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Polymorphisms of interleukin-1β in Japanese patients with hepatitis B virus infection

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Background/Aims: **Hepatitis B virus (HBV) induces liver cirrhosis (LC) and hepatocellular carcinoma (HCC) mainly by causing chronic necro-inflammatory hepatic disease. Our aim was to investigate the relationships between the polymorphisms of the interleukin-1B (*IL-1B*) promoter region and the interleukin-1 receptor antagonist gene (*IL-1RN*) and disease progression in an HBV-infected Japanese population.**

Methods: **Genomic DNA was extracted from the peripheral blood of 237 HBV carriers. Polymorphisms in *IL-1B* and *IL-1RN* were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and PCR with confronting two-pair primers (PCR-CTPP) methods. These polymorphic sites include the promoter regions of *IL-1B* at positions −511 and −31, and *IL-1RN* variable tandem repeats.**

Results: **The *IL-1B* −31 and −511 loci were in complete linkage disequilibrium, and the frequency of the *IL-1B* −31 T carrier (*IL-1B* −31 T/T or T/C) was significantly higher in HBV carriers with LC compared to those without LC (LC; 86.1% vs non-LC; 72.1%, $P = 0.009$). There was no difference in the genotype distribution of the *IL-1RN* polymorphism.**

Conclusions: **This is the first report describing the association between *IL-1B* polymorphism and HBV-related hepatic fibrosis, and our data suggest that *IL-1B* polymorphisms may be related to disease progression of HBV-related hepatitis in Japan.**

Keywords: Cytokines; Hepatitis B virus; Interleukin-1β; Liver cirrhosis; Polymorphism; Hepatic fibrosis

1. Background

Recent studies have emphasized that hepatic satellite cells (HSC) play an important role in the pathogenesis of hepatic fibrosis [1]. Following liver injury, HSC produce matrix metalloproteinase (MMPs) resulting in the development of hepatic fibrosis [2]. IL-1 has been implicated in the regulation of MMPs production by HSC [3]. IL-1β is known to be a proinflammatory cytokine and mediate several immune responses [4]. It is encoded by the *IL-1B* gene with several promoter elements, including a TATA box, a typical motif of includible genes [5,6]. The *IL-1B* gene has diallelic polymorphisms at −511, −31 base pairs (bp) from the transcriptional start site [7]. IL-1-receptor antagonist (*IL-1RN*) is an anti-inflammatory molecule that competes for receptor binding with IL-1β [8,9]. The *IL-1RN* gene contains an 86-bp variable number tandem repeat (VNTR) polymorphism in intron 2 [10]. These polymorphisms are, therefore, of

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Abbreviations: HBV, hepatitis B virus; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1B, interleukin-1B; IL-1RN, interleukin-1 receptor antagonist gene.

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Table 1
Baseline characteristics of HBV carrier

	ASC ($n = 65$)	CH ($n = 58$)	LC ($n = 65$)	HCC ($n = 49$) (CH:6, LC:43)
Mean age (yr)	55.7 ± 17.6	42.6 ± 14.4	58.2 ± 9.3	62.9 ± 9.4
Sex (M/F)	34/31	37/21	50/15	40/9
Mean ALT (U/L)	21.2 ± 8.1	134.7 ± 159.1	71.4 ± 89.3	59.2 ± 62.8
Mean PLT ($10^3/\mu l$)	197.8 ± 52.2	199.5 ± 51.1	125.9 ± 55.2	121.6 ± 84.0
Mean albumin (g/dl)	4.6 ± 0.3	4.5 ± 0.4	4.2 ± 0.6	3.8 ± 1.0

Abbreviations: ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; PLT, platelet.

potential functional importance in their modulation of IL-1β protein production and its biological activity.

Increasing evidences indicate that genetic factors influence the natural history of chronic liver diseases [11]. Recent studies have proposed that a number of gene polymorphisms influence the progression of fibrosis in patients with hepatitis C virus (HCV) infections, autoimmune chronic cholestasis, and alcohol-induced liver disease [12–14]. The aim of the present study was to elucidate the association of *IL-1B* and *IL-1RN* loci polymorphism as host genetic factors with an increased risk of developing HBV-related liver diseases in a Japanese population.

2. Patients and methods

2.1. Patients

A total of 237 patients who were positive for hepatitis B surface antigen (HBsAg) visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between August, 1999, and June, 2004. As controls, 63 healthy Japanese volunteers (33 men and 30 women, 22–66 years old, with a mean age of 36.6 ± 7.7 years) without any history liver disease were enrolled in the study after obtaining informed consent. The patients were regularly followed, with measurements of serum ALT and HBV markers such as HBsAg, HBeAg, anti-HBe using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan), and HBV DNA. Tumor markers such as alpha fetoprotein and/or des-γ-carboxy-prothrombin were also measured every month, and with ultrasonography or computed tomography of the liver every 3 months to detect HCC in a early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle biopsy specimens, if needed in all patients. All patients did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. The study protocol was approved by the Ethics Committees of both Nagasaki University Hospital and National Nagasaki Medical Center, and informed consent was obtained from each individual.

Of the 237 HBV carriers, 65 patients were considered to be asymptomatic carriers (ASC) based on sustained normalization of the serum ALT levels together with seropositivity for anti-HBe throughout the study (Table 1). On the other hand, 172 of the 237 HBV carriers were considered to have chronic progressive liver disease (CPLD) such as chronic hepatitis (58), cirrhosis (65), or hepatocellular carcinoma (49) manifested by elevated ALT levels and by clinical or histological findings on examination of liver tissue during the follow-up period (Table 1). Of the 49 patients with HCC, 6 (12%) were found to have chronic hepatitis; 43 (88%) had cirrhosis. The clinical data, including bilirubin, albumin, prothrombin time and the presence of ascites or hepatic encephalopathy, were collected and Child–Pugh score was calculated in LC patients. Of 237 HBV carriers, 79 had undergone liver biopsy during the study period to assess the degree of liver fibrosis using the METAVIR system [15]. Liver biopsy was not performed in parts of patients who had apparent biochemical, endoscopic and ultrasound features of LC.

2.2. DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd., UK).

2.3. Genotyping

The polymerase chain reaction (PCR) amplification was conducted using the primers listed in Table 2.

2.3.1. IL-1RN

The *IL-1RN* intron 2 contains a VNTR of an 86-bp length of DNA. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Allele 1 (four repeats) was 410 bp, allele 2 (two repeats) was 240 bp, allele 3 (three repeats) was 325 bp, allele 4 (five repeats) was 500 bp, and allele 5 (six repeats) was 595 bp [16].

