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ORIGINAL ARTICLE VIROLOGY

Hepatitis B virus subgenotype C2 is the most prevalent subgenotype in northeast China

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Abstract

The geographical distribution of hepatitis B virus (HBV) subgenotypes and their clinical implications in patients with acute and chronic hepatitis B in the Heilung-kiang province of northeast China were investigated. Nested PCR and multiplex PCR were performed with genotype-specific primers and with subgenotype-specific primers to identify genotypes and subgenotypes from serum samples of 412 HBV infections including 69 with acute self-limited hepatitis (ASH) and 343 with chronic hepatitis (CH). A total of 361 samples were genotyped and 304 were further subgenotyped. The most common HBV genotype was C (93.63%, 338/361), with subgenotype group C2 (83.73%, 283/338) predominating. Genotype B was also found and subgenotype B2 predominated within this genotype. Out of 69 infected patients with ASH, 48 were identified as genotype C and all belonged to subgenotype C2. Of 343 infected patients with CH, 313 were genotyped and 256 were subgenotyped; amongst these, C2 (91.80%, 235/256), B2 (7.42%, 19/256) and mixed subgenotypes B2 and C2 (0.78%, 2/256) were found. In HBV subgenotype C2 infections, ASH had a higher ratio of women than CH patients. These results show that HBV subgenotypes C2 and B2 were found in Heilung-kiang province of northeast China. In ASH and CH groups, the distributions of subgenotypes were coincident with C2, the predominant subgenotype. Analysis of the association between subgenotype and the outcomes of HBV infection was inconclusive in our study.

Keywords: Acute hepatitis B, chronic hepatitis B, genotype, hepatitis B virus, multiplex PCR, nested PCR, subgenotype
Original Submission: 14 October 2008; **Revised Submission:** 5 January 2009; **Accepted:** 21 January 2009
Editor: E. Gould
Article published online: 18 May 2009
Clin Microbiol Infect 2010; **16:** 477–481
10.1111/j.1469-0691.2009.02834.x

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Introduction

Hepatitis B virus (HBV) infection is associated with a wide variety of clinical outcomes, including acute hepatitis (AH), asymptomatic carrier status, and chronic hepatitis (CH). Many adult patients with acute hepatitis B have naturally self-limiting infections [1]. Some chronically infected individuals develop cirrhosis or hepatocellular carcinoma. There are currently an estimated 400 million chronic HBV infections worldwide [2]. HBV infection in China is currently prevalent at 10–20% [3].

HBV is classified into at least eight genotypes (A–H), based on nucleotide sequence divergence among strains of >8% [4–7]. HBV genotypes can be further divided into subgenotypes based on a >4% diversity in the complete nucleotide sequence. These subgenotypes may vary in geographical distribution, viral characteristics, and relationship to clinical outcomes [8,9]. In Asia, HBV genotype B and C are highly prevalent. Genotype B includes seven geographically segregated subgenotypes [10,11]. Subgenotype B1 circulates in Japan, B2 in Asia, B3 in Indonesia, B4 and B5 in Vietnam, and recently B6 and B7 were found in Arctic indigenous populations and in the Nusa Tenggara islands in eastern Indonesia. The six subgenotypes of genotype C are also geographically segregated [12–15]. The subgenotype C1 circulates in Southeast Asia and Southern China, C2 in East Asia, C3 in Oceania, C4 appears predominantly in Australian Aboriginals, C5 circulates in Vietnam and the Philippines and recently C6 has been found in Indonesia. In China, the largest country in Asia, the most common HBV genotypes are B and C. The distributions of HBV genotypes differ greatly in different regions in China, with a North–South divide in the prevalence of genotype C and B, respectively. In the northern regions of China genotype C is predominant and genotype B is more frequently found in southern China. In some regions of northern China, subgenotype C2 is predominant, whereas subgenotype C1 is more prevalent than C2 in southern China [16–18].

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There are no current studies describing subgenotype distributions in northeast China and very few studies describe the clinical outcomes of acute HBV infection based on genotype or subgenotype worldwide. This study investigated HBV subgenotype distribution and clinical implications in acute and chronic HBV patients in the Heilung-kiang province of northeast China.

