สัญญาเลขที่ RSA4580024

รายงานวิจัยฉบับสมบูรณ์

โครงการ ความชุกและความสำคัญทางคลินิกของสายพันธุ์และการกลายพันธุ์ของไวรัสตับอักเสบบี

Prevalence and Clinical Significance of Hepatitis B Viral Genotypes and Mutations

โดย

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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กิตติกรรมประกาศ

โครงการวิจัยเรื่อง "ความชุกและความสำคัญทางคลินิกของสายพันธุ์และการกลายพันธุ์ของไวรัสตับอักเสบบี" ได้รับทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย ตามสัญญาเลขที่ RSA4580024 ระยะเวลาดำเนินการ 3 ปี ตั้งแต่ 15 สิงหาคม 2545 ถึงวันที่ 14 สิงหาคม 2548 ผู้รับทุนขอกราบขอบพระคุณสำนักงานฯ ที่ให้การสนับสนุนอย่างเต็มที่จนทำให้โครงการวิจัยสำเร็จลุล่วงด้วยดี ขอขอบคุณบุคคลต่อไปนี้ที่มีส่วนสำคัญยิ่งในความสำเร็จของโครงการได้แก่ ศาสตราจารย์นายแพทย์ยง ภู่วรวรรณ ศาสตราจารย์ ปิยะรัตน์ โตสุโขวงศ์ ศาสตราจารย์ ดร. จิระพันธ์ กรึงไกร รองศาสตราจารย์แพทย์หญิงวโรชา มหาชัย อาจารย์นายแพทย์ปิยะวัฒน์ โกมลมิศร์ (คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) และผู้ช่วยวิจัยที่มีส่วนช่วยเหลือให้โครงการวิจัยสำเร็จลงได้แก่ นางสาวอภิรดี เทียมบุญเลิศ, นางสาวพจนาถ จันทรรัศมี และนางสาววีราภา จันทรสุพิศ รวมทั้งหน่วยวิจัยไวรัสตับอักเสบและภาควิชาชีวเคมี คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในฐานะสถาบันต้นสังกัดที่ให้การสนับสนุนโดยอำนวยความสะดวกในโครงการอย่างดียิ่ง

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รหัสโครงการ: RSA4580024

ชื่อโครงการ: ความชุกและความสำคัญทางคลินิกของสายพันธุ์และการกลายพันธุ์ของไวรัสตับอักเสบบี

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โครงการวิจัย:

ไวรัสตับอักเสบบีเป็นเชื้อไวรัสที่เป็นสาเหตุสำคัญของเกิดโรคตับอักเสบชนิดเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับ ปัจจุบันมีประชากรทั่วโลกมากกว่า 400 ล้านคนหรือเท่ากับประมาณร้อยละ 5 ของประชากรโลกที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง ความแตกต่างของสายพันธุ์และการกลายพันธุ์ของยีนในบางตำแหน่งของไวรัสอาจมีความสำคัญต่อการดำเนินโรคที่แตกต่างกัน จุดมุ่งหมายของโครงการนี้เพื่อศึกษาความชุกและความสำคัญทางคลินิกของสายพันธุ์และการกลายพันธุ์บริเวณพรีคอร์ คอร์และเอกซ์ยีนของไวรัสตับอักเสบบีในตัวอย่างเลือดของกลุ่มประชากรไทยที่มีติดเชื้อไวรัสแบบเรื้อรังโดยเฉพาะผู้ป่วยมะเร็งตับ ผลการศึกษาอณูชีววิทยาของไวรัสตับอักเสบบีพบว่าเชื้อไวรัสสายพันธุ์ที่พบมากที่สุดในประเทศไทยคือสายพันธุ์ซีและบีตามลำดับ โดยไวรัสสายพันธุ์ซีทำให้เกิดตับอักเสบที่รุนแรงกว่าไวรัสสายพันธุ์บี นอกจากนี้การกลายพันธุ์บางอย่างที่บริเวณคอร์และเอกซ์ยีนของเชื้อไวรัสอาจมีส่วนทำให้โรคตับมีความรุนแรงมากขึ้นและอาจเกี่ยวข้องกับกลไกการเกิดมะเร็งตับ ผลการวิจัยจากโครงการนี้น่าที่จะเป็นประโยชน์ในทางระบาดวิทยาและทางคลินิก เนื่องจากข้อมูลการศึกษาในประเทศไทยยังมีค่อนข้างน้อย นอกจากนี้ความชุกและความสัมพันธ์ทางคลินิกของสายพันธุ์และการกลายพันธุ์ของไวรัสตับอักเสบบีอาจมีความแตกต่างกันตามภูมิภาคต่างๆของโลก

ผลงานที่ศึกษาในช่วงเวลา 2 ปีที่ได้รับทุนสนับสนุน มีผลงานได้รับการตีพิมพ์ในวารสารนานาชาติจำนวน 4 ผลงานและกำลังเรียบเรียงเพื่อส่งตีพิมพ์อีก 1 ผลงาน

คำหลัก: ไวรัสตับอักเสบบี มะเร็งตับ สายพันธุ์ไวรัส การกลายพันธุ์

Abstract

Project Code: RSA4580024
Project Title: Prevalence and Clinical Significance of Hepatitis B Viral Genotypes and Mutations
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Project Period: 15 August 2002-14 August 2005

Project Description:

Hepatitis B virus (HBV) infection is a major public health problem, with more than 400 million HBV carriers estimated worldwide. Chronic HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). There have been increasing lines of evidence to indicate influences of HBV genotypes and mutations on the outcome of liver disease, particularly the development of HCC. The project is aimed to study the prevalence and clinical significance of genotypes and mutations in Precore/core and X regions of HBV in Thai patients. Our study demonstrated that genotype C and B were the predominant strains, accounting for approximately 75 and 20% of patients, respectively. Patients with HBV genotype C, compared to those with genotype B, had a higher positive rate of HBeAg and exhibited earlier progression of cirrhosis and HCC. However, there was no difference in the risk of developing HCC and its prognosis between patients with genotypes B and C. Furthermore, certain X gene mutations and, particularly, CP mutations in young patients may contribute to the development of HCC As the genetic variability of HBV differs geographically and the data available in Thailand are still limited, our study will provide useful information regarding the epidemiology and clinical relevance of HBV genotypes and mutations in Thai populations.

Keywords: Hepatitis B virus, hepatocellular carcinoma, genotypes, mutations

Project Description

1. Introduction and Rationale

Hepatitis B virus (HBV) infection is a major public health problem, with more than 400 million HBV carriers estimated worldwide[1]. Chronic HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). In HBV endemic areas such as Southeast Asia and sub-Saharan Africa, more than 60% of HCC cases are attributable to chronic infection with the virus[2],. Although the association between chronic HBV infection and HCC is well established, the virological factors contributing to tumor development are not yet fully understood.

HBV, a member of the family *Hepadnaviridae*, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, core, polymerase and X genes (Figure1). Based on a comparison of complete genomic sequences, HBV has currently been classified into 8 genotypes, designated A to H [3]. HBV genotypes appear to show varying geographic patterns in their distribution. For instance, genotypes A and D are common in Europe and North America, whereas genotypes B and C prevail in Southeast Asia, China and Japan. Genotypes F and H are restricted to Central and South America. Genotype E is found predominantly in West Africa and genotype G is found in the USA and Europe.

Besides the differences in geographical distribution, there is growing evidence that the viral genotypes may influence the clinical outcomes of patients with chronic HBV infection. Among Asian patients who constitute approximately 75% of HBV carriers worldwide, it has been shown that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B[4-7]. Genotype C is also associated with a lower rate of hepatitis B e antigen (HBeAg) seroconversion and a lower response rate to alpha interferon therapy compared to genotype B[8, 9]. However, the association between HBV genotype and the risk of developing HCC is still controversial [4, 10-12]. In addition, the impact of HBV genotype on clinical features and prognosis of patients with HCC remains unclear.

HBV has a high mutation rate compared with other DNA viruses because it lacks of proofreading capacity during the replication via reverse transcription of its pregenomic RNA[13, 14]. The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A), which abolishes hepatitis B e antigen (HBeAg) production, and the dual mutation in the basal core promotor (CP) region (A1762T/G1764A), which down-regulates HBeAg production[14]. These mutations have been reported in up to 50-80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia[15]. While the role of the PC mutant to the course of chronic HBV infection is still controversial, the CP mutants have been linked to the severity of liver diseases, particularly HCC [16, 17].

The X-ORF encodes a 154 amino acid protein called hepatitis B virus X protein (HBx). HBx plays an important role in the regulation of viral genome expression, and has also been implicated in hepatocarcinogenesis[18]. HBx is a promiscuous *trans*-activator and has been shown to *trans*-activate many virus and host genes through a wide variety of *cis*-elements[19]. HBx can deregulate cell cycle checkpoint controls and stimulate DNA synthesis and cell proliferation[20]. It has been shown that the X gene is frequently integrated into the host genome and is expressed in most HCC cases [21]. However, its specific role and that of this mutant protein in the pathogenesis of liver cancer have yet to be elucidated.

2. Objectives

The project was aimed at studying the prevalence of genotypes and the frequency, location and nature of mutations in precore/core and X regions of HBV in Thai patients who are chronically infected with this virus. We also aimed to determine whether the HBV genotypes and certain types of the mutations might influence the severity of chronic liver disease and the development of HCC. As the genetic variability of HBV differs geographically and the data available in Thailand are still limited, our study will provide useful information regarding the epidemiology and clinical relevance of HBV genotypes and mutations in Thai populations.

3. Methodology

3.1 Subjects

Serum samples for HBV genotyping were obtained from 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 2004. All patients were positive for Hepatitis B s antigen (HBsAg). 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. Patients who had previously been treated with antiviral therapy 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. The degree of hepatic inflammation and fibrosis was graded according to modified Knodell histology index[22]. Cirrhosis was diagnosed based on histological examinations and/or imaging studies, and subsequently classified its severity based on Child's criteria. 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 IU/ml. The staging of HCC was classified according to CLIP criteria.

To investigate whether mutations within the PC/CP and X genes could be associated with the development of HCC, a case-control study was conducted by selecting 50 patients with HCC and 50 patients without HCC, who were matched for sex and age, as well as the distribution of hepatitis B e antigen (HBeAg) and HBV genotypes.

Serum samples were collected from each patient at the time of their clinical evaluation and stored at -70^{0}C until the above-mentioned tests were performed.

3.2 HBV DNA extractions

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

3.3 HBV DNA detection for genotyping

HBV DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ), using the primer sequences previously described[23]. The forward primer was P1 (nt. 2823-2845: 5'-TCACCATATTCTTGGGAACAAGA); the reverse primer was P2 (nt. 80-61: 5'-TTCCTGAACTGGAGCCACCA). The primers were

located in conserved genomic regions 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 μL of 2.5X Eppendorf MasterMix (Hamburg, Germany), 1μM P1, 1 μM P2 and sterile water, in a final volume of 50 μL. 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 (10μL) 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.

3.4 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 μL of PCR product were mixed with 1.5 μL of 10X buffer, 3 μL of sterile water and 0.5 μL (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 [23].

3.5 Amplification of the X/CP/PC regions

Partial gene covering 752 nt. (from nt. 1287 to 2038) of the X/CP/PC region was amplified by PCR using primer pair Xi1: 5'AGCTTGTTTTGCTCGCAGC3' (forward primer, nt. 1287-1305), and Ci 1: 5' TTCCGGAGACTCTAAGGCC 3' (reverse primer, nt. 2020-2038). The PCR reaction was performed as described above.

3.6 Nucleotide sequencing and phylogenetic analysis

For automated DNA sequencing, the bands of PCR amplified products were purified from the gel using Gel Extraction Kit (Perfectprep Gel Cleanup, eppendorf, Hamburg, Germany) according to the manufacturer's specifications. The sequencing reaction was performed using the Gene Amp PCR System 9600 (Perkin-Elmer, Boston, USA). The sequencing product was subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston, USA). Nucleotide sequences were multiple-aligned, analyzed using the CLUSTAL X program (version 1.8). Nucleotide consensus sequences of X/CP/PC regions of HBV genotypes B and C were taken from GenBank for multi-alignment and mutant analysis. Tree construction was analyzed by using TREEVIEW (version 1.5).

3.7 Serological assays

HBsAg, HBeAg and anti-HCV were determined by enzyme-linked immunosorbent assay (ELISA), using commercial available kits from Abbott (Abbott Laboratories, Chicago, IL).

3.8 Quantitative assay of viral load

Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection range of this assay was 2.7 to 8.7 log genome equivalents/ml (LGE/ml).

3.9 Statistical analysis

Data were presented as percentage, mean and standard deviation. Chi-square test, unpaired *t* test, and ANOVA analysis were used to assess the statistical significance of the difference between groups where appropriate. Survival curves were established using the Kaplan-Meier method and differences between curves were verified using the log-rank test. Cox regression analysis was performed to identify which independent variables would have a significant influence on the overall survival. *P* values below 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 10.0 software for Windows (SPSS, Inc., Chicago, IL).

4. Results

4.1 Distribution of HBV genotypes in patients with chronic HBV infection

Of the 470 patients enrolled in this study, HBV DNA was detected in 332 patients (70.6%). The most common HBV genotypes were genotypes C and B, which were found 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. The demographic and clinical data of 332 patients with different stages of chronic HBV infection are shown in Table 1. Mean age was significantly higher in patients with cirrhosis and HCC than in the other two groups (P=0.001), and positive HBeAg rate was significantly higher in patients with chronic hepatitis than in other groups (P=0.001). Although genotype C was the most common genotype in each group, no significant differences were observed with respect to the distribution of the genotypes in various stages of chronic HBV infection (P=0.16).

Table 1 Demographic and clinical data of 332 patients with chronic HBV infection

Diagnosis	*n*	Sex (m/f)	Age (yr)	ALT (U/L)	HBeAg positive	Genotype			
						A	B	C	U
Carrier	93	57/36	30.9±10.6	27.5±4.5	42/82 (51.2)	2 (2.2)	16 (17.2)	73 (78.4)	2 (2.2)
CH	103	81/19	36.2±10.1	157.4±103.8	61/92 (66.3)	5 (4.9)	20 (19.4)	76 (73.8)	2 (1.9)
Cirrhosis	60	47/13	48.8±13.8	135.0±90.9	26/53 (49.1)	2 (3.3)	10 (16.7)	44 (73.3)	4 (6.7)
HCC	76	60/16	51.4±12.9	107.8±107.4	15/71 (21.1)	2 (2.6)	23 (30.3)	50 (65.8)	1 (1.3)

CH, chronic hepatitis; HCC, hepatocellular carcinoma; U, unclassified genotype. Quantitative variables are expressed as mean±SD, categorical variables are expressed as *n* (%).

4.2 Clinicopathological differences between genotypes B and C in chronic hepatitis

Because the number of patients with genotype A was small, only genotypes B and C were included for further analysis of clinicopathological differences between genotypes. As shown in Table 2, patients with genotypes B and C were comparable with respect to sex, age and total bilirubin. The rate of positive HBeAg in patients with genotype B was significantly lower than that in patients with genotype C (44.4% *vs.* 71.6%, respectively, P=0.03). Mean ALT level was also significantly lower in patients with genotype B than those in patients with genotype C (119.8±58.5 IU/L and 159.8±106.4 IU/L, respectively; P=0.03), but HBV DNA levels were comparable between them (7.25±1.74 and 7.10±1.34 LGE/ml, respectively; P=0.78). Patients with

genotype B had a lower score of both necroinflammation activity and fibrosis than those with genotype C, but the differences were not statistically significant (5.8±2.1 and 1.5±1.0 *vs.* 6.7±2.1 and 1.8±0.9, *P*=0.51 and 0.47, respectively).

Table 2 **Demographic and clinical data of patients with chronic hepatitis**

Characteristics	Genotype B (*n* = 20)	Genotype C (*n* = 76)	*P*
Age (yr)	35.1±9.6	36.4±10.3	NS
Sex (male/female)	16/4	65/11	NS
Total bilirubin (mg/dL)	0.8±0.2	0.7±0.3	NS
ALT (IU/L)	119.8±58.5	159.8±106.4	0.03
HBeAg positive	8/18 (44.4)	53/74 (71.6)	0.03
HBV DNA (LGE/mL)	7.25±1.74	7.10±1.34	NS
HAI inflammation	5.8±2.1	6.7±2.1	NS
HAI fibrosis	1.5±1.0	1.8±0.9	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as *n* (%). HAI inflammation, sum of necroinflammatory scores of histology activity index. HAI fibrosis, sum of fibrosis scores of histology activity index.

4.3 Clinical differences between genotypes B and C in cirrhosis

There were no significant differences in gender, total bilirubin, serum albumin, and Child classification between groups of patients, as shown in Table 3. The mean age of patients with genotype B tended to be older than those with genotype C (54.7±9.2 *vs.* 47.4±14.4 years, respectively, *P*=0.06). The positive rate of HBeAg in patients with genotype B was significantly lower than that in patients with genotype C (11.1% *vs.* 56.8%, respectively, *P*=0.01). Patients with genotype B tended to have lower mean ALT and HBV DNA levels than those with genotype C, but the difference was not statistically significant (101.5±49.0 IU/L and 6.59±1.20 LGE/ml *vs.* 133.7±93.4 IU/L and 7.05±2.50 LGE/ml, *P*=0.14 and 0.12, respectively).

Table 3 **Demographic and clinical data of patients with cirrhosis**

Characteristics	Genotype B (*n* = 10)	Genotype C (*n* = 44)	*P*
Age (yr)	54.7±9.2	47.4±14.4	0.06
Sex (male/female)	8/2	35/9	NS
Total bilirubin (mg/dL)	1.4±0.8	2.2±1.2	NS
ALT (IU/L)	101.5±49.0	133.7±93.4	NS
Albumin (g/dL)	3.7±0.4	3.8±0.5	NS
HBeAg positive	1/9 (11.1)	25/44 (56.8)	0.01
HBV DNA (LGE/mL)	6.59±1.20	7.05±2.50	NS
Child classification (A/B/C)	7/2/1	24/15/5	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as *n* (%).

4.4 Clinical differences between genotypes B and C in HCC

The clinical data of 73 patients with HCC were compared according to HBV genotype, as shown in Table 4. Between the two groups, there were no significant differences in gender, total bilirubin, ALT, serum albumin, AFP and HBV DNA levels, tumor staging according to CLIP criteria. However, the mean age of patients with genotype B was significantly older than those with genotype C (61.1±9.8 *vs.* 51.3±13.1 years, respectively, P=0.001). Four of 22 patients (18.1%) with genotype B were positive for HBeAg, whereas 11 of 49 patients (22.4 %) with genotype C were positive for this marker, but the difference was not statistically significant (P=0.68). When patients with HCC were stratified by age (Figure 1), none of the patients with genotype B was younger than 40 years, whereas 9 patients (18 %) with genotype C were younger than 40 years (P=0.03). On the contrary, 13 patients (56.5%) with genotype B were older than 60 years, whereas 13 patients (26 %) with genotype C were older than 60 years (P=0.01).

Table 4 **Demographic and clinical data of patients with HCC**

Characteristics	Genotype B (n = 23)	Genotype C (n = 50)	P
Age (yr)	61.1±9.8	51.3±13.1	0.001
Sex (male/female)	20/3	38/12	NS
Total bilirubin (mg/dL)	2.1±2.0	2.9±4.9	NS
ALT (IU/L)	95.5±102.6	121.4±126.1	NS
Albumin (g/dL)	3.4±0.7	3.5±0.5	NS
HBeAg positive	4/22 (18.1)	11/49 (22.4)	NS
HBV DNA (LGE/mL)	6.71±1.62	6.52±2.63	NS
AFP (IU/mL)	42 485.2±95 590.9	42 032.6±89 382.8	NS
CLIP score (0-1/2-3/4-6)	5/7/11	8/25/17	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as n (%).

