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Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma

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***Background/Aims*: Hepatitis B virus genotype C (HBV/C) has been classified into two geographically distinct subgenotypes; HBV/C1/Cs (Southeast Asia) and HBV/C2/Ce (East Asia).**

***Methods*: Viral differences in enhancer II/core promoter and precore regions between the subgenotypes and their association with hepatocellular carcinoma (HCC) were assessed in a matched cross-sectional control study of 118 carriers (from Hong Kong) with HBV/C1/Cs (48.0 years, 81% male, 40% HBeAg+, 44% HCC) and 210 HBV/C2/Ce (172 from Japan, 38 from Hong Kong) (50.2 years, 78% male, 30% HBeAg+, 46% HCC).**

***Results*: Univariate analyses showed that mutation V1753 was predictive for HCC among HBeAg-positive-C1/Cs-carriers ($P = 0.0055$), and T1653 among HBeAg-positive-C2/Ce-carriers ($P = 0.018$), and T1653 or V1753 or T1762/A1764 among HBeAg-negative-C2/Ce-carriers ($P < 0.05$). In the multivariate analysis on all HBV/C subjects, independent predictive factors for HCC were subgenotype C2/Ce (odds ratio, 4.21; 95% confidence interval, 1.07–16.23), T1653 (3.64; 1.93–6.86), V1753 (3.07; 1.66–5.65) and T1762/A1764 (2.58; 1.21–5.49) mutations, age (≥50 years), gender (male) and HBeAg (positive).**

***Conclusions*: Our data indicate that T1653 and/or V1753 mutations in addition to T1762/A1764 are differently associated with HCC in context of HBeAg status among HBV/C1/Cs and C2/Ce-carriers. HBV/C subgenotypes have specific mutation patterns, which is probably responsible for increased carcinogenesis of HBV/C2/Ce.**

***Keywords*: Hepatitis B virus; Hepatocellular carcinoma; Subgenotype C; Enhancer II; Core promoter; Precore genome**

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]. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j"

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Abbreviations: HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; RFLP, restriction fragment length polymorphism; RTD-PCR, real-time detection polymerase chain reaction.

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for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Recently, a 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, Thailand and Hong Kong (named HBV/C1) and the other for strains from (Far) East Asia including Japan and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [8], and by recent independent studies in Hong Kong [9] and Japan [10]. The latter papers designated the 2 subgenotypes as HBV/Cs in Southeast Asia and HBV/Ce in the (Far) East Asia that have not only different epidemiological distributions but also different virological findings in precore regions [9,10].

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore region at nt 1896 (A1896) are associated with HBe antigen seroconversion (SC) and viral replication. It is noteworthy that the both BCP and precore stop-codon mutations are often found in patients with advanced liver disease such as hepatocellular carcinoma (HCC) [11–14]. Beyond these mutations, the C to T mutation in the upstream regulatory sequence (C1653T) is associated with fulminant hepatitis [15] and located in the alpha box, which is a strong activating element of both enhancer II and core promoter [16]. Takahashi et al. [17,18] reported that C-to-T1653 and T-to-V(not T)1753 mutants were more closely associated with the progression of liver disease from chronic hepatitis to cirrhosis and/or HCC in HBeAg-positive patients, compared with the BCP double mutation. Our recent case-control study supports that the addition of T1653 mutation in enhancer II to the BCP mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19].

To evaluate clinical and virological significances between HBV/C1/Cs and HBV/C2/Ce, in the present study, we performed a multi-center cross-sectional matched control study among HBV/C carriers [inactive carriers (IC), chronic hepatitis (CH), HCC] and determined the specific HBV mutations associated with disease progression.

2. Materials and methods

2.1. Serum samples

A total of 328 sera were obtained from chronic HBV/C carriers who visited Nagoya City University Hospital, Musashino Red Cross Hospital, Osaka National Hospital in Japan and 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 carrier.

2.2. Serological assays for HBV markers

HBeAg and anti-HBe were detected by Chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse f, FUJIREBIO INC., Tokyo, Japan). HBV Genotypes were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed to distinct epitopes on the preS2-region [20,21], with use of commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan).

