Clinical and Virological Differences between Hepatitis B Virus Genotypes B and C: A Case-Control Study

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Objective : *The pathogenic significance of hepatitis B virus (HBV) genotypes is undefined. The aim of this study was to elucidate the differences in clinical and virological features between HBV genotypes B and C by conducting a case-control study in Thai patients who were chronically infected with the virus.*
Patients and Method : *HBV genotyping was assessed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method in stored sera of 470 patients with chronic hepatitis B. Among these, 65 patients with HBV genotype B were enrolled and matched individually to those with HBV genotype C according to sex, age, and distribution of liver disease which included asymptomatic carrier, chronic hepatitis, cirrhosis and hepatocellular carcinoma.*
Results : *Serum alanine aminotransferase (ALT) was significantly higher in patients with genotype C than those with genotype B. Hepatitis B e antigen (HBeAg) was significantly more frequent in genotype C than genotype B patients (50.8 and 30.8%, respectively, p=0.03), but the levels of HBV DNA were comparable between them. Among patients who were positive for HBeAg, the mean age of genotype C patients tended to be older than genotype B patients.*
Conclusion : *The present study demonstrated that patients with HBV genotype C had a significantly higher rate of HBeAg, experienced delayed HBeAg seroconversion and exhibited more severe liver disease compared to those with genotype B.*

Keywords*: Hepatitis B, Genotype, HBeAg*

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Hepatitis B virus (HBV) infection is a major public health problem, with more than 350 million HBV carriers estimated worldwide [1]. Chronic infection with the virus is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier status, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[2]. The liver damage in chronic HBV infection is thought to be due to the host immune response rather than a cytopathic effect of the virus itself [3]. Accordingly, the virus and host factors that influence the immune response play essential roles in the pathogenesis of HBV-associated liver injury and its clinical outcome[4]. Hence, identification of such factors may have important clinical implications in the management of patients with chronic HBV infection.

HBV, a member of hepadnaviridae, is a circular double-stranded DNA virus. On the basis of a comparison of complete genomic sequences, HBV has currently been classified into 8 genotypes, designated A to H [5]. HBV genotypes appear to show a geographic pattern in their distribution. Genotype A is found in North America, northern Europe and some parts of Africa. Genotypes B and C are common in Southeast Asia, whereas genotype D is found across the world. Genotype E is restricted to Africa; genotype F is found in Central and South America, and genotype G has been detected in France and North America. Recently, genotype H has been reported from Central America[6]. Besides the differences in geographical distribution, there is growing evidence indicating that the viral genotypes may influence the clinical outcomes of patients with chronic HBV infection. Several studies in Asia have suggested that HBV genotype C is associated with a lower rate of hepatitis B e antigen (HBeAg) seroconversion and more severe liver disease compared with genotype B [7-10]. However, most of the current available data have been conducted from case

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series, in which confounding effects from selection bias could not be excluded. Accordingly, case-control studies on the association between HBV genotypes and their clinical implications are still required. Therefore, the aim of this study was to elucidate the differences in clinical and virological features between genotypes B and C by conducting a case-control study in Thai patients with chronic HBV infection.

Patients and Method

Patients

Serum samples were obtained from 470 patients with chronic HBV infection who had undergone long-term follow-up at Chulalongkorn Memorial Hospital (Bangkok, Thailand), and the National Blood Center, Thai Red Cross, between August 1997 and August 2003. All patients were positive for Hepatitis B s antigen (HBsAg), as deter-mined using a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL). Of these, patients who were positive for hepatitis C virus antibody (anti-HCV) and those who had another potential cause of chronic liver disease were excluded. The patients were clinically classified into 4 groups including asymptomatic carrier, chronic hepatitis, cirrhosis and HCC. Asymptomatic carrier was diagnosed by persistent normal serum alanine aminotransferase (ALT) level for at least 1 year. Chronic hepatitis was diagnosed by the presence of prolonged elevation of serum ALT level, and confirmed by histological examinations. Cirrhosis was diagnosed based on histological examinations and/or imaging studies. HCC was established by histopathology and/or a combination of mass lesions in the liver on hepatic imaging and serum alpha-fetoprotein (AFP) levels above 400 ng/ml.

Serum samples were collected from each patient at the time of their initial clinical evaluation and stored at -70^{0}C until further tests were performed. All patients had been informed as to its purpose of exterminating the etiologies of liver disease and had given their written consent. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

To compare the clinical and virological differences between patients with genotypes B and C, a case-control was conducted by selecting patients infected with each genotype, who were matched for sex and age ($\pm$ 5 years), as well as the distribution of asymptomatic carrier, chronic hepatitis, cirrhosis and HCC.

