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Expression profiles of transcription factors for special CD4+ T-cell subsets in peripheral blood mononuclear cells from patients with hepatitis B virus infection

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Abstract

This study is to characterize the transcription factor expression profiles for the peripheral CD4+ T-cell subsets, and analyze its associations with the clinical measures of the hepatitis B virus (HBV) infection.

Totally 275 subjects were included. The expression levels of transcription factors (T-bet, GATA-3, Foxp3, RORγt, and Bcl-6) in the peripheral blood mononuclear cells (PBMCs) were determined by the real-time fluorimetry quantitative PCR (FQ-PCR).

Lowest expression levels of all these transcription factors were observed for the HBsAb(–) group, which were higher in the HBsAb (+) and RHB groups. The T-bet/GATA-3 ratios in the CHB and RHB groups were significantly lower than the HBsAb(–) group, whereas the RORγt/Foxp3 ratios in the AHB and RHB groups were significantly higher than the CHB and HBsAb(+) groups. Furthermore, the RORγt mRNA expression levels were significantly different among groups with different disease severities or with different alanine aminotransferase (ALT) levels. The asymptomatic carrier (AsC) group and the group with ALT $\leq$ 40 had the highest express level. The mRNA expression levels of T-bet, GATA-3, Foxp3, and RORγt varied along with the aspartate aminotransferase (AST) levels, with AST $\leq$ 40 having the highest expression levels. In addition, significant differences were observed in the transcription factor expression levels between the group with the serum HBV DNA load of (1.000–9.999) $\times$ 10^4 copies/mL and other groups.

Expression profile of critical transcription factors for peripheral CD4+ T-cell subsets may indicate clinical outcomes of HBV infection.

Abbreviations: AHB = acute HBV infection, ALT = alanine aminotransferase, AsC = asymptomatic carrier, AST = aspartate aminotransferase, Bcl-6 = B cell lymphoma 6, CHB = chronic HBV infection, CHE = cholinesterase, Ct = comparative cycle threshold, FoxP3 = Forkhead/winged helix transcription factor, FQ-PCR = fluorimetry quantitative PCR, GATA-3 = guanine adenine thymine adenine sequence-binding protein 3, HAV = hepatitis A virus, HBV = hepatitis B virus, HCV = hepatitis C virus, HDV = hepatitis D virus, HIV = human immunodeficiency virus, MICHB = mild CHB, MOCHB = moderate CHB, PBMCs = peripheral blood mononuclear cells, PTA = prothrombin activity, RHB = recessive self-limited HBV infection, RORγt = retinoic acid receptor-related orphan receptor, SCHB = severe CHB, TBIL = total bilirubin, Tfh = follicular helper T, Th1 = T helper 1.

Keywords: CD4+T-cell subsets, hepatitis B virus, infection, peripheral blood mononuclear cells, transcription factors

1. Introduction

Hepatitis B virus (HBV) infection is a global health concern, affecting approximately 30% of the people worldwide.[1–3] The clinical outcomes of HBV infection are variable. Most HBV infection (acute or recessive) in adults ends up with spontaneous recovery. Approximately 5% of the HBV-infected adults become chronic infection, representing an important risk factor for liver cirrhosis and hepatocellular carcinoma.[2,4,5] The variability of HBV infection outcomes is due to different CD4+T cell responses, which is considered to play a pivotal role in the anti-HBV immunity.[6–8]

CD4+ T cells included the following six subsets: the T helper 1 (Th1) cells, T helper 2 (Th2) cells, regulatory T (Treg) cells, T helper 17 (Th17) cells, follicular helper T (Tfh) cells, and T helper 9 (Th9). Different CD4+ T cell subsets secrete different cytokines, which are regulated by different transcription factors.[6–8] Th1 cells, characterized by the T-box expressed in T cells (T-bet) and the production of IFN-γ, are crucial for viral clearance.[8,9] Th2 cells, featured by the guanine adenine thymine adenine sequence-binding protein 3 (GATA-3) and the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13, may be associated with viral persistence.[8,10]

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Chronic HBV infection (CHB) often have the Th1/Th2 imbalance, with defect in the Th1 cells.[8,11]

Th17 cells represent a pro-inflammatory T cell subset, which produce cytokines of IL-17A, IL-17F, and TNF-α. The Th17 cell development is dependent on the retinoic acid receptor-related orphan receptor (RORγt).[6–8] Frequency of circulating Th17 increases with the disease progression of CHB, and the Th17 cells are enriched in both the peripheral blood and liver in the CHB patients.[8,12–14] Treg cells exert immunoregulatory effects by expressing CD4, CD25, CD45RO, and CTLA-4. Forkhead/winged helix transcription factor (FoxP3) has been demonstrated to be a unique marker for Treg cells.[6–8] CHB patients always have increased frequency of Treg cells in the peripheral blood or liver, which can inhibit the HBV-specific immune response.[15–18] Tfh cells express chemokine receptor CXCR5, ICOS, PD-1, and IL-21, with the critical transcription factor of the B cell lymphoma 6 (Bcl-6). High frequency of $CD4^+CXCR5^+$ Tfh cells has been detected in the CHB patients,[19–21] indicating active immune status,[19] which facilitates the HBeAg seroconversion by producing IL-21.[21] Th9 cells can produce IL-9, with the critical transcription factor of STAT6. However, Th9 cells are unlikely to be involved in the pathogenesis of HBV infection.[7,22]

Transcription factors are critical for the expansion, differentiation, and cytokine production of the $CD4^+$ T cell subsets.[6,7] Many studies have characterized the $CD4^+$ T-cell subsets in the HBV infection at the cellular levels, based on the phenotype/frequency analysis by flow cytometry and cytokine measurement by ELISA/ELISPOT. However, there are a few systematic studies characterizing the specific transcription factors of $CD4^+$ T-cell subsets in correlation with different clinical outcomes of HBV infection. In this study, with the real-time fluorimetry quantitative PCR (FQ-PCR), the expression profiles of transcription factors of the $CD4^+$ T-cell subsets (T-bet, GATA-3, RORγt, Foxp3, and Bcl-6) were measured, and their potential association with the disease clinical measures were also evaluated.

