Table 1. Demographics of HBV carriers with or without HCC

	Cases without HCC	Cases with HCC
Total number of HBV carriers	661	30
Sex (male:female)	343:318	21:9
Median age (years, range)	41.9 (16~80)	47.4 (35~67)
Male	41.3 (16~75)	47.0 (35~65)
Female	42.9 (19~80)	48.6 (35~67)
Median follow-up period (years, range)	10 (2~26)	8 (2~15)
Male	9 (2~26)	8 (2~15)
Female	10 (2~26)	9 (4~13)
Serum transaminase levels (mean ± SD)		
AST (IU/L)	24.2 ± 23.0*	54.3 ± 41.2
ALT (IU/L)	29.9 ± 23.6*	38.3 ± 33.3

* $p<0.05$ (compared to cases with HCC)

with or without HCC are shown in Table 1.

The study protocol was approved by the Human Ethics Review Committee of Iwate Medical University, and was permitted by the Committee of Iwate Health Service Association and Iwate Medical Association.

2. Methods

We used the initial serum samples for determination of HBV genotypes which were stored at −20℃ in the institute of Iwate Health Service Association.

HBV genotypes were determined using an enzyme linked immunosorbent assay (ELISA) kit (Institute of Immunology Co., Ltd., Tokyo, Japan) according to the method previously reported by Usuda, et al.[17]. Briefly, 10 μl of a serum sample was placed on a plate fixed with monoclonal antibodies against epitope *b* (located in the pre-S antigen of HBV, and common to all genotypes), epitope m (specific to genotype B), and epitopes *k*, *s* and *u* (associated with several genotypes). A reactive enzyme was then added for color development, and absorbency was measured to determine HBV genotype.

HBsAg was determined using commercial hemagglutination assay kits (MyCell, Institute of Immunology Co. Ltd., Tokyo, Japan). HBeAg and anti-HB e antibody (anti-HBe) were also determined using commercial enzyme immunoassay kits.

Serum levels of ALT, asparate aminotransferase (AST) and γ-glutamyltransferase (γ-GTP) were examined using a routine automatic analyzer.

3. Statistical analysis

Data are expressed as mean±standard deviation (SD) or median (range). Comparisons between the groups were performed by Chi-square test of Fisher's exact test. Probabilities of less than 0.05 were considered statistically significant.

III. Results

1. Distribution of HBV genotypes and their relationship with age

Of 680 cases, 19 (2.9%) were genotype A, 297 (44.9%) were genotype B, and 345 (52.2%) were genotype C. The 30 cases with HCC showed 9 (30%) cases of genotype B and 21 (70%) cases of genotype C. Genotype A was not detected in HCC cases.

Table 2. The relationship between positive rate of HBeAg in each genotype and age at first examination

	Genotype A	Genotype B	Genotype C
Total numbers	19	297	345
Sex (male:female)	13:6	157:140	173:172
Numbers of HBeAg positive (%) #	0 (0)	11 (3.7)	115 (33.3) $^{++}$, **
Sex (male:female)	0:0	8:3	66:49
>29 years	0 (0)	7 (24.1)	29 (43.9)
30 ~39	0 (0)	2 (3.3)	37 (33.0)
40 ~49	0 (0)	0 (0)	28 (28.9)
50 ~59	0 (0)	1 (1.2)	13 (26.5)
60 ~69	0 (0)	1 (2.9)	7 (36.8)
70<	0 (0)	0 (0)	1 (50.0)
Liver function tests			
AST (IU/L)	22.4 ± 11.1	25.8 ± 12.7	33.8 ± 29.9^{+}, *
ALT (IU/L)	29.3 ± 22.1	27.7 ± 17.5	40.8 ± 50.0
g-GTP (IU/L)	29.4 ± 22.3	28.9 ± 40.9	36.2 ± 47.2

$^{+}$, * $p<0.01$ (compared to genotypes A and B, respectively)
$^{++}$, ** $p<0.001$ (compared to genotypes A and B, respectively)
Percentage is the rate of HBeAg positive cases among the total number of HBV carriers in each age group

The relationship between each HBV genotype and age is shown in Figure 1. When the age of HBV carriers was compared based on age in 2003, genotype A was found only in the 20～30-year-old carriers. The rate of genotype B gradually increased as age increased and was the highest in carriers over 70 years old. The rate of genotype C was higher in 40 to 60 years old, but lower in carriers over 70 years old.

2. Positive rate of HBeAg in each HBV genotype

The positive rate of HBeAg for each genotype of HBV was 0% in genotype A, 3.7% in genotype B and 33.3% in genotype C. The positive rate of HBeAg in genotype C was significantly higher ($p<0.001$) than that in genotypes A and B. The relationship between

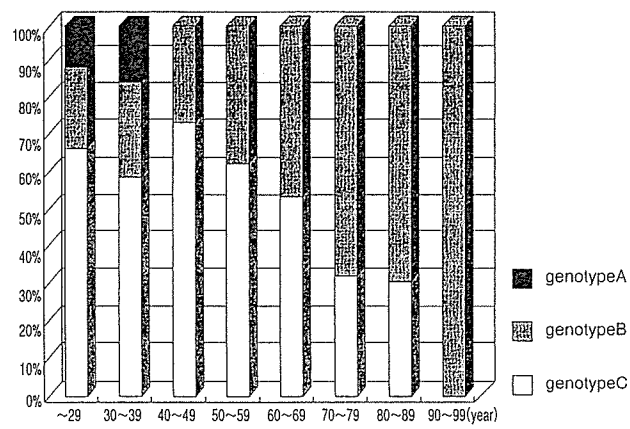

Fig. 1. The relationship between each HBV genotype and age

Table 3. Profiles of HCC cases with genotype B or C

	Genotype B (9)	Genotype C (21)
Age developed to HCC (years)	64 ± 13*	52.7 ± 9
Numbers of HBeAg positive (%)	0 (0%)	6 (27.3%)
Serum ALT levels during follow-up	39.4 ± 22.8	65.5 ± 52.8

* $p<0.05$ (compared to genotype C)

the positive rate of HBeAg in genotypes B and C and age is shown in Table 2. In 20- to 30-year-old genotype B carriers, 11 carriers exhibited a relatively higher positive rate of HBeAg. On the other hand, in genotype C, the percentages of HBeAg positive cases were higher than genotype B at all ages, in particular, in the carriers in their 20s.

3. Changes in the HBeAg/anti-HBe system in genotype B and C carriers during follow-up

Six (54.5%) of 11 genotype B carriers who were positive for HBeAg at the first examination seroconverted to anti-HBe during follow-up, while 5 (45.5%) carriers remained HBeAg positive during follow-up. Of the 115 genotype C carriers who were positive for HBeAg at the first examination, 24 (20.9%) carriers seroconverted to anti-HBe and 12 (10.4%) carriers were positive for alternately between HBeAg and anti-HBe, while 79 (68.7%) carriers remained HBeAg positive during follow-up. Duration until the seroconversion from HBeAg to anti-HBe was 4.5±2.3 years (range; 2 ~ 8 years) in genotype B, and 6.0±4.2 years (1 ~15 years) in genotype C.

4. Relationship between HBV genotypes and serum ALT levels during follow-up

Serum ALT levels during a long-term follow-up were compared among the three HBV genotypes. Serum ALT levels of each HBV genotype were 29.3± 22.1 IU/L in genotype A, 27.7±17.5 IU/L in genotype B, 40.8±50.0 IU/L in genotype C. The levels of serum ALT in the genotype C were significantly higher ($p<0.05$) than those of genotype B, but no significant differences were observed between genotypes A and B.

5. Analysis of HBV carriers complicated with HCC

Comparison between HCC cases with genotype B or C is shown in Table 3. Cases with genotype B were significantly older compared to the cases with genotype C. All cases with genotype B exhibited a negative rate of HBeAg at the start of follow-up, while HBeAg was positive in 27.3% of cases with genotype C. Serum ALT levels during follow-up were significantly higher in genotype C than in genotype B.

IV. Discussion

In Iwate Prefecture, the immunoprophylaxis of perinatal transmission of HBV was started in 1981 and covered more than 60% of all babies by 1986 when it became a mandatory national program[18]. Briefly, 20.7% of HBV carrier mothers were positive for HBsAg and their babies received immunoprophylaxis. As a result of this program, the prevalence of HBsAg decreased from 0.75% in children born between 1978 and 1980 to 0.23% in those born between 1981 and 1985, and further to 0.04% in those born between 1986 and 1990. Therefore, it is believed that the prevention of

perinatal HBV transmission influences not only mother-to-baby transmission but also horizontal transmission from HBV carrier children who might be infected during infancy. However, there are still many HBV carriers who have not received immunoprophylaxis under the national program for prevention of HBV infection.

