され、わが国では、エンテカビルが推奨されている。

エンテカビル(バラクルード®) 0.5mg 1日1回 空腹時

- 食事によりAUCが20%、C_{max}が40～50%低下するため、食前後2時間は空けること。
- 腎機能障害がある場合は、投与間隔の調節が必要である。Ccr(mL/min)30以上50未満では2日に1回、10以上30未満では3日に1回、10未満、および血液透析または持続携行式腹膜透析を行っている場合は7日に1回である。

2 エンテカビルの副作用は少ない

- 下痢、悪心、便秘、上腹部痛、倦怠感などが挙げられているが、頻度は数%程度で少ない。また、重篤な副作用としてアナフィラキシー様症状、乳酸アシドーシスなども報告されているがまれである。

3 抗ウイルス薬開始後は1～3ヵ月ごとにHBV-DNAを測定する

- 抗ウイルス薬開始後は1～3ヵ月ごとにHBV-DNAを測定し、ウイルス量が10倍以上(1.0 logcopy/mLの上昇で10倍になる)、増加していないかどうかを確認する。

C B型肝炎の既往への対処のコツ

1 HBV-DNAが検出感度以上であった場合

- 抗ウイルス薬(エンテカビル)の投与を行う。

2 HBV-DNAが検出感度未満であった場合

- 1～3ヵ月ごとにHBV-DNAを測定し、再活性化していないかどうかを確認する。
- 既往感染者でのHBVの再活性化の予防に関する検討は十分に行われていない。ただし、HBV-DNAが検出されてから肝障害・肝炎が出現するまでに平均4～5ヵ月ほど先行するといわれており、HBV-DNAを1～3ヵ月に1回モニタリングして、HBV-DNAが検出感度以上になってから抗ウイルス薬の投与を行っても、肝炎の重症化は予防可能と推察されている。

3 B型肝炎のワクチン接種歴があり、HBs抗体のみ陽性の場合

- 既往感染にはならず、Dと同様に、特別な対応は不要となる。

D HBs抗原が陰性で、HBc抗体またはHBs抗体が陰性の場合

- 再活性化のリスクはほぼないと判断し、特別な対応は不要である。
- ただし、化学療法開始後にHBc抗体やHBs抗体を測定した場合や免疫抑制剤を使用している場合には、抗体価が低下していることもあるので、HBV-DNAなどでの精査が望ましい。

E 再活性化した場合にはどうしたらよいか?

1 再活性化の定義

- 一般に表1のように定義されている。

2 HBs抗原陽性の場合の対応

- 抗ウイルス薬をきちんと服用しているかどうかを確認する。服用していなければ、直ちに服用するように指導する。
- きちんと抗ウイルス薬を服用していても再活性化の定義を満たすことがある。そのなかで頻度の高いのは、HBV-DNAの自然変動であり、後日、再測定すると改善していることもある。
- それでも、HBV-DNAが上昇する場合は、抗ウイルス薬に対する耐性株の出現が疑われるため、肝臓専門医に相談することが望ましい。

3 HBs抗原陰性、HBc抗体またはHBs抗体陽性の場合の対応

- HBV-DNAが検出感度以上になったら、抗ウイルス薬(エンテカビル)の投与を開始する。通常、速やかにHBV-DNAは低下する。

表1 再活性化の定義

HBs抗原陽性例
・HBV DNAが10倍以上の上昇
・HBe抗原陰性例で、HBe抗原が陽性化
HBs抗原陰性で、HBc抗体またはHBs抗体陽性例・HBs抗原が陽性化
・HBV DNA検出感度以下の例でHBV DNAが検出感度以上となった場合

予防投与した抗ウイルス薬の中止のタイミング

投与の中止を考慮する場合は、化学療法終了後12カ月以降にする

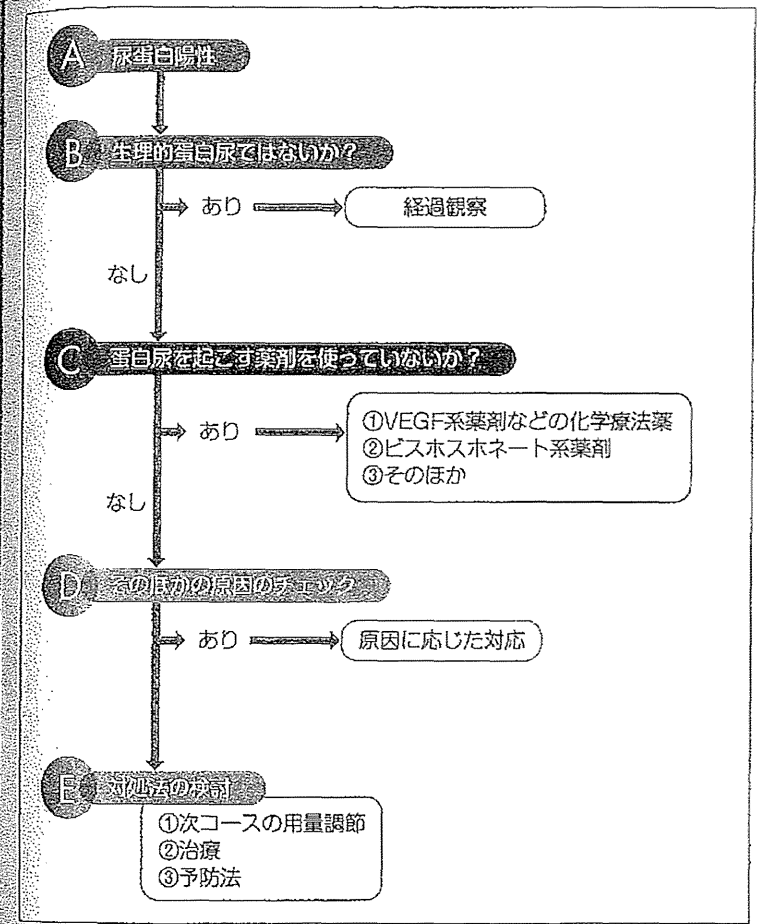
- 通常、化学療法終了後12カ月は、抗ウイルス薬の予防投与は継続することが推奨されている。
- HBV-DNAが検出感度以下の症例で、化学療法終了後12カ月以降に抗ウイルス薬の投与の中止を考慮してもよいが、抗ウイルス薬の投与中止後にも再活性化が起こることも念頭に置いておく必要がある。

中止時には肝臓専門医と相談し、中止後1年間は経過観察を行う

- 中止する場合には、中止後の再活性化のリスクを勘案し、肝臓専門医と相談のうえで、抗ウイルス薬の投与を中止する。
- 抗ウイルス薬投与中止後1年間は4週ごとにHBV-DNA定量と肝機能(AST、ALT)による経過観察を行い、HBV-DNAが検出感度以上になった場合は、直ちに抗ウイルス薬の投与を再開する。

(池田公史)

蛋白尿 —蛋白尿の原因となる疾患や薬剤はないか？抗VEGF抗体薬の蛋白尿にも注意—



HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Risk Factors for Long-Term Persistence of Serum Hepatitis B Surface Antigen Following Acute Hepatitis B Virus Infection in Japanese Adults

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The proportion of patients who progress to chronicity following acute hepatitis B (AHB) varies widely worldwide. Moreover, the association between viral persistence after AHB and hepatitis B virus (HBV) genotypes in adults remains unclear. A nationwide multicenter study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in patients with AHB. For comparing factors between AHB patients with viral persistence and those with self-limited infection, 212 AHB patients without human immunodeficiency virus (HIV) coinfection were observed in 38 liver centers until serum hepatitis B surface antigen (HBsAg) disappeared or a minimum of 6 months in cases where HBsAg persisted. The time to disappearance of HBsAg was significantly longer for genotype A patients than that of patients infected with non-A genotypes. When chronicity was defined as the persistence of HBsAg positivity for more than 6 or 12 months, the rate of progression to chronicity was higher in patients with genotype A, although many cases caused by genotype A were prolonged cases of AHB, rather than chronic infection. Multivariate logistic regression analysis revealed only genotype A was independently associated with viral persistence following AHB. A higher peak level of HBV DNA and a lower peak of alanine aminotransferase (ALT) levels were characteristics of AHB caused by genotype A. Treatment with nucleotide analogs (NAs) did not prevent progression to chronic infection following AHB overall. Subanalysis suggested early NA initiation may enhance the viral clearance. *Conclusion*: Genotype A was an independent risk factor for progression to chronic infection following AHB. Our data will be useful in elucidating the association between viral persistence after AHB, host genetic factors, and treatment with NAs in future studies. (HEPATOLOGY 2014;59:89-97)

Abbreviations: AHB, acute hepatitis B; ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg; HBeAg, hepatitis B e-antigen; CLIA, chemiluminescent enzyme immunoassay; EIA, enzyme immunoassay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IgM, immunoglobulin M; anti-HBe, antibody to HBeAg; NAs, nucleotide analogs; RPHA, reverse passive hemagglutination.