Materials and Methods

Patients

A total of 412 samples from patients infected with HBV (309 male and 103 female, mean age 38.8 ± 11.7 years) were collected from the following five hospitals of Heilung-kiang Province: Harbin Infectious Diseases Hospital (n = 32); The Chinese People's Liberation Army 211th hospital (n = 1) and the First, Second and Fourth Affiliated Hospitals, Harbin Medical University (n = 2, n = 376 and n = 1, respectively) from November 2006 to April 2008. All patients in our study demonstrated hepatitis B surface antigen (HBsAg), HBV DNA ($\geq 1.0 \times 10^2$ copies/mL) and the absence of antibodies against hepatitis A, hepatitis C, hepatitis D, and human immunodeficiency viruses (HIV). Patients were subdivided into the two groups: acute self-limited hepatitis (ASH) and CH. The demographics and detailed descriptions of the serology can be seen in Table 1. Individuals in the ASH group demonstrated high-titre IgM against the HBV core antigen, and had all recovered from their liver disease, had lost HBsAg, and most had scored positive for antibodies to HBsAg during ≥24 weeks after their initial presentation.

Individuals in the CH group, who were hospitalized or were being seen in the clinical service at the Infectious Disease Department of the Second Affiliated Hospital of Harbin Medical University, had shown persistent HBsAg for >6 months. The study protocol conformed to the 1975 Declaration of Helsinki regulations. The procedures were approved by our ethics committee and informed, written consent was obtained from each participant.

TABLE 1. The demographic and serological description of HBV infections with ASH and CH

Characteristics	ASH (n = 69)	CH (n = 343)
Age (mean ± SD, years)	38.5 ± 12.3	38.8 ± 11.6
Sex (male:female)	2:1 (46:23)	3.2:1 (263:80)
ALT level (mean U^{-1} ± SD)	1528.8 ± 1219.6	209.6 ± 382.7
AST level (mean U^{-1} ± SD)	923.4 ± 696.2	127.0 ± 237.5
HBV DNA (mean ± SD, copies/mL)	$1.2 \times 10^6 \pm 5.7 \times 10^6$	$4.7 \times 10^7 \pm 1.3 \times 10^8$
Positive for HBeAg	23 (33.3%)	265 (77.3%)
Positive for HBeAb	40 (58.0%)	36 (10.5%)

Positive scores for HBeAg and HBeAb are given as the number of patients (%). HBV, hepatitis B virus; ASH, acute self-limited hepatitis; CH, chronic hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBeAb, antibody to hepatitis B e antigen.

Laboratory assays

HBV serology and antibodies against hepatitis A, hepatitis C, hepatitis D and HIV were determined by ELISA using commercial test kits (Kehua Co., Shanghai, China). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured in a clinical laboratory (Modular P800; Roche, Basel, Switzerland) with commercial test kits (Olympus Diagnostica GmbH; Lismeehan, O'Callaghans's Mills, County Clare, Ireland). Serum HBV DNA was quantified by real-time fluorimetry PCR (GmbH LightCycler1.5; Roche Diagnostics, Mannheim, Germany) using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology, Shenzhen, China).

HBV genotyping and subgenotyping

Serum HBV DNA was extracted with a commercial kit (TIANamp, Beijing, China) according to the manufacturer's instructions, suspended in 30 μL distilled water and 2 μL was used as template for HBV DNA amplification. First, nested PCR (Bioer XP Cycler; Bioer, Hangzhou, China) was performed using HBV A–F genotype-specific primer pairs [19]. For the samples that were successfully genotyped, the HBV subgenotypes were further determined by multiplex PCR with HBV subgenotype-specific primers (for subgenotype C1, C2, B1 and B2) as described by Chen *et al.* [20]. *Taq* DNA polymerase and the primers were purchased from MBI Fermentas (Glen Burnie, MD, USA) and Genecore Biological Technology Limited Company (Shanghai, China), respectively. The thermocycler was changed to an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 94°C for 60 s, 48°C for 60 s, and 68°C for 60 s. The final elongation step was 72°C for 8 min. Amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and analysed by the Gel image system (GIS2010; Tanon, Shanghai, China).

Statistical analysis

The χ^2 and t-tests were used for statistical analysis, and statistical significance was assigned at p values <0.05.

Results

Distribution of HBV subgenotypes in Heilung-kiang province

A total of 87.6% (361/412) samples were successfully genotyped and 84.2% (304/361) of those samples were

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subgenotyped. The distributions of HBV genotypes were as follows: C, 93.6% (338/361); B, 5.8% (21/361) and mixed genotypes B and C, 0.6% (2/361). No other genotypes (A, or D–F) were found in our study. The distributions of HBV subgenotypes were as follows: C2, 93.1% (283/304); B2, 6.3% (19/304) and mixed subgenotypes B2 and C2, 0.7% (2/304). Subgenotypes C1 and B1 were not found in our study. PCR reactions in which no amplification product was obtained accounted for 12.4% (51/412) of the genotype and 15.8% (57/361) of the subgenotype determinations. The results suggest that subgenotype C2 is predominant in Heilung-kiang province.

