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Original Paper

The Influence of B and T Lymphocyte Attenuator Genetic Variants on Susceptibility to Chronic Hepatitis B Virus Infection

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Key Words

B and T lymphocyte attenuator • Single nucleotide polymorphisms • Chronic HBV infection

Abstract

Background/Aims: B and T lymphocyte attenuator (BTLA) is an immune inhibitory receptor involved in the pathogenesis of chronic viral infections. Little is known about the effects of BTLA gene polymorphisms on chronic hepatitis B virus (HBV) infections. In this study, we investigated whether the polymorphisms of BTLA are associated with the progression of chronic HBV infection. ***Methods:*** A total of 382 chronic HBV carriers and 170 healthy individuals in the same region were recruited for this study. The chronic HBV carriers were divided into three groups: asymptomatic HBV carriers (ASC), moderate chronic hepatitis B group (MCHB), and severe chronic hepatitis B group (SCHB). Two BTLA functional single nucleotide polymorphisms (SNPs; rs76844316 and rs9288952) were genotyped by polymerase chain reaction and sequenced directly. ***Results:*** The results showed that the frequency of the G allele of rs76844316 was significantly lower in the SCHB group than in the other three groups. Subjects bearing at least one G allele (TG or GG genotype) at rs76844316 had decreased susceptibility to severe chronic hepatitis B compared with those bearing the TT genotype. Haplotype analysis of the two SNPs revealed that the frequency of the G-G haplotype was significantly lower in SCHB patients than in controls. Moreover, in the SCHB group, patients carrying the G allele of rs76844316 tended to have lower ALT levels than those without it. ***Conclusion:*** Our findings suggest that the genetic variants of rs76844316 in BTLA influence the susceptibility to severe chronic hepatitis B and might play a protective role against the progression of chronic hepatitis B.

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Introduction

Chronic hepatitis B virus (HBV) infection is a major public health problem around the world. It is estimated that more than 240 million people are chronically infected with HBV, and every year about 650, 000 die of HBV-associated liver cirrhosis or hepatocellular carcinoma [1, 2]. Whether HBV infections persist is mainly determined by the extent and quality of the host immune response. Viral clearance often occurs in individuals who develop broad and strong immune responses [3]. Over the past years, genetic variants of immune molecules have been found to be one of the major factors associated with the different clinical outcomes of HBV infections, including single nucleotide polymorphisms (SNPs) of various genes, such as cytokines and co-signaling molecules [4-7].

B and T lymphocyte attenuator (BTLA) is a coinhibitory receptor belonging to the immunoglobulin superfamily. In structure and function, BTLA resembles programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4), which are other coinhibitory receptors [8]. BTLA is widely expressed on both hematopoietic and non-hematopoietic cells, such as T and B lymphocytes, dendritic cells, natural killer cells, and other cells in the lymphoid compartment. Unlike CTLA-4 and PD-1, the BTLA ligand is not a member of the B7 family but a tumor necrosis factor receptor named herpesvirus-entry mediator (HVEM). Ligation of BTLA with HVEM favors Treg cell induction, the inhibition of T and B cell proliferation, and the production of cytokines [9-11].

As a negative immune regulator, BTLA is crucial not only for the maintenance of peripheral tolerance but also for the pathogenesis of infectious diseases [12, 13]. In chronic hepatitis B patients, the expression of BTLA increases in intrahepatic specific CD8+ T cells and is associated with the dysfunction of cytotoxic T cells [14-16]. Blockade of the BTLA pathway can partially restore the function of viral specific CD8+ T cells and enhance their proliferation [15, 17]. Upregulation of BTLA on T cells is also correlated with clinical outcomes in chronic hepatitis B, such as liver cirrhosis and hepatocellular carcinoma [18]. Both BTLA and HVEM have been detected via immunochemistry in the livers of HBV-related acute-on-chronic liver failure patients, whereas they are absent in the livers of patients with chronic HBV and healthy people [19]. The above studies suggest that the BTLA-HVEM pathway plays an important role in chronic HBV infections.

Based on the importance of BTLA in chronic HBV infections, we asked whether gene polymorphisms might be a genetic factor affecting the clinical outcomes of chronic HBV infections. In this study, we detected two functional SNPs in the exons of BTLA and evaluated their association with susceptibility to the progression of chronic HBV infections.