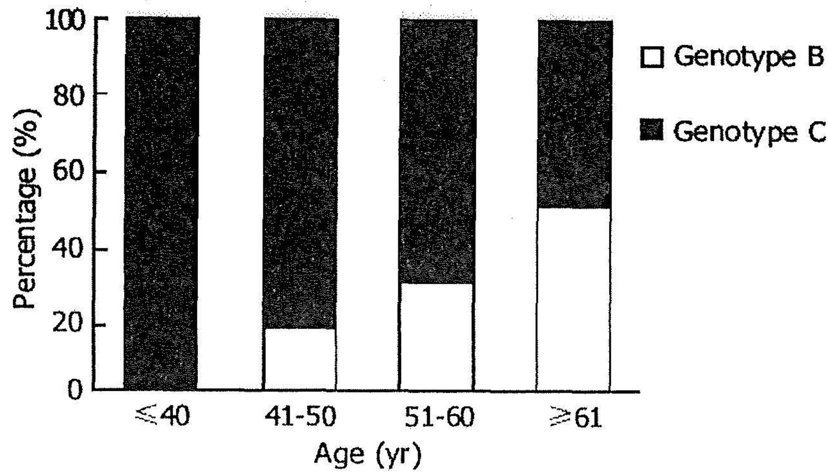

Figure 1 HBV genotypes B and C in 73 patients with HCC.

The Kaplan-Meier survival curves demonstrated that the overall median survival for patients with genotypes B and C were 5.5 and 7.3 months, respectively (P=0.81, using log-rank test) (Figure 2). For patients who were treated with any specific therapeutic modality, the median survival for the genotype B and C groups were 11.5 and 12.4 months, respectively (P=0.97). In the untreated cases, the median survival of the genotype B and C groups were 4.5 and 4.0 months, respectively (P=0.85).

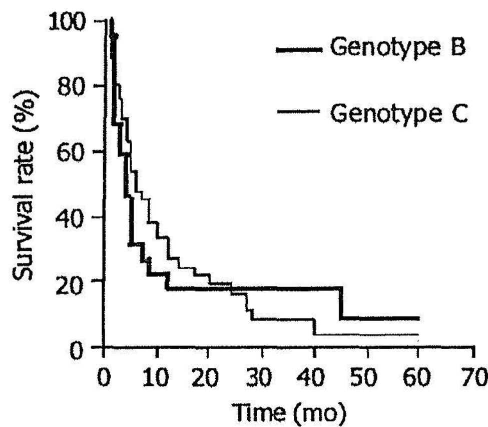

Figure 2 Overall survival of HCC patients with HBV genotypes B and C.

HBV genotype was entered into Cox regression analysis together with other variables that would influence prognosis. These included sex, age, HBeAg, HBV DNA level, CLIP stage and therapy for HCC. The multivariate analyses revealed that independent unfavorable factors of overall survival included CLIP stage and lack of therapy for HCC (Table 5). However, the HBV genotype was not selected as an independent predictor of survival.

Table 5 **Multivariate analysis of unfavorable factors of survival in patients with HCC, by using Cox regression analysis**

Factors	Risk ratio	95%CI	P
CLIP stage	4.63	1.70–12.62	0.003
No therapy	6.22	2.54–15.22	0.001

4.5 Sequence analyses for mutational patterns of the PC/CP and X gene

To investigate the mutations within PC/CP and X genes, the case-control study was conducted by selecting 50 patients with HCC and 50 patients without HCC, who were matched for sex and age, as well as the distribution of hepatitis B e antigen (HBeAg) and HBV genotypes, as shown in Table 6. The obtained sequences span the region from nt 1287 to 2038 which included the entire X-protein ORF (nt. 1374-1836), the Enh2 region (nt. 1685-1773), the basal core promoter (BCP) (nt. 1742-1849), direct repeat 1 (DR1) (nt. 1824-1834), direct repeat 2 (DR2) (nt.1590-1600), the precore ORF (nt. 1814-1901), and a part of the core region (nt. 1901-2038).

After alignment of these regions with recently published HBV-genotypes using BLAST search program and CLUSTAL X, we found that 14/50 (28%) and 12/50 (24%) of the obtained fragments from the HCC and control groups, respectively, corresponded to genotype B. The remaining 36/50 (72%) of the HCC group and 38/50 (76%) of the control group were clustered in genotype C (Figure 3, 4).

	Case	Control	*P Value*
Number of	50	50	
Sex M:F	44 : 6	44 : 6	
Age mean	54.20	50.98	0.152
Age medain	54.50	50.50	0.196
Genotype			
Genotype B	14 (28%)	12 (24%)	
Genotype C	36 (72%)	38 (76%)	0.820
HBeAg Status			
Negative	34 (68%)	32 (64%)	
Positive	16 (32%)	18 (36%)	0.833

Table 6. Demographic, clinical and virological characteristics of patients with HCC (case) and patients with non-HCC (control)

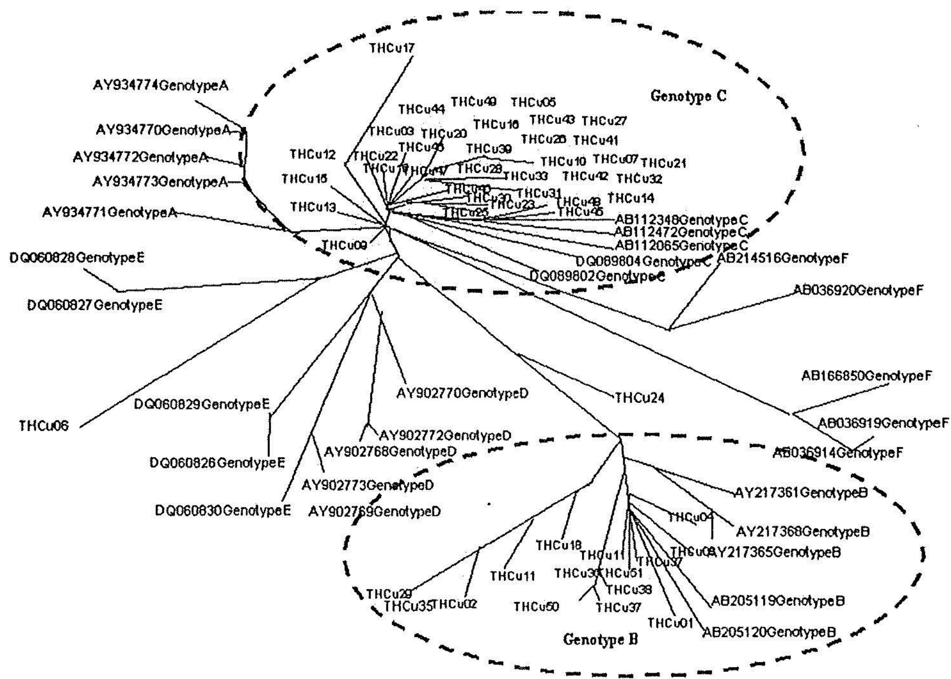

Figure 3. Phylogenetic tree of patients with HCC

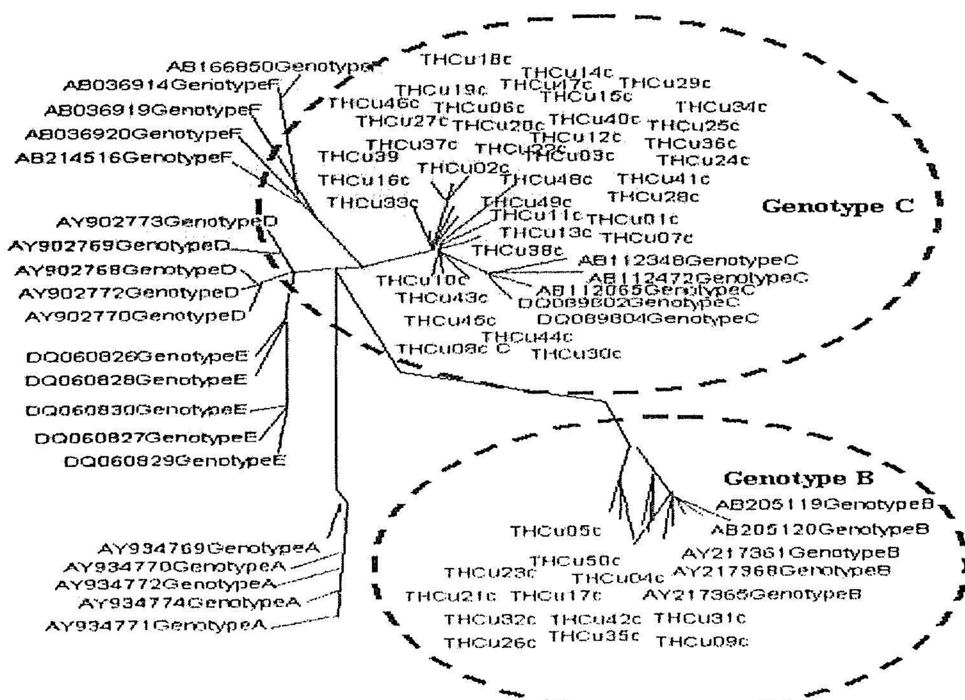

Figure 4. Phylogenetic tree of patients with non-HCC

4.6 Precore mutations in patients with HCC

In the precore region, the PC stop codon mutant (G1896A) was found more frequently in patients with HCC than non-HCC, but there was no significant difference (48% and 36%, respectively, *P*=0.31). There was also no significant difference in the percentage of patients with PC mutations between the HCC group and the control group, regarding to patients' mean age or HBV genotypes. However, the frequency of the mutation at nt. 1899 (G1899A) was found to be significantly higher in the HCC group than the control group (40% and 14%, respectively, *P*=0.007) (Table 7, and Figure 5-7).

Mutation	Case	Control	*P*	*OR.(95% CI)*
G1896A (n=50)	24 (48%)	18 (36%)	0.311	1.641 (0.737-6.355)
G1896A Age <= 50 yrs (Case n=23, Control n=25)	18 (34.8%)	6 (24%)	0.615	1.689(0.481-5.933)
G1896A Genotype B (Case n=14, Control n=12)	9 (64.3%)	10 (83.3%)	0.391	0.360(0.055-2.338)
G1896A Genotype C (Case n=36, Control n=38)	15 (41.7%)	8 (21.1%)	0.096	2.679(0.963-7.453)
T1858 C1858	47 (94%) 3 (6%)	50 (100%)	-	-
G1899A (n=50)	20 (40%)	7 (14%)	0.0007	4.095(1.539-10.90)

Table 7. Precore mutations in patients with HCC and non-HCC

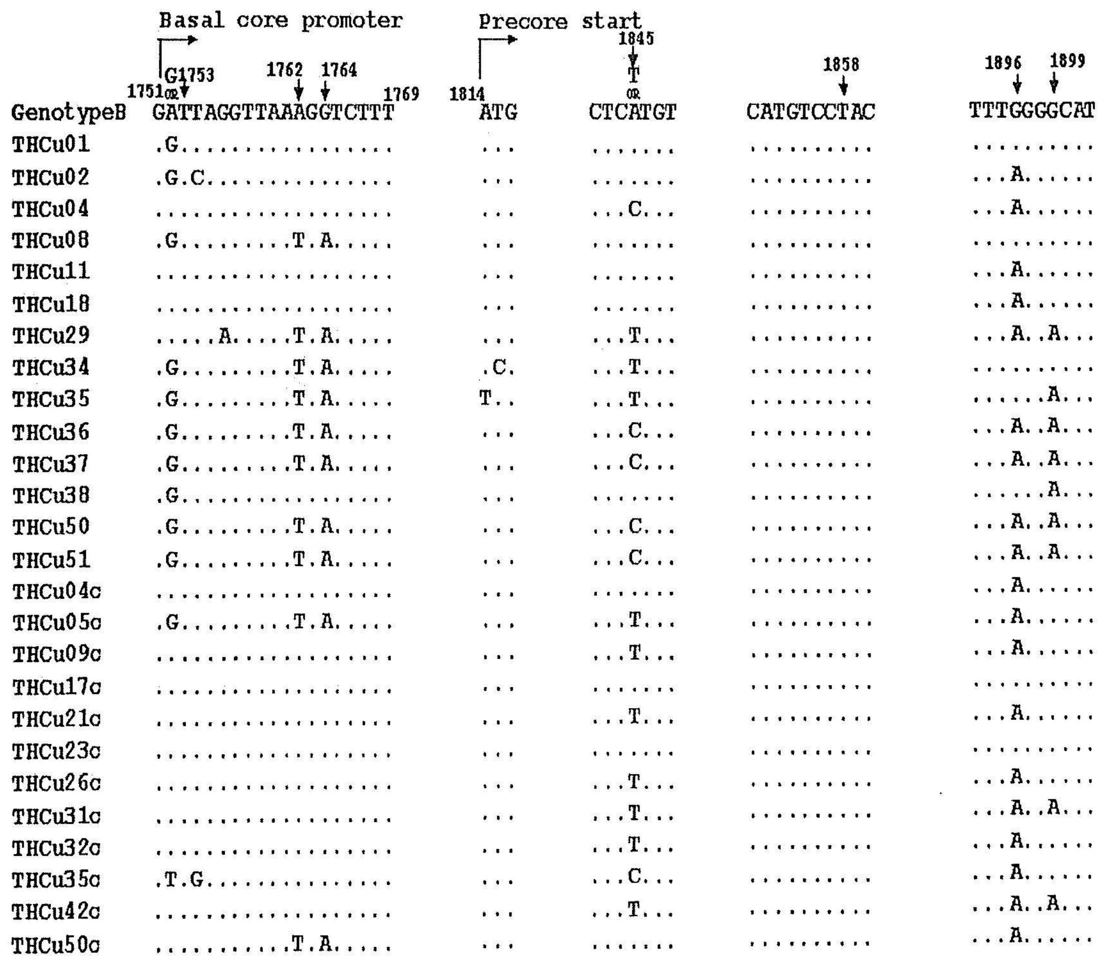

Figure 5. Alignment of HBV basal core promoter, and precore region from patients with genotype B (cases: THCu01-51; controls THCu04C-50c)

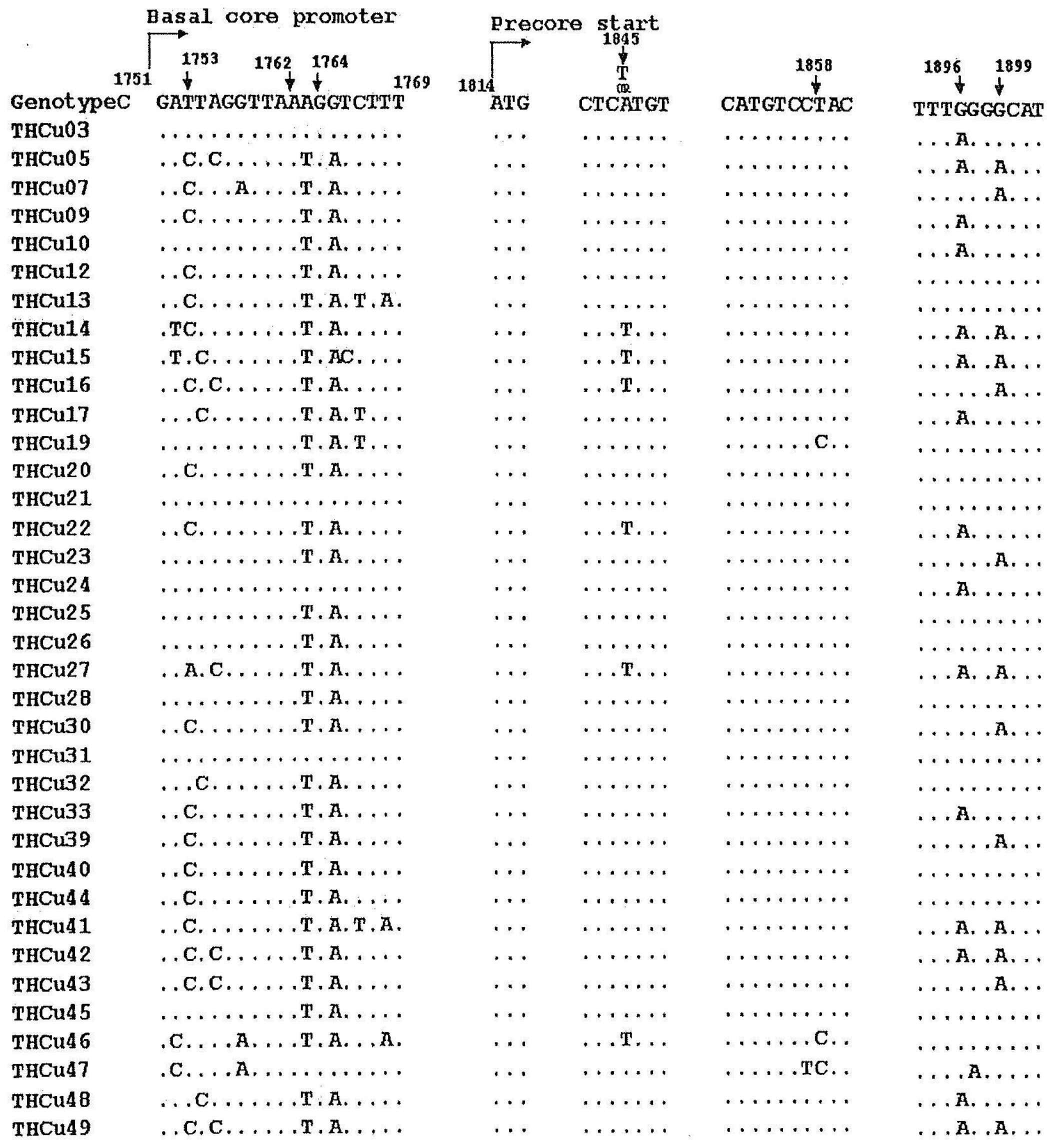

Figure 6. Alignment of HBV basal core promoter, and precore region from patients with HCC, who were infected with HBV genotype C

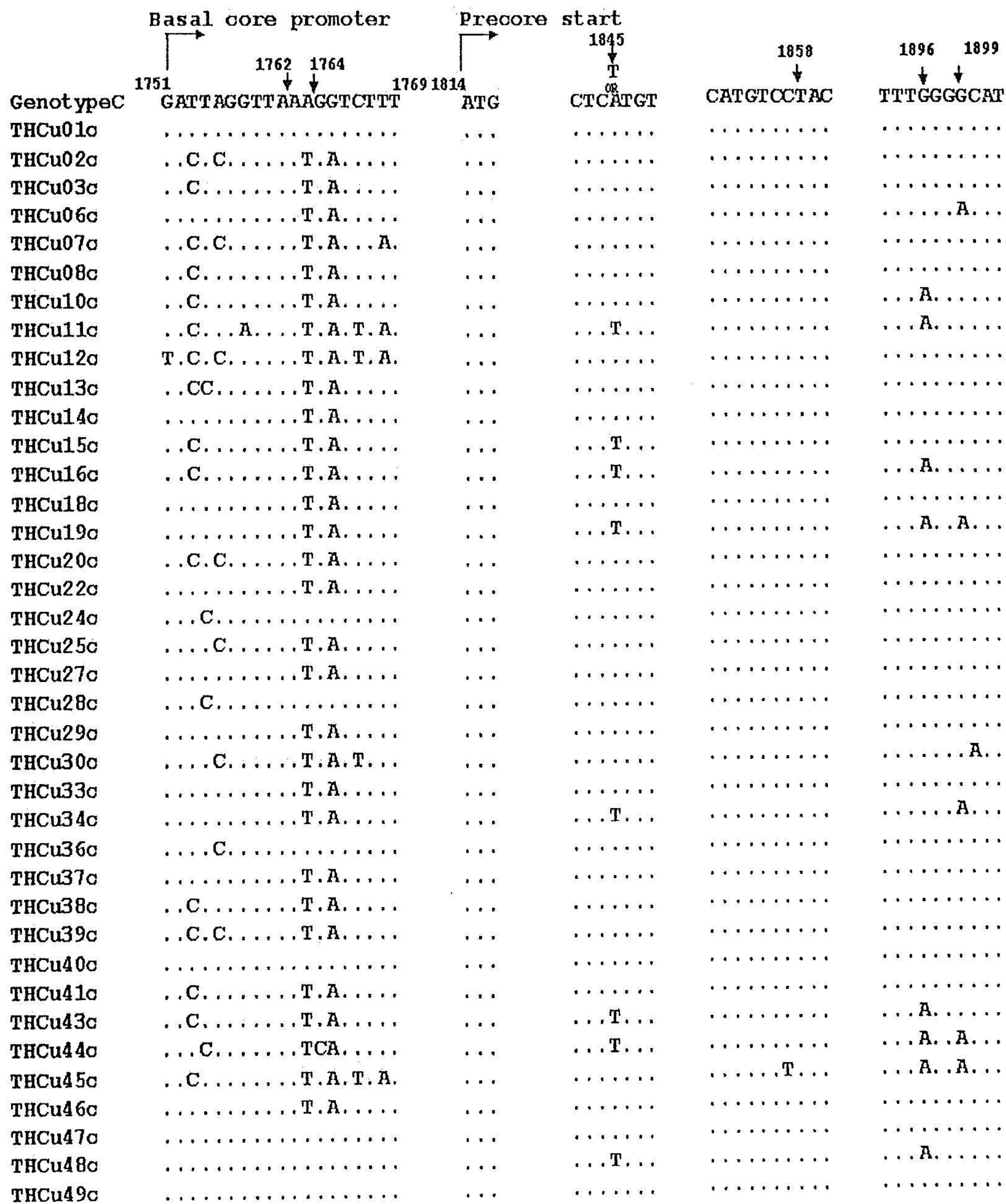

Figure 7. Alignment of HBV basal core promoter, and precore region from patients with non-HCC, who were infected with HBV genotype C

4.7 Core promoter mutations in patients with HCC

In the CP region, there was a higher percentage of patients with double mutations (A1762T/G1764A) in the HCC group (80%) compared with that of the control group (64%), but the difference was not statistically significant (*P*=0.12). However, among patients who were younger than 50 years, there was a significantly higher prevalence of the double mutations in HCC patients compared with control cases (82.6% and 48%, respectively, *P*=0.002). Furthermore, when HBV genotypes were taken into account, there was a significantly higher prevalence of these mutations in HCC patients compared with control patients with genotype B (51.5% and 16.7%, respectively, *P*=0.045), but there was no significant difference between HCC and control patients with genotype C (88.9% and 81.6%, respectively, *P*=0.0.58). Similarly, there was significantly higher prevalence of the double mutations in HCC patients compared with control patients who were younger than 50 years and were infected with HBV genotype B (66.7% and 0%, respectively, *P*=0.02).