HBV DNA Extractions

DNA was extracted from 100 mL serum with proteinase-K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 30 mL sterile water and directly subjected to PCR-based amplification.

HBV DNA Detection

HBV DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ), using the primer sequences described previously [11]. The forward primer was P1 (nt. 2823-2845: 5'-TCA CCATATTCTTGGGAACAAGA); the reverse primer was P2 (nt. 80-61: 5'-TTCCTGAACTGGAGCCACCA). The primers were located in conserved genomic regions of the preS1 gene to ensure a high sensitivity for the amplification of all HBV genotypes.

Two microlitres of DNA sample were combined with a reaction mixture containing 20 mL of 2.5X Eppendorf MasterMix (Hamburg, Germany), 1mM P1, 1 mM P2 and sterile water, in a final volume of 50 mL. PCR was performed under the following conditions: After an initial 2 min denaturation step at 94 C, 35 cycles of amplification were performed, each including 30 sec denaturation at 94 C, 30 sec annealing at 55 C and 30 sec extension at 72 C, followed by a final 10 min extension at 72 C. Each amplified DNA sample (10mL) was added to loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME) at 100 Volt for 60 min. The 479-bp product stained with ethidium bromide on preparation was visualized on a UV transilluminator.

PCR-RFLP analysis for genotyping

PCR products were subjected to RFLP analysis, using restriction endonuclease *Ava*II and *Dpn*II (New England Biolabs, Beverly, MA) to determine the HBV genotype. Briefly, 10 mL of PCR product were mixed with 1.5 mL of 10X buffer, 3 mL of sterile water and 0.5 mL (5U) of *Ava*II and *Dpn*II, respectively, in separate reactions and incubated at 37 C for 3.5 hours. After incubation, the samples were run on a composite gel containing 2% NuSieve agarose (FMC BioProducts, Rockland, ME) and 1% standard agarose. The sizes of the RFLP products, visible under UV light as a result of prior ethidium bromide staining, served to identify the various HBV genotypes based on the polymorphism patterns [11].

Serological and Virological Assays

Hepatitis B e antigen (HBeAg) was determined

using commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL). Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Data were presented as percentage, mean and standard deviation.The Fisher's exact test and unpaired *t* test were used to assess the statistical significance of the difference between groups as appropriate. A *p*-value < 0.05 for a two-tailed test was considered statistically significant.

Results

Of the 470 patients enrolled in the present study, HBV DNA was detected in 332 patients (70.6%). The most common HBV genotypes in the present study were genotype C and B, which were detected in 243 (73.2%) and 69 (20.8%) patients, respectively. The remaining 20 cases included 11 (3.3%) with genotype A and 9 (2.7%) with unclassified genotype. Among these, 65 patients with genotype B were enrolled and matched individually to those with HBV genotype C with respect to sex (male: female ratio; 10:3 in both groups), age (43.7±15.8 and 43.6±16.1 years, respectively), and the severity of liver disease.

Table 1 compares clinical and virological features between patients with genotypes B and C. There were no significant differences in total bilirubin (TB) and albumin. However, mean serum alanine aminotransferase (ALT) was significantly higher in patients with genotype C than those with genotype B (152.4±108.0 and 102.5±73.6 U/l, respectively, $p = 0.02$). HBeAg was also significantly more frequent in patients with genotype C than those with genotype B (50.8 and 30.8%, respectively, $p = 0.03$), although HBV DNA levels were comparable between them.

Table 2 compares clinical and virological features between patients with genotypes B and C, who were sub-classified by the HBeAg status (Table 2a, HBeAg positive; Table 2b, HBeAg negative). Among HBeAg positive patients, the mean age of patients with genotype C tended to be older than those with genotype B, although the difference was not statistically significant (39.7±14.2 and 33.4±17.7 years, respectively, $p = 0.07$). Among patients who were negative for HBeAg, mean serum ALT level was significantly higher in patients with genotype C than

Table 1. Clinical and virological features of patients with HBV genotypes B and C