2.3. PCR-RFLP for distinguishing between subgenotypes C1/Cs and C2/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/C1/Cs or C2/Ce [10]. The first-round PCR was performed with a sense primer (HBV964F) and an antisense primer (HBV1272R) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2) and an antisense primer (HBV1272R). To determine HBV/C1/Cs, a portion (5 μL) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *AseI* at 37 °C and *BstEII* at 60 °C for 1 h each. For HBV/C2/Ce digestion, *NciI* was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (wt/vol) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.4. Detection and quantification of serum HBV DNA

HBV DNA sequences spanning the S gene were amplified with real-time detection polymerase chain reaction (RTD-PCR) according to the method of Abe et al. [22] with a forward primer (HBSF2), a reverse primer (HBSR2), and Taq Man probe (HBSP2′) with an additional G at the 3′-end of the original HBSP2 [23]. The detection limit of this method was 100 copies/mL.

2.5. 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 [24], with slight modifications [23]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer. The sequences covered enhancer II/core promoter (Fig. 1A) and precore genes (Fig. 1B), which could be associated with HBeAg production, viral replication and disease progression.

2.6. A cross-sectional control study for clinical and virological differences between HBV/C1/Cs and C2/Ce

The clinical diagnosis was established after serum biochemistry tests ultasonography, computerized tomography (CT), the magnetic resonance imaging (MRI), and the liver biopsy. To compare the clinical differences between HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 210$), age-, sex-, HBeAg status-matched HBV carriers were enrolled (Table 1). The carriers were also matched according to the severity of liver disease in each group. The HBsAg-positive individuals with normal alanine aminotransferase (ALT) levels over 2 years (examined at least four times at 3-month intervals), and without the presence of portal hypertension were defined as IC. Individuals with persistent elevation of ALT levels (>1.5× upper limit of normal) [35 U/L] over

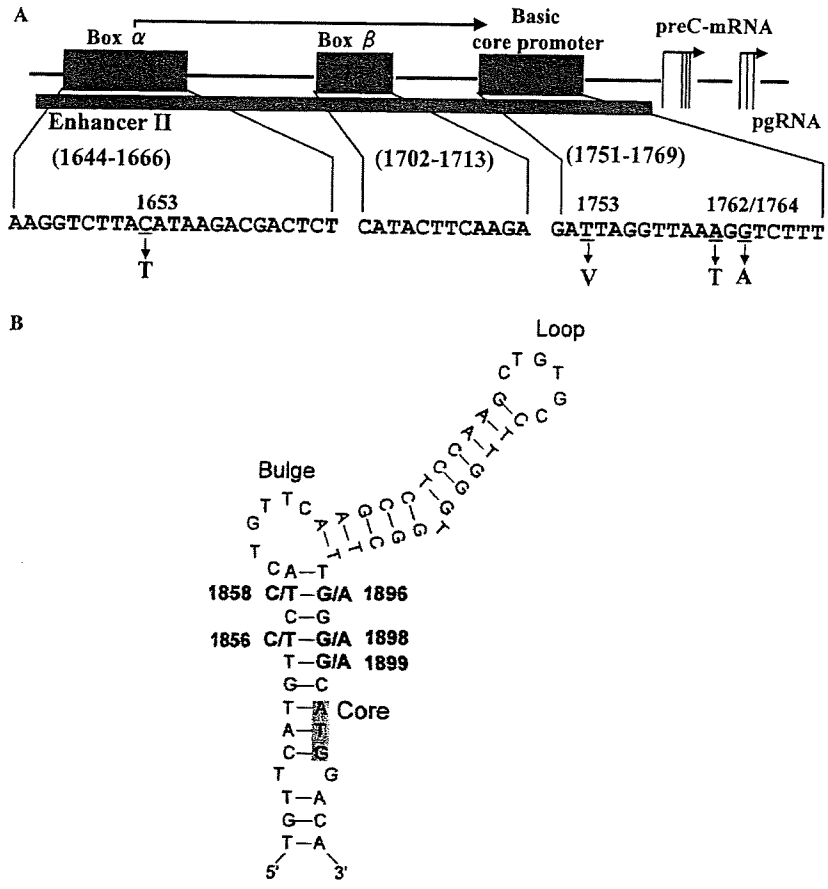

Fig. 1. (A) C1653T, T1753V (not T), A1762T/G1764A mutations in the enhancer II/core promoter region, and (B) ε loop structure of the encapsidation signal in precore genome. pgRNA, pregenomic RNA.

a 6-month period (at least three readings at 2-month intervals) without decrease of platelet count, albumin and hypersplenism (splenomegaly on ultrasonography) were defined as CH. Patients with established hepatocellular carcinoma according to the clinical biochemical investigation [Alpha-fetoprotein (AFP) and/or serum protein induced by vitamin K absence named antagonist II (PIVKA-II)], CT and/or MRI, and liver biopsy were included in HCC group. Patients with hepatitis C virus or human immunodeficiency virus co-infection were excluded, and none had received antiviral treatment during the follow-up period.

