Table 1 Baseline characteristics at the initiation of add-on ADV therapy in LAM-resistant CHB patients based on HBeAg status

Baseline characteristics	HBeAg positive $n = 99$	HBeAg negative $n = 59$
Age (years)	51.6 (25.5–80.4)	59.3 (33.3–76.9)
Sex (male/female)	73/26	36/23
Liver disease (CH/cirrhosis)	79/20	38/21
Duration of LAM therapy (months)	29.8 (6.0–82.4)	39.3 (8.4–91.2)
History of IFN therapy (months)	39	15
HBV DNA (log copies/mL)	7.5 (2.1–7.6)	5.9 (2.1–7.6)
≤6	15	31
6–7.5	38	21
>7.5	46	7
Total bilirubin (mg/dL)	0.8 (0.3–5.2)	0.9 (0.41–3.7)
AST (IU/L)	60 (18–959)	60 (17–464)
ALT (IU/L)	80 (11–697)	86 (17–724)
γ-GTP (IU/L)	38 (12–325)	53 (10–740)
Albumin (g/dL)	4.3 (2.6–5.4)	4.3 (2.7–5.2)
Platelet count ($\times 10^3/mm^3$)	15.5 (3.7–50.0)	12.3 (1.7–33.2)

Continuous variables are expressed in median (range) and categorized variables in number.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; γ-GTP, γ-glutamyl transpeptidase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LAM, lamivudine.

dichotomized. The hazards ratio (HR) and the odds ratio (OR) are presented with 95% confidence intervals (CI) and *P*-values, with less than 0.05 being considered statistically significant. All data analyses were processed using the R statistical software ver. 2.13.

RESULTS

IN THIS RETROSPECTIVE nationwide analysis of add-on ADV therapy in Japan, a total of 158 patients were enrolled from 2003–2010, consisting of 99 HBeAg positive and 59 HBeAg negative patients. Table 1 summarizes the baseline characteristics of the study population; most were HBV genotype C. At the time of this analysis, the median total duration of ADV treatment was 41 months (range, 6–84), and the median time of LAM monotherapy, prior to initiation of ADV, was 34 months (range, 6–91).

VR

Figure 1 shows a Kaplan–Meier curve displaying the cumulative probability of VR based on HBV DNA levels among HBeAg positive and negative patients. Patients with a lower HBV DNA level displayed earlier VR than those with a higher HBV DNA level among both HBeAg positive and negative patients ($P < 0.001$, $P = 0.002$,

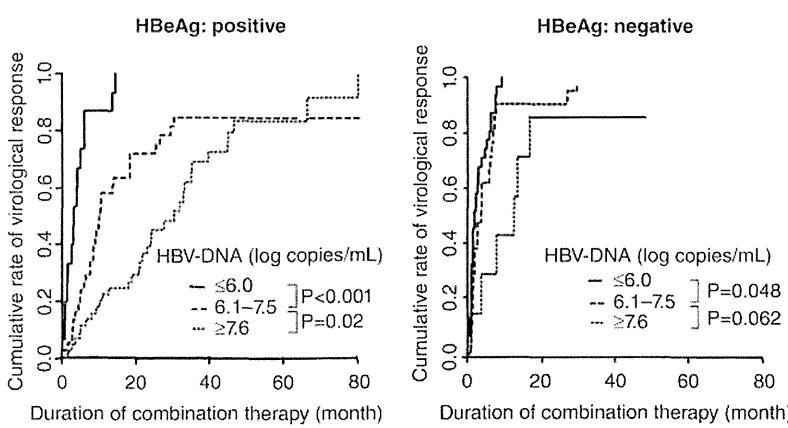

Figure 1 Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response compared with HBeAg positivity or higher HBV replication. —, ≤6.0; ---, 6.1–7.5; ·····, ≥7.6.

Table 2 Univariate and multivariate Cox's regression analysis of predictors of virological response

Variable	HBeAg positive $n = 99$				HBeAg negative $n = 59$	
	Univariate		Multivariate		Univariate	
	HR	*P*-value	HR	*P*-value	HR	*P*-value
Age (years) (<45/45≤)	0.91	0.69			0.66	0.34
Sex (male/female)	1.07	0.86			0.71	0.21
Liver disease (CH/cirrhosis)	0.61	0.069			1	0.99
Duration of LAM therapy (months) (<34/34≤)	0.92	0.76			1.72	0.076
History of IFN therapy (−/+)	0.83	0.43			0.89	0.73
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.28	<0.001	<0.001	<0.001	0.44	0.012
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.66	0.067	1.73	0.06	1.54	0.13
AST (IU/L) (<100/100≤)	1.57	0.061			1.11	0.71
ALT (IU/L) (<130/130≤)	1.51	0.085			1.05	0.87
γ-GTP (IU/L) (<70/70≤)	1.53	0.113			1.33	0.3
Albumin (g/dL) (<4.1/4.1≤)	0.51	0.011	0.48	0.0065	1.41	0.32
Platelet count ($\times 10^4/mm^3$) (<15/15≤)	0.93	0.77			1.1	0.74