Among the HBV carriers residing in Japan, a small number of HBeAg positive carriers or carriers with abnormal levels of serum ALT are recognized as a high-risk group for developing CH and LC or HCC[5, 14-16]. However, clinical features and outcome in the majority of HBV carriers with different HBV subtypes remain unclarified.

In this large-scale survey three genotypes were found among HBV carriers residing in Iwate Prefecture who were identified during a health-screening program between 1977 and 2004. HBV genotypes B and C were the most common, followed by genotype A. In a previously published report by Orito and colleagues[6], concerning the geographic distribution of HBV genotypes among patients with chronic liver disease in Japan, the prevalence of genotype B in the Tohoku area including Iwate Prefecture was reported to be higher (22.9%) than that of the other mainland areas. However, the rate of HBV genotype B in this study was higher than expected (44.5%), even if a high rate of HBV genotype B was considered to be endemic in the Tohoku area. A possible reason for this is that our subjects lived without any medical management.

Generally, in HBV carrier residents with genotype B, seroconversion from HBeAg to anti-HBe occurs at a young age (10-20 years after birth), resulting in stabilization of liver function. Therefore, the majority of HBV genotype B carriers live as asymptomatic carriers who do not require medical management or follow-up[16]. Actually, the positive rate of HBeAg in carrier residents with genotype B was extremely low compared to carrier residents with genotype C at the start of follow-up. On the other hand, it has been found that seroconversion from HBeAg to anti-HBe at a young age is not frequent in genotype C carriers, and in most patients abnormal serum ALT levels remain[5, 6, 16]. Therefore, the HBV carriers with genotype C have many opportunities to visit the hospital and receive the medical management, resulting in high prevalence of genotype C. In the present study, the prevalence in every age group of genotype C carriers were significantly lower than rates of anti-HBe of genotype B carriers, while the serum AST and ALT levels were higher in genotype C. Ishikawa, et al.[16] previously showed that the seroconversion from HBeAg to anti-HBe was less likely to occur in genotype C carriers, especially in carriers in their 40s. These subjects were also more likely to develop chronic liver disease, because their serum transaminase levels fluctuated and their HBV-DNA levels were high.

Interestingly, in the present study we found that the prevalence rate of genotype A was relatively high among young people from 20 to 30 years old. Genotype A is the predominant genotype in Europe and the United States[2]. A previous report concerning the geographic distribution of HBV genotypes in Japan showed a low prevalence rate (1.7%) of HBV genotype A[6]. A recent report has suggested that acute hepatitis patients infected with HBV genotype A often transfer to a persistent

HBV carrier state[19]. Also, in Europe, most HBV infections are genotypes A and D, and significantly more genotype A carriers developed chronic liver disease when compared with genotype D carriers[20]. The reason for the increased prevalence rate of genotype A in Iwate Prefecture among the young generation is unclear, and it is therefore necessary to follow these carriers over the long term.

HBV is one of the major causative agents of HCC in Japan. In particular, HBV genotypes B and C are frequently seen in patients with HCC. Previous reports in Japan showed that the mean age is higher in HCC patients with genotype B than in those with genotype C, although results in Taiwan and another Asian countries are controversial [5, 11-13]. In general, genotype B is less prevalent than genotype C among patients with liver cirrhosis, because HBV genotype B is associated with earlier seroconversion from HBeAg to the corresponding anti-HBe and with lower histological activity scores. In the present study, we also demonstrated that HBV carriers with genotype B and HCC were significantly older than cases with genotype C. In addition, genotype B carriers showed lower serum ALT levels during follow-up than genotype C carriers. Therefore, these results suggest that genotype C carriers might have a tendency for persistent fluctuation of abnormal serum ALT levels over the long-term, accelerating the development of HCC.

Recently, lamivudine, an oral cytosine nucleoside analogue, which potently inhibits HBV replication by interfering with HBV reverse transcriptase activity, has been used clinically for the treatment of chronic HBV infection[21-23]. This therapy for chronic HBV infection induced a marked decrease in HBV-DNA and ALT levels, resulting in histological improvement, although lamivudine-resistant HBV strains have appeared in long-term lamivudine therapy[24, 25]. Therefore, this therapy is expected to change the natural course of HBV carriers with persistent abnormal liver function.

In conclusion, the prevalence of genotypes B and C were equal in HBV carriers residing in Iwate Prefecture. Differences between HBV genotypes, in particular genotypes B and C, were closely associated with positive rate of HBeAg, fluctuating serum ALT levels, and clinical outcomes of these carriers.

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内容自抄

B型肝炎ウイルス（HBV）には８つの遺伝子型（A～H）が存在しているが，HBVキャリア住民の遺伝子型頻度や自然歴は十分に検討されていない．そこで，検診受診者を対象にHBVキャリアの遺伝子型とその臨床的特徴を検討した．岩手県予防医学協会でHBVキャリアと診断された661例と岩手県癌登録事業より肝細胞癌で死亡が確認された30例を対象とした．遺伝子型の測定はELISA法を用いた．岩手県のHBVキャリア住民の遺伝子型の頻度は，各遺伝子型と年齢との関係をみると，Aは20～30歳代でのみ認められ，Bは70歳以上で高く，Cは40～60歳代では半数以上を占めた．各遺伝子型のHBe抗原陽性率の頻度は，CがA，Bより高率であった．経過観察期間中の血清ALT値は，Cが，A，Bに比較して有意に高値であった．肝癌例ではBでの発癌年齢はCに比較して有意に高齢であり，経過観察期間中の血清ALT値も低値であった．

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Differences of Hepatocellular Carcinoma Patients with Hepatitis B Virus Genotypes of Ba, Bj or C in Japan

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Key Words

Hepatocellular carcinoma, epidemiology · Subtypes Ba/Bj, hepatitis B · Hepatitis B virus, genotypes B/C

Abstract

Hepatitis B virus (HBV) genotypes B (HBV/B) and C (HBV/C) are prevalent in Asia. Recently HBV/B has been classified into two subtypes, HBV/Ba which is ubiquitously found in Asia, and HBV/Bj which is specific in Japan. In addition, the frequency of positive HBeAg has been reported to be higher in patients with HBV/Ba than those with HBV/Bj. However, little is known about the differences between patients with various genotypes who developed hepatocellular carcinoma (HCC). In 296 serum samples of HCC patients collected from all over Japan, HBV genotypes were determined with the restriction fragment length polymorphism. HBV/A was detected in 1.0%, HBV/Ba in 4.4%, HBV/Bj in 7.4%, and HBV/C in 86.5%. In the Tohoku district and Okinawa, HBV/Ba, HBV/Bj and HBV/C were found in 6.7, 40.0 and 48.9%, compared to 4.0, 1.6 and 93.2% in the other districts in Japan. HBV/Bj patients were more frequently found in the group older than 65 years while HBV/Ba patients were found in all age groups. The frequency of positive HBeAg in HBV/Bj patients was significantly low compared to that in the other patients. More than 60% of the patients with HCC had cirrhosis as the underlying liver diseases. However, in HBV/Ba patients aged 50 years or younger, 80% of them had chronic hepatitis, while 87.5% of those aged older than 50 years had cirrhosis. These data suggest that great differences exist among patients with HCC infected with different genotypes.

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Introduction

In Japan, in more than two thirds of the patients with hepatocellular carcinoma (HCC) the disease is associated with hepatitis C virus (HCV). However, hepatitis B virus (HBV) is the major causative agent of HCC in Asian countries. All strains of HBV isolated from various countries can be classified into 8 HBV genotypes, HBV genotype A (HBV/A) to HBV/H, according to their phylogenetic relationships [1–3]. It has been reported that the clinical and virologic manifestations of patients with chronic HBV infection show significant differences among the different HBV genotypes [4–6]. In addition, specific distributions of HBV genotypes have been demonstrated among areas and countries [4, 7]. In south-east Asian countries, such as Japan, Taiwan, or China, HBV/B and HBV/C are prevalent [5, 7, 8].

In Japanese patients with HCC, the patients with HBV/B are rare and their mean age is high [7, 9]. However, in Taiwanese patients with HCC, a high proportion of younger patients have HBV/B. Until now, it is still unclear why younger Taiwanese patients with HBV/B develop HCC while Japanese patients with HBV/B rarely develop HCC, only in older age.

