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Original Article

High prevalence of the B2+C2 subgenotype mixture in patients with chronic hepatitis B in Eastern China

Jun ZHONG, Yue-qiu GAO*, Xue-hua SUN, Xiao-jun ZHU, Man LI

Shanghai University of Traditional Chinese Medicine Affiliated Shanghai Shuguang Hospital, Department of Infectious Diseases, Key Laboratory of Liver and Kidney Diseases, Shanghai University of Traditional Chinese Medicine, Ministry of Education E-institute of Shanghai Municipal Education Commission, Shanghai 200021, China

Aim: To investigate the prevalence of hepatitis B virus (HBV) genotype mixtures among patients with chronic hepatitis B (CHB) in Eastern China.
Methods: A total of 4908 chronic HBV patients from Eastern China were enrolled. HBV genotypes and subgenotypes were determined using a multiplex PCR technique. Serum viral loads and hepatitis B e antigen (HBeAg) levels detected using real-time fluorescent quantitative PCR and ELISA assay, respectively. The presence of precore/basic core promoter (PC/BCP) mutations was examined with PCR and direct sequencing of the amplified products.
Results: HBV genotypes B, C, D, B+C, and B+D were found in 19.21%, 64.75%, 1.49%, 13.63%, and 0.92% of the patients, respectively. In 669 patients with the genotype mixture B+C, the subgenotypes B2+C2 and B2+C1 accounted for 68.13% and 31.87%, respectively, no other subgenotypes were identified. HBV B+C was more frequent in the patients with moderate CHB than in patients with mild CHB. In patients with moderate CHB, the subgenotype mixture B2+C2 was lower than B2+C1 (51.97% *vs* 63.38%), while the opposite situation was found in patients with severe CHB (22.15% *vs* 15.49%). The highest average viral load was found in patients with the genotype B+C mixture. The prevalence of HBV B2+C2 increased in patients from 50 to 59 years of age and was significantly different from the proportion of patients in the same age group with genotype B (23.2% *vs* 15.2%). A double mutation (G1896A) in the PC was significantly more common in subgenotype B2+C2 than in subgenotype B2+C1.
Conclusion: The HBV B2+C2 subgenotype was prevalent in CH patients with a high HBV replication status and correlated with a more severe course of the disease.

Keywords: liver disease; chronic hepatitis; hepatitis B virus; genotype mixture; subgenotype; mutation; Eastern China

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Introduction

Hepatitis B virus (HBV) genotypes have distinct geographical distributions and are associated with different clinical outcomes, prognoses, and responses to interferon treatment[1]. Ten HBV genotypes (A–J) and 34 HBV subgenotypes have been identified to date[2]. In China, subgenotypes B2, C2 and C1 are the most common strains[3–5].

Infection with more than one HBV genotype often results in a genotype mixture, *ie*, co-infection or super-infection with multiple HBV genotypes in an infected patient[2]. With the use of multiplex polymerase chain reaction (PCR), genotype mixtures, including A+C, B+C, C+D, A+B+C, C+E, B+C+D, A+D, and A+B, have frequently been identified in HBV-infected subjects[6–8]. The B+C genotype mixture is common in China[8], and A+D is common in Pakistan[9, 10] and Romania[11]. Subgenotype B2 (formerly Ba) is prevalent throughout Asia, including China, whereas the prevalence of subgenotype B1 (Bj) is restricted to Japan. C1 (Cs) was described in Southern Asia, whereas C2 (Ce) was prevalent in the Far East. In China, the prevalence of genotype mixtures varies in different geographic regions. For example, genotype mixtures account for 10.6% of hepatitis cases in Shanghai[2], 22.7% in Jiangsu province[12], 9.6% in Hubei province[13], 1.6% in Yunnan province[14] and 0.78% in Heilongjiang province[15].

The impact of HBV B+C on the natural course of chronic hepatitis B (CHB) infection and the severity of liver damage is still unknown. Yin *et al* reported that genotype mixtures are associated with higher viral load and a more severe course of the disease than HBV genotype C alone[8]. However, the impact of HBV genotype mixture-related factors, such as

* To whom correspondence should be addressed.
E-mail gaoyueqiu@hotmail.com
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serum viral load and HBeAg expression, on the course of infection and the development of liver disease has not yet been fully characterized.