2. Materials and methods

2.1. Patients

Blood samples were collected from 16 patients with acute HBV infection (AHB) at early stage, 137 patients with CHB, and 62 patients with recessive self-limited HBV infection (RHB), respectively, who were admitted to the Second Affiliated Hospital, Harbin Medical University and the Hospital for Infectious Diseases in Harbin, Heilongjiang, China, from December 2011 to October 2013. In these 137 patients with CHB, there were 36 asymptomatic carriers (AsCs), 49 patients with mild CHB (MICHB), 40 patients with moderate CHB (MOCHB), and 12 patients with severe CHB (SCHB). The diagnostic standards for AHB, RHB, CHB, and AsCs were described in details previously.[23,24]

SCHB cases were diagnosed as the CHB patients exhibiting obvious or persistent hepatitis symptoms, with the serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) > 3 times of the normal level (0–40 IU/L), total bilirubin (TBIL) > 5 times of the normal level (1.6–20.6 μmol/L), and albumin ≤ 32 g/L, as well as the ratio of albumin to globulin (A/G) ≤ 1.0, gamma globulin ≥ 26%, prothrombin activity (PTA) between 40% to 60%, and cholinesterase (CHE) ≤ 4500 U/L. Moreover, SCHB could be directly diagnosed when one of the following items was confirmed: albumin ≤ 32 g/L, TBIL > 85.5 μmol/L, PTA between 40% to 60%, and CHE < 2500 U/L. On the other hand, diagnostic criteria for MICHB included the mild clinical signs and symptoms, such as the serum levels of ALT or AST < 120 IU/L, TBIL < 34.2 μmol/L, albumin ≥ 32 g/L, A/G ≥ 1.4, gamma globulin ≤ 21%, PTA > 70%, and CHE > 5400 U/L. Clinical symptoms, signs, and laboratory examination results between the mild and severe CHB cases were diagnosed as MOCHB.[23,24]

All these included patients were demonstrated the absence of antibodies against hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis D virus (HDV), or human immunodeficiency virus (HIV). Other causes of chronic liver damages were also excluded. None of these patients had received anti-HBV agents or steroids within 6 months before sampling. In addition, 16 healthy individuals without vaccine injection [HBsAb(–)], and 44 healthy individuals with vaccine injection [HBsAb(+)] were enrolled as controls. The basic characteristics of these subjects were listed in Table 1. Study protocol was approved by our ethics committee and the written informed consent was obtained from each participant.

2.2. Laboratory assays

HBV serology and antibodies against HAV, HCV, HDV, and HIV were determined by ELISA using commercially available kits (Kehua Co., Ltd., Shanghai, China). AST, ALT, and TBIL levels were measured in a clinical laboratory (Modular P800; Roche, Mannheim, Germany) with commercially available kits (Roche, Mannheim, Germany). Serum HBV DNA was quantified by FQ-PCR (Stratagene Mx3000p; Agilent Technologies, Waldbronn, Germany) using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit with a detection limit of 500 copies/ml (QIAGEN, Shenzhen, Guangzhou, China).

2.3. Cell preparation

Peripheral blood mononuclear cells (PBMCs) were obtained out of the fresh EDTA anti-coagulated peripheral blood from subjects by standard Ficoll-Hypaque density centrifugation (Solarbio Science & Technology Co., Ltd., Beijing, China).

Table 1

Clinical characteristics of included subjects.

	AHB	CHB	RHB	HBsAb(+)	HBsAb(-)	*n*	*P*
Male/female	9/7	94/43	32/30	22/22	6/10	163/112	.020
Age, ys	36.31 ± 11.93	37.28 ± 11.65	41.31 ± 15.51	39.20 ± 8.47	41.38 ± 8.97	38.68 ± 12.15	.140
ALT, μ/L	1045.06 ± 1086.09	167.35 ± 284.23	25.58 ± 20.99	15.55 ± 5.73	15.94 ± 5.99	153.36 ± 398.94	<.001
AST, μ/L	562.31 ± 677.06	110.88 ± 214.70	26.21 ± 14.91	17.16 ± 4.38	16.96 ± 4.22	97.55 ± 251.60	<.001
TBIL, μmol/L	162.04 ± 157.75	35.85 ± 86.13	12.20 ± 5.38	11.98 ± 3.54	14.24 ± 16.44	32.78 ± 78.95	<.001
HBVDNA, IU/mL)	$3.33 \times 10^6 \pm 1.09 \times 10^7$	$(1.78 \pm 4.85) \times 10^7$				$(1.63 \pm 4.61) \times 10^7$	.002

AHB = acute self-limiting HBV infection, CHB = patients with chronic HBV infection, HBsAb(–) = subjects with non-vaccine immunity, HBsAb(+) = subjects with vaccine immunity, RHB = patients with recessive self-limiting infection.

Table 2

Sequences of primers and probes used in FQ-PCR.

