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Hepatitis B virus subgenotype C2- and B2-associated mutation patterns may be responsible for liver cirrhosis and hepatocellular carcinoma, respectively

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Abstract

The objective of this study was to examine hepatitis B virus (HBV) subgenotypes and mutations in enhancer II, basal core promoter, and precore regions of HBV in relation to risks of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in Southeast China. A case-control study was performed, including chronic hepatitis B (CHB; n = 125), LC (n = 120), and HCC (n = 136). HBV was genotyped by multiplex polymerase chain reaction and subgenotyped by restriction fragment length polymorphism. HBV mutations were measured by DNA sequencing. HBV genotype C (68.2%) predominated and genotype B (30.2%) was the second most common. Of these, C2 (67.5%) was the most prevalent subgenotype, and B2 (30.2%) ranked second. Thirteen mutations with a frequency >5% were detected. Seven mutation patterns (C1653T, G1719T, G1730C, T1753C, A1762T, G1764A, and G1799C) were associated with C2, and four patterns (C1810T, A1846T, G1862T, and G1896A) were associated with B2. Six patterns (C1653T, G1730C, T1753C, A1762T, G1764A, and G1799C) were obviously associated with LC, and 10 patterns (C1653T, G1730C, T1753C, A1762T, G1764A, G1799C, C1810T, A1846T, G1862T, and G1896A) were significantly associated with HCC compared with CHB. Four patterns (C1810T, A1846T, G1862T, and G1896A) were significantly associated with HCC compared with LC. Multivariate regression analyses showed that HBV subgenotype C2 and C2-associated mutation patterns (C1653T, T1753C, A1762T, and G1764A) were independent risk factors for LC when CHB was the control, and that B2-associated mutation patterns (C1810T, A1846T, G1862T, and G1896A) were independent risk factors for HCC when LC was the control.

Key words: Hepatitis B virus; Genotype; Core promoter; Precore; Mutation; Advanced liver disease

Introduction

More than 350 million people are chronically infected with hepatitis B virus (HBV) worldwide. Asia and Africa are high endemic areas. It is estimated that approximately 15 to 40% of patients with chronic infection will eventually progress to liver cirrhosis (LC), liver failure, and hepatocellular carcinoma (HCC) (1). Chronic HBV infection is a major cause of LC and HCC, but the pathogenesis remains elusive. In addition to the consumption of poisons and host determinants, the virological characteristics of HBV should be widely studied.

HBV has been classified into eight well-characterized genotypes (A to H) based on a sequence divergence of greater than 8% over the entire HBV genome, which reflect distinctive geographical distributions (2). Genotype A is prevalent in Northwestern Europe, sub-Saharan Africa, North America, and India. Genotypes B and C are predominant in Asia. Genotype D has a worldwide prevalence, but it is the most common genotype in the Mediterranean region. Genotype E is mainly restricted to Western Africa, and genotypes F and H have been found in South and Central America, respectively. Finally, genotype G has been identified in Europe as well as in North and Central America (3,4). Genotypes are further categorized into subgenotypes based on nucleotide sequence divergence between 4 and 8%. For example, genotype B is divided into subgenotypes B1 to B6, and genotype C includes the subgenotypes C1 to C5. Both the B and C subgenotypes are mainly prevalent in East and Southeast Asia (5). Importantly, some studies indicated that the HBV genotype/subgenotype could influence the progression of liver disease, hepatitis B e-antigen (HBeAg) seroconversion, and response to antiviral therapy (6-8).

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HBV contains four genes with partially overlapping open reading frames encoding the viral surface, precore/core, polymerase, and X. The enhancer II (enhII; nucleotides 1685-1773) and basal core promoter (BCP; nucleotides 1742-1849) are located within the core promoter (CP) region (nucleotides 1643-1849), which overlaps the X gene (nucleotides 1374-1836) (9). The CP region is thought to play an important role in viral replication. The precore region (nucleotides 1814-1900) encodes HBeAg (10), which is used clinically as an indicator of active viral replication. HBV generates mutation rates more than 10-fold higher than those of other DNA viruses because its polymerase lacks a proofreading function (11). Multiple mutations have been identified in the enhII/BCP/precore regions, of which the BCP mutations (A1762T/G1764A) and the precore stop codon mutation (G1896A) have been extensively studied. The A1762T/G1764A mutations are associated with severe liver disease, including liver failure, LC, and HCC, and are considered potential biomarkers for HCC (12,13). The G1896A mutation correlates with increased risk of HCC in a newly updated meta-analysis (14). In addition, study of C1653T, T1753V, G1899A, and other mutations in the enhII/BCP/precore regions has just begun (14).