Another frequent spot of mutation in the CP region found in this study was T1753A. There was no significant difference in the percentage of patients with this mutation between the HCC and control groups (38% and 32%, respectively, *P*=1.00) (Table 8, and Figure 5-7).

Mutations	Case	Control	*P*	OR.(95% CI)
A1762T/G1764A (n=50)	40 (80%)	32 (64%)	0.119	2.250 (0.913-5.545)
A1762T/G1764A Age < or 50 yrs (Case n=23, Control n-25)	19 (82.6%)	12 (48%)	0.028	5.146 (1.357-19.524)
A1762T/G1764A Genotype B (Case n-14, Control n=12)	8 (57.1%)	2 (16.7)	0.050	6.667 (1.047-42.431)
A1762T/G1764A Genotype C (Case n=26, Control n=38)	32 (88.9%)	30 (81.6%)	0.399	2.133 (0.582-7.824)
A1762T/G1764A Genotype B & Age < or 50 yrs (Case n=6, Control n=7)	4 (66.7%)	0 (0%)	0.021	4.500 (1.326-15.277)
T1753C/A (n=50)	19 (38%)	16 (32%)	1.000	1.092 (0.480-2.484)

Table 8. Core promoter mutations in patients with HCC and non-HCC

4.8 X gene mutations in patients with HCC

Single nucleotide mutations were present in the X gene, but with a generally scattered distribution, and without significant difference between the HCC and control groups. However, two of the HCC group had a 24-bps insertion at nt 1674 and a 2-bps deletion at nt 1721-1722, respectively, leading to a frameshift mutation of amino acid followed by new stop codons. The resulting proteins would therefore be predicted shorter than the full-length X protein (Table 9, and figure 8-13).

X gene mutation (n=50)	Case	Control	*P* value
C13T	4 (8%)	3 (6%)	1.000
C31T	2 (4%)	0	0.485
T/G/C88A	8 (16%)	16 (32%)	0.101
C/T118G	2 (4%)	0	0.485
T/G/Cnt124A WT : Mu	4 (8%)	0	0.117
Cnt131T WT : Mu	2(4%)	0	0.485
Tnt193C WT : Mu	4 (8%)	0	0.117
Gnt240A (G1613A)	16(32%)	18 (36%)	0.726
C256T	8(16%)	5(10%)	0.552
C/A/G259T	4 (8%)	4 (8%)	1.000
C280T (C1653T)	12 (24%)	9 (18%)	0.623
T426C	8 (16%)	3 (6%)	0.201

Table 9. X gene mutations in patients with HCC and non-HCC

```
                     13     31        131   193          240   280   285 288
GenotypeB   ATG GGCTG ATCCT     GTACC TCATC      GGAGA TTGCA AGAGG
THCu01      ... ..... .....     ..... .....      ..... ..... .....
Case02      ... ..... .....     ..T.. .....      ...A. ..... .....
THCu04      ... ..... .....     ..... .....      ..... ..... .....
THCu08      ... ..... .....     ..... .....      ..... ..... .....
THCu11      ... ..... .....     ..T.. .....      ..... ..... .....
THCu18      ... ..... .....     ..... .....      ..... ..A.. .....
THCu29      ... ..... .....     ..... .....      ..... ..... .....
THCu34      ... ..... .....     ..... .....      ..... ..... .....
THCu35      ... ..... .....     ..... .....      ...A. ..... ....A
THCu36      ... ..T.. ..T..     ..... ...C.      ...A. ...T. .....
THCu37      ... ..T.. .....     ..... ...C.      ...A. ...T. .....
THCu38      ... ..... .....     ..... .....      ..... ..... .....
THCu50      ... ..T.. ..T..     ..... ...C.      ...A. ...T. .A...
THCu51      ... ..T.. .....     ..... ...C.      ...A. ...T. .....
THCu04C     ... ..... .....     ..... .....      ...A. ..... ....A
THCu05C     ... ..... .....     ..... .....      ..... ..... .....
THCu09C     ... ..... .....     ..... .....      ..... ...T. .....
THCu17C     ... ..... .....     ..... .....      ..... ..A.. .....
THCu21c     ... ..... .....     ..... .....      ...A. ..... .....
THCu23c     ... ..T.. .....     ..... .....      ..... ..A.. .....
THCu26c     ... ..... .....     ..... .....      ..... ...T. .....
THCu31c     ... ..... .....     ..... .....      ..... ..... .....
THCu32c     ... ..... .....     ..... .....      ..... ...T. .....
THCu35c     ... ..... .....     ..... .....      ..... ..... .....
THCu42c     ... ..... .....     ..... .....      ...A. ..... .....
THCu50C     ... ..... .....     ..... .....      ..... ..... .....
```

Figure 8. Alignment of HBV X region from patients with HCC and non-HCC, who were infected with HBV genotype B

	301	24 bps insertion	302	348	G OR380	389 391	426	465
GenotypeB	T	-	C	GTGTT	GATTA	AAGGT	TGTGT	TAA
THCu01	.	-	.	.....	.G...	.....	.....	...
THCu02	.	TGCATAAGAGGACTCTTGGACTTT	.	.....	.G.C.	.....	.....	...
THCu04	.	-	.	.....	.....	.....	.....	...
THCu08	.	-	.	.....	.G...	.T.A.	..C..	...
THCu11	.	-	.	.....	.....	.....	..C..	...
THCu18	.	-	.	.....	.....	.....	.....	...
THCu29	.	-	.	.....	.....	.T.A.	.....	G..
THCu34	.	-	.	.....	.G...	.T.A.	..C..	...
THCu35	.	-	.	.....	.G...	.T.A.	..C..	...
THCu36	.	-	.	..A..	.G...	.T.A.	..C..	...
THCu37	.	-	.	..A..	.G...	.T.A.	..C..	...
THCu38	.	-	.	.....	.G...	.....	.....	...
THCu50	.	-	.	..A..	.G...	.T.A.	..C..	...
THCu51	.	-	.	.....	.G...	.T.A.	..C..	...
THCu04c	.	-	.	.....	.....	.....	..C..	...
THCu05c	.	-	.	.....	.G...	.T.A.	..C..	...
THCu09c	.	-	.	.....	.....	.....	.....	...
THCu17c	.	-	.	.....	.....	.....	.C...	...
THCu21c	.	-	.	.....	.....	.....	.....	...
THCu23c	.	-	.	.....	.....	.....	.C...	...
THCu26c	.	-	.	.....	.....	.....	.....	...
THCu31c	.	-	.	.....	.....	.....	.....	...
THCu32c	.	-	.	.....	.....	.....	.....	...
THCu35c	.	-	.	.....	.T.G.	.....	..C..	...
THCu42c	.	-	.	.....	.....	.....	.....	...
THCur50c	.	-	.	.....	.....	.T.A.	.....	...

Figure 9. Alignment of HBV X region from patients with HCC and non-HCC, who were infected with HBV genotype B (cont)

```
          1    13    31    88    118   124   131
GenotypeC ATG  GGCTG ATCCT CCGTC CCCCT CTGCG TGCCG
THCu03    ...  ..... ..... ..T.. ..... ..... .....
THCu05    ...  ..... ..... ..T.. ..... ..T.. .....
THCu07    ...  ..... ..... ..C.. ..... ..T.. .....
THCu09    ...  ..... ..... ..C.. ..G.. ..T.. .....
THCu10    ...  ..... ..... ..... ..... ..A.. .....
THCu12    ...  ..... ..... ..A.. ..... ..... .....
THCu13    ...  ..... ..... ..C.. ..... ..... .....
THCu14    ...  ..... ..... ..A.. ..... ..... .....
THCu15    ...  ..... ..... ..C.. ..T.. ...T. .....
THCu16    ...  ..... ..... ..T.. ..... ..T.. .....
THCu17    ...  ..... ..... ..A.. ..... ..T.. .....
THCu19    ...  ..... ..... ..... ..... ..T.. .....
THCu20    ...  ..... ..... ..... ..... ..T.. .....
THCu21    ...  ..... ..... ..... ..... ..... .....
THCu22    ...  ..... ..... ..... ..... ..T.. .A...
THCu23    ...  ..... ..... ..... ..... ..... .....
THCu24    ...  ..... ..... ..C.. ..G.. ..C.. ...T.
THCu25    ...  ..... ..... ..... ..... ..T.. ..A..
THCu26    ...  ..... ..... ..A.. ..... ..T.. .....
THCu27    ...  ..... ..... ..... ..... ..T.. .....
THCu28    ...  ..... ..... ..A.. ..... ..... .....
THCu30    ...  ..... ..... ..... ..... ..A.. .....
THCu31    ...  ..... ..... ..C.. ..... ..... .....
THCu32    ...  ..... ..... ..... ..... ..T.. .....
THCu33    ...  ..... ..... ..C.. ..... ...A. .....
THCu39    ...  ..... ..... ..C.. ..... ..A.. .....
THCu40    ...  ..... ..... ..A.. ..... ..... .....
THCu41    ...  ..... ..... ..C.. ..... ..... .....
THCu42    ...  ..... ..... ..T.. ..... ..T.. .....
THCu43    ...  ..... ..... ..T.. ..... ..T.. .....
THCu44    ...  ..... ..... ..A.. ..... ..T.. .....
THCu45    ...  ..... ..... ..A.. ..... ..A.. .....
THCu46    ...  ..... ..... ..... ..... ..T.. .....
THCu47    ...  ..... ..... ..... ..... ..T.. .....
THCu48    ...  ..... ..... ..C.. ..... ..T.. .A...
THCu49    ...  ..... ..... ..T.. ..... ..T.. .....
```

Figure 10. Alignment of HBV X region from patients with HCC, who were infected with HBV genotype C

	240	256	280	380	389 391	437	465
GenotypeC	**GGAGA**	**CCCGC**	**TTACA**	**GATTA**	**AAGGT**	**AGCAC**	**TAA**
THCu03	.....	.....	.....	.....	.....	.....	...
THCu05	.....	..T..	.....	..C.C	.T.A.	.....	...
THCu07	.....	.....	.....	..C..	.T.A.	.....	...
THCu09	.....	..T..	.....	..C..	.T.A.	.....	...
THCu10	.....	.....	...T.	.....	.T.A.	.....	...
THCu12	.....	.....	.....	..C..	.T.A.	.....	...
THCu13	.....	.....	...T.	..C..	.T.A.	..T..	...
THCu14	...A.	.....	.....	.TC..	.T.A.	.....	...
THCu15	...A.	.....	.....	.T.C.	.T.AC	.....	...
THCu16	.....	..T..	.....	..C.C	.T.A.	.....	...
THCu17	.....	.....	.....	...C.	.T.A.	.....	...
THCu19	...A.	.....	...T.	.....	.T.A.	.....	...
THCu20	.....	..A..	.....	..C..	.T.A.	.....	...
THCu21	.....	..A..	.....	.....	.....	.....	...
THCu22	...A.	.....	....T	..C..	.T.A.	.....	...
THCu23	.....	.....	.....	.....	.T.A.	.....	...
THCu24	...A.	A..A.	..G..	.....	.....	.....	...
THCu25	.....	.....	.....	.....	.T.A.	.....	...
THCu26	.....	.....	.....	.....	.T.A.	.....	...
THCu27	...A.	.....	.....	..A.C	.T.A.	.....	...
THCu28	...A.	.....	.....	.....	.T.A.	.C...	...
THCu30	.....	..T..	.....	..C..	.T.A.	G.T..	...
THCu31	.....	.....	.....	.....	.....	.....	...
THCu32	.....	.....	.....	...C.	.T.A.	.....	...
THCu33	.....	.....	.....	..C..	.T.A.	.....	...
THCu39	.....	..T..	.....	..C..	.T.A.	G.T..	...
THCu40	.....	.....	.....	..C..	.T.A.	.....	...
THCu44	.....	.....	...T.	..C..	.T.A.	.AT..	...
THCu41	.....	..T..	.....	..C.C	.T.A.	.....	...
THCu42	.....	.....	....T	..C.C	.T.A.	.....	...
THCu43	...A.	...A.	.....	..C..	.T.A.	.....	...
THCu45	...A.	.....	...T.	.....	.T.A.	.....	...
THCu46	.....	.....	...T.	.C...	.T.A.	.....	...
THCu47	...A.	.....	...T.	.C...	.....	.....	...
THCu48	.....	.....	.....	...C.	.T.A.	.....	...
THCu49	.....	..T..	.....	..C.C	.T.A.	.....	...

Figure 11. Alignment of HBV X region from patients with HCC, who were infected with HBV genotype C (cont)

	1	13	31	88	118	124	131
GenotypeC	ATG	GGCTG	ATCCT	CCGTC	CCCCT	CTGCG	TGCCG
THCu01c	...	.....	.....	.....	.....	..T..	.....
THCu02c	...	.....	.....	..T..	.....	..T..	.....
THCu03c	...	.....	.....	..A..	.....	.....	.....
THCu06c	...	.....	.....	.....	.....	..T..	.....
THCu07c	...	.....	.....	..A..	.....	..T..	.....
THCu08c	...	.....	.....	.....	.....	.....	.....
THCu10c	...	.....	.....	.....	.....	..T..	.....
THCu11c	...	.....	.....	.....	.....	..T..	.....
THCu12c	...	.....	.....	..A..	.....	..T..	.....
THCu13c	...	.....	.....	..C..	.....	..T..	.....
THCu14c	...	.....	.....	..A..	.....	.....	.....
THCu15c	...	.....	.....	.....	.....	..T..	.....
THCu16c	...	.....	.....	.....	.....	..T..	.....
THCu18c	...	.....	.....	..C..	.....	.....	.....
THCu19c	...	.....	.....	..C..	.....	.....	.....
THCu20c	...	..G..	.....	..C..	..A..	..T..	.....
THCu22c	...	..T..	.....	..C..	..A..	.....	.....
THCu24c	...	.....	.....	..T..	.....	..C..	.....
THCu25c	...	..G..	.....	..A..	.....	..T..	.....
THCu27c	...	.....	.....	..A..	.....	..T..	.....
THCu28c	...	.....	.....	..A..	.....	..T..	.....
THCu29c	...	.....	.....	..A..	.....	..T..	..A..
THCu30c	...	.....	.....	..A..	.....	..T..	.....
THCu33c	...	.....	.....	..A..	.....	..T..	.....
THCu34c	...	.....	.....	.....	.....	..T..	.....
THCu36c	...	..T..	.....	..C..	..A..	..T..	.....
THCu37c	...	.....	.....	.....	.....	..T.A	.....
THCu38c	...	.....	.....	..A..	.....	.....	.....
THCu39c	...	.....	.....	..T..	.....	..T..	.....
THCu40c	...	.....	.....	..A..	.....	.....	.....
THCu41c	...	.....	.....	..A..	.....	.....	.....
THCu43c	...	.....	.....	..C..	.....	.....	.....
THCu44c	...	.....	.....	..A..	.....	..T..	.....
THCu45c	...	.....	.....	..T..	.....	..C..	.....
THCu46c	...	.....	.....	..A..	.....	..T..	.....
THCu47c	...	.....	.....	..A..	.....	..T..	.....
THCu48c	...	.....	.....	..C..	..G..	..C..	..GT.
THCu49c	...	.....	.....	..A..	.....	..T..	.....

