

T3098C and T53C Mutations of HBV Genotype C Is Associated with HBV Infection Progress¹

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Objective To analyze the association between mutation(s) in preS region of HBV and hepatitis B disease progress in Chinese patients with genotype C chronic HBV infection. **Methods** Ninety-three patients with chronic genotype C HBV infection, including 24 asymptomatic carriers (ASC), 26 patients with chronic hepatitis B (CHB), 22 patients with liver cirrhosis (LC) and 21 HCC patients were investigated. Levels of HBV DNA, HBeAg, alanine aminotransferase (ALT), aspartate transaminase (AST) were measured. HBV preS region was analyzed by PCR direct sequencing. **Results** The prevalence of preS T3098C and T53C mutations of genotype C HBV was significantly higher in LC and HCC patients than ASC and CHB patients. The rate of T3098C mutation in ASC, CHB, LC, and HCC patients were 0.00% (0/24), 3.85% (1/26), 9.09% (2/22), and 30.77% (8/22), respectively ($P=0.0015$), while the rate of T53C mutation was 12.50% (3/24), 3.85% (1/26), 40.91% (9/22), and 42.31% (11/26), respectively ($P=0.0012$). **Conclusion** The frequency of genotype C HBV preS T3098C and T53C mutations is associated with hepatitis B infection progression.

Key words: Hepatitis B virus (HBV); Genotype C; pre S; Mutation; Hepatocellular carcinoma (HCC)

INTRODUCTION

Hepatitis B virus (HBV) infection is highly endemic in China, with 93-million individuals infected with chronic HBV^[1-2]. The clinical spectrum of chronic HBV infection may range from biochemically and histologically inactive chronic HBsAg carrier state to active chronic hepatitis, HBV-related liver cirrhosis and/or hepatocellular cancer (HCC).

Based on a nucleotide sequence divergence larger than 8% in the complete viral genome, or 4% in S gene sequence, HBV has been classified into eight genotypes, designated as A-H^[3-6]. The HBV genotype distribution shows clear geographic and ethnic features^[7-12]. Genotypes C and B are prevalent in most areas of China, accounting for 90% of the total hepatitis B cases^[12-14]. It has been revealed that HBV genotype might contribute to the clinical features, the disease outcome and the response to antiviral therapy^[15-19]. Patients with genotype C infection have more progressive clinical phenotypes and less response to interferon than those with

genotype B^[18-20].

Although the pathogenesis of hepatitis B has not been fully understood, the importance of HBV genotype and genomic mutation in liver disease progression has been demonstrated. HBV preS mutants prevail in the countries where HBV infection is highly endemic^[21], and are often associated with severe liver diseases^[21-24]. In addition, It has been reported that the preS deletion mutants are more frequently found in individuals infected with genotype C HBV than in those infected with genotype B^[19-20]. Genotype C is the most prevalent HBV genotype in China. Our previous study showed that HBV preS deletion mutations are closely related to HCC in Chinese infected with genotype C HBV^[25]. Further analysis of preS sequences revealed that the rate of T3098C and T53C mutations was significantly higher in HCC patients. Therefore, the present study was to investigate the impact of HBV T3098C and T53C mutations on the development of HCC in Chinese infected with solely genotype C HBV and to reveal the impact of preS mutations rather than the genotype on the disease progress.

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MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of Peking University Health Science Center and an informed consent was obtained from each patient. Ninety-three patients genotype C HBV, including 24 asymptomatic carriers (ASC), 26 patients with chronic hepatitis B (CHB), 22 patients with liver cirrhosis (LC), and 21 patients with hepatocellular carcinoma (HCC), mainly acquired through mother-to-infant transmission, were enrolled in this study. Most of the patients were recruited from infectious disease hospitals in Beijing. All the patients were local Chinese residents. No patients studied had accepted any antiviral therapy.