The aims of the present study were to identify the distribution of HBV genotype mixture in Eastern China and to explore the relationship between genotype mixture and gender, age, clinical spectrum of chronic HBV infection, and viral replicative activity.

Materials and methods

Enrollment of subjects

Clinical diagnosis was based on liver function tests, hepatitis virus markers, autoantibodies, tumor markers, ultrasonography, and liver histopathology. The patients met the following criteria: HbsAg-positive for at least 6 months to establish chronic HBV infection and free from other concomitant causes of liver disease (*eg*, hepatitis C or D, HIV infection, alcohol consumption >60 g/d) or relatively rare liver diseases (*eg*, autoimmune hepatitis and metabolic liver disease). None of the patients used drugs or had hepatotoxin exposure. Serum samples were collected from all inpatients and outpatients with chronic hepatitis B and stored at -80 °C until analysis.

Serum samples

A total of 4908 serum samples were obtained from chronic HBV patients who visited the Shanghai Shuguang Hospital from June 2007 to July 2009. All subjects signed an informed consent form and participated in the study voluntarily. All patients were chronic HBV carriers (seropositive for the HBV surface antigen for at least 6 months) and were seronegative for hepatitis C and hepatitis D. All samples were stored at -80 °C until analysis.

Quantitative detection of HBV DNA

The serum HBV DNA loads in patients with chronic HBV infection were measured with real-time fluorescent quantitative PCR using a LightCycler PCR system (FQD-33A, BIOER, Hangzhou, China) with a lower limit of detection of approximately 1000 viral genome copies/mL. The reverse transcription products were denatured at 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 40 s. The handling procedures were performed strictly as described by the instructions in the reagent kit (Shenzhen PG Biotech Co, Ltd, China).

Detection of HBeAg

HBeAg was measured at a virological laboratory using an enzyme-linked immunosorbent assay (ELISA) kit as recommended by the manufacturer (Sino-American Biotech Co, Ltd, Shanghai, China). HBV DNA was extracted from 100 μL of serum using the QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and suspended in 50 μL distilled water.

HBV genotyping

HBV genotype and subgenotype were determined using a multiplex PCR technique that had been previously developed in our laboratory[16]. The specificity of the multiplex PCR was improved by raising the annealing temperature from 56 °C to 63 °C and adding betaine (Sigma, St Louis, MO, USA) to a final concentration of 1 mol/L.

Mutations within the basal core promoter and precore regions

PCR and direct sequencing of the amplified products were performed as described previously using primers that flanked the basal core promoter and precore regions[17].

Statistical analysis

The data were expressed as the mean±standard deviation (SD) for normally distributed variables. Categorical data were compared by a two-tailed Chi-square test with Yates's correction or Fisher's exact test when the expected number in the cell was below 5. Continuous data were compared by a two-tailed Student's *t*-test or one-way ANOVA, as appropriate. We used R software version 2.5.1 and the SPSS 12.0 statistical package (Statistical Software, Chicago, IL, USA). All reported *P* values were two-tailed, and *P* values <0.05 were considered statistically significant.

Results

Distribution of HBV genotypes in CH patients

A total of 4908 DNA-positive CHB patients were investigated, including 143 hepatocellular carcinoma (HCC) patients, 1697 patients with mild CHB, 1627 patients with moderate CHB and 1441 patients with severe CHB. HBV genotypes B, C, D, B+C, and B+D were found in 19.21%, 64.75%, 1.49%, 13.63%, and 0.92% of these patients, respectively (Table 1). Genotype mixture B+C was more common in the patients with moderate and severe CHB than was genotype B, indicating that HBV coinfection with two HBV genotypes promotes a more severe course of the disease. Genotype C was the most prevalent, followed by B and B+C. Among 669 B+C patients, subgenotype mixture B2+C2 (68.13%, 456) was more common than B2+C1 (31.87%, 213). No other subgenotype mixtures were identified (Table 2).