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Received January 5, 2013; accepted July 10, 2013.

Hepatitis B virus (HBV) infection is one of the most common persistent viral infections in humans. Approximately 2 billion people worldwide have been exposed to HBV and 350 million of them remain persistently infected.[1] The incidence of HBV infection and the patterns of transmission vary greatly worldwide among different population subgroups.[2] In Western countries, chronic HBV infection is relatively rare and acquired primarily in adulthood by way of sexual transmission or the use of injectable drugs. Meanwhile, in Asia and most of Africa, the majority of infections are the result of transmission from an infected mother to her newborn. However, very few studies of acute hepatitis B (AHB) in adults have been reported.

HBV genomic sequences vary worldwide and have been classified into at least eight genotypes (A through H) based on an intergroup divergence of 8% or more over the complete nucleotide sequence.[3,4] These genotypes have distinct geographic distributions.[5-7] In particular, genotype A is predominant in Northwestern Europe, the United States, Central Africa, and India.[8, 9] The Japanese have been infected with genotypes B and C since prehistoric times.[10] Recently, many lines of evidence have revealed among the Japanese an increase in acute infection with HBV genotype A following sexual transmission.[11,12] As a result of this increasing transmission of genotype A, the distribution of HBV genotypes in Japan clearly differs among patients with acute and chronic infections.[13] Moreover, recent studies suggest that acute infection with HBV genotype A may be associated with an increased risk of progression to persistent infection.[15] Indeed, the prevalence of HBV genotype A in chronic hepatitis B patients doubled in Japan between 2000-2001 and 2005-2006 (1.7% versus 3.5%).[11]

Human immunodeficiency virus (HIV)-1 infection results in an immunodeficient state, with the virus sharing routes of transmission with HBV. HIV-related immunodepletion influences the natural history of HBV infection, and epidemiological studies have revealed that HIV-positive patients are more likely to have a prolonged acute illness following HBV infection and lower rates of hepatitis B e-antigen (HBeAg) clearance.[16] Therefore, in this study patients with coinfection of HIV were excluded to examine the influence of HBV genotype directly without the confounding influence of HIV.

From 2005 to 2010, a multicenter cohort study was conducted throughout Japan on 212 patients with AHB. The aim of this cohort study was to assess the influence of clinical and virological factors, including HBV genotypes and treatment with nucleotide analogs (NAs), on AHB patients who became persistently infected.

Patients and Methods

Patients With AHB. The multiple-source cohort included 212 randomly selected AHB patients without coinfection of HIV. From 2005 through 2010, the study participants were recruited from 38 liver centers throughout Japan. The cohort included patients who were admitted to the hospital because of AHB and who visited the hospital every month after being discharged. The diagnosis of AHB was contingent on the rapid onset of clinical symptoms accompanied by elevated serum alanine aminotransferase (ALT) levels, the detection of serum hepatitis B surface antigen (HBsAg), and a high-titer antibody to hepatitis B core antigen (anti-HBc) of the immunoglobulin M (IgM) class. Patients with initial high-titer anti-HBc (>10.0 S/CO) were diagnosed as having an exacerbation of chronic hepatitis B and were excluded. If the patient had been tested previously, the absence of serum HBsAg and anti-HBc before admission was verified from the medical record to discriminate a new infection from an acute exacerbation of a persistent infection. Patients with acute hepatitis A, hepatitis C, and drug- or alcohol-induced acute hepatitis were also excluded; hepatitis D virus infection was not determined because of its extreme rarity in Japan. The study protocol conformed to the 1975 Declaration of

Supported by Japanese Ministry of Health, Labor and Welfare grant H21-003 and Japanese Ministry of Education, Culture, Sports, Science and Technology grant 22790679.

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DOI 10.1002/hep.26635

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Table 1. Characteristics of Patients With Genotype A or a Non-A Genotype Acutely Infected With Hepatitis B Virus

Features	Genotype A (n = 107)	Non-A Genotypes (n = 105)*	*P* Value
Age (years)	36.3 ± 12.0	40.7 ± 14.3	0.032
Male sex	102 (95.3)	75 (71.4)	<0.001
HBeAg positive	104 (97.2)	79 (75.2)	<0.001
ALT (IU/L)	1210 ± 646	2225 ± 2851	0.045
Total bilirubin (mg/dL)	9.9 ± 9.4	7.5 ± 6.7	0.115
HBV DNA (log copies/mL)	7.0 ± 1.5	5.8 ± 1.5	<0.0001
Duration until disappearance of HBsAg (month)	6.7 ± 8.5	3.4 ± 6.5	<0.0001
Persistence of HBsAg positivity more than 6 months	25 (23.4)	9 (8.6)	0.003
Persistence of HBsAg positivity more than 12 months	8 (7.5)	1† (0.9)	0.018
Sexual transmission	81/84 (96.4)‡	71/79 (89.9)§	0.095
Treatment with NAs	61 (57.0)	42 (40.0)	0.013

Data are presented as n (%), mean ± standard deviation. HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; NAs, nucleotide analogs.

*Non-A genotypes include genotypes B, C, D, F and H (n = 25, 77, 1, 1, and 1, respectively).

†One patient had genotype C.

‡Transmission routes were unknown for 23 patients.

§Transmission routes were unknown for 26 patients.

Helsinki and was approved by the Ethics Committees of the institutions involved. Every patient gave informed consent for this study.

Serological Markers of HBV Infection. HBsAg, HBeAg, antibodies to HBsAg (anti-HBs), HBeAg (anti-HBe), and HBcAg, and anti-HBc of the IgM class were tested by a chemiluminescent enzyme immunoassay (CLIA) by ARCHITECT (Abbott Japan, Tokyo, Japan). HBV DNA measurements were performed using a real-time polymerase chain reaction (PCR) assay (Cobas TaqMan HBV Auto; Roche Diagnostics, Tokyo, Japan).

Genotyping of HBV. The six major HBV genotypes (A through F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan). This method is based on the pattern of detection by monoclonal antibodies of a combination of epitopes on preS2-region products, which is specific for each genotype.[17,18] Samples for which EIA could not determine the genotype were examined by direct sequencing of the pre-S2/S gene, followed by phylogenetic analysis.

Treatment With NAs. Treatments with NAs were performed using lamivudine or entecavir for more than 3 months. The individual clinicians determined if NAs were administered to patients, and when the treatment was to be started. The time to onset of treatment with NAs was measured in days from onset of AHB.

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 0.05 was considered significant. Multivariate analysis was performed using a backward stepwise logistic regression model to determine independent factors for viral persistence following AHB. Variables in the multivariate analysis were selected based on variables that were marginally significant with $P < 0.1$ in univariate analysis. Maintenance of HBsAg positivity was analyzed using the Kaplan-Meier method and significance was tested with the log-rank test. STATA Software (StataCorp, College Station, TX) v. 11.0 was used for analyses.

Results

Comparison of Characteristics Between Genotype A and Non-A Genotype AHB Patients. A total of 107 AHB patients (50.5%) were infected with genotype A while 105 AHB patients (49.5%) were infected with non-A genotypes, including genotypes B (25 [11.8%]), C (76 [35.8%]), D (1 [0.5%]), F (1 [0.5%]), and H (1 [0.5%]). Compared to those infected with non-A genotypes, genotype A patients were significantly younger (36.3 ± 12.0 versus 40.7 ± 14.3 years, $P = 0.032$), predominantly men (95.3% versus 71.4%, $P < 0.001$), and more frequently positive for HBeAg (97.2% versus 75.2%, $P < 0.001$). Moreover, genotype A patients had a lower peak ALT levels (1,210 ± 646 versus 2,225 ± 2,851 IU/L, $P = 0.045$) and a higher peak level of HBV DNA (6.7 ± 8.5 versus 3.4 ± 6.5 log copies/mL, $P < 0.0001$). A significantly higher percentage of genotype A patients were treated with NAs (57% versus 40%, $P = 0.013$). These data are summarized in Table 1.