Blood samples taken from the patients were kept at -80 °C as aliquots. HBV-infected patients enrolled in this study fulfilled the following criteria: HBsAg positive (EIA, Abbott, USA), serum HBV DNA positive (fluorescence quantitative PCR, Shenzhen PG Biotechnology Development Co. Ltd, China), genotype C HBV infection (confirmed by genotyping assay, see later description), antibodies against HAV, HCV and HDV all negative (EIA, Abbott, USA), anti-HEV negative (EIA, Genelabs, Singapore), anti-EBV and anti-CMV negative (EIA, Dade Behring, Marburg, Germany), with the exclusion of autoimmune liver disease, drug-related hepatitis, alcoholic hepatitis and obstructive jaundice. The clinical diagnosis of HBeAg (+) and HBeAg (-) CHB conformed to the EASL criteria^[26]. The criteria for the diagnosis of ASC are HBsAg positive >6 months, serum HBV DNA-positive, persistently normal ALT/AST levels^[27], and those for the diagnosis of CHB are HBsAg positive >6 months, serum HBV DNA-positive, persistently or intermittently elevated ALT/AST levels, or histological changes of necroinflammatory score (Knodell) ≥4. All chronic hepatitis B patients with or without liver cirrhosis were diagnosed by liver biopsy. Twenty-one HCC patients were diagnosed by ultrasonography and computed tomography, and confirmed by biopsy during liver transplantation or autopsy.

Biochemical, Serological, and Serum HBV DNA Assays

Alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured with a Hitachi automatic clinical analyzer 7180 (Hitachi High-Technologies Co. Ltd, Japan), using chemical reagents purchased from Roche (Switzerland). Serum HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc were detected with a commercially available EIA kit

(Abbott, USA; Shanghai Kehua, China) according to its manufacture's instructions. Serum HBV DNA levels were measured by fluorescence quantitative polymerase chain reaction (FQ-PCR) (Shenzhen PG, China) with the lowest detection level of $\sim 2.5 \times 10^2$ copies/mL of serum HBV DNA.

HBV DNA Extraction

HBV DNA was extracted from serum samples using a RNAgents total RNA isolation system (Promega, USA) with slight modifications. In brief, a 60 µL denaturing solution was added into a 50 µL serum sample and kept at 37 °C for 10 min, and then a 60 µL solution (phenol: chloroform: isoamyl alcohol = 50:49:1) was added into the mixture, which was centrifuged at 14 000 rpm for 10 min at 4 °C after mixing. The top aqueous phase was transferred into another tube, and added with an equal volume of isopropanol and a 10 µL of 2 mol/L pH 4.0 NaAc, and kept at -20 °C for at least 2 h. The mixture was centrifuged at 14 000 rpm for 15 min at 4 °C, and the supernatant was discarded. The pellet was rinsed with a 500 µL of pre-chilled 75% ethanol, precipitated by centrifugation at 14 000 rpm for 10 min at 4 °C, air-dried, dissolved in 20 µL nuclease-free water, and stored at -20 °C.

HBV Genotyping by Nested PCR

Genotypes of HBV were identified by PCR with type-specific primers based on preS1 and S gene sequences as previously described^[28]. Briefly, HBV DNA was amplified by nested PCR with the high fidelity Taq polymerase (Sangon, Shanghai, China). The first round PCR was done with the universal primers, and the second round PCR with type-specific primers. The specificity of this assay system was confirmed by nucleotide sequencing plus phylogenetic analysis as previously described^[29].

Sequencing of PreS Genes of HBV Genome

PreS genes of HBV genome were amplified by nested PCR and the purified PCR products were sequenced directly. The sequences of primers used for the first and second rounds of PCR are 5'-ACATACTCTGTGGAAGGCTG-3' (forward, nt2750-2769) and 5'-TTGAGAGAAGTCCACCACGA-3' (reverse, nt273-254), and 5'-GGAAGGCTGGCATTCTATAT-3' (forward, nt2761-2780) and 5'-CTGTGGTATTGTGAGGATTC-3' (reverse, nt244-225), respectively.

PCR was performed in 50 µL reaction volume.

For the first round, the reaction contained 10 μL of DNA extract, 1 μL of each primer (20 $\mu\text{mol/L}$), 0.2 μL of the high fidelity Taq polymerase (5 U/ μL) and 5 μL of 10 \times DNA polymerase buffer (Sangon, Shanghai, China), 2 μL of dNTP mixture (2.5 mmol/L of each of dATP, dGTP, dCTP, and dTTP, respectively) (SABC, China), and 30.8 μL dH₂O. For the second round, the reaction contained 5 μL of the first round PCR product, 1 μL of each primer (20 $\mu\text{mol/L}$), 0.2 μL of the high fidelity Taq polymerase (5 U/ μL), and 5 μL of 10 \times DNA polymerase buffer, 1 μL of dNTP mixture (2.5 mmol/L of each of dATP, dGTP, dCTP and dTTP, respectively), and 36.8 μL dH₂O.

