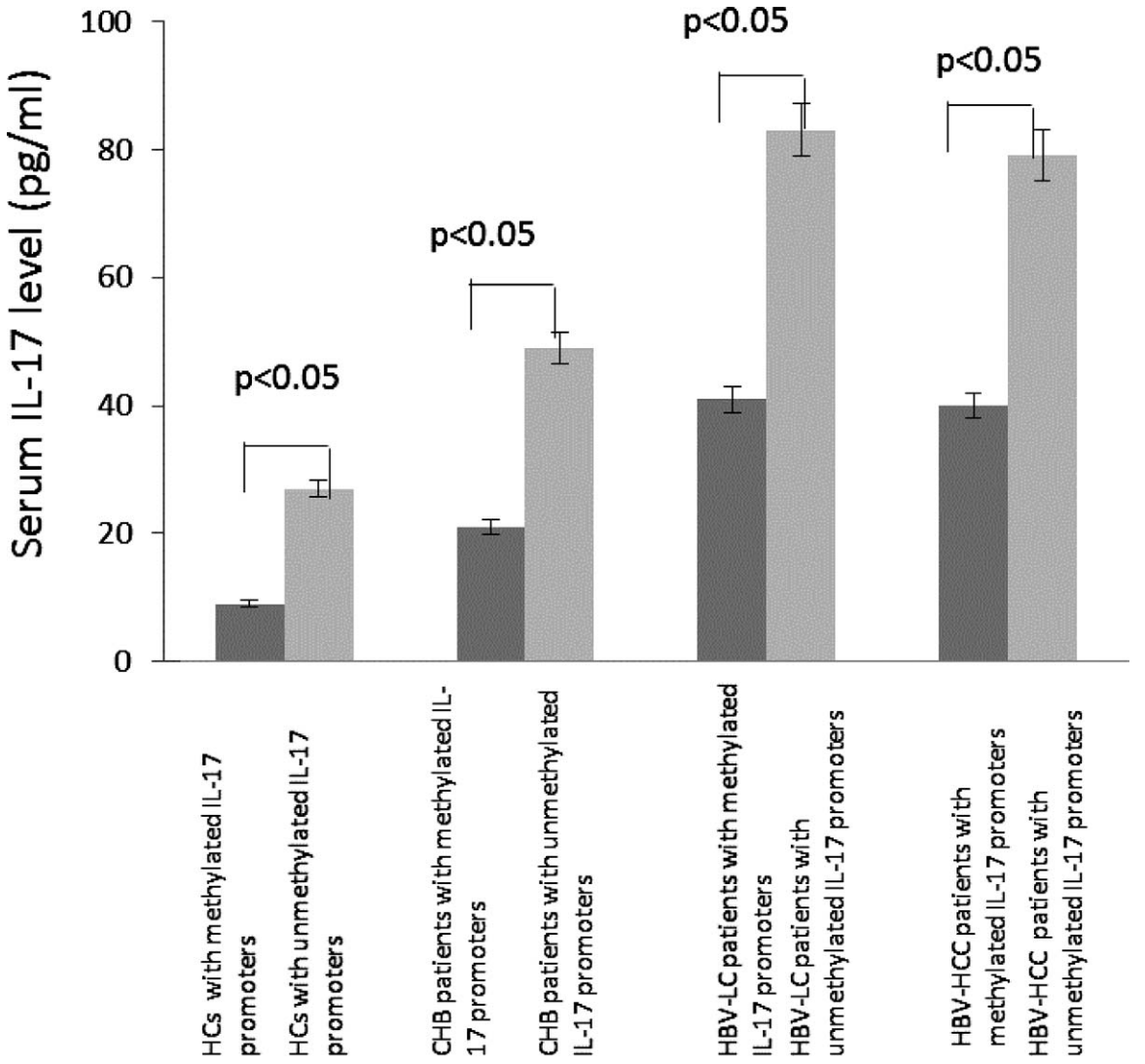

Figure 5. Serum IL-17 levels in methylated DNA groups and unmethylated DNA groups. Notes: All data are summarized as the mean and standard deviations. $P < .05$ was considered statistically significant. Significant difference ($P < .05$, methylated vs unmethylated). CHB = chronic hepatitis B, HBV-HCC = hepatitis B-associated hepatocellular carcinoma, HBV-LC = hepatitis B-associated liver cirrhosis, HC = healthy controls.