2.7. Statistical evaluation

Statistical analyses were performed using chi squared test and Fisher's exact test for categorical variables. Mann–Whitney *U* test was used for continuous variables where appropriate. The univariate general linear modeling (GLM) was used to test the effects of specific HBV mutations on the HCC and non-HCC groups of the HBV-carriers in the context of their HBeAg status. Multivariate analyses with logistic regression were used to determine the independent factors associated with

Table 1
Demographic and clinical characteristics of patients with HBV subgenotype C1/Cs and C2/Ce who were matched for age, sex, HBeAg status and clinical states

Characteristics	C1/Cs ($n = 118$)	C2/Ce ($n = 210$)	*P* value
Country (Japan/Hong Kong)	0/118	172/38	<0.0001
Age, mean ± SD	48.0 ± 13.7	50.2 ± 10.7	Matched
Sex, male (%)	96 (81%)	163 (78%)	Matched
HBeAg positive (%)	47 (40%)	62 (30%)	Matched
HBV DNA (log copies/mL), mean ± SD	5.7 ± 1.7	5.4 ± 1.4	NS
ALT (U/L), median (range)	51 (4–1154)	46 (8–773)	NS
IC/CH/HCC*	19/47/52	29/85/96	Matched
HCC (%)	52 (44%)	96 (46%)	Matched

* IC, inactive carriers; CH, chronic hepatitis; HCC, hepatocellular carcinoma; NS, not significant.

HCC. Differences were considered significant for P values less than .05. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 12.0 for windows, SPSS Inc., Chicago, IL).

3. Results

For comparative analyses, HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 211$) carriers' groups were matched in respect to age, sex, HBeAg status, and clinical states. Table 1 shows comparative summary for HBV/C1/Cs and C2/Ce groups according to the origin country of the enrolled carriers, age, sex, HBe Ag status, HBV DNA levels, ALT levels and clinical states (IC/CH/HCC); no significant differences demonstrated with exception for the original country. HBV/C1/Cs is found in Hong Kong, whereas HBV/C2/Ce is predominant in Japan.

When HCC patients in HBV/C1/Cs ($n = 52$) and C2/Ce ($n = 96$) groups were compared, no significant difference was observed in mean age, sex, HBeAg positivity, HBV DNA, and ALT levels (Table 2). However, the frequency of C-to-T1653 mutation in the box alpha (Fig. 1A) was significantly higher in HBV/C2 (C1/Cs = 23%, C2/Ce = 48%, $P = 0.0055$), whereas T-to-V1753 mutation was significantly prevalent in HBV/C1/Cs (C1/Cs = 75%, C2/Ce=39%, $P < 0.0001$). The prevalence of T1762/A1764 was high in both of these groups with no significant difference (Table 2). In the precore region including encapsidation signal (ε) (Fig. 1B), the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pairing within ε structure, was frequently found in HBV/C2/Ce strains (40/96, 42%), whereas another precore mutation (A1898), accompanied by a C-to-T substitution at nt 1856, was found only in HBV/C1/Cs strains (18/52, 35%) (Table 2). A1899 mutation was prevalent in HBV/C2/Ce (C1/Cs, 12% vs. C2/Ce, 27%) as well as A1896 mutation.