Table 2
PCR condition for IL-1B and IL-1RN

Polymorphism	Primers	PCR conditions
IL-1B, T to C at −31	F1 5′-AATGTGGACATCAACTGCA-3′ R1 5′-CTCCCTCGCTGTTTTTATA-3′ F2 5′-ACTTCTGCTTTTGAAAGCC-3′ R2 5′-TCAGCTGTTAGATAAGCAG-3′	PCR-CTPP (confronting two-pair primers) 10 min at 95 °C, 30 cycles of 1 min at 95 °C, 48 °C and 72 °C and 5 min at 72 °C
IL-1B, C to T at −511	5′-GCCTGAACCCTGCATACCGT-3′ 5′-GCCAATAGCCCTCCCTGTCT-3′	PCR-RFLP (*Ava I*) 10 min at 94 °C, 5 cycles of 30 sec at 94 °C, 65 °C and 72 °C and 30 cycles of 30 sec at 94 °C, 60 °C and 72 °C and 5 cycles of 30 sec at 94 °C, 55 °C and 72 °C and 7 min 72 °C
IL-1RN 86 bp VNTR at intron 2	F 5′-CTCAGCAACACTCCTAT-3′ R 5′-TCCTGGTCTGCAGGTAA-3′	10 min at 95 °C, 35 cycles of 1 min at 95 °C, 55 °C and 72 °C and 5 min at 72 °C

Table 3
IL-1RN genotype frequencies in HBV carriers

Variables	Patients with HBV					Healthy volunteers ($n = 63$) (%)
	Total ($n = 237$) (%)	ASC ($n = 65$) (%)	CH ($n = 58$) (%)	LC ($n = 65$) (%)	HCC ($n = 49$) (%)	
IL-1RN						
1/1	216 (91.1)	59 (90.8)	53 (91.4)	58 (89.2)	46 (93.9)	53 (84.1)
1/2	13 (5.5)	5 (7.7)	1 (1.7)	6 (9.2)	1 (2.0)	7 (11.1)
1/3	1 (0.4)	0	1 (1.7)	0	0	0
1/4	4 (1.7)	0	2 (3.4)	1 (1.5)	1 (2.0)	2 (3.2)
2/2	1 (0.4)	0	0	0	1 (2.0)	0
2/4	2 (0.8)	1 (1.5)	1 (1.7)	0	0	1 (1.6)

Note. The genotype are shown as frequency (percentage).
Abbreviations: HBV, hepatitis B virus; ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1RN, IL-1 receptor antagonist.

2.3.2. *IL-1B −511*

A fragment containing the *Ava*I polymorphic site at position −511 of the *IL-1B* gene was amplified by PCR. Fragments were separated by electrophoresis on 3% agarose with ethidium bromide staining using appropriate commercially available size markers for comparison. The C allele was designated if two bands of 92 and 63 bp were obtained, and the T allele was designated if a signal band of the undigested 155 bp was obtained. Genotypes were designated as follows: C/C, two bands of 92 and 63 bp: C/T, three bands of 155, 92, and 63 bp; and T/T, a single band of 155 bp [17].

2.3.3. *IL-1B −31*

For the polymorphisms at −31 of *IL-1B*, a new method named PCR-CTPP (PCR with confronting two-pair primers) was applied, which does not require a step to digest DNA products for single nucleotide polymorphism genotyping [18]. All PCR products were visualized on a 2% agarose gel with ethidium bromide staining.

2.4. *Statistical analysis*

Results are expressed as means ± SD. Comparisons were made by Fisher's extract probability test and the χ^2 test. All p values were two-tailed, and P values <0.05 were considered to indicate statistical significance.

3. Results

3.1. *IL-1RN gene polymorphisms*

Six *IL-1RN* genotypes (1/1, 1/2, 1/3, 1/4, 2/2, and 2/4) were included in our study. Genotype 1/1(4/4 repeats) was the most common genotype in the HBV carrier patients (91.1%). In contrast to Caucasian populations, the homozygote allele 2* (2/2 repeat) was found in only one patient with HCC. The heterozygote allele 2* (1/2, 2/2, and 2/4) was found in 9.2% of ASC, 3.4% CH, 9.2% LC, and 4.0% of the HCC group (Table 3). The present study found no significant difference in *IL-1RN* genotype frequencies among various liver diseases of HBV carriers.

3.2. *IL-1B gene polymorphisms*

Table 4 shows the genotype frequencies of *IL-1B* gene polymorphism. Since *IL-1B* −511C/T was in complete linkage equilibrium with *IL-1B* −31T/C, only *IL-1B* −31T/C was described in this haplotype analysis. Although no statistical difference was found in allelic frequencies between liver cirrhosis patients and healthy subjects, *IL-1B* −31C/C homozygotes were less frequently seen in HBV carriers with LC or HCC. The proportion of the C/C genotype of *IL-1B* −31 in patients with HCC (14.3%) was not different from that in patients with LC (15.4%). However, when cases were subdivided according to the presence of LC (Table 5), the frequency of the C/C genotype in patients with LC (13.9%) was significantly lower than that in patients without LC (27.9%), while inversely the frequency of

Table 4
IL-1B genotype frequencies in HBV carriers

Variables	Patients with HBV					Healthy volunteers ($n = 63$) (%)
	Total ($n = 237$) (%)	ASC ($n = 65$) (%)	CH ($n = 58$) (%)	LC ($n = 65$) (%)	HCC ($n = 49$) (%)	
IL-1B −511						
C/C	69 (29.1)	17 (26.2)	18 (31.0)	17 (26.2)	17 (34.7)	20 (31.8)
C/T	117 (49.4)	27 (41.5)	27 (46.6)	38 (58.5)	25 (51.0)	29 (46.0)
T/T	51 (21.5)	21 (32.3)	13 (22.4)	10 (15.4)	7 (14.3)	14 (22.2)
IL-1B −31						
C/C	51 (21.5)	21 (32.3)	13 (22.4)	10 (15.4)	7 (14.3)	14 (22.2)
C/T	117 (49.4)	27 (41.5)	27 (46.6)	38 (58.5)	25 (51.0)	29 (46.0)
T/T	69 (29.1)	17 (26.2)	18 (31.0)	17 (26.2)	17 (34.7)	20 (31.8)

Note. The genotype are shown as frequency (percentage).
Abbreviations: HBV, hepatitis B virus; ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IL-1B, interleukin-1B.

Table 5
Differential distribution of IL-1B genotypes in HBV carriers

Locus	Genotype	Non-LC ($n = 129$)	LC ($n = 108$)	OR (95% CI)	P
IL-1B −31	T/C, T/T	93 (72.1%)	93 (86.1%)	2.40 (1.23–4.68)	0.0089
	C/C	36 (27.9%)	15 (13.9%)		

IL-1B −31 T carrier (*IL-1B* −31 T/T or T/C) was significantly higher in HBV carriers with LC compared to those without LC (LC; 86.1% vs non-LC; 72.1%, $P = 0.009$). When the LC patients were divided using Child–Pugh classification, there was no significant difference in *IL-1B* gene genotype frequencies between LC patients with Child–Pugh classification A and Child–Pugh classification B or C (Table 6A). In *IL-1RN* genotypes, *IL-1RN* 1/1 genotype was the most common genotype and 1/2 genotype was increased in LC patients with Child–Pugh classification A. However, there was no significant difference in genotype of *IL-1RN* among these LC patient groups (Table 6B). Furthermore, we examined the relationship between *IL-1B* gene genotype and the stage of hepatic fibrosis (Table 7). The frequency of the C/C genotype was reduced in patients with fibrosis stage F3–F4 (11.8%) compared to those with F0–F2 (28.6%), however, the number of patients who had undergone liver biopsy was limited and statistically significant difference was observed.