Figure 12. Alignment of HBV X region from patients with non-HCC, who were infected with HBV genotype C

```
            240   256     280   380  389 391   437    465
GenotypeC   GGAGA CCCGC   TTACA GATTA AAGGT    AGCAC  TAA
THCu01c     ..... .....   ..... ..... .....    ...... ...
THCu02c     ..... .....   ..... ..C.C .T.A.    ...... ...
THCu03c     ..... .....   ..... ..C.. .T.A.    ...... ...
THCu06c     ...A. .....   ...T. ..... .T.A.    ...... ...
THCu07c     ..... .....   ..... ..C.C .T.A.    ...... ...
THCu08c     ..... .....   ..... ..C.. .T.A.    ...... ...
THCu10c     ...A. .....   ..... ..C.. .T.A.    ...... ...
THCu11c     ..... .....   ..... ..C.. .T.A.    ...... ...
THCu12c     ...A. ..T..   ...T. T.C.C .T.A.    ...... ...
THCu13c     ..... .....   ..... ..CC. .T.A.    ...... ...
THCu14c     ..... .....   ..... ..... .T.A.    ...... ...
THCu15c     ...A. ...A.   ..... ..C.. .T.A.    ...... ...
THCu16c     ...A. .....   ..... ..C.. .T.A.    ...... ...
THCu18c     ...A. .....   ...T. ..... .T.A.    ...... ...
THCu19c     ...A. ..T..   ...T. ..... .T.A.    ...... ...
THCu20c     ..... .....   ..... ..C.C .T.A.    ...... ...
THCu22c     ...A. .....   ..... ..... .T.A.    ...... ...
THCu24c     ..... .....   ..... ...C. .....    ...... ...
THCu25c     ...A. ..T..   ...T. ....C .T.A.    ...... ...
THCu27c     ..... .....   ..... ..... .T.A.    ...... ...
THCu28c     ..... .....   ..... ...C. .....    ...... ...
THCu29c     ..... .....   ..... ..... .T.A.    ...... ...
THCu30c     ..... T.T..   ..... ....C .T.A.    ...... ...
THCu33c     ..... .....   ..... ..... .T.A.    ...... ...
THCu34c     ...A. .....   ...T. ..... .T.A.    ...... ...
THCu36c     ..... .....   ..... ....C .....    ...... ...
THCu37c     ..... .....   ..... ..... .T.A.    ...... ...
THCu38c     ..... .....   ..... ..C.. .T.A.    ...... ...
THCu39c     ..... .....   ..... ..C.C .T.A.    ...... ...
THCu40c     ...A. ..T..   ..... ..... .....    ...... ...
THCu41c     ..... .....   ..... ..C.. .T.A.    ...... ...
THCu43c     ...A. .....   ..... ..C.. .T.A.    ...... ...
THCu44c     ...A. A....   ..... ...C. .TCA.    ...... ...
THCu45c     ..... .....   ..... ..C.. .T.A.    ...... .G.
THCu46c     ...A. .....   ..... ..... .T.A.    ...... ...
THCu47c     ..C.. .....   ..... ..... .....    ...... .G.
THCu48c     ..... ...A.   ..G.. ..... .....    ...... ...
THCu49c     ..... .....   ..... ..... .....    ...... ...
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Figure 13. Alignment of HBV X region from patients with non-HCC, who were infected with HBV genotype C (cont)

5. Discussion

Identification of host and viral factors leading to severe liver damage and to the development of HCC may have important clinical implications in the management of patients with chronic HBV infection. There are now increasing data suggesting that HBV genotypes and mutations may play an important role in causing different disease profiles in chronic HBV infection. Regarding the roles of HBV genotypes, most studies have been performed in Asia and restricted to comparisons between genotypes B and C, which are the two most common HBV genotypes in this region accounting for more than 90% of cases [6, 24, 25]. Current available data from this region demonstrate that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B [4-7]. In addition, patients with genotype C infection, compared to those with genotype B, are more frequently HBeAg positive and display higher HBV DNA levels that may contribute to multiple episodes of acute flares and progression of liver disease [26]. Taken together, these data suggest that patients with genotype C have a tendency to exhibit more severe liver disease than those with genotype B.

In agreement with previous studies [25, 27], our study demonstrated that genotype C and B were the predominant strains, accounting for approximately 75 and 20% of patients, respectively. In this respect, it would appear that the prevalence of HBV genotypes in Thailand is comparable to that reported from Japan and China [6, 24], but differs from the distribution observed in Taiwan, where HBV genotype B is more common than genotype C [4, 28]. Interestingly, the prevalence of genotype B and C in patients with HCC in our study was comparable to that in asymptomatic carrier, chronic hepatitis and cirrhosis. The equal distribution of genotypes B and C among various stages of chronic liver disease is consistent with previous reports conducted in Japan [6, 11], but it contradicts the observations from other studies[4, 7, 24]. Hence, our data suggest that although genotype C is the most prevalent strain in Thailand, the risk of development of HCC may not be different between genotypes B- and C-related chronic liver disease.

The predominance of HBV genotypes B and C allows the comparison of clinical outcomes of patients who are chronically infected with these two HBV strains. Our results showed that the mean ages among asymptomatic carriers and chronic hepatitis were comparable between patients with genotypes B and C. However, the mean age of patients with genotype C tended to be older than those with genotype B in cirrhotic group. Interestingly, the divergence in the mean age of patients with genotypes B and C was more noticeably in those with HCC. 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, regardless of the viral genotype. Our results also showed that patients with genotype C had a tendency of higher ALT, necroinflammatory scores and HBV DNA levels than patients with genotype B. Moreover, patients with genotype C had a significantly higher prevalence of HBeAg positivity, particular among patients with chronic hepatitis and cirrhosis, but the difference seemed to be disappearing upon the disease progression to HCC. Although HBeAg is a marker of active viral replication, the disappearance of HBeAg with or without seroconversion of antibody to HBeAg during the course of chronic infection does not always imply disease remission [29]. Collectively, it is reasonable to speculate that, at least in our populations, patients with genotype C has a trend for delayed HBeAg seroconversion and more prolonged necroinflammatory process causing earlier development of cirrhosis and HCC.

Nonetheless, it would appear that there is no difference in the risk between patients with genotypes B and C in the progression to liver cancer.

It is currently unclear whether a certain HBV genotype is associated with a greater risk for progression of cirrhosis to HCC. Studies from Taiwan and Japan have demonstrated an increase in HCC development among patients with HBV genotype C compared to genotype B [4, 6, 12, 28]. Similarly, a study conducted in China has suggested that genotype C may predispose to HCC, whereas genotype B has a relatively better prognosis [24]. On the contrary, recent studies from Hong Kong and Japan have shown that there is no difference in the risk of developing HCC between patients with genotypes B and C [11, 30]. Moreover, a potential correlation between HBV genotype and the age of the patients with HCC has been debated. Intriguingly, the report from northern Taiwan has observed that genotype B is associated with the development of HCC in patients younger than 35 years of age, while those with genotype C more frequently develop the cancer after 50 years of age [4]. This observation, however, has not been confirmed by subsequent studies from southern Taiwan and Japan [6, 11, 28]. In southern Taiwan, for example, there is no significant difference in the mean age between HCC patients with genotypes B and C [28]. By remarkable contrast, the mean age of Japanese patients with genotype B is approximately 70 years compared to 55 years of those with genotype C [6]. In the present report, the mean age of HCC patients with genotype B was significantly older than those with genotype C (61 and 51 years, respectively). Thus, the age distribution of HBV genotypes in Thai patients with HCC seems to correspond with the report from Japan, but differs from those studies from Taiwan.

The molecular virological factors responsible for this discrepancy among countries remain largely unknown. It has been postulated that the difference in the mean age between Taiwanese and Japanese patients with HCC may be partially influenced by the divergence of HBV subtypes distributed among different geographic areas[31]. Recently, two subtypes of HBV genotype B, namely Ba and Bj, have been identified based on the phylogenetic analysis. It has shown that genotype Ba consists of the recombination with the precore/core region originating from genotype C, whereas genotype Bj does not [32]. Genotype Bj is exclusively found in Japan, while genotype Ba is ubiquitous in other countries in Asia, including Thailand[33]. Based on our data, however, this postulation could not clarify the similarity in the mean age between Thai and Japanese patients with HCC, and the diversity between Thai and Taiwanese patients. Thus, it is likely that other virological factors, such as CP or X gene mutations might act as potential variables influencing the development of HCC in patients with chronic liver disease, even though they are infected with HBV of the same genotype. In addition, discrepancies regarding the role of HBV genotype might be related to variability of host and environmental factors in different geographic areas, such as genetic polymorphism and aflatoxin exposure.

Regarding the impact of HBV genotypes on the prognosis of HCC, a prospective study in Japan demonstrates that patients with genotype C tend to have relatively poor clinical outcomes after transcatheter arterial embolization (TACE) therapy compared with those with genotype B[34]. This finding is consistent with a recent report from Taiwan indicating that patients with genotype C exhibit a greater recurrent rate after curative resection of the tumor compared with those with genotype B [35]. However, our findings showed that the clinical features at presentation and overall survival of patients with HCC did not depend on the HBV genotype. The similarity of the tumor characteristics and clinical outcome of patients with genotypes B and C in this study was supported by the data of a recent case-control report

conducted in Hong Kong[36]. Therefore, it would appear that in Thai populations, once HCC has developed, the course of the disease might be independent from the underlying HBV infection because the proportion of surviving patients is similar, irrespective of the infecting genotype. It should also be noted that overall median survival observed in this study was much shorter compared to other reports. The low survival rates in Thai patients with HCC in this study were in part resulted from advanced stages of the cancer at the time of the diagnosis, while only minorities had an early detection in the course of follow-up programs.

A point mutation at nt. 1896 (G1896A) in the precore region is well documented [37]. The occurrence of the A1896 variant is restricted to HBV genotype carrying T1858 and is thus widespread in China, Japan and Mediteranean basin, where the predominant genotypes are B, C, and D, but rare in North America and Europe, where the predominant genotype A has almost exclusively C1858 [38]. The results presented in this study supported the predominance of genotype B and C associated with T1858 in Thai patients, which corresponds to the pattern observed in the other regions. However, this mutation alone might not account for the increased risk of HCC, as shown in our case-control study. Furthermore, mutation at nt. 1899 (G1899A) was found to be significantly higher in the HCC group than the control group. Such mutation in the encapsidation signal would stabilize base paring of the stem-loop structure of the viral pregenomic RNA. That is, this mutation alters a G-U pair of the stem structure to a more stable A-U pair[39]. Interestingly, this mutation has shown low prevalence in other reports, mainly in patients with chronic hepatitis[39, 40], but was found frequently in our patients with HCC. The reason for this discrepancy is unclear, though it could probably be from the differences in the clinical stage of HBV infection or could be related to the oncogenic role of the virus. Further studies are needed to clarify the role of this mutant in HBV-related hepatocarcinogenesis.

Double mutations in the BCP at nt.1762 and 1764 have been previously shown to be associated with the clinical outcome of chronic HBV infection[16, 17]. Our data indicate that in an endemic area like Thailand, the frequency of these mutations is high, particularly in patients with HCC. These findings are consistent with previous studies in China and Africa, but not with other studies in Vietnam and Brazil[17, 40-42]. We also showed that patients infected with HBV genotype B harboring these mutations were at increased risk for HCC. In addition, the prevalence of A1762T/G1764A mutations in younger HCC patients was comparable with older HCC patients, but was significantly higher than that in aged-matched non-HCC patients. The findings in this case-control study also excluded the possibility of cohort effect that patients with chronic HBV infection are prone to have these mutations in advanced age. Accordingly, our data suggested that patients infected with genotype B harboring the CP mutant might be at higher risk for the early development of HCC than those without such mutant.

It has been reported recently that mutation in the X gene may contribute in HBV-related hepatocarcinogenesis[18]. Our results are consistent with those studies in that frameshift mutation that should result in the production of truncated HBx proteins were more prevalent in HCC patients[40, 43]. In addition, our data showed that a point mutation frequently detected in the X gene in serum samples of patients with HCC. However, the prevalence of these mutants was similar between the HCC and control groups, suggesting that emergence of these mutants did not immediately lead to developing of HCC. Instead, these mutants likely occurred during a long-standing inflammatory process of chronic HBV infection because the viral infection in Thailand occurred mostly in the early childhood.

6. Conclusion and Future Prospect

Our study demonstrated that patients with HBV genotype C, compared to those with genotype B, had a higher positive rate of HBeAg and exhibited earlier progression of cirrhosis and HCC. Genotype by itself, however, might not be responsible for an increased oncogenic effect because there was no difference in the risk of developing HCC and its prognosis between patients with genotypes B and C. Instead, certain X gene mutations and, particularly, CP mutations in young patients may contribute to the development of HCC. Further large-scale prospective studies, which offer advantages over cross-sectional investigations, are needed to establish the existence of these observations in the future.

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Output

Publications from the project

4 published articles, and 1 expected publication

1. Hepatitis B virus genotypes and hepatocellular carcinoma. World J Gastroenterol 2005; 11: 2238-43.
2. Clinical and virological differences between hepatitis B virus genotypes B and C: a case-control study. J Med Assoc Thai 2004; 87 (supple 2): S223-7.
3. Simultaneous quantitation and genotyping of hepatitis B virus by real-time PCR and melting curve analysis. J Virol Methods 2004; 120: 131-40.
4. A novel recombinant of hepatitis B virus genotype G and C isolated from a Thai patient with hepatocellular carcinoma. J Gen Virol 2005; 86: 3027-30.
5. Precore, core promoter and X gene mutations of hepatitis B virus in Thai patients with hepatocellular carcinoma: a case-control study. (manuscript in preparation)

บทความสำหรับการเผยแพร่

ไวรัสตับอักเสบบีเป็นสาเหตุสำคัญของเกิดโรคตับอักเสบชนิดเรื้อรัง ซึ่งอาจมีการดำเนินของโรคต่อไปเป็นตับแข็งและมะเร็งตับ ปัจจุบันมีประชากรทั่วโลกมากกว่า 400 ล้านคนหรือเท่ากับประมาณร้อยละ 5 ของประชากรโลกที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง และในแต่ละปีมีผู้เสียชีวิตเนื่องจากโรคตับแข็งและมะเร็งตับจากการติดเชื้อไวรัสตับอักเสบบีไม่น้อยกว่า 1 ล้านคน ประเทศไทยเป็นประเทศที่มีความชุกชุมของไวรัสตับอักเสบบีสูงเช่นเดียวกับประเทศในภูมิภาคเอเชียตะวันออกเฉียงใต้ แม้ว่าอัตราการติดเชื้อในปัจจุบันจะมีแนวโน้มลดลงโดยเฉพาะในเด็กเนื่องจากความสำเร็จของการฉีดวัคซีนในการป้องกันโรคของประเทศ แต่อัตราการติดเชื้อในผู้ใหญ่ยังคงไม่ลดลงมากนัก ดังนั้นไวรัสตับอักเสบบีและโรคตับที่เกี่ยวข้องโดยเฉพาะมะเร็งตับจึงยังคงเป็นปัญหาสำคัญทางการแพทย์และสาธารณสุขของประเทศไทยในปัจจุบัน

ผู้ติดเชื้อไวรัสตับอักเสบบีแต่ละรายมีการดำเนินโรคแตกต่างกัน ทั้งนี้อาจขึ้นอยู่กับปัจจัยของผู้ป่วยเองเช่นการติดเชื้อตั้งแต่อายุน้อย ผู้ป่วยเพศชาย หรือผู้ป่วยที่มีการตอบสนองทางภูมิคุ้มกันผิดปกติ นอกจากนี้ปัจจัยที่เกี่ยวข้องกับเชื้อไวรัสโดยตรงอาจมีส่วนทำให้ความรุนแรงของโรคแตกต่างกัน องค์ความรู้ในระดับอณูชีววิทยาของไวรัสบีจากการศึกษาวิจัยของโครงการเรื่อง "ความชุกและความสำคัญทางคลินิกของสายพันธุ์และการกลายพันธุ์ของไวรัสตับอักเสบบี" แสดงให้เห็นว่าเชื้อไวรัสสายพันธุ์ซีซึ่งสายพันธุ์ที่พบบ่อยที่สุดในประเทศไทยก่อให้เกิดความรุนแรงของโรคมากกว่าสายพันธุ์บี นอกจากนี้การกลายพันธุ์ของเชื้อไวรัสในบางตำแหน่งอาจเกี่ยวข้องกับการกลไกการเกิดโรคมะเร็งตับ ความแตกต่างของสายพันธุ์และการกลายพันธุ์เชื้อไวรัสที่มีผลต่อการดำเนินของโรคและความรุนแรงของโรคนี้กำลังเป็นที่สนใจกันมากทั่วโลก

ผลของการศึกษาวิจัยดังกล่าวข้างต้นนี้นอกจากจะเป็นประโยชน์อย่างยิ่งทั้งในด้านระบาดวิทยาของประชากรไทยโดยใช้เป็นข้อมูลเปรียบเทียบกับกลุ่มประชากรต่างๆทั่วโลกแล้ว ยังก่อให้เกิดองค์ความรู้ที่เกี่ยวกับกลไกการเกิดโรคและความรุนแรงของโรคของผู้ป่วยตับอักเสบเรื้อรังจากเชื้อไวรัสตับอักเสบบี ซึ่งอาจมีประโยชน์ในการป้องกันและรักษาโรคมะเร็งตับต่อไปในอนาคต

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World J Gastroenterol 2005;11(15):2238-2243
World Journal of Gastroenterology ISSN 1007-9327
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Hepatitis B virus genotypes and hepatocellular carcinoma in Thailand

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Received: 2004-08-30 Accepted: 2004-10-06

Abstract

AIM: The role of hepatitis B virus (HBV) genotypes on the clinical features and prognosis of patients with hepatocellular carcinoma (HCC) is currently unknown. The aim of the present study was to evaluate the distribution of HBV genotypes and their clinical relevance in Thai patients.

METHODS: HBV genotypes were determined by PCR-RFLP in stored sera of 93 asymptomatic carriers, 103 patients with chronic hepatitis, 60 patients with cirrhosis and 76 patients with HCC. The clinical data were analyzed in relation to the HBV genotype.

