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Clinical and Virological Characteristics of Hepatitis B Virus Subgenotypes Ba, C1, and C2 in China[▽]

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Hepatitis B virus (HBV) subgenotypes Ba, C1 (Cs), and C2 (Ce) are the most prevalent HBV variants in China. To investigate the virological characteristics of these subgenotypes and their clinical implications, we enrolled a cohort of 211 patients in the Guangdong Province of China, including 132 with chronic hepatitis B virus infection (CH), 32 with liver cirrhosis (LC), and 47 with hepatocellular carcinoma (HCC) according to clinical examination, liver function test, and ultrasonograph results. Overall, HBV Ba was found in 51.2% (108/211), HBV C1 in 33.6% (71/211), and HBV C2 in 15.2% (32/211) of the cases. The distribution of HBV genotype C was greater among patients in the LC and HCC groups than among patients in the CH group, while the distribution of HBV genotype B was greater among the CH patients than among the LC and HCC patients. No significant differences in clinical features were found among patients with HBV Ba, C1, and C2. Virologically, HBV C1 had the strongest association with the A1762T G1764A double mutation, while the mutation at position 1896 resulting in A (1896A) was uncommon. In contrast, HBV Ba had the highest frequency of 1896A but the lowest of A1762T G1764A, and HBV C2 had intermediate frequencies of these mutations. Mutations of 1653T and 1753V were specifically associated with HBV C2 and C1, respectively. Multivariate analyses showed that the 1653T, 1753V, and A1762T G1764A mutations and patient age significantly increased the risk of HCC development. In conclusion, HBV Ba, C1, and C2 have different mutation patterns in the enhancer II/core promoter/precore region. Therefore, genotyping and detecting the 1653T and 1753V mutations, in addition to the A1762T G1764A double mutation, might have important clinical implications as predictive risk factors for hepatocarcinogenesis.

Hepatitis B virus (HBV) infection remains an important cause of morbidity and mortality worldwide, especially in developing countries. HBV can be classified into at least eight genotypes (A to H) based on a divergence in the entire nucleotide sequence greater than 8% (1, 21, 22, 26). HBV genotypes have a distinct geographical distribution. Genotypes A and D are commonly found in Europe, the Mediterranean region, and the Middle East. Genotypes B and C are highly prevalent in Asia (2, 19).

HBV exhibits a mutation rate more than 10-fold higher than that of other DNA viruses for the lack-of-proofreading function of the polymerase (3). A variety of mutations in the precore/core region were identified. The most prevalent and well defined are the precore stop codon mutation at nucleotide (nt) 1896 resulting in A (1896A) and the basal core promoter double mutation (A1762T G1764A). Previous studies have shown that the A1762T G1764A and 1896A mutations are involved in the mechanism of HBV e antigen (HBeAg)-negative HBV infections and have distinct regional differences (12, 16, 18). Studies also confirmed the presence of a correlation between HBV genotypes and the development of 1896A and A1762T G1764A mutations (15, 18). The 1896A mutation was commonly found in genotypes B, D, and E and sometimes in genotype C but rarely in genotype A (14). However, the A1762T G1764A double mutation was commonly detected in HBV genotypes A and C but less frequently in genotypes B and D (30, 35). Beyond these mutations in core promoter/precore regions, the mutations of C to T at nt 1653 (C1653T) in the box alpha and T to V(C/A/G) at nt 1753 (T1753V) in the basic core promoter may increase the risk of hepatocellular carcinoma (HCC) in patients with HBV genotype C infections (10, 29, 31).

Based on a divergence of greater than 4% but less than 8% in the complete nucleotide sequence, HBV genotypes have been divided into subgenotypes. Until now, five subgenotypes of genotype C have been identified (6, 9, 20, 25). Subgenotype C1 (Cs) was found in Southeast Asia, C2 (Ce) in East Asia, C3 in Polynesia, C4 in Aborigines from Australia, and C5 in the Philippines and Vietnam. A recent report of patients from Hong Kong showed that HBV subgenotype C1 has a comparable frequency of the A1762T G1764A mutation but a lower frequency of the 1896A mutation than does subgenotype C2 (6). A comparison of the A1762T G1764A and 1896A mutations developed among subgenotypes Ba, C1, and C2 is still unclear. Whether the differing tendencies to develop the A1762T G1764A and 1896A mutations in different genotypes will impact clinical outcomes needs to be confirmed. The impact of HBV genotype C subgenotypes on the natural course of chronic HBV infection and the severity of liver damage remains unknown.