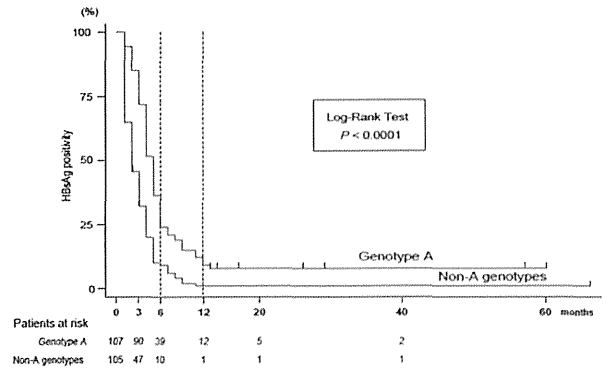

Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

Cumulative Maintenance of HBsAg Positivity During Follow-up in Patients With Genotype A and Non-A Genotypes. In the patients infected with genotype A and non-A genotypes, the mean durations of HBsAg positivity maintenance were 6.7 ± 8.5 and 3.4 ± 6.5 months, respectively ($P < 0.0001$; Table 1, Fig. 1). For 6 months after AHB onset, the number of patients with genotype A and non-A genotypes maintaining HBsAg positivity were 39/107 (36.4%) and 10/105 (9.5%), respectively ($P < 0.001$). However, in many patients HBsAg disappeared between 7 and 12 months after AHB onset; that is, HBsAg disappeared in 31/107 (29.0%) of patients with genotype A and in 9/105 (8.6%) of patients with non-A genotypes during this time period. However, in some patients HBsAg never disappeared after persisting for more than 12 months following AHB onset. When chronicity after AHB was defined as the persistence of HBsAg for more than 12 months, chronicity developed in 7.5% (8/107) of patients with genotype A and in 0.9% (1/105) of patients with non-A genotypes ($P = 0.018$).

Comparison of Characteristics Between Patients in Whom HBsAg Persisted More Than 6 or 12 Months and Those With Self-Limited AHB Infection. Table 2 compares the demographic and clinical characteristics between patients in whom HBsAg disappeared within 6 months and those in whom HBsAg persisted for more than 6 months from AHB. The peak ALT levels ($1,882 \pm 2,331$ versus $1,018 \pm 696$ IU/L, $P = 0.0024$) and peak HBV DNA levels (6.3 ± 1.6 versus 7.4 ± 1.6 mg/dL, $P = 0.0004$) were significantly higher and lower in the former group than in the latter group, respectively. Moreover, marked differences were present in the distribution of genotypes between the two groups. The percentage of the HBV genotype A (46.1% versus 73.5%, $P = 0.003$) was significantly higher among patients in whom HBsAg was persistent for more than 6 months. In addition, we compared the demographic and clinical characteristics between patients in whom HBsAg disappeared within 12 months and those in whom HBsAg persisted for more than 12 months from AHB. Peak ALT ($1,787 \pm 2,118$ versus 775 ± 513 IU/L, $P = 0.0089$) and peak total bilirubin (8.7 ± 8.2 versus 3.8 ± 6.6 mg/dL, $P = 0.0039$) levels were significantly higher in the former group than in the latter group. In contrast, the peak HBV DNA levels (6.4 ± 1.6 versus 7.9 ± 1.4 mg/dL, $P = 0.0046$) were significantly lower

Table 2. Comparison Between Patients With Chronicity Following Acute Hepatitis B and Those With Self-Limited Acute Infections Determined by the Persistence of HBsAg for More Than 6 or 12 Months

Features	Disappearance of HBsAg Within 6 Months (n = 178)	Persistence of HBsAg for More Than 6 Months From AHB (n = 34)	*P* Value	Disappearance of HBsAg Within 12 Months (n = 203)	persistence of HBsAg for More Than 12 Months From AHB (n = 9)	*P* Value
Age (years)	38.2 ± 13.1	40.0 ± 14.5	0.454	38.1 ± 13.2	46.7 ± 14.0	0.061
Male sex	147 (82.6)	30 (88.2)	0.416	169 (83.3)	8 (88.9)	0.677
HBeAg positive	150 (84.3)	32 (94.1)	0.131	175 (86.2)	8 (88.9)	0.815
ALT (IU/L)	1882 ± 2331	1018 ± 696	0.0024	1787 ± 2118	775 ± 513	0.0089
Total bilirubin (mg/dL)	8.6 ± 7.5	8.7 ± 11.3	0.137	8.7 ± 8.2	3.8 ± 6.6	0.0039
HBV DNA (log copies/mL)	6.3 ± 1.6	7.4 ± 1.6	0.0004	6.4 ± 1.6	7.9 ± 1.4	0.0046
HBV genotype						
Non-A	96 (53.9)	9 (26.5)		104 (51.2)	1 (11.1)	
A	82 (46.1)	25 (73.5)	0.003	99 (48.8)	8 (88.9)	0.018
Sexual transmission	128/137 (93.4)*	24/26 (92.3)†	0.711	146/157 (93.0)‡	6/6 (100.0)§	0.356
NAs treatment (+)	82 (46.1)	21 (61.8)	0.093	98 (48.3)	8 (88.9)	0.017

Data are presented as n (%) and mean ± SD. HBsAg, hepatitis B surface antigen; AHB, acute hepatitis B, HBeAg, hepatitis B e-antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; NAs, nucleotide analogs.

*Transmission routes of 41 patients were unknown.

†Transmission routes of 8 patients were unknown.

‡Transmission routes of 46 patients were unknown.

§Transmission routes of 3 patients were unknown.

Table 3. Multivariate Analysis of Factors Independently Associated With Persistence of HBsAg Positivity Following Acute Hepatitis B

	Persistence of HBsAg More Than 6 Months From AHB		
Factors	Odds Ratio	95% CI	*P* Value
ALT (per 1 IU/L increase)	1.000	0.999-1.000	0.035
HBV DNA (per 1 log copy/mL increase)	1.176	0.931-1.484	0.173
Genotypes			
Non-A	1.00		
A	4.224	1.853-9.631	0.001

95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBV, hepatitis B virus.

in the former group than in the latter group. The percentages of HBV genotype A (48.8% versus 88.9%, $P = 0.018$) and NAs treatment (+) (48.3% versus 88.9%, $P = 0.017$) were significantly higher among patients in whom the HBsAg persisted for more than 12 months.

Factors Independently Associated With Viral Persistence Following AHB. A stepwise logistic regression model was used to perform multivariate analysis which explains relationships between some factors and persistence of HBsAg positivity more than 6 months following AHB. Peak ALT level, peak HBV DNA level, genotype A, and treatment with NAs were retained in the final multivariate logistic model in a backward stepwise manner ($P < 0.1$). For predicting the persistence of HBsAg for more than 6 months, only genotype A was independently associated with progression of AHB to the persistence of HBsAg (odds ratio [OR]: 4.224, $P = 0.001$, Table 3).

Characteristics of Patients Who Progressed to Chronicity That Was Defined as the Persistence of HBsAg for More Than 12 Months Following Acute Hepatitis B. Table 4 shows the clinical and virological characteristics of nine patients who progressed to chronicity defined as the persistence of HBsAg for more than 12 months following AHB. Among the nine patients who progressed to chronicity from AHB, eight (88.9%) were men and eight (88.9%) were HBeAg-positive. In general, among the patients who progressed to chronicity following AHB, the peak HBV DNA levels were high, and the peak total bilirubin and ALT levels were low. In eight (88.9%) patients, entecavir was administered; however, the duration until the onset of NA treatment from AHB onset was long (75-570 days).

Early Onset of Treatment With NAs Was Able to Prevent Viral Persistence After AHB Caused by Genotype A. The cumulative proportion maintaining HBsAg positivity during follow-up, expressed in terms of time after AHB onset, were significantly longer in patients with NAs treatment than in those without NAs treatment ($P = 0.046$, Fig. 2A). Table 5 shows the percentages of patients in whom HBsAg persisted for more than 6 or 12 months among patients categorized based on the period of time (i.e., duration) until the onset of NAs treatment. For patients in whom the onset of NAs treatment was less than 4 weeks from the onset of AHB, 12.7% of the patients showed persistent HBsAg for more than 6 months, while none showed HBsAg positivity for more than 12 months. For patients in whom the onset of NAs treatment was at 5-8 weeks, 37.5% of the patients showed persistent HBsAg for more than 6 months, whereas none showed persistent HBsAg for more than 12 months. For all groups, the period of HBsAg positivity in patients starting NAs treatment within 8 weeks from AHB onset was significantly shorter than that in patients beginning NAs treatment after more than 8 weeks from AHB onset ($P < 0.0001$, Fig. 2B). Patients starting NAs treatment within 8 weeks from AHB onset never progressed to chronicity after AHB caused by genotype A.

Table 4. Characteristics of Patients Who Progressed to Chronicity Following Acute Hepatitis B

Case	Age	Gender	HIV	HBeAg	HBV DNA (log copies/mL)	Total Bilirubin (mg/dL)	ALT (IU/L)	Observation Period (Months)	NAs Treatment	Duration Until NAs Treatment (Days)	Transmission Routes	Genotype
1	23	Male	(−)	(+)	7.6	1.7	1271	26	ETV	570	Heterosexual	A
2	40	Male	(−)	(−)	8.8	1.4	568	13	ETV	240	Heterosexual	A
3	45	Male	(−)	(+)	7.7	0.9	867	57	ETV	135	Heterosexual	A
4	37	Male	(−)	(+)	7.6	3.4	384	29	ETV	75	Unknown	A
5	54	Male	(−)	(+)	9	2	455	17	ETV	155	Homosexual	A
6	45	Male	(−)	(+)	4.8	21.2	512	60	(−)	(−)	Homosexual	A
7	61	Male	(−)	(+)	9.1	1.5	804	17	ETV	88	Unknown	A
8	56	Male	(−)	(+)	9.0	1.1	1820	14	ETV	118	Unknown	A
9	31	Female	(−)	(+)	7.4	0.8	296	66	ETV	150	Blood transfusion	C

HIV, human immunodeficiency virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; NAs, nucleotide analogs; ETV, entecavir.