4. Discussion

Liver fibrogenesis is initiated by hepatocyte damage and the subsequent recruitment and activation of inflammatory cells [19]. These inflammatory cells produce fibrogenic cytokines and growth factors that activate hepatic satellite cells (HSC) [20]. The role of cytokine gene polymorphism in the progression of liver fibrosis or development of cirrhosis in patients with chronic liver diseases has been investigated extensively. Yee et al. indicated that TNF2 allele (−238A) and TNF3 allele (−308A) are more frequently found in patients with cirrhosis in chronic HCV infection [21]. Polymorphisms of TGF-β gene are thought to be one of the determinants of fibrosis progression in viral hepatitis [11]. Therefore, cytokine polymorphism could be involved in fibrosis progression in HBV infection.

Table 6A
Distribution of IL-1B genotype and Child–Pugh classification in LC patients

Locus	Genotype	Child–Pugh classification		
		A ($n = 79$) (%)	B ($n = 20$) (%)	C ($n = 9$) (%)
IL-1B −31	C/C	10 (12.7)	4 (20.0)	1 (11.1)
	C/T	44 (55.7)	12 (60.0)	5 (55.6)
	T/T	25 (31.6)	4 (20.0)	3 (33.3)

Recent studies have indicated that HSC play an important role in hepatic fibrogenesis and that IL-1 is a potent cytokine that induces the myofibroblastic activation of HSC [3]. Our data indicate that the frequency *IL-1B* −31 T carrier (*IL-1B* −31 T/T or *IL1B* −31 T/C) was significantly higher in HBV carriers with LC compared to in those without LC. These results suggest that the *IL-1B* genotype may influence fibrotic progression in HBV-related hepatitis. On the other hand, the frequency of the *IL-1RN* (intron 2, VNTR)* A2 allele was extremely uncommon in the study subjects and was not significantly different between HBV carriers with or without liver cirrhosis.

In our study, *IL-1B* −511 was in a complete linkage disequilibrium (LD) with IL-1B −31 in the Japanese population. Therefore, the effect of the *IL-1B* −511 C/T may be due to LD with *IL-1B* −31 T/C. There are some confusions regarding the 1*L*-1*B* (−31) alleles in earlier literatures. El-Omar et al. reported that −31C alleles increased the risk of gastric cancer [5,6]. They considered the *IL-1B* −31C allele but not the T allele as a pro-inflammatory gene. The *IL-1B* −31 T/C polymorphism is situated on a TATA box in the promoter region. However, the mutation of T to C in the TATA box in the *IL-1B* gene promoter (−31) will result in down regulation of the *IL-1B* gene in electrophoretic mobility-shift assay [5,6]. Xuan et al. found that *IL-1B* polymorphisms (*IL-1B* −511 C/C and −31T/T) enhanced IL-1β production in the gastric body of Japanese patients [22]. Similarly, the *IL-1B* −31 T/T genotype has been shown to be associated with an increased risk for HCC in Japanese patients with HCV infection [23]. Therefore, it is possible that the *IL-1B* −31T/T allele could be implicated in inflammatory processes. Our finding that HBV carriers harboring an *IL-1B* −31C/C genotype were less frequent in LC patients

Table 6B
Distribution of IL-1RN genotype and Child–Pugh classification in LC patients

Variables	Child–Pugh classification		
	A ($n = 79$) (%)	B ($n = 20$) (%)	C ($n = 9$) (%)
IL-1RN			
1/1	72 (91.1)	19 (95.0)	8 (88.9)
1/2	6 (7.6)	0	0
1/3	0	0	0
1/4	1 (1.3)	1 (5.0)	0
2/2	0	0	1 (11.1)
2/4	0	0	0

Table 7
Distribution of IL-1B genotypes and METAVIR score in HBV carriers

Locus	Genotype	Stage of fibrosis		OR (95% CI)	*P*
		F0–F2 ($n = 28$) (%)	F3–F4 ($n = 51$) (%)		
IL-1B −31	T/C, T/T	20 (71.4)	45 (88.2)	3.0 (0.92–9.8)	0.061
	C/C	8 (28.6)	6 (11.8)		

than non-LC patients is in accord with these findings. IL-1 is a proinflammatory cytokine which is involved in the fibrotic response. IL-1 causes tissue injury, which induces the fibrotic response, by producing chemotactic molecules, such as chemokines [24]. IL-1 is also implicated in the proliferation of HSC [25] and the regulation of the expression of various matrix metalloproteinases, which play a key role in the turnover and the deposition of extracellular matrix (ECM) [3]. Therefore, it is possible that genetic polymorphism of *IL-1B* gene may influence the progression of hepatic fibrosis by affecting the hepatic expression of IL-1 during the process of liver injury. Since this is the first report of the association between *IL-1B* polymorphism and hepatic fibrosis in HBV carriers, further investigation is required to confirm and extend our findings.

Several studies reported that *IL-1B* −31T is a risk haplotype for the development of cancer. Hirankarn et al. reported that IL-1B- 511C (−31T) allele is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population [26]. More recently, Chen et al. reported that in the presence of the *IL-1RN*2* allele, a ~5-fold increased risk of HCC was found for HBV carriers harboring the *IL-1B* −31 T/T or IL-1B −511 C/C genotype compared with those harboring the IL-1B −31 C/C or *IL-1B* −511 T/T genotype [27]. The frequencies of the *IL-1B* −31 genotype between HCC and non-HCC patients were insignificant in our study. However, our study included only 49 HCC patients and could not assess the interaction between polymorphisms of the *IL-1RN* and *IL-1B* genes due to the infrequency of the *IL-1RA*2* allele among Japanese subjects. Further investigations using large-scale case-control studies are needed to elucidate the relationship between the HCC risk and the *IL-1B* gene polymorphism.

Takamatsu et al. reported that the presence of the *IL-1B* −31C/C genotype was found at a significantly higher frequency in patients with liver cirrhosis than in those without cirrhotic alcoholic liver disease [28]. The reason for this discrepancy between our data and those of this previous report is unclear, although one possible explanation is a difference of the pathogenesis of liver cirrhosis.

In summary, the findings of the present study suggest that polymorphism in the promoter region of the *IL-1B* gene (−31) is implicated in the regulation of liver fibrosis in patients with HBV infection. The interactions between HBV viral factors and host factors including cytokine polymorphisms may contribute to disease progression in HVB carriers. The interaction between *IL-1B* polymorphisms and liver fibrogenesis deserves further study.

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Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan

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Abstract

Genotypes of hepatitis B virus (HBV) were determined in 485 patients with acute hepatitis B from all over Japan. They were A in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Sexual contacts were the main route of transmission in them. Overall,

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HBV persisted in only 5 of the 464 (1%) followed patients. Genotypes C accounted for more than 68% in northern as well as southern areas, contrasting with genotype A accounting for 34% in and around the Metropolitan areas. During 24 years from 1982 to 2005, genotype A increased from 5% to 33%, while genotype B gradually decreased from 26% to 8%. Fulminant hepatitis was significantly more frequent in infection with genotype Bj (41%) than those with the other genotypes ($p < 0.01$). The core-promoter double mutation (T1762/A1764) and precore stop-codon mutation (A1896) were more frequent in patients with fulminant than acute self-limited hepatitis (57% versus 15% and 58% versus 10%, respectively, $p < 0.01$ for both). In conclusion, genotype A distributes unevenly over Japan, prevails in younger patients through sexual transmission and has increased with years. Furthermore, fulminant outcome was more frequent in patients with genotype Bj than those with the other genotypes.