Gene	Accession No.	Sequence (5' to 3')	Product length (bp)
Tbet	NM_013351	Forward: CTTGGTGTGGACTGAGATTGC Reverse: ACTGGAAGGATAGGGGGACA Probe: ATTCAGGACTGGGCGAAGGAGACTCT	103 bp
GATA-3	XM_005252443	Forward: AGACCACCACAACCACACTCT Reverse: GATGCCTTCCTTCTTCATAGTCA Probe: ATGCCAATGGGGACCCTGTCTGC	122bp
RORγt	XM_005245425	Forward: CCAGCTCCAGCTGTCTTCTACC Reverse: ACATCTGCTTCCTTCTCCACAAC Probe: AAGCAGAAGTCGCTCGCACTGGTCA	147bp
Foxp3	XM_005272612	Forward: AAGGAAAGGAGGATGGACG Reverse: CAGGCAAGACAGTGGAAACC Probe: AAACTGGTGGGAGGCAGAGGTGGT	123bp
Bcl-6	NM_001130845	Forward: TTTAGAGTGCTCATTTGGTTTTG Reverse: ATTAAGGTTGAGAAGAACATCACTG Probe: CTGTGAAGCAAGGCATTGGTGAAGAC	135bp
β-actin	XM_005249820	Forward: CTGGAACGGTGAAGGTGACA Reverse: CACCTCCCCTGTGTGGACTT Probe: AGCGAGCATCCCCCAAAGTTCACA	215bp

2.4. FQ-PCR analysis

Total RNA was extracted from each frozen sample using lysate RL (Bioteke, Beijing, China), according to the manufacturer's instructions. The total mRNA was converted to cDNA using the supermoIII RT Kit (Bioteke) for FQ-PCR. PCR primers and probes were designed based on the reported cDNA sequences and synthesized by Gemma Company (Shanghai, China). Sequences of primers and FAM-labeled probes were summarized in Table 2. PCR was performed using Premix ExTaq (Bioneer, Daejeon, Korea) on the real-time PCR machine in a final volume of 20 μL, with the following thermal conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All PCR assays were performed in duplicate, and data were analyzed using the comparative cycle threshold (Ct) method. The relative expression levels of target genes were determined with the $2^{-\Delta\Delta Ct}$ method, which were normalized to β-actin.

2.5 Statistical analysis

Data were analyzed with the SPSS 17.0 software (SPSS, Chicago, IL). One-way ANOVA and student *t* test were used for comparison of parametric quantitative data. Kruskal–Wallis and Mann–Whitney tests were used for comparison of nonparametric data. Spearman correlation analysis was performed between the transcription factor mRNA expression for peripheral $CD4^+$ T-cell subsets and the clinical measures. All tests used were 2-tailed. $P < .05$ was considered statistically significant.

3. Results

3.1. Demographics and serological analysis of enrolled subjects

Demographics and serological analysis results of the enrolled subjects were shown in Table 1. Our results showed that the significant difference was observed in the sex ratio between these groups ($P = .024$). Because the percentage of males and females enrolled in the CHB group was clearly higher than the other groups. There was no significant difference in the ages of the enrolled subjects between these groups ($P = .140$). On the other hand, significant differences were observed in the ALT levels, HBV loads, and positive ratios of other serological markers, suggesting different status and stages of HBV infection. These results indicate that the enrolled subjects meet the requirements for the following investigation.

3.2. Different expression profiles of transcription factors among these groups

To determine the expression profiles of $CD4^+$ T-cell subsets in the AHB, RHB, CHB, HBsAb(+), and HBsAb(–) groups, the expression levels of transcription factors in the PBMCs were investigated by FQ-PCR, including T-bet, GATA-3, Foxp3, RORγt, and Bcl-6. As shown in Table 3 and Figure 1, significant

Table 3

Transcription factor relative expression levels of subjects.

	AHB *n*=16	CHB *n*=137	RHB *n*=62	HBsAb(+) *n*=44	HBsAb(-) *n*=16	*P*
T-bet	0.36±0.60	0.55±0.80	0.78±1.00	1.03±1.04	0.42±0.61	.001
GATA-3	0.27±0.47	0.39±0.73	0.66±0.86	0.89±1.00	0.15±0.20	.001
Foxp3	0.61±1.35	0.73±2.09	1.38±2.23	2.33±3.73	0.09±0.17	.007
RORγt	0.37±1.00	0.16±0.44	0.57±0.87	0.69±1.27	0.02±0.02	.017
Bcl-6	1.52±3.14	1.08±1.78	1.31±2.12	1.64±2.45	0.54±0.99	.360
T/G	4.85±4.61	2.70±2.40	2.54±2.11	2.39±2.40	3.66±1.79	.061
R/F	0.55±0.39	0.35±0.33	0.98±1.61	0.30±0.26	0.31±0.26	.000

AHB = acute HBV infection, Bcl6 = the B cell lymphoma 6, CHB = chronic HBV infection, FoxP3 = Forkhead/winged helix transcription factor, GATA-3 = the guanine adenine thymine adenine sequence-binding protein 3, HBsAb(–) = healthy individuals without vaccine injection, HBsAb(+) = healthy individuals with vaccine injection, R/F = RORγt/ Foxp3, RHB = recessive self-limited HBV infection, RORγt = the retinoic acid receptor-related orphan receptor, T/G = T-bet/ GATA-3, T-bet = the T-box expressed in T cells. $P < .05$ was considered statistically significant.