As the above mutations in the enhancer II/core promoter and precore regions could be associated with subgenotypes as well as HBeAg status, we examined the mutations predictive for HCC among all HBV/C1/Cs and C2/Ce patients in the context of HBeAg status. The prevalence of HBV mutations such as C1653T, T1753V, A1762T/G1764A and G1896A was compared among HBeAg-positive, and -negative patients with and without HCC within the C1/Cs and C2/Ce groups (Table 3). As summarized in Table 3, V1753 was frequently found among HCC patients infected with C1/Cs, when compared to those without HCC. Interestingly, the difference was greater in HBeAg-positive group ($P = 0.0055$), whereas in HBeAg-negative group the trend was only remaining ($P = 0.051$). When C2/Ce infected patients with and without HCC were compared, T1653 was frequently found among HCC patients in both HBeAg-positive and -negative groups ($P = 0.018$, 0.012, respectively), and V1753 or T1762/A1764 was also frequent in HBeAg-negative group ($P = 0.046$, 0.024, respectively). The univariate GLM confirmed the above results; V1753 mutation was predictive for HCC in the HBeAg-positive-C1/Cs ($P = 0.0092$), T1653 mutation in the HBeAg-positive-C2/Ce ($P = 0.0056$), both T1653 ($P = 0.0046$) and V1753 mutations ($P = 0.016$) in the HBeAg-negative-C2/Ce group. On the other hand, A1896 mutation was negatively correlated with HCC ($P = 0.0015$).

The factors possible attributable for HCC; age, sex, HBeAg positivity, HBV DNA level, ALT (two groups divided by median values), subgenotypes and mutations; T1653, V1753, T1762/A1764, T1856, T1858, A1896, A1898 and A1899 were tested in multiple logistic regression analysis for all 328 HBV-carriers (Table 4). Age

Table 2
Clinical and virologic characteristics of HCC patients infected with HBV subgenotype C1/Cs and C2/Ce

Characteristics	C1/Cs ($n = 52$)	C2/Ce ($n = 96$)	P value
Age, mean ± SD	54.2 ± 11.9	53.7 ± 9.0	NS
Sex, male (%)	43 (83%)	81 (84%)	NS
HBeAg-positive (%)	24 (46%)	36 (38%)	NS
HBV DNA (log copies/mL), mean ± SD	5.5 ± 1.6	5.2 ± 1.4	NS
ALT (U/L), median (range)	50 (4–1154)	53 (16–473)	NS
Mutation in the box alpha			
T1653	12 (23%)	46 (48%)	0.0055
Mutations in the core promoter			
V(not T)1753	39 (75%)	37 (39%)	<0.0001
T1762/A1764	47 (90%)	88 (92%)	NS
Mutation in the precore region			
T1856	21 (40%)	0	<0.0001
T1858	2 (4%)	96 (100%)	<0.0001
A1896	2 (4%)	40 (42%)	<0.0001
A1898	18 (35%)	0	<0.0001
A1899	6 (12%)	26 (27%)	0.05

NS, not significant.

Table 3
HBV/C1 and C2 mutations among patients with and without HCC in context of HBeAg

No.	T1653	V1753	1762/A1764	A1896
C1/CS				
HBeAg (+)				
Non-HCC ($n = 23$)	2 (8.7%)	7 (30.4%)	16 (69.6%)	4 (17.4%)
HCC ($n = 24$)	6 (25.0%)	**18 (75.0%)***	22 (91.7%)	1 (4.2%)
HBeAg (−)				
Non-HCC ($n = 43$)	8 (18.6%)	21 (48.8%)	33 (76.7%)	10 (23.2%)
HCC ($n = 28$)	6 (21%)	21 (75%)	25 (89%)	1 (4%)
C2/Ce				
HBeAg (+)				
Non-HCC ($n = 26$)	1 (3.8%)	7 (26.9%)	17 (65.4%)	6 (23.1%)
HCC ($n = 36$)	**10 (27.8%)**	15 (41.7%)	31 (86.1%)	9 (25.0%)
HBeAg (−)				
Non-HCC ($n = 88$)	33 (37.5%)	18 (20.5%)	71 (80.7%)	57 (64.8%)
HCC ($n = 60$)	**36 (60.0%)***	**22 (36.7%)***	**57 (95.0%)***	31 (51.7%)

* Non-HCC vs. HCC, $P < 0.05$ (Yates corrected chi-square). Significant data are shown in bold.