RESULTS: HBV genotypes C and B were predominant in Thailand, accounting for 73% and 21%, respectively. The distributions of genotypes B and C were similar in HCC patients compared to the other groups. Genotype C was significantly more common in HCC patients who were under 40 years old than genotype B (18% *vs* 0%, $P = 0.03$), but was significantly less common in patients older than 60 years (26% *vs* 56.5%, $P = 0.01$). The positive rate of hepatitis B e antigen (HBeAg) in patients with genotype C was significantly higher than that in patients with genotype B (71.6% *vs* 44.4%, $P = 0.03$ in chronic hepatitis; 56.8% *vs* 11.1%, $P = 0.01$ in cirrhosis). There were no differences between HCC patients with genotypes B and C regarding tumor staging by CLIP criteria and the overall median survival. Multivariate analyses showed that HBV genotype was not an independent prognostic factor of survival in HCC patients.

CONCLUSION: Patients with genotype C had a higher positive rate of HBeAg and exhibited earlier progression of cirrhosis and HCC than those with genotype B. However, there were no differences in the risk of developing HCC and its prognosis between patients with these genotypes.

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Key words: HBV; Genotype; Hepatocellular carcinoma

Tangkijvanich P, Mahachai V, Komolmit P, Fongsarun J, Theamboonlers A, Poovorawan Y. Hepatitis B virus genotypes and hepatocellular carcinoma in Thailand. *World J Gastroenterol* 2005; 11(15): 2238-2243
http://www.wjgnet.com/1007-9327/11/2238.asp

INTRODUCTION

Hepatitis B virus (HBV) infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[1]. HBV, a member of the hepadnaviridae, is a relaxed circular double-stranded DNA virus, and has currently been classified into eight genotypes, designed A-H based on a comparison of entire genomic sequences[2,3]. HBV genotypes appear to show varying geographic patterns in their distribution. For instance, genotypes A and D are predominant in Western countries and India, whereas genotypes B and C prevail in Southeast Asia, China and Japan. Genotype E is restricted to Africa and genotype F is found in Central and South America.

Besides the differences in geographical distribution, there is growing evidence that the viral genotypes may influence the clinical outcomes of patients with chronic HBV infection. Among Asian patients who constitute approximately 75% of HBV carriers worldwide, it has been shown that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B[4-7]. Genotype C is also associated with a lower rate of hepatitis B e antigen (HBeAg) seroconversion and a lower response rate to alpha interferon therapy compared to genotype B[8,9]. However, the association between HBV genotype and the risk of developing HCC is still controversial[4,10-12]. In addition, the impact of HBV genotype on clinical features and prognosis of patients with HCC remains unclear. Thus, the aim of this study was to determine the role of HBV genotypes on clinical severity of patients with chronic liver disease, particularly those with HCC.

MATERIALS AND METHODS

Subjects

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 determined by the use of 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. Patients who had previously been treated with antiviral therapy were excluded. The patients were clinically classified into four groups including asymptomatic carrier, chronic hepatitis, cirrhosis and HCC. Asymptomatic carrier was diagnosed by periodical examination of 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 histologic examinations. The degree of hepatic inflammation and fibrosis was graded according to modified Knodell histology index[13]. Cirrhosis was diagnosed based on histologic examinations and/or imaging studies, and its severity was subsequently classified based on Child's criteria. 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 IU/mL. The staging of HCC was classified according to CLIP criteria[14].

Serum samples were collected from each patient at the time of their clinical evaluation and stored at -70 ℃ until further tests were performed. All patients were informed regarding the purpose of exterminating the etiologies of liver disease and their written consent were obtained. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

HBV-DNA extractions

DNA was extracted from 100 μL serum with proteinase-K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 30 μL 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 previously described[15]. The forward primer was P1 (nt 2823-2845: 5'-TCACCATATTCTTGGGAACAAGA); the reverse primer was P2 (nt 80-61: 5'-TTCCTGAACTGGAGCCACCA). The primers were located in conserved genomic regions to ensure a high sensitivity for the amplification of all HBV genotypes. Two microliters of DNA sample were combined with a reaction mixture containing 20 μL of 2.5×Eppendorf MasterMix (Hamburg, Germany), 1 μmol/L P1, 1 μmol/L P2 and sterile water, in a final volume of 50 μL. PCR was performed under the following conditions: After an initial 2-min denaturation step at 94 ℃, 35 cycles of amplification were performed, each including 30-s denaturation at 94 ℃, 30-s annealing at 55 ℃ and 30-s extension at 72 ℃, followed by a final 10-min extension at 72 ℃. Each amplified DNA sample (10 μL) was added to a loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME) at 100 V 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 μL of PCR product was mixed with 1.5 μL of 10×buffer, 3 μL of sterile water and 0.5 μL (5U) of *Ava*II and *Dpn*II, respectively, in separate reactions and incubated at 37 ℃ for 3.5 h. 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[15].

Serological and virological assays

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). The detection range of this assay was from 2.7 to 8.7 log genome equivalents/mL (LGE/mL).

Statistical analysis

Data were presented as percentage, mean and standard deviation. χ^2 test, unpaired t test, and ANOVA were used to assess the statistical significance of the difference between groups where appropriate. Survival curves were established using the Kaplan-Meier method and differences between curves were verified using the log-rank test. Cox regression analysis was performed to identify which indepe-ndent variables would have a significant influence on the overall survival. P values below 0.05 were considered statis-tically significant. All statistical analyses were performed using SPSS 10.0 software for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Distribution of HBV genotypes in patients with chronic HBV infection

Of the 470 patients enrolled in this study, HBV-DNA was detected in 332 patients (70.6%). Our data showed that the most common HBV genotypes were genotypes C and B, which were found 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. The demographic and clinical data of 332 patients with different stages of chronic HBV infection are shown in Table 1. Male-to-female ratio of asymptomatic carriers was significantly lower than the other groups (P = 0.002). Mean age was significantly higher in patients with cirrhosis and HCC than in the other two groups (P = 0.001), and positive HBeAg rate was significantly higher in patients with chronic hepatitis than in other groups (P = 0.001). Although genotype C was the most common genotype in each group, no significant differences were observed with respect to the distribution

Table 1 Demographic and clinical data of 332 patients with chronic HBV infection

Diagnosis	n	Sex (m/f)	Age (yr)	ALT (U/L)	HBeAg positive	Genotype			
						A	B	C	U
Carrier	93	57/36	30.9±10.6	27.5±4.5	42/82 (51.2)	2 (2.2)	16 (17.2)	73 (78.4)	2 (2.2)
CH	103	84/19	36.2±10.1	157.4±103.8	61/92 (66.3)	5 (4.9)	20 (19.4)	76 (73.8)	2 (1.9)
Cirrhosis	60	47/13	48.8±13.8	135.0±90.9	26/53 (49.1)	2 (3.3)	10 (16.7)	44 (73.3)	4 (6.7)
HCC	76	60/16	54.4±12.9	107.8±107.4	15/71 (21.1)	2 (2.6)	23 (30.3)	50 (65.8)	1 (1.3)

CH, chronic hepatitis; HCC, hepatocellular carcinoma; U, unclassified genotype. Quantitative variables are expressed as mean±SD; categorical variables are expressed as *n* (%).

of the genotypes in various stages of chronic HBV infection (P = 0.16).

Clinicopathologic differences between genotypes B and C in chronic hepatitis

Because the number of patients with genotype A was less, only genotypes B and C were included for further analysis of clinicopathologic differences between genotypes. As shown in Table 2, patients with genotypes B and C were comparable with respect to sex, age and total bilirubin. The rate of positive HBeAg in patients with genotype B was significantly lower than that in patients with genotype C (44.4% *vs* 71.6%, respectively, P = 0.03). Mean ALT level was also significantly lower in patients with genotype B than those in patients with genotype C (119.8±58.5 and 159.8±106.4 IU/L, respectively; P = 0.03), but HBV-DNA levels were comparable between them (7.25±1.74 and 7.10±1.34 LGE/mL, respectively; P = 0.78). Patients with genotype B had a lower score of both necroinflammation activity and fibrosis than those with genotype C, but the differences were not statistically significant (5.8±2.1 and 1.5±1.0 *vs* 6.7±2.1 and 1.8±0.9, P = 0.51 and 0.47, respectively).

Clinical differences between genotypes B and C in cirrhosis

There were no significant differences in gender, total bilirubin, serum albumin, and child classification between groups of patients, as shown in Table 3. The mean age of patients with genotype B tended to be older than those with genotype C (54.7±9.2 *vs* 47.4±14.4 years, respectively, P = 0.06). The positive rate of HBeAg in patients with genotype B was significantly lower than that in patients with genotype C (11.1% *vs* 56.8%, respectively, P = 0.01). Patients with genotype B tended to have lower mean ALT and HBV-DNA levels than those with genotype C, but the difference was not statistically significant (101.5±49.0 IU/L and 6.59±1.20 LGE/mL *vs* 133.7±93.4 IU/L and 7.05±2.50 LGE/mL, P = 0.14 and 0.12, respectively).

Clinical differences between genotypes B and C in HCC

The clinical data of 73 patients with HCC were compared according to HBV genotype, as shown in Table 4. Between the two groups, there were no significant differences in gender, total bilirubin, ALT, serum albumin, AFP and HBV-DNA levels, tumor staging according to CLIP criteria. However, the mean age of patients with genotype B was significantly older than those with genotype C (61.1±9.8 *vs* 51.3±13.1 years, respectively, P = 0.001). Four of 22 patients (18.1%) with genotype B were positive for HBeAg, whereas 11 of 49 patients (22.4 %) with genotype C were positive for this marker, but the difference was not statistically significant (P = 0.68). When patients with HCC were stratified by age (Figure 1), none of the patients with genotype B was younger than 40 years, whereas nine patients (18 %) with genotype C were younger than 40 years (P = 0.03). On the contrary, 13 patients (56.5%) with genotype B were older than 60 years, whereas 13 patients (26 %) with genotype C were older than 60 years (P = 0.01).

The Kaplan-Meier survival curves demonstrated that the overall median survival for patients with genotypes B and C were 5.5 and 7.3 mo, respectively (P = 0.81, using log-rank test) (Figure 2). For patients who were treated with any specific therapeutic modality, the median survival for the genotype B and C groups were 11.5 and 12.4 mo, respectively (P = 0.97). In the untreated cases, the median survival of the genotype B and C groups were 4.5 and 4.0 mo,

Table 2 Demographic and clinical data of patients with chronic hepatitis

Characteristics	Genotype B (n = 20)	Genotype C (n = 76)	P
Age (yr)	35.1±9.6	36.4±10.3	NS
Sex (male/female)	16/4	65/11	NS
Total bilirubin (mg/dL)	0.8±0.2	0.7±0.3	NS
ALT (IU/L)	119.8±58.5	159.8±106.4	0.03
HBeAg positive	8/18 (44.4)	53/74 (71.6)	0.03
HBV DNA (LGE/mL)	7.25±1.74	7.10±1.34	NS
HAI inflammation	5.8±2.1	6.7±2.1	NS
HAI fibrosis	1.5±1.0	1.8±0.9	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as *n* (%). HAI inflammation, sum of necroinflammatory scores of histology activity index. HAI fibrosis, sum of fibrosis scores of histology activity index.

Table 3 Demographic and clinical data of patients with cirrhosis

Characteristics	Genotype B (n = 10)	Genotype C (n = 44)	P
Age (yr)	54.7±9.2	47.4±14.4	0.06
Sex (male/female)	8/2	35/9	NS
Total bilirubin (mg/dL)	1.4±0.8	2.2±1.2	NS
ALT (IU/L)	101.5±49.0	133.7±93.4	NS
Albumin (g/dL)	3.7±0.4	3.8±0.5	NS
HBeAg positive	1/9 (11.1)	25/44 (56.8)	0.01
HBV DNA (LGE/mL)	6.59±1.20	7.05±2.50	NS
Child classification (A/B/C)	7/2/1	24/15/5	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as *n* (%).

respectively (P = 0.85).

HBV genotype was entered into Cox regression analysis together with other variables that would influence prognosis. These included sex, age, HBeAg, HBV-DNA level, CLIP stage and therapy for HCC. The multivariate analyses revealed that independent unfavorable factors of overall survival included CLIP stage and lack of therapy for HCC (Table 5). However, the HBV genotype was not selected as an independent predictor of survival.

DISCUSSION

Identification of host and viral factors leading to severe liver damage and to the development of HCC may have important clinical implications in the management of patients with 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 studies on HBV genotype and its clinical relevance have been performed in Asia and restricted to comparisons between genotypes B and C, which are the two most common HBV genotypes in this region accounting for more than 90% of cases[6,16,17]. Current available data from this region demonstrate that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B[4-7]. In addition, patients with genotype C infection, compared to those with genotype B, are more frequently HBeAg positive and display higher HBV-DNA levels that may contribute to multiple episodes of acute flares and progression of liver disease[18]. Taken together, these data suggest that patients with genotype C have a tendency to exhibit more severe liver disease than those with genotype B.

In agreement with previous studies[17,19], our study demonstrated that genotype C and B were the predominant strains, accounting for approximately 75% and 20% of patients, respectively. In this respect, it would appear that the prevalence of HBV genotypes in Thailand is comparable to that reported from Japan and China[6,16], but differs from the distribution observed in Taiwan, where HBV genotype B is more common than genotype C[4,20]. Interestingly, the prevalence of genotype B and C in patients with HCC in our study was comparable to that in asymptomatic carrier, chronic hepatitis and cirrhosis. The equal distribution of genotypes B and C among various stages of chronic liver disease is consistent with previous reports conducted in Japan[6,11], but it contradicts the observations from other studies[4,7,16]. Hence, our data suggest that although genotype C is the most prevalent strain in Thailand, the risk of development of HCC may not be different between genotypes B- and C-related chronic liver disease.

The predominance of HBV genotypes B and C allows the comparison of clinical outcomes of patients who are chro-nically infected with these two HBV strains. Our results showed that the mean ages among asymptomatic carriers and chronic hepatitis were comparable between patients with genotypes B and C. However, the mean age of patients with genotype C tended to be older than those with genotype B in cirrhotic group. Interestingly, the divergence in the mean age of patients with genotypes B and C was more noticeably in those with HCC. 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

Table 4 Demographic and clinical data of patients with HCC

Characteristics	Genotype B (n = 23)	Genotype C (n = 50)	P
Age (yr)	61.1±9.8	51.3±13.1	0.001
Sex (male/female)	20/3	38/12	NS
Total bilirubin (mg/dL)	2.1±2.0	2.9±4.9	NS
ALT (IU/L)	95.5±102.6	121.4±126.1	NS
Albumin (g/dL)	3.4±0.7	3.5±0.5	NS
HBeAg positive	4/22 (18.1)	11/49 (22.4)	NS
HBV DNA (LGE/mL)	6.71±1.62	6.52±2.63	NS
AFP (IU/mL)	42 485.2±95 590.9	42 032.6±89 382.8	NS
CLIP score (0-1/2-3/4-6)	5/7/11	8/25/17	NS

Quantitative variables are expressed as mean±SD. Categorical variables are expressed as n (%).

Table 5 Multivariate analysis of unfavorable factors of survival in patients with HCC, by using Cox regression analysis

Factors	Risk ratio	95%CI	P
CLIP stage	4.63	1.70-12.62	0.003
No therapy	6.22	2.54-15.22	0.001

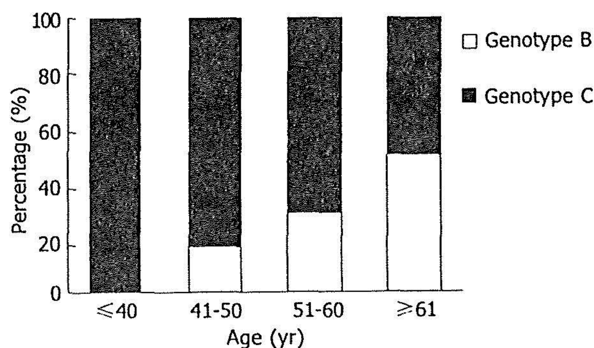

Figure 1 HBV genotypes B and C in 73 patients with HCC.

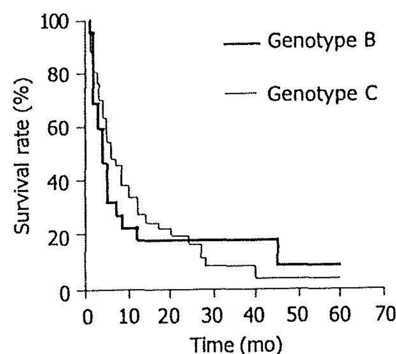

Figure 2 Overall survival of HCC patients with HBV genotypes B and C.

surrogate for the duration of HBV infection, regardless of the viral genotype. Our results also showed that patients with genotype C had a tendency of higher ALT, necroinflammatory scores and HBV-DNA levels than patients with genotype B. Moreover, patients with genotype C had a significantly higher prevalence of HBeAg positivity, particularly among patients with chronic hepatitis and cirrhosis, but the difference seemed to be disappearing upon the disease progression to HCC. Although HBeAg is a marker of active viral replication, the disappearance of HBeAg with or without seroconversion of antibody to HBeAg during the course of chronic infection does not always imply disease remission[21]. Collectively, it is reasonable to speculate that, at least in our populations, patients with genotype C have a trend for delayed HBeAg seroconversion and more prolonged necroinflammatory process causing earlier development of cirrhosis and HCC. Nonetheless, it would appear that there is no difference in the risk between patients with genotypes B and C in the progression to liver cancer.