Table 5. Proportion of Patients in Whom HBsAg Persisted for More Than 6 or 12 Months Among Patients Categorized Based on the Number of Weeks Until the Onset of NAs Treatment

Duration Until Onset of NAs Treatment (Weeks)	Persistence of HBsAg for More Than 6 Months	Persistence of HBsAg for More Than 12 Months	Total Patients
<4 weeks (n, %)	9 (12.7)	0 (0)	71
5-8 weeks (n, %)	6 (37.5)	0 (0)	16
9-12 weeks (n, %)	1 (33.3)	1 (33.3)	3
13-16 weeks (n, %)	4 (100)	1 (25.0)	4
>17 weeks (n, %)	9 (100)	6 (66.7)	9
Total	29	8	103

HBsAg, hepatitis B surface antigen; NAs, nucleotide analogs.

Discussion

A multicenter nationwide study was conducted throughout Japan to evaluate the influence of clinical and virological factors on chronic outcomes in Japanese patients who contracted AHB in adulthood. The study was feasible in Japan, where a universal vaccination program for HBV has not been implemented because of the extremely high efficacy of the immunoprophylaxis that is given to babies born to carrier mothers. The implementation of this program has resulted in a decrease in the persistent HBV carrier rate from 1.4% to 0.3%.[19] Selective vaccination means that Japanese are more likely to be infected with HBV by way of horizontal transmission since the percentage of the population possessing anti-HBs is much lower than that in countries in which universal vaccination programs have been established.[20] In addition, Japan is faced with the ever-increasing impacts of globalization: as many as 17 million Japanese travel abroad and over 7 million people visit Japan from overseas each year. This "population mixing" may help to explain the increased prevalence in Japan of AHB due to genotype A, which is transmitted through indiscriminate sexual contact. Consequently, Japan may be the only country in the world where the influences of HBV genotypes, including genotype A (as is predominant in Western countries) and genotypes B and C (as are predominant in Asian countries), on chronic outcomes after AHB can be compared.

Currently, the persistence of HBsAg in serum for more than 6 months is considered to represent a progression to chronic infection.[21] However, our data showed that HBsAg frequently disappeared between 7 to 12 months after the onset of AHB in patients with genotype A (31/107 [29.0%]) and non-A genotypes (9/105 [8.6%]) (Fig. 1). These patients were considered to exhibit prolonged cases of AHB, rather than persistent infection. This finding reflects the higher sensitivity of the most up-to-date assays for HBsAg as compared with previous methods. In the present study, HBsAg was measured by CLIA, which has been reported to be about 150 times more sensitive in the detection of HBsAg than reverse passive hemagglutination (RPHA)-HBsAg, which has been used for the last 30 years in Japan.[22] The use of a more sensitive assay for HBsAg results in a longer period during which HBsAg may be detected. In this study, HBsAg did not disappear in nine patients after remaining continuously detectable for more than 12 months. Therefore, the persistence of HBsAg for more than 12 months, as measured with a highly sensitive method for detecting HBsAg, may be suitable for defining the progression of AHB to chronicity; however, further study is necessary to determine whether this definition is appropriate worldwide.

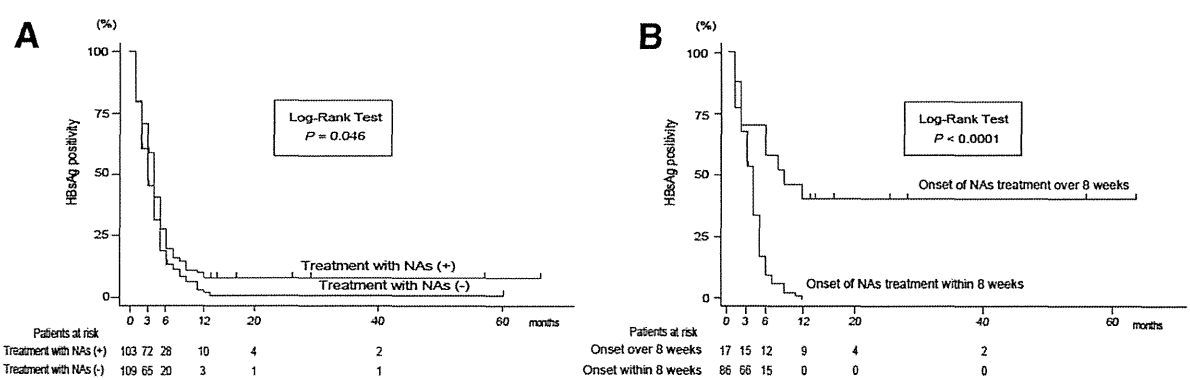

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the cumulative proportion of AHB patients in genotype A maintaining HBsAg positivity between treatment onset with NAs within 8 weeks and treatment onset with NAs over 8 weeks after onset of AHB, as analyzed using the Kaplan-Meier test. $P < 0.0001$, treatment onset with NAs over 8 weeks: red line, treatment onset with NAs within 8 weeks: blue line.

It has been reported that ~10% of patients who contract HBV as adults do not clear HBsAg from their serum and become carriers.[23] Meanwhile, a wide variation has been seen in the rate of persistence after AHB infection in adults. For example, viral persistence following AHB was seen in 0.2% (1/507) of adults in Greece,[24] 7.7% (5/65) of adult Alaskan Eskimos, and 12.1% (7/58) of adults in Germany.[25] The difference in the proportion of patients progressing from AHB to chronicity in different regions may be attributable to virological and host factors. In this study, 4.2% (9/212) of patients progressed to chronicity after AHB: 7.5% (8/107) of those infected with genotype A and 0.9% (1/105) of those infected with non-A genotypes. The non-A genotypes included genotypes B, C, D, F, and H (n = 25, 77, 1, 1, and 1, respectively). Genotypes B and C are predominant in eastern Asian countries, where the majority of those infected with HBV acquired the virus during the perinatal period by way of vertical transmission.[26] On the other hand, genotype A is predominant in Western countries, where the main route is horizontal transmission later in life.[26,27] Because HBeAg persists long after the infection in the genotype C as compared to other genotypes, this genotype has been shown to be a risk factor for perinatal and horizontal transmission in newborns and children.[28] The predominance of genotype A in Western countries may be attributable to a higher chronicity rate following AHB by way of horizontal transmission in adults.

In this study the characteristics of AHB associated with genotype A were a higher peak level of HBV DNA and a lower peak level of ALT. These findings were similar to those for patients with HBV-HIV coinfection.[29] Such characteristics of genotype A or coinfection with HIV are assumed to be attributable to milder hepatitis associated with weaker cellular immune responses. More slowly replicating viruses have been reported to evoke weaker cellular responses, enhancing the likelihood of persistence.[30] Indeed, our prior study showed that the replication of genotype A was significantly slower than that of genotype C in immunodeficient, human hepatocyte chimeric mice.[31] Moreover, variation among genotypes in the expression pattern of HBeAg may affect the progression of AHB to chronicity. Another previous study of ours revealed that a single form of HBeAg was detected by western blot analysis in serum samples from patients infected with genotypes B through D, but that two additional larger forms of HBeAg were detected in patients with genotype A.[32] Milich and Liang[33] reported that HBeAg may modulate the host immune response as a tolerogen to promote chronicity. Therefore, the different expression pattern of HBeAg by genotype A HBV may contribute to chronicity following AHB.

Early NAs initiation appeared to enhance the viral clearance across genotypes, although treatment with NAs did not show any overall benefit in duration of HBsAg. Previous studies examining the efficacies of NAs for preventing progression to chronic infection after AHB have reported conflicting results. Some small-scale studies have suggested the efficacy of lamivudine and entecavir in preventing the progression of AHB to chronic hepatitis.[34,35] Another study showed a lower seroconversion rate of HBsAg in lamivudine users.[36] Further, a randomized placebo-controlled trial showed no significant difference in clinical outcomes.[37] However, these previous studies did not mention the prevalence of HBV genotypes in the respective study populations. Although this was a retrospective study, our study included data on the prevalence of HBV genotypes. Additionally, our findings suggested that larger prospective randomized studies for every HBV genotype should be performed to determine whether early treatment with NAs prevented the progression of AHB to a chronic state.

In conclusion, in Japan genotype A was an independent risk factor for progression to chronic infection following AHB in adults. Confirmation of this association in patients with AHB in other countries is desirable and may provide insight into the pathogenetic mechanisms underlying this association. Early NA treatment appeared to reduce the likelihood of chronicity but this potentially important intervention needs to be prospectively studied before recommendations can be made.