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

respectively; log-rank test). HBeAg negative patients displayed higher VR rates than HBeAg positive patients at month 12 (89.9% vs 45.5%), month 24 (95.0% vs 61.5%), month 36 (98.4% vs 79.6%) and month 48 (98.4% vs 86.4%) of treatment. Even at a higher HBV DNA level (HBV DNA ≥7.0 log copies/mL), HBeAg negative patients displayed more rapid VR than HBeAg positive patients ($P < 0.001$). Seven patients did not achieve VR during the 4-year treatment, and one HBeAg positive patient developed ADV-resistant mutations without VR at month 44 of treatment. According to the results of the univariate Cox regression model, HBV DNA level and Alb level were associated with VR in HBeAg positive patients, while only the HBV DNA level was in HBeAg negative patients (HR = 0.44, 95% CI = 0.24–0.84, $P = 0.012$). In multivariate analysis, both lower HBV DNA level and lower Alb level were independent predictive factors associated with VR in HBeAg positive patients (HR = 0.26, 0.48, 95% CI = 0.15–0.44, 0.28–0.81, $P < 0.001$, $P = 0.0065$, respectively) (Table 2), while only the HBV DNA level was selected by a stepwise analysis for HBeAg negative patients.

HBeAg clearance or HBeAg seroconversion

Among 99 HBeAg positive patients, HBeAg clearance and seroconversion were achieved by 17.1% and 11.0% at month 24, by 24.3% and 14.3% at month 36 of treatment, and by 34.0% and 16.0% by the end of follow up, respectively. Except for a history of IFN therapy (OR = 2.46, 95% CI = 0.94–6.6, $P = 0.047$), none of the other baseline variables were significantly associated with HBeAg clearance, according to the results of the univariate logistic regression analysis. In multivariate analysis, serum ALT level and history of IFN therapy were independent predictive factors for HBeAg clearance (Table 3). No patient experienced a reappearance of HBeAg or reverse seroconversion to HBeAg positive status during this treatment.

Normalization of ALT levels

The mean ALT level declined from 138.2 to 24.7 IU/L by add-on ADV therapy. Furthermore, addition of ADV to LAM-resistant CHB led to normalization of ALT levels in 75.2%, 79.5% and 82.7% of the patients at months 24 and 36, and at the final follow up, respectively. We next estimated the predictive factors for ALT normalization. Univariate logistic regression analysis revealed that only the baseline Alb level was significantly related to the ALT normalization. In the multivariate model, female patients (OR = 0.19, $P = 0.037$) and lower Alb level (OR = 0.19, $P = 0.0017$) were found to be independent predictors of ALT normalization.

DISCUSSION

ADD-ON ADV therapy has been a standard rescue treatment for patients with LAM-resistant HBV, but the overall benefits of long-term add-on ADV therapy

Table 3 Univariate and multivariate logistic regression analysis of predictors of HBeAg clearance and ALT normalization

Variable	HBeAg loss, $n = 99$				ALT normalization			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value
Age (years) (<45/45≤)	0.42	0.065			0.94	0.85		
Sex (male/female)	3.02	0.075	2.99	0.081	0.4	0.34	0.19	0.037
Liver disease (CH/cirrhosis)	0.76	0.59			0.54	0.73		
Duration of LAM therapy (months) (<34/34≤)	1.1	0.97			0.59	0.39		
History of IFN therapy (−/+)	2.46	0.047	2.67	0.041	1.2	0.78		
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.49	0.15			0.32	0.21		
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.03	0.83			1.83	0.72		
AST (IU/L) (<100/100≤)	1.52	0.47			3.99	0.075		
ALT (IU/L) (<130/130≤)	2.44	0.061	2.74	0.043	3.71	0.13		
γ-GTP (IU/L) (<70/70≤)	2.16	0.17			1.29	0.98		
Albumin (g/dL) (<4.4/4.4≤)	0.9	0.99			0.17	0.0047	0.19	0.0017
Platelet count ($\times 10^4/mm^3$) (<15/15≤)	1.21	0.82			0.52	0.39		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

have not yet been fully assessed. In this multicenter study of 158 patients from 21 hospitals over a mean follow-up period of 43.5 months, we tried to evaluate the long-term efficacy of add-on ADV therapy to LAM-resistant patients, and also to investigate which baseline factors were associated with VR, HBeAg clearance and ALT normalization. We found long-term add-on ADV treatment produced long-term virological and biochemical improvement. In addition, each outcome had different predictive factors; baseline HBV DNA and Alb level were predictive factors for VR in HBeAg positive patients, history of IFN therapy and ALT level for HBeAg clearance, and sex and Alb level for ALT normalization.