Materials and Methods

Patients and control subjects

We enrolled 382 patients with chronic HBV infections from the First Affiliated Hospital of Zhejiang University (Hangzhou, China). Chronic HBV infection was diagnosed by seropositivity for HBsAg for at least 6 months via a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL). Based on alanine aminotransferase (ALT)/aspartate aminotransferase (AST), total bilirubin (TBil), and the international normalized ratio (INR), we divided the patients into three groups. The asymptomatic HBV carriers (ASC) group comprised 142 patients who had normal serum ALT/AST levels and were negative for HBeAg and HBVDNA, with no previous history of hepatitis B or any other clinical symptoms for 1 year prior to the study. The moderate chronic hepatitis B (MCHB) group comprised 127 patients with a previous history of hepatitis B, continuously elevated levels of ALT/AST, serum total TBil of less than 10 times the upper limit of normal (171 μM), and/or an INR of less than 1.5. The severe chronic hepatitis B (SCHB) group comprised 113 patients with TBil of more than 10 times the upper limit of normal and/or an INR exceeding 1.5. Patients coinfected with other types of hepatitis virus, alcoholic liver disease, or other immune-related diseases were excluded. As controls, 170 healthy volunteers who were negative for serum hepatitis markers

were recruited from the same geographical area. The subjects provided informed consent and the study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang University.

Genotyping of BTLA polymorphisms

Ethylenediaminetetraacetic acid-treated peripheral blood samples were collected from the patients and healthy controls. Genomic DNA from the samples was extracted with the whole blood genomic DNA extraction mini-kit according to the manufacturer's instructions (Hangzhou SIMGEN Biotechnology Co., Ltd., Hangzhou, China). Two SNPs, rs76844316 and rs9288952, located in exon4 and exon5, respectively, were investigated in our study. Primers exon4S 5'-TCCCTCCCCTTCCTTTTAGA-3' and exon4AS 5'-TGCCTGGCACATGGTGTTAT-3' were used for the amplification of exon4 (rs 76844316), and primers exon5S 5'-TACCATGGCCGTAAGTGTCA-3' and exon5AS 5'-GAGCCCAGACAATGATGTCA-3' were used for the amplification of exon 5 (rs9288952). The primers were designed according to the BTLA DNA sequence (NCBI Reference Sequence: NC_000003.12), and synthesized and purified by Invitrogen (Shanghai, China). A 5.0-μL quantity of genomic DNA was amplified in each reaction containing 0.25 μL of rTaq (Takara Biotechnology Co. Ltd., Dalian, China). The PCR procedure was initiated at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and extended at 72°C for 5 min. PCR products were then directly sequenced using ABI 3730 sequencer by BGI Co., Ltd. (Hangzhou, China).

Detection of HBV markers, HBV DNA, and liver function in sera

Serum HBV markers including HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc were detected by electro-chemiluminescence immunoassay (Abbott GmbH & Co. KG, Ludwigshafen, Germany). Serum HBV DNA was assayed by real-time fluorescent quantitative PCR (Shanghai ZJ Bio-Tech Co., Ltd). The levels of ALT and AST were measured by colorimetric analysis via a biochemistry analyzer (7600 Series automatic analyzer, Hitachi, Tokyo, Japan)

Statistical analysis

We checked the Hardy–Weinberg equilibrium for each polymorphism via a Chi-square test. The distributions of the genotypes and allele frequencies were compared by Chi-square analysis. The associations of the genotypes and allele frequencies in chronic HBV infections were analyzed by unconditional logistic regression after adjustment for age and sex. The haplotype frequencies were analyzed using the SHEsis program [20]. Since the distribution was skewed and there was great variability, the HBV DNA and ALT data were transformed logarithmically and evaluated by t-tests. A *P*-value of less than 0.05 was considered statistically significant. All statistical analysis was performed by SPSS software version 11.5 (SPSS, Inc., Chicago, IL).

Results

Clinical characteristics of the study subjects

All genotypes in both patients and controls were in Hardy-Weinberg equilibrium ($P>0.05$). The demographic and laboratory characteristics in the study subjects are shown in Table 1. It was noted that the patients with SCHB were younger than those in the other groups (Table 1, $P<0.05$). There were more male patients in the MCHB and SCHB groups than in the healthy control and ASC groups (controls vs SCHB, $P<0.01$; ASC vs MCHB, $P<0.05$; ASC vs SCHB, $P<0.01$). However, no significant differences were found in either age or sex between the control and ASC groups ($P>0.05$). All the above data indicated that differences in