Many cross-sectional and case-control studies have suggested that HBV genotype/subgenotype and enhII/BCP/precore mutations have a significant impact on the progression of chronic hepatitis B (CHB) (6,7,12-18), but there are also contradictory observations (9,10,19-22). Meanwhile, data are limited on the relationship between HBV subgenotypes, the mutation hot spots in the enhII/BCP/precore, and clinicopathological characteristics in chronically infected patients. In this regard, an accurate estimation of the prevalence and distribution of HBV subgenotypes in Southeast China was urgently needed because of the high incidence rates of LC and HCC in this region. In this study, our objective was to elucidate the clinical and virological differences between HBV subgenotypes, as well as the associated risk factors for the progression of chronic HBV infection.

Material and Methods

Patients

This study was approved by the Medical Ethics Committee of the Affiliated Hangzhou Hospital, Nanjing Medical University, Hangzhou, China, and all participants completed an informed consent process. A total of 381 patients who visited the Affiliated Hangzhou Hospital of Nanjing Medical University from June 2010 to December 2012 were enrolled. All patients came from Southeast China, including the Provinces of Zhejiang, Jiangsu, and Fujian. All participants were positive for hepatitis B surface antigen (HBsAg) and were divided into three age- and gender-matched groups (CHB, LC, and HCC). One hundred and twenty-five CHB patients (98 males and 27 females, mean age 48.8 ± 11.9 years, range 32-67 years) met the diagnostic criteria based on the guidelines of prevention and treatment for CHB (2010 version) of the Chinese Society of Hepatology and Chinese Society of Infectious Diseases, Chinese Medical Association. All were without LC and HCC as detected by liver ultrasound. All CHB patients agreed to a liver biopsy for increased accuracy of estimating liver tissue damage by Scheuer's classification (23). One hundred and twenty LC patients (86 males and 34 females, mean age 49.1 ± 10.2 years, range 33-65 years) were diagnosed by liver ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). All had accompanying portal hypertension and hypersplenism. One hundred and thirty-six HCC patients (101 males and 35 females, mean age 49.3 ± 10.3 years, range 32-71 years) who met the diagnostic criteria for HCC confirmed by histopathological examination were examined by liver ultrasound, CT, MRI, and serum α-fetoprotein (AFP) levels. There were no significant differences among the three groups with respect to gender and age. In addition, none of the participants received anti-viral treatment. Patients presenting other liver diseases, such as autoimmune hepatitis, alcoholic hepatitis, Wilson disease, and other types of hepatitis virus infection, were excluded from this study. Two milliliters of serum was collected from each patient and stored at -70°C until use.

Serological markers and quantitation of HBV DNA

Serum alanine aminotransferase (ALT), albumin, and total bilirubin (TBIL) were measured by standard procedures. HBV DNA levels were determined using a real-time polymerase chain reaction (PCR) kit (DaAn Gene Diagnostic Co., China) with a lower limit of detection of 500 copies/mL. HBeAg was detected by enzyme-linked immunosorbent assay kits (Kehua Bio-engineering Co., China). Serum hyaluronic acid (HA) and AFP levels were measured by a chemiluminescent assay (Bayer Co., Germany).

Determination of HBV genotypes/subgenotypes and enhII/BCP/precore mutations

HBV DNA was extracted using a commercial viral genomic extraction kit (Sunbiotech Co., China). HBV was genotyped by multiplex PCR and subgenotyped by PCR and restriction fragment length polymorphism (RFLP) as described previously (24,25). In addition, 26 serum samples, including 10 with B2 subgenotype, 10 with C2 subgenotype, and 6 with B/C mixed genotypes based on multiplex PCR and RFLP, were identified by sequencing according to Liu et al. (26).