The rate of VR was 90.8% at 4 years of treatment. The strongest predictive factor for VR in both HBeAg positive and negative patients were confirmed by previous observations showing that add-on ADV therapy achieves more rapid and higher rates of VR when ADV is initiated in LAM-resistant patients with low viral replication levels.[11–17] We also found that lower Alb level was an independent predictive factor for VR in HBeAg positive patients. In fact, baseline Alb correlated with PLT counts ($r = 0.51$, $P < 0.001$) and T-Bil ($r = -0.38$, $P < 0.001$), indicating that a lower Alb level reflected progression of liver disease. Little attention has been given to the relation of Alb level with VR – further studies will be needed to confirm our findings and understand its underlying mechanisms – but progression of chronic hepatitis might be predictive of VR under the add-on ADV treatment. This is the first report to show the significance of baseline Alb levels as we used a time-to-event method for large populations, which is a more powerful and informative method to assess the association of factors to time-to-event outcomes.

The rate of HBeAg clearance was 34% at the end of follow up, which was compatible with previous observations.[10,18] According to the results of multivariate analysis, IFN history was the strongest predictor of HBeAg clearance. Of the 37 patients, 17 (46%) who had previously received IFN therapy achieved HBeAg loss, suggesting that previous IFN therapy might have some immune modulatory effect on the ongoing combination therapy. IFN-induced HBeAg loss has been reported to be durable after a follow-up period of 4–8 years.[19–21] In addition, baseline ALT levels were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline ALT levels to be the strongest predictor of HBeAg seroconversion in response to IFN therapy[22] as well as nucleos(t)ide analog therapy.[23,24]

Alanine aminotransferase normalization was achieved in 82.7% of the patients. ALT normalization and VR were independent of each other. Actually, among 24 patients who did not achieve ALT normalization, only seven had not achieved VR, suggesting that ALT elevation after sustained suppression of HBV replication might be associated with some conditions other than CHB. In addition, lower baseline Alb was revealed

to be an independent and positive predictive factor for ALT normalization. Considering that patients who did achieve ALT normalization had lower Alb levels than patients with elevated ALT at the final follow up (4.4 vs 4.6 g/dL, $P < 0.01$), and Alb levels are significantly higher in non-alcoholic fatty liver disease,[25] we speculate that fatty liver disease is related to the abnormal ALT. To clarify this, further studies by liver biopsy and/or ultrasonography will be needed.

In conclusion, long-term ADV treatment was highly effective in LAM-resistant CHB patients in terms of virological and biochemical response. In addition, the emergence of resistance to the add-on ADV therapy appears to be delayed and infrequent, in contrast to LAM. Furthermore, lower HBV DNA level and lower Alb level were significant predictive factors for better outcomes. Even though add-on ADV therapy in LAM-resistant CHB patients was highly effective in the long term, CHB patients with LAM or entecavir monotherapy need to be carefully followed-up and the optimal timing of ADV intervention should be determined on the basis of HBV DNA level and progression of liver disease.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Appendix S1 Relationship of liver cirrhosis with virological response on the basis of fibrosis, using 60 out of 158 patients liver biopsy had been performed. Fibrosis was related with platelet counts but neither with albumin levels nor with the virological response.