The PCR programs used were as follows. For the first round, PCR program involved denaturing at 94 $^{\circ}\text{C}$ for 4 min followed by 35 cycles of denaturing at 94 $^{\circ}\text{C}$ for 30 s, annealing at 60 $^{\circ}\text{C}$ for 30 s and elongation at 72 $^{\circ}\text{C}$ for 60 s, then a final extension at 72 $^{\circ}\text{C}$ for 7 min. For the second round, PCR program involved denaturing at 94 $^{\circ}\text{C}$ for 5 min followed by 35 cycles of denaturing at 94 $^{\circ}\text{C}$ for 30 s, annealing at 60 $^{\circ}\text{C}$ for 30 s and elongation at 72 $^{\circ}\text{C}$ for 60 s, then a final extension at 72 $^{\circ}\text{C}$ for 7 min.

The second round PCR products were electrophoretically separated on 1.5% agarose gels with 0.5 $\mu\text{g/mL}$ ethidium bromide in 1 \times Tris-acetic acid-EDTA (TAE) buffer. The predicated length of target sequence was 699 bp. The purified target PCR products were sequenced using primers for the second round PCR in both directions commercially (Shanghai Sangon Biotechnology Co., Ltd., China).

Statistical Analysis

Statistical analysis of the data was done using SPSS version 10.0. Statistical differences were evaluated using rank sum test, χ^2 test and multiple logistic regression analysis where appropriate. $P < 0.05$ was considered statistically significant. The nucleotide sequence data were analyzed using BioEdit (version 7.0) and DNASTar Lasergene (version 7.1) softwares.

RESULTS

Comparison of Demographic, Biochemical and Virological Characteristics of Different Clinical Groups of Patients with Chronic HBV Infection

All the 93 patients were confirmed to be infected with genotype C HBV. The demographic, biochemical and virological characteristics of the four clinical groups are summarized in Table 1. The mean

age of ASC group, CHB group, LC group, and HCC group showing the disease severity-related increasing pattern was 20 \pm 1 years, 28 \pm 10 years, 48 \pm 9 years, and 56 \pm 13 years, respectively ($P=0.0000$). The same pattern was also observed in the percentage of males (50.00% in ASC, 61.54% in CHB, 81.82% in LC and 85.71% in HCC, $P=0.0276$). Serum ALT and AST levels were significantly higher in patients with CHB ($P=0.0000$). PreS T53C point mutation was obviously increased in both LC and HCC groups, while the frequency of preS T3098C point mutation was significantly higher in patients with HCC than in those with either ASC, and CHB or LC ($P=0.0015$) (Fig. 1a).

Analysis of HBV T3098C and T53C mutations

The coexistence of T3098C and T53C mutations was mainly found in HCC patients (28.57%, 6/21), while no such coexistent mutations were detected both in 24 ASC and in 26 CHB patients (Table 1). The features of HBV patients with or without T3098C mutation are shown in Table 2. The patients with T3098C were older (56.7 \pm 17 years vs 35 \pm 16 years, $P < 0.01$), less likely HBeAg positive (20.00% vs 56.62%, $P=0.0258$), had more severe HCC (70.00% vs 16.87%, $P < 0.0001$) and a higher frequency of T53C mutation (70.00% vs 19.28%, $P=0.0017$) than those without T3098C. The same pattern was found in patients harboring HBV T53C mutation who were older (49 \pm 17 years vs 33 \pm 16 years, $P < 0.01$), had more severe HCC (43.48% vs 15.71%, $P < 0.01$) and a higher frequency of T3098C (30.43% vs 4.29%, $P=0.0017$) than those without T53C. Interestingly, the circulating HBV DNA level was lower in patients with T53C mutation than in those without T53C mutation (6.09 \pm 1.58 vs 5.01 \pm 1.80, $P=0.0310$). The HBeAg positive rate was about 20% in patients with either mutation while more than 50% of patients without such mutations were HBeAg positive. No significant difference was observed in gender and ALT levels between the patients with or without either T3098C or T53C mutation. The association of HBV T3098C and T53C mutations with HCC was evaluated by multiple logistic regression analysis. With 95% confidence interval, the odd ratio (OR) was 22.48 (1.86-271.93, $P=0.014$) for T3098C and HCC, or 28.4 (3.18-253.52, $P=0.0002$) for T3098C/T53C and HCC, indicating that the T3098C and T3098C/T53C mutations are higher in HCC patients. However, the T53C mutation rate was also higher both in LC and in HCC patients (Fig. 1b).