To assess the mutations in the enhII/BCP/precore, sequencing of amplified DNA by nested PCR was carried out following the method of Yin et al. (27). PCR products were directly sequenced by the dideoxy chain termination

method using Big Dye Terminator in an ABI 3730xl DNA analyzer (Applied Biosystems, USA). The sequences were aligned by ClustalW, an online program used for multiple sequence alignment (http://www.genome.jp/tools/clustalw/).

After alignment, the nucleotide with the highest frequency at each site in the enhII/BCP/precore regions from the asymptomatic HBsAg carrier (ASC) state was defined as the wild-type nucleotide. Substitutions with other nucleotides at each site were defined as mutations. The HBV mutation data for ASCs were organized as in Yin et al. (27).

Statistical analysis

The chi-square test or the Fisher exact test was used to distinguish the differences of frequency distribution in the groups. Statistical significance between two groups with normal distributions was detected by the Student *t*-test, and non-normal distributed data were analyzed using the Mann-Whitney U-test. Associations are reported as odds ratios or as risk estimates with 95% confidence intervals. Independent risk factors were analyzed by multiple logistic regressions. All statistical tests were two-sided, and a probability of $P<0.05$ was considered to be statistically significant. Data analysis was performed using the SPSS 11.0 software (SPSS Inc., USA).

Results

The results of 26 samples showed perfect congruence between both techniques for genotype/subgenotype classification. In a total of 381 patients, HBV genotypes C, B, and B/C mixed genotype were detected in 260 (68.2%), 115 (30.2%), and 6 (1.6%) patients, respectively. The prevalence of HBV genotypes and subgenotypes is summarized in Table 1. All genotype B subgenotypes (115/115) were B2. Genotype C2 (257/260) predominated in genotype C, and the remainder were C1 (3/260). Overall, genotype C2 (67.5%, 257/381) was the most prevalent subgenotype in this population in Southeast China, followed by B2 (30.2%, 115/381). The prevalence of genotype B in CHB was higher than in LC or HCC, and the prevalence of genotype C in LC or HCC was higher than in CHB ($P<0.05$), but no difference in prevalence of either genotype in LC and HCC was found. The distributions of subgenotypes B2 and C2 among the three groups were the same as their genotypes (Table 1).

In comparison with the patients infected with B2, those with C2 in the CHB, LC, and HCC groups had higher serum HA levels ($P<0.05$). In the HCC group, the patients with C2 were more prone to HBeAg seropositivity. However, the differences in serum levels of ALT, TBIL, albumin, AFP, and HBV DNA were not significant (Table 2).

In this study, all the CHB and HCC patients had complete clinicopathological data. As Table 3 shows, the CHB patients infected with the C2 virus staged higher for fibrosis than those with B2 HBV ($P<0.05$); no significant difference in inflammation grading was observed. There were no significant differences in tumor number, histological grade, TNM stage, or lymph node metastasis between the patients with B2 or C2 in the HCC group. Interestingly, the patients with B2 had larger tumors ($\geqslant$5 cm), while those with C2 were more commonly accompanied by cirrhosis ($P<0.05$; Table 3).

Mutations with a frequency $>5\%$ were selected in this study. A total of 13 mutations were detected (Table 4). The frequencies of six mutation patterns in the CP region (C1653T, G1730C, T1753C, A1762T, G1764A, and G1799C) were higher in LC patients than in CHB patients ($P<0.05$). The frequencies of 10 mutation patterns (C1653T, G1730C, T1753C, A1762T, G1764A, G1799C, C1810T, A1846T, G1862T, and G1896A) were higher in HCC patients than in CHB patients ($P<0.05$). The frequencies of four mutation patterns close to the precore region (C1810T, A1846T, G1862T, and G1896A) were higher in HCC patients than in LC patients ($P<0.05$; Table 4).

To evaluate the associations between the 13 mutations and HBV subgenotype B2/C2 infection, the frequency of each mutation in the patients with B2 or C2

Table 1. HBV genotype frequency distributions in 381 patients with chronic HBV infection.