Table 1. The demographic data of all studied groups. (ASC: asymptomatic HBV carriers; MCHB: moderate chronic hepatitis B; SCHB: severe chronic hepatitis B. ALT: alanine aminotransferase. Compared with controls, * P <0.05, ** P <0.01. Compared with ASC, $^{\Delta}$ P <0.05, $^{\Delta\Delta}$ P <0.01. Compared with MCHB, $^{\Diamond}$ P<0.05)

			chronic HBV carriers	
Variables	Controls(n=170)	ASC (n=142)	MCHB (n=127)	SCHB (n=113)
Mean age (years)	46.9±15.8	45.8±15.4	44.2±12.8	41.0±12.3$^{**\Delta\Delta\Diamond}$
Male (%)	113(66.5)	87(61.3)	94(74.0) $^{\Delta}$	93(82.3)$^{**\Delta\Delta}$
HBeAg(+/-)	0/170	0/142	57/70	56/57
Mean ALT(IU/L)	23.5±11.5	27.0±9.4	247.0±384.4	480.3±518.0
Bilirubin(μmol /L)	13.1±4.8	14.4±4.9	63.7±91.9	280.3±159.1
HBVDNA(+/-)	0/170	0/142	95/32	84/29

both sex and age influenced the pathological outcomes of HBV infections, which is in agreement with previous studies [21, 22].

Associations of BTLA polymorphism with susceptibility to HBV infection

To determine whether the two SNPs of BTLA gene were associated with susceptibility to chronic HBV infection, PCR products containing the SNPs were obtained and directly sequenced. The genotype and allele frequencies of the two BTLA SNPs (rs76844316 and rs9288952) in the patient and control groups are summarized in Tables 2–5. As shown in Table 2, there were no significant differences in either the genotype or allele frequencies of rs9288952 among the control, ASC, MCHB, and SCHB groups. For rs76844316 T>G, our results showed that the frequencies of TG and GG genotypes were significantly lower in the SCHB group than in the healthy control group (Table 3, P=0.008), and the frequency of the G allele was also significantly lower in the SCHB group than in the other three groups (Table 4, SCHB vs controls, P=0.008; SCHB vs ASC, P=0.042; SCHB vs MCHB, P=0.018). However, the genotype and allele frequencies of rs76844316 among the control, ASC, and MCHB groups were not significantly different. As sex and age are sensitive factors related to the different outcomes of HBV infections, we further analyzed the data by unconditional logistic regression after an adjustment for age and sex. It was found that subjects with at least 1G allele (rs76844316) had approximately a 35% lower risk of severe chronic hepatitis B than those with the TT genotype (for the dominant model, odds ratio [OR]: 0.356, 95% confidence interval [CI]: 0.160–0.792, P =0.011; for the additive model, OR: 0.363; 95% CI: 0.163–0.810, P =0.013; Table 5). Thus, the genetic variant rs76844316 in BTLA gene was associated with decreased susceptibility to severe chronic hepatitis B after HBV infection, and the rare allele G might play a protective role against disease progression.

Table 2. The distribution of rs9288952 genotype and allele frequencies in patients and controls. (For genotype and allele analyses, comparison between controls and chronic HBV infected groups)

	Genotypes (%)				Alleles (%)		
Groups	AA	AG	GG	P	A	G	P
Controls(170)	86(50.6)	67(39.4)	17(10.0)		239(70.3)	101(29.7)	
ASC (142)	67(47.2)	65(45.8)	10(7.0)	0.426	199(70.1)	85(29.9)	0.951
MCHB (127)	61(48.0)	57(44.9)	9(7.1)	0.516	179(70.5)	75(29.5)	0.962
SCHB (113)	65(57.5)	39(34.5)	9(8.0)	0.508	169(74.8)	57(25.2)	0.244

Table 3. The distribution of rs76844316 genotype and allele frequencies in patients and controls. (For genotype analysis, comparison of TT with TG+GG between cntrols and chronic HBV infected groups. *P<0.05 between controls and chronic HBV infected groups)

	Genotypes (%)				Alleles (%)		
groups	TT	TG	GG	P	T	G	P
Controls (170)	137(80.6)	32(18.8)	1(0.6)		306(90.2)	34(9.8)	
ASC (142)	120(84.5)	20(14.1)	2(1.4)	0.366	260(91.5)	24(8.5)	0.507
MCHB (127)	106(83.5)	18(14.2)	3(2.3)	0.525	230(90.6)	24(9.4)	0823
SCHB (113)	104(92.0)	9(8.0)	0(0.0)	0.008*	217(96.0)	9(4.0)	0.008*

Table 4. The differences of rs76844316 genotype and allele frequencies between SCHB and other groups. (For genotype analysis, comparison of TT with TG+GG between the two indicated groups. *P<0.05 between the two indicated groups)