Clinical and virological differences of the HBeAg-positive and HBeAg-negative patients infected with mixed B+C genotype

The clinical and virological differences of 669 samples infected with mixed B+C were tested, including 341 HBeAg-positive and 328 HBeAg-negative samples. The mean age, DNA load, HBeAg-positive, and frequencies of the G1896A, 1762T/1764A, 1896A/1858T mutations were significantly different ($P<0.05$) between the HBeAg-positive and HBeAg-negative groups.

The mean age of the patients in the HBeAg-negative group was significantly higher than in HBeAg-positive group ($P<0.001$). The male-to-female ratio was lower in the HBeAg-negative group than in the HBeAg-positive group, but this difference was not significant ($P=0.492$). Patients with mixed B+C in the HBeAg-negative group showed a higher tendency than those in the HBeAg-positive group to develop G1896A, 1762T/1764A, 1896A/1858T mutations (Table 3).

Table 1. Distribution of HBV genotypes and genotype mixtures.

	B+C	B	C	D	B+D
n=4908	13.63% (669)	19.21% (943)	64.75% (3178)	1.49% (73)	0.92% (45)
Age	38.06±14.42	42.83±14.20	35.26±11.93	31.86±12.73	37.66±17.12
Male/female	1.98	2.71	2.51	2.01	1.77
Viral load	5.57±1.89	4.80±1.90	4.16±1.49	4.21±1.88	5.01±1.96
HBeAg(+) rate	50.97% (341)	40.7% (384)	18.2% (578)	22.4% (16)	31.7% (14)
CH	665	932	3050	73	45
CHB-mild	23.77% (159)	77.09% (727)	10.56% (336)	84.00% (61)	59.00% (27)
CHB-moderate	55.61% (372)	20.78% (196)	65.26% (2074)	16.00% (12)	38.00% (17)
CHB-severe	20.03% (134)	0.95% (9)	20.15% (641)	0.00% (0)	2.00% (1)
LC/HCC	0.60% (4)	1.17% (11)	4.03% (128)	0.00% (0)	0.00% (0)

Table 2. Clinical relevance of HBV subgenotype mixture B+C.

Subgenotype	B2+C2	B2+C1	X^2	*P*
n=669	68.13% (456)	31.87% (213)		
Age	38.11±13.99	37.90±13.95		
Male/female	1.98 (303/153)	2.44 (151/62)	1.31	0.250
Viral load	5.44±1.98	5.62±1.91		
HBeAg(+) rate	48.23% (220/236)	56.90% (121/92)	4.25	0.039
G1896A	12.58% (57/399)	6.45% (14/199)	5.38	0.02
1762T/1764A	17.74% (81/375)	14.78% (31/182)	1.07	0.30
1896A/1858T	5.45% (25/431)	2.34% (5/208)	3.33	0.068
CHB-mild	25.00% (114)	21.13% (45)	1.20	0.273
CHB-moderate	51.97% (237)	63.38% (135)	7.65	0.006
CHB-severe	22.15% (101)	15.49% (33)	4.02	0.045
LC/HCC	0.88% (4)	0.00% (0)		

Table 3. Clinical and virological differences of the HBeAg-positive and HBeAg-negative patients infected with mixed B/C genotype.

B+C	HBeAg(+)	HBeAg(−)	X^2	*P*
n=669	50.97% (341)	49.03% (328)		
Age	34.03±11.02	46.02±13.37		
Viral load (LEG/mLb)	6.49±1.51	4.54±1.58		
Male/female	2.04 (229/112)	1.83 (212/116)	0.47	0.492
G1896A	7.92% (27/314)	13.41% (44/284)	5.32	0.492
1762T/1764A	13.20% (45/296)	20.43% (67/261)	6.27	0.012
1896A/1858T	3.52% (12/329)	5.49% (18/310)	1.51	0.219

Clinical features of CH patients with subgenotype mixture B2+C2
HBV B+C was less frequent in HCC patients (0.60%, 4), while all HCC patients with mixed genotypes were infected with HBV B2+C2 (Table 2). HBV B2+C2 was more common in patients with moderate CHB than in patients with mild CHB (51.97% *vs* 25.00%, *P*=0.000). After adjustment for age, HBV B2+C2 was more frequent in patients with severe CHB than those with mild CHB in the <30 years old and 50–59 years old groups (*P*=0.048, 0.008, respectively).