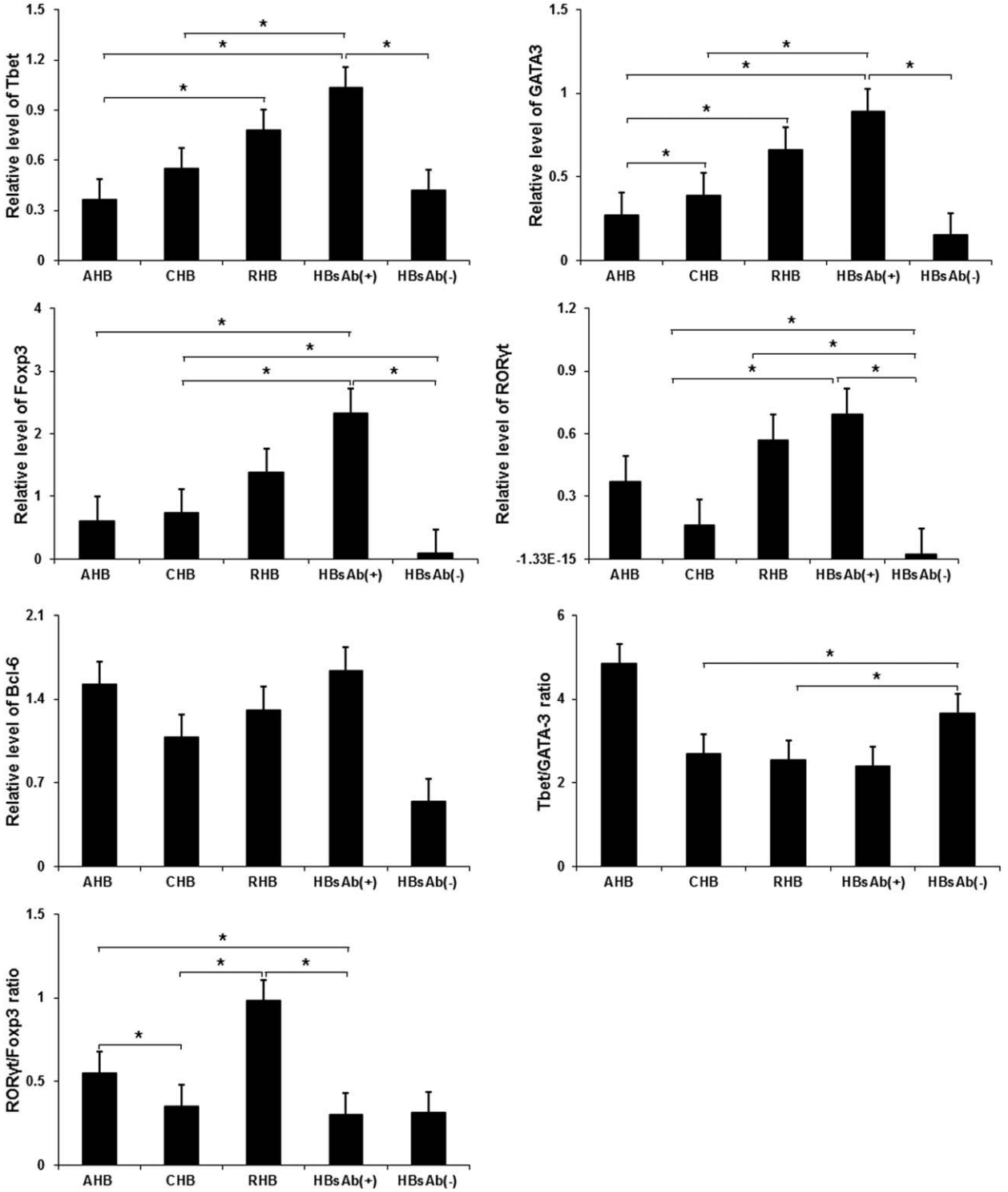

Figure 1. Basic expression levels of T-bet, GATA-3, Foxp3, RORγt, and Bcl-6 in subjects. The ratios of T-bet/GATA-3 and RORγt/Foxp3 were also shown. $^{*}P < .05$.

differences were observed in the relative expression levels of these transcription factors between these 5 groups.

The T-bet mRNA relative expression levels were significantly different to among these 5 groups ($P = .001$). In specific, the T-bet mRNA relative expression level in the HBsAb(+) group was significantly higher than the HBsAb(–) group (u = –2.77, $P = .006$) and the CHB group (u = –3.57, $P < .001$). Moreover, the T-bet mRNA relative expression level in the AHB group was significantly lower than the RHB (u = –2.36, $P = .018$) and HBsAb(+) (u = –3.21, $P = .001$) groups. The GATA-3 mRNA relative expression levels were significantly different to among the 5 groups ($P = .001$). In specific, the GATA-3 mRNA relative expression level in the HBsAb (+) group was significantly higher than the HBsAb(–) group (u = –2.671, $P = .008$) and the CHB group (u = –3.04, $P = .002$).

Moreover, the GATA-3 mRNA relative expression level in the AHB group was significantly lower than the CHB (u=–2.02, $P=.043$), RHB (u=–2.70, $P=.007$), and HBsAb(+) (u=−3.24, $P=.001$) groups. Compared with the HBsAb(−) group, significantly lower Tet/GATA-3 ratios were observed for the CHB (u=−2.24, $P=.025$) and RHB (u=−2.09, $P=.037$) groups, which was critically lower for the HBsAb(+) group ($t=1.92$, $P=.060$). Moreover, compared with the HBsAb(+) group, the T-bet/GATA-3 ratio in the AHB group was to among higher (u=−1.79, $P=.074$).