Recently, we demonstrated that HBV/B strains should be divided into two subtypes, HBV/Ba and HBV/Bj, according to their genetic relationship, and that HBV/Ba is found ubiquitously in Asian countries while HBV/Bj is found only in Japan [10, 11]. It was reported that HBeAg was found more frequently in patients with chronic infection with HBV/Ba than in those with chronic infection with HBV/Bj (32 vs. 9%) [12]. However, it is still unknown whether etiological and virologic differences are found between the HCC patients with HBV/Ba and HBV/Bj. Thus, in the patients with HCC, the difference between the subtypes of HBV/Ba and HBV/Bj might explain the etiological or clinical differences between Japan and Asia where HBV/Bj and HBV/Ba are endemic, respectively.

So, the aim of this study was to investigate the differences in the etiological, virologic and clinical characteristics among Japanese HCC patients with different HBV genotypes, such as HBV/Ba, HBV/Bj or HBV/C.

Patients and Methods

Patients with HCC

Two hundred and ninety-six patients with HCC were consecutively collected from 19 hospitals throughout Japan during January 2001 to December 2002. All the patients were chronically positive for HBsAg, and negative for anti-HDV, anti-HCV and anti-HIV. The diagnosis of HCC was reached clinically with ultrasound, computerized tomography, magnetic resonance imaging, angiography, tumor markers and biopsy if possible. The diagnoses of chronic hepatitis (CH) and liver cirrhosis (LC) were principally done by liver biopsy. However, a proportion of patients with ascites, jaundice or severe thrombocytopenia were diagnosed by ultrasound, computerized tomography and liver function tests. The serum samples and clinical data were collected from these patients with written informed consent. This study was conducted according to the ethical guidelines in our hospitals.

Virologic Assays

In all serum samples, HBsAg (CLIA, Fujirebio, Japan, detection limit 0.13 ng/ml), HBeAg (CLIA, Fujirebio, Japan) and anti-HBe (CLIA) were tested. Serum HBV DNA was detected by nested polymerase chain reaction (PCR) with the primers derived from the S gene. The patients were not enrolled in this study if the serum HBV DNA was not detected by PCR. The HBV genotype was determined by restriction fragment length polymorphism as described previously [13]. In brief, the S gene of HBV DNA was amplified by nested PCR. Then the products were sequentially digested by the restriction enzyme, *Alw*I, *Ear*I, *Hph*I, *Nci*I and *Nla*IV, respectively. The HBV genotype was determined by the size of the digested PCR product which was electrophoresed on agarose gel. When the test results were inconclusive, the sequences of the S region were determined directly, then the genotype was decided by phylogenetic analysis [13, 14]. When patients were found to have HBV/B, the subtypes Ba and Bj were determined by restriction fragment length polymorphism [11]. In brief, at nucleotide position 1838 in the pre-core region, only A was found in patients with HBV/Ba while only G was found in those with HBV/Bj. The restriction enzyme detection system was established targeting the discrimination of this difference in nucleotides with the restriction enzyme, *Spe*I and *Mse*I after the pre-core region was amplified by PCR.

Statistical Analysis

The data were statistically analyzed by Student's t test, nonparametric Mann-Whitney test, and χ^2 test where appropriate. A p value of <0.05 was regarded as statistically significant.

Results

HBV Genotypes and Clinical Findings

Of the 296 patients, 223 were male and 73 were female. The mean age was 55.1 ± 10.8 (range 26–81) years. The clinical findings are shown in table 1. Thirty-five percent of the patients were positive for HBeAg. Regarding the HBV genotypes, 3 patients (1.0%) were HBV/A, 13 (4.4%) HBV/Ba, 22 (7.4%) HBV/Bj, 256 (86.5%) HBV/C, and 2 (0.7%) of mixed genotype (HBV/B and C). The clinical findings by HBV genotype are shown in table 2. There were no significant differences in the mean levels of total bilirubin, AST and ALT among patients with different HBV genotypes. However, the mean ALP level and γ-

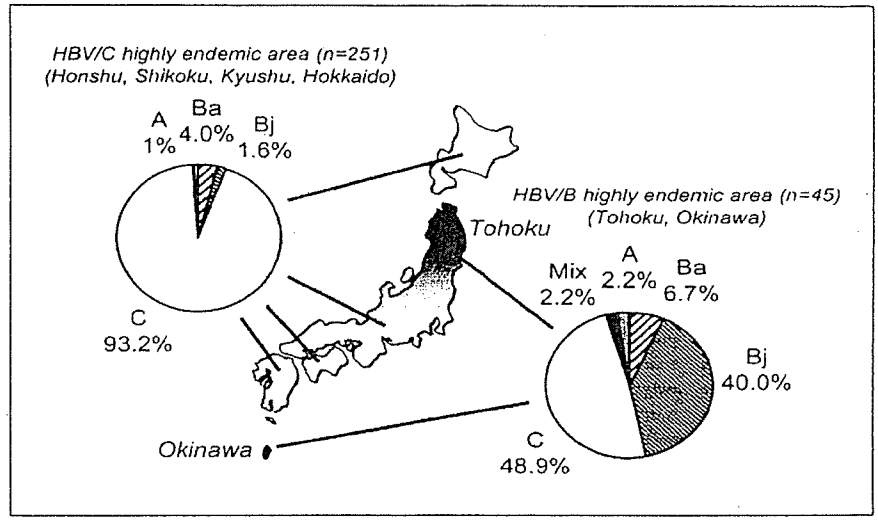

Fig. 1. The geographic distribution of HBV genotypes in Japan. In the Tohoku district, the northern area of mainland Japan, and Okinawa, the most southern islands, 48.9% of HCC patients were HBV/C, 6.7% were HBV/Ba, and 40.0% were HBV/Bj. In contrast, in other parts of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 93.2% were HBV/C, 4.0% were HBV/Ba and 1.6% were HBV/Bj.

Table 1. Characteristics of 296 HBsAg-positive Japanese patients with HCC collected from all over Japan

Male:female	223:73
Age, years	55.1 ± 10.8[a]
Total bilirubin, mg/dl	1.5 ± 1.9
AST, IU/l	78.5 ± 103.9
ALT, IU/l	63.0 ± 69.8
ALP, IU/l	321.1 ± 225.4
γ-GTP, IU/l	108.4 ± 174.4
HBeAg, % positive	35.0
Anti-HBe, % positive	64.8
HBV genotype	
HBV/A	3 (1.0%)
HBV/Ba	13 (4.4%)
HBV/Bj	22 (7.4%)
HBV/C	256 (86.5%)
Mix	2 (0.7%)

[a] Mean ± SD.

Table 2. Clinical findings of the HCC patients with HBV genotypes of Ba, Bj or C

	HBV genotype		
	Ba	Bj	C
Age, years	55.4 ± 12.9	66.6 ± 10.6	54.0 ± 10.7
		$p < 0.01$	$p < 0.01$
Total bilirubin, mg/dl	1.0 ± 0.4	1.2 ± 0.7	1.5 ± 2.0
AST, IU/l	173.9 ± 352.6	51.6 ± 42.1	82.6 ± 113.4
ALT, IU/l	102.4 ± 162.9	33.9 ± 16.8	66.5 ± 74.9
ALP, IU/l	147.7 ± 126.6	209.8 ± 95.4	343.9 ± 238.0
		$p < 0.05$	
γ-GTP, IU/l	78.6 ± 55.9	63.1 ± 45.9	110.5 ± 186.7
			$p < 0.05$

GTP level of the HBV/C patients was significantly higher than those with HBV/Ba and HBV/Bj, respectively ($p < 0.05$).

Geographic Distribution of HBV Genotypes

The geographic distribution of HBV genotypes was area-specific in Japan (fig. 1). This specific distribution of HCC patients was in accord with that of all the patients including asymptomatic carriers, CH and LC patients, as described previously [7]. Namely, in the Tohoku district, the northern area of the Japanese mainland, and Okinawa, the most southern islands, 22 (48.9%) of HCC patients were HBV/C, 3 (6.7%) were HBV/Ba, and 18 (40.0%) were HBV/Bj. In contrast, in other areas of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 234 (93.2%) were HBV/C, 10 (4.0%) were HBV/Ba, and 4 (1.6%) were HBV/Bj ($p < 0.01$).