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In China, genotypes B and C are the most common HBV genotypes (36). In northern China, more than 90% of patients were infected with subgenotype C2, while in southern China, the predominant subgenotype was C1 (33). In the present study, we aimed to investigate the clinical and virological differences of subgenotypes Ba, C1, and C2, as well as the possible significant factors in HCC development.

MATERIALS AND METHODS

Patients. A total of 211 patients (176 men and 35 women, 12 to 73 years in age; mean age ± standard deviation, 37.5 ± 14.0 years) infected with HBV were enrolled in this study. All patients were from the Guangdong Province of China and were positive for the HBV surface antigen. All patients were chronic HBV carriers (serum positive for the HBV surface antigen for at least 6 months) and were seronegative for hepatitis C and hepatitis D viruses. All serum samples were stored at −30°C until analysis.

Laboratory assays. Aalanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin (TBIL), and HBeAg tests were performed in a clinical laboratory using commercial test kits (Olympus Diagnostica GmbH, Lismeehan, O′Callaghans's Mills, County Clare, Ireland; AxSYM; Abbott Laboratories, Wiesbaden, Germany). For patients with chronic hepatitis B virus infections (CH), HBV DNA levels were determined using the COBAS Amplicor Monitor test, and 99 had a liver biopsy. Liver histology was evaluated independently by two pathologists who were blind to the patients' clinical data, using the Knodell score with a little modification as described previously (24).

HBV genotyping and subgenotyping. HBV DNA was extracted from 100 μl serum using a QIAamp DNA blood mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and suspended in 50 μl distilled water. Three microliters were used as a template for HBV DNA amplification. HBV genotypes and subgenotypes were determined using PCR-restriction fragment length polymorphism as described by Zeng et al. (37), Tanaka et al. (32), and Sugauchi et al. (27).

Amplification and sequencing of the preC/C gene. All samples were subjected to heminested PCR to find the specific mutations in the enhancer II/core promoter and precore/core regions and to confirm the PCR-restriction fragment length polymorphism results of the genotypes/subgenotypes as previously reported (33). Amplified products were directly sequenced in both the forward and reverse directions using an ABI 3700 sequencer and commercial kit (Applied Biosystems, Foster City, CA).

Statistical analyses. All data were analyzed by using the statistical package SPSS (version 12.0; SPSS, Inc., Chicago, IL). Chi-square, Fisher's exact, and Student's *t* tests were used as appropriate. Multivariate analyses with logistic regression were used to determine the independent factors associated with HCC. A *P* value of <0.05 was considered statistically significant.

RESULTS

HBV genotypes/subgenotypes in patients with different clinical diagnoses. In this study, all patients were grouped according to diagnosis based on clinical examination, liver function test, and ultrasonograph results. Thirty-two patients (15.2%), including 26 men and 6 women with a mean age of 50.2 ± 12.0 years, were diagnosed with liver cirrhosis (LC), and 47 patients (22.3%), including 42 men and 5 women with a mean age of 49.8 ± 11.6 years, were diagnosed with HCC. The remaining 132 patients (62.5%; 108 men and 24 women; mean age, 30.0 ± 9.1 years) were diagnosed with CH. For all 211 patients, 108 (51.2%) were infected with genotype B, and 103 (48.8%) were infected with genotype C. All genotype B infections were of the subgenotype Ba. When 103 genotype C samples were subgenotyped, 71 subgenotype C1 (Cs) and 32 subgenotype C2 (Ce) samples were identified. The ratio of C1 samples to C2 samples was approximately 2 to 1. Significant differences were observed in the distributions of genotypes B and C between HCC patients and CH ($P < 0.001$) or LC ($P = 0.003$) patients, while no significant differences were observed in the distributions between CH and LC patients ($P = 0.71$). The proportion of genotype C infections increased according to disease progression, as shown in 40.2% (53/132) of patients with CH, 43.7% (14/32) of patients with LC, and 76.6% (36/47) of patients with HCC. The proportion of HBV C1 infections tended to increase with disease progression, especially in HCC patients. A similar upward trend was also observed in HBV C2 infections, and the ratios of HBV C1 infections to HBV C2 infections in CH, LC, and HCC patients were not different ($P = 0.874$) (Fig. 1).