The T-bet mRNA relative expression levels were significantly different to among these 5 groups ($P=.001$). In specific, compared with the HBsAb(–) group, significantly higher RORγt mRNA relative expression levels were observed in the CHB (u=−2.35, $P=.019$), RHB (u=−2.35, $P=.019$), and HBsAb(+) (u=−2.84, $P=.004$) groups. Moreover, the RORγt mRNA relative expression level in the CHB group was significantly lower than the HBsAb (+) group (u=−2.26, $P=.024$). The Foxp3 mRNA relative expression levels were significantly different among these 5 groups ($P=.007$). In specific, compared with the HBsAb(−) group, the Foxp3 mRNA relative expression levels were significantly higher in the CHB (u=−2.50, $P=.012$) and HBsAb(+) (u=−2.89, $P=.004$) groups. Moreover, compared with the HBsAb(+) group, the Foxp3 mRNA relative expression levels were significantly lower in the AHB (u=−2.29, $P=.022$) and CHB (u=−2.47, $P=.014$) groups. In addition, the RORγt/Foxp3 ratios in the AHB and RHB groups were significantly higher than the CHB group (u=−2.59, $P=.009$ and u=−4.32, $P<.001$ for the AHB and RHB groups, respectively) and the HBsAb(+) group ($t=-2.75$, $P=.008$ and u=−3.67, $P<.001$ for the AHB and RHB groups, respectively). Although the highest Bcl-6 mRNA relative expression level was observed in the HBsAb(+) group, Bcl-6 is not found statistically significantly different between these groups.

Taken together, among these 5 groups, the lowest relative expression levels of all these transcription factors (T-bet, GATA-3, Foxp3, RORγt, and Bcl-6) was observed in the HBsAb(−) group. Moreover, the transcription factor relative expression levels were higher in the HBsAb(+) and RHB groups than other groups. However, the T-bet/GATA-3 ratios in the CHB and RHB groups were significantly lower than the HBsAb(−) group, and the RORγt/Foxp3 ratios in the AHB and RHB groups were significantly higher than the CHB and HBsAb(+) groups.

3.3. Different expression profiles of transcription factors in CHB with different severities

Based on the clinical and laboratory findings, these 137 CHB patients were divided into the following 4 groups: the SCHB, MOCHB, MICHB, and AsCs groups. The expression levels of transcription factors in PBMCs (ie, T-bet, GATA-3, Foxp3, RORγt, and Bcl-6) were tested by FQ-PCR. As shown in Table 4, these transcription factors showed significantly different relative expression levels among these four groups. Out of these 5 transcription factors, only the RORγt mRNA relative expression levels were significantly different among the 4 groups ($P=.025$). In specific, compared with the MOCHB group, the RORγt mRNA expression levels were significantly higher in the SCHB (Z=−2.521, $P=.012$) and AsCs (Z=−2.645, $P=.008$) groups. For all the 5 transcription factors, their expression levels were high in the AsCs group. In particular, the Foxp3 and Bcl-6 mRNA relative expression levels in the AsCs group were significantly higher than the MOCHB (Z=−2.003, $P=.045$) and MICHB (Z=−2.015, $P=.044$) groups.

3.4. Correlation between critical transcription factor expressions for CD4+ T-cell subsets and disease severity markers in CHB patients

These CHB patients were divided into 3 groups based on the levels of ALT, AST, and TBIL to investigate whether the expression levels of transcription factors for CD4+T-cell subsets were correlated with their levels. As shown in Table 5, the RORγt mRNA expression levels were significantly different ($P=.045$) among groups with different ALT levels. The RORγt mRNA expression level in the group with ALT $\leq$ 40 was significantly higher than the groups with ALT of 41–120 (Z=–2.062, $P=.039$) and >120 (Z=−2.176, $P=.030$). Moreover, the mRNA expression levels of T-bet, GATA-3, Foxp3, and RORγt were significantly different ($P=.013$; $P=.001$; $P=.014$; and $P=.011$, respectively) among groups with different AST levels. The mRNA expression levels of these transcription factors in the group with AST $\leq$ 40 were significantly higher than the groups with AST of 41–120 (Z=−2.846, $P=.004$; Z=−3.655, $P=.000$; Z=−2.856, $P=.004$; and Z=−2.845, $P=.004$, respectively).

3.5. Correlation between transcription factor expressions for CD4+ T-cell subsets and HBV DNA load in CHB patients

To investigate whether the expression levels of transcription factors for CD4+ T-cell subsets were correlated with the HBV replication level, the serum viral titers were measured in the CHB patients. These patients were divided into six groups based on the serum HBV DNA load. As shown in Table 6, the GATA-3, Foxp3, and RORγt mRNA expression levels in the group with

Table 4

Transcription factor relative expression levels of CHB groups with different severities.

	SCHB $n=12$	MOCHB $n=40$	MICHB $n=49$	AsCs $n=36$	P
T-bet	0.4194±0.4732	0.5199±0.9487	0.4649±0.5292	0.7537±0.9995	.308
GATA-3	0.2903±0.4633	0.3991±0.9695	0.2985±0.3818	0.5325±0.8439	.226
Foxp3	0.2859±0.4693	0.5069±1.2403	0.3634±0.9797	1.6080±3.5336	.222
RORγt	0.1366±0.2715	0.0689±0.1996	0.1033±0.2326	0.3511±0.7405	.025
Bcl-6	0.4235±0.3897	1.0691±1.8772	0.9364±1.6384	1.4902±2.0779	.111
T/G	1.9954±2.2803	3.1335±2.9253	2.5507±2.4575	2.6000±1.5606	.397
R/F	0.4479±0.5198	0.2928±0.3101	0.3725±0.3376	0.3336±0.2794	.387

AsCs = asymptomatic carriers, Bcl6 = the B cell lymphoma 6, CHB = Chronic HBV infection, FoxP3 = forkhead/winged helix transcription factor, GATA-3 = the guanine adenine thymine adenine sequence-binding protein 3, MICHB = mild CHB, MOCHB = moderate CHB, R/F = RORγt/Foxp3, RORγt = the retinoic acid receptor-related orphan receptor, SCHB = severe CHB, T/G = T-bet/GATA-3, T-bet = the T-box expressed in T cells. $P<.05$ was considered statistically significant.