	Genotype		Allele	
	$\chi 2$	P	$\chi 2$	P
SCHB vs controls	7.038	0.008*	7.003	0.008*
SCHB vs ASC	3.340	0.068	4.152	0.042*
SCHB vs MCHB	4.016	0.045*	5.582	0.018*

Table 5. The association test of rs76844316 polymorphisms between controls and SCHB group. (a: Comparison TT with TG+GG. b: Comparison TT with TG. OR: odds rate CI: confidence intervals. *P<0.05 between controls and SCHB)

Genotypes	SCHB	Controls	Dominant model[a]		Additive model[b]	
	(n=115)	(n=170)	P	OR (95%CI)	P	OR(95%CI)
TT	104 (92.0%)	137 (80.6%)	0.011*	0.356	0.013*	0.363
TG	9 (8.0%)	32 (18.8%)		(0.160~0.792)		(0.163~0.810)
GG	0 (0%)	1 (0.6%)				

Haplotype analysis of BTLA SNPs between control and SCHB groups

Since we found a significant association between BTLA SNP (rs76844316) and severe chronic hepatitis B, we further conducted haplotype analysis of the two functional BTLA SNPs (rs76844316 and rs9288952) in both the control and patient groups. Haplotypes

Table 6. Haplotype analysis of the investigated BTLA gene polymorphisms between the controls and SCHB group

rs76844316	rs9288952	SCHB(%)	Controls(%)	x^2	P	OR (95%CI)
T	A	170(75.2)	239(70.3)	1.644	0.200	1.283(0.876~1.878)
T	G	47(20.8)	67(16.7)	0.101	0.751	1.070(0.704~1.625)
G	G	9 (4.0)	34(10.0)	7.005	0.008*	0.373(0.175~0.794)

with frequencies of >3% in each SNP were recruited for the analysis. The haplotype frequencies in both the ASC and MCHB groups were not significantly different from those in the control group (data not shown). However, one haplotype, G-G, was found to be associated with the SCHB group. As shown in Table 6, there were three major estimated haplotypes, namely, T-A, T-G, and G-G, among the patients and controls. T-A was the most frequent haplotype observed in both patients and controls (75.2% and 70.3%, respectively). The frequency of the G-G haplotype was significantly lower in SCHB patients than in the controls (OR: 0.373, 95% CI: 0.175–0.794, *P*=0.008; Table 6), which indicated that G-G haplotype was associated with a low tendency toward severe chronic hepatitis B after HBV infection.

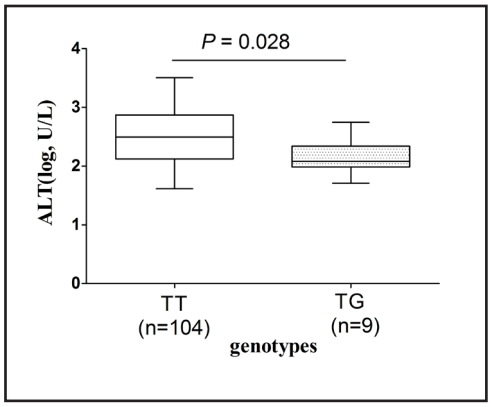

Fig. 1. The comparison of ALT serum levels in severe chronic hepatits between rs76844316 TT and TG carriers.

Association between BTLA polymorphism, viral replication, and liver inflammation

In our study, the G allele of BTLA SNP rs76844316 was found to be associated with decreased susceptibility to severe chronic hepatitis. Therefore, we asked whether this genetic SNP might also be associated with HBV viral replication and hepatic inflammation. Two replication parameters, HBV DNA and HBeAg, were analyzed in 240 chronic hepatitis patients (including the MCHB and SCHB groups). However, there were no significant differences in either the positivity rates or levels of HBV DNA and HBeAg between the genotypes (TT vs TG + GG; data not shown). In addition, the levels of serum ALT, which may reflect the extent of liver inflammation to some degree [23], were also compared between patients with and without the G allele. Since there was great variability among the ALT data, we transformed the data logarithmically and performed a *t*-test. As shown in Fig. 1, the serum ALT levels in patients with TT genotypes were significantly higher than those of patients with TG genotypes in the SCHB group (no GG genotypes were found in the SCHB group). In the MCHB group, no significant differences were found between the patients with TT and those with TG/GG genotypes (data not shown). The results indicated an association between the G allele and a lower level of liver inflammation in severe chronic hepatitis, which further validated the conclusion that the G allele of rs76844316 plays a protective role during exacerbations of chronic hepatitis B.