Appendix

Members of the Japanese AHB Study Group include Yasuharu Imai (Ikeda Municipal Hospital), Norie Yamada, Hideaki Takahashi (St. Marianna University School of Medicine), Koji Ishii (Toho University School of Medicine), Hideyuki Nomura (Shin-Kokura Hospital), Jiro Nishida (Tokyo Dental Collage Ichikawa General Hospital), Shigeru Mikami (Kikkoman Hospital), Tsuneo Kitamura (Juntendo University Urayasu Hospital), Akihito Tsubota (Kashiwa Hospital Jikei University School of Medicine), Noritomo Shimada (Shinmatsudo Central General Hospital), Tetsuya Ishikawa (Nagoya University Graduate School of Medicine), Yoshiyuki Ueno (Tohoku University Graduate School of Medicine), Tomoyoshi Ohno (Social Insurance Chukyo Hospital), Etsuro Orito (Nagoya

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Hepatology Research

Hepatology Research 2014; 44: 365–367 doi: 10.1111/hepr.12295

Special Report

Classification of the etiologies of acute liver failure in Japan: A report by the Intractable Hepato-Biliary Diseases Study Group of Japan

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The Intractable Liver Diseases Study Group of Japan, supported by the Ministry of Health, Labor and Welfare, established novel diagnostic criteria for "acute liver failure" in 2011. In these criteria, patients without histological findings of hepatitis are included in the disease entity of "acute liver failure", as in Europe and the USA. In this report, classification criteria for the etiologies of "acute liver failure" in Japan are proposed.

Key words: autoimmune hepatitis, drug-induced liver injury, fulminant hepatic failure, fulminant hepatitis, HBV

Hepatitis Viral Infection is the most important and common cause of acute liver failure in Japan. Acute liver failure is typically represented by fulminant hepatitis in Japan, and the diagnostic criteria for "fulminant hepatitis" were established by the Inuyama Symposium in 1981.[1] The etiology of fulminant hepatitis includes viral infection, autoimmune hepatitis, drug allergy-induced liver injury and hepatitis of indeterminate etiologies.[2] In contrast, in the USA, Trey and Davidson proposed criteria for the diagnosis of "fulminant hepatic failure" in 1970,[3] which includes liver failure caused by drug toxicity, circulatory disturbances, metabolic diseases, acute fatty liver of pregnancy and postoperative liver damage, none of which is included in the etiological factors of the disease entity of "fulminant hepatitis" in Japan. Then, Polson and Lee published an American Association for the Study of Liver Diseases position paper in 2005,[4] and "fulminant hepatic failure" was replaced by "acute liver failure", although the etiological factors of the disease entity of "acute liver failure" have not been changed until now, either in Europe or in the USA.

The diagnostic criteria for "fulminant hepatitis" in Japan need to be revised to correspond to those for "acute liver failure" in Europe and the USA. Thus, the Intractable Liver Diseases Study Group of Japan, supported by the Ministry of Health, Labor and Welfare, established novel diagnostic criteria for "acute liver failure", which include the disease entity of "fulminant hepatitis" in 2011.[5,6] According to these criteria, patients showing prothrombin time values of 40% or less of the standardized values or international normalized ratios of 1.5 or more caused by severe liver damage developing within 8 weeks of the onset of symptoms are diagnosed as having "acute liver failure", with the liver function prior to the current onset of liver damage being

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Received 3 December 2013; revision 24 December 2013; accepted 25 December 2013.

Table 1 Classification of the etiologies of acute liver failure

I. Viral infection: Those satisfying the following criteria for laboratory data, showing clinical features consistent with viral infection.
I-① HAV: Positive test result for anti-HAV (IgM).
I-② HBV: Positive test result for either HBsAg or anti-HBc (IgM), but care should be exercised in rare cases in which the test result for serum HBV DNA is positive whereas all of the serum markers for HBV are negative.*
I-②-1 Transient HBV infection; when any of the following three situations is satisfied:
- Negative test result for HBsAg preceding the onset of liver injury in the absence of immunosuppressive and/or anticancer therapies in the previous 12 months.
- High levels of anti-HBc (IgM).
- Low levels of anti-HBc (IgG).

I-②-2 Acute exacerbation in HBV carriers; when any of the following four situations is satisfied:
- Positive test result for HBsAg preceding the onset of liver injury (A).
- Low levels of anti-HBc (IgM) (B).
- High levels of anti-HBc (IgG) (C).
- Negative test result for HBs antigen, but positive test results for anti-HBc or anti-HBs preceding the onset of liver injury, in cases with a history of immunosuppressive and/or anticancer therapies in the previous recent 12 months (D).

I-②-2-i Asymptomatic or inactive HBV carriers without drug exposure; those satisfying A, B or C above in the absence of immunosuppressive and/or anticancer therapies in the previous 12 months.
I-②-2-ii Reactivation in asymptomatic or inactive HBV carriers receiving immunosuppressive and/or anticancer drugs; those with a history of immunosuppressive and/or anticancer therapies in the previous 12 months satisfying A, B or C above.
I-②-2-iii Reactivation by immunosuppressants and/or anticancer drugs in patients with resolved HBV (de novo HBV hepatitis); those satisfying D.
I-②-3 Indeterminate HBV infection; those with HBV infection, but not fulfilling the criteria shown in I-②-1 and I-②-2.
*To bear in mind that in general, hepatitis due to HBV is associated with high levels of serum HBV DNA, except in HBeAg positive asymptomatic carriers.
I-③ HCV: Positive for anti-HCV and/or HCV RNA.
I-④ HEV: Positive for anti-HEV (IgA) and/or HEV RNA.
I-⑤ Other viruses: demonstration of transient infection or reactivation of EBV, cytomegalovirus and other viruses through measurements of serological markers and viral genomes.
II. Autoimmune hepatitis; those satisfying "Criteria for Diagnosis of Autoimmune Hepatitis" proposed by the International Autoimmune Hepatitis Group, or those positive for antinuclear antibody or serum IgG concentrations 1.1-times the upper limit of the normal range at each institution or greater.**
**To bear in mind that patients with autoimmune hepatitis may be confused with those having drug-induced liver injuries or hepatitis of indeterminate etiology. Patients with the possibility of this condition should be treated as soon as possible as cases for autoimmune hepatitis.
III. Drug-induced liver injuries; those consistent with drug-induced liver injurybased on their clinical courses.
III-① Drug allergy-induced hepatitis.***
III-② Drug toxicity-induced liver injury (excluded from hepatitis).***
***Differential diagnosis between drug allergy-induced hepatitis and drug toxicity-induced liver injuries is based on the types and doses of the drugs and the clinical features of the patients.
IV. Liver injuries without the histological findings of hepatitis; diagnosis is based on the clinical features of the patients.
IV-① Circulatory disturbance.****
IV-② Metabolic diseases; Wilson's disease, anorexia nervosa, acute fatty liver of pregnancy, Reye's syndrome and others.
IV-③ Infiltration of the liver by malignant cells.
IV-④ Liver injuries after liver resection and transplantation.
IV-⑤ Miscellaneous etiologies.
****Liver injuries after operation other than liver resection and transplantation; those due to bacterial infection, DIC and heat stroke are in general classified as being caused by circulatory disturbance
V. Indeterminate etiology despite adequate examinations.
VI. Unclassified due to inadequate examinations.

DIC, disseminated intravascular coagulation; EBV, Epstein–Barr virus; HAV, hepatitis A virus; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; Ig, immunoglobulin.

estimated to be normal. Patients without histological findings of hepatitis are included in the disease entity of "acute liver failure", as in Europe and the USA. In this report, classification criteria for the etiologies of "acute liver failure" in Japan are proposed (Table 1).

ACKNOWLEDGMENTS

THIS STUDY WAS supported by Health Labor Sciences Research Grant, Research on Measures for Intractable Diseases, Ministry of Health Labor and Welfare of Japan.

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Hepatology Research

Hepatology Research 2014; 44: 1130–1141 doi: 10.1111/hepr.12238

Original Article

Novel hepatitis B virus strain developing due to recombination between genotypes H and B strains isolated from a Japanese patient

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Aim: In Japan, genotypes B and C are the predominant genotypes isolated from patients with chronic hepatitis B, while genotype A predominates in patients with acute hepatitis B. Globalization, however, appears to have changed the distribution of the hepatitis B virus (HBV) genotypes. Thus, the viral characteristics of HBV genotypes other than genotypes A, B and C were examined.

Methods: Screening of genotypes was performed by enzyme immunoassay and/or polymerase chain reaction INVADER method in 222 patients with HBV. The full-length nucleotide sequences of unusual strains were compared to those in the database, followed by construction of a phylogenetic tree.