Distribution of HBV subgenotypes in ASH and CH groups

All 412 patients were subdivided into the two groups, ASH (n = 69) and CH (n = 343). In the ASH group, 69.6% (48/69) of samples were successfully genotyped; C accounted for 100% (48/48) and all belonged to subgenotype C2. In the CH group, 91.3% (313/343) of samples were successfully genotyped, and the genotypes found at the following frequencies: C, 92.7% (290/313); B, 6.7% (21/313) and mixed genotypes B and C, 0.64% (2/313). A total of 81.79% (256/313) of the samples were successfully subgenotyped, in which the frequencies of the different subgenotypes were C2, 91.8% (235/256); B2, 7.4% (19/256); and mixed subgenotypes B2 and C2, 0.8% (2/256). The distribution of subgenotype C2 in ASH and CH groups was not significantly different statistically (χ^2 = 2.7, p 0.10). These results suggest that the distributions of HBV subgenotypes were coincident in ASH and CH patients and show that the subgenotype C2 was predominant in the two groups.

Comparison between ASH and CH patients infected with subgenotype C2

The demographic and clinical data on subgenotype C2 infections in ASH and CH groups are shown in Table 2. When compared with CH patients, ASH patients had a higher ratio of women (ASH male:female, 31:17 vs. CH, 185:50, p 0.02), higher HBeAb (antibody to hepatitis B e antigen) positive rate (35.4% vs. 9.8%, p <0.001), higher serum ALT and AST levels (1524.7 ± 848.1 ul vs. 182.5 ± 304.1 ul, p <0.001; 990.8 ± 675.5 ul vs. 107.6 ± 146.2 ul, p <0.001, respectively), lower HBeAg (hepatitis B e antigen) positive rates (31.2% vs. 77.9%, p <0.001) and lower serum HBV DNA levels ($1.7 \times 10^6 \pm 6.9 \times 10^6$ vs. $4.4 \times 10^7 \pm 1.3 \times 10^8$, p <0.001).

Comparison between subgenotype C2 and B2 infections in the CH group

The demographic and clinical data from subgenotype C2 and B2 infections are shown in Table 3. No significant differences were observed between subgenotype B2 and C2 infections when age, HBeAg positive rate, HBeAb positive rate, serum HBV DNA, ALT or AST levels were compared (p >0.05). Mixed subgenotypes B2 and C2 were identified in two CH infections and these data were

TABLE 2. Comparison between ASH and CH patients infected with subgenotype C2

Characteristics	ASH (n = 48)	CH (n = 235)	p-value
Age (mean ± SD, years)	39.7 ± 12.2	38.2 ± 10.6	0.37
Sex (male:female)	1.8:1 (31:17)	3.7:1 (185:50)	0.02
ALT level (mean U^{-1} ± SD)	1524.7 ± 848.1	182.5 ± 304.1	0.00
AST level (mean U^{-1} ± SD)	990.8 ± 675.5	107.6 ± 146.2	0.00
HBV DNA (mean ± SD, copies/mL)	$1.7 \times 10^6 \pm 6.9 \times 10^6$	$4.4 \times 10^7 \pm 1.3 \times 10^8$	0.00
Positive for HBeAg	15 (31.2%)	183 (77.9%)	0.00
Positive for HBeAb	17 (35.4%)	23 (9.8%)	0.00

Chi-square test was used when comparing sex ratios HBeAg and HBeAb positive rates; *t*-tests were used for age, ALT, AST and HBV DNA levels.
ASH, acute self-limited hepatitis; CH, chronic hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBeAb, antibody to hepatitis B e antigen.

TABLE 3. Comparison between subgenotype C2 and B2 infections in the CH group

Characteristics	CH (n = 235)	B2 (n = 19)	p-value
Age (mean ± SD, years)	38.2 ± 10.6	36.4 ± 16.5	0.69
Sex (male:female)	3.7:1 (185:50)	2.2:1 13:6	0.45
ALT level (mean U^{-1} ± SD)	182.5 ± 304.1	673.7 ± 1145.2	0.30
AST level (mean U^{-1} ± SD)	107.6 ± 146.2	494.9 ± 884.3	0.29
HBV DNA (mean ± SD, copies/mL)	$4.4 \times 10^7 \pm 1.3 \times 10^8$	$1.1 \times 10^8 \pm 2.7 \times 10^8$	0.36
Positive for HBeAg	183 (77.9%)	14 (73.7%)	0.96
Positive for HBeAb	23 (9.8%)	3 (15.8%)	0.66

CH, chronic hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBeAb, antibody to hepatitis B e antigen.