APPENDIX I

THE LIVER DISEASE Network Group of the National Hospital Organization consists of the following physicians and their institutions: Hiromi Ishibashi, Hiroshi Yatsuhashi, Department of Clinical Research Center, Nagasaki Medical Center; Makoto Nakamuta, Department of Gastroenterology, Kyushu Medical Center; Michiyasu Yagura, Department of Gastroenterology, Tokyo National Hospital; Hirotsugu Takano, Department of Gastroenterology, Kure Medical Center; Takeaki Satoh, Center for Liver Disease, Kokura Medical Center; Toyokichi Muro, Department of Gastroenterology, Oita Medical Center; Hideo Nishimura, Department of Gastroenterology, Asahikawa Medical Center; Hiroshi Mano, Department of Gastroenterology, Sendai Medical Center; Fujio Makita, Department of Gastroenterology, Nishigunma National Hospital; Akira Saitou, Department of Gastroenterology, Nishisaitama-chuo National Hospital; Masahiko Takahashi, Department of Gastroenterology, Tokyo Medical Center; Shigeo Hayashi, Department of Gastroenterology, National Disaster Medical Center; Tatsuji Komatsu, Department of Gastroenterology, Yokohama Medical Center; Yukio Watanabe, Department of Gastroenterology, Sagamihara National Hospital; Masakazu Kobayashi, Department of Gastroenterology, Matsumoto Medical Center; Hideo Morimoto, Hajime Ohta, Department of Gastroenterology, Kanazawa Medical Center; Masaaki Shimada, Department of Gastroenterology, Nagoya Medical Center. Toshiki Komeda, Department of Gastroenterology, Kyoto Medical Center; Taizo Hijioka, Department of Gastroenterology, Osaka Minami Medical Center; Haruhiro Yamashita, Department of Gastroenterology, Okayama Medical Center; Eiichi Takesaki, Department of Gastroenterology, Higashi Hirosima Medical Center; Toru Hayashi, Department of Gastroenterology, Zentsuji National Hospitail; Mitsuaki Koga, Department of Gastroenterology, Ureshino Medical Center; Kazuhiro Sugi, Departoment of Gastroenterology, Kumamoto Medical Center; Hironori Sakai, Department of Gastroenterology, Beppu Medical Center; Tetsuo Yamamoto, Department of Gastroenterology, Yonago Medical Center; Yukio Oohara, Department of Gastroenterology, Hokkaidou Medical Center; Michio Kato, Department of Hepatology, Minamiwakayama Medical Center; Naohiko Masaki, Department of Gastroenterology, Kohnodai Hospital.

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ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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ABSTRACT

Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.

Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.

Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991–1996, 29.3% during 1997–2002, and 50.0% during 2003–2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.

Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?

- In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
- However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
- Infection with genotype A more often persists than infection with other genotypes.
- Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?

- This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
- The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
- Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5–6 years later.
- About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
- Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A–J, based on a >8% divergence in the full-genome sequence.[1–7] Different genotypes are associated with distinct clinical manifestations, such as severity and progression of liver disease, as well as response to antiviral treatments.[8–10] Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)[11–13];

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.
- Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.
- Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)[14 15]; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).[16] Subgenotypes also influence the replication of HBV and clinical manifestation.[15 17 18]

According to a report from Japan in 2001,[19] genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.[20 21] Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.[22 23] These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

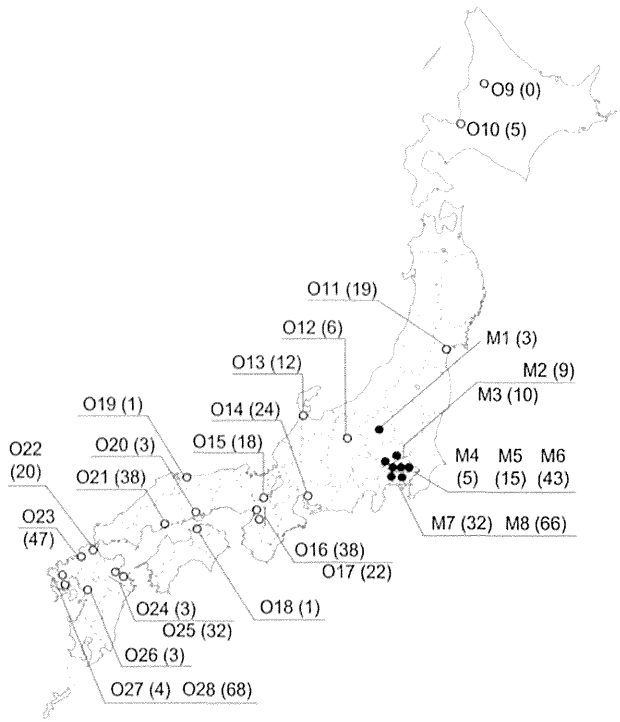

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1–M8) are indicated by eight closed circles, and those in other regions (O9–O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagamihara Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

PATIENTS AND METHODS

Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991–1996), 2nd (1997–2002) and 3rd (2003–2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at −20°C.