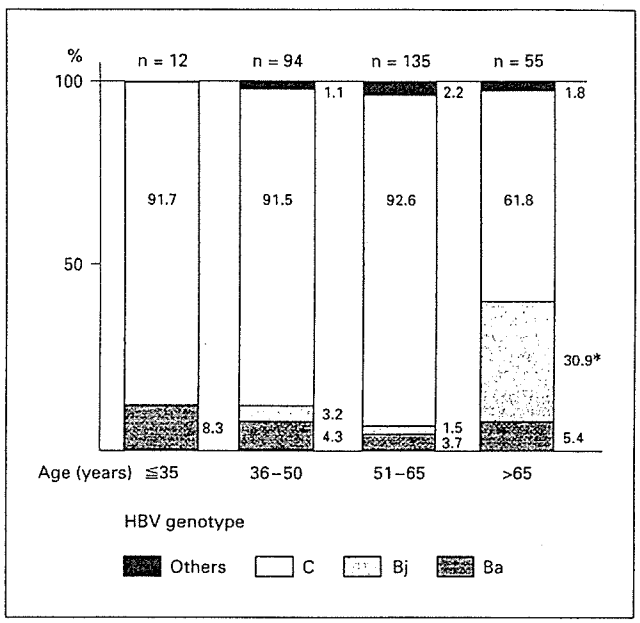

Fig. 2. The distribution of HBV genotypes in each age group. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of patients had HBV/C while 30.9% had HBV/Bj (* $p < 0.01$, group aged older than 65 years vs. other age groups). More patients with HBV/Ba were in the younger aged group, although the number of patients with HBV/Ba was small in all the groups.

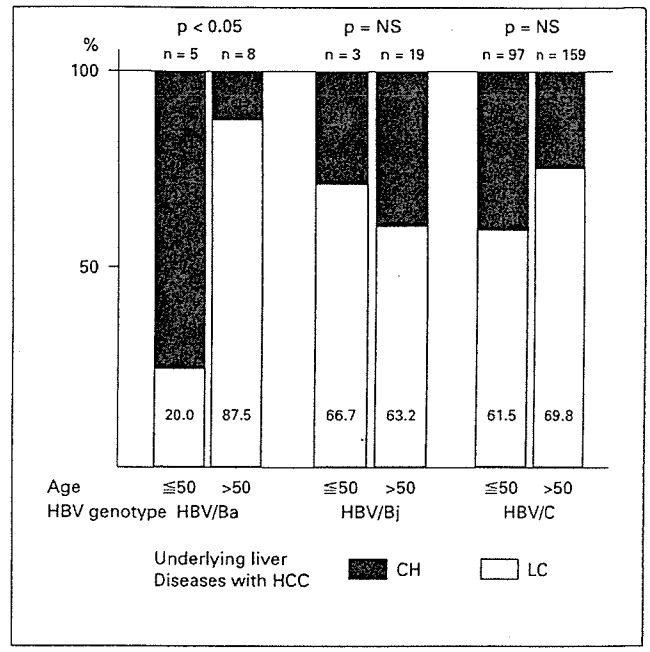

Fig. 4. The underlying liver diseases, chronic hepatitis (CH) or liver cirrhosis (LC), in HCC patients. In patients with HBV/Ba, only 25.0% of the group aged 50 years or younger had LC, while 85.7% of the group aged older than 50 years had LC ($p < 0.01$). However, in patients with HBV/Bj or HBV/C, the ratios of the underlying liver diseases were approximately identical even when compared by age.

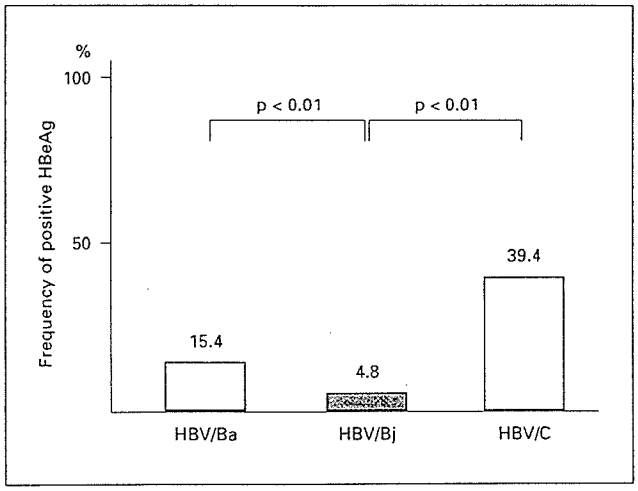

Fig. 3. The frequency of patients with positive HBcAg in each HBV genotype. The frequency of positive HBcAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$).

Mean Age and Frequency of Positive HBeAg among Patients with Each Genotype

The mean age of HBV/Bj patients (66.6 ± 10.6 years) was significantly higher than those with HBV/Ba (55.4 ± 12.9 years, $p < 0.01$) and HBV/C (54.0 ± 10.7 years, $p < 0.01$; table 2). The distribution of HBV genotypes in each age group is shown in figure 2. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of the patients had HBV/C while 30.9% had HBV/Bj ($p < 0.01$, group aged older than 65 years vs. other age groups). HBV/Ba tended to be found in the younger age group although the number of patients with HBV/Ba was small in all groups.

The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$; fig. 3).

Underlying Liver Diseases

All HCC patients had underlying chronic liver diseases, such as CH or LC. We compared the underlying liver diseases among those aged 50 years or younger and those aged older than 50 years by HBV genotype (fig. 4). In 13 patients with HBV/Ba, only 1 (20.0%) of the 5 patients aged 50 years or younger had LC, while 7 (87.5%) of the 8 patients aged older than 50 years had LC ($p < 0.05$). However, in patients with HBV/Bj or HBV/C, the ratios of underlying liver diseases were approximately identical even when compared by age.

Discussion

The clinical and virologic features of patients with chronic HBV infection are specific according to their HBV genotypes [4, 15]. However, to date, there has been no report on the relationship between the HBV genotypes of Ba, Bj and C, and the clinical characteristics of HCC patients. We therefore analyzed the relationship between the clinical characteristics of Japanese HCC patients identified throughout Japan, and their HBV genotypes, including the HBV subtypes of Ba and Bj. In this study, we demonstrated that HBV/Ba (4.4%), HBV/Bj (7.4%) and HBV/C (86.5%) were found in Japanese HCC patients, and that there were distinct clinical differences among the three HBV genotypes, in geographic distribution, age distribution, and the frequency of positive HBeAg.

Of the Japanese patients with chronic HBV infection, including asymptomatic carriers, CH, LC and HCC, 1.7% were HBV/A, 12.2% HBV/B, 84.7% HBV/C, 0.4% HBV/D, and the others 1.0%, as reported previously [7]. In this study, we collected 296 serum samples from patients with HCC throughout Japan. In addition, we recently developed a new method for detecting HBV/Ba and HBV/Bj with restriction fragment length polymorphism [11]. Thus, we showed that 1.0% was HBV/A, 4.4% HBV/Ba, 7.4% HBV/Bj, 86.5% HBV/C, and mixed genotype 0.7% in Japanese HCC patients. This prevalence in HCC patients is almost identical to that in all patients with chronic HBV infection [7]. In addition, the geographic distribution of HBV/B and HBV/C in HCC patients is also identical to that in all patients. However, when we analyzed the HBV subtypes of HBV/Ba and HBV/Bj in patients with HBV/B, a high proportion of patients with HBV/Bj is found in the highly endemic HBV/B area, the Tohoku district and Okinawa, while the prevalence of HBV/Ba is approximately identical between the highly endemic HBV/C area, the other areas of Japan, and the highly endemic HBV/B area. Thus, HBV/Bj is specifically distributed in the Tohoku district and Okinawa.

As reported previously, HBV/Ba is ubiquitous in all Asian countries including Japan, although HBV/Bj is specific to Japan and is not found in other countries [11]. In Okinawa, it is reported that a high proportion of patients with chronic HBV infection have HBV/B and a good prognosis compared with patients with HBV/C [16, 17]. In contrast, in Taiwan, close to Japan, a higher proportion of patients aged 50 years or younger with HBV/B have HCC and CH [15]. The underlying liver diseases in those who developed HCC were compared among each HBV genotype group. In the HBV/Ba group, up to 75% of the patients aged 50 years or younger had CH as the underlying liver disease, compared with patients aged over 50 years. On the other hand, in the group with HBV/Bj or HBV/C, more than 60% of the patients had LC regardless of their age. The mean age of the patients with HBV/Ba in Japan is more than 10 years younger than those with HBV/Bj. So, more younger patients with HBV/Ba tend to have CH than the other patients. However, the molecular mechanism is unclear why patients with HBV/Ba develop HCC at a younger age and often have CH.