Table 5

Transcription factor relative expression levels of CHB groups with different ALT, AST, and TBIL levels.

Group	N	T-bet	GATA-3	Foxp3	RORγt	Bcl-6	T/G	R/F
ALT ≦ 40	51	0.65 ± 0.89	0.44 ± 0.75	1.22 ± 3.09	0.30 ± 0.65	1.46 ± 2.29	2.88 ± 2.30	0.41 ± 0.41
ALT: 41-120	41	0.44 ± 0.54	0.29 ± 0.40	0.39 ± 1.06	0.08 ± 0.20	0.75 ± 1.06	2.32 ± 1.98	0.30 ± 0.24
ALT > 120	45	0.55 ± 0.91	0.42 ± 0.93	0.52 ± 1.18	0.08 ± 0.19	0.97 ± 1.79	2.82 ± 2.83	0.32 ± 0.31
P		.409	.690	.775	.045	.127	.467	.349
AST ≦ 40	61	0.70 ± 0.86	0.46 ± 0.69	1.09 ± 2.82	0.26 ± 0.60	1.40 ± 2.11	2.51 ± 1.86	0.34 ± 0.31
AST: 41-120	43	0.40 ± 0.74	0.21 ± 0.40	0.34 ± 1.05	0.07 ± 0.20	0.57 ± 0.56	3.44 ± 3.04	0.38 ± 0.38
AST > 120	33	0.48 ± 0.76	0.49 ± 1.05	0.58 ± 1.29	0.09 ± 0.22	1.13 ± 2.03	2.06 ± 2.16	0.31 ± 0.32
P		.013	.001	.014	.011	.169	.090	.557
TBIL ≦ 20.6	98	0.56 ± 0.80	0.40 ± 0.80	0.82 ± 2.35	0.17 ± 0.48	1.11 ± 1.76	2.80 ± 2.41	0.31 ± 0.26
TBIL: 20.7-103	32	0.50 ± 0.57	0.44 ± 0.57	0.57 ± 1.26	0.13 ± 0.28	1.12 ± 2.02	2.34 ± 2.34	0.39 ± 0.42
TBIL > 103	7	0.24 ± 0.21	0.06 ± 0.04	0.11 ± 0.10	0.18 ± 0.35	0.41 ± 0.45	2.72 ± 2.73	0.62 ± 0.64
P		.625	.150	.844	.258	.447	.403	.465

ALT = alanine aminotransferase, AST = aspartate aminotransferase, Bcl6 = the B cell lymphoma 6, CHB = chronic HBV infection, FoxP3 = forkhead/winged helix transcription factor, GATA-3 = the guanine adenine thymine adenine sequence-binding protein 3, R/F = RORγt/Foxp3, RORγt = the retinoic acid receptor-related orphan receptor, T/G = T-bet/GATA-3, T-bet = the T-box expressed in T cells, TBIL = total bilirubin. $P < .05$ was considered statistically significant.

the serum HBV DNA load of $(1.000–9.999) \times 10^4$ copies/mL were significantly higher than the groups with the serum HBV DNA load of $\geq 1.000 \times 10^7$ copies/mL ($Z = -2.748$, $P = .006$; $Z = -3.206$, $P = .001$; and $Z = -1.959$, $P = .050$, respectively). Moreover, the GATA-3 and Foxp3 mRNA expression levels in the group with the serum HBV DNA load of $(1.000–9.999) \times 10^4$ copies/mL were significantly higher than the groups with the serum HBV DNA load of $\leq 1.000 \times 10^3$ copies/mL ($Z = -2.117$, $P = .034$ and $Z = -2.414$, $P = .016$, respectively) and $(1.000–9.999) \times 10^6$ copies/mL ($Z = -2.369$, $P = .018$ and $Z = -2.463$, $P = .014$, respectively). Furthermore, the Foxp3 and RORγt mRNA expression levels in the group with the serum HBV DNA load of $(1.000–9.999) \times 10^4$ copies/mL were significantly higher than the group with the serum HBV DNA load of $(1.000–9.999) \times 10^5$ copies/mL ($Z = -2.515$, $P = .012$; $Z = -1.782$, $P = .075$).

The included patients in the CHB group were also divided into the sAg+eAg+ and sAg+eAg- groups. No significant differences were observed in the expression levels of any transcription factors between these groups ($P > .05$) (data not shown).

4. Discussion

$CD4^+$ T cells play pivotal roles in the adaptive immune response to HBV infection in human beings.[8] However, relatively less is known about the effects of transcription factor expression for peripheral $CD4^+$ T-cell subsets on the clinical outcomes of HBV infection. In the present study, the mRNA expression levels of transcription factors (T-bet, GATA-3, RORγt, Foxp3, and Bcl-6) were analyzed and compared in the patients with different HBV infection outcomes. The associations between the transcription factor expressions and clinical parameters were evaluated. Our results found that the transcription factor profile of $CD4^+$ T-cell subsets may be used as potential marker for the evaluation of the clinical outcomes of HBV infection.

Our results showed that higher expression levels of T-bet and GATA-3 might be associated with slight liver injuries. It has been widely accepted that the Th1 and Th2 immunity are important in the HBV infection, and the balance of Th1/Th2 influences the outcomes of HBV infection. A predominant shift of the immune response profile towards Th1 has been shown to be crucial for the eradication of HBV, while a prevalent Th2 response seems to favor the viral persistence.[25,26] In this study, the mRNA expression levels of both T-bet and GATA-3 were lower in the AHB and HBsAb(−) groups, when compared with others. However, the ratio of T-bet/GATA-3 was markedly increased in the AHB and HBsAb(−) groups than other groups, indicating that the ratio of T-bet/GATA-3 might represent an important indicator for the disease outcomes. The expression levels of T-bet and GATA-3, as well as the ratio of T-bet/GATA-3, in CHB patients different severities were further investigated, and their associations with severity markers or viral load were analyzed. Our results showed significant differences in the T-bet and GATA-3 mRNA expression levels between the groups with AST ≤ 40 and 41 to 120.