Indeed, it is currently unclear whether a certain HBV genotype is associated with a greater risk for progression of cirrhosis to HCC. Studies from Taiwan and Japan have demonstrated an increase in HCC development among patients with HBV genotype C compared to genotype B[4,6,12,20]. Similarly, a study conducted in China has suggested that genotype C may predispose to HCC, whereas genotype B has a relatively better prognosis[16]. On the contrary, recent studies from Hong Kong and Japan have shown that there is no difference in the risk of developing HCC between patients with genotypes B and C[11,22]. Moreover, a potential correlation between HBV genotype and the age of the patients with HCC has been debated. Intriguingly, the report from northern Taiwan has observed that genotype B is associated with the development of HCC in patients younger than 35 years of age, while those with genotype C more frequently develop cancer after 50 years of age[4]. This observation, however, has not been confirmed by subse-quent studies from southern Taiwan and Japan[6,11,20]. In southern Taiwan, for example, there is no significant difference in the mean age between HCC patients with genotypes B and C[20]. By remarkable contrast, the mean age of Japanese patients with genotype B is approximately 70 years compared to 55 years of those with genotype C[6]. In the present report, the mean age of HCC patients with genotype B was significantly older than those with genotype C (61 and 51 years, respectively). Thus, the age distribution of HBV genotypes in Thai patients with HCC seems to correspond with the report from Japan, but differs from those studies from Taiwan.

The molecular virological factors responsible for this discrepancy among countries remain largely unknown. It has been postulated that the difference in the mean age between Taiwanese and Japanese patients with HCC may be partially influenced by the divergence of HBV subtypes distributed among different geographic areas[23]. Recently, two subtypes of HBV genotype B, namely Ba and Bj, have been identified based on the phylogenetic analysis. It has shown that genotype Ba consists of the recombination with the precore/core region originating from genotype C, whereas genotype Bj does not[24]. Genotype Bj is exclusively found in Japan, while genotype Ba is ubiquitous in other countries in Asia, including Thailand[25]. Based on our data, however, this postulation could not clarify the similarity in the mean age between Thai and Japanese patients with HCC, and the diversity between Thai and Taiwanese patients. Thus, it is likely that other as yet unrecognized virological factors might act as potential variables influencing the development of HCC in patients with chronic liver disease, even though they are infected with HBV of the same genotype. In addition, discrepancies regarding the role of HBV genotype might be related to variability of host and environmental factors in different geographic areas, such as genetic polymorphism and aflatoxin exposure.

Regarding the impact of HBV genotypes on the prognosis of HCC, a prospective study in Japan demonstrates that patients with genotype C tend to have relatively poor clinical outcomes after transcatheter arterial embolization (TACE) therapy compared with those with genotype B[26]. This finding is consistent with a recent report from Taiwan indicating that patients with genotype C exhibit a greater recurrent rate after curative resection of the tumor compared with those with genotype B[27]. However, our findings showed that the clinical features at presentation and overall survival of patients with HCC did not depend on the HBV genotype. The similarity of the tumor characteristics and clinical outcome of patients with genotypes B and C in this study was supported by the data of a recent case-control report conducted in Hong Kong[28]. Therefore, it would appear that in Thai populations, once HCC has developed, the course of the disease might be independent of the underlying HBV infection because the proportion of surviving patients is similar, irrespective of the infecting genotype. It should also be noted that overall median survival observed in this study was much shorter compared to other reports. The low survival rates in Thai patients with HCC in this study in part resulted from advanced stages of the cancer at the time of the diagnosis, while only minorities had an early detection in the course of follow-up programs.

In summary, our study demonstrated that patients with HBV genotype C, compared to those with genotype B, had a higher positive rate of HBeAg and exhibited earlier progression of cirrhosis and HCC. Genotype by itself, however, might not be responsible for an increased oncogenic effect because there was no difference in the risk of developing HCC and its prognosis between patients with genotypes B and C. Further large-scale prospective studies, which offer advantages over cross-sectional investigations, are needed to establish the existence of these observations in the future.

ACKNOWLEDGMENTS

This study was supported by the Thailand Research Fund [Research Scholar (PT), and Senior Research Scholar (YP)], and Center of Excellence, Viral Hepatitis Research Unit, Chulalongkorn University.

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Science Editor Li WZ Language Editor Elsevier HK

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*

J Med Assoc Thai 2004; 87 (Suppl 2): S223-7
e-Journal: *http://www.medassocthai.org/journal*

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°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 (± 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.

Acknowledgements

The authors wish to thank the center of excellence fund, Rachadaphisek Somphot Endowment, Chulongkorn University and the Thailand Research Fund for supporting this research.

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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*

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Journal of Virological Methods

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Simultaneous quantitation and genotyping of hepatitis B virus by real-time PCR and melting curve analysis

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Received 28 January 2004; received in revised form 30 March 2004; accepted 15 April 2004

Available online 7 June 2004

Abstract

Hepatitis B virus (HBV) genotype and HBV DNA levels have been implicated in clinical evaluation and prognosis of patients with chronic HBV infection. The aim of the present study was to develop a rapid and sensitive method for simultaneous HBV DNA quantitation and differentiation between HBV genotypes B and C in a single-step reaction by real-time PCR and melting curve analysis using SYBR Green I fluorescent dye. The genotypes obtained by this method were compared with those examined by PCR-RFLP and direct sequencing on 52 serum samples of patients with chronic HBV infection. Using the results obtained by direct sequencing and phylogenetic analysis as the reference, the accuracy of HBV genotyping by PCR-RFLP and melting curve analysis was 90.38 and 92.31%, respectively. The geometric mean of HBV DNA levels was 3.42×10^6, 2.10×10^6, 1.19×10^5 and 3.10×10^4 copies/μl in asymptomatic carriers, patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma, respectively. It is concluded that this method has the advantages of rapidity, reproducibility and accuracy, which would be feasible and attractive for large-scale analysis, particularly in regions where HBV genotypes B and C are prevalent.
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Keywords: Real-time PCR; Melting analysis; Genotypes; HBV

1. Introduction

Hepatitis B virus (HBV) infection is a public heath problem worldwide with an estimated 350 million people are infected chronically with the virus (Maddrey, 2000). Chronic HBV infection is associated with a diverse clinical spectrum, ranging from asymptomatic carrier status over chronic hepatitis and cirrhosis to hepatocellular carcinoma (HCC). HBV, a member of the hepadnaviridae, has a relaxed-circular, partially double stranded DNA genome of approximately 3200 nucleotides, and has been classified into eight genotypes (A–H) based on an inter-group divergence of more than 8% in the entire genomic sequence (Kidd-Ljunggren et al., 2002; Arauz-Ruiz et al., 2002). It has been shown that most HBV genotypes have distinct geographical distributions. For instance, genotypes A and D are predominant in Western countries and India, whereas genotypes B and C prevail in Southeast Asia, China and Japan. Genotype E is restricted to Africa and genotype F is found in Central and South America.

Besides the differences in geographical distribution, there is growing evidence that the viral genotypes may influence the clinical outcomes of chronic HBV infection. In particular, among Asian patients who constitute approximately 75% of HBV carriers worldwide, it has been shown that HBV genotype C is associated with more severe liver diseases (Lindh et al., 1999; Kao et al., 2000a; Orito et al., 2001a; Chan et al., 2003), and it also has a lower response rate to antiviral treatment than genotype B (Kao et al., 2000b; Wai et al., 2002). These data suggest that the determination of HBV genotypes, especially genotypes B and C may constitute an essential part of the clinical evaluation in patients with chronic HBV infection. Currently, HBV genotyping is performed mainly by restriction fragment length polymorphism (RFLP) (Lindh

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doi:10.1016/j.jviromet.2004.04.012

et al., 1998), post-PCR hybridization or line probe assay (Grandjacques et al., 2000), genotype-specific primers (Naito and Abe, 2001) and direct sequencing (Norder et al., 1990). However, all these methods are time-consuming and require post-PCR manipulations. Thus, they are not suitable for routine clinical practices when population-based testing is required. As a result, there is a need for rapid, high-throughput and accurate assays to identify HBV genotypes.

Determination of HBV DNA concentrations in serum has also become an essential tool to identify patients with high viral replication, and to predict whether antiviral therapy will be successful (Mommeja-Marin et al., 2003). There is mounting evidence indicating that quantitative assays of HBV DNA using real-time polymerase chain reaction (PCR) are highly reproducible and much more sensitive than the currently used branched-chain DNA (bDNA) method (Abe et al., 1999; Pas et al., 2000; Loeb et al., 2000; Chen et al., 2001; Jardi et al., 2001; Yates et al., 2001). In this study, a method for simultaneous quantitation of HBV DNA and genotyping to distinguish between genotypes B and C was performed by real-time PCR using SYBR Green I fluorescent dye. This novel assay allows real-time detection of PCR products and genotype determination by melting curve analysis. The accuracy of HBV genotyping ascertained by this method was compared to that obtained by RFLP and direct sequencing. The data suggest that this new method has the advantages of rapidity, reproducibility and accuracy, which would be both feasible and attractive for large-scale testing, particularly in regions where genotypes B and C are highly prevalent.

2. Materials and methods

2.1. Clinical samples

Serum samples investigated in this study were obtained from patients with chronic HBV infection, who had attended The King Chulalongkorn Memorial Hospital, Bangkok, Thailand. All specimens were stored at −70 °C until tested. The use of the sera was approved by the University Ethics Committee, and written consent was obtained from all patients. To validate the genotyping assay, 52 samples were randomly selected and divided into the four groups listed below based on the clinical spectrum of chronic HBV infection: 12 samples from HBV asymptomatic carrier, 16 samples from patients with chronic hepatitis B, 12 samples from patients with cirrhosis and 12 samples from patients with HCC.

2.2. HBV DNA extractions

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

2.3. HBV DNA detection

HBV DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg, NJ), using the primer sequences described previously (Lindh et al., 1998). The forward primer was P1 (nt. 2823–2845: 5′-TCACCATATTCTTGGGAACAAGA); the reverse primer was P2 (nt. 80–61: 5′-TTCCTGAACTGGAGCCACCA). The primers were located in conserved genomic regions 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 μL of 2.5× Eppendorf MasterMix (Hamburg, Germany), 1 μM P1, 1 μM P2 and sterile water, in a final volume of 50 μL. 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 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C, followed by a final 10 min extension at 72 °C. Each amplified DNA sample (10 μL) was added to loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME) at 100 V for 60 min. The 479-bp product stained with ethidium bromide on preparation was visualized on a UV transilluminator.

2.4. 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 μL of PCR product were mixed with 1.5 μL of 10× buffer, 3 μL of sterile water and 0.5 μL (5U) of *Ava*II and *Dpn*II, respectively, in separate reactions and incubated at 37 °C for 3.5 h. 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 (Lindh et al., 1998).

2.5. Direct sequencing

For automated DNA sequencing, the PCR products of interest were purified from the gel using the Gel Extraction Kit (Perfectprep Gel Cleanup, Eppendorf, Hamburg, Germany) according to the manufacturer's specifications. The sequencing reaction was performed using the Gene Amp PCR System 9600 (Perkin-Elmer, Boston, USA). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston, USA). The results were analyzed and HBV genotypes were determined by BLAST

analysis. HBV DNA sequences were also subjected to phylogenetic analysis.

2.6. Phylogenetic analysis and HBV genotyping

New sequences obtained in this study have been submitted to GenBank and assigned accession numbers: AY486238-AY486289 (S1–S52). The following genome sequences of eight different HBV genotypes (A–H) were obtained from GenBank and used as reference sequences for the phylogenetic tree: (A: AJ344115, B: AB33554, AY167101, C: AF157113, AY206393, D: AJ344116, E: AB106564, F: AB036919, G: AF405706 and H: AY090460).

Nucleotide sequences were multiple aligned using the CLUSTAL X program (version 1.8). TREEVIEW (version 1.5) was run for phylogenetic tree construction. Those sample sequences appearing on the same node with reference sequences were interpreted to be of the same genotype.

2.7. Quantitation of HBV DNA and melting curve analysis

Samples previously identified as HBV genotypes B and C served as controls for developing the genotyping assay based on real-time PCR with melting curve analysis. The HBV plasmid DNA standard was constructed by inserting the *PreS* region (nt. 2814–80) into pGEM-T Easy Vector (thereafter called pGEM-*PreS*) through T-A cloning strategy. The concentration of the pGEM-*PreS* plasmid was determined by measurement of OD_{260} and verified by agarose gel electrophoresis (data not shown). Serial 100-fold dilutions of the pGEM-*PreS* plasmid DNA from 10^2 to 10^{10} copies/μL were detected by real-time PCR assay and used to prepare the standard curve for quantitation of HBV DNA from patient specimens. The standard plasmid DNA for quantitation and control plasmid DNA for genotyping were included in each run as an external standard of the real-time PCR assay.

The primers used in this analysis allowed the amplification of a 280-bp product in *PreS*1 region. The forward primer was *PreS*1F (nt. 2814–2835: 5′-GGGTCACCATATTCT TGGGAAC-3′); the reverse primer was *PreS*1R (nt. 3094–3075: 5′-CCTGAGCCTGAGGGCTCCAC-3′). The primers are conserved among over 150 known sequences obtained from GenBank corresponding to different genotypes. The combination of 1.0 μL of DNA sample with a reaction mixture containing 10 μL of 2× QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 0.5 μM *PreS*1F, 0.5 μM *PreS*1R and RNase-free water were used in a final volume of 20 μL (the final $MgCl_2$ concentration was 2.5 mM per reaction).

Real-time PCR amplification was carried out in a LightCycler™ (Roche, Basel, Switzerland). After a preincubation step at 95 °C for 15 min in order to activate the HotStarTaq DNA polymerase, amplification was performed during 40 cycles including denaturation (94 °C, 15 s with a temperature transition rate of 20 °C/s), annealing (60 °C, 20 s, with a temperature transition rate of 20 °C/s) and extension (72 °C, 25 s with a temperature transition rate of 20 °C/s). A single fluorescent signal was obtained once per cycle at the end of the extension step with detection channel F1. After amplification, melting curve analysis was performed on the products by heating to 95 °C for 5 s, cooling to 75 °C for 15 s, followed by a temperature increase to 98 °C with a temperature transition rate of 0.05 °C/s while continuously collecting the fluorescent signal.

To analyze the data of the real-time PCR assay, base line adjustment was carried out in the 'Arithmetic' mode and fluorescence curve analysis was carried out in the 'Fit Points' mode of the LightCycler data analysis software, version 3.5 (Roche, Basel, Switzerland). A standard curve was created automatically in each run by plotting the threshold cycle number against the copy numbers of each standard, and HBV DNA quantitation of unknown samples was deduced from the regression line. The derivative melting curves were also obtained with the LightCycler data analysis software, version 3.5 (Roche). The melting temperature (T_m) of the melting curve in each sample was used to identify the HBV genotype. The samples whose melting curves shared the same T_m point with the control genotypes were interpreted as belonging to the same genotype.

2.8. Data analysis

All samples were genotyped blindly by RFLP, sequencing and melting curve analysis without any conscious bias. HBV genotypes determined by different methods were also compared. The results of direct sequencing were used as a standard in the comparison with the other methods. To confirm the melting curve analysis, the test was repeated on some of the specimens and yielded identical results. HBV DNA levels corresponding to different clinical phases were analyzed and presented as geometric means. Data were analyzed using SPSS for Windows version 11.5 software package. Statistical analyses were performed using Mann–Whitney test for comparison of HBV DNA levels among each group. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Preliminary real-time PCR genotyping assay

Three preliminary real-time PCR assays with melting curve analysis suggested that a pair of primers (*PreS*1F/*PreS*1R), which yield a 280-bp product amplified from the *PreS*1 region was effective to discriminate between the melting peaks of genotypes B and C. In addition, the reaction occurred without any unexpected PCR product or primer dimers (data not shown). There were no unwanted fluorescent signals that interfered with the genotyping assay. The mean T_m values for genotypes B and C were 85.56 and 83.86 °C, respectively. Therefore, the cut-off value was