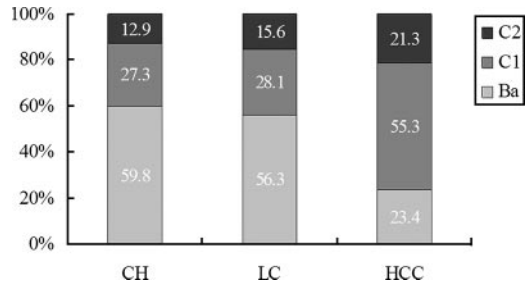

FIG. 1. Prevalences of subgenotypes Ba, C1, and C2 in different patient groups. The distributions of subgenotypes Ba, C1, and C2 in HCC patients were significantly different from those in CH ($P < 0.001$) and LC patients ($P = 0.011$), but no significant differences were observed between the distributions of these subgenotypes in CH and LC patients ($P = 0.886$). The prevalences of C1 and C2 increased similarly with disease progression and were not significantly different ($P = 0.874$) in CH, LC, and HCC patients.

Clinical differences among different subgenotypes. There were no significant differences in clinical features among patients infected with HBV Ba, C1, or C2 in this study, specifically in age, sex ratio, HBeAg-positive rate, inflammation and fibrosis scores, and serum levels of ALT, AST, ALB, TBIL, and HBV DNA (Table 1).

Virological differences among different subgenotypes. Significant differences were observed among HBV Ba, C1, and C2 when their virological characteristics were compared (Table 1). Compared with HBV Ba and HBV C2, HBV C1 was associated with the highest tendency to develop A1762T G1764A and T1753V mutations but the lowest prevalence of the 1896A mutation and T at nt 1858 (1858T). As a contrast, HBV Ba showed the highest frequency of the 1896A mutation but the lowest frequency of the A1762T G1764A, C1653T, and T1753V mutations. HBV C2 had an intermediate prevalence of the 1896A, A1762T G1764A, and T1753V mutations but had the highest frequency of the C1653T mutation. 1856C, 1858T, and 1898G were found exclusively among HBV Ba and HBV C2 strains. However, about 87% of the HBV C1 strains had 1858C. The A1898G mutation and 1856T were specific to HBV C1.

Further statistical analyses between every two subgenotypes showed significant differences in the frequencies of the A1762T G1764A (Ba versus C1, $P < 0.001$; Ba versus C2, $P = 0.008$; C1 versus C2, $P = 0.013$) and T1753V (Ba versus C1, $P < 0.001$; Ba versus C2, $P = 0.005$; C1 versus C2, $P = 0.034$) mutations. Significant differences were also found in the numbers of precore 1896A mutations between Ba and C1 ($P < 0.001$) or C1 and C2 ($P = 0.001$), but no difference in number was observed between Ba and C2 ($P = 0.289$). HBV Ba was