Zhang et al[27] have reported that both the AHB and CHB patients have significantly higher Th17 responses than subjects

Table 6

Transcription factor relative expression levels of CHB groups with different HBV DNA levels.

HBVDNA (copies/mL)	N	T-bet	GATA-3	Foxp3	RORrt	Bcl-6	T/G	R/F
$< 1.000 \times 10^3$	36	0.61 ± 0.79	0.32 ± 0.55	0.72 ± 1.93	0.22 ± 0.56	0.78 ± 0.85	2.65 ± 2.20	0.31 ± 0.25
$(1.000–9.999) \times 10^3$	12	0.56 ± 0.64	0.74 ± 1.10	1.61 ± 4.25	0.20 ± 0.35	1.95 ± 2.90	2.21 ± 2.59	0.49 ± 0.57
$(1.000–9.999) \times 10^4$	15	0.50 ± 0.40	0.57 ± 0.59	1.35 ± 2.18	0.30 ± 0.60	2.04 ± 3.09	2.33 ± 2.55	0.36 ± 0.41
$(1.000–9.999) \times 10^5$	19	0.75 ± 1.08	0.37 ± 0.50	0.43 ± 0.89	0.07 ± 0.21	0.88 ± 1.53	2.82 ± 1.83	0.35 ± 0.40
$(1.000–9.999) \times 10^6$	26	0.46 ± 0.88	0.46 ± 1.17	0.79 ± 2.54	0.17 ± 0.51	1.11 ± 1.98	2.16 ± 1.92	0.34 ± 0.27
$\geq 1.000 \times 10^7$	29	0.47 ± 0.75	0.20 ± 0.30	0.23 ± 0.57	0.07 ± 0.17	0.70 ± 0.62	3.54 ± 3.08	0.33 ± 0.28
F or *U*	137	3.324	8.180	10.128	4.692	3.770	1.090	0.453
P		.650	.147	.072	.455	.583	.369	.994

Bcl6 = the B cell lymphoma 6, CHB = Chronic HBV infection, FoxP3 = Forkhead/winged helix transcription factor, GATA-3 = the guanine adenine thymine adenine sequence-binding protein 3, R/F = RORγt/Foxp3, RORγt = the retinoic acid receptor-related orphan receptor, T/G = T-bet/ GATA-3, T-bet = the T-box expressed in T cells. $P < .05$ was considered statistically significant.

with AsCs, and the Th17 responses are significantly higher in the AHB patients compared with the CHB patients. In consistence with that, our results showed that the RORγt mRNA expression level was increased in the AHB group compared with the CHB group, although without statistical significance. In the CHB group, the RORγt mRNA expression level was significantly higher compared with the HBsAb(–) group, but was significantly or markedly lower than the HBsAb(+) and RHB groups. These data indicate that Th17 responses often occur in the case of HBV infection. Previous studies concerning CHB patients have suggested that Th17 is highly enriched in the peripheral blood and liver, and the increased Th17 response is correlated with the liver injury in the HBV-infected individuals.[12,27–29] Our further stratification analysis revealed that the RORγt mRNA expression levels were markedly increased in the SCHB and AsCs patients compared with the MOCHB patients, with the highest level for the AsCs patients. However, a previous study has shown that that the SCHB patients have a significantly increased Th17 frequency compared with the patients with MICHB and HCs.[12] Moreover, our results showed that the increased mRNA expression level of RORγt was accompanied by normal serum AST and ALT levels ($\leq 40\,\mu$/L) in the CHB patients. Hou et al[30] have indicated that Th17 cells up-regulate the anti-apoptotic molecules and increase the persistent infection by promoting the survival of virus-infected cells. However, in the present study, correlation between the higher RORγt mRNA expression level and lower serum HBV DNA load was found in the CHB patients. Therefore, further in-depth studies are still needed to find out whether Th17 participates in the viral persistent infection. Taken together, RORγt seems to be involved in light or none liver injuries, which represents a candidate for the evaluation of clinical outcomes of HBV infection.

In this study, our results suggested that like RORγt, FoxP3 may also be one of the regulatory targets of HBV infection and immune response. Previous studies have shown higher frequencies of Treg cells in patients with chronic infection than healthy controls or convalescent patients.[16,31–34] TrehanPati et al[35] have reported higher number of Treg cells in the AHB patients compared with the CHB patients. Several studies have also shown that the mRNA expression level of Foxp3 was increased in the CHB patients.[36,37] Based on these findings, Foxp3 is involved in the pathogenesis of CHB, influencing the disease clinical outcomes. In the present study, our results showed that the Foxp3 mRNA expression level was markedly increased only in the CHB patients compared with the healthy controls without vaccine injection [HBsAb(−)]. However, lower frequencies of Treg cells were noted in patients with chronic infection than healthy controls with vaccine injection [HBsAb(+)] and convalescent patients who spontaneously cleared infection. Prior studies have found correlation between the liver injury severity and the Treg activity enhancement in the CHB patients.[16,32,38] Two independent groups have also reported that SCHB patients have elevated Foxp3 mRNA expression levels,[16,38] indicating that the increased Foxp3 expression is associated with the poor disease prognosis. In the present study, association was noted only between the higher Foxp3 mRNA expression and normal serum AST level. Previous studies have shown that intrahepatic Foxp3 expression is strongly correlated with the HBV DNA load,[39] and the frequencies of circulating Treg cells are correlated with the concentration of HBV DNA load.[16,31–34,37] In the present study, the Foxp3 mRNA expression levels in the CHB patients with different HBV DNA loads were also investigated. Higher Foxp3 mRNA expression level was only found in the group with the serum HBV DNA load of (1.000–9.999) $\times 10^4$ copies/mL, and there was a positive correlation between the Foxp3 mRNA expression and serum HBV DNA load.