Keywords: Chronic hepatitis; Fulminant hepatitis; Hepatitis B e antigen; Hepatitis B surface antigen; Sexual transmission

1. Introduction

Mass vaccination with plasma-derived or recombinant vaccines has been effective in Asian countries hyperendemic with hepatitis B virus (HBV), as well as in the United States and France. In Japan, perinatal transmission of HBV from mothers with hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in serum used to be the principal route to establish the persistent carrier state [1]. Hence, passive and active immunoprophylaxis of babies born to carrier mothers with HBeAg by combined hepatitis B immunoglobulin and vaccine was mandated since 1986, and extended to carrier mothers without HBeAg in 1995. As the results, HBsAg has become rare in the Japanese born after 1986, and is detected in merely 0.3% of the first-time blood donors younger than 19 years at present [2].

There is an increasing trend, however, for acute HBV infection to occur preferentially in young men through promiscuous sexual contacts [3–7]. Foreign sexual workers from neighboring Asian countries are suspected as significant sources of de novo HBV infection in them [4,8]. Thus, patients with acute hepatitis visiting hospitals are increasing all over Japan. Since the majority of acute HBV infections ran subclinical courses, hospitalized cases of acute hepatitis B would represent the tip of an iceberg.

Eight genotypes have been detected by the sequence divergence >8% in the entire HBV genome composed of approximately 3200 nucleotides (nt), and designated by capital alphabet letters from A to H in the order of documentation [9–12]. They have distinct geographical distribution and are associated with severity of liver disease as well as response to antiviral therapies [13–15]. Furthermore, subgenotypes have been reported for HBV/A, B and C, and named Aa (Asian/African type) and Ae (European type) [16], Bj (Japanese type) and Ba (Asian type) [17], as well as Ce (east Asian type) and Cs (southeast Asian type) [18]. There have been increasing lines of evidence that Aa and Ae, as well as Ba and Bj, influence the replication of HBV and bear clinical relevance [19–21].

Over 24 years from 1982 to 2005, a multicenter study was conducted throughout Japan on 547 patients with acute hepatitis B. Genotypes were determined on viral isolates recovered from them, and analyzed for distributions changing with time and in place. The results highlighted foreign HBV genotypes represented by HBV/A which have increased through sexual contacts, and HBV/Bj prevalent in patients with fulminant hepatitis.

2. Patients and methods

2.1. Patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals throughout Japan, of them, 147 and 336 cases are overlapping with previous report from Yotsuyanagi et al. [7] and Ozasa et al. [22], respectively, and 64 cases were newly registered in this study. These hospitals were grouped into the following eight areas: Hokkaido, Tohoku, Kanto, Koshin, Tokai, Kinki, Chugoku/Shikoku and Kyushu/Okinawa. The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. The great majority of them were followed for clinical outcomes until the disappearance of HBsAg through 24 weeks or longer after the presentation. HBV genotypes were determined in sera stored at −40 °C, and their geographical distributions and chronological changes were analyzed. Further, they were correlated with sources of infection and clinical outcomes of acute hepatitis. The study protocol conformed to the 1975 declaration of Helsinki, and was approved by Ethics Committees of institutions. Every patient or his/her next of kin gave an informed consent on the purpose of this study.

2.2. Serological markers of HBV infection

HBsAg was determined by hemagglutination (MyCell, Institute of Immunology Co. Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (AxSYM, Abbott Japan, Tokyo, Japan), and HBeAg by ELISA (ELISA, F-HBe, Kokusai Diagnostic, Kobe, Japan). Anti-HBc of IgM class was determined by ELISA (HBc-antiM RIA, Dainabot, Tokyo, Japan).

2.3. Genotypes and subgenotypes of HBV

The six major HBV genotypes (A–F) were determined serologically by ELISA using commercial kits (HBV GENO-

TYPE EIA, Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies that is specific for each of them [23,24]. Genotypes were confirmed by restriction fragment length polymporphisim (RFLP) when required [25].

HBV/Bj (Japanese type) without the recombination with genotype C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP involving on specific nucleotide substitutions, by the method described previously [26].

2.4. Point mutations in the precore resion and basic core-promoter (BCP)

Mutations in the precore region for A1896 and BCP for T1762/A1764 were detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostic, Tokyo, Japan) according to the manufacturer's instructions, or by sequencing the precore region and BCP using the method described previously [27]. The results were recorded as "the wild-type" or "the mutant-type" expressed dominantly by HBV isolates.

2.5. Statistical analysis

Categorial variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorial variables by the Mann–Whitney's U-test. A p-value less than 0.05 was considered significant.

3. Results

3.1. Clinical profiles of patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals from all over Japan. Genotypes of HBV were unclassifiable in 40 (7%) and sufficient clinical data not available in 22 (4%) of them. Exclusive of these 62 patients, 485 (89%) were left for the evaluation of geographic distribution of HBV genotypes, as well as their changes with time, transmission routes and relevance with clinical outcomes.

The 485 patients with acute hepatitis B had the mean ± S.D. age of 35.7 ± 13.7 years, and included 338 (70%) men. Their peak alanine aminotransferase (ALT) averaged 2576 ± 1673 IU/L and peak total bilirubin 9.5 ± 9.5 mg/dL. They all possessed anti-HBc of IgM class in high titers, and HBeAg was detected in sera from 338 (70%) of them at the presentation. Fulminant hepatitis with coma of grade >II and prothrombin time <40% developed within 8 weeks after the onset in 45 (9%) of them.

Fulminant hepatitis led to death in 18 (5%) patients, and 3 (1%) received liver transplantation. Exclusive of these 21 patients, HBV persisted in only 5 of the 464 (1%) studied patients with acute hepatitis. They represented 3 of the 92 (3%) infected with HBV/A, 1 of the 58 (2%) with B and 1 of the 330 (0.3%) with C. None of the five patients with chronic outcome had received antiviral or steroid treatment during their acute phase of illness.

3.2. Geographic distribution of HBV genotypes in patients with acute hepatitis B

Overall, HBV/A was detected in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Distribution of HBV genotypes over Japan is illustrated in Fig. 1. They dispersed unevenly in place. HBV/C accounted for more than 68% in both northern and southern areas, contrasting with HBV/A accounting for 34% in and around the Metropolitan area. Among HBV/B infections, the proportion of HBV/Ba was higher in Koshin, Kinki and Kyushu/Okinawa (14/17, 1/1 and 2/3), while that of HBV/Bj was higher in Hokkaido, Tohoku, Kanto and Tokai (4/4, 6/7, 13/21 and 5/5).