Results: Unusual HBV strains were isolated from two patients: a 27-year-old Japanese bisexual man with acute hepatitis B with HIV co-infection and a 52-year-old Japanese man with chronic hepatitis B. The former strain was classified as genotype H, showing an overall identity of 99.8% to the Thailand strain (EU498228), while the nucleotide sequence of the latter strain showed similarity to the genotype B strains isolated in Malaysia (JQ027316) and Indonesia (JQ429079) between DR2 and DR1 in the X region, with identities of 96.9%. However, this strain was classified as genotype H by full-length sequence analysis, and the sequence between nt2023 and nt2262 showed no similarity to that in any previously reported strains.

Conclusion: HBV strains showing recombination between genotype B and H strains were found even in chronic hepatitis patients in Japan. Globalization may yield HBV strains of possible novel genotypes containing novel nucleotide sequences in the precore/core region.

Key words: genotype, globalization, hepatitis B virus, nucleotide sequence, recombination

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a global health problem with an estimated 400 million people worldwide showing persistent infection.[1] These patients are at a serious risk of developing the complication of liver cirrhosis and hepatocellular carcinoma (HCC),[2] and approximately 1 million deaths per year are attributed to cirrhosis and HCC caused by HBV infection.[3] In Japan, more than 30 000 people die of HCC each year,[4] and in 15% of these cases, the etiology has been shown to be HBV infection.[5] On the other hand, patients with persistent HBV infection serve as a source of HBV transmission to the healthy population, resulting in the occurrence of acute liver diseases with fatal outcomes. According to a nationwide survey of fulminant hepatitis and late-onset hepatic failure in Japan, acute liver failure is caused by HBV infection, either transient infection or acute exacerbation of persistent infection, in approximately 40% of cases.[6–8]

Hepatitis B virus is a double-stranded DNA virus belonging to the *Hepadnaviridae* family; the genome is composed of approximately 3200 nucleotides organized into four open reading frames (ORF) for the P, C, S and X genes.[9] According to the results of full-length nucleotide sequence analysis of the entire genome, HBV has been classified into at least eight genotypes, A–H,

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Received 7 August 2013; revision 4 September 2013; accepted 4 September 2013.

showing nucleotide differences of more than 8% from each other.[10] The frequency of each genotype among isolates from patients with HBV infection differs depending on the geographic area of the world;[11] genotype A HBV strains prevail in Africa, Europe and India, while genotype B and C strains are frequent in Asia, and genotype E strains in sub-Saharan Africa. On the other hand, genotype D strains are distributed all over the world, and genotype F and H strains are found exclusively in Central and South America. It has been demonstrated that the clinical features of patients with HBV infection, including their responses to antiviral therapies, differ depending on the genotype of the viral strain causing the infection,[12] suggesting that identification of the HBV genotype causing the infection, in addition to determination of the serum HBV DNA levels and mutation profile of the viral genome is crucial to establish the therapeutic strategy in patients with both acute and chronic liver diseases caused by HBV.

However, it has been reported recently that globalization of the world may have altered the geographic distribution of HBV genotypes, including in Asian countries. In Japan, genotypes B1/Bj and C2 strains are the predominantly isolated strains from patients with both acute and chronic liver diseases caused by HBV infection; the distribution of the HBV genotypes has been reported to differ depending on the geographic areas even within Japan; genotype B strains are found more frequently in Okinawa islands and northeastern areas of Honshu island, while genotype C strains are more prevalent in other areas of Japan.[13] It has been suggested that such a distribution pattern may be upset in the near future, because genotype A strains have begun to be isolated more frequently from patients with acute liver diseases caused by HBV infection in Japan, especially in metropolitan cities such as Tokyo, Osaka and Nagoya,[14,15] and this genotype strain is known to produce persistent infection even in elderly patients contracting the infection.[16] Furthermore, the occurrence of recombination among different genotypes may also influence the geographic distribution patterns. HBV strains resulting from genome recombinations among genotype A, C and G strains have been found in Laos and Vietnam, and been tentatively proposed as "genotype I" strains.[17,18] Moreover, a HBV strain positioned between the human and ape genotypes on the phylogenetic tree has been isolated from a Japanese patient with HCC who had previously lived in Borneo.[19]

Thus, we screened the genotypes of the HBV strains isolated from patients with acute and chronic liver diseases caused by HBV, and the full-length nucleotide sequences of the strains other than genotype A, B and C strains found in the screening examination were analyzed and compared with those in the database. In the present paper, we report on the viral characteristics of such unusual strains detected in Japanese patients with HBV infection.

METHODS

Patients and experimental designs

THE SUBJECTS WERE 222 Japanese patients with acute or chronic hepatitis seen first between May 2011 and December 2012 at the outpatient clinic of Saitama Medical University Hospital. All the patients tested positive for serum hepatitis B surface antigen (HBsAg), and the HBV genotypes were screened by enzyme immunoassay (EIA)[20,21] or the polymerase chain reaction (PCR)-INVADER method.[22] The full-length nucleotide sequence was analyzed when genotypes other than A, B or C were identified from the patients. The screening examinations for the HBV genotypes were done under the assurance of national health insurance coverage. Written informed consent was obtained from each of the patients prior to the analysis of the full-length nucleotide sequences of the isolated HBV strains. The characteristics of the viral genotypes other than A, B or C identified through the screening examination were analyzed after obtaining the approval of the institutional review board of Saitama Medical University Hospital.

DNA extraction and direct nucleotide sequencing of the HBV strains

Nucleic acids were extracted from 200 μL of serum samples QIAamp MinElute Virus Spin Kits (Qiagen, Tokyo, Japan). The virus DNA was eluted in RNase-free water at a volume of 100 μL and maintained at −20°C until use. To obtain a full-length nucleotide sequence of HBV DNA, a long-distance nested PCR was performed to amplify two overlapping fragments according to the methods of Takahashi *et al.*[23] using oligonucleotide primers shown in Table S1.

A fragment with a length of 3040 bases (WA2) corresponding to oligonucleotides from 1908–1780 nt of a standard genotype C HBV isolate was amplified using two primer sets, external WA-L (1859–1882 nt) and WA-R (1805–1828 nt) primers and internal WA2-L (1887–1908 nt) and WA2-R (1780–1801 nt) primers, and PrimeSTAR GXL DNA Polymerase (TaKaRa, Shiga, Japan) with the primer annealing at 60°C for 35 cycles

in the first PCR and 30 cycles in the second PCR. A fragment with a length of approximately 378 bases (gN2) corresponding to the residue from 1702–2081 nt was amplified similarly using two primer sets, external gN1-L (1606–1625 nt) and gN1-FR/gN1-HR (2160–2179 nt) primers and internal gN2-L/gN2-HL (1683–1702 nt) and gN2-FR/gN2-HR (2081–2100 nt) primers, and TaKaRa Ex Taq Hot Start Version (TaKaRa) with the primer annealing at 55°C for 35 cycles in the first PCR and 30 cycles in the second PCR. PCR conditions for PrimeSTAR GXL DNA Polymerase and PrimeSTAR GXL DNA Polymerase were specified according to the protocol of the manufacturer.

Both WA2 and gN2 fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the BigDye Teminator version 3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) using the internal primers shown in Table S1, according to the protocol of the manufacturer. The nucleotide sequences of the amplified products were directly sequenced with a 3130 Genetic Analyzer (Applied Biosystems), and the obtained data for nucleotide sequences were connected using ATGC version 7 (GENETYX, Tokyo, Japan).

Whole-genome cloning of HBV strains

To obtain a whole-genome clone of HBV strains, an additional PCR and In-Fusion reactions were performed. The WA2 and gN2 fragments were amplified using Prime STAR MAX DNA Polymerase (TaKaRa) and primer sets, WA2-Sap I-L (1943–1960 nt) and WA2-Sap I-R (1689–1708 nt) primers and gN2-Sap I-L (1704–1723 nt) and gN2-Sap I-R (1940–1957 nt) primers, respectively (Table S1), with the primer annealing at 55°C for 35 cycles. T-Vector pMD20 (TaKaRa) was amplified using a primer set, pMD20-Sap I-L (1705–1708 nt) and pMD20-Sap I-R (1704–1707 nt) primers, at conditions similar to that in amplification of both fragments. All PCR conditions were specified according to the protocol of the manufacturer. Both fragments and the vector were purified using the QIAquick PCR Purification Kit (Qiagen). WA2-Sap I fragment (100 ng), 50 ng of gN2-Sap I fragment and 100 ng of T-Vector pMD20-Sap I were mixed in a tube with In-Fusion HD Enzyme Premix (Clontech, Mountain View, CA, USA) at a total volume of 10 μL. The reaction mixture was incubated at 50°C for 15 min, and then transferred to ice. Reaction mixture (2.5 μL) was transformed into Stellar Competent Cell (Clontech) followed by mini-prepping and was subjected to nucleotide sequencing. Both conditions for In-Fusion reaction and transformation were specified according to the protocol of the manufacturer.