Most HBV B2+C2 patients were males, and the male-to-female ratio of HBV B2+C2 decreased with age, although there was no statistically significant difference in the distribution of genotypes between genders (Figure 1).

Patients with genotype C were younger on average than those with genotype B (Table 1). The average age of HBV B2+C2 patients was close to the average age of all patients. The prevalence of HBV B2+C2 increased in patients from 50–59 years of age and was significantly different from the proportion of patients in that age group with genotype B (23.2% *vs* 15.2%, *P*=0.024) (Figure 1). HBV B2+C2 was associated with

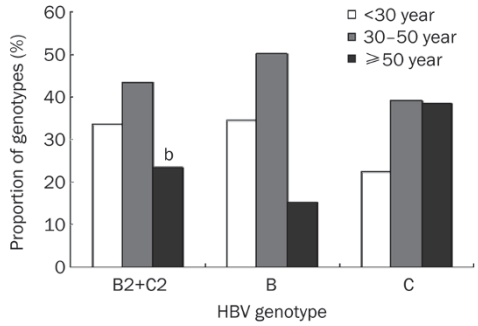

Figure 1. Age-related proportion of genotypes in CH patients infected with HBV B, C, and subgenotype mixture B2+C2. The proportion of patients with HBV B2+C2 went up at 50–59 years stage, significantly different from those with genotype B (23.2% *vs* 15.2%, P=0.024). ^bP<0.05.

a more severe course of the disease than for genotype B alone (6.15% *vs* 3.70%, P=0.016) in young CH patients (<30 years) and for genotype C alone (22.15% *vs* 17.29%, P=0.041) in older CH patients (50–59 years).

The average viral load was highest in patients with the genotype mixture B+C (5.57±1.89 log10 copies/mL). The prevalence of B+C varied with the degree of HBV replication, reaching 20.03%, 23.77%, and 55.61% in the severe, mild and moderate replication level groups, respectively. The HBeAg-positive rate was highest in the B+C group and was significantly different from the rates in the genotype B and C groups (P=0.000).

Viral load decreased with age in the group of all HBV patients, while the viral load of HBV B2+C2 patients increased at 50–59 years of age (Figure 2). The prevalence of the G1896A mutation in the HBV B2+C2 group (12.58%) was higher than that of the HBV B2+C1 group (6.45%) (P=0.02). The

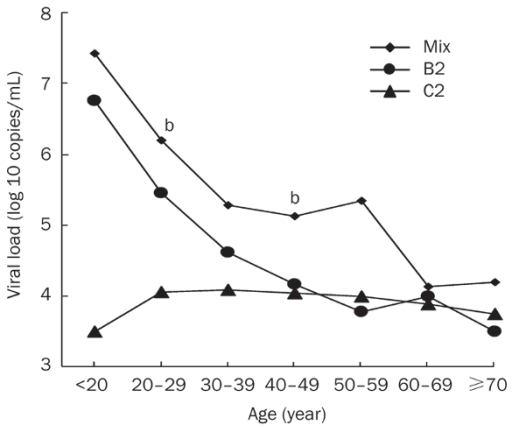

Figure 2. Age-related changes of serum viral load in CH patients infected with HBV B, C, and genotype mixture B2+C2 (mix). Average viral loads in CH patients with HBV B2+C2 were higher than those with B and C in all stages of age. Serum viral load in CH patients decreased with age. However, viral load in patients with HBV B2+C2 went up in 50–59 years group, significantly different from those with B and C (*vs* B: 5.34 *vs* 3.78; P=0.0000; *vs* C: 5.34 *vs* 4.00; P=0.0000, respectively). ^bP<0.05.