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excluded from further analysis because of insufficient numbers of patients.

Discussion

In China, the distributions of HBV genotypes in the CH group have been investigated in many regions [16,18,21,22]. These results suggest that genotype C predominates in northern China, genotype B predominates in southern China, genotype D is prevalent in Xinjiang, genotype A is rare, and genotypes E, F, G and H are not found. Up to now, the distribution of HBV genotypes in ASH groups has not been reported in China. We analysed a cohort of 69 patients with ASH, and found that genotype C was the most prevalent; the same was true of the distribution of genotypes in CH group in this region. For the distribution of subgenotypes, Wang *et al.* [17,23] and Li *et al.* [18] reported that in CH patients infected with genotype C, the majority were of subgenotype C2 in northern and southeastern China. By contrast, subgenotype C1 was more prevalent in southern China.

Within genotype B, subgenotype B2 was more prevalent than B1. No other subgenotypes were detected in these studies. Heilung-kiang province is located further north than Changchun, and the distribution of HBV subgenotypes in that province has not been reported before. We investigated the distribution of subgenotypes in 412 HBV infections including 69 with ASH and 343 with CH, subgenotypes C2, B2 and mixed subgenotypes C2 and B2 were found and C2 was the most common in both ASH (100%) and CH (91.8%) groups.

Many studies from Asia have reported that genotype C takes a more aggressive disease course than genotype B [23–25]. Genotype B was more prevalent in patients with ASH [26,27], suggesting that genotype B was associated with a lower rate of CH infections. Another study, from Japan, indicated that the less common subgenotypes (A2, C1, B2) were detected more frequently in acute HBV infections [28], and that persistence of HBV after acute infection was rare and occurred more often in patients infected with subgenotype A than others [29].

In our study, the distribution of genotype B/B2 was obviously lower than C/C2 in the CH group, but in the ASH group, only genotype C/C2 was detected; the distribution of (sub) genotypes in ASH and CH groups was consistent. For this reason, the investigation into the association between (sub) genotype and the outcomes of HBV infection was inconclusive in our study. As to why the same subgenotype C2 is associated with two completely different clinical outcomes in our study, we have considered several possible factors. First, there may be some differences present in the full HBV genomic sequences in subgenotype C2, not detected by the subgenotyping protocol; sequence differences have been described in a previous report [30] suggesting that basal core promoter and precore mutations are associated with progression to fulminant hepatitis in acute hepatitis B patients infected with subgenotype C2. Second, perhaps host factors should be evaluated together with subgenotype when assessing the outcomes of HBV infection. For example, the Human Leukocyte Antigen, HLA- DRB1*13 allele [31,32] has been reported to be associated with ASH or spontaneous viral clearance after HBV infection, whereas the DRB1*12 allele [33,34] might be correlated with viral persistence. Third, the higher female ratio in the ASH group in our study may also contribute to the different outcomes of HBV subgenotype C2 infections.

Our results also highlight the different clinical features between ASH and CH patients infected with subgenotype C2. The different disease process of HBV infections during enrolment may have contributed to these results. Most CH patients included in this study were rarely hospitalized with light clinical symptoms. Conversely, most ASH patients were hospitalized with intense clinical symptoms and achieved peak serum ALT and AST levels.

In conclusion, we describe the distribution and clinical implications of HBV subgenotypes in ASH and CH patients in the Heilung-kiang province of northeast China. Our results reveal that subgenotypes C2 and B2 were found in Heilung-kiang province of northeast China. In ASH and CH groups, the distributions of subgenotypes were coincident and C2 was predominant. The association between subgenotypes and the outcomes of HBV infection was inconclusive in our study.

Acknowledgements

The authors thank Dr Z. Z. Jin, Dr J. Q. Zhang and Dr P. Shi from the Harbin Infectious Diseases Hospital, Heilung-kiang, China, for their help in collecting samples for this study.

Transparency Declaration

This work was supported by a grant for the China Postdoctoral Science Foundation funded project (No. 20070420891), the Natural Science Foundation of the Heilung-kiang Province (No. D2007-18), the Doctor Research Fund of the Hospital (No. BS2007-07) and the Harbin Medical University Graduate Innovation Fund of China (No. HCXS2008012).

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