Features	Genotype B (n = 65)	Genotype C (n = 65)	Differences
Sex (male: female)	50:15	50:15	Matched
Age (mean ± SD)	43.7±15.8	43.6±16.1	Matched
Liver disease			
Asymptomatic carrier	16 (24.6%)	16 (24.6%)	Matched
Chronic hepatitis	20 (30.8%)	20 (30.8%)	Matched
Cirrhosis	10 (15.4%)	10 (15.4%)	Matched
Hepatocellular carcinoma	19 (29.2%)	19 (29.2%)	Matched
Total bilirubin (mg/dl)	1.8±1.8	3.0±5.3	NS
Alanine transaminase (U/L)	102.5±73.6	152.4±108.0	0.02
Albumin (g/dl)	3.7±0.7	3.6±0.5	NS
HBeAg	20 (30.8%)	33 (50.8%)	0.03
HBV DNA (log.copies/ml)	6.42±1.76	6.78±1.52	NS

Table 2. Comparison between patients with HBV genotypes B and C based on HBeAg status

a) HBeAg positive

Features	Genotype B (n=20)	Genotype C (n=33)	Differences
Sex (male)	17 (85%)	25 (75.8%)	NS
Age (mean ± SD)	33.4±17.7	39.7±14.2	NS
Liver disease			NS
Asymptomatic carrier	7 (35%)	8 (24.2%)	
Chronic hepatitis	8 (40%)	14 (42.5%)	
Cirrhosis	1 (5%)	7 (21.2%)	
Hepatocellular carcinoma	4 (20%)	4 (12.1%)	
Alanine transaminase (U/L)	107.1±79.1	135.1±74.7	NS
HBV DNA (log.copies/ml)	7.12±1.25	6.97±2.47	NS

b) HBeAg negative

Features	Genotype B (n=45)	Genotype C (n=32)	Differences
Sex (male)	33 (73.3%)	25 (78.1%)	NS
Age (mean ± SD)	48.7±16.5	45.6±14.6	NS
Liver disease			NS
Asymptomatic carrier	9 (20%)	8 (25%)	
Chronic hepatitis	12 (26.7%)	6 (18.7%)	
Cirrhosis	9 (20%)	3 (9.4%)	
Hepatocellular carcinoma	15 (33.3%)	15 (46.9%)	
Alanine transaminase (U/L)	99.5±87.6	168.0±135.7	0.01
HBV DNA (log.copies/ml)	6.14±1.35	6.61±2.12	NS

those with genotype B (168.0±135.7 and 99.5±87.6 U/l, respectively, $p = 0.01$). Besides these two aspects, there were no differences in sex distribution, severity of liver disease, and HBV DNA levels between patients infected with genotypes B and C, regardless of HBeAg positive or negative in serum.

Discussion

Several circumstances, including host and HBV-related factors, have been recognized as important determinations of the highly variable outcome of chronic HBV infection. There are now increasing data suggesting that HBV genotypes may play an important role in causing different disease profiles in chronic HBV infection. Most of the current data demonstrate that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B [7-10, 12, 13]. Genotype C is also associated with a lower rate of HBeAg seroconversion and a lower response rate to alpha interferon therapy compared to genotype B [14, 15]. Despite this information, case-control studies regarding patients with HBV genotypes B and C infection are rather limited thus far. For the reason that the HBeAg status depends largely upon sex and age of the patients[16], case-control studies would be necessary for investigation of the clinical relevance of HBV genotypes in terms of the HBeAg status.

In the present study, 65 patients infected with HBV genotypes B and C were compared clinically and virologically. Although they were matched for sex and age, as well as the stage of chronic liver disease, the duration of infection among them could not be specified. Given that the majority of Thai patients acquire HBV infection vertically from their mothers at birth or horizontally during early childhood from carrier family members, their age would probably serve as a reasonable surrogate for the duration of HBV infection.

In accordance with previous reports, the present data confirmed that patients with genotype C had a significantly higher prevalence of HBeAg at presentation than those with genotype B. These results indicate that genotype C, compared to genotype B, is associated with a lower rate of HBeAg seroconversion. Moreover, among HBeAg positive patients, the mean age of patients infected with genotype C tended to be older than those infected with genotype B, suggesting a delayed HBeAg seroconversion of HBV genotype C. Although HBeAg is not essential for HBV replication and not necessary linked to low replication or viremia, early HBeAg seroconversion in the immune clearance phase usually indicates a favorable outcome in the natural course of chronic HBV infection, because it is frequently associated with the cessation of virus replication and non-progressive liver disease[17]. In contrast, late HBeAg seroconversion after multiple episodes of reactivation and remission may accelerate the progression of chronic hepatitis and, thus, have a relatively poor clinical outcome[18]. In this respect, studies conducted in Hong Kong and Taiwan demonstrated that spontaneous HBeAg seroconversion in patients with genotype B occurred approximately 1 decade earlier compared to patients with genotype C [19, 20]. Hence, one explanation for the more severe liver disease associated with HBV genotype C, as indicated with higher elevation of ALT level in the present study, may be related to a delayed HBeAg seroconversion and longer duration of viral replication.