HBV genotype	CHB (n = 125)	LC (n = 120)	HCC (n = 136)	P[a]	P[b]	P[c]
B	56 (44.8)	25 (20.8)	34 (25.0)	0.006	0.005	NS
B1	0	0	0			
B2	56 (44.8)	25 (20.8)	34 (25.0)	0.006	0.005	NS
C	67 (53.6)	92 (76.7)	101 (74.3)	0.002	0.003	NS
C1	1 (0.8)	2 (1.7)	0			
C2	66 (52.8)	90 (75.0)	101 (74.3)	0.003	0.002	NS
B + C mixture	2 (1.6)	3 (2.5)	1 (0.7)			

Data are reported as number (%). CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; NS: non-significant. [a]LC *vs* CHB; [b]HCC *vs* CHB; [c]HCC *vs* LC. P values for HBV subgenotype C1 and the genotype mixture are not shown because of the small sample size. The chi-square test was used for statistical analysis.

Table 2. Serological and virologic markers for chronically infected patients with HBV subgenotype B2/C2.

Markers	HBV/ B2	HBV/ C2	P
CHB	n = 56	n = 66	
ALT (U/L)	102.59 ± 90.25	97.85 ± 69.26	NS
TBIL (μM)	48.84 ± 35.43	50.07 ± 46.47	NS
Albumin (g/L)	37.31 ± 8.20	39.94 ± 10.52	NS
HA (ng/mL)	148.47 ± 48.63	169.58 ± 52.04	0.023
AFP (ng/mL)	8.91 ± 5.67	8.17 ± 6.39	NS
HBeAg (+)	30 (53.6)	38 (57.6)	NS
HBV DNA (log_{10} copies/mL)	5.96 ± 1.28	5.78 ± 1.34	NS
LC	n = 25	n = 90	
ALT (U/L)	78.28 ± 46.57	73.13 ± 39.46	NS
TBIL (μM)	74.87 ± 45.08	76.34 ± 42.76	NS
Albumin (g/L)	30.38 ± 11.52	29.24 ± 12.97	NS
HA (ng/mL)	497.16 ± 172.90	594.25 ± 186.12	0.021
AFP (ng/mL)	47.90 ± 16.34	45.37 ± 13.54	NS
HBeAg (+)	12 (48.0)	53 (58.9)	NS
HBV DNA (log_{10} copies/mL)	5.83 ± 1.16	5.68 ± 1.43	NS
HCC	n = 34	n = 101	
ALT (U/L)	64.32 ± 43.27	61.97 ± 46.38	NS
TBIL (μM)	29.12 ± 16.70	26.87 ± 19.26	NS
Albumin (g/L)	33.46 ± 14.84	34.08 ± 16.36	NS
HA (ng/mL)	314.69 ± 94.85	367.18 ± 114.67	0.018
AFP (ng/mL)	796.30 ± 406.67	772.53 ± 397.48	NS
HBeAg (+)	12 (35.3)	56 (55.5)	0.038
HBV DNA (log_{10} copies/mL)	5.98 ± 1.06	5.71 ± 1.37	NS

Data are reported as means ± SD or number (%). CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; ALT: alanine aminotransferase; TBIL: total bilirubin; HA: hyaluronic acid; AFP: α-fetoprotein; HBeAg: HBV e-antigen; NS: non-significant. The Student *t*-test, the Mann-Whitney U-test and the chi-square test were used for statistical analysis.

Table 3. Clinicopathological characteristics in CHB and HCC patients with HBV subgenotype B2/C2 infection.

Characteristics	HBV/B2	HBV/C2	P
CHB	n = 56	n = 66	
Grade			
1-2	31 (55.4)	30 (45.5)	NS
3-4	25 (44.6)	36 (54.5)	
Stage			
0-2	43 (76.8)	37 (56.1)	0.025
3-4	14 (25.0)	29 (43.9)	
HCC	n = 34	n = 101	
Tumor number			
Single	22 (64.7)	74 (73.3)	NS
Multiple	12 (35.3)	27 (26.7)	
Tumor size			
<5 cm	11 (32.4)	53 (52.5)	0.037
⩾5 cm	23 (67.6)	47 (46.5)	
Histological grade			
I + II	24 (70.6)	68 (67.3)	NS
III + IV	10 (29.4)	33 (32.7)	
TNM stage			
I + II	27 (79.4)	86 (85.1)	NS
III + IV	7 (20.6)	15 (14.9)	
Lymph node metastasis			
Present	12 (35.3)	27 (26.7)	NS
Absent	22 (64.7)	74 (73.3)	
Cirrhosis			
Present	8 (23.5)	43 (42.6)	0.048
Absent	26 (76.5)	58 (57.4)	