1762T/1764A mutations in HBV B2+C2 were also frequently found in older patients (50–59 years), but there was no significant difference from the prevalence in the HBV B2+C1 group. The distributions of subgenotype mixture B2+C2 and B2+C1 were significantly different in patients with moderate and severe CHB (P=0.006 and 0.045, respectively) (Table 2).

Discussion

There have been many studies addressing whether HBV genotypes mixtures are associated with specific clinical features, but most previous studies have examined relatively small cohorts[12–14, 18, 19]. This study revealed that the HBV genotypes B, C, D, B+C, and B+D existed in Eastern China. Our study also revealed that subgenotype mixture B2+C2 was prevalent in mixed-genotype patients from Eastern China, where both the HBV C2 and B2 subgenotypes are prevalent[2], consistent with previous reports. These results indicate that similar to single-genotype infections, subgenotype mixtures are associated with the patient's place of birth.

Little is known about the molecular characteristics of HBV genotype mixtures from CHB patients or their association with disease progression. In the present study, HBV B2+C2 was more common than B2+C1 in patients with severe CHB. After adjustment for age, HBV B2+C2 was more frequent in patients with severe CHB than those with mild CHB in the <30 years old and 50–59 years old groups. Genotype B was prevalent in young CH patients and genotype C was prevalent in older CH patients. HBV B2+C2 was associated with a more severe course of the disease than for genotype B alone in young CH patients (<30 years) and for genotype C alone in older CH patients (50–59 years). These results indicated that co-infection or superinfection with multiple genotypes was associated with a poorer prognosis than was single-genotype infection.

The most significant features of HBV B2+C2 were the higher viral load and HBeAg expression in young CH patients (<30 years). Replicative HBV infection stimulates the host immune response that leads to chronic hepatocyte destruction and regeneration with the development of fibrosis and eventually cirrhosis[20]. As an immunoregulatory protein, HBeAg could promote HBV chronicity[21]. HBV genotypes also play a role in viral replication. HBV genotype C exhibits less replication activity in young patients than does genotype B[5]. Our results also confirmed that the average DNA load of patients with genotype B was higher than that in patients with genotype C. These results indicated that HBV B2+C2 in young CH patients (<30 years) was associated with severe liver damage, most likely because of high viral load and the presence of subgenotype B2.

It was notable that the viral load of patients with B2+C2 increased in patients at 50–59 years of age, while viral load and HBeAg expression decreased with age overall. In this study, the prevalence of the G1896A mutation in the HBV B2+C2 group was higher than that of the HBV B2+C1 group. The G1896A mutation was frequently found in subgenotype HBV B2, where it serves as an alternative mechanism for HBeAg seroconversion[22]. Tillmann *et al* have observed

the G1896A mutation among patients with more active liver disease[23], while most investigators did not confirm the correlation between the G1896A mutation and higher viral load[21, 24, 25]. The G1896A mutation most likely develops with time, as it appears to be more common among older subjects. Further experiments will be required to determine whether the G1896A mutation increases the replicative competency of HBV B2+C2.

The 1762T/1764A mutations in HBV B2+C2 were also frequently found in the older patients (50–59 years), but there was no significant difference between patients with mild and severe CHB. The accumulation of 1762T/1764A mutations might be associated with the aging of patients. An *in vitro* study showed that the 1762T/1764A mutations might increase HBV replication activity[25]. Above all, we assumed that mutations in the precore/core promoter region might be partially responsible for the advanced liver disease and elevated viral load in older patients with HBV B2+C2.

In conclusion, HBV B2+C2 was prevalent in CH patients from Eastern China with high HBV replication. Subgenotype HBV B2+C2 was significantly correlated with a more severe course of the disease.

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Author contribution

Jun ZHONG performed the majority of experiments; Yue-qiu GAO, Xue-hua SUN, Xiao-jun ZHU, and Man LI provided vital reagents and analytical tools and were also involved in editing the manuscript; Yue-qiu GAO coordinated the study and collected of all the human material in addition to providing financial support for this work; and Jun ZHONG designed the study and wrote the manuscript.

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