Discussion

Previous research has found that BTLA gene polymorphisms are associated with susceptibility to autoimmune diseases and cancers, including rheumatoid arthritis (RA), breast cancer, renal cell carcinoma, and colorectal cancer [24-29]. However, few studies have focused on the association of BTLA SNPs with chronic viral infections. In the present study, we conducted a case-control study on the relationship between two functional BTLA SNPs

and chronic hepatitis B. Our results first demonstrated that the genetic variant rs76844316 T>G of BTLA was associated with susceptibility to chronic hepatitis B, and might play a protective role in severe chronic hepatitis B. Haplotype analysis of the two SNPs found that the frequency of the G-G haplotype was significantly lower in patients with severe chronic hepatitis B than in the controls. Moreover, patients carrying the G allele of rs76844316 tended to have lower ALT levels than those without it among patients with severe chronic hepatitis B. These finding suggested that BTLA polymorphisms are important factors associated with the clinical outcomes of chronic hepatitis B.

In humans, the BTLA gene is located on chromosome 3q13.2 and composed of five exons and four introns. Structurally, BTLA contains three parts: an extracellular immunoglobulin domain, a transmembrane region, and an intracellular region. In the intracellular region, there are two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Tyrosine phosphorylation of ITIM motifs induces the recruitment of the phosphatases SHP-1 and SHP-2, which leads to inhibitory effects [28, 30]. We selected two functional SNPs located in exons 4 and 5. The genetic variant rs9288952 A>G results in amino acid substitution from proline to leucine at position 267 in exon 5, and is reported to be associated with susceptibility to breast cancer [25]. However, there was no significant difference in the genotypes or alleles of this SNP among the controls and chronic HBV carriers (Table 2). In exon 4, a single nucleotide mutation rs76844316 T>G causes an asparagine-to-threonine substitution at position 197 of BTLA, located in the intracellular region of BTLA, which is associated with susceptibility to RA [28]. Although the amino acids encoded by these two functional SNPs are not within the ITIM motifs of BTLA [31, 32], our results showed that rs76844316 T>G was strongly associated with a lower risk of severe chronic hepatitis. We hypothesize that this substitution may alter the posttranslational modifications of BTLA, such as glycosylation of asparagine, or phosphorylation of threonine by serine/threonine kinase, which may influence the strength of BTLA signaling and ultimately regulate the immune response. In fact, the BTLA-HVEM pathway and its functions are very complicated. In different disease models, BTLA signaling may produce counteractive effects even in the same type of immune cells. Some studies have shown that BTLA signaling plays not only an inhibitory role in T cells, but also enhances T cell effector functions in some types of inflammation [33, 34]. Although many studies have focused on the function of BTLA signaling in T cells, its effects on other types of cells are largely unknown. Therefore, the exact regulation of BTLA rs76844316 variants on the immunopathogenesis of chronic HBV infections needs further study.

Coinhibitory immunoreceptors, such as CTLA-4 and PD-1, are known to regulate T-cell activation and Th1/Th2 cytokine production and are involved in the immune response to HBV infection. Their gene polymorphisms are reported to influence viral clearance and persistent infection [5, 35, 36]. Thus, we want to clarify the relationship between viral replication and rs76844316 variants of BTLA. However, no significant differences in both the positivity rates and levels of HBV DNA and HBeAg in chronic hepatitis B were found between TT and TG/GG genotypes of rs76844316 in the present study, which might indicate that this BTLA genetic variant has no obvious effects on HBV replication. As the vigor of T-cell responses to HBV infection are regulated by coinhibitory receptors, gene polymorphisms may have some effects on liver inflammation. In the clinical evaluation of hepatic inflammation, serum ALT is currently the most widely used non-invasive index, so we compared serum ALT levels between the TT and TG/GG genotypes of rs76844316. In the SCHB group, it was shown that serum ALT levels were significantly higher in patients with TT genotypes than in those with non-TT genotypes. Combined with the above results, which showed that the frequency of the G allele at rs76844316 was significantly lower in the SCHB group, it is strongly suggested that this genetic variant of BTLA might confer lower susceptibility to severe chronic hepatitis B. In addition, it should be noted that, due to the limited number of patients and ethnic differences among the gene polymorphisms, the results of our study should be further validated in large-scale investigations.

In summary, our study has demonstrated for the first time that the rs76844316 polymorphism is associated with lower susceptibility to severe chronic HBV infection,

which indicates that the polymorphisms of BTLA gene could play important roles in the host immune response to HBV infection. This genetic variant of BTLA may have protective effects against the progression of chronic hepatitis B, and might be helpful for evaluating the clinical outcomes of chronic HBV infection.

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Disclosure Statement

The authors declare no conflicts of interest.

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