Data are reported as number (%). The pathological examination for liver biopsy in chronic hepatitis B (CHB) patients was according to Scheuer's classification; the histological assay was determined by referencing the standard of Edmondson grade; tumor stage was classified according to the TNM criteria of the International Union Against Cancer. HBV: hepatitis B virus; HCC: hepatocellular carcinoma; NS: non-significant. The chi-square test was used for statistical analysis.

subgenotype was compared. Seven mutation patterns (C1653T, G1719T, G1730C, T1753C, A1762T, G1764A, and G1799C) in the CP region were more prevalent in the patients with the C2 than with the B2 subgenotype ($P<0.05$). Four mutation patterns (C1810T, A1846T, G1862T, and G1896A) close to the precore region were more prevalent in the patients with the B2 than with the C2 subgenotype ($P<0.001$; Table 5).

Table 6 shows seven combined mutation patterns (>5% frequency) that were all double or triple combined mutations. The frequencies of all combined mutations in LC or HCC were higher than in CHB ($P<0.05$), but no difference was found between LC and HCC. The A1762T/G1764A double mutation was highly prevalent, occurring in 36.9% of CHB patients, 63.6% of LC patients, and 60.0% of HCC patients. Each of the combined mutations detected in this study included the A1762T and/or the G1764A variant.

To analyze the independent risk factors for the progression of chronic liver disease, the multiple variants were evaluated by multivariate regression, including HBeAg status, HBV subgenotype, and the presence of C1653T, G1730C, T1753C, A1762T, G1764A, G1799C, C1810T, A1846T, G1862T, or G1896A mutations (Table 7). When CHB patients were the control, HBV subgenotype C2 infection and C2-associated mutation patterns (C1653T, T1753C, A1762T, and G1764A) in the CP region were independent risk factors for LC or C2 infection, and eight mutation patterns (C1653T, T1753C, A1762T, G1764A, C1810T, A1846T, G1862T, and G1896A) were independent risk factors for HCC. When LC patients were the control, HBV subgenotype B2-associated mutation patterns (C1810T, A1846T, G1862T, and G1896A) close to the precore region were independent risk factors for HCC.

Table 4. Thirteen mutations in the enhII/BCP/precore regions of HBV in chronically infected patients.

Substitution	ASC_S (n = 844)	CHB (n = 125)	LC (n = 120)	HCC (n = 136)	P[a]	P[b]	P[c]
C1653T	68 (8.1)	8 (6.4)	22 (18.3)	32 (23.5)	0.004	<0.001	NS
T1674C	64 (7.6)	25 (20.0)	34 (28.3)	36 (26.5)	NS	NS	NS
G1719T	150 (17.8)	65 (52.0)	67 (55.8)	75 (55.1)	NS	NS	NS
G1730C	214 (25.4)	73 (58.4)	88 (73.3)	97 (71.3)	0.014	0.029	NS
T1753C	49 (5.8)	8 (6.4)	25 (20.8)	30 (22.1)	0.001	<0.001	NS
A1762T	145 (17.2)	51 (40.8)	70 (58.3)	81 (59.6)	0.006	0.002	NS
G1764A	186 (22.0)	55 (44.0)	80 (66.7)	86 (63.2)	<0.001	0.002	NS
G1799C	183 (21.7)	70 (56.0)	94 (78.3)	101 (74.3)	<0.001	0.002	NS
C1810T	0	2 (1.6)	7 (5.8)	26 (19.1)	NS	<0.001	0.002
A1846T	67 (7.9)	31 (24.8)	34 (28.3)	55 (40.4)	NS	<0.001	<0.001
G1862T	0	7 (5.6)	7 (5.8)	30 (22.1)	NS	0.002	0.010
G1896A	150 (17.8)	51 (40.8)	52 (43.3)	81 (59.6)	NS	0.002	0.010
G1899A	0	22 (17.6)	25 (20.8)	17 (12.5)	NS	NS	NS