It is unclear why Japanese patients with HBV/B have a good prognosis while Taiwanese patients with HBV/B often have more advanced liver diseases, such as HCC. The frequency of patients positive for HBeAg in the HBV/Ba and HBV/C groups was higher than in the HBV/Bj group. So, the viral activity of HBV may be higher in patients with HBV/Ba or HBV/C than those with HBV/Bj. Thus, these differences in subtypes of HBV/Ba and Bj could be one of the reasons why the discrepancy in prognosis exists between Japanese and Taiwanese patients with HCC.

The differences in DNA sequences between HBV/Ba and HBV/Bj can be characterized in the core gene [10]. It has been reported that HBV/Ba, not HBV/Bj, recombines with HBV/C in the core gene. The product of the core gene is reported to be a cytotoxic T-cell epitope [18], suggesting that patients with HBV/Ba and HBV/C may be exposed to severe immune responses for destroying hepatocytes compared with those with HBV/Bj. In addition, patients with HBV/Ba more often have core promoter mutations at nucleotide 1762/1764 than those with HBV/Bj [11], which is associated with more advanced liver diseases [6, 19]. Taken together, these facts may indicate a poor prognosis in patients with HBV/Ba compared to those with HBV/Bj.

In the patients with HBV/C, the mean ALP and γ-GTP levels were higher than those with the other genotypes. In this study, there may exist some bias of regarding the tumor size of HCC between patients with HBV/C and the other patients. It is considered that more patients with a rather large size of HCC were found in the patients with HBV/C, resulting in elevation in ALT and γ-GTP levels.

To investigate the hepatocarcinogenesis and risk factors of HCC, it is important to study the differences in host, environmental and viral factors. The various genetic alterations, such as mutations of cancer-associated genes or loss of some chromosomes, are found in the HCC cells [20]. However, the genetic polymorphism varies among populations [21]. The differences in host genomes are still unknown between Japanese and other Asian populations. The association of environmental factors, such as air, water and food contaminated with some chemical agents, and HCC is still unclear, although aflatoxin affects the mutation of p53 in HCC [22]. However, with respect to the viral factors, a survey of the distribution of HBV genotypes or subtypes will be important clues for solving these problems.

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Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism

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Abstract

Recently hepatitis B virus genotype C (HBV/C) has been classified into geographically typical two subtypes (subgenotypes); HBV/C1 in Southeast Asia (Cs) and HBV/C2 in East Asia (Ce). Our aim is to develop a rapid subtyping assay and to examine the virological features of these two subtypes. Based on 171 HBV/C strains retrieved from the database, 17 single nucleotides polymorphisms (SNPs) were found between two subtypes. Taking advantage of five SNPs in non-overlapping polymerase region, a restriction fragment length polymporphism method with three endonucleases was newly developed for distinguishing between HBV/Cs and HBV/Ce. The method was applied to 49 HBV/C carriers from Japan and Hong Kong. The 24 in Hong Kong were classified into HBV/Cs, and the 25 in Japan were HBV/Ce, confirmed by sequencing. Some specific mutations were detected in the encapsidation signal; precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858, was found in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs. Especially, two closely linked mutations (A1896 and A1899) in HBV/Ce could stabilize the epsilon loop structure more efficiently and influece viral replication. Hence, these virological differences between the two subtypes might influence clinical features.

Keywords: Hepatitis B virus; Single nucleotides polymorphisms; Subgenotypes

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. HBV strains even of the same genotype may differ both virologically and clinically. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Additionally, there have been some lines of evidence for virological and clinical differences between HBV/Aa in Africa and HBV/Ae in Europe and the US [8,9]. Infection with HBV/Aa is associated with low serum levels of HBV DNA as well as low prevalence of hepatitis B e antigen (HBeAg) in serum, and is implicated in the high incidence of HBV-induced hepatocellular carcinoma (HCC) in Africa [10,11].

Recently, phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from southeast Asia including Vietnam, Myanmar and Thailand (named HBV/C1) and the other for strains from

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(far) East Asia including Japan, Korea and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [12], and by a recent independent study in Hong Kong [13]. The latter paper designated the two subtypes (subgenotypes) as HBV/Cs in Southeast Asia and HBV/Ce in the (far) East Asia that have different epidemiological distributions [13]. However, further studies are required to evaluate clinical and virological significance between HBV/C1 (Cs) and HBV/C2 (Ce), and development of a simple and efficient method for classification is essential.

In this study, we investigated single nucleotides polymorphisms (SNPs) between HBV/Cs and HBV/Ce at complete genome levels, and developed a novel polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method in the non-overlapping polymerase region involving five SNPs to distinguish between HBV/Cs and HBV/Ce precisely.

2. Materials and methods

2.1. Subjects

A total 49 sera containing HBV/C determined by the ELISA on preS2-region products [14,15], with the results confirmed by PCR-RFLP of the S gene [16], were obtained from chronic carriers of HBV who visited Nagoya City University hospital in Japan or Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each HBV carrier. To determine SNPs between HBV/Cs and HBV/Ce, 34 HBV/Cs and 137 HBV/Ce complete sequences were additionally recruited from DDBJ/EMBL/GenBank database.

2.2. PCR-RFLP for distinguishing between subtypes (subgenotypes) Cs and Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/Cs or Ce. The first-round PCR was performed with a sense primer (HBV964F: 5′-ATT AGA CCT ATT GAT TGG AAA GT-3′ [nt 964-986]) and an antisense primer (HBV1272R: 5′-AGT ATG GAT CGG CAG AGG AG-3′ [nt 1272-1253]) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2: 5′-CCT ATT GAT TGG AAA GTA TGT CA-3′ [nt 970-992]) and an antisense primer (HBV1272R). To determine HBV/Cs, a portion (5 μl) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *BstE*II at 60 °C for 1 h each. For HBV/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (w/v) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.3. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [17], with slight modifications. In brief, the first round of PCR was performed with sense primer (HB7F-2: 5′-CAT GGA GAC CAC CGT GAA CGC-3′ [nt 1607–1627]) and antisense primer (HB8R-2: 5′-ATA GGG GCA TTG GTC T-3′ [nt 2314–2299]) for 40 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min [6 min in the last cycle]) in a 96-well cycler (GeneAmp 9700, Perkin-Elmer Cetus, Norwalk, CA). The second round of PCR was performed with sense primer (HB7F-2) and antisense primer (HB7R-2: 5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072–2052]) for 35 cycles, under the same conditions as in the first-round PCR. The standard precautions for avoiding contamination during PCR were exercised carefully, and a negative control serum was included in each run of tests to ensure the specificity. Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

2.4. Molecular evolutionary analyses of HBV

Reference sequences were retrieved from the DDBJ/EMBL/GenBank database and their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the six-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method with the mid-point rooting option.

3. Results

3.1. SNPs for distinguishing between HBV/Cs and HBV/Ce in complete genomes

When the 171 HBV/C (34 HBV/Cs and 137 HBV/Ce) strains, retrieved from the DDBJ/EMBL/GenBank database were compared over the complete genomes, 17 SNPs were found between two subtypes (subgenotypes) (Table 1). Of them, five SNPs in non-overlapping polymerase region include restricted enzyme sites: *BstE*II site (nt 1041 of T [T1041] and C1044), *Ase*I site (A1050 and A1053) and *Nci*I site (C1155). Interestingly, the 34 HBV/Cs strains possessed *BstE*II site (G/GTNACC [nt 1039–1045]) and/or *Ase*I