3.3. Demographic and clinical differences of patients infected with various genotypes

Table 1 compares demographic and clinical characteristics of patients with different HBV genotypes. Patients with HBV/D were excluded from the analysis due to their small numbers. The mean age was lower in patients with HBV/A (31.8 ± 10.9 years) than HBV/Ba (40.7 ± 10.9, $p < 0.01$), HBV/Bj (41.2 ± 17.0, $p = 0.01$) and HBV/C (35.8 ± 13.9, $p < 0.03$); it was higher in patients with HBV/Ba than HBV/C (40.7 ± 10.9 versus 35.8 ± 13.9, $p = 0.02$). The proportion of patients aged <30 years was significantly greater in HBV/A (58%) than HBV/Ba (12%, $p < 0.01$), HBV/Bj (38%, $p < 0.04$) or HBV/C infection (42%, $p < 0.01$). Men predominated ($p < 0.01$) in infections with HBV/A and HBV/Ba (92% and 88%, respectively) than those with HBV/Bj and HBV/C (56% and 64%, respectively). The peak ALT level was higher in HBV/Bj (3371 ± 2342 IU/L) than HBV/A (2051 ± 1009, $p = 0.04$) or HBV/C (2650 ± 1747, $p < 0.03$) infection. HBeAg was detected in 84% of patients with HBV/A at the frequency much higher than that in those with HBV/Ba (54%, $p < 0.01$), HBV/Bj (59%, $p < 0.01$) or HBV/C (60%, $p < 0.01$).

The routes of transmission were sexual contacts in 216 (45%) patients, followed by medical accidents in 14 (8%), blood transfusion in 4 (1%) and drug in 1 (0.2%); transmission routes were not identified in the remaining 245 (51%) patients. Sexual transmission was the most frequent cause of infection in 57% of HBV/A, 73% of HBV/Ba, 34% of HBV/Bj and 40% of HBV/C infections.

Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (41%) than the other genotypes ($p < 0.01$); it occurred in 2 of the 5 (40%) patients with HBV/D, also. In reflection of severe clinical courses, the peak ALT level tended to be high in patients with HBV/Bj.

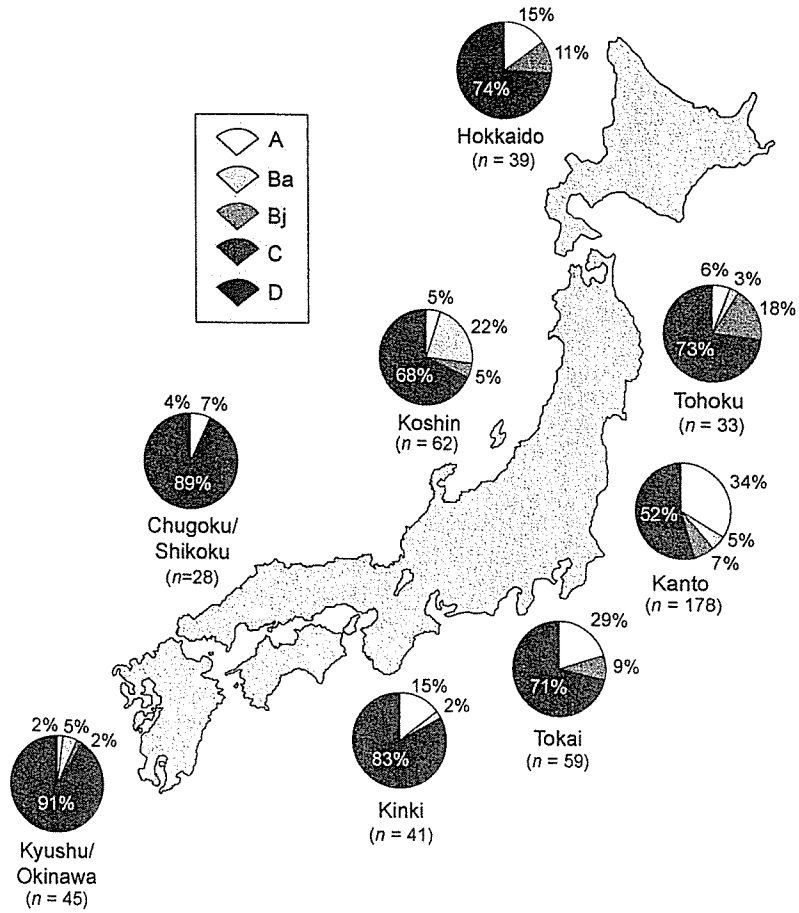

Fig. 1. Geographic distribution of HBV genotypes among 485 patients with acute hepatitis in Japan during 1982–2005.

The core-promoter double mutation (A1762T/G1764A) was more frequent in patients with fulminant than acute self-limited hepatitis infected either with HBV/Bj (70% versus 0%, $p<0.01$) or HBV/C (50% versus 18%, $p<0.01$). Also, precore stop-codon mutation (G1896A) was more offen in patients with fulminant than acute self-limited hepatitis who were infected with HBV/Ba (100% versus 5%, $p=0.01$), HBV/Bj (60% versus 0%, $p<0.01$) or HBV/C (50% versus 14%, $p<0.01$).

3.4. Changes in the distribution of HBV genotype with the age and time

Fig. 2 depicts the distribution of HBV genotypes stratified by the age. Prevalence of HBV/A decreased with the age, and was higher in the patients aged <30 years (26%) than in the 40s (14%, $p=0.03$) or aged ≥50 years (8%, $p<0.01$). Prevalence of HBV/B increased with the age, in converse, and was lower in the patients aged <30 years (7%) than in the 40s (18%, $p=0.01$) or aged ≥50 years (17%, $p=0.02$). No significant differences were observed in the distribution of HBV/Ba or HBV/Bj among all age groups.

Fig. 3 illustrates changes in HBV genotypes through the four time spans covering 24 years. HBV/A accounted for 5% (2/42) in 1982–1990, 5% (4/78) in 1991–1995 and 14% (18/133) in 1996–2000, and thereafter increased to 33% (58/176) in 2001–2005. There was significant difference between 1982–1995 and 1996–2005 ($p<0.01$). HBV/B accounted for 26% (11/42) in 1982–1990, 17% (13/78) in

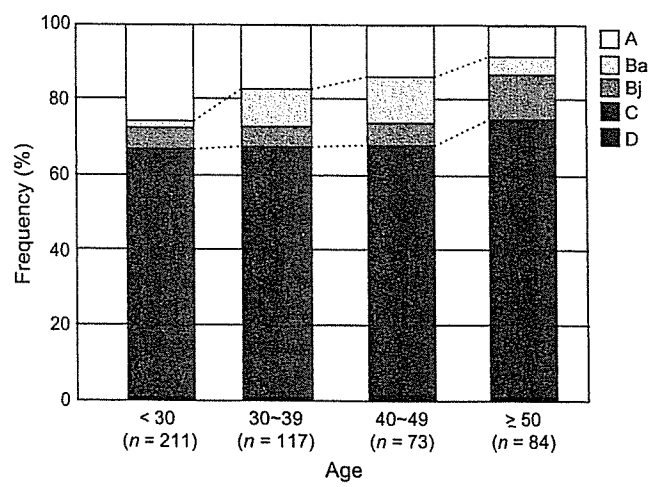

Fig. 2. Age-specific prevalence of HBV genotypes among 485 patients with acute hepatitis.