TABLE 1. Clinical and virological differences of HBV subgenotypes Ba, C1, and C2

Characteristic	Value for subgenotype group[a]			*P* value[b]
	Ba (n = 108)	C1 (n = 71)	C2 (n = 32)	
Age (yr)	36.2 ± 3.8	38.2 ± 15.4	40.1 ± 11.0	0.327
Sex (no. of males/no. of females)	91/17	58/13	27/5	0.877
No. (%) of HBeAg-positive patients	62 (57.4)	34 (47.9)	16 (50)	0.434
Liver function indicators				
ALT (U/liter)	182.5 ± 223.8	136.7 ± 113.3	153.3 ± 162.0	0.256
AST (U/liter)	118.9 ± 140.5	96.3 ± 67.3	119.4 ± 108.7	0.407
ALB (g/liter)	42.7 ± 6.4	40.7 ± 6.7	41.3 ± 6.3	0.209
TBIL (μmol/liter)	25.4 ± 20.2	29.0 ± 28.4	26.5 ± 19.4	0.676
No. (%) of patients with viral mutation(s):				
C1653T	1 (0.9)	6 (8.5)	5 (15.6)	0.002
T1753V	6 (5.6)	31 (43.7)	7 (21.9)	<0.001
A1762T G1764A	30 (27.8)	55 (77.5)	17 (53.1)	<0.001
1856T	0 (0.0)	30 (42.3)	0 (0.0)	<0.001
1858T	108 (100.0)	9 (12.7)	32 (100.0)	<0.001
1896A	45 (41.7)	4 (5.6)	10 (31.3)	<0.001
1898A	0 (0.0)	11 (15.5)	0 (0.0)	<0.001
1899A	12 (11.1)	7 (9.9)	2 (6.3)	0.819
HBV DNA (log no. of copies/ml)[c]	8.4 ± 2.1 (79)	8.1 ± 1.7 (36)	7.7 ± 1.1 (17)	0.394
Histology[c]				
Grade	2.4 ± 1.0 (61)	2.3 ± 0.9 (25)	2.7 ± 0.9 (13)	0.496
Stage	2.0 ± 0.9 (61)	2.1 ± 0.9 (25)	2.3 ± 0.9 (13)	0.60

[a] Data are given as the means ± standard deviations or no. (%) of patients except for the characteristic "sex."
[b] *P* values are for values between Ba, C1, and C2 subgenotype groups.
[c] Data given are means ± standard deviations (no. of patients). Grade, liver inflammation status graded on a scale from 1 to 4; stage, fibrosis status indicated on a scale from 1 to 4, with 4 representing established cirrhosis.

associated with an incidence of the 1653T mutation significantly lower than that in HBV C1 ($P = 0.015$) or HBV C2 ($P = 0.002$), while HBV C1 and HBV C2 had no significant difference in incidence ($P = 0.287$).

Clinical and virological differences among patients with different clinical diagnoses. When clinical and virological data were compared among CH, LC, and HCC patient groups, the differences were significant (Table 2). CH patients showed a

TABLE 2. Clinical and virological features of patients in different HBV clinical groups

Characteristic	Value for disease group			*P* value[a]
	CH (n = 132)	LC (n = 32)	HCC (n = 47)	
Mean age ± SD (yr)	30.0 ± 9.1	50.2 ± 12.0	49.8 ± 11.6	<0.001
Sex (no. of males/no. of females)	108/24	26/6	42/5	0.461
No. (%) of HBeAg-positive patients	94 (71.2)	10 (31.3)	8 (17.0)	<0.001
Liver function indicators (mean ± SD)				
ALT (U/liter)	201.7 ± 202.5	146.7 ± 183.7	63.9 ± 41.6	<0.001
AST (U/liter)	117.1 ± 121.3	142.5 ± 141.9	74.2 ± 60.4	0.023
ALB (g/liter)	46.1 ± 4.9	34.7 ± 3.1	38.3 ± 4.6	<0.001
TBIL (μmol/liter)	13.9 ± 6.7	51.4 ± 20.5	35.4 ± 28.5	<0.001
No. (%) of patients with viral mutation(s):				
C1653T	2 (1.5)	1 (3.1)	9 (19.1)	<0.001
T1753V	13 (9.8)	6 (18.8)	25 (53.2)	<0.001
A1762T G1764A	44 (33.3)	18 (56.3)	40 (85.1)	<0.001
1856T	17 (12.9)	4 (12.5)	9 (19.1)	0.554
1858T	100 (75.8)	25 (78.1)	24 (51.1)	0.004
1896A	36 (27.3)	6 (18.8)	17 (36.2)	0.233
1898A	2 (1.5)	1 (3.1)	8 (17.0)	0.001
1899A	7 (5.3)	4 (12.5)	10 (21.3)	0.006

[a] *P* values are between values for CH, LC, and HCC patient groups.