Spearman grade correlation analysis revealed that IL-17 promoter methylation was negatively correlated with IL-17 mRNA expression, indicating that methylation modification inhibits expression of the IL-17 gene. Low methylation is key for *IL-17* gene activation, which may lead to an increase in IL-17 secretion; hypomethylation of the *IL-17* gene promotes gene transcription and increases protein synthesis, which promotes differentiation of $CD4^+$ T cells into Th17 cells. Most importantly, this study revealed that the frequency of IL-17 promoter methylation in the HBV-HCC group was significantly lower than that in the CHB and HBV-LC groups. However, the mechanism of IL-17 overexpression is not well studied, and the occurrence and development of malignant tumors involve the inactivation or activation of many genes. As gene mutation or promoter methylation can determine gene activation or inactivation,[33] this finding indicates that methylation frequency has potential value in prognostic evaluations and as a new marker for targeted gene therapy.

We found that methylation and unmethylation of the *IL-17* gene frequently coexist. MSP can only detect methylation, but not quantitatively, and this restricts accurate evaluation of the relationship between gene methylation and mRNA expression. It is possible that methylation at different densities initiates transcription to varying degrees. Therefore, methylation of non-CpG islands upstream of the first exon of the *IL-17* gene may not regulate transcription. It is generally accepted that a certain proportion of CpG island methylation (60%) can completely inhibit gene expression but that a low proportion of methylation can only reduce gene transcription, which needs to be quantified to further elucidate the mechanism by which transcription is inhibited. The recent application of microarray technology and pyrophosphate sequencing technology in gene methylation research will enable a better understanding of the methylation status and allow for developing a better DNA methylation spectrum, which is expected to provide new targets for clinical diagnosis and treatment.

Of course, our research has limitations. First, the limited sample size makes it impossible for us to further improve. Moreover, the CHB group included light, moderate and severe CHB, and the HBV-LC group included compensated and decompensated LC. Therefore, more refined grouping is necessary. Third, as our study was not a prospective study, we did not include resolved HBV cases and patients with/after treatment. In addition, due to the practical difficulties in obtaining tissues from living individuals (liver biopsy is not widely accepted in China), methylation levels were assessed in PBMCs, and there was no analysis of IL-17 promoter methylation or IL-17 expression in HCC tissue or HCC cell lines.

In conclusion, this study suggests that epigenetic phenomena represented by DNA methylation play an important role in the pathogenesis of CHB. Treatment that suppresses hypomethylation of the IL-17 promoter region may inhibit the occurrence and development of CHB. Moreover, IL-17 promoter status may help clinicians initiate the correct treatment strategy at the CHB stage. In addition, these findings suggest that IL-17 promoter methylation may serve as a biomarker for HBV-HCC. However, the mechanism leading to the change in methylation status is not yet clear, and this change still needs to be further explored in the future.

Acknowledgments

We greatly appreciate the time and effort of the participants of this study.

Author contributions

Yan Yang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Yan Yang participated in the study concept and design and revised the manuscript. Cui-Huan Tian and Jun Dai contributed to the conception of the study, collection and analysis of the data, and drafting of the manuscript. The analysis and interpretation of the data were performed by Wei Zhang and Yan Liu. All authors read and approved the final manuscript. The authors have also agreed to be accountable for all aspects of the work in regard to ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Cui-Huan Tian and Jun Dai contributed equally to this study and should be regarded as co-first authors.