Table 1
Demographic and clinical differences among patients infected with HBV of distinct genotypes

	Genotypes			
	A ($n=92$)	Ba ($n=26$)	Bj ($n=32$)	C ($n=330$)
Mean age (years)	31.8 ± 10.9[a]	40.7 ± 10.9[b]	41.2 ± 17.0	35.8 ± 13.9
Age <30 years	54 (58%)[c]	3 (12%)[d]	12 (38%)	140 (42%)
Male	85 (92%)[e]	23 (88%)[f]	18 (56%)	210 (64%)
Peak ALT (IU/L)	2051 ± 1009[g]	2536 ± 1104	3371 ± 2342[h]	2650 ± 1747
Peak total				
Bilirubin (mg/dL)	10.3 ± 10.4	8.9 ± 5.8	10.5 ± 8.1	9.3 ± 9.7
HBeAg positive	78 (84%)[i]	14 (54%)	19 (59%)	199 (60%)
Transmission routes				
Sexual	52 (57%)	19 (73%)	11 (34%)	134 (40%)
Medical accident	0	0	3 (9%)	11 (3%)
Drug/tattoo	0	0	0	1 (1%)
Blood transfusion	0	0	1 (4%)	3 (1%)
Unknown	40 (43%)	7 (23%)	17 (53%)	181 (55%)
Metropolitan areas	60 (65%)[i]	8 (31%)	13 (41%)	93 (28%)
Fulminant hepatitis	1 (1%)	2 (8%)	13 (41%)[j]	29 (9%)
Mutations in HBV DNA				
BCP (1762T/1764A)				
Acute self-limited	2/67 (3%)	4/22 (18%)	0/13 (0%)[k]	41/223 (18%)[k]
Fulminant	0/1 (0%)	0/2 (0%)	7/10 (70%)	10/20 (50%)
Precore (1896A)				
Acute self-limited	1/67 (1%)	1/22 (5%)[k]	0/13 (0%)[l]	31/223 (14%)[k]
Fulminant	1/1 (100%)	2/2 (100%)	6/10 (60%)	10/20 (50%)

$p<0.01$, acute vs. fulminant.
[a] $p<0.01$, A vs. Ba. $p=0.01$, A vs. Bj. $p=0.03$, A vs. C.
[b] $p=0.02$, Ba vs. C.
[c] $p<0.01$, A vs. Ba. $p<0.04$, A vs. Bj. $p<0.01$, A vs. C.
[d] $p<0.01$, Ba vs. C. $p<0.04$, A vs. Bj. $p<0.01$, A vs. C.
[e] $p<0.001$, A vs. Bj. $p<0.01$, A vs. C.
[f] $p<0.01$, Ba vs. Bj. $p<0.01$, Ba vs. C.
[g] $p=0.04$, A vs. Ba. $p<0.01$, A vs. B1. $p<0.01$, A vs. C.
[h] $p=0.03$, Bj vs. C.
[i] $p<0.01$, A vs. Ba, Bj or C.
[j] $p<0.01$, Bj vs. A, Ba or C.
[k] $p<0.01$, acute vs. fulminant.
[l] $p=0.01$, acute vs. fulminant.

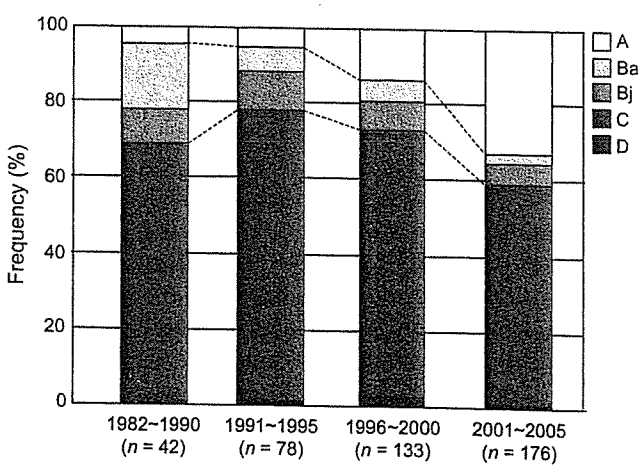

Fig. 3. Chronological changes in the distribution of HBV genotypes among 429 patients with acute hepatitis.

1991–1995 and 14% (18/133) in 1996–2000, and thereafter decreased to 8% (14/176) in 2001–2005; there was significant difference between 1982–1990 and 2001–2005 ($p<0.01$). However, no significant differences were observed in the distribution of HBV/Ba or HBV/Bj among the four time spans.

4. Discussion

As in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission and horizontal infection during the infancy in Japan, until 1986 when combined active and passive immunoprophylaxis was started in the national program. There still are approximately million HBV carriers estimated by the prevalence of HBsAg in the first-time blood donors [28]; they had been infected with HBV before the prophylaxis started. The age-specific prevalence is high in the elderly, and some of them develop chronic liver disease culminating in cirrhosis and hepatocellular carcinoma. By far the majority of genotypes in

patients with persistent HBV infection are HBV/B (approximately 30%) and HBV/C (about 70%) [4,29]. In patients with acute hepatitis in hospitals in the Metropolitan Tokyo, however, HBV/A was detected in 23% with HBV/Ae prevailing [5], in contrast to HBV/B in 14% and HBV/C in 44% [4]. It is not certain if a high prevalence of HBV/A in acute HBV infection in Tokyo is extrapolated to the other areas in Japan.

The present study was performed on 547 patients with acute hepatitis B visiting 25 hospitals in Japan during 24 years from 1982 to 2005. They include the 147 (27%) patients reported by Yotsunangi et al. [7] and the 336 (61%) by Ozasa et al. [22]; the remaining 64 (12%) patients were recruited anew. Yotsuyanagi et al. [7] dealt with a peculiar distribution of HBV/A clustering in and around Metropolitan areas and clinical manifestation dependent on genotypes, while Ozasa et al. [22] focused on the influence of HBV genotypes on clinical outcomes, in terms of fulminant hepatitis and viral persistence, as well as the occurrence of precore stop-codon mutation. The assortment of these cohorts of patients have given us the power to precisely picture the epidemiology of genotypes in acute HBV infection all over Japan and shift thereof during the past quarter of century. In addition, it confirmed the results of previous studies on the precore mutations, and extended them to the core-promoter double mutation that can make differences in fulminant or self-limited outcome of acute HBV infection under the influence of HBV genotypes. In the present multicenter study on patients with acute hepatitis B, HBV/C was the most frequent and HBV/A was the second most common in central areas in Japan harboring big cities (Fig. 1). Along with HBV/A, the other foreign genotypes (Ba and D) accounted for 123 of the 485 (25%) acute HBV infections. Furthermore, foreign origins of acute HBV infections would increase further, if overseas origins of HBV/C (Ce and Cs) are examined in the future.