TABLE 3. Independent risk factors predictive for HCC development in HBV patients as determined by multivariate analysis

Factor	Odds ratio (95% CI)	*P* value
Age		
<50 yr	1	0.001
≥50 yr	14.25 (3.16–64.16)	
Sex		
Female	1	0.81
Male	0.83 (0.18–3.91)	
HBeAg status		
Negative	1	0.07
Positive	0.32 (0.10–1.10)	
Genotype		
B	1	0.591
C	1.50 (0.34–6.56)	
C1653T mutation		
Absence	1	0.007
Presence	20.62 (2.33–182.36)	
T1753V mutation		
Absence	1	0.013
Presence	5.63 (1.45–21.84)	
A1762T G1764A mutation		
Absence	1	0.008
Presence	5.50 (1.58–19.18)	
1896A mutation		
Absence	1	0.17
Presence	2.52 (0.67–9.41)	
1898A mutation		
Absence	1	0.551
Presence	2.05 (0.19–21.81)	
1899A mutation		
Absence	1	0.966
Presence	1.03 (0.22–4.84)	

significantly higher rate of HBeAg positivity than LC ($P < 0.001$) or HCC ($P < 0.001$) patients. The incidence of A1762T G1764A mutations increased along with the progression of liver disease, and significant differences were found between every two patient groups (CH versus LC, $P = 0.016$; CH versus HCC, $P < 0.001$; LC versus HCC, $P = 0.004$). Interestingly, the lowest prevalence of 1896A was observed in LC patients, compared with that in CH and HCC patients. In addition to A1762T G1764A, C1653T and T1753V mutations were also associated with HCC development ($P < 0.001$).

Possible risk factors associated with HCC development. Data obtained from 47 patients with HCC and 132 with CH were used as input in the multivariate statistical analyses to find the possible significant risk factors for HCC development. An age of ≥50 years (odds ratio at a 95% confidence interval [CI], 14.25 [range, 3.16 to 64.16]; $P = 0.001$) and the presence of the HBV mutations A1762T G1764A (95% CI, 5.50 [range, 1.58 to 19.18]; $P = 0.008$), C1653T (95% CI, 20.62 [range, 2.33 to 182.36]; $P = 0.007$), and T1753V (95% CI, 5.63 [range, 1.45 to 21.84]; $P = 0.013$) were significantly associated with the development of HCC (Table 3).

DISCUSSION

HBV genotypes B and C are the most prevalent strains in southeast Asia. Several studies from this region have shown a higher rate of early spontaneous HBeAg seroconversion, less-active liver disease, and a lower likelihood of developing HCC with genotype B than with genotype C (4, 8, 23). Contradictory findings reported by Sumi et al. suggested that, despite an earlier HBeAg seroconversion and slower development of HCC among genotype B-infected patients, the lifelong risks for HCC development may not differ significantly among patients with genotypes B and C (28). In the present study, a cohort of 211 patients with chronic HBV infection was analyzed. Fifty-one percent of patients (108/211) were infected with HBV genotype B and 49% (103/211) with genotype C. However, their distributions in the different diagnosis patient groups (CH, LC, and HCC) showed a significant difference, suggesting that Chinese patients with genotype C have a higher risk of developing HCC, which is in agreement with previous reports (4, 34). Kao et al. observed that HBV B infection was more closely associated with the development of HCC in young Taiwanese patients than was HBV C infection (11). In this study, however, HCC patients with HBV Ba infections were not as young as those with HBV C infections.

The impact of HBV C subgenotypes on the natural course of chronic HBV infection and the severity of liver damage remains unknown. In agreement with our previous reports (35, 36), no significant differences in clinical features were found among patients infected with HBV subgenotypes Ba, C1, and C2 in the present study. A previous report has indicated that HBV C strains with TCC at nt positions 1856 to 1858, corresponding to the precore region, appear to have higher prevalences of liver cirrhosis than those with CCC and have a higher prevalence of HBeAg positivity and higher ALT levels than those with CCT (7). In our study, approximately 40% of patients with HBV C1 infections had TCC at nt 1856 to 1858. Conclusions about the influence of HBV genotypes on clinical outcomes might require lifelong observation.