TABLE 1

Characteristics of Individuals Infected with Genotype C HBV in Different Clinical Groups

Characteristics	ASC (n=24)	CHB (n=26)	LC(n=22)	HCC (n=21)	P-value	P-value (Non-HCC vs. HCC)
Demographic Characteristics						
Male/Female (% Male)	12/12 (50.00)	16/10 (61.54)	18/4 (81.82)	18/3 (85.71)	0.0276	
Age $\bar{x} \pm s$	20±1	28±10	48±9	57±12	0.0000	
Biochemical Characteristics						
ALT (IU/L) $\bar{x} \pm s$	27±7	218±252	125±175	51±22	0.0000	
AST (IU/L) $\bar{x} \pm s$	26±4	162±225	159±202	70±46	0.0000	
Virological Characteristics						
LogDNA $\bar{x} \pm s$	5.87±2.19	6.68±1.02	5.07±1.74	5.35±1.39	0.0084	
HBeAg Positive (%)	15 (62.50)	19 (70.08)	10 (45.45)	5 (23.80)	0.0052	
T3098C Mutants (%)	0 (0.00)	1 (3.85)	2 (9.09)	7 (33.33)	0.0015	<0.0001
T53C Mutants (%)	3 (12.50)	1 (3.85)	9(40.91)	10 (47.62)	0.0012	0.0067
T3098C & T53C Mutants (%)	0 (0.00)	0 (0.00)	1 (4.55)	6 (28.57)	0.0370	<0.0001

Note. ASC: asymptomatic carrier; CHB: chronic hepatitis B; HCC: hepatocellular carcinoma; HBeAg: hepatitis B e antigen; ALT: alanine aminotransferase; AST: aspartate aminotransferase; s: standard deviation.

TABLE 2

Characteristics of Individuals Infected with Genotype C HBV with or without T3098C and T53C Mutants

Characteristics	T3098C Mutants			T53C Mutants		
	Positive (n=10)	Negative (n=83)	P-value	Positive (n=23)	Negative (n=70)	P-value
Demographic Characteristics						
Male/female (% Male)	9/1 (90.00)	55/28 (66.27)	0.0989	17/4 (80.95)	47/25 (65.28)	0.3804
Age $\bar{x} \pm s$	56.7±17	35±16	0.0004	49±17	33±16	0.0011
Advanced Liver Disease HCC (%)	7 (70.00)	14 (16.87)	<0.0001	10 (43.48)	11 (15.71)	0.0057
Biochemical Characteristics						
ALT (IU/L) $\bar{x} \pm s$	106±152	110±178		77±77	120±196	
AST (IU/L) $\bar{x} \pm s$	147±164	104±170		86±63	114±190	
Virological Characteristics						
LogDNA $\bar{x} \pm s$	5.23±1.64	5.94±1.68	0.2150	5.01±1.80	6.09±1.58	0.0310
HBeAg Positive (%)	2 (20.00)	47 (56.62)	0.0258	5 (21.74)	44 (62.86)	0.0006
T53C Mutants (%)	7 (70.00)	16 (19.28)	0.0017	-	-	-
T3098C Mutation (%)	-	-	-	7 (30.43)	3 (4.29)	0.0017

Note. HCC: hepatocellular carcinoma; HBeAg: hepatitis B e antigen; ALT: alanine aminotransferase; AST: aspartate aminotransferase; s: standard deviation.

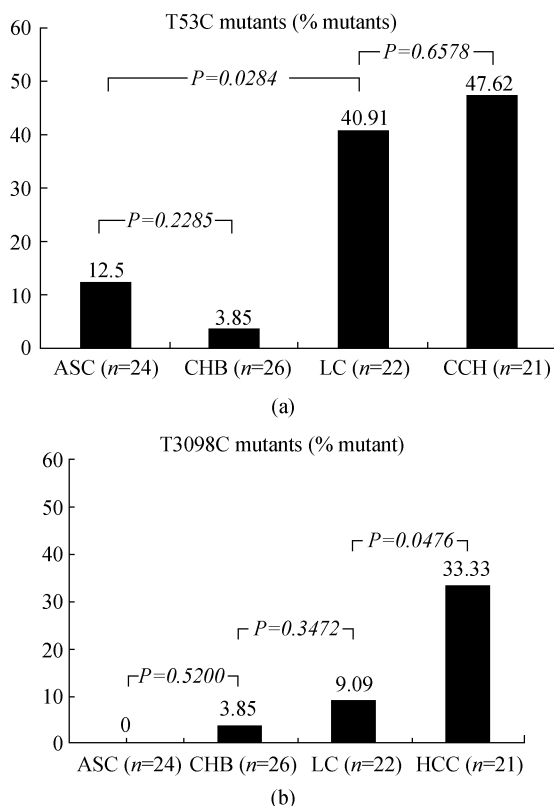


FIG. 1. Prevalence of T3098C mutation in CHB patients stratified by clinical status with the frequency of T3098C mutation being statistically significant according to χ^2 test (a) and the prevalence of T53C mutation in CHB patients stratified by clinical status with the frequency of T53C mutation being statistically significant according to χ^2 test (b).