SimPlot analysis and construction of the phylogenetic tree

The complete full-genome sequences of the isolated HBV strains were compared with those of the 35 reference sequences retrieved from the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank database. The full-genome sequences of the following HBV strains shown in the database (represented by their accession numbers) were used in the SimPlot analysis, followed by construction of the phylogenetic tree: genotype A, *AB076678*, *AF090838* and *M57663*; genotype B, *AB010291*, *AB033554*, *AF121249*, *D00329* and *D50521*; genotype C, *AB049609*, *AB049610*, *AB112063*, *AB112066*, *AB112471* and *AB115417*; genotype D, *AB033559*, *AB126581* and *Z35716*; genotype E, *AB091255*, *AB106564* and *X75657*; genotype F, *AB166850*, *AY090459* and *X69798*; genotype G, *AB056513*, *AB064310* and *AF160501*; genotype H, *AB179747*, *AY090454*, *AY090457* and *AY090460*; genotype I, *EU833891*, *GU357844*, *JF899337* and *JF899338*; and genotype J, *AB486012*.

The nucleotide sequences were multiple-aligned using GENETYX for Windows version 11 software (GENETYX) and the genotype was specified using Kimura's two-parameter method.[24] A phylogenetic tree was constructed by the neighbor-joining method.[25] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and resampling were carried out 1000 times. The subtypes of the strains used for the comparison were obtained from published articles.[26,27] Moreover, the recombination of the HBV genomes among strains of different genotypes was examined by the SimPlot program (available at http://sray.med.som.jhmi.edu/SCRoftware/) and boot scanning analysis.[25,28]

RESULTS

Genotypes of HBV strains obtained from patients with acute and chronic liver diseases

THE HBV STRAINS isolated from the 222 patients were classified according to the screening examinations carried out by EIA and/or the PCR-INVADER method as follows: genotype A, 21 (9.4%) strains; genotype B, 66 (29.7%) strains; and genotype C, 112 (50.5%) strains. The HBV genotype was indeterminate in 21 patients (9.4%) due to the low titers of serum HBsAg and/or HBV DNA. When the total subject population was stratified further, genotypes A, B, C and the

indeterminate genotype were found in 15 (50.0%), three (10.0%), 11 (36.7%) and zero (0%) of the 30 patients with acute liver diseases, and six (3.1%), 63 (32.8%), 101 (52.6%) and 21 (11.0%) of the 192 patients with chronic liver diseases, respectively. In contrast, one each of the patients (1.0%) with acute (case 1) and chronic (case 2) liver diseases had a HBV genotype other than A, B or C. The demographic and clinical features of the two patients were as follows.

A 27-year-old bisexual man (case 1) working in the adult entertainment industry was diagnosed as having acute hepatitis caused by HBV, and the genotype of the infecting HBV strain was identified as genotype H by the PCR-INVADER method. He received highly active antiretroviral therapy because of co-infection with HIV, and the serum HBV DNA titers decreased to less than the detectable level, with positivity for serum anti-HBs antibody developing 25 months later.

A 57-year-old man (case 2) was diagnosed as having chronic hepatitis caused by HBV, and the infecting HBV strain was classified as genotype F by the PCR-INVADER method, despite the genotype being classified as indeterminate by the EIA method. His deceased father had lived in Brazil in his youth and his elder brother had been diagnosed as being a HBV carrier at another hospital. He received oral entecavir at a daily dose of

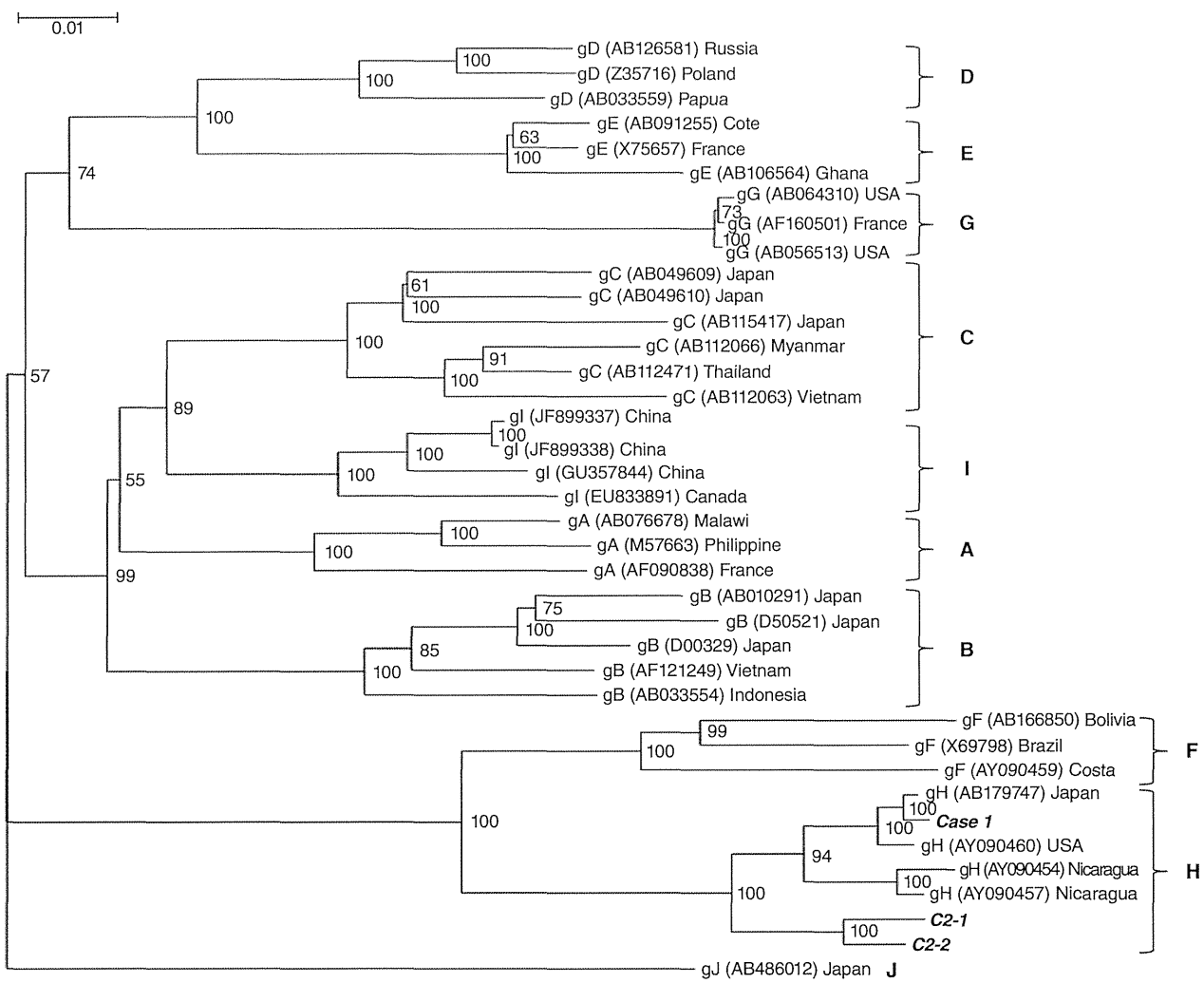

Figure 1 A phylogenetic tree constructed based on the full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 and case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance.

0.5 mg, and the serum HBV titers decreased from 5.3 log copies/mL to a level less than 2.1 log copies/mL by 3 months of treatment.

Full-length nucleotide sequences of the isolated HBV strains that were different from genotypes A, B and C

The nucleotide sequences of the HBV strains isolated from cases 1 and 2 were analyzed. A phylogenetic tree constructed based on the full-length sequence of HBV genome led to classification of the HBV strain isolated from case 1 as genotype H, showing an overall identity of 99.8% (3210/3215 bp) to the Thailand strain of genotype H (EU498228) (Figs 1,2). A similar analysis using a phylogenetic tree led to classification of the HBV strain isolated from case 2 as genotype H (Figs 1,3) despite it being classified as indeterminate and genotype F by EIA and PCR-INVADER assay, respectively. The full-length nucleotide sequence analysis showed an overall identity of 97.1% (3125/3218 bp) to genotype H strain isolated from a patient in Mexico (AB375164).