Table 1
Intra- and inter-assay variation of $T_m s$ for tested specimens

Genotype	Variation of T_m (°C)					
	Intra-assay		Inter-assay			
	Interval	Range	Interval	Range	Mean (SD)	CV
B	84.98–85.49	0.51	84.95–85.87	0.92	85.29 (0.29)	0.34
C	83.46–84.33	0.87	83.40–84.33	0.93	84.02 (0.25)	0.30

set at 84.71 °C (mid-point between B and C) and used it to interpret the genotype of unknown samples.

3.2. Variability of T_m for genotypes B and C

The variation in T_ms found for each HBV genotype tested is presented in Table 1. The intra-assay variation was indicated from runs with more than one sample of a specific genotype. This analysis included all samples with concordant results between melting curve analysis and sequencing method. The coefficient of variation (CV) of $T_m s$ (inter-assay) was 0.34 and 0.30 for genotype B and C samples, respectively.

3.3. HBV genotyping by three different methods

Among 52 samples tested by RFLP, one was untypeable, three were typed as A, 11 were typed as B and 37 were typed as C. According to the genotyping results by melting curve analysis, 11 samples were typed as B and 41 samples were typed as C. Fig. 1 shows a representative genotyping experiment by real-time PCR with melting curve analysis using SYBR Green I. Based on the results of direct sequencing, one sample was typed as A, 14 samples were typed as B and 37 samples were typed as C. The data obtained from direct sequencing were aligned with the reference genotype sequences and analyzed; the phylogenetic tree is shown in Fig. 2.

3.4. Comparative HBV genotyping assays

To compare this new method with RFLP and sequencing, 52 patient samples were blindly tested by all three methods. Table 2 shows the results of genotyping by RFLP, direct sequencing and melting curve analysis. The results obtained by direct sequencing were used as a standard to compare with the other methods. Among 52 samples tested, one was untypeable (S22) and four were wrongly typed (S3, S29, S32 and S33) by RFLP when comparing the results with direct sequencing. Discrepancies between the real-time PCR with melting curve analysis and the direct sequencing method occurred in four of 52 samples (S3, S22, S33 and S36). Therefore, the accuracy of HBV genotyping by RFLP and melting curve analysis was 90.38 and 92.31%, respectively.

As an illustration of the different results obtained from melting curve analysis and direct sequencing, samples S3,

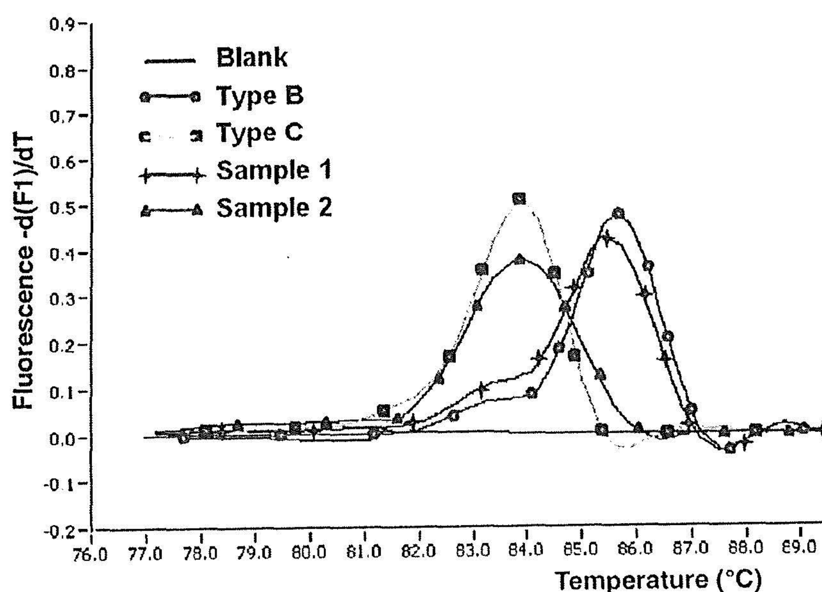

Fig. 1. Representative genotyping experiment by melting analysis based on real-time PCR using SYBR Green I. The melting point peaks were clearly separated between genotypes B and C using *PreS1*F/*PreS1*R at 2.5 mM $MgCl_2$.

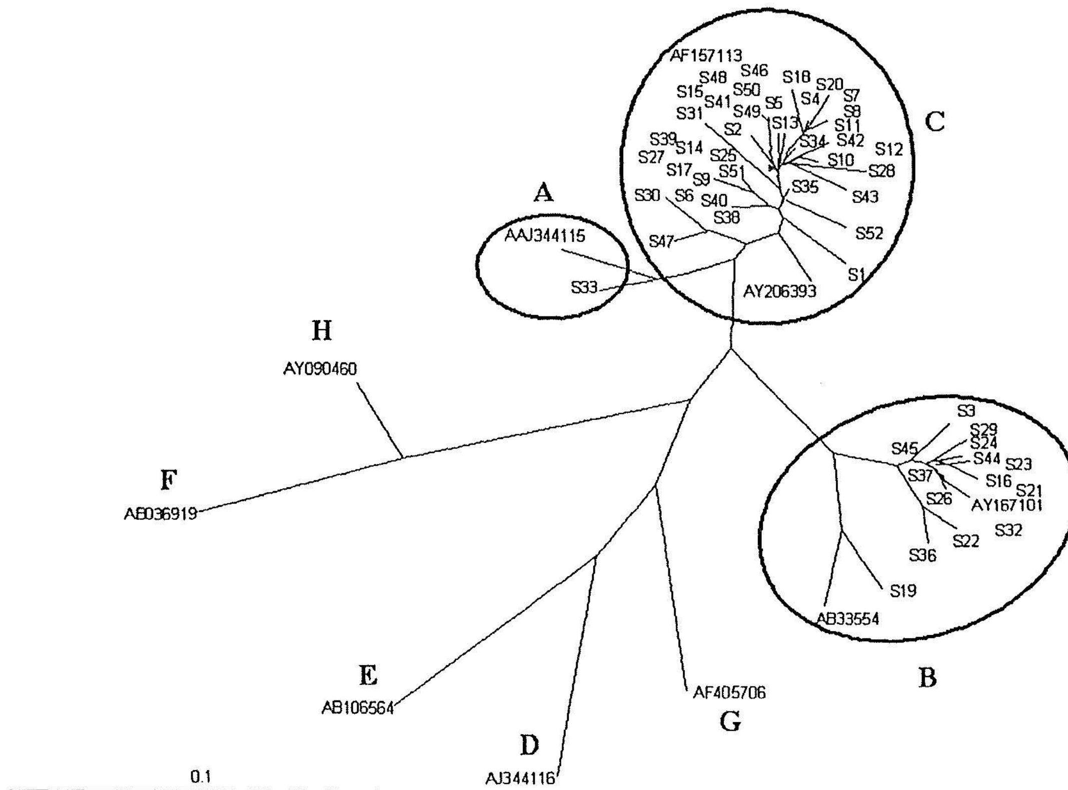

Fig. 2. Phylogenetic tree analysis of the *PreS1* region (nt. 2881–3216) of 52 sample sequences used in this study. Sample numbers are identical to those indicated in Table 2.

S22 and S36 were identified as genotype B by direct sequencing but were determined as genotype C by melting curve analysis. For sample S33, melting analysis indicated genotype C, whereas direct sequencing yielded genotype A. Furthermore, the percentage of GC content (between nt. 2881–3094) was calculated in each sample based on the DNA sequence. The percentages of GC content were 57.94 and 56.07 for the controls of genotypes B and C, respectively. With the incongruous sample S3, the percentage of GC content was 57.01 ($T_m = 84.59$) and thus, the melting curve indicated genotype C instead of genotype B. In the same way, S22 and S36 had a 56.07 and 56.54% of GC content, respectively. Therefore, these samples displayed discrepancy of genotyping results due to GC content variations.

3.5. Analysis of the percentages of GC content from GenBank sequences

To evaluate whether melting curve analysis is useful for discriminating between HBV genotypes B and C in other parts of Asia, GenBank sequences obtained from Japan, Taiwan, China, Indonesia, Vietnam, Myanmar and the Philippines were analyzed (accession numbers AB106884, AY167098, AY169097, AY167093-4, AY206390-1, AY518556, AF282917-8, and AB033555 for genotype B; accession numbers AB033552, AB115418, AY167099, AY167095-6, AY167091-2, AY206388, AF461363, AB112065, AB111946, AB112063, AB112348, AB112408, and AB112066 for genotype C). Based on the analysis of these sequences, the mean percentages of GC content were 58.24 ± 0.43 and 55.86 ± 1.13 for genotypes B and C, respectively, while the mean percentages of GC content in this study were 58.03 ± 0.50 and 55.88 ± 0.56, respectively. Therefore, expected T_m for genotypes B and C in other regions should be comparable with the values described in this study. These data suggest that the method described here could also be applied in other parts of Asia.

Since genotypes A and D may occur in regions where genotypes B and C are prevalent, some GenBank sequences obtained from other countries in Asia were also analyzed for their GC content (accession numbers AB116081, AB166080, AB116079, AB116086-7, AY373429, AB116082-4, and AB116091-4 for genotype A; accession numbers AB116266, and AY373430 for genotype D). In this respect, the mean percentages of GC content were 56.61 ± 0.60 and 57.24 ± 0.43 for genotypes A and D, respectively. However, it is expected that genotype D may have a T_m value different from those of other genotypes due to a 33-bp deletion in its *PreS1* region.

Table 2
HBV genotypes of 52 samples determined by three different methods

Sample no.	Group	HBV eAg	RFLP	Sequence	Melting	T_m (°C)	%GC	DNA levels (copies/μl)	Accession no.
S1	HCC	–	C	C	C	84.20	56.07	1.35×10^3	AY486238
S2	CH	+	C	C	C	84.17	56.07	4.49×10^5	AY486239
S3	HCC	+	A	B	C	84.59	57.01	9.52×10^5	AY486240
S4	CH	+	C	C	C	84.17	56.07	3.02×10^5	AY486241
S5	CH	+	C	C	C	83.97	56.07	4.40×10^5	AY486242
S6	LC	+	C	C	C	84.33	56.07	4.05×10^7	AY486243
S7	LC	+	C	C	C	84.04	56.07	1.98×10^5	AY486244
S8	CH	+	C	C	C	84.17	56.07	1.01×10^6	AY486245
S9	LC	+	C	C	C	83.49	55.61	6.34×10^2	AY486246
S10	LC	+	C	C	C	83.88	56.07	2.68×10^3	AY486247
S11	LC	+	C	C	C	83.85	55.14	2.52×10^4	AY486248
S12	LC	+	C	C	C	84.20	55.61	2.00×10^4	AY486249
S13	LC	+	C	C	C	84.23	56.07	1.88×10^5	AY486250
S14	HCC	–	C	C	C	83.46	56.54	8.03×10^3	AY486251
S15	CH	+	C	C	C	84.19	55.61	1.53×10^6	AY486252
S16	CH	+	B	B	B	85.42	58.41	3.31×10^6	AY486253
S17	CH	+	C	C	C	84.14	56.07	5.46×10^6	AY486254
S18	CH	+	C	C	C	84.10	55.61	2.34×10^5	AY486255
S19	CH	+	B	B	B	84.98	57.48	1.24×10^7	AY486256
S20	LC	+	C	C	C	84.07	55.61	3.27×10^6	AY486257
S21	LC	–	B	B	B	85.16	57.94	9.83×10^6	AY486258
S22	LC	+	Unt	B	C	84.23	56.07	3.01×10^6	AY486259
S23	LC	–	B	B	B	84.95	57.48	1.94×10^4	AY486260
S24	CH	–	B	B	B	84.95	57.94	3.15×10^4	AY486261
S25	CH	+	C	C	C	84.00	55.61	1.66×10^6	AY486262
S26	CH	–	B	B	B	85.26	58.41	6.17×10^7	AY486263
S27	CH	+	C	C	C	84.14	56.07	1.12×10^7	AY486264
S28	HCC	ND	C	C	C	84.33	56.07	5.65×10^7	AY486265
S29	ASC	–	A	B	B	85.42	57.48	4.76×10^5	AY486266
S30	HCC	–	C	C	C	84.62	57.94	9.46×10^5	AY486267
S31	CH	+	C	C	C	84.01	54.67	2.69×10^7	AY486268
S32	CH	+	A	B	B	85.00	57.48	1.40×10^5	AY486269
S33	ASC	+	B	A	C	84.49	57.01	3.19×10^7	AY486270
S34	ASC	+	C	C	C	84.10	55.14	8.43×10^6	AY486271
S35	ASC	+	C	C	C	84.17	56.07	2.83×10^7	AY486272
S36	ASC	+	B	B	C	84.20	56.54	3.20×10^6	AY486273
S37	ASC	+	B	B	B	85.42	58.41	6.40×10^7	AY486274
S38	ASC	+	C	C	C	84.23	56.07	1.29×10^7	AY486275
S39	ASC	+	C	C	C	84.23	55.61	1.21×10^8	AY486276
S40	ASC	+	C	C	C	84.20	56.07	5.77×10^5	AY486277
S41	ASC	+	C	C	C	84.23	56.54	2.12×10^5	AY486278
S42	ASC	–	C	C	C	84.04	55.61	3.45×10^3	AY486279
S43	LC	–	C	C	C	83.68	55.14	3.39×10^3	AY486280
S44	HCC	–	B	B	B	85.10	58.41	8.63×10^3	AY486281
S45	ASC	ND	B	B	B	85.71	58.88	5.28×10^6	AY486282
S46	HCC	–	C	C	C	84.04	55.14	4.38×10^5	AY486283
S47	HCC	–	C	C	C	83.62	56.54	1.61×10^3	AY486284
S48	HCC	ND	C	C	C	83.59	55.61	1.07×10^4	AY486285
S49	CH	+	C	C	C	84.07	55.14	2.09×10^8	AY486286
S50	HCC	–	C	C	C	84.07	55.61	1.89×10^3	AY486287
S51	HCC	–	C	C	C	83.40	56.07	1.12×10^3	AY486288
S52	HCC	+	C	C	C	84.30	56.54	1.05×10^4	AY486289

Abbreviations: ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Unt, untypeable; ND, not done.

3.6. Quantitation of HBV DNA by real-time PCR

Plasmid pGEM-*PreS* diluted serially from 10^2 to 10^{10} copies/μl was detected by real-time PCR assay. Fig. 3 shows that a series of fluorescence amplification plots were obtained with nearly equal intervals. A standard curve was then constructed plotting cycle threshold (Ct) values against the known copy number of each standard sample (Fig. 4). The standard curve showed good regression between copy number and Ct value ($r = -1.00$). The amount of HBV DNA in an unknown sample could be calculated by measuring the Ct value through the standard curve within an eight order of

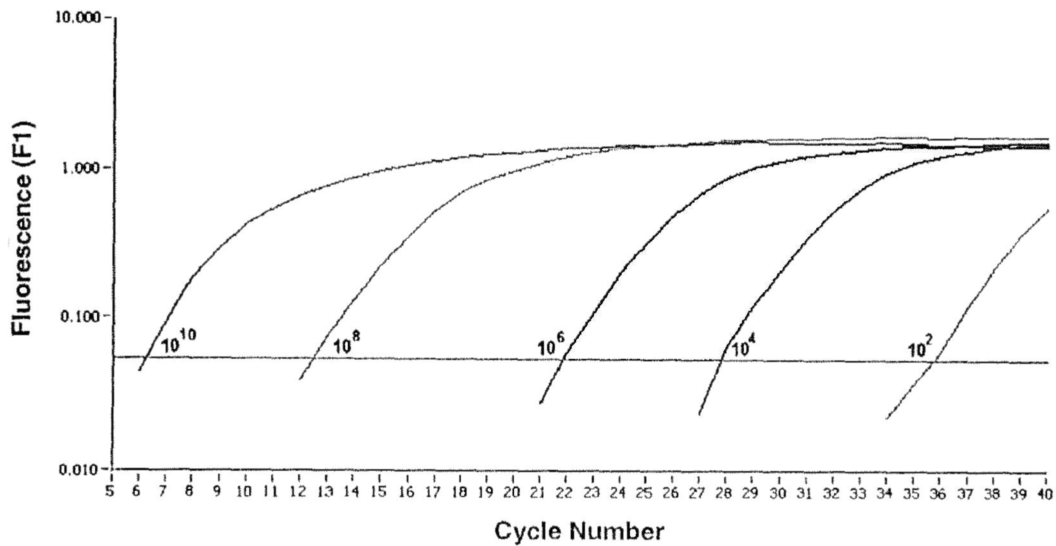

Fig. 3. Real-time PCR fluorescence curve derived from serial diluted standard concentration plasmid. Each plot corresponds to a particular input target copy number ranging from 10^{10} to 10^2. The x-axis indicates the PCR cycle number. The y-axis indicates the fluorescence intensity over the background.

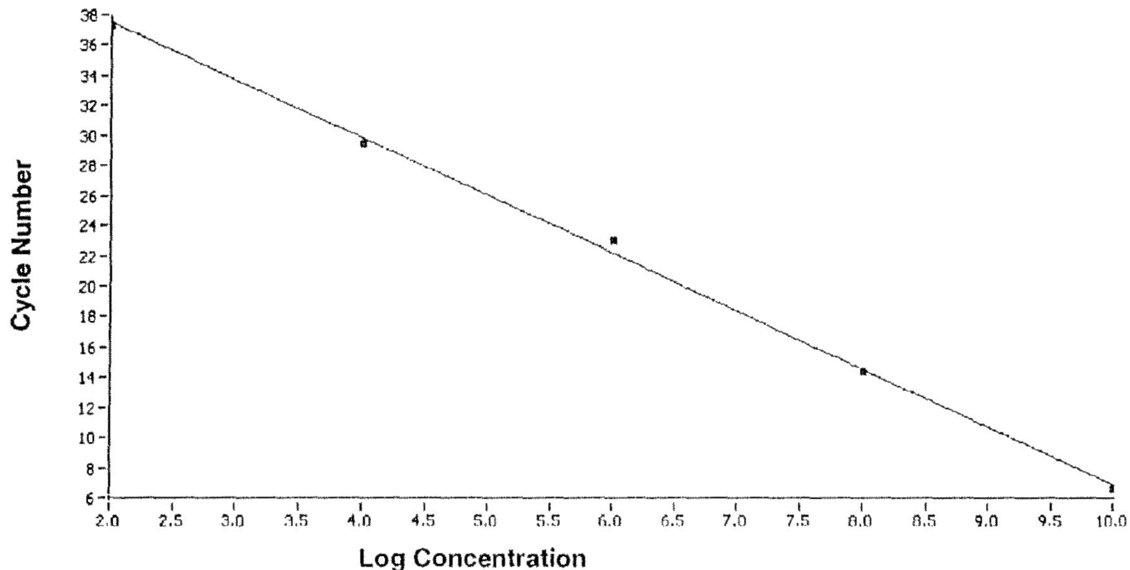

Fig. 4. Standard curve for real-time PCR. Serially diluted pGEM-*PreS* plasmid was amplified and analysed in real time. The threshold cycle (Ct) values were plotted against copy number to construct the standard curve, $r = -1.00$.

magnitude wide linear range. A minimum of 10^2 copies/μl of the standard plasmid could be constantly detected by the system. The results of the tested specimens were reported and confirmed reproducibility.

Fig. 5 shows comparative HBV DNA levels in different clinical phases of disease. The geometric means were 3.42×10^6, 2.10×10^6, 1.19×10^5 and 3.10×10^4 copies/μl in asymptomatic carriers, patients with chronic hepatitis, cirrhosis and HCC, respectively. The data demonstrated that HBV DNA levels tended to decrease with increasing severity of the diseases. In this respect, HBV DNA levels in patients with cirrhosis and HCC were statistically significant lower than those detected in asymptomatic carriers ($P < 0.05$).

4. Discussion

There are now substantial data suggesting that HBV genotypes may play an important role in causing different disease profiles in chronic HBV infection. However, almost all these studies on HBV genotype and its clinical relevance have been performed in Asia and restricted to comparisons between genotypes B and C, which are the two most

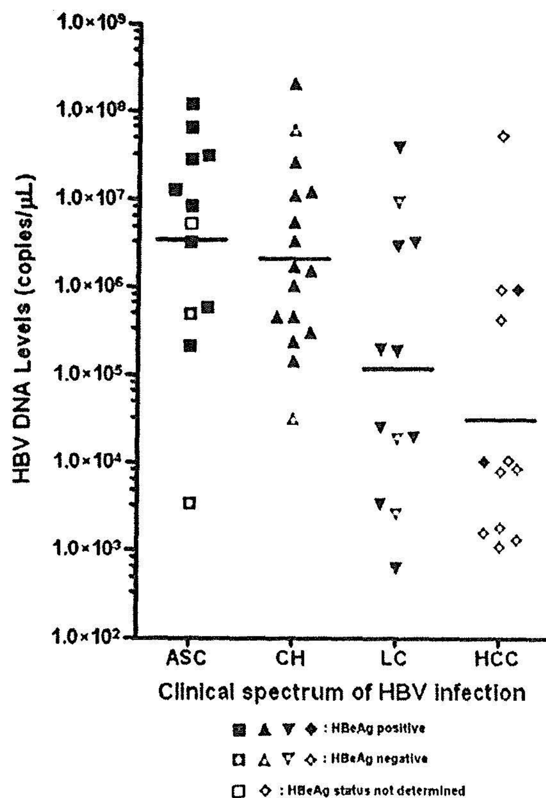

Fig. 5. Comparative geometric mean of HBV DNA levels among different clinical spectrum of chronic HBV infection: asymptomatic carrier (ASC), chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma. The horizontal lines indicate geometric mean of HBV DNA levels. The levels of HBV DNA in LC and HCC patients were significantly lower than those found in ASC patient ($P < 0.05$).

common HBV genotypes in this region accounting for more than 90% of cases (Theamboonlers et al., 1999; Kao et al., 2000a; Orito et al., 2001a; Ding et al., 2001). Current data demonstrate that HBV genotype C is more commonly associated with severe liver diseases and the development of cirrhosis compared to genotype B (Lindh et al., 1999; Kao et al., 2000a; Orito et al., 2001b; Chan et al., 2003). Although the association between HBV genotype and the risk of progression to HCC is still conflicting (Kao et al., 2000a; Orito et al., 2001b; Fujie et al., 2001), a recent study indicates that HCC patients with genotype C have a relatively poor clinical outcomes compared to patients with genotype B (Tsubota et al., 2001). Genotype C is also associated with a higher frequency of core promoter mutation and a lower response rate to alpha interferon therapy (Kao et al., 2000b; Wai et al., 2002). In addition, patients with genotype C infection, compared to those with genotype B, are more frequently HBeAg positive and display higher HBV DNA levels that may contribute to multiple episodes of acute flares and progression of liver disease (Kao et al., 2002). Taken together, these data emphasize the need for a simple genotyping method to discriminate between HBV genotypes B and C, particularly in regions where these genotypes are highly predominant.

In contrast to the methods used in prior studies, the technique employed in this study permitted genotyping and quantitation of HBV in a single-tube PCR reaction. Currently, HBV quantitation and genotyping have been evaluated by separated assays, which have increased both time and expenditure. For quantitation of HBV DNA, the bDNA assay has been so far the method applied most frequently mainly due to its reproducibility (Hendricks et al., 1995), but its low sensitivity is a major limitation. The commercial quantitative PCR assay has a higher sensitivity (Noborg et al., 1999), but the variation within the lower detection range is high, and the linear range is relatively narrow requiring pre-dilution of high-titer samples. Thus, the development of quantitative techniques that are sensitive and reproducible, such as real-time PCR, offers a new tool for precise and linear quantitation of HBV DNA over a wide range of concentrations. Likewise, assays for genotyping based on conventional PCR such as RFLP, line probe assay and direct sequencing are time-consuming and may increase the risk of cross-contamination. To solve these problems, the method described here has, for the first time, allowed simultaneous quantitation of HBV DNA by real-time PCR and determination of HBV genotypes B and C on the basis of melting curve analysis.

Melting curve analysis in conjunction with real-time PCR was first introduced in 1997 (Riric et al., 1997; Lay and Wittwer, 1997). Following this technique, detection of amplification products and analysis of the melting curves can either be performed by hybridization probes or by applying the nonspecific DNA binding fluorescent dye SYBR Green I. Genotyping assays based on hybridization probes are theoretically accompanied by higher specificity, but the requirement for oligonucleotides with fluorescent label significantly increases the expenditure. In contrast, SYBR Green I is less expensive and could be developed in an economical manner. In addition, this simpler method can be performed with any established PCR primers with only minor modifications of the described protocols. Hence, genotyping assay based on real-time PCR using SYBR Green I fluorescence may be more appropriate for large-scale molecular analysis compared to hybridization-based methods. Using the LightCycler system, amplification products can be detected by the dye being intercalated in minor groove of double-stranded DNA, and genotype determination can be performed by analysis of the melting curves. As shown in this study, the T_m values are directly related to the nucleotide sequence variations (percentage of GC contents) of corresponding genotypes, if other external and internal factors such as $MgCl_2$ concentration and the length of the PCR products are controlled (Wittwer et al., 1997).

For genotyping by melting curve analysis, a set of primers (*PreS*1F/*PreS*1R) was used to amplified the *preS*1 region, which has been shown to be the most variable part of the viral genome yet, remarkably conserved within each genotype (Lindh et al., 1998). The preliminary results demonstrated that HBV genotypes B and C could be discriminated by

means of distinct T_m values (85.56 and 83.86 °C, respectively). Moreover, the T_m values of corresponding genotypes were highly reproducible with relatively small intra- and inter-assay variability in the study of an additional 52 samples. In this report, the coefficient of variation (CV) for the T_m values of HBV genotypes B and C were as low as 0.34 and 0.30, respectively. The accuracy of the assay was further validated by comparison with the results obtained by RFLP and direct sequencing. In fact, RFLP is currently regarded as the most commonly used method, whereas direct sequencing is the "gold standard" for identifying HBV genotypes. Using the results obtained by direct sequencing as the reference, the data showed that the accuracy of HBV genotyping by melting curve analysis was comparable to that determined by conventional RFLP analysis (approximately 92.3% and 90.4%, respectively). Altogether, these data suggest that the method described above is reliable for differentiating between HBV genotypes B and C, which represent the two most common genotypes worldwide.

Despite comparable in their accuracies, the real-time PCR-based technique offers several advantages over RFLP analysis in determining HBV genotypes. First, techniques based on RFLP analysis need PCR conditions of sufficient specificity to produce a clean amplification product that can be enzymatically digested and unambiguously analyzed by electrophoresis. Thus, interpretation using this method may be complicated by incomplete digestion of the amplicons. Secondly, real-time PCR-based genotypic assays are more amenable to high-throughput screening, as they do not require extensive post-amplification manipulation. Since the reaction is carried out in a single step in a closed system, the risk of carryover contamination following PCR amplification is considerably reduced. Thirdly, the time saved by the present approach is considerable. Using the real-time PCR technique, less than 2 h were required to complete setting up and performing a 40-cycle amplification and data analysis. Finally, the method described here also facilitates simultaneous quantitation of HBV DNA. As mentioned previously, measurement of HBV DNA concentrations in serum has become an essential tool to identify patients with high viral replication, and to predict the response to antiviral therapy (Mommeja-Marin et al., 2003). In this regard, the quantitative real-time PCR assay is highly sensitive as it can detect samples ranging from 10^2 to 10^{10} copies/μl as shown in this study, while the widely used bDNA method is limited to a range of approximately 10 to 10^6 copies/μl (Abe et al., 1999; Loeb et al., 2000). Thus, this quantitative PCR assay certainly assists in defining accurately levels of viral replication among patients with HBV infection.