Data are reported as number (%). Hepatitis B virus (HBV) mutation data for asymptomatic HBsAg carrier (ASC) state were organized as in the study by Yin et al. (27). A nucleotide with the highest frequency at each site in the enhII/BCP/precore regions of HBV from ASCs was defined as the wild-type nucleotide. Nucleotide substitutions at each site were defined as mutations. EnhII: enhancer II region of HBV; BCP: basal core promoter region of HBV; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; NS: non-significant. [a]LC *vs* CHB; [b]HCC *vs* CHB; [c]HCC *vs* LC. The chi-square test or the Fisher exact test was used for statistical analysis.

Discussion

It is generally accepted that HBV genotypes vary in geographic distribution and show a distinct association with the clinical progression of liver disease (6-10,14,16). A cross-sectional study of 270 Taiwanese patients with chronic HBV infection demonstrated that HBV genotype C was associated with more severe liver disease and that genotype B may be associated with the development of HCC in young Taiwanese (28). A survey from North China reported that HBV genotypes B (14.6%), C (84.2%), and D (1.2%) were detected in 1301 chronically infected patients. Among them, subgenotypes C2 (77.2%) and B2 (17.8%) were identified. It was suggested that genotype C infection was likely to be associated with a longer disease duration and more severe impairment of liver function than genotype B infection (29). Another report from Southern China revealed that genotype B accounted for more than 50% of cases and that subgenotypes C1 (33.6%), B2 (51.2%), and C2 (15.2%) were endemic (16). These data revealed that the prevalence of HBV genotype C and subgenotype C2 decreased, whereas the prevalence of genotype B, and subgenotypes B2 and C1 increased from north to south in China.

Table 5. Determination of subgenotype-associated mutations in the enhII/BCP/precore regions of HBV.

Substitution	Subgenotype B2 (n = 115)	Subgenotype C2 (n = 257)	P
C1653T	10 (8.7)	51 (19.8)	0.006
T1674C	8 (7.0)	32 (12.5)	NS
G1719T	17 (14.8)	151 (58.8)	<0.001
G1730C	25 (21.7)	233 (90.7)	<0.001
T1753C	6 (5.2)	57 (22.2)	<0.001
A1762T	24 (20.9)	173 (67.3)	<0.001
G1764A	23 (20.0)	178 (69.3)	<0.001
G1799C	6 (5.2)	246 (95.7)	<0.001
C1810T	24 (20.9)	5 (1.9)	<0.001
A1846T	57 (49.6)	38 (14.8)	<0.001
G1862T	24 (20.9)	25 (9.7)	<0.001
G1896A	81 (70.4)	84 (32.7)	<0.001
G1899A	25 (21.7)	43 (16.7)	NS

Data are reported as number (%). enhII: enhancer II region of hepatitis B virus (HBV); BCP: basal core promoter region of HBV; NS: non-significant. The chi-square test was used for statistical analysis.

In our study, HBV genotype C (68.2%) predominated, and genotype B (30.2%) was the second most common in chronically infected patients. Of these, C2 (67.5%) was the most prevalent subgenotype followed by B2 (30.2%), and C1. The proportion of HBV subgenotype C2 increased from CHB to HCC and LC, while that of B2 decreased. Subgenotype C2 was associated with LC and HCC, whereas B2 was associated with CHB in univariate analyses.

In each of the three patient groups, serum HA levels were significantly higher in participants with subgenotype C2 than in those with subgenotype B2, which indicated that patients with C2 were more likely to progress to severe liver fibrosis and cirrhosis than patients with B2. Additionally, HCC group patients with subgenotype C2 had a higher HBeAg seropositivity rate than those with

Table 6. Combined mutations in the enhII/BCP/precore regions of HBV.