($\geqslant 50$) [odds ratio (95% CI): 2.90 (1.72–4.89), $P < 0.0001$], sex (male) [2.29 (1.18–4.43), $P = 0.014$], HBeAg (positive) [2.39 (1.34–4.28), $P = 0.003$] and subgenotype (C2/Ce) [4.21 (1.07–16.23), $P = 0.039$] were significantly associated with the development of HCC. HBV mutations found in strong association with HCC were T1653 [3.64 (1.93–6.86)], V1753 [3.07 (1.66–5.65)], and T1762/A1764 [2.58 (1.21–5.49)]. A1896 stop-codon mutation was negatively correlated with HCC [0.31 (0.16–0.62), $P = .001$] in this population (Table 4).

4. Discussion

In this study, we focused on HBV/C, which is prevalent in Asia and possibly contribute to progressive liver disease and poor clinical outcomes in HBV-carriers [3,25]. Previous reports containing epidemiological and phylogenetic analyses of the HBV/C complete genome determined at least 4 subgenotypes (C1–4) with different geographic distribution [10,26]. HBV/C1 was found only in Southeast Asia, and HBV/C2 was found in Far East Asia; while remaining two were rarely found in most Asian countries, and probably represent isolated local epidemics; HBV/C3 found in Pacific countries and HBV/C4 strains were isolated from Aborigines in Northeast Australia [24]. In the present study, we examined the clinical and virological differences between C1/Cs and C2/Ce.

The multivariate analysis in this study showed that the following factors were predictive for HCC; subgenotype C2/Ce, and mutations in the enhancer II/core promoter; T1653, V1753 and T1762/A1764. In agreement with the previous reports [27–29], the elder age ($\geqslant 50$), male sex and HBeAg positive were also independent risk factors for HCC. The T1653 and V1753 mutations had been first reported by Takahashi et al. [17]; these specific mutations were prevalent among Japanese HCC patients. Our recent age-, sex-matched case-control study also confirmed that the T1653 mutation in the box alpha in addition to the T1762/A1764 double mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19]. The T1762/A1764 mutation had been highly frequent in the elder HBV/C carriers ($\geqslant 50$) regardless of the clinical states [19]; however, these results do not contradict that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis of HBV/C compared to HBV/B (Ba and Bj) correlated with high prevalence of T1762/A1764 [3,7,11]. A prospective cohort of 1638 high-risk individuals in Qidong (China) showed that the T1762/A1764 mutation was detected in 8 of the 15 HCC cases (53.3%) before cancer [30], suggesting that the T1762/A1764 double mutation would indicate a high potential risk for hepatocarcinogenesis. Hence, T1653 and/or V1753 mutations in addition to T1762/A1764 are strongly associated with HCC development.

Buckwold et al. reported that the emerging T1762/A1764 dramatically decreases the affinity with the liver-enriched transcription factors resulting in the reduction of the HBeAg expression [31]. Thereafter, Li et al. reported that this mutation not only affects the nuclear receptor binding site but also creates a new HNF1 transcription factor binding site [32]. In the previous study, it was demonstrated that the box alpha elements (1646–1668) individually stimulate the promoter activity for more than 100-fold [16]. The T1653 mutation converts the alpha box binding site for C/EBP and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and enhancer II/core promoter activity. Hence, the T1653 mutation could influence the HBeAg production and viral replication through the BCP activity. Although a number of studies have reported the role of the BCP mutations in the viral features, the exact mechanisms of HCC development still remains unclear,

Table 4
Variables with independent predictive value for HCC in the multivariate analysis

Factor	Total ($n = 328$)	
	Odds ratio (95% CI)	P value
Age*		
<50	1	
⩾50	2.90 (1.72–4.89)	$P < 0.0001$
Sex		
Female	1	
Male	2.29 (1.18–4.43)	$P = 0.014$
HBeAg		
Negative	1	
Positive	2.39 (1.34–4.28)	$P = 0.003$
HBV DNA (log copies/ml)*		
<5.5	1	
⩾5.5	0.74 (0.25–2.86)	NS
ALT (U/L)*		
<50	1	
⩾50	1.75 (0.56–6.63)	NS
HBV subgenotypes		
C1/Cs	1	
C2/Ce	4.21 (1.07–16.23)	$P = 0.039$
T1653 mutation		
Absence	1	
Presence	3.64 (1.93–6.86)	$P < 0.0001$
V(not T)1753 mutation		
Absence	1	
Presence	3.07 (1.66–5.65)	$P < 0.0001$
T1762/A1764 mutation		
Absence	1	
Presence	2.58 (1.21–5.49)	$P = 0.014$
T1856 mutation		
Absence	1	
Presence	0.41 (0.10–1.69)	NS
T1858 mutation		
Absence	1	
Presence	0.32 (0.07–1.43)	NS
A1896 mutation		
Absence	1	
Presence	0.31 (0.16–0.62)	$P = 0.001$
A1898 mutation		
Absence	1	
Presence	3.31 (0.72–15.35)	NS
A1899 mutation		
Absence	1	
Presence	1.14 (0.57–2.29)	NS