Clinical relevance of HBV genotypes, in terms of severity of liver disease and response to antiviral treatment, has been evaluated mostly in patients with chronic liver disease [27,30,31]. Due to uneven distributions of HBV genotypes over the world, however, comparison is largely restricted to only two genotypes prevailing in each country. All in all, it would be reasonable to state that patients with HBV/A or HBV/B fare better than those with HBV/D or HBV/C [21,27,30–32]. Influence of HBV genotypes on acute hepatitis B is also reported [33]. In the present study, in which patients with acute hepatitis infected with HBV/A, HBV/Ba, HBV/Bj or HBV/C were compared, there were significant differences in age, gender, peak ALT levels, HBeAg positivity and outcome (Table 1). These results indicate that HBV genotypes influence the clinical profile in not only chronic, but also acute hepatitis B.

Fulminant hepatitis developed rather frequently in 45 of the 485 (9%) patients, although it would be overrepresented in them who visited hospitals due to overt liver disease. Remarkably, chances for patients infected with HBV/Bj to develop fulminant hepatitis were significantly higher than those with HBVA, HBV/Ba or HBV/C (41% versus 1%, 8% or 9%, $p < 0.01$). These results were consistent with those in the previous study [22]. In patients with acute hepatitis B from Chiba in Japan [34], HBV/B was more frequent in those with fulminant than self-limited hepatitis (63% versus 31%, $p = 0.027$); however, some cases of acute exacerbation of persistent HBV infection were included in their study.

The precore stop-codon mutation (G1896A) was detected significantly more frequently in patients with fulminant than acute self-limited hepatitis, in confirmation of our previous report [22]. Furthermore, the BCP double mutation (A1762T/G1764A) was detected more often in fluminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of BCP mutation was much higher in the patients with fulminant (70%) than that reported in those with chronic hepatitis (16%) [27]. Precore and BCP mutations are very frequent in patients with fulminant hepatitis in Asia [35–37] and the Middle East [38], but rare in Western countries [39–41]. These differences may be explained by distinct geographical distributions of HBV genotypes.

An extremely wide range (0–12%) has been reported in the rate of persistence after acute HBV infection in the adulthood [42–49]. Why such a big difference arises needs to be clarified, because the HBV genotype is implicated in a high chronicity rate in European countries where HBV/A is predominant. In accordance with this view, HBV/A was more frequent than HBV/D in 32 patients with chronic active hepatitis (80% versus 11%), while the reverse was the case in acute hepatitis (10% versus 80%) in Switzerland [33]. In Japan, also, infection with HBV/A tends to prolong longer than that with HBV/B or HBV/C in the adulthood (23% [3/13] versus 13% [1/8] or 12% [3/25]) [4]. In the present series on 464 patients with acute hepatitis B in Japan, for whom genotyping was feasible, HBV infection persisted in only 5 (1%) representing 3 of the 92 (3%) with HBV/A, 1 of the 58 (2%) with HBV/B and 1 of the 330 (0.3%) with HBV/C. Hence the infection with HBV/A would persist longer than those with the other genotypes.

The present study highlighted the recent rapid increase in cases of acute hepatitis B in Japan, which gained about five-fold from 4.7 per year before 1990 to 33.1 after 2000 in participant hospitals. Primary HBV infection in the adulthood can occur by homo/heterosexual contacts, intravenous drug use, medical accidents and blood transfusion [50–54]. Acute HBV infection in the 485 patients studied was transmitted by sexual contacts in 216 (45%), medical accidents in 14 (3% (none since 2001)) and blood transfusion in 4 (1% (none since 2001)). The route is unknown for the remaining 245 (51%) patients; some of them might have been infected by undeclared sexual contacts and/or intravenous drug use, although recreational drugs are still uncommon in Japan. It comes as a surprise that the overall proportion of foreign genotypes (A, Ba and D) in patients with acute hepatitis B was high at 25%. Of these foreign genotypes, Asian genotypes such as HBV/Ba have decreased gradually, while HBV/A has increased in converse (Fig. 3). Furthermore,

some of "domestic" HBV/C infections that accounted for the majority (68%) may well have been imported by sexual workers from foreign countries where this genotype is prevalent. Combined, a substantial part of acute HBV infection in Japan does seem to have been imported.

In conclusion, the distribution of HBV genotypes in patients with acute hepatitis B is different geographically, and has changed with time in Japan. Recently, acute HBV infection is increasing among the Japanese adults, although it rarely becomes chronic. A significant part of it is transmitted sexually with foreign HBV genotypes. Furthermore, a fulminant outcome is frequent after infection with HBV/Bj having precore and/or BCP mutations. These facts should be made open to the public for making them aware of the risk and taking measures to prevent it.

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Influence of Genotypes and Precore Mutations on Fulminant or Chronic Outcome of Acute Hepatitis B Virus Infection

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The outcome of acute hepatitis B virus (HBV) infection is variable, influenced by host and viral factors. From 1982 through 2004, 301 patients with acute HBV infection entered a multi-center cross-sectional study in Japan. Patients with fulminant hepatitis (n = 40) were older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P < .0017$), less predominantly male (43% vs. 71%, $P = .0005$), less positive for hepatitis B e antigen (HBeAg) (23% vs. 60%, $P < .0001$), less infected with subgenotype Ae (0% vs. 13%, $P < .05$), and more frequently with Bj (30% vs. 4%, $P < .0001$) than those with acute self-limited hepatitis (n = 261). Precore (G1896A) and core-promoter (A1762T/G1764A) mutations were more frequent in patients with fulminant than acute self-limited hepatitis (53% vs. 9% and 50% vs. 17%, $P < .0001$ for both). HBV infection persisted in only three (1%) patients, and they represented 2 of the 23 infected with Ae and 1 of the 187 with the other subgenotypes (9% vs. 0.5%, $P = .032$); none of them received antiviral therapy. In multivariate analysis, age 34 years or older, Bj, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and G1896A mutation were independently associated with the fulminant outcome. In *in vitro* transfection experiments, the replication of Bj clone was markedly enhanced by introducing either G1896A or A1762T/G1764A mutation. In conclusion, persistence of HBV was rare (1%) and associated with Ae, whereas fulminant hepatitis was frequent (13%) and associated with Bj and lack of HBeAg as well as high replication due to precore mutation in patients with acute HBV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006; 44:326-334.)

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBc, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ALT, alanine aminotransferase.

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Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are persistently infected with it.[1] Acute infection with HBV resolves in the great majority but can induce fulminant hepatitis or go on to become chronic. Host and viral factors may influence fulminant or chronic outcome of acute HBV infection, but they are not fully defined.

Eight genotypes have been detected by a sequence divergence greater than 8% in the entire HBV genome of approximately 3,200 nucleotides (nt), and designated by capital alphabet letters from A (HBV/A) to H in the order of documentation.[2-5] They have distinct geographical distributions associated with severity of liver disease as well as response to antiviral therapies.[6-8] Furthermore, subgenotypes have been reported for HBV/A, B, and C and named Aa/A1 (Asian/African type) and Ae/A2 (European type),[9] Bj/B1 (Japanese type) and Ba/B2 (Asian type),[10] as well as Cs/C1 (Southeast Asian type) and Ce/C2 (East Asian type).[11-13] Increasing lines of evidence indicate that subgenotypes of HBV/A and B influence the replication of HBV and bear clinical relevance.[14-16] Furthermore, genotypes affect mutations in precore region and core promoter, thereby influencing the expression of hepatitis B e antigen (HBeAg).[8,17]

During the 23 years from 1982 to 2004, a multi-center cross-sectional study was conducted throughout Japan on 301 patients with acute hepatitis B. We examined the influence of genotypes/subgenotypes on their fulminant or chronic outcome. Furthermore, the influence of G1896A or A1762T/G1764A on replication of HBV was evaluated in an *in vitro* replication model.