Combined mutation patterns	CHB (n = 122)	LC (n = 115)	HCC (n = 135)	P[a]	P[b]	P[c]
C1653T + A1762T	2 (1.6)	11 (9.6)	16 (11.9)	0.003	<0.001	NS
C1653T + G1764A	3 (2.5)	14 (12.2)	17 (12.6)	0.002	<0.001	NS
A1762T + G1764A	45 (36.9)	73 (63.5)	81 (60.0)	<0.001	<0.001	NS
C1653T + A1762T + G1764A	2 (1.6)	10 (8.7)	16 (11.9)	0.004	<0.001	NS
T1753C + A1762T + G1764A	8 (6.6)	27 (23.5)	28 (20.7)	<0.001	<0.001	NS
A1762T + 1764A + G1799C	28 (23.0)	57 (49.6)	65 (48.1)	<0.001	<0.001	NS
A1762T + 1764A + G1896A	8 (6.6)	17 (14.8)	30 (22.2)	0.039	<0.001	NS

Data are reported as number (%). BCP: basal core promoter; HBV: hepatitis B virus; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; NS: non-significant. [a]LC *vs* CHB; [b]HCC *vs* CHB; [c]HCC *vs* LC. The chi-square test or the Fisher exact test was used for statistical analysis.

subgenotype B2, which was not observed in CHB and LC patients. This result suggests that the difference in capacity for HBeAg seroconversion between patients with subgenotype B2 or C2 did not appear until chronic liver disease progressed to HCC.

Previous studies suggested that the HBV genotype may affect the clinical behavior of HCC (30,31). However, the influence of HBV subgenotype on the clinicopathological features of liver disease in Southeast China is not well understood. In this study, we found that CHB patients infected with subgenotype C2 had a higher fibrosis staging than those with the B2 subgenotype, according to Scheuer's classification. This result was supported by the serological assay results for HA levels. Interestingly, HCC patients with B2 accounted for a higher proportion of tumors ⩾5 cm, whereas those with the C2 subgenotype were more likely to progress to cirrhosis. This finding has rarely been reported in the published literature, and extensive supporting clinical data are still needed.

HBV mutation data for ASCs from the study by Yin et al. (27) are applicable to our data because, in that study, the subjects were from Southeast China, the sample size was large enough, frequencies of HBV genotypes/subgenotypes in the total sample were similar to our results, and mutational analysis was achieved by sequencing. In our study, we detected 13 mutations in the enhII/BCP/precore regions of HBV. Univariate analyses showed that 6 mutation patterns (C1653T, G1730C, T1753C, A1762T, G1764A, and G1799C) located in the CP region were associated with LC compared with CHB ($P<0.05$). Ten mutation patterns (C1653T, G1730C, T1753C, A1762T, G1764A, G1799C, C1810T, A1846T, G1862T, and G1896A) were associated with HCC compared with CHB ($P<0.05$), and 4 mutation patterns (C1810T, A1846T, G1862T, and G1896A) located close to the precore region were associated with HCC compared with LC ($P<0.05$).

Seven of 13 mutation patterns (C1653T, G1719T, G1730C, T1753C, A1762T, G1764A, and G1799C) in the CP region were associated with C2 infection, and 4 mutation patterns (C1810T, A1846T, G1862T, and G1896A) close to the precore region were associated with B2 infection.

The prevalence and clinical implication of the combined mutations in the enhII/BCP/precore regions have been published (21,32). Seven combined mutations detected by this study were associated with the development of chronic HBV infection. Each combined mutation containing A1762T and/or G1764A occurred in the early stage of advanced liver disease, which means that the A1762T and/or G1764A mutation may stimulate the occurrence of the other mutations in the enhII/BCP/precore regions.

Multivariate analysis revealed that HBV subgenotype C2 infection and C2-associated mutation patterns (C1653T, T1753C, A1762T, and G1764A) contributed to the development of LC from CHB, and that B2-associated mutation patterns (C1810T, A1846T, G1862T, and G1896A) contributed to the development of HCC from LC. We speculate that HBV subgenotype C2 and C2-associated mutations may be related to the progression from liver necroinflammation to cirrhosis, whereas B2-associated mutations may be involved in hepatocarcinogenesis.