Th17 and Treg cells share reciprocal developmental pathways in immune responses. There exists an imbalance of Th17/Treg in the CHB patients.[40] Zhai et al[41] have reported that the Th17/Treg ratio was inversely associated with the survival of acute-on-chronic liver failure (ACLF) patients, and the Treg/Th17 ratio in the CHB patients was negatively correlated with the inflammation degree.[40,42] In addition, entecavir-induced suppression of viral replication results in a profound decreased Treg/Th17 ratio, indicating that the imbalance of Treg to Th17 might play an important role in the HBV persistence.[39] In the present study, our results showed that the RORγt/Foxp3 ratios in the AHB and RHB groups were significantly higher than the CHB and HBsAb (+) groups. Moreover, the RORγt/Foxp3 ratio in the RHB group was the highest among all the groups. However, no correlation was observed between the ratio and disease severity or HBV DNA load. Thus, we speculate that the balance between Th17 and Treg cells might be a crucial indicator for immune homeostasis, which, at least partially, reflects the balance between pro-inflammation and anti-inflammation processes. Taken together, the Th17/Treg ratio may be a useful marker for the evaluation of disease severities,[43,44] and the RORγt/Foxp3 ratio may represent a potential prognostic marker.

Although contradictory results exist, high frequency of Tfh might be associated with the immunity against HBV infection. Several studies have indicated that Tfh participates in the HBV-related immune responses.[19–21] Xing et al[11] have found that the increased Tfh level was detected in the CHB patients, and Feng et al[19] have reported that high frequency of Tfh may be a biomarker for the active immune stage of CHB. In the present study, the expression levels of Bcl-6 were higher in the AHB, CHB, RSH, and HBsAb(+) groups, compared with the HBsAb(−) group, though without statistical significance, implicating its potential role in the HBV-related immune responses. Moreover, our results also showed that the Bcl-6 mRNA expression level was significantly increased in the AsCs patients. However, no significant differences were observed concerning the hepatic injury markers and HBV DNA loads in the CHB patients. Our results were inconsistent with previous findings showing that the frequency of Tfh is significantly correlated with the AST levels, but not the levels of HBV DNA load in the immune-active CHB patients.[19] Besides, higher levels of serum HBV DNA, ALT, and TBIL in patients infected with HBV genotype C may be related to less Tfh cells in the peripheral blood.[45] Further in-depth studies are still needed in the future.

In the present study, one likely relationship between the transcription factor expressions for $CD4^+$T-cell subsets and hepatitis B markers was found. Our results showed that the no significant differences were observed in the mRNA expression levels of any transcription factor between the sAg+eAg+ and sAg +eAg- groups. This finding was contradictory with a previous study showing that Treg cells are significantly higher in the HBeAg(+) CHB patients than the HBeAg(−) patients.[45] Previous studies have also showed an association between the serum HBeAg content and increased Treg percentage,[16,31,32,36,46] suggesting that HBeAg might induce Treg cells. In terms of Tfh cells, in contrast with our study, previous studies has shown that HBeAg(−) patients represent higher frequencies of Treg cells than HBeAg(+) patients and HCs.[31,32,46] Moreover, higher frequency of circulating Tfh cells has also

been demonstrated in patients achieving HBeAg seroconversion, and Tfh might play a role in facilitating the HBeAg seroconversion in CHB patients via regulating IL-21 secretion.[21] Despite the contradictory findings, our results suggest that the expression levels of these transcription factors, especially T-bet and GATA-3, may be valuable prognostic biomarkers for the evaluation of immune status in CHB patients and the prediction of clinical outcomes of HBV infection.

The expression levels of transcription factors are different for different $CD4^+$ T-cell subsets in the PBMCs, making it a practical method to measure the transcription factor expression levels in the total PBMCs, without isolating the different cell subsets. Our results for the mRNA expression levels of transcription factors were in line with previous findings about cell frequencies determined by flow cytometry. Nevertheless, there were several limitations for this study, such as the limited sample sizes and the lack of functional study of transcription factors for $CD4^+$ T-cell subsets in the pathogenic processes with different clinical outcomes. Further detailed investigation of the expression and function of intrahepatic transcription factors for $CD4^+$ T-cell subsets with enlarged sample sizes are still needed.

In conclusion, our results showed that the expression profiles of critical transcription factors for $CD4^+$T-cell subsets in different clinical outcomes of HBV infection, which may predict that the cellular immune level of recessive self-limited HBV infection is on the high side and equal to the level after the vaccine; and chronic infected cell immunity level is also higher than normal group without the vaccine, but lower than the recessive self-limited person, indicating low cellular immune response. Notably, the expression profiles of critical transcription factors for $CD4^+$T-cell subsets in different severities of CHB were revealed, as well as their correlations with liver injury, viral replication, and immune status. The expression profile may be used as potential marker for evaluating the clinical outcomes of HBV infection. Detection of the transcription factor mRNA expression for peripheral $CD4^+$T-cell subsets may become a widely used technique for monitoring cellular immune responses in the CHB patients. Our results have implications for optimizing immunotherapeutic approaches in the treatment of HBV infection in future.

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