Remarkably, differences among HBV subgenotypes Ba, C1, and C2 were observed in virological characteristics, including mutations at positions 1653, 1753, 1762 and 1764, 1856, 1858, 1896, and 1898. HBV C2 was strongly associated with C1653T and HBV C1 with T1753V. The 1858T mutation was found exclusively among HBV Ba and HBV C2 patients, while approximately 87% of HBV C1 patients have 1858C. The 1896A mutation was frequently found in HBV Ba; as an alternative mechanism for HBeAg seroconversion, the A1762T G1764A mutations were more common in HBV C1. This result supports the hypothesis that the A1762T G1764A double mutation is preferentially selected in patients infected with HBV genotypes that preclude the development of an 1896A mutation (5). Interestingly, HBV C2 usually has 1858T (7), but the rate of occurrence of the 1896A mutation is slightly lower than in HBV Ba and significantly higher than in HBV C1 ($P = 0.001$). In contrast, the rate of occurrence of the double A1762T G1764A mutation in HBV C2 is significantly lower than in HBV C1 ($P = 0.013$) and higher than in HBV Ba ($P = 0.008$).

Notably, the A1762T G1764A mutation rate remarkably increased in patients with advanced liver disease, although the

clinical characteristics of patients harboring HBV with and without the A1762T G1764A mutations were not significantly different among CH, LC, and HCC patients (data not shown). An in vitro study showed the A1762T G1764A mutations may increase HBV virulence by upregulating viral replication through removing a nuclear receptor binding site in the core promoter and creating a hepatocyte nuclear factor 1 (HNF1) binding site (13). It has recently been suggested that the A1762T G1764A double mutation, rather than genotype C, is the genuine risk factor in hepatocarcinogenesis (17, 35), but no difference was found in the prevalences of A1762T G1764A mutations between HCC and control patients with genotype C (35). In our patients, a high prevalence of A1762T G1764A mutations (approximately 85%) in HCC patients was also observed, but this increased proportion of A1762T G1764A mutations in HCC patients might be explained by the remarkable increase in the proportion of patients infected with genotype C, which has a stronger association with A1762T G1764A than does genotype B. Moreover, the accumulation of A1762T G1764A mutations might be associated with the aging of patients and the duration of the infection, and HCC represents an older group among chronically HBV-infected patients.

Multivariate analysis showed that older age and C1653T, T1753V, and A1762T G1764A mutations significantly increased the risk of HCC development. The C1653T and T1753V mutations are nonsynonymous and cause changes in the amino acids of the X gene and may also affect the activity of the enhancer II/core promoter. Our recent studies also confirmed that C1653T and T1753V mutations may increase the risk of HCC (10, 31). But it is notable that the ratios of HBV C1 occurrence to HBV C2 occurrence in CH, LC, and HCC patients showed no significant difference ($P = 0.874$), though HBV C1 has a markedly higher tendency to develop A1762T G1764A mutations than HBV C2 in this population. As for hepatocarcinogenesis, additional factors, such as host immune response, integration of HBV DNA into the hepatocyte chromosomes, HBV DNA levels, and transmitted pattern of HBV, should be considered.

In conclusion, we investigated the relationship between HBV subgenotypes and the severity of liver disease. Genotype C was more frequently present in HCC patients than in other patients, though no significant differences in clinical features were found between subgenotypes Ba, C1, and C2. HBV C1 had the highest prevalence of A1762T G1764A mutations, while the 1896A mutation was more common in HBV Ba. In addition to older age, the C1653T, T1753V, and A1762T G1764A mutations in the enhancer II/core promoter region are significantly associated with HCC. Further studies are required to investigate the mechanisms of action of the C1653T, T1753V, and A1762T G1764A mutations on hepatocarcinogenesis.

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