The nucleotide sequence of the HBV strains isolated from case 2 was further analyzed depending on the ORF, because the identity of the full-length nucleotide sequences to that of previously reported strains was less in case 2 than that in case 1. Consequently, the nucleotide sequence between DR2 (1590–1600 nt) and DR1 (1824–1834 nt) in the X region showed a similarity to that of the corresponding region of a genotype B strain isolated in Malaysia (JQ027316) and Indonesia (JQ429079), with identities of 98.4% (241/245 bp) and 98.0% (240/245 bp) (Fig. 4a). Moreover, analysis of the nucleotide sequence between 2023 and 2262 nt in the precore/core regions revealed that several different clones existed as quasispecies among HBV strains isolated from case 2, and two major clones, C2-1 and C2-2, were separated following cloning and sequencing of whole-genome nucleotides. Both C2-1 and C2-2 clones

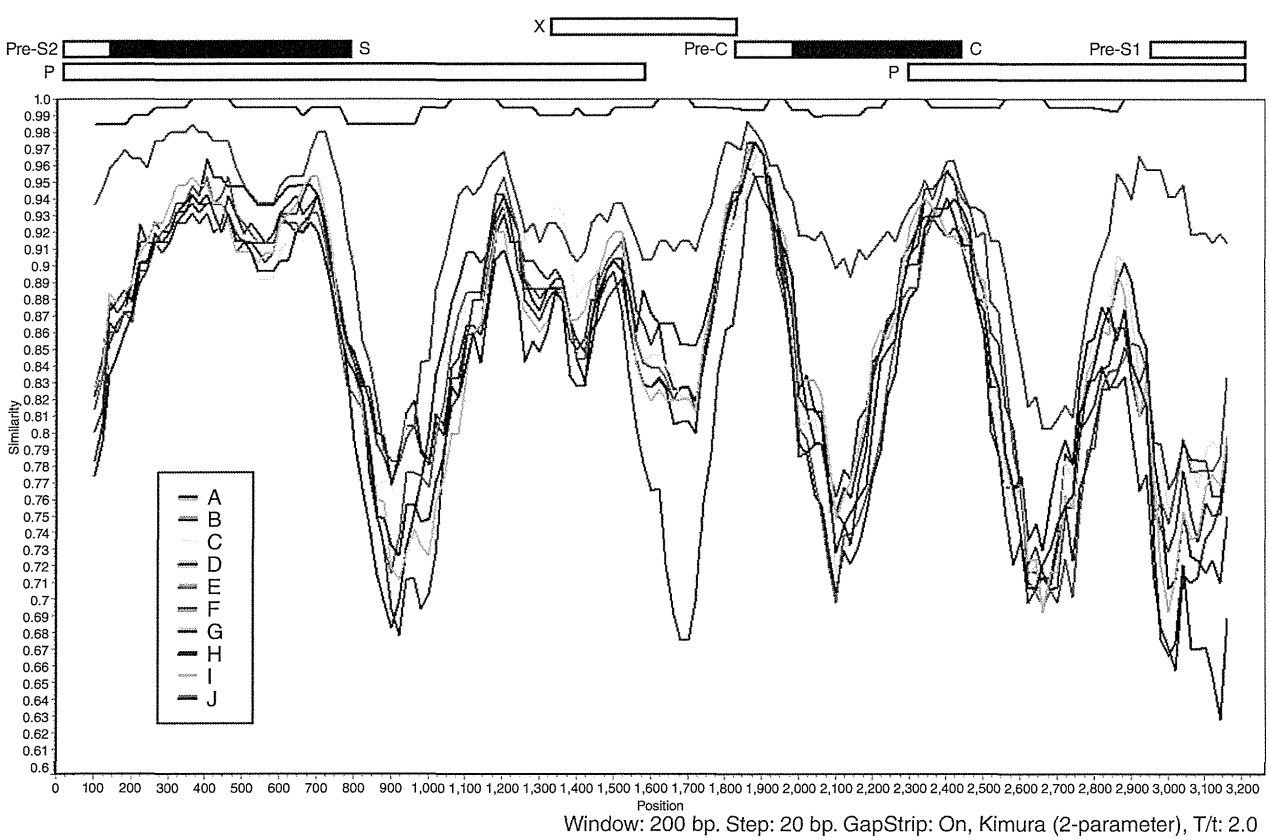

Figure 2 Nucleotide similarity comparison of a full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size and gap-stripped alignments).

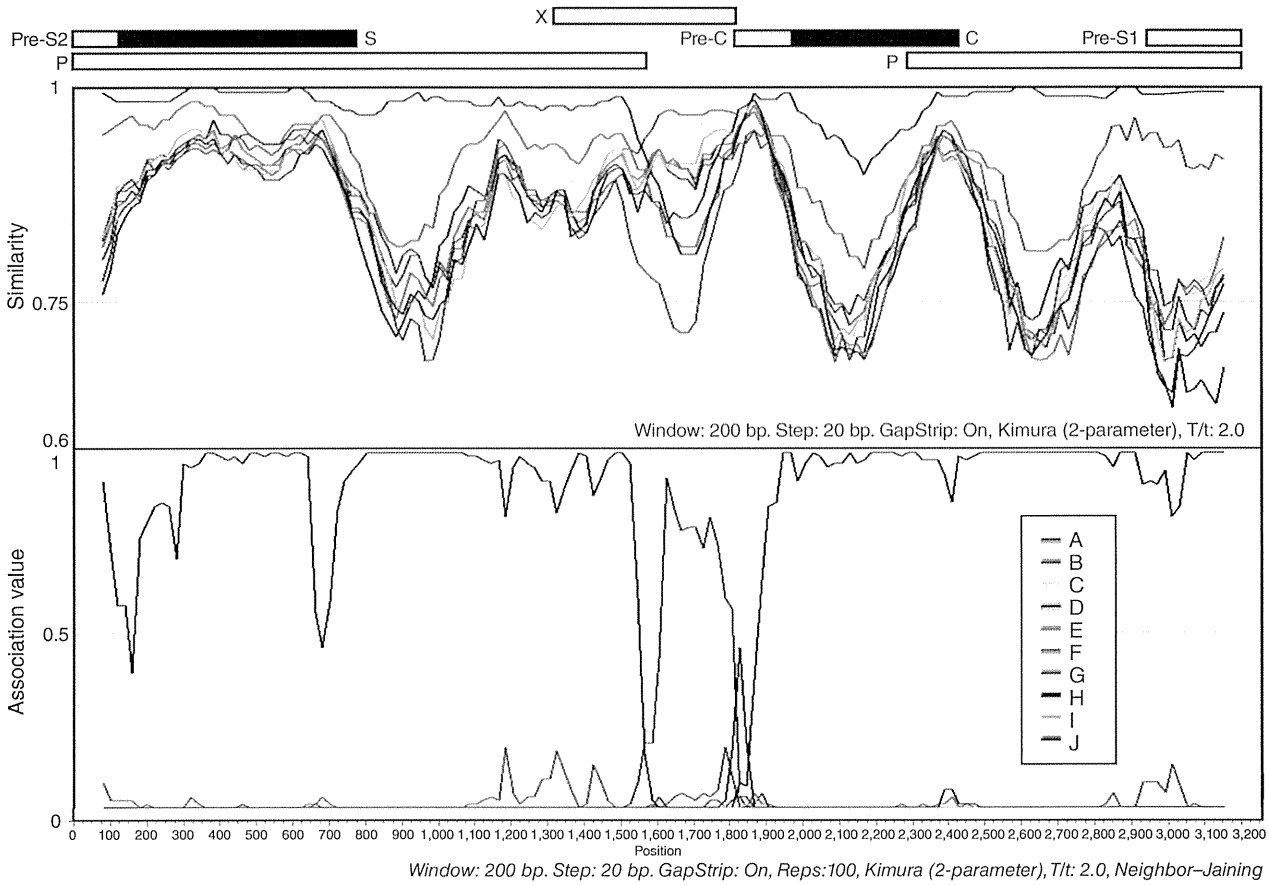

Figure 3 Nucleotide similarity comparison of the full-length sequence of the hepatitis B virus (HBV) strain isolated from case 2 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size, 100 bootstrap replicates, gap-stripped alignments and neighbor-joining algorithm).

were classified as genotype H according to full-length nucleotide sequence analysis, with an identity of 96.4% to 95.8% to each other, and as genotype B based on analysis of the nucleotide sequence between DR2 and DR1, with an identity of 96.9% to 95.8%, respectively. However, the nucleotide sequence between 2023 and 2262 nt in the precore/core regions showed no similarity to that of any previously reported HBV strains. In these regions, the C2-1 and C2-2 clones showed nucleotide sequences with an identity of 98.6% to each other, and the nucleotide divergences in comparison to strains of genotypes A–J ranged 9.6–30.0% in the C2-1 clone and 8.1–28.5% in the C2-2 clone (Table 1). A phylogenetic tree constructed based on these regions revealed that both strains may be classified into the novel cluster of HBV (Fig. 4b). Also, the amino acid sequence divergences from previously reported HBV strains ranged from 18.1% to 27.9% in the C2-1 clone and 17.1% to 26.9% in the C2-2 clone.

The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession number AB818694 for case 1, AB819065 for the C2-1 and AB819066 for the C2-2 strain.

DISCUSSION

IN THE PRESENT paper, the genotypes of the HBV strains isolated from 222 patients with acute and chronic hepatitis B were evaluated by EIA and/or PCR-INVADER assay, and HBV genotype A strains, commonly isolated in Africa, Europe and India, were found in 9.4% of the patients; genotype A strains were isolated from 50.0% of patients with acute liver diseases and 3.1% of patients with chronic liver diseases. These values were almost in line with those reported from other institutions in Japan.[11–13] HBV genotype A strains are known to be frequently isolated from patients with