Table 1
Subtype-specific mutations in the complete genomes of HBV/Cs and HBV/Ce

SNPs no.	Nucleotide position	Cs ($n=34$)	Unmatched	Amino acids/region	Ce ($n=137$)	Unmatched	Amino acids/region	Enzymes
1	166	C	0	Thr/S, **His/P**	A	1	Thr/S, **Asn/P**	
2	312	T	2	**Leu/S**, Phe/P	C	1	**Ser/S**, Phe/P	
3	400	C	3	Ile/S, **Leu/P**	A	0	Ile/S, **Ile/P**	
4	**1041**	**T**	**6**	**Gly/P**	**C**	**10**	**Gly/P**	*BstE*II
5	**1044**	**C**	**0**	**Thy/P**	**T**	**1**	**Thy/P**	*BstE*II
6	1047	A	0	Pro/P	T	3	Pro/P	
7	**1050**	**A**	**2**	**Ala/P**	**C**	**20**	**Ala/P**	*Ase*I
8	**1053**	**A**	**1**	**Leu/P**	**A/G**	**1**	**Leu/P**	*Ase*I
9	**1155**	**T**	**0**	**Ala/P**	**C**	**9**	**Ala/P**	*Nci*I
10	1721	A	1	Val/X	G	0	**Leu/X**	
11	2065	A	0	Leu/C	C	7	Leu/C	
12	2158	A	2	Val/C	C	5	Val/C	
13	2559	A	0	Lys/P	C	3	**Gln/P**	
14	2561	A	1	Lys/P	G	5	**Gln/P**	
15	2633	G	0	Leu/P	A	0	Leu/P	
16	2958	T	2	**Phe/P**, Asn/PreS1	C	4	**Leu/P**, Asn/PreS1	
17	3008	C	1	**Ser/P, Ala/PreS1**	A	5	**Arg/P, Asp/PreS1**	

(AT/TAAT [nt 1050–1055]), while the 137 HBV/Ce strains had neither *BstE*II nor *Ase*I sites. On the other hand, 128 of 137 (93%) HBV/C2 strains possessed *Nci*I site (CC/SGG [nt 1154–1158]) and none of the HBV/C1 strains had *Nci*I site due to T1151. Additionally, according to the SNPs, eight amino acids differences were found between two subtypes (subgenotypes) (Table 1).

3.2. PCR-RFLP for distinguishing between HBV/Cs and HBV/Ce

Geographically, typical genetic representatives for HBV/Cs and HBV/Ce (eight strains each) were selected. The partial genome sequence alignment including restriction sites is shown in Fig. 1. HBV/Cs strains were obtained

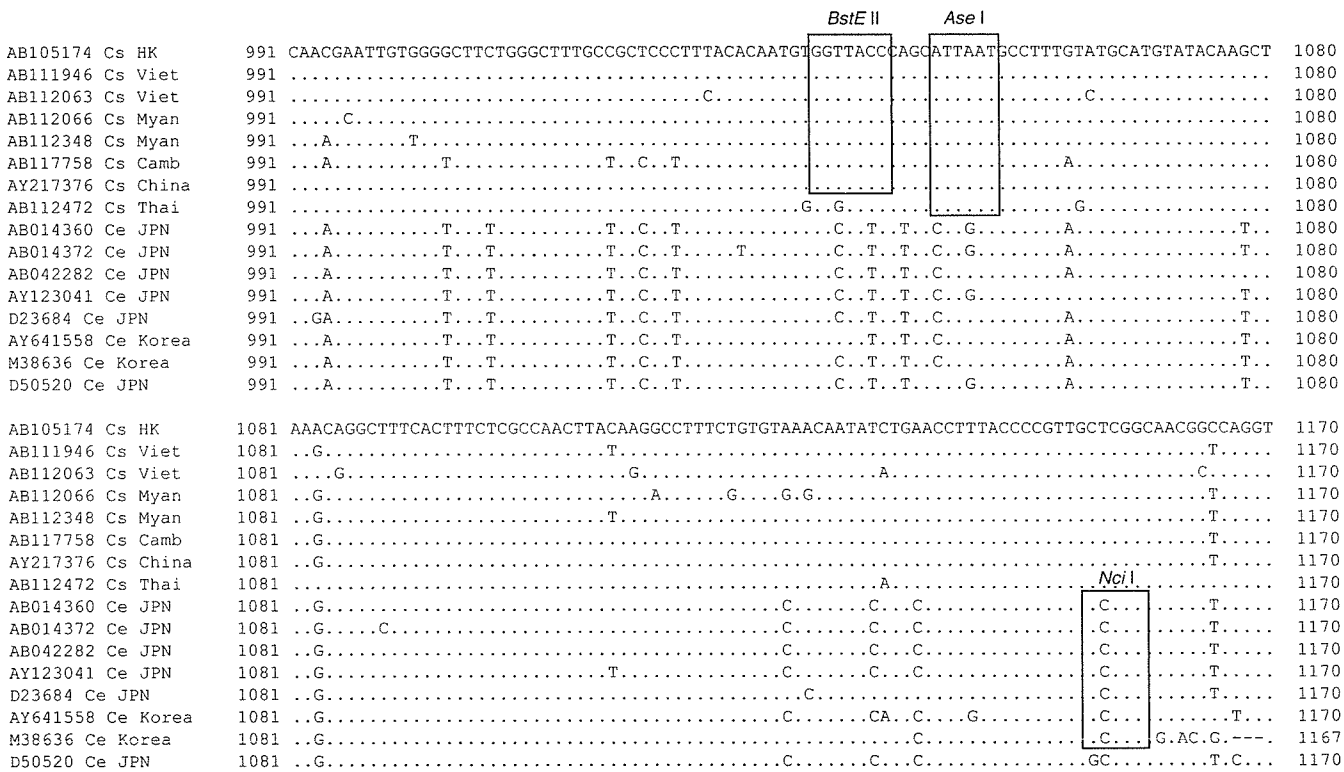

Fig. 1. Alignment of 8 HBV/Cs (C1) and 8 HBV/Ce (C2) sequences in non-overlapping polymerase region. The specific *BstE*II and *Ase*I sites are specific for HBV/Cs strains, while *Nci*I site is found in HBV/Ce strains. All sequences from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet).

from Vietnam, Thailand, Myanmar, China, Hong Kong and HBV/Ce from Japan, Korea. The subtypes (subgenotypes) of the 16 strains were confirmed by a phylogenetic analysis of the complete genome (Fig. 2a). Taking advantage of the five SNPs of T1041, C1044, A1050, A1053 and C1155, a RFLP method with three endonucleases was developed for distinguishing between HBV/Cs and HBV/Ce. PCR products of 309 bp in size (nt 964–1272), amplified on HBV/Cs strains, were split by *Ase*I digestion into two fragments of 88 and 221 bp and/or *BstE*II digestion into two fragments of 76 and 233 bp (Fig. 3), while those on HBV/Ce strains were not. In contrast, the products of 309 bp, amplified on HBV/Ce strains, were broken down by *Nci*I digestion into two fragments of 192 and 117 bp, while those on HBV/Cs strains were not.

Total 49 HBV/C samples, consisting of 24 in Hong Kong and 25 in Japan, were examined for the specificity of the novel PCR-RFLP method. Based on the PCR-RFLP, the 24 strains from Hong Kong were classified into HBV/Cs, and the 25 from Japan were HBV/Ce. To confirm the reliability of the PCR-RFLP method, the precore region plus core gene was sequenced directly on all 49 samples. All the 24 HBV/Cs and 25 HBV/Ce samples determined by PCR-RFLP were