Patients and Methods

Patients With Acute Hepatitis B. During 1982 through 2004, 336 consecutive cases of acute hepatitis B were registered in 16 hospitals throughout Japan. These hospitals were from the following eight areas: Hokkaido (represented by J.-H. K. and S.H.), Tohoku (T.K. and K.S.), Kanto (H.T., Y.A. and K.I.), Koshin (E.T. and S.O), Tokai (A.O., Y.T., E.O., M.S., R.U., M.M., and S.K.), Kinki (T.O.), Honshu/Shikoku (Y.M., K.H., and M.O.), and Kyushu (H.Y. and H.S.). The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. Patients with initial high-titered anti-HBc (≥90% inhibition by a 1:200 diluted serum) were excluded; they were diagnosed as exacerbation of chronic hepatitis B. Patients with acute hepatitis A, hepatitis C, or human immunodeficiency virus co-infection, and drug- or alcohol-induced acute hepatitis also were excluded; hepatitis D virus infection was not examined because of its extreme rarity in Japan.[18] Most of them were followed for clinical outcomes until the disappearance of hepatitis B surface antigen (HBsAg) during 24 weeks or longer after the presentation. The criteria of fulminant hepatitis are based on the report by Trey et al.,[19] with a slight modification in 1981 (Inuyama symposium, Aichi, Japan): coma of grade II or higher and prothrombin time less than 40% developing within 8 weeks after the onset. Serum samples were collected at the presentation and had been stored at −80°C. HBV genotypes, HBV DNA, and HBeAg were determined, and clinical outcomes of acute hepatitis were analyzed. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave an informed consent for this study.

Serological Markers of HBV Infection. HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme immunoassay (EIA) (Axsym; Abbott Japan, Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe; Kokusai Diagnostic, Kobe, Japan) or chemiluminescent EIA (Fujirebio Inc., Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan).

Genotypes and Subgenotypes of HBV. The six major HBV genotypes (A-F) were determined serologically by EIA using commercial kits (HBV GENOTYPE EIA; Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies, which is specific for each of them.[20] HBV/G was determined by a slight modification of the polymerase chain reaction (PCR) with specific primers.[21]

Subgenotypes of HBV/A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia,9 which corresponds to subgroup A′ originally reported by Bowyer et al.,[22] were determined by PCR restriction fragment length polymorphism (RFLP) involving nucleotide conversions in an immediate upstream of the precore region that are specific for each of them.[16,23] HBV/Bj (Japanese type) lacking the recombination with C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP based on specific nucleotide substitutions, after the methods described previously.[15,24]

Subgenotypes of HBV/C, Cs (Southeast Asian type) found only in Southeast Asia, including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong, and Southern China, and Ce (East Asian type), found in Far

East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.[12]

Quantification of HBV DNA and Sequencing. HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,[25] with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.[10] Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5′-CTC CAC AGT AGC TCC AAA TTC TTT A-3′). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA. Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100 μL serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html.

Cell Culture and DNA Transfection. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hours of culture, cells were transfected with 5 μg DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1 μg reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization. HBV DNA samples from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

Results

Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes. Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ($P = .0001$), Aa ($P < .01$), Bj or Cs ($P < .05$ for each) and Ce than Ba ($P < .05$). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ($P < .05$).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ($P < .005$), Ce ($P < .001$) or Ba ($P < .05$). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.[21] HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ($P < .005$) or Bj ($P < .05$) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ($P < .05$). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ($P < .05$); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D[a] (n = 5)	G[a,b] (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3[d]	41.5 ± 10.7[e]	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%)[f]	19 (86%)[g]	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%)[h]	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113[i]	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks)[c]	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7[j]	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08[k]	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

[a]Patients with HBV genotype D or G were not included in the analysis.
[b]All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.
[c]Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.
[d]$P = .0001$, Ae vs. Ba. $P < .01$, Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.
[e]$P < .05$, Ba vs. Ce.
[f]$P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.
[g]$P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[h]$P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.
[i]$P < .05$, Ae vs. Bj.
[j]$P < .01$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[k]$P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

Clinical Outcome of Patients With Acute Hepatitis B. Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% 2/23 vs. 0.5% 1/187, $P = .032$).

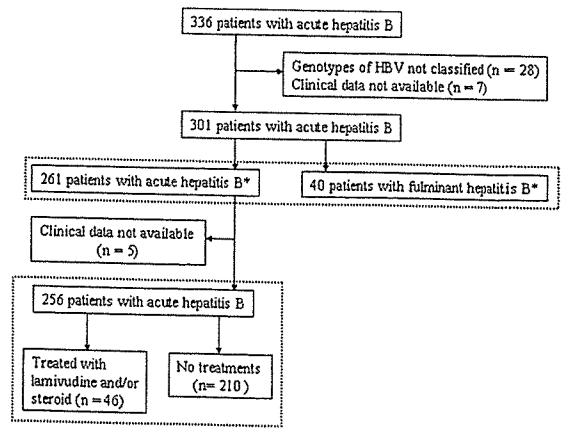

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8)[a]	Ae (n = 32)[a]	Ba (n = 21)[a]	Bj (n = 10)[a]	Cs (n = 10)[a]	Ce (n = 167)[a]	D (n = 3)[a]	G (n = 5)[a]
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%)c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36)[b]	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16)[b]	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%)c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations[a]			
nt 1753 and/or nt1754[b]	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	P Value
Age (yr)			
<34[a]	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200[a]	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0[a]	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00[a]	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (−)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (−)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754[b]			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1..013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

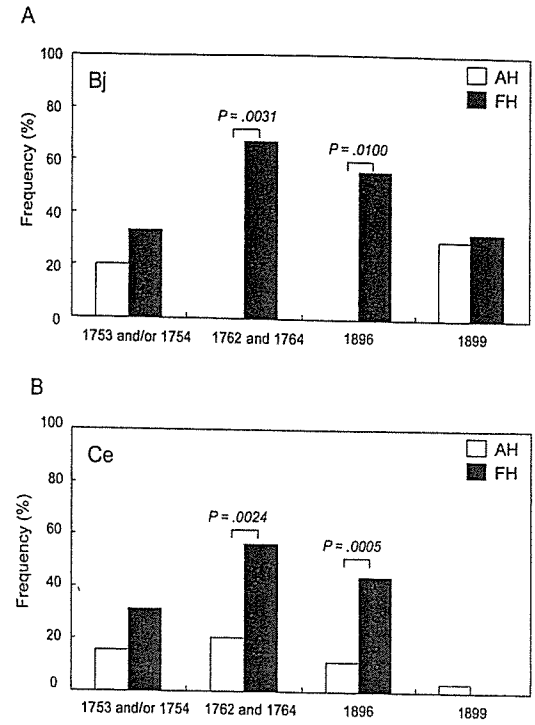

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares