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

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**These authors contributed equally to this work.*

Address reprint requests to: Masashi Mizokami, M.D., Ph.D., Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1, Konodai, Ichikawa 272-8516, Japan. E-mail:mmizokami@hospk.ncgm.go.jp; fax: +81-(0)47-375-4766.

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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.

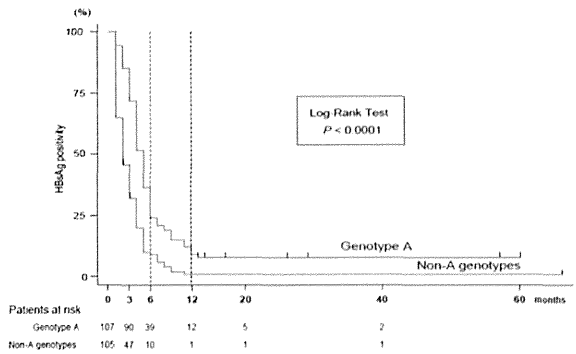

Fig. 1. Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between genotype A and non-A genotypes, analyzed using the Kaplan-Meier test. $P < 0.0001$, genotype A: red line, non-A genotypes: blue line.

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.

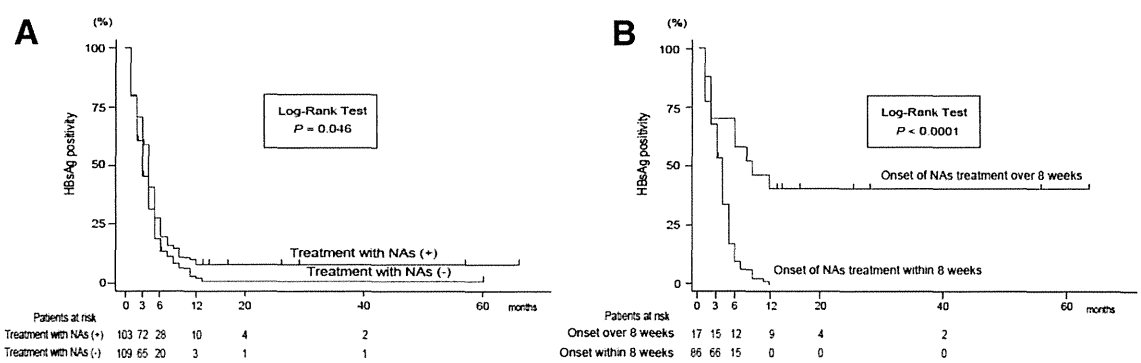

Fig. 2. (A) Comparison of the cumulative proportion of AHB patients maintaining HBsAg positivity between treatment with NAs (+) and treatment with NAs (−), as analyzed using the Kaplan-Meier test. $P = 0.046$, treatment with NAs (+): red line, treatment with NAs (−): blue line. (B) Comparison of the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

Daini Red Cross Hospital), Michihiro Suzuki (Kawasaki Municipal Tama Hospital), Hitoshi Takagi (Gunma University Graduate School of Medicine), Eiichi Tomita (Gifu Municipal Hospital), Kumada Takashi (Ogaki Municipal Hospital), Toshihiko Mizuta (Saga University Faculty of Medicine), Tetsuya Mine (Tokai University School of Medicine), Jong-Hon Kang (Teine-Keijinkai Hospital), Katsuji Hirano (Juntendo University Shizuoka Hospital), Hirohito Tsubouchi (Kagoshima University Graduate School of Medical and Dental Sciences), Akito Nozaki (Yokohama City University Medical Center), Akito Sakai (Kanazawa University Graduate School of Medical Science), Shuhei Nishiguchi (Hyogo College of Medicine), Akihiro Tamori (Osaka City University Graduate School of Medicine), Satoru Hagiwara (Kinki University School of Medicine), Takahide Nakazawa (University of Kitasato East Hospital), Michio Sata (Kurume University School of Medicine), Toshiro Kamoshida (Hitachi General Hospital) Atsushi Takahashi (Fukushima Medical University School of Medicine), Satoshi Kakizaki (Gunma University Graduate School of Medicine), Yoshimasa Kobayashi (Hamamatsu University School of Medicine), Shigeru Sasaki (Sapporo Medical University), Tadashi Ikegami (Tokyo Medical University Ibaraki Medical Center), Yoichi Hiasa (Ehime University Graduate School of Medicine), Kenji Nagata (University of Miyazaki), Tomoyuki Kubota (Saiseikai Niigata Daini Hospital), Hiroshi Mitsui (Tokyo Teishin Hospital), Norihiko Yamamoto (Mie University School of Medicine), Masataka Tsuge (Hiroshima University), Shuichi Sato (Shimane University Hospital), Yoshito Ito (Kyoto Prefectural University of Medicine), Wataru Sato (Akita University School of Medicine), Shigeharu Uchida (Japanese Red Cross Society), Yuki Tada (National Institute of Infectious Diseases), Toshiaki Mizuochi (National Institute of Infectious Diseases), Norihiro Furusho (Kyushu University), and Shuhei Hige (Hokkaido University Graduate School of Medicine).

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Prediction of early HBeAg seroconversion by decreased titers of HBeAg in the serum combined with increased grades of lobular inflammation in the liver

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Sung Kwan Bae[1,2ABCDEF], Hiroshi Yatsuhashi[1,2ABDEF], Satoru Hashimoto[1,2BD], Yasuhide Motoyoshi[1,2BD], Eisuke Ozawa[1,2BD], Shinya Nagaoka[1,2BD], Seigo Abiru[1,2BD], Atsumasa Komori[1,2BD], Kiyoshi Migita[1,2D], Minoru Nakamura[1,2D], Masahiro Ito[1,2BD], Yuzo Miyakawa[3DEF], Hiromi Ishibashi[1,2ABDEF]

[1] Clinical Research Center, National Hospital Organization (NHO), Nagasaki Medical Center, Nagasaki, Japan
[2] Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[3] Miyakawa Memorial Research Foundation, Tokyo, Japan

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Summary

Background: Hepatitis B e antigen (HBeAg) seroconversion is an important hallmark in the natural course of chronic hepatitis B. This study was designed to predict early HBeAg seroconversion within 1 year, by not only biochemical and virological markers, but also pathological parameters in patients with chronic hepatitis B.

Material/Methods: In a retrospective cohort study, 234 patients with HBeAg were reviewed for demographic, biochemical, virological and pathological data at the time of liver biopsy. Then, the patients who accomplished HBeAg seroconversion within 1 year thereafter were compared with those who did not, for sorting out factors predictive of early HBeAg seroconversion.

Results: Early HBeAg seroconversion occurred in 58 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: alanine aminotransferase (ALT) (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p=0.001), core-promoter mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). In multivariate analysis, only serum HBeAg levels <100 Paul Ehrlich Institute (PEI) U/ml and grades of lobular inflammation >2 were independent factors for early HBeAg seroconversion (odds ratio 8.430 [95% confidence interval 4.173–17.032], p<0.001; and 4.330 [2.009–9.331], p<0.001; respectively).

Conclusions: HBeAg levels < 100 PEIU/ml combined with grades of lobular inflammation >2 are useful for predicting early HBeAg seroconversion. In patients without liver biopsies, high ALT levels (>200 IU/L) can substitute for lobular inflammation (grades >2).

key words: **alanine aminotransferase • chronic hepatitis • hepatitis B virus • hepatitis B e antigen • lobular inflammation • seroconversion**

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Author's address: Hiroshi Yatsuhashi, Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan, e-mail: yatsuhashi@nmc.hosp.go.jp

Background

Worldwide, an estimated 350 million people are infected with hepatitis B virus (HBV) persistently [1,2]. HBV infection is a major global concern, because up to 40% of patients can develop grave complications, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [3]. In the natural course of chronic hepatitis B, HBeAg seroconversion, defined by the loss of HBeAg and development of the corresponding antibody (anti-HBe), is an important hallmark, because it is highly correlated with a favorable long-term outcome. Seroconversion is usually followed by sustained suppression of HBV DNA, normalization of alanine aminotransferase (ALT) levels, and clinical remission accompanied by ameliorated necro-inflammatory activities in the liver [4–6].

To date, a number of factors have been found to predispose patients to spontaneous HBeAg seroconversion [7–19]. However, few studies have evaluated pathological factors for predicting early HBeAg seroconversion. In a small series of patients from Spain, the Knodell's index of histological activity was one of the independent predictors of early HBeAg seroconversion [14]. Recently, novel markers of the replication of HBV were introduced, such as levels of HBsAg, HBeAg and HBcrAg (HBV core-related antigen), which can replace HBV DNA levels. These serological markers of HBV replication have been evaluated for sensitive and reliable prediction of early HBeAg seroconversion [20–23]. In the present study, an attempt was made to select factors predictive of early HBeAg seroconversion, from among many biochemical, virological and pathological parameters, based on the data of 234 HBeAg-positive patients with chronic hepatitis B.

Material and Methods

Patients and study design

This is a retrospective cohort study with use of stored sera and liver biopsy specimens from patients with chronic hepatitis B who were taken care of in the Hepatology Department, Nagasaki Medical Center, Japan, during 1991 through 2005. The clinical database was reviewed to identify consecutive patients who underwent liver biopsies and had been followed for longer than 1 year. The inclusion criteria were presence of hepatitis B surface antigen (HBsAg) for 6 months or longer, positivity for HBeAg at the time of liver biopsy, and lack of antiviral treatments before receiving liver biopsies. The exclusion criteria were co-infection with hepatitis C virus (HCV) or human immunodeficiency virus type-1, serological markers suggestive of autoimmune disease, daily intake of alcohol >50 g, recent exposure to hepatotoxic drugs, and no stored sera available. They were followed every 3 months or more frequently, if indicated clinically, and their serum samples were monitored for liver biochemistry and serologic markers of HBV infection, including HBsAg, HBeAg, anti-HBe, HBV DNA and HBcrAg. Serum samples had been stored at –20°C until use.

Antiviral therapy was commenced immediately in the patients with: (1) significant fibrosis/cirrhosis detected by liver biopsy; and (2) evidence of decompensation, such as ascites, varices and hepatic encephalopathy.

To identify predictors of early HBeAg seroconversion, clinical, biological, virological and pathological data at the time of liver biopsy were compared between patients who did and who did not achieve early HBeAg seroconversion, within 1 year after receiving liver biopsies, by univariate and multivariate analyses. Further, patients were stratified by independent factors for HBeAg seroconversion, and the cumulative incidence of HBeAg seroconversion was compared between groups using the Kaplan-Meier method. The study protocol complied with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the review board of the institution. Each patient gave a written informed consent before participating in this study.

Routine laboratory tests for HBV markers

Quantitative measurements of HBsAg and HBeAg were carried out using commercial enzyme-linked immunosorbent assay (ELISA) kits in the ARCHITECT ANALYSER i2000 (Abbott Japan Co., Ltd., Tokyo, Japan) in accordance with the manufactures' instructions in Nagasaki Medical Center. The sensitivity of HBsAg assay ranged from 0.05 to 250 IU/ml. Sera with HBsAg >250 IU/ml were serially diluted 100-fold so as to include them within the dynamic range. HBeAg was quantified by a two-step immunoassay with use of chemiluminescence microparticles. Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe. After a washing step, conjugate and reactants were added for exciting emission of the light that is proportional to the concentration of HBeAg. The result was expressed by the ratio of relative light unit (RLU) of the sample to the cut-off RLU (S/CO). Samples with S/CO values >1.0 were regarded positive for HBeAg. Then, serial dilutions of the reference standard of PE HBeAg (Paul-Ehrlich Institute, Langen, Germany) were used to define the linear range of the assay and create a reference curve for linear regression. The linear range was 0.024–100 PEIU/ml. A standard curve was produced, and linear regression was used to convert assay results into appropriate units (PEIU/ml). For samples that fell outside the linear range of the assay, the assay was performed on serial dilutions to ensure the linearity.

HBV DNA and HBcrAg

HBV DNA was determined by the COBAS Taqman HBV test (Roche Diagnostics K.K., Tokyo, Japan). Values under or over the detection range were recorded as 2.1 or 9.1 log copies/ml. HBcrAg was measured by the CLEIA HBcrAg assay kit (Fujirebio, Inc., Tokyo, Japan) in a fully automated analyzer (Lumipulse system, Fujirebio, Inc.). Values under or over the detection range were recorded as 3.0 or 7.0 log copies/ml. Assays for HBV DNA and HBcrAg were performed in a commercial clinical laboratory (SRL, Inc., Tokyo, Japan). Sera with values over the detection range were diluted to include them within the dynamic range.

Interferon-inducible protein 10 (IP-10)

IP-10 was quantified by the Invitrogen Human IP-10 ELISA (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol in Nagasaki Medical Center.

HBV genotyping

HBV DNA was extracted from serum (100 µl) with use of the SMITEST EX R&D extraction kit (MBL Co., Ltd., Nagoya, Japan). It was amplified for determination of genotypes by

Table 1. Histological evaluation of liver biopsy specimens.

(A) Fibrosis staging	
Stage	**Fibrosis**
0	None
1	Enlarged, fibrotic portal tracts
2	Periportal or portal-portal septa but intact architecture
3	Fibrosis with architectural distortion without obvious cirrhosis
4	Probable or definite cirrhosis

(B) Inflammation grading			
Grade	**Portal/periportal activity**		**Lobular inflammation**
	Piecemeal necrosis	**Lymphocyte aggregation**	
0	None or minimal	None	None
1	Inflammation only	< 1/3 in portal triad	Inflammation alone
2	Mild	1/3–2/3 in portal areas	Focal necrosis or acidphil bodies
3	Moderate	> 2/3 in portal areas	Severe focal cell damages
4	Severe	Entire portal triad	Damage with bridging necrosis

the SMITEST HBV Genotyping Kit (MBL Co., Ltd.) based on hybridization with type-specific probes immobilized on a solid-phase support [24].

Precore stop codon (G1896A) and core promoter (A1762T/G1764A) mutations

A1896 mutation in the precore (PreC) region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA, Roche Diagnostics, Tokyo, Japan), and mutations in the core promoter (CP) region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit, Roche Diagnostics K.K.). The results were recorded as "the wild-type" and "mutant types" dominantly expressed by HBV isolates [25].

Histological examination

Liver biopsy was taken by fine-needle aspiration (16G sonopsy) guided by ultrasonography. Biopsy specimens were fixed in 10% neutral formalin, cut at 3- to 4-μm thickness, and stained with Hematoxyline-Eosin and Azan-Mallory, as well as for silver to visualize reticuline fibers. Tissue sections were examined independently by two senior liver pathologists. For each biopsy specimen, a protocol was filled out for grading necro-inflammation and staging fibrosis by the criteria of Desmet et al. [26] and Scheuer [27] (Table 1). As for the portal activity, not only piecemeal necrosis, but also lymphocytic aggregation was categorized into 5 (0–4) grades in the respective area involved.

Statistical analysis

Continuous variables were compared between groups by the Mann-Whitney *U* test, and categorical variables by χ^2 and Fisher's exact tests. The cumulative incidence of HBeAg seroconversion was calculated using the Kaplan-Meier method, and the difference was evaluated by the log-rank test. Multiple logistic regression analysis was performed to identify independent factors in significant association with early HBeAg seroconversion. A p value <0.05 was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of patients

Among the 673 patients with HBsAg who had received liver biopsies in our hospital during 1991 through 2005, 234 (34.8%) patients who met the inclusion criteria were enrolled in this study. Demographic and laboratory characteristics at the time of liver biopsy are listed in Table 2. They had a median age of 37 years (range: 12–74), and 161 (69%) were men. Of them, 231 (99%) were infected with HBV of genotype C. The median serum ALT level at the baseline was 141 IU/l (range: 13–2644 IU/l), and the median duration of follow-up was 86.5 months (range: 12.0–213.0 months). During the follow-up, 91 (39%) received antiviral treatment, with interferon (IFN) or lamividine, or the combination thereof.

Comparison of clinical features between patients with and without early HBeAg seroconversion

Early HBeAg seroconversion, within 1 year after receiving liver biopsies, was achieved by 58 of the 234 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: ALT (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p<0.001), CP mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). Other factors including age, albumin, platelets, AFP, PreC mutation, cell infiltration and

Table 2. Baseline characteristics of patients.

Features	Total (n=234)
Demographic data	
Age (years)	37 (12–74)
Men (%)	161 (69)
Biolochemical markers	
Albumin (g/dl)	4.1 (2.5–5.0)
Platelets ($\times 10^3/mm^3$)	179 (43–338)
ALT (IU/l)	141 (13–2644)
AFP (ng/ml)	7 (0–1863)
IP-10 (ng/ml)	214 (66–3253)
Virological markers	
HBV genotypes: A/B/C (%)	1/2/231 (0/1/ 99)
HBsAg (IU/ml)	8039 (2–261647)
HBeAg (PEIU/ml)	245.3 (0.01–3179.7)
HBV DNA (log copies/ml)	7.7 (3.6–8.9)
HBcrAg (log U/ml)	7.8 (5.4–9.2)
PC mutations: wild/mix/ mutant (%)	132/100/2 (56/43/1)
CP mutations: wild/mix/ mutant/others (%)	55/50/126/3 (24/21/54/1)
Pathological features	
Fibrosis stages: 0/1/2/3/4 (%)	15/73/54/38/54 (7/31/23/16/ 23)
Lymphocytic aggregation: 0/1/2/3/4 (%)	6/65/107/45/11 (2/28/46/19/5)
Piecemeal necrosis: 0/1/2/3/4 (%)	59/52/57/58/8 (25/22/24/25/4)
Lobular inflammation: 0/1/2/3/4 (%)	4/91/104/32/3 (2/39/44/14/1)
Antiviral treatments	
Within 1 year of biopsy (%)	91 (39)
Antiviral agents: 1/2/3/4* (%)	44/33/13/1 (49/36/14/1)
Duration of follow up (months)	86.5 (12.0–213.0)

Qualitative variables are expressed in the number with percentage in parentheses, and quantitative variables are expressed in the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

piecemeal necrosis in the liver, as well as treatments within 1 year after the entry and type of antiviral agents, were not associated with early HBeAg seroconversion (Table 3).

Evaluation of HBV markers for predicting early HBeAg seroconversion

HBV markers were compared for sensitivity and specificity in predicting early HBeAg seroconversion by the receiver operating characteristic analysis (Figure 1). HBeAg at the time of liver biopsy was the best predictor of early HBeAg seroconversion, with the widest area under the curve of 0.750; it was larger than those of HBcrAg (0.708), HBV DNA (0.650) and HBsAg (0.630). Hence, HBeAg was selected as the best HBV marker predictive of early seroconversion. Based on the receiver operating characteristic curve, HBeAg titers were dichotomized by 100 PEIU/ml in the immunoassay.

Independent predictors for early HBeAg seroconversion

A multivariate logistic regression analysis was performed to select independent predictors of early HBeAg seroconversion from among variables significant in the univariate analysis (Table 4). Of all factors, including histological characteristics, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation remained as independent factors predictive of early HBeAg seroconversion (Table 4A). Of factors exclusive of histological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/ml remained as independent factors for early HBeAg seroconversion (Table 4B).

Combinations of two independent factors for predicting early HBeAg seroconversion

Two combinations of independent factors were evaluated for the performance in predicting early HBeAg seroconversion. The patients who had two predictors in combination, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation, achieved early HBeAg seroconversion in the highest frequency at 66.0% (31/47). In a remarkable contrast, merely 6.9% (4/58) of the patients without either of these predictors achieved early HBeAg seroconversion (Figure 2A).

Likewise, early seroconversion was achieved by 18 of the 30 (60.0%) patients with the other combination of independent factors, exclusive of pathological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/l. By contrast, only 6 of the 99 (6.1%) patients without either of them achieved early HBeAg seroconversion (Figure 2B).

Sensitivity, specificity, positive predictive value and negative predictive value of predicting early HBeAg seroconversion are: 74.5% (31/58), 90.9% (160/176), 66.0% (31/47) and 85.6% (160/187), respectively, for the combination of HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation; and 31.0% (18/58), 93.2% (164/176), 60.0% (18/30) and 80.4% (164/204), respectively, for the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l.

Long-term clinical outcomes

Besides the 58 patients with early HBeAg seroconversion, an additional 97 patients achieved HBeAg seroconversion during a median follow-up period of 86.5 months. Cumulative

Table 3. Univariate analysis of risk factors for early HBeAg seroconversion.

Variables	Early HBeAg seroconversion		p value
	Achieved (n=58)	Not achieved (n=176)	
Demographic data			
Age (years)	36 (17–69)	37 (12–74)	0.303
Men (%)	41 (71)	120 (68)	0.721
Biolochemical markers			
Albumin (g/dl)	4.1 (2.8–4.8)	4.1 (2.5–5.0)	0.877
Platelets ($\times 10^3/mm^3$)	171 (43–291)	186 (57–338)	0.487
ALT (IU/l)	227 (18–2072)	121 (13–2644)	0.002
AFP (ng/ml)	12 (1–1863)	6 (0–683)	0.070
IP-10 (ng/ml)	259 (77–1743)	204 (66–3253)	0.029
Virological markers			
HBV genotypes A/B/C (%)	0/0/58 (0/0/100)	1/2/173 (1/1/98)	1
HBsAg (IU/ml)	5127 (8–261647)	9033 (2–128511)	0.003
HBeAg (PEIU/ml)	20.9 (0.01–1985.0)	377.1 (0.01–3179.7)	<0.001
HBV DNA (log copies/ml)	7.2 (3.7–8.7)	7.8 (3.6–8.9)	0.001
HBcrAg (log U/ml)	7.2 (5.7–9.2)	8.0 (5.4–9.1)	<0.001
PC mutations: wild/mix/mutant (%)	26/31/1 (45/53/2)	106/69/1 (60/39/1)	0.075
CP mutations: wild/mix/mutant/others (%)	8/9/40/1 (14/15/69/2)	47/41/86/2 (27/23/49/1)	0.040
Pathological features			
Fibrosis stage: 0/1/2/3/4 (%)	1/12/18/14/13 (2/21/31/24/22)	14/61/36/24/ 41 (8/35/20/14/23)	0.033
Lymphocytic aggregation: 0/1/2/3/4 (%)	0/11/27/17/3 (0/19/47/29/5)	6/54/80/28/8 (3/31/45/16/5)	0.087
Piecemeal necrosis: 0/1/2/3/4 (%)	7/12/18/19/2 (12/21/31/33/3)	52/40/39/39/6 (30/23/22/22/3)	0.068
Lobular inflammation: 0/1/2/3/4 (%)	0/13/29/15/1 (0/22/50/26/2)	4/78/75/17/2 (2/44/43/10/1)	0.002
Antiviral treatments within 1 year after biopsy (%)	28 (48)	63 (36)	0.091
Antiviral agents: 1/2/3/4* (%)	18/5/5/0 (64/18/18/0)	26/28/8/1 (41/44/13/2)	0.051

Qualitative variables are expressed by the number of patients with percentage in parentheses, and quantitative variables are expressed by the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up >10 years after liver biopsies (Figure 3). Of note, HCC developed in 18 of the 234 (7.7%) patients during the follow-up.

Figure 4A compares cumulative HBeAg seroconversion rates stratified by HBeAg titers and grades of lobular inflammation. The patients, who had the combination of HBeAg <100 PEIU/ml and lobular inflammation grades ≥2, gained an HBeAg seroconversion rate higher than those having 3 other combinations. Likewise, cumulative HBeAg seroconversion rates stratified by HBeAg titers and ALT levels are compared in Figure 4B. HBeAg seroconversion rate of the patients, who had the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l, was higher than those with 3

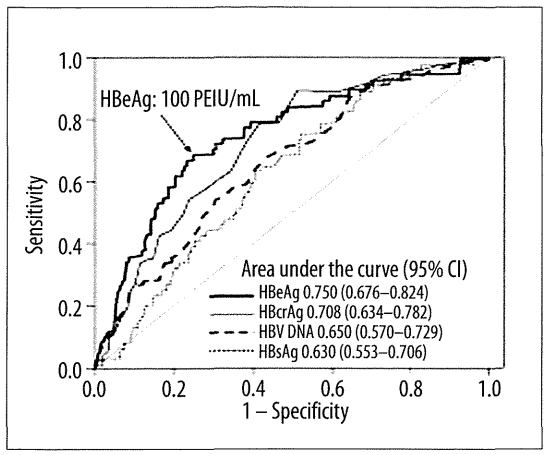


Figure 1. Receiver operating characteristic curves for evaluation of the power of predicting early HBeAg seroconversion.

other combinations, with definitive (p=0.003 and p<0.001) or marginal (p=0.061) significance.

Discussion

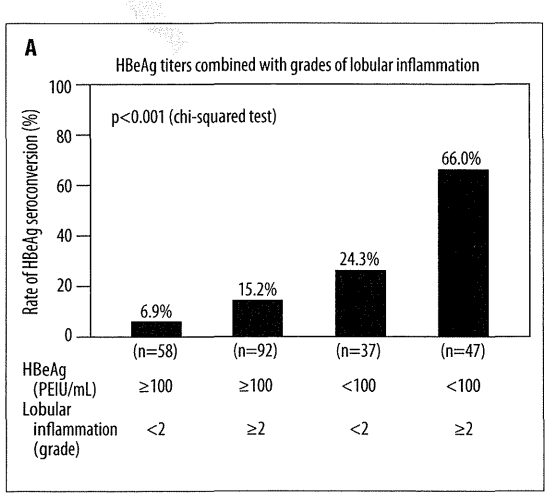
HBeAg seroconversion is important as a clinical target in the management of chronic hepatitis B. In the absence of therapeutic interventions, HBeAg seroconversion occurs spontaneously at a rate of 0.8–15% per year [28]. To date, many factors have been found in association with HBeAg seroconversion, including older age, high ALT levels, genotype B (compared with C), the Knodell's index of histologic activities, the amount of HBV core antigen in the liver, high serum AFP levels, increased immunoglobulin-M anti-HBc titers, increased serum β_2-microglobulin concentrations, enhanced expression of HLA-antigens on the membrane of hepatocytes, non-vertical transmission modes, low HBV DNA levels, and high serum levels of IL-10 as well as IL-12 [7–19].

It would be clinically useful to predict early HBeAg seroconversion, because antiviral treatments can be withheld in the patients in whom HBeAg disappears and anti-HBe develops within a certain time limit, perhaps 1 year. In the present study, the majority of patients (99% of the 234 examined) were infected with HBV of genotype C. Patients with persistent HBV infection in Japan are infected with HBV of either genotype B or C, with an increasing gradient of C toward the south [29,30]. All

Table 4. Multivariate analysis for the risk of early HBeAg seroconversion.

Variables	Odds ratio	95% confidence interval	p value
(A) All factors including histological characteristics			
HBeAg (<100 PEIU/ml)	8.430	4.173–17.032	<0.001
Lobular inflammation (≥2)	4.330	2.009–9.331	<0.001
(B) Factors exclusive of histological characteristics			
HBeAg (<100 PEIU/ml)	7.327	3.703–14.497	<0.001
ALT (≥200 IU/l)	3.093	1.562–6.127	0.001

HBeAg – hepatitis B e antigen; ALT – alanine aminotransferase.



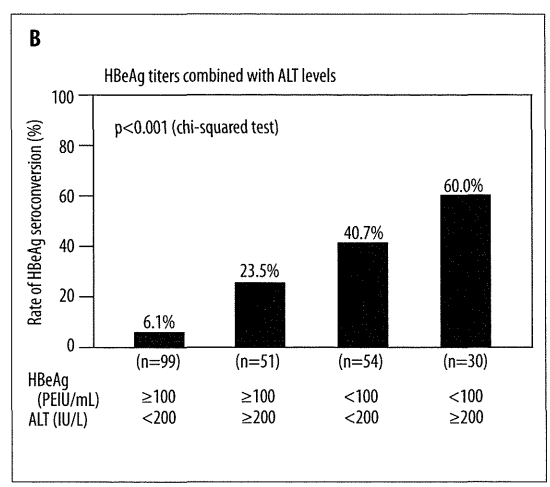


Figure 2. Probability of early HBeAg seroconversion. (**A**) The rate of early HBeAg seroconversion assessed by HBeAg titers and grades of lobular inflammation. (**B**) The rate of early HBeAg seroconversion assessed by HBeAg titers and ALT levels.

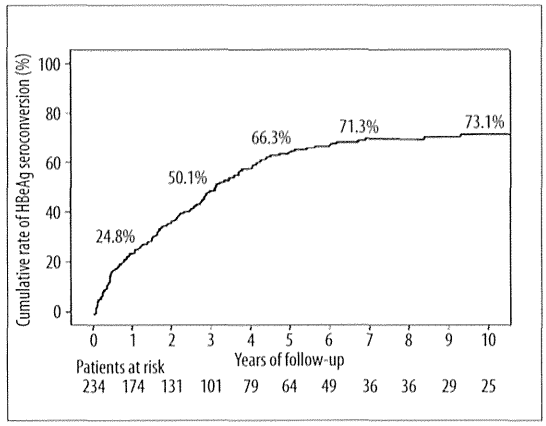


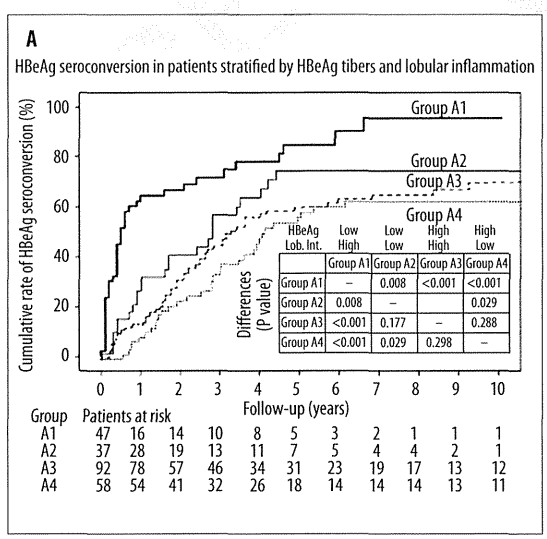
Figure 3. Cumulative rates of HBeAg seroconversion in the 234 patients during 10 years. Cumulative rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up.

the 234 patients had received liver biopsies before they were started to be followed for HBeAg seroconversion. The present study is unique in that, not only serological variables, but also histological parameters were evaluated for the association with early HBeAg seroconversion within 1 year. By univariate analysis, many factors that have been reported in association with HBeAg seroconversion predicted early HBeAg seroconversion. Among them, only HBeAg (<100 PEIU/ml) and lobular inflammation (grades >2) remained as independent factors for early HBeAg seroconversion by multivariate analysis.

Previous clinical studies have indicated that serial monitoring of HBsAg, HBeAg and HBV DNA levels during antiviral treatments is useful for predicting HBeAg seroconversion [20–23]. Although the determination of HBV DNA in sera remains as an important tool for monitoring outcomes of patients with chronic hepatitis B, it is technically challenging, costly, and subject to inconsistency. Hence, three serological markers of HBV replication, HBsAg, HBeAg and HBcrAg, were quantitated for evaluating the performance in predicting early HBeAg seroconversion, in comparison with HBV DNA levels. In the receiver operating characteristic analysis, HBeAg levels performed the best amongst these four replication markers, with an area under curve wider than those of the other three. Since the quantitation of HBeAg is relatively easy, fast, and inexpensive, HBeAg would be qualified as a sensitive and practical predictor of early HBeAg seroconversion [20–23].

The histological activity has been reported to predict early HBeAg seroconversion in previous studies [14,31]. Therefore, pathological parameters including the stage of fibrosis, as well as grades of portal inflammation, piecemeal necrosis and lobular inflammation, were evaluated in this study. By multivariate analysis, lobular inflammation of grades >2, represented by focal necrosis or acidophil bodies, was identified as an independent factor for early seroconversion. Hence, portal inflammation without necrosis would not be enough, but instead, severe lobular inflammation may be required for predicting early seroconversion.

Many previous studies have identified a variety of factors associated with HBeAg seroconversion [7–19], but a combination of serum markers of HBV with pathological parameters was evaluated rarely. Therefore, the combination of HBeAg <100 PEIU/ml and grades >2 lobular inflammation was evaluated for the predictability of early HBeAg seroconversion. Patients with neither HBeAg <100 PEIU/ml nor grades >2 lobular inflammation had a minimal chance for early HBeAg seroconversion (6.9% [4/58]), whereas a high proportion of patients with both of these predictors did accomplish early seroconversion (66.0% [31/47]) (Figure 2A). Thus, the combination of histologic activity and serum HBV marker would be very useful for predicting early HBeAg seroconversion, and serve in decision making whether or not



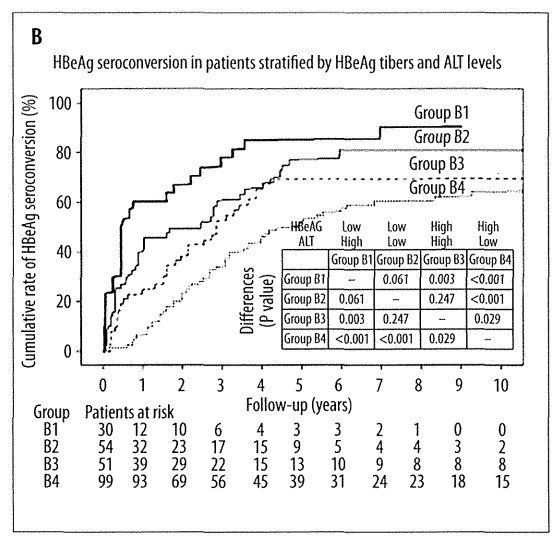


Figure 4. Cumulative rates of HBeAg seroconversion in four groups of patients. (**A**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and grades of lobular inflammation. (**B**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and ALT levels. HBeAg titers were dichotomized into low (<100 PEIU/ml) or high (≥100 PEIU/ml); lobular inflammation grades into low (<2) or high (≥2); and ALT levels into low (<200 IU/l) or high (≥200 IU/l).

to commence antiviral treatments in HBeAg-positive patients with chronic hepatitis B. Although some patients received antiviral treatments, they would not have influenced the evaluation to any serious extent. Within the first 1 year of follow-up, antiviral treatments were given comparably frequently to patients with and without early HBeAg seroconversion (48% *vs.* 36%, p=0.091). In addition, HBeAg seroconversion is achieved by at most 12–27% of patients who had received antiviral treatments during the first year [28].

Although liver biopsy is essential for defining the stage of disease progression, it has some limitations, in that it is invasive and accompanies the risk of complications. By multivariate analysis, exclusive of pathological factors, ALT ≥200 IU/l remained as an independent factor (Table 4). ALT ≥200 (IU/l), corresponding to 5 × the upper limit of normal [ULN], coincided with the cut-off point recognized by the receiver operating characteristic curve (data not shown). In previous studies, also, ALT levels ≥5 × ULN were predictive of early HBeAg seroconversion [19,32–33]. Present results are in line with these observations, and point to the capability of ALT ≥200 IU/l to replace lobular inflammation of grades ≥2 in the patients in whom liver biopsy is not feasible.

Conclusions

The results of this study indicate that the combination of low HBeAg titers and high grades of lobular inflammation is clinically useful for predicting early HBeAg seroconversion in patients with chronic hepatitis B. When and if liver biopsy is not to be performed, ALT can substitute for lobular inflammation. The combination of low HBeAg titers, with either high grades of lobular inflammation or elevated ALT levels, predicted not only early, but also long-term HBeAg seroconversion.

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GENETIC POLYMORPHISM-DISEASE ASSOCIATION

HLA-DP gene polymorphisms and hepatitis B infection in the Japanese population

KIYOSHI MIGITA, SEIGO ABIRU, MASASHI OHTANI, YUKA JIUCHI, YUMI MAEDA, SUNG KWAN BAE, SHIGEMUNE BEKKI, SATORU HASHIMOTO, KAKHARMAN YESMEMBETOV, SHINYA NAGAOKA, MINORU NAKAMURA, ATSUMASA KOMORI, TATSUKI ICHIKAWA, KAZUHIKO NAKAO, HIROSHI YATSUHASHI, HIROMI ISHIBASHI, and MICHIO YASUNAMI

OMURA AND NAGASAKI, JAPAN

The mechanisms underlying the different outcomes of hepatitis B virus (HBV) infection are not fully understood.[1] Kamatani et al[2] identified an association of the single nucleotide polymorphisms (SNPs) human leukocyte antigen (*HLA*)-*DPA1* (rs3077) and *HLA-DPB1* (rs9277535) with chronic HBV infection in a genome-wide association study (GWAS). Additional studies confirmed that rs3077 and rs9277535 were associated with chronic HBV infection in the Han-Chinese population and strengthened the findings from previous GWAS.[3-6] Furthermore, Hu et al[7] reported that SNPs in *HLA-DP* (rs3077 and rs9277535) were associated with both HBV clearance and hepatocellular carcinoma (HCC) development. To investigate the association of these *HLA-DP* variants with the disease progression of HBV infection, we genotyped the 2 SNPs (rs3077 and rs9277535) in different clinical stages of liver disease in Japanese HBV carriers.

CLINICAL SUMMARY

A total of 241 HBV carriers (positive for hepatitis B surface antigen) who visited the clinics for liver diseases at the Nagasaki University Hospital or Nagasaki Medical Center between 1999 and 2007 were enrolled. As controls, 143 healthy Japanese volunteers (56 men and 87 women aged 16–63 years, with a mean age of 31.3 ± 8.9 years) without any history of liver disease were enrolled. All patients did not have any other types of liver diseases, such as chronic hepatitis C, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The study protocol was approved by the Ethics Committees of National Nagasaki Medical Center, and informed consent was obtained from each individual. Of the 241 HBV carriers, 69 were considered to be asymptomatic carriers on the basis of sustained normalization of the serum alanine aminotransferase (ALT) levels together with seropositivity for anti-hepatitis Be antigen throughout the study. On the other hand, 172 of the 241 HBV carriers were considered to have chronic liver disease, such as chronic hepatitis (57), cirrhosis (65), or HCC (50) manifested by elevated ALT levels and by clinical or histologic findings on examination of liver tissue during the follow-up period. Of the 50 patients with HCC, 6 (12%) were found to have chronic hepatitis and 44 (88%) had cirrhosis. All patients were regularly followed with measurements of serum ALT and HBV markers, such as hepatitis B surface antigen, hepatitis Be antigen, anti-hepatitis Be antibody, and HBV-DNA. A total of 79 patients had undergone liver biopsy during the study to assess the degree of liver fibrosis. However, liver biopsy was not performed in patients who had apparent biochemical, endoscopic, and ultrasound features of liver cancer. Tumor markers such as alpha-fetoprotein and des-γ-carboxy-prothrombin were measured with ultrasonography of the liver every 6 months to detect HCC in an early stage. The diagnosis of HCC was made by several imaging modalities in all patients and confirmed histologically by sonography-guided fine-needle tumor biopsy specimens. The genotype of rs3077 (*HLA-DPA1*) and rs9277535 (*HLA-DPB1*) was determined by direct sequencing. The apolipoprotein B mRNA-editing enzyme catalytic peptide 3G (*APOBEC3G* H186R) genotyping was performed on the basis of the report by An et al.[8]

The frequencies of the 2 SNPs of *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) are listed in Table I. There was a significant difference in the frequencies between these 2 SNPs between Japanese HBV carriers and healthy subjects, as described previously.[3] We divided HBV carriers into 2 groups: a nonadvanced group (asymptomatic carriers or chronic hepatitis, n = 115) and an advanced group (liver cirrhosis or HCC, n = 126). The frequencies of CC (rs3077) or GG (rs9277535) genotypes were higher in the advanced group compared with those in the nonadvanced group; however, the difference was not significant (Table I). Next, we stratified the HBV carriers for the presence or absence of the *APOBEC3G* H186R variant and examined the effects of *HLA-DP* polymorphisms on the progression of HBV-related liver disease. Both C and G alleles of rs3077 and rs9277535 significantly increased the risk for advanced liver disease in HBV carriers lacking the H186R variant (Table II).

A 2-stage GWAS identified SNPs including rs3077 and rs9277535 located in *HLA-DPA1* and *HLA-DPB1*, which were associated with a susceptibility to chronic HBV infection.[2] After the first Japanese GWAS, 5 studies replicated the association of these 2 *HLA-DP* SNPs (rs3077 and rs9277535) and chronic HBV infection in the Han-Chinese population.[3-7] Among these studies, an association between HBV-related HCC and rs9277535 or rs3077 was demonstrated.[7] In this study, we examined whether these 2 SNPs (rs3077 and rs9277535) in *HLA-DP* genes were associated with the disease progression and susceptibility to HBV infection in a Japanese population. As demonstrated previously, we reconfirmed that rs3077 and rs9277535 in the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with HBV infection. Although some differences in the frequencies of rs3077 and rs9277535 genotypes between HBV carriers with advanced liver disease (liver cirrhosis and HCC) and those without advanced liver disease were observed, these differences were not statistically significant.

Recent evidence suggests that APOBEC3G inhibits HBV production by interfering with HBV replication through hypermutation of the majority of the HBV genome.[8] Because of the *APOBEC3G* gene's ability to regulate HBV replication, mutations of the gene may cause a deleterious variation that may affect the outcome of HBV infection. Among the SNPs identified in the *APOBEC3G* gene, H186R variant was strongly associated with a decline in $CD4^+$ T-cell numbers and accelerated progression to acquired immune deficiency syndrome–defining conditions in human immunodeficiency virus–infected individuals.[9,10] Viral disease outcome is influenced by host variability in immune response genes and genes that control viral replication or mutation rate.[11] *APOBEC3G* coding region variant might influence the progression of HBV infection by inducing the replication of HBV.[12] Therefore, genetic diversity of immune response genes, such as *HLA*, and genes that control viral replication, such as *APOBEC3G*, could contribute to the variability in outcome of HBV infection. To minimize the effects

From the Clinical Research Center, NHO Nagasaki Medical Center, Omura, Japan; Department of Gastroenterology, Nagasaki University Hospital, Nagasaki, Japan; Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

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Reprint requests: Kiyoshi Migita, MD, Clinical Research Center, NHO Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8652, Japan; e-mail: migita@nmc.hosp.go.jp.

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Table I. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and HBV infection

SNP ID	HBV carrier n = 241 (%)	Healthy subjects n = 143 (%)	*P* value*	OR (95% CI)	Advanced HBV carrier n = 115 (%)	Nonadvanced HBV carrier n = 126 (%)	*P* value*	OR (95% CI)
rs3077								
C/C	148 (61.4)	47 (32.9)			77 (67.0)	71 (56.3)		
C/T	79 (32.8)	72 (50.3)			33 (28.7)	46 (36.5)		
T/T	14 (5.8)	24 (16.8)			5 (4.3)	9 (7.1)		
C allele (allele frequencies)	375 (77.8)	166 (58.0)	<0.0001	2.533 (1.843–3.483)	187 (81.3)	188 (74.6)	0.077	1.480 (0.957–2.290)
rs9277535								
G/G	143 (59.3)	45 (31.5)			73 (63.5)	70 (55.6)		
A/G	82 (34.0)	72 (50.3)			36 (31.3)	46 (36.5)		
A/A	16 (6.6)	26 (18.2)			6 (5.2)	10 (7.9)		
G allele (allele frequencies)	368 (76.3)	162 (56.6)	<0.0001	2.471 (1.804–3384)	182 (79.1)	186 (73.8)	0.170	1.345 (0.880–2.056)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were calculated using the chi-square test.

Table II. Association between *HLA-DP* polymorphisms (rs3077, rs9277535) and the outcome of HBV infection in HBV carrier without H186R variant

SNP ID	Advanced HBV carrier n = 90 (%)	Nonadvanced HBV carrier n = 108 (%)	*P* value*	OR (95% CI)
rs3077				
C/C	64 (71.1)	60 (55.6)		
C/T	22 (24.4)	40 (37.0)		
T/T	4 (4.4)	8 (7.4)		
C allele (allele frequencies)	150 (83.3)	160 (74.1)	0.026	1.750 (1.065–2.874)
rs9277535				
G/G	5 (5.6)	10 (9.3)		
A/G	24 (26.7)	39 (36.1)		
A/A	61 (67.8)	59 (54.6)		
G allele (allele frequencies)	146 (81.1)	157 (72.7)	0.049	1.614 (1.000–2.604)

Abbreviations: CI, confidence interval; *HBV,* hepatitis B virus; *OR,* odds ratio; *SNP,* single-nucleotide polymorphism.
**P* values were culculated using the chi-square test.

of viral factors, such as APOBEC3G-mediated HBV editing, and evaluate the effect of *HLA-DP* more precisely, we focused on the subjects without the H186R variant. Because the *APOBEC3G* coding region variant might influence the progression of HBV infection,[11] we investigated the effect of *HLA-DP* polymorphisms on the outcome of HBV infection in HBV carriers lacking the H186R variant.

Our results showed that *HLA-DP* polymorphisms were associated with the progression of HBV infection and that this association was significant in Japanese HBV carriers lacking H186R variants. Our data demonstrated that *HLA-DP* polymorphisms are important in determining the susceptibility and the progression of HBV infection in the Japanese population.

One limitation of our study is the lack of information of HBV genotypes in the patients studied. Another limitation is that the number of HBV carriers (n = 241) is relatively small. Larger studies are needed to confirm the results of our study.

CONCLUSIONS

We confirmed that rs3077 and rs9277535 SNPs in the *HLA-DP* locus are associated with the susceptibility and progression of HBV infection in the Japanese population. Further functional analyses are warranted to validate the biological plausibility of these SNPs in chronic HBV infection.

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Original Article

Long-term outcomes of add-on adefovir dipivoxil therapy to ongoing lamivudine in patients with lamivudine-resistant chronic hepatitis B

Takashi Toyama,[1] Hisashi Ishida,[1] Hiromi Ishibashi,[2] Hiroshi Yatsuhashi,[2] Makoto Nakamuta,[3] Masaaki Shimada,[4] Hajime Ohta,[5] Takeaki Satoh,[6] Michio Kato,[7] Taizo Hijioka,[8] Hirotsugu Takano,[9] Toshiki Komeda,[10] Michiyasu Yagura,[11] Hiroshi Mano,[12] Yukio Watanabe,[13] Masakazu Kobayashi[14] and Eiji Mita[1]

[1]*Department of Gastroenterology and Hepatology, NHO, Osaka National Hospital, Osaka,* [2]*Department of the Clinical Research Center, NHO, Nagasaki Medical Center, Nagasaki,* [3]*Department of Gastroenterology, NHO, Kyushu Medical Center, Kyushu,* [4]*Department of Gastroenterology, NHO, Nagoya Medical Center, Nagoya,* [5]*Department of Gastroenterology, NHO, Kanazawa Medical Center, Kanazawa,* [6]*Center for Liver Disease, NHO, Kokura Medical Center, Kokura,* [7]*Department of Hepatology, NHO, Minamiwakayama Medical Center, Minamiwakayama,* [8]*Department of Gastroenterology, NHO, Osaka Minami Medical Center, Osaka,* [9]*Department of Gastroenterology, NHO, Kure Medical Center, Kure,* [10]*Department of Gastroenterology, NHO, Kyoto Medical Center, Kyoto,* [11]*Department of Gastroenterology, NHO, Tokyo National Hospital, Tokyo,* [12]*Department of Gastroenterology, NHO, Sendai Medical Center, Sendai,* [13]*Department of Gastroenterology, NHO, Sagamihara National Hospital, Sagamihara, and* [14]*Department of Gastroenterology, NHO, Matsumoto Medical Center, Matsumoto, Japan*

Aim: Add-on adefovir dipivoxil (ADV) therapy has been a standard rescue treatment for patients with lamivudine (LAM)-resistant chronic hepatitis B, but the overall benefits of long-term add-on ADV therapy are still limited. The aim of this study was to evaluate the long-term efficiency of add-on ADV treatment and to explore predictive factors associated with it.

Methods: A total of 158 patients with LAM-resistant chronic hepatitis B were included in this retrospective, multicenter, nationwide study in Japan. After confirming LAM resistance, ADV was added to LAM treatment. Three types of events were considered as outcomes: virological response, hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization. Virological response was defined as serum hepatitis B virus (HBV) DNA levels of less than 3 log copies/mL. Baseline factors contributing to these outcomes were examined by univariate and multivariate analyses.

Results: The median total duration of ADV treatment was 41 months (range, 6–84). The rate of virological response was 90.8% at 4 years of treatment; HBeAg clearance and ALT normalization were achieved by 34.0% and 82.7%, respectively, at the end of follow up. Each outcome had different predictive factors: baseline HBV DNA and albumin level were predictive factors for virological response, history of interferon therapy and ALT level for HBeAg clearance, and sex and baseline albumin level for ALT normalization.

Conclusion: Long-term add-on ADV treatment was highly effective in LAM-resistant chronic hepatitis B patients in terms of virological and biochemical responses. Lower HBV replication and lower albumin level at baseline led to better outcomes.

Key words: adefovir dipivoxil, alanine aminotransferase normalization, chronic hepatitis B, hepatitis B e antigen clearance, lamivudine resistance, virological response

INTRODUCTION

CHRONIC HEPATITIS B (CHB) is an important cause of morbidity and mortality worldwide.[1–3] The main goals of therapy in CHB patients are to prevent the development of liver failure, due to subsequent liver

Correspondence: Dr Eiji Mita, Department of Gastroenterology and Hepatology, NHO, Osaka National Hospital, 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan. Email: eijim67@onh.go.jp
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cirrhosis, and the emergence of hepatocellular carcinoma (HCC). All of these are likely be achieved by suppressing hepatitis B virus (HBV) replication, which thereby leads to remission of liver disease.[4]

Lamivudine (LAM) treatment has been used to prevent the progression of CHB and the development of HCC.[5] LAM is an effective and well-tolerated treatment for patients with CHB, but it has the major limitation of drug-resistant mutants arising at a rate of 16–32% during the first year of treatment and increasing by 15% with each additional year of treatment.[6–8] The widespread use of LAM monotherapy in CHB patients before introduction of entecavir, which is more potent, has progressively increased the numbers of patients with LAM-resistant HBV mutant strains.

Adefovir dipivoxil (ADV) has been reported to be effective in suppressing HBV replication and approved as a standard therapy in LAM-resistant patients.[9,10] However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients are still limited. The aims of this study were to evaluate the long-term efficiency of ADV add-on treatment based on virological response (VR), hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization, and to explore the predictive factors associated with ADV add-on treatment.

METHODS

Patients

A TOTAL OF 158 patients (109 males and 49 females) were included in this retrospective study from 21 medical centers of the National Hospital Organization (NHO) in Japan. Both HBeAg positive and negative CHB patients were considered eligible if they had documented LAM resistance confirmed by detection of mutations in the YMDD motif of the reverse transcriptase gene of the virus (genotypic resistance), elevated serum HBV DNA levels (≥4 log copies/mL and/or >1 log copies/mL elevation from the LAM on-treatment nadir) and/or elevated serum ALT levels (>40 IU/L). Patients were excluded if they had decompensated liver cirrhosis, HCC at the initiation of ADV, or if they had co-infections (human immunodeficiency virus, hepatitis C virus) or other concomitant liver diseases such as autoimmune liver disease. Patients with no available clinical, biochemical, serological or virological data at baseline as well as every 6 months during treatment were also excluded.

Patient records were extracted from each institutional database. All data were labeled with their respective institution and pooled. In total, 20 variables were examined to evaluate the long-term responses. The following variables were used as baseline factors: sex, HBeAg status, liver disease, age, body mass index, duration of LAM monotherapy, history of interferon (IFN) therapy, serum HBV DNA level, aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase (γ-GTP), platelet (PLT) counts, and total bilirubin (T-Bil), albumin (Alb), prothrombin time (PT) and α-fetoprotein (AFP) levels. All were measured at the initiation of ADV therapy. For each variable, it was not used in the stepwise analysis if missing data accounted for more than 10% of the cases.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients and approval of this study was obtained from the NHO.

Statistical analysis

Three types of events were considered as outcomes: (i) VR; (ii) HBeAg clearance; and (iii) ALT normalization. VR was defined as serum HBV DNA levels of less than 3 log copies/mL by a quantitative real-time polymerase chain reaction assay, and ALT normalization was defined as a decrease in ALT levels to less than 31 IU/L during the on-treatment follow-up period. Baseline factors that could have an impact in the prediction of VR, HBeAg clearance as well as ALT normalization were investigated. The predictive value of several baseline parameters for VR was evaluated using time-to-event methods, because of the varying length of follow up. Time-to-event analysis was carried out using Kaplan–Meier estimates to draw cumulative incidence curves, compared by log–rank tests, as well as using univariate and multivariate Cox's proportional hazards models in combination with stepwise regression analysis. Factors contributing to HBeAg clearance and ALT normalization during ADV add-on therapy were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A stepwise variable selection procedure was used for variables that were at least marginally associated with the outcomes.

Covariates included in these analyses were binomial or continuous variables. Quartile analysis was initially performed separately for each continuous variable to make the decision regarding cut-off points. At first, we divided each continuous data into quarters to convert numerical values into four categorical values. Then, we estimated whether there was a regular trend among these four ordinal categorical data with outcome and selected a cut-off point among the 25th, 50th and 75th percentiles so that these variables could be appropriately