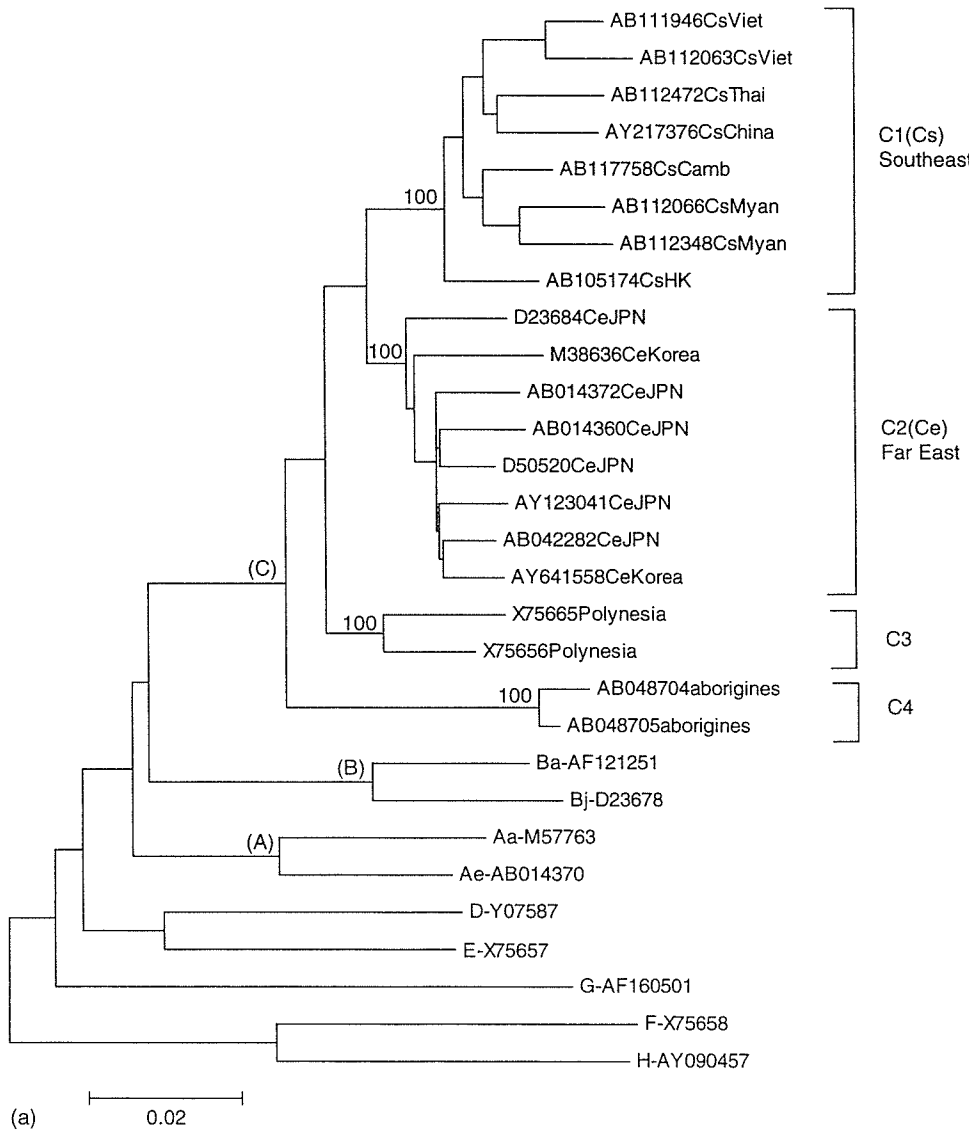

Fig. 2. (a) A phylogenetic tree constructed on the complete genome sequences of 29 HBV strains. Eight HBV/C1 (Cs) and eight HBV/C2 (Ce) strains (shown in Fig. 1) are compared along with four other HBV/C (C3 and C4) and nine HBV strains representative of the other seven genotypes (Aa, Ae, Ba, Bj, D–H). (b) A phylogenetic tree constructed on the X gene, precore and core gene sequences spanning 398 bp. Together with the above 29 representative sequences retrieved from database, 24 HBV/C1 (Cs) strains determined by PCR-RFLP belong to HBV/C1 (Cs) and 25 HBV/C2 (Ce) strains by PCR-RFLP had a cluster with the representative HBV/C2 (Ce) strains from database. All strains in this study are shown in bold. Each representative strain from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet). The length of the horizontal bar indicates the number of nucleotide substitution per site.

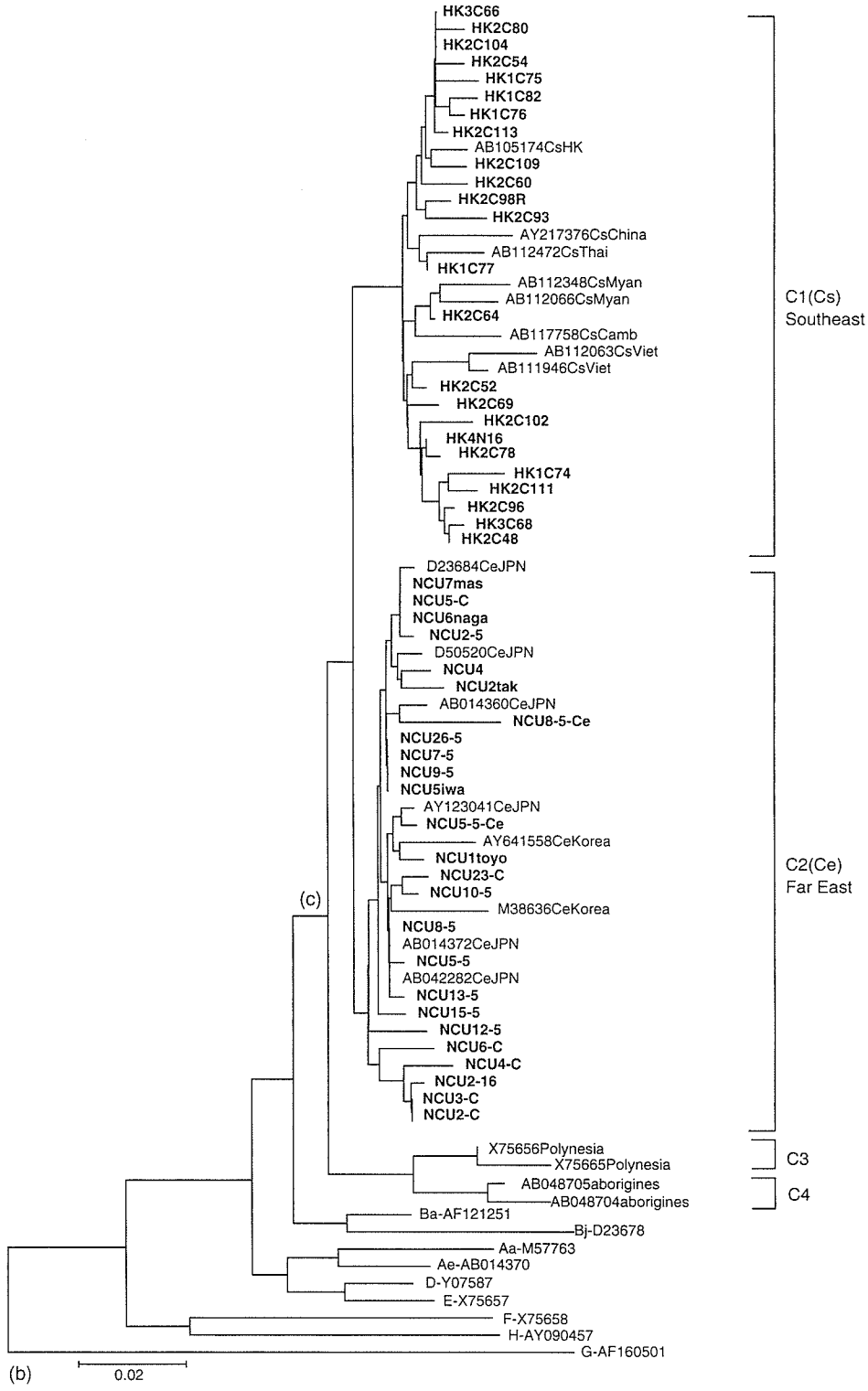

Fig. 2. (*Continued*).

completely classified into each subtype (subgenotype) by sequencing. To evaluate the sensitivity of the method, serial dilution of each HBV/Cs and HBV/Ce clones was used for the hemi-nested PCR, and its detection limit was five copies per assay.

3.3. Mutations in the enhancer, BCP and precore region in patients infected with HBV/C1 and C2

An alignment of sequences covering the BCP and the encapsidation signal (ε) in HBV/Cs and HBV/Ce allowed