* Two groups were divided by each median value. NS, not significant.

particularly in respect to reflection of the mutations on the X protein. The T1653 mutation responsible for an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be somehow associated with hepatocarcionogenesis. Similarly, the changes of amino acids from isoleucine to asparagine/serine/threonine by V1753 mutation may also affect the function of X protein.

Many previous studies on the HBV encapsidation sequence focused on the configuration of nucleotide 1858 [33,34]. Of note, all HBV/C2/Ce strains possessed T1858 and most HBV/C1/Cs had C1858. A previous study carried among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [35]. Although the polymorphism of C or T at nucleotide 1858 affects the development of the precore stop-codon mutation, it does not seem to influence disease activity [4,11,36,37]. A recent report showed that HBV carriers bearing TCC at nucleotides 1856–1858 had higher HBeAg positivity and ALT levels than those with CCT; but similar prevalence of liver cirrhosis was observed between them [37]. The precore stop-codon mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found mainly in HBV/C2/Ce, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/C1/Cs strains. 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 [38]. Although 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, the multivariable analysis in this study showed that A1896 stop-codon mutation was negatively correlated with HCC development. The result remains controversial [36,39,40] and the mechanism of HCC development in association with A1896 mutation remains unclear.

HBeAg positivity was one of independent predictive factor for HCC in this study, which was consistent with a previous prospective study by Yang et al. [41]. The biologic function of HBeAg remains controversial. HBeAg is not required for viral replication; but it appears to be necessary for the establishment of chronic infection in animal models [42]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/C2/Ce strains possessed T1858 and most C1/Cs had C1858, the C1/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of C1/Cs [37]. The mechanisms of HBeAg seroconversion and its association with HCC development remain unclear. Assuming that different mechanism may exist leading to carcinogenesis in context of HBeAg status of patients, in the present cross-sectional control study, we examined patients divided into four subgroups in respect to subgenotypes/HBeAg status. In univariate analysis the V1753 mutation was confirmed as predictor for HCC in the HBeAg-positive-C1/Cs, and T1653 mutation in the HBeAg-positive-C2/Ce. Interestingly, in the HBeAg-negative-C2/Ce group, T1653 or V1753 or T1762/A1764 mutations

appeared to be significantly associated with HCC development, which supported the previous reports [17–19]. These data might indicate that different HBV mutation patterns might be predictive for HCC in HBV/C1/Cs and C2/Ce-infected carriers in the context of HBeAg status.

In this cross-sectional study, however, HBV DNA level was retracted from predictive factors for HCC. One of the reasons is that HBV DNA data were available only at the time of diagnosis of HCC, when it has already decreased. A recent prospective study in Taiwan indicated that high HBV DNA levels at baseline and genotype C were independent predicting factors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than that at baseline [29]. Our recent cross-sectional case-control study [19] also showed that HBV DNA level was retracted from predicting factor for HCC.

In conclusion, the present multi-center cross-sectional control study indicated that subgenotype C2/Ce, T1653, V1753 and T1762/A1764 mutations in the enhancer II/core promoter are independent factors strongly associated with HCC development as well as the elder age, male sex and HBeAg positivity. The mutation patterns are associated with subgenotypes and HBeAg, suggesting clinical importance of the HBV/C subgenotyping and detection of the mutation pattern for the prediction of HCC. Further prospective studies in countries where HBV genotype C is endemic are required to confirm whether the accumulation of these mutations during the follow-up causes clinical and virological differences between HBV-infected carriers with HBV/C1/Cs and C2/Ce subgenotypes.

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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.