Nonetheless, there were some discrepancies between HBV genotyping obtained by melting curve analysis and direct sequencing. These differences could be explained by the fact that sequence variations in the *preS1* region may complicate melting curve analysis for HBV genotyping by producing T_m values that differ from the values expected for genotypes B or C. Also, it could be argued that this method excludes the detection of other HBV genotypes, as it is designed to detect only HBV genotypes B and C. Moreover, due to the close proximity between the T_m values of genotypes A and C, and to the melting peaks exhibiting slight variations between different runs, discrimination between these two genotypes may sometimes prove difficult. Thus, this assay may not be suitable in some geographical regions, such as North America where different HBV genotypes are found as a result of immigration from countries with a high prevalence of HBV infection (Chu et al., 2003). These disadvantages are, however, negligible for clinical purposes when studies are performed in Asia. Considering that the prevalence of other genotypes in this region is very low and their clinical significance is still unclear, determination of HBV genotypes other than genotypes B and C appears to be irrelevant for therapeutic decisions. Notably, the method described here could also be applied in other geographical regions, such as Western Europe and India, where HBV genotypes A and D are highly prevalent. In fact, current data suggest that the viral genotypes in these areas may correlate with differences in clinical features among patients chronically infected with the virus. For instance, one recent study in Spain demonstrates that biochemical remission, clearance of HBV DNA, and clearance of HBsAg are more common among patients with genotype A than genotype D (Sanchez-Tapias et al., 2002). Likewise, a recent study in India has shown that genotype D is associated with more severe liver disease and may predict occurrence of HCC in younger patients (Thakur et al., 2002). Taking into consideration that HBV genotype D is characterized by a 33-bp deletion in the *PreS1* region (Locarnini et al., 2003), it is reasonable to speculate that such a deletion should facilitate the identification of this genotype by producing a T_m value very different from that of genotype A.

In conclusion, the real-time PCR-based method described in this study provides a rapid and effective way for simultaneous quantitation and genotyping of HBV, particularly for distinction between genotypes B and C, which prevail in Asia. Additional investigations performed on serum samples from other geographic regions are warranted to explore further the clinical value of this method.

Acknowledgements

This study was supported by the Thailand Research Fund (Senior Research Scholar (YP), Research Scholar (PT) and Royal Golden Jubilee Ph.D. Program (SP)) and Center of Excellence, Viral Hepatitis Research Unit, Chulalongkorn University for their generous support. We also would like to express our gratitude to the staff of the Viral Hepatitis Research Unit, Chulalongkorn University and Hospital for their dedication in the study. Lastly, we would like to thank Ms. Petra Hirsch and venerable Dr. Mettanando Bhikkhu of Wat Nakprok, Bangkok for reviewing the manuscript. The

authors have no conflicts of interest that are directly relevant to the content of this manuscript.

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Journal of General Virology (2005), 86, 3027–3030 DOI 10.1099/vir.0.81241-0

Short Communication

A novel recombinant of *Hepatitis B virus* genotypes G and C isolated from a Thai patient with hepatocellular carcinoma

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Received 6 June 2005
Accepted 19 August 2005

Genomic recombination between different genotypes of *Hepatitis B virus* (HBV) resulting in hybrid strains has been increasingly documented. In this study, a novel recombinant of HBV genotypes G and C isolated from a Thai patient with hepatocellular carcinoma is reported. Based on phylogenetic analyses of the S, P and X genes and the entire genome, the HBV isolate clustered on a branch within genotype G, but clustered with genotype C on analysis of the C gene. Using the program SIMPLOT and bootscanning analysis, the recombination breakpoints were located at nt 1860 and 2460 of the precore/core region. The hallmarks of the original genotype G, including a 36 bp insertion in the core region and dual stop codons in the precore region, were not identified in this isolate. These data should encourage further investigations on the epidemiological and virological characteristics of HBV genotype G involved in recombination with other genotypes.

Hepatitis B virus (HBV) is one of the major causes of chronic liver diseases, including chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC), which affect more than 350 million people worldwide (Ganem & Prince, 2004). HBV, a member of the family *Hepadnaviridae*, is a circular double-stranded DNA virus of approximately 3200 bp that encodes four overlapping open reading frames (ORFs). Based on sequence divergence in the entire genome of >8 %, HBV is currently classified into eight genotypes, designated A to H (Weber, 2005). The genotypes of HBV have distinct geographical distributions and may influence the clinical outcomes of patients with chronic infection (Schaefer, 2005). Genotypes A and D are common in Europe and North America, whereas genotypes B and C are highly prevalent in Asia. Genotypes F and H are restricted to Central and South America. Genotype E is found predominantly in West Africa and genotype G is found in the USA and Europe.

Hybrid HBV strains resulting from genomic recombination between different genotypes have been increasingly documented (Bollyky *et al.*, 1996; Bowyer & Sim, 2000; Fares & Holmes, 2002; Morozov *et al.*, 2000). For instance, phylogenetic analysis revealed that hybrids between HBV genotypes B and C, which have sites of recombination over the precore/core region, were found ubiquitously in Asian countries, except for Japan (Luo *et al.*, 2004; Sugauchi *et al.*, 2002). Similarly, hybrids between HBV genotypes A and D have been reported in Italy and South Africa (Morozov *et al.*, 2000; Owiredu *et al.*, 2001) and an aberrant recombinant between genotype C and a subgroup of genotype A was isolated in Vietnam (Hannoun *et al.*, 2000). Recently, hybrids of HBV genotypes C and D have been identified in Tibet and China (Cui *et al.*, 2002; Wang *et al.*, 2005).

In a previous study, the distribution of HBV genotypes in 332 Thai patients with chronic HBV infection was investigated by using PCR–restriction fragment-length polymorphism analysis (Tangkijvanich *et al.*, 2005). Our data showed that the most common HBV genotypes in this group were C, B and A, accounting for 73, 21 and 3 %, respectively. However, the genotypes of the remaining isolates could not be specified by using this technique. As a result, samples that could not be genotyped were sent for direct sequencing of the preS1 gene. Among these samples, an aberrant HBV strain belonging to genotype G was recovered from a Thai patient with HCC. Through a more extensive analysis, it was revealed that the isolate represented a novel HBV hybrid with genotype C in the precore/core region.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is DQ078791.

Here, the complete genomic sequence of this HBV isolate is reported. The isolate was from a patient (male, 47 years old) who was a resident in southern Thailand and had undergone follow-up at Chulalongkorn Memorial Hospital (Bangkok, Thailand) between February and April 1998. The patient was diagnosed with HCC by the presence of mass lesions in the liver on hepatic imaging and serum α-fetoprotein levels above 400 ng ml^{-1}. The patient was seropositive for HBsAg (ELISA; Abbott Laboratories), but negative for HBeAg (ELISA; Abbott Laboratories) and anti-HCV (ELISA; Ortho Diagnostic Systems). To determine the complete nucleotide sequence of the HBV isolate, DNA was extracted from 100 μl stored serum (−70 °C) by using proteinase K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. DNA pellets were dissolved in 30 μl sterile water and subjected directly to PCR-based amplification. PCR was performed by using a set of primers to amplify six overlapping fragments of the HBV genome as follows. In fragment 1, sense and antisense primers were CORE1 (5′-GAGTGTGGATTCGCACTCCTCC-3′; nt 2268–2289) and R1 (5′-TGTAACACGAGCAGGGGTCCTA-3′; nt 201–180), respectively. In fragment 2, sense and antisense primers were F2 (5′-CATCCTCAGGCCATGCAGTGGA-3′; nt 3193–3214) and R4 (5′-ATGGCACTAGTAAACTGAGCC-3′; nt 689–669), respectively. In fragment 3, sense and antisense primers were F4 (5′-GTCCTCCAATTTGTCCTGG-3′; nt 348–366) and R6 (5′-GGCGAGAAAGTGAAAGCCTG-3′; nt 1103–1084), respectively. In fragment 4, sense and antisense primers were F6 (5′-ATATGGATGATGTGGTATTGGG-3′; nt 737–758) and MD26 (5′-GTTCACGGTGGTCTCCAT-3′; nt 1625–1608), respectively. In fragment 5, sense and antisense primers were Xi1 (5′-AGCTTGTTTTGCTCGC-AGC-3′; nt 1287–1305) and Xi3 (5′-GGCACAGCTTGGA-GGCTTG-3′; nt 1883–1865), respectively. In fragment 6, sense and antisense primers were X101 (5′-TCTGTGCC-TTCTCATCTG-3′; nt 1552–1569) and CORE2 (5′-CCCA-CCTTATGAGTCCAAGG-3′; nt 2476–2457), respectively. Amplicons were purified with a gel-extraction kit (Perfect-prep Gel Cleanup; Eppendorf). DNA sequencing analysis of the PCR products was performed with a Perkin-Elmer 310 sequencer.

For phylogenetic analysis, nucleotide sequences were multiply aligned by using the program CLUSTAL_X (version 1.83). Alignments were then fed into the software program PHYLIP (version 3.5c). SEQBOOT, DNADIST and NEIGHBOR were used for bootstrapping of a 1000-replicate dataset; CONSENSE was used to compute a consensus tree. The phylogenetic trees were visualized by using TREEVIEW software (version 1.6.6) (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

An entire HBV genome of 3213 nt was obtained, which was designated CU400. Analysis of the whole genome sequence after running through the BLAST program and comparing with sequences in GenBank indicated that the isolate belonged to genotype G. Phylogenetic analysis of the S gene sequences showed that the isolate clustered on a genotype G branch (Fig. 1a); this was also observed on analysis of the P and X genes and the full genome sequences (data not shown). However, in trees based on the C gene, the sequence clustered with genotype C, instead of genotype G (Fig. 1b). Phylogenetic analyses of the S, P and X genes of this hybrid exhibited a close sequence similarity (96·8–97·3 %) to the original isolates of genotype G, but <88·7 % similarity to any isolates of genotypes A to F. Taken together, the clustering of sequences in discordant positions following phylogenetic analysis provided evidence of a novel recombinant between genotypes G and C.

To locate the breakpoints of genomic recombination more accurately, the program SIMPLOT (version 2.5) (Lole *et al.*, 1999) and bootscanning analysis (Salminen *et al.*, 1995) were used. The SIMPLOT program was applied to identify phylogenetically informative sites supporting alternative tree topologies. This method was performed by considering four sequences at a time: one putative recombinant sequence, two reference sequences of original G (GenBank accession no. AB056513) and C (GenBank accession no. X04615) genotypes, and one sequence of a known outgroup (genotype E; GenBank accession no. X75664). Each informative site supports one of three possible phylogenetic relationships among the four taxa. Bootscanning and cluster analysis maximizing the χ^2 parameter were used to identify breakpoints in the intergenotypic recombinants; the *P* value for the subsequent division of the sequence into genotypes was calculated by using Fisher's exact test. Following these methods, the similarity plots of the complete sequence showed regions of higher similarity to genotype G alternating with regions of higher similarity to genotype C within the precore/core region (Fig. 2a). The recombination breakpoints were estimated at positions nt 1860 and 2460, which are located in the terminal areas of the precore and core regions, respectively (Fig. 2b).

Unlike other HBV genotypes, the authentic genotype G would not be able to encode HBeAg because of stop codons at positions 2 and 28 (Stuyver *et al.*, 2000) in the precore region, which can abort translation of the HBeAg precursor made of 10 aa encoded by the 3′-terminal part of the precore region and 149 aa encoded by the 5′-terminal part of the C gene (Okamoto *et al.*, 1990). When the nucleotide sequence of the new isolate was aligned, a translational codon was detected at codon 2 (C1817T) in the precore region, but the stop codon at codon 28 could not be identified. Thus, the presence of a translational stop mutation at codon 2 indicates that this strain would not express HBeAg in the serum. In contrast to the original strain of HBV genotype G (Stuyver *et al.*, 2000), the core region did not possess an insertion of 36 bp located at the 5′ end. However, a 3 bp deletion at position 3 in the preS1 region, which is a typical characteristic of genotype G, was found. In the HBsAg region, amino acids at position 122, 127 and 160 were lysine (K), proline (P) and lysine (K), respectively. Hence, the serological subtype of this HBV strain was expected to be *adw2*.

The most interesting result of the present study is that a

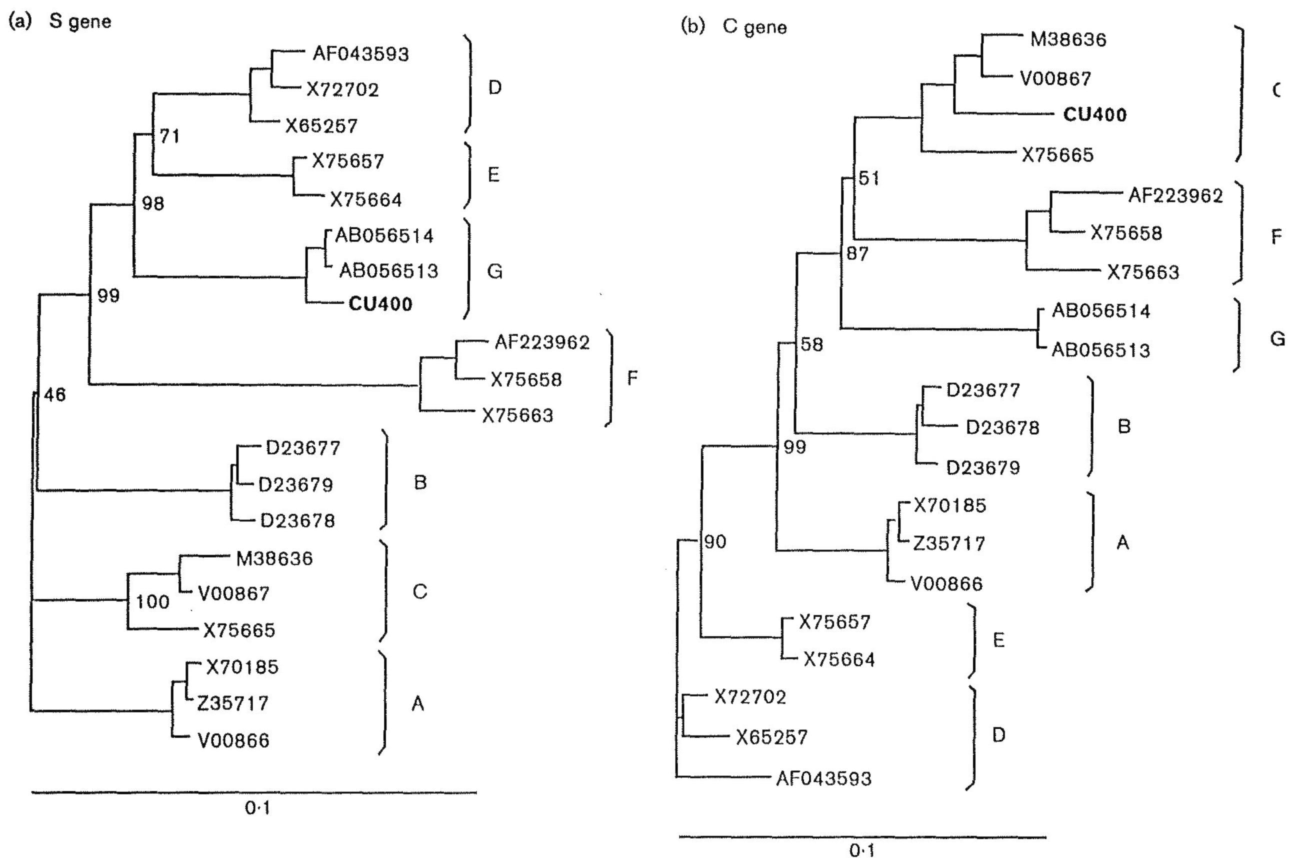

Fig. 1. Phylogenetic analysis of isolate CU400 compared with reference strains; GenBank accession numbers are given. Bootstrap values (%) are shown at the nodes. (a) Analysis of the surface S (surface) gene ORF (including preS1/preS2/HBsAg). (b) Analysis of the C (core) gene region.

novel hybrid HBV strain resulting from recombination between genotypes G and C has been described at length for the first time. This mosaic strain was recognized because it produced an unusual genotyping pattern by nucleotide sequencing and phylogenetic analysis. Indeed, strong evidence suggests that genotype G is frequently co-infected with other genotypes. For instance, all of the isolates of genotype G recovered in San Francisco were co-infected with genotype A (Kato *et al.*, 2002b). Similarly, all of the genotype G isolates from Canada were co-infected with either genotype A or genotypes A and C (Osiowy & Giles, 2003). Given evidence for the high frequency of co-infection with other genotypes, it is uncertain whether genotype G is competent to replicate by itself or mainly depends on other genotypes for replication (Kato *et al.*, 2002a). Moreover, whether the recombinant strain of genotypes G and C would facilitate the evasion of immune surveillance and have an advantage for persistence in hosts over the authentic genotype G is unclear.

It should be mentioned that the recombination breakpoints identified in this study occurred in the vicinity of the DR1 region and encapsidation signal of the HBV pregenome. In an *in vitro* recombination assay, fragments containing the region spanning DR1, which is believed to be the origin of virus replication and a preferred site in the viral genome for integration, enhanced recombination in the presence of extracts from actively dividing cells (Hino *et al.*, 1991). Of relevance to this evidence, it was demonstrated that the region encompassing nt 1600–2000 achieved a recombination-site density that was almost fivefold higher than that of the remaining part of the genome (Pineau *et al.*, 1998). Thus, it is possible that the genomic region covering DR1 would be responsible for intergenotypic recombination of genotype G, as well as for integration between HBV and the host genome. However, additional data on the recombination of HBV genotype G are still required before any conclusions can be drawn. Furthermore, the possibility that such a recombination might contribute to the development of HCC in this case needs further investigation.

Acknowledgements

This study was supported by the Thailand Research Fund and Center of Excellence, Viral Hepatitis Research Unit, Chulalongkorn University and the International Joint Research Program, Research Fund Project on Health Sciences focusing on Drug Innovation of Japan.

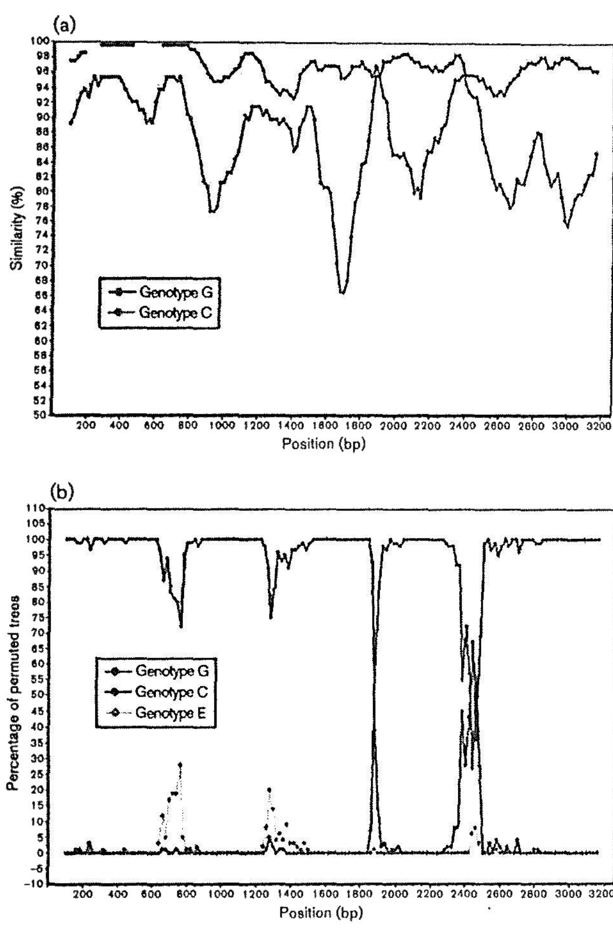

Fig. 2. (a) Nucleotide similarity comparison of the complete genome of CU400 with those of genotype G (GenBank accession no. AB056513) and genotype C (GenBank accession no. X04615), showing a window size of 200 bp and a step size of 20 bp. (b) Bootscan plots showing the likelihood of clustering of the putative G/C recombinant sequence (CU400) with the reference sequences of genotypes G and C, as well as genotype E as the outgroup (GenBank accession no. X75664).

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Manuscript in preparation

Precore, core promoter and X gene mutations of hepatitis B virus in Thai patients with hepatocellular carcinoma: a case-control study.

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Summary

Chronic hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). However, the pathogenesis of HBV-associated HCC is not fully understood. To investigate whether mutations within the precore, core promoter and X genes of HBV could be associated with the development of HCC, a case-control study was conducted by selecting 50 patients with HCC and 50 patients without HCC, who were matched for sex and age, as well as the distribution of hepatitis B e antigen (HBeAg) and HBV genotypes. HBV mutations in serum samples were determined by PCR with specific primers at nucleotide (nt) 1287–2038, and direct sequencing. Our data showed that precore stop codon mutation (PC) and double mutations in basal core promoter (A1762T/G1764A) (CP) were found more frequently in the HCC group than in the control group, but the differences were not statistically significant (PC 48% vs. 36%, P=0.33; CP 80% vs. 64%, P=0.12). However, the prevalence of CP in HCC patients who were younger than 50 years or who were infected with HBV genotype B were significant higher than that of the controls (82% vs. 48% P=0.03, and 57.1% vs 16.7% P=0.04, respectively). In addition, single nucleotide mutations were present in the X gene, but with a generally scattered distribution, and without significant difference between groups. However, two of the HCC group had a 24-bps insertion at nt 1674 and a 2-bps deletion at nt 1721-1722, respectively, leading to a frameshift mutation of amino acid. We conclude that certain X gene mutations and, particularly, CP mutations in young patients who are infected with genotype B may contribute to the development of HCC.