DISCUSSION

Sharing the same carboxy-termini but not amino-termini with small envelope protein, preS proteins play an important role in the pathogenesis of HBV-related liver diseases. The preS1 protein contains the hepatocyte-binding site (aa21-47) and is known to be essential for virion assembly and transportation of virions out of the hepatocyte^[30]. The preS2 protein has been found to bear the binding site for polymerized human serum albumin, which is believed to be involved in the attachment of HBV to the human hepatocyte membrane^[31]. The preS protein contains both B- and T-cell epitopes, thus playing an important role in the interaction between HBV and immune defense. It has been shown to induce protective immune response in chimpanzees and mice^[31-34]. In the pressure of immune selection, HBV

isolates with preS mutations can avoid the attack of human immune system and become the dominant virus population *in vivo*.

PreS mutations are frequently found in patients with chronic HBV infection, and seem to be correlated with the progress of liver disease^[22-24]. Huy *et al.*^[21] studied the prevalence of HBV preS mutants in 12 countries and found that the mutation rate is significantly higher in HCC patients than in those with other liver diseases, suggesting that the frequency of preS mutations is genotype-related, and the mutants occur more frequently in patients infected with HBV genotype C than in those infected with HBV genotype B^[35]. Genotype C is associated with more severe liver damage than genotype B, and preS mutations may exert some effects on liver disease progression.

In this study, two point mutations were identified within the HBV preS region. The detection rate of T3098C (33.33%) was considerably higher in HCC patients than in either ASC (%), CHB (3.85%), and LC patients (9.09%) ($P < 0.001$), suggesting that T53C mutation is related to HCC^[29]. In the present study, the rate of T53C mutation was significantly increased even in LC group. However, it was higher LC and HCC patients (4/50, 8.00%) with their disease progressed than in those with their disease no progressed, including ASC and CHB patients (19/43, 44.19%) ($P < 0.0001$). The multiple logistic regression analysis in this study demonstrated that the presence of preS T3098C and T3098C/T53C mutations was positively correlated with HCC, suggesting that T3098C and T53C mutations may be the valuable biomarkers for more severe liver diseases of patients infected with HBV genotype C.

PreS deletion of mutants accelerates the accumulation of envelope proteins in endoplasmic reticulum (ER) and causes ER stress. ER stress will induce oxidative DNA damage and mutagenesis, potentially resulting in genomic DNA instability, which enhances HCC development^[36-37]. PreS2 protein is unnecessary for virion formation, secretion, and infectivity^[38-40]. However, previous reports and this study suggested that infection with preS2-defective HBV variants is often associated with severe liver diseases^[21, 37-39, 41]. The nt3098 is located in preS1 region, which induces the change of the 84-site amino acid, from isoleucine to threonine, while T53C mutation leads to the change of aa22 of preS2 protein, from phenylalanine to leucine. The changes of secondary structure of preS protein have also been analyzed, and two extra β sheet structures are generated by T3098C and T53C mutations, one between aa70 and aa80 of preS1 protein, the other

between aa22 and aa31 of preS2 protein. Whether these changes of secondary structure have some effects on the function of protein needs further study.

In this study, the average circulating HBV DNA load was lower in the 23 patients with T53C mutation (5.01 ± 1.80 vs 6.09 ± 1.58 , $P=0.0310$), as shown in Table 2. Although the mean circulating HBV DNA level was also lower in the 10 patients with T3098C mutation than in those without. However, 7 out of 10 patients also had T53C mutation. It would be worthwhile to test *in vitro* whether these mutations located in the spacer region affect HBV DNA polymerase activity.

The precise mechanism of hepatocarcinogenesis in chronic HBV infection remains obscure. It has been demonstrated that HBV preS mutants play a certain role in the development of HCC. However, this study has an intrinsic limitation because of a cross-sectional observation. Further studies are required to evaluate the influence of HBV preS mutants on the development of severe liver diseases in a longitudinal observation.

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