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References

[1] Liaw YF, Chu CM. Hepatitis B virus infection. Lancet 2009;373:582–92.
[2] Tanaka M, Katayama F, Kato H, et al. Hepatitis B and C virus infection and hepatocellular carcinoma in China: a review of epidemiology and control measures. J Epidemiol 2011;21:401–16.
[3] Tacke F, Hammerich L, Heymann F. Role of IL-17 and Th17 cells in liver diseases. Clin Dev Immunol 2011;2011:345803.
[4] Zhang JY, Zhang Z, Lin F, et al. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. Hepatology 2010;51:81–91.
[5] Jhun JY, Lee SH, Kim HY, et al. HMGB1/RAGE induces IL-17 expression to exaggerate inflammation in peripheral blood cells of hepatitis B patients. J Transl Med 2015;13:310.
[6] Ma HY, Xu J, Liu X, et al. The role of IL-17 signaling in regulation of the liver-brain axis and intestinal permeability in alcoholic liver disease. Curr Pathobiol Rep 2016;4:27–35.
[7] Kobayashi M, Higuchi S, Mizuno K, et al. Interleukin-17 is involved in α-naphthylisothiocyanate-induced liver injury in mice. Toxicology 2010;275:50–7.
[8] Kim N, Yoon YI, Yoo HJ, et al. Combined detection of serum IL-10, IL-17, and CXCL10 predicts acute rejection following adult liver transplantation. Mol Cells 2016;39:639–44.
[9] Shulin Z, Hong'An X, Tianyan C, et al. The balance between intrahepatic IL-17 + T cells and Foxp3 + regulatory T cells plays an important role in HBV-related end-stage liver disease. BMC Immunol 2011;12:47–147.
[10] Meng F, Wang K, Aoyama T, et al. Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. Gastroenterology 2012;143:765–76.
[11] Amara S, Lopez K, Banan B, et al. Synergistic effect of pro-inflammatory TNFα and IL-17 in periostin mediated collagen deposition: potential role in liver fibrosis. Mol Immunol 2015;64:26–35.
[12] Rui L, Jian S, Han W, et al. High expression of IL-17 and IL-17RE associate with poor prognosis of hepatocellular carcinoma. J Exp Clin Cancer Res 2013;32:3–13.
[13] Punt S, Langenhoff JM, Putter H, et al. The correlations between IL-17 vs. Th17 cells and cancer patient survival: a systematic review. OncoImmunology 2015;4:e984547.
[14] Klutstein M, Nejman D, Greenfield R, et al. DNA methylation in cancer and aging. Cancer Res 2016;76:3446–50.
[15] Nishida N, Yada N, Hagiwara S, et al. Unique features associated with hepatic oxidative DNA damage and DNA methylation in non-alcoholic fatty liver disease. J Gastroenterol Hepatol 2016;31:1646–53.
[16] Cheng-Gang J, Qun C, Lina W, et al. The innovative regularity and role of p16 methylation in blood during HCC development. J Cancer 2018;9:1925–31.
[17] Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–92.
[18] Søes S, Daugaard IL, Sørensen BS, et al. Hypomethylation and increased expression of the putative oncogene ELMO3 are associated with lung cancer development and metastases formation. Oncoscience 2014;1:367–74.
[19] Lok AS, McMahon BJ. Chronic hepatitis B: update. Hepatology 2009;50:661–2.
[20] Bruix J, Sherman M. American Association for the Study of Liver DManagement of hepatocellular carcinoma: an update. Hepatology 2011;53:1020–2.
[21] Raimondo G, Allain JP, Brunetto MR, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol 2008;49:652–7.
[22] Pollicino T, Saitta C, Raimondo G. Hepatocellular carcinoma: the point of view of the hepatitis B virus. Carcinogenesis 2011;32:1122–32.
[23] Yang B, Wang Y, Zhao C, et al. Increased Th17 cells and interleukin-17 contribute to immune activation and disease aggravation in patients with chronic hepatitis B virus infection. Immunol Lett 2013;149:41–9.
[24] Du WJ, Zhen JH, Zeng ZQ, et al. Expression of interleukin-17 associated with disease progression and liver fibrosis with hepatitis B virus infection: IL-17 in HBV infection. Diagnos Pathol 2013;8:40.
[25] Goldberg DS, Olthoff KM. Standardizing MELD exceptions: current challenges and future directions. Curr Transplant Rep 2014;1:232–7.
[26] Rochtus A, Izzi B, Vangeel E, et al. DNA methylation analysis of Homeobox genes implicates HOXB7 hypomethylation as risk factor for neural tube defects. Epigenetics 2015;10:92–101.
[27] Augello C, Gianelli U, Falcone R, et al. PDGFB hypomethylation is a favourable prognostic biomarker in primary myelofibrosis. Leuk Res 2015;39:236–41.
[28] Smith IM, Glazer CA, Mithani SK, et al. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PloS One 2009;4:e4961.
[29] Johnson ND, Wiener HW, Smith AK, et al. Non-linear patterns in age-related DNA methylation may reflect CD4(+) T cell differentiation. Epigenetics 2017;12:492–503.
[30] Saxena R, Kaur J. Th1/Th2 cytokines and their genotypes as predictors of hepatitis B virus related hepatocellular carcinoma. World J Hepatol 2015;7:1572–80.

[31] Rios DA, Valva P, Casciato PC, et al. Chronic hepatitis C liver microenvironment: role of the Th17/Treg interplay related to fibrogenesis. Sci Rep 2017;7:13283.
[32] Catherine L, Marissa W, Chuan JH, et al. DNA methylation as a noninvasive epigenetic biomarker for the detection of cancer. Dis Markers 2017;2017:1–3.
[33] Mazzon I, Favilli A, Grasso M, et al. Is 'cold loop' hysteroscopic myomectomy a better option for reproduction in women with diffuse uterine leiomyomatosis? A case report of successful repeated pregnancies. J Obstet Gynaecol Res 2015;41: 474–7.