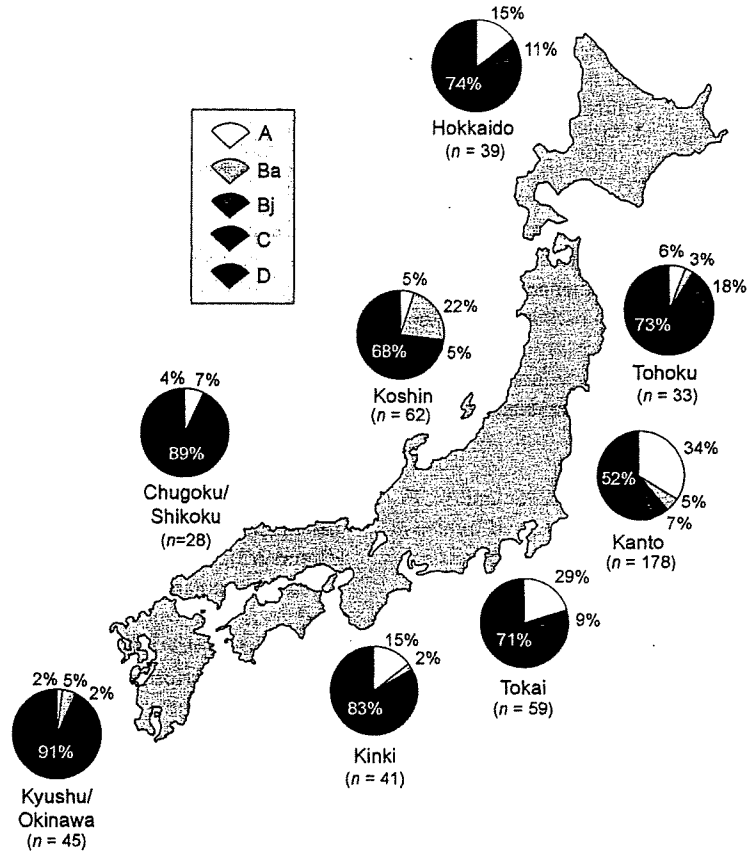

Fig. 1. Geographic distribution of HBV genotypes among 485 patients with acute hepatitis in Japan during 1982–2005.

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

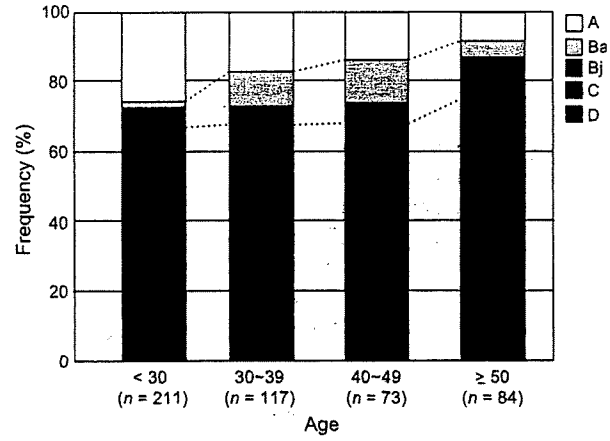

Fig. 2. Age-specific prevalence of HBV genotypes among 485 patients with acute hepatitis.

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.

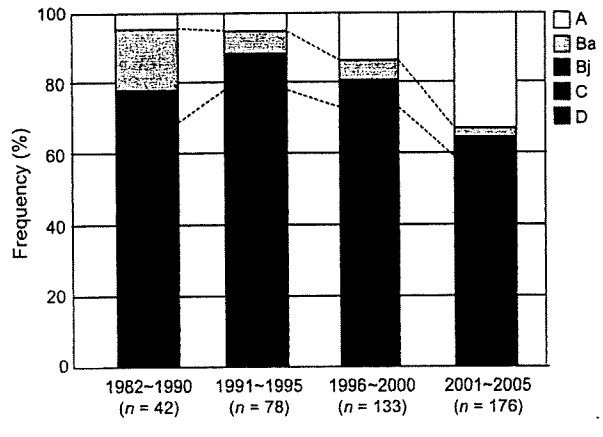

Fig. 3. Chronological changes in the distribution of HBV genotypes among 429 patients with acute hepatitis.

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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The nucleotide sequences of HBV DNA isolates used in this study have been deposited in the international DNA database under accession numbers AB249373-AB249636.

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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,