The underlying mechanisms of the mutations in the enhII/BCP/precore regions of HBV and their impact on the occurrence of LC and HCC are unclear. The C1653T mutation occurs at the center of the immunodominant antigenic domain and may be involved in inflammatory processes (33). The T1753C mutation enhances viral replication, leading to persistent infection (34). The A1762T and G1764A mutations are associated with diminished HBeAg production at the transcriptional level, enhanced host immune response, and viral replication (34). The C1810T and G1862T mutations affect the expression of HBeAg at the translational and post-translational levels, respectively (35). The G1896A mutation produces a stop codon for HBeAg expression (36), while the role of the A1846T mutation in the regulation of HBeAg expression requires further study. In addition, the

Table 7. Multivariate analysis of independent risk factors for LC and HCC, respectively.

Factor	LC (CHB as control)		HCC (CHB as control)		HCC (LC as control)	
	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
HBV subgenotype						
B2	1		1		1	
C2	2.368 (1.082-4.157)	0.002	2.401 (1.136-4.308)	0.001	0.807 (0.430-1.514)	NS
HBeAg						
Negative	1		1		1	
Positive	0.969 (0.561-1.673)	NS	0.925 (0.493-1.617)	NS	0.964 (0.526-1.705)	NS
C1653T						
C	1		1		1	
T	2.350 (1.071-5.212)	0.020	2.974 (1.466-6.094)	0.001	1.371 (0.715-2.636)	NS
G1730C						
G	1		1		1	
C	1.609 (0.932-2.812)	NS	1.339 (0.794-2.262)	NS	0.904 (0.503-1.624)	NS
T1753C						
T	1		1		1	
C	2.753 (1.273-6.021)	0.005	1.985 (0.992-4.005)	0.036	1.075 (0.567-2.043)	NS
A1762T						
A	1		1		1	
T	1.713 (1.024-2.870)	0.029	1.669 (1.016-2.723)	0.026	1.052 (0.619-1.787)	NS
G1764A						
G	1		1		1	
A	1.668 (0.997-2.792)	0.038	1.764 (1.077-2.891)	0.017	0.860 (0.497-1.487)	NS
G1799C						
G	1		1		1	
C	0.843 (0.516-1.420)	NS	1.597 (0.938-2.725)	NS	0.898 (0.629-1.582)	NS
C1810T						
C	1		1		1	
T	0.614 (0.442-1.416)	NS	15.012 (3.395-92.783)	<0.001	2.935 (1.275-7.086)	0.005
A1846T						
A	1		1		1	
T	1.457 (0.832-2.563)	NS	2.175 (1.261-3.764)	0.003	1.752 (0.991-3.106)	0.038
G1862T						
G	1		1		1	
T	1.588 (0.568-4.567)	NS	4.797 (1.943-2.360)	<0.001	4.569 (1.818-1.965)	<0.001
G1896A						
G	1		1		1	
A	1.408 (0.836-2.375)	NS	2.217 (1.256-3.165)	0.002	1.926 (1.136-3.270)	0.008

CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HbeAg: HBV e-antigen; CI: confidence interval; OR: odds ratio; NS: non-significant. Multiple logistic regressions were used for statistical analysis.

C1653T, T1753C, A1762T, and G1764A mutations lead to H94Y, I127T, K130M, and V131I amino acid substitutions in HBx. These changes, located in regions D (~85-119 amino acids) and E (~120-140 amino acids) of the HBx functional region, could abrogate the transactivation of HBx, which is associated with hepatocarcinogenesis (37). The C1653T, T1753C, A1762T, and G1764A mutations may play critical roles in persistent viral infections and inflammatory processes, and therefore may be responsible for severe liver fibrosis. The C1810T, A1846T, G1862T, and G1896A mutations most likely result in crude expression of HBeAg and accumulation of the HBeAg precursor in the endoplasmic reticulum/Golgi apparatus of the cell (38). This accumulation could affect various cellular pathways that contribute to the development of HCC.

In conclusion, our study systemically investigated the relationship of HBV genotypes/subgenotypes and enhII/BCP/precore mutations to the progression of chronic HBV infection. We found that the HBV subgenotypes C2 and B2 predominated in chronically infected patients in

Southeast China. Our results showed that HBV subgenotype C2 and C2-associated mutation patterns (C1653T, T1753C, A1762T, and G1764A) were independent risk factors for LC when CHB was the control and that B2-associated mutation patterns (C1810T, A1846T, G1862T, and G1896A) were independent risk factors for HCC when LC was the control.

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