The mechanisms responsible for the difference in rate of HBeAg seroconversion between HBV genotypes B and C are not clear. One study showed that the mean serum HBV DNA levels were higher among patients with genotype C compared to those with genotype B [9], but this finding may be related to a higher prevalence of HBeAg among patients with genotype C. As shown in the present study, the mean HBV DNA levels were comparable between genotype B and C in either HBeAg positive or negative patients, in keeping with the suggestion that each genotype has a comparable replicative activity with the same HBeAg status. Another study reported that the precore stop codon variant ($G_{1896}A$), which abrogates HBeAg production was more commonly found in patients with genotype B than those with genotype C [7]. However, this finding was not confirmed in another study[9]. In addition, a recent report revealed that although patients with genotype B were more likely to exhibit the precore mutation, the presence of this variant was not an independent predictor of spontaneous HBeAg seroconversion[20], suggesting that factors other than selection of the precore stop codon mutation may be more important in HBeAg seroconversion.

In summary, the present case-control study demonstrated that HBV genotype C had a significantly higher rate of HBeAg and exhibited more severe liver disease compared to genotype B in Thai patients with chronic HBV infection. In addition, HBeAg seroconversion tended to occur later in patients infected with genotype C than patients with genotype B. Due to the limitation of the present study for being a cross-sectional observation, further analysis in large-scale longitudinal studies is warrant to clarify the influence of HBV genotypes on the clinical course of chronic liver disease.

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ความแตกต่างทางคลินิกและไวรัสวิทยาระหว่างสายพันธุ์บีและซีของไวรัสตับอักเสบบี

พิสิฐ ตั้งกิจวานิชย์, วโรชา มหาชัย, ปิยะวัฒน์ โกมลมิศร์, จุฑาทิพย์ ฟองศรัณย์, อภิรดี เทียมบุญเลิศ, ยง ภู่วรวรรณ

วัตถุประสงค์ *: ความสำคัญทางคลินิกของสายพันธุ์ของไวรัสตับอักเสบบียังไม่ทราบแน่ชัด การศึกษาแบบ case-control นี้มีวัตถุประสงค์เพื่อเปรียบเทียบความแตกต่างทางคลินิกและไวรัสวิทยาของสายพันธุ์บี และซีของไวรัสตับอักเสบบีในผู้ป่วยไทยที่มีการติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง*

วิธีการ *: ตรวจสายพันธุ์ของไวรัสตับอักเสบบีในเลือดของผู้ป่วยจำนวน 470 รายโดยวิธี polymerase chain reaction (PCR) และ restriction fragment length polymorphism (RFLP) หลังจากนั้นจับคู่ผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์บีและซี เพศเดียวกันที่มีอายุใกล้เคียงกันและมีระยะของโรคซึ่งได้แก่ระยะการเป็นพาหะ เป็นตับอักเสบเรื้อรัง ตับแข็ง และมะเร็งตับเหมือนกันจำนวนกลุ่มละ 65 คน*

ผลการศึกษา *: ผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์ซีมีระดับเฉลี่ยของค่า alanine aminotransferase (ALT) สูงกว่าผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์บี รวมทั้งยังตรวจพบ Hepatitis B e antigen (HBeAg) ในเลือดบ่อยกว่าผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์บีอย่างมีนัยสำคัญ (ร้อยละ 50.8 และ 30.8 ตามลำดับ p = 0.03) แต่ระดับของ HBV DNA ไม่แตกต่างกัน นอกจากนี้พบว่าผู้ป่วยไวรัสสายพันธุ์ซีมีอายุเฉลี่ยสูงกว่าผู้ป่วยไวรัสสายพันธุ์บีในกลุ่มที่มี HBeAg*

สรุป *: ผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์ซีมีการตรวจพบ HBeAg บ่อยกว่า มี HBeAg seroconversion ที่อายุเฉลี่ยสูงกว่า และมีความรุนแรงของโรคมากกว่าผู้ป่วยที่ติดเชื้อไวรัสสายพันธุ์บี*