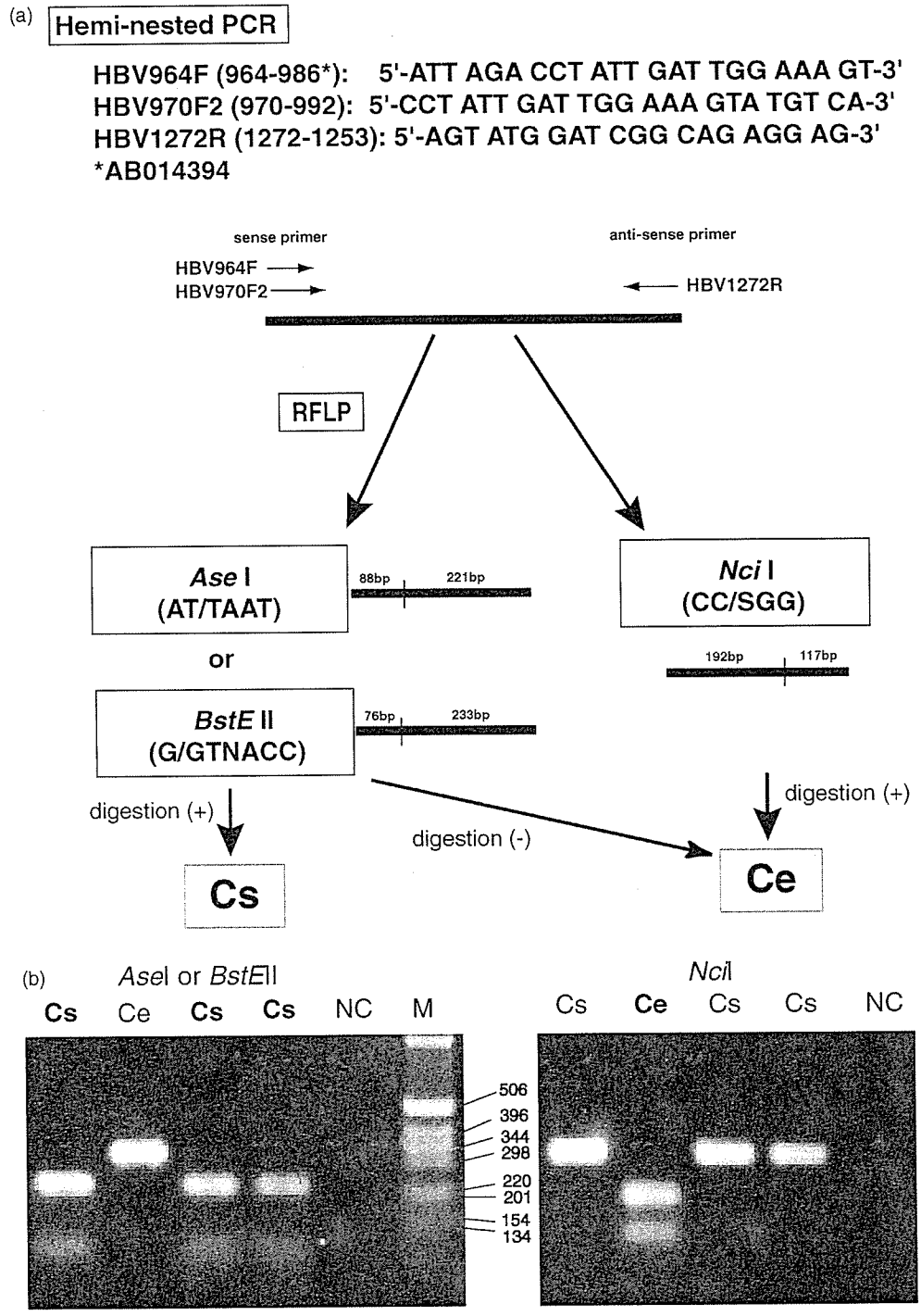

Fig. 3. (a) The strategy of a novel subtyping assay of HBV/C based on PCR-RFLP with *BstE*II, *Ase*I, *Nci*I restriction enzymes. HBV/Cs is digested by *BstE*II and/or *Ase*I, while HBV/Ce is digested by only *Nci*I. (b) Identification of restriction patterns obtained by restriction endonuclease digestion. Using hemi-nested PCR followed by cleavage with three kinds of restrict enzyme, it was possible to distinguish between HBV/Cs and HBV/Ce.

the identification of specific substitutions for HBV/C1 and HBV/C2 strains at nt 1721, 1757, 1775, 1856 and 1858 (Table 2). The prevalence of T1653, A1896 and A1899 substitutions was significantly higher in HBV/Ce than that in HBV/Cs, while the prevalence of A1727 and A1898 substitutions was higher in HBV/Cs. Double mutation in BCP (T1762/A1764) was highly prevalent in both subtypes (subgenotypes). Interestingly, the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains (45/162, 28%), whereas no mutation was found in HBV/Cs strains due to C1858. Another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs strains (7/58, 12%) (Table 2).

Table 2
Subtype-specific mutations in basic core promoter and encapsidational signal of HBV/Cs and Ce strains

Nucleotide position	Cs	This study ($n=24$)	Database ($n=34$)	Ce	This study ($n=25$)	Database ($n=137$)	P-value
1653	T	2 (8%)	0	T	7 (28%)	37 (27%)	<.0001
1721	A	22 (92%)	33 (97%)	G	24 (96%)	137 (100%)	<.0001
1727	A	19 (79%)	30 (88%)	A	13 (52%)	59 (43%)	<.0001
1757	A	13 (54%)	10 (29%)	G	25 (100%)	137 (100%)	<.0001
1762/1764	T/A	21 (88%)	13 (38%)	T/A	20 (80%)	65 (47%)	NS
1775	G	15 (63%)	28 (82%)	A	25 (100%)	132 (96%)	<.0001
1856	T	9 (38%)	6 (18%)	C	25 (100%)	137 (100%)	<.0001
1858	C	23 (96%)	23 (68%)	T	25 (100%)	137 (100%)	<.0001
1896	A	0	0	A	6 (24%)	39 (28%)	<.0001
1898	A	7 (29%)	0	A	0	0	<.0001
1899	A	1 (4%)	1 (3%)	A	4 (16%)	19 (14%)	0.029

4. Discussion

Chronic patients infected with HBV/C have a more aggressive clinical course than those infected with HBV/B [3,18]. In this study, we focused on HBV/C because it is prevalent mainly in Asia and seems to contribute to progressive liver disease and poor clinical outcomes in infected patients. Phylogenetic analyses of the complete genome show at least 4 subtypes (subgenotypes) of HBV/C (C1–4) with different geographic distribution (Fig. 2a) [19,20]. HBV/C1 was found only in Southeast Asia including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong and southern China, while HBV/C2 was found in far East Asia including Japan, Korea and northern China. Additionally, two another subtypes (subgenotypes) of HBV/C were named as C3 and C4 [19,20]. C3 was found in a large area of the Pacific from New Zealand to Polynesia, while C4 was isolated from Aborigines in Northeast Australia [17]. However, as C3 and C4 strains were rarely found in most Asian countries, we focused the classification between Cs (C1) and Ce (C2) in the present study.

A total of 118 complete genome sequences of the HBV/C strains isolated in the different geographic regions were analyzed phylogenetically in the recent study [13]; the phylogenetic subclusters within HBV/C were subsequently designated respectively to the geographic regions, i.e. "Cs" for Southeast Asian (Vietnam, Thailand, Myanmar and Southern China), and "Ce" for far East Asia (Korea, Japan, and Northern China). According to this classification, 80% of the patients in Hong Kong were belonged to the Cs and 20% to the Ce [13]. When taken in account both facts, i.e. evident geographic origins of these subtypes (subgenotypes) and the phylogenetic confirmation, the designation using the small letters (indicating possible origins) appears to be logical, similarly to the previously reported Asian "Ba" and Japanese "Bj" [5,7], Africa/Asian Aa and European Ae [8]. Hence, "Cs" and "Ce" designation was applied to the present study.

Based on five SNPs between HBV/Cs and HBV/Ce, we developed a novel PCR-RFLP method for distinguishing between HBV/Cs and HBV/Ce with high reliability. All 49 samples examined were completely classified by the PCR-RFLP. This method allows the classification between these subtypes (subgenotypes) without using expensive, labor- and time-consuming methods such as sequencing and molecular evolutionary analyses. Examining additional 171 complete sequences from database, only 9 sequences of HBV/Ce have exceptional mutations at the restriction site of *NciI*, indicating that less than 5% of the strains known up to date are unclassified by this method, and require sequencing as previously described [17].

Some specific mutations were detected in the encapsidation signal site; the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/Cs strains (Fig. 4). These mutations could stabilize the ε loop structure and the former HBeAg-negative mutants bearing a TAG stop codon mutation at codon 28 (A1896) uniformly replicate at least 20-fold better than mutants bearing a TGA stop codon at the same amino acid position enhance viral replication [21]. This C1858 variant was frequntly found in HBV/A and HBV/F [22]. Additionally, A1899 mutation was more prevalent in the HBV/Ce. As previously reported, the effects caused by these two closely linked mutations (A1896 and A1899) on viral replication are not independent each other [21]. The stringent selection for a highly efficient RNA encapsidation element may play a crucial role in the natural occurrence of these two closely linked precore mutations. Our replication model also shows that the combined mutations can induce higher replication in vitro (unpublished data). Hence, these several virological differences between the two subtypes (subgenotypes) might influence clinical outcomes such as fulminant hepatitis or hepatocarcinogemesis.

The biologic function of HBeAg remains controversial. Although HBeAg is not required for viral replication, it appears to be necessary for the establishment of chronic infection in animal models [23]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/Ce strains possessed T1858 and most HBV/Cs had C1858, the HBV/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of HBV/Cs. The clinical significance of C1858 and T1858 among HBV/C