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) ≤40% and hepatic encephalopathy of grade ≤I. Fulminant hepatitis (FH) was diagnosed from PT ≤40% and hepatic encephalopathy of grade ≥II. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

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determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μl) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.[24]

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

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

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: http://clustalw.ddbj.nig.ac.jp/top-j.html). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean±SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p<0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,[26 27] the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

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

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, $p<0.05$; or 56.0%, $p<0.0001$), and in those with genotype B than C (79.2% vs 56.0%, $p<0.05$).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6±7.6 vs 7.1±6.2 mg/dl, $p<0.05$), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C ($p<0.05$). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, $p<0.05$; A vs C, $p<0.0001$). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, $p<0.001$; A vs C, $p<0.0001$)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted

Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

Feature	Total (n=547)	HBV genotypes			
		A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6†† *	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd (p<0.05), 2nd and 3rd (p<0.0001), and 1st and 3rd (p<0.0001). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, p<0.0001). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods (p<0.05), 2nd and 3rd (p<0.05), and 1st and 3rd (p<0.0001). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and 33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, p<0.0001; 2nd vs 3rd, p<0.0001).

Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

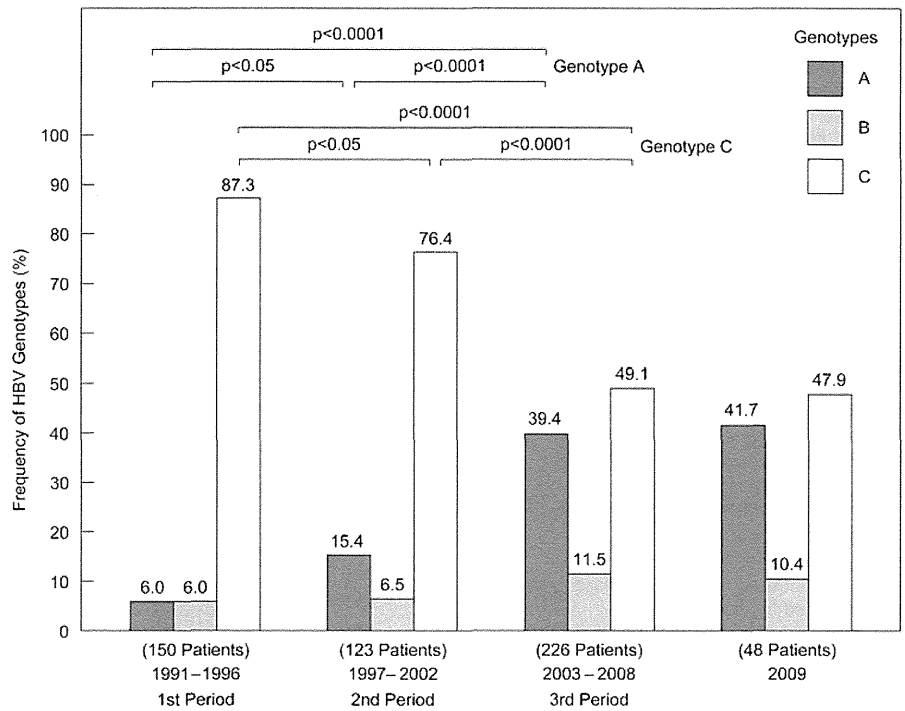

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in 43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-gene sequences from these 43 isolates, along with those from 25 isolates of genotype B retrieved from the database (figure 4). Of the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. In a pair-wise comparison, the sequence divergence among 10 subgenotype B1 isolates ranged from 0.4% to 1.4%, and that among 28, two and three isolates of subgenotypes B2, B3 and B4 spanned 0–1.7%, 0.5% and 0.6–0.8%, respectively. The inter-subgenotype divergence among B1–B4 ranged from 0.6% to 4.4%.

One 'identical group' made up of five isolates was detected among the 28 of subgenotype B2; it was named 'IX'. In contrast, no 'identical group' was found in 10, two or three isolates of subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained from patients who had travelled to foreign countries in the recent past (7/28 (25.0%) patients with B2 to China and other countries; 1/2 (50.0%) patients with B3 to a country unknown; and 1/3 (33.3%) patients with B4 to Vietnam). However, none of the 10 subgenotype B1 isolates was associated with travel to foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical groups was higher for subgenotype A2 (64.4%) than for A1, B1, B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging to the seven identical groups than the non-identical group of subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among the isolates in the seven identical groups of subgenotype A2, those in groups I, III and VII clustered locally during short periods of 2–7 years. In contrast, subgenotype A2 isolates in groups II and VI were scattered widely over longer periods of 11–16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission from mother to baby and horizontal infection during infancy. In 1986, a national prevention programme was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this was extended to babies born to HBeAg-negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[28 29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991–1996)	2nd Period (1997–2002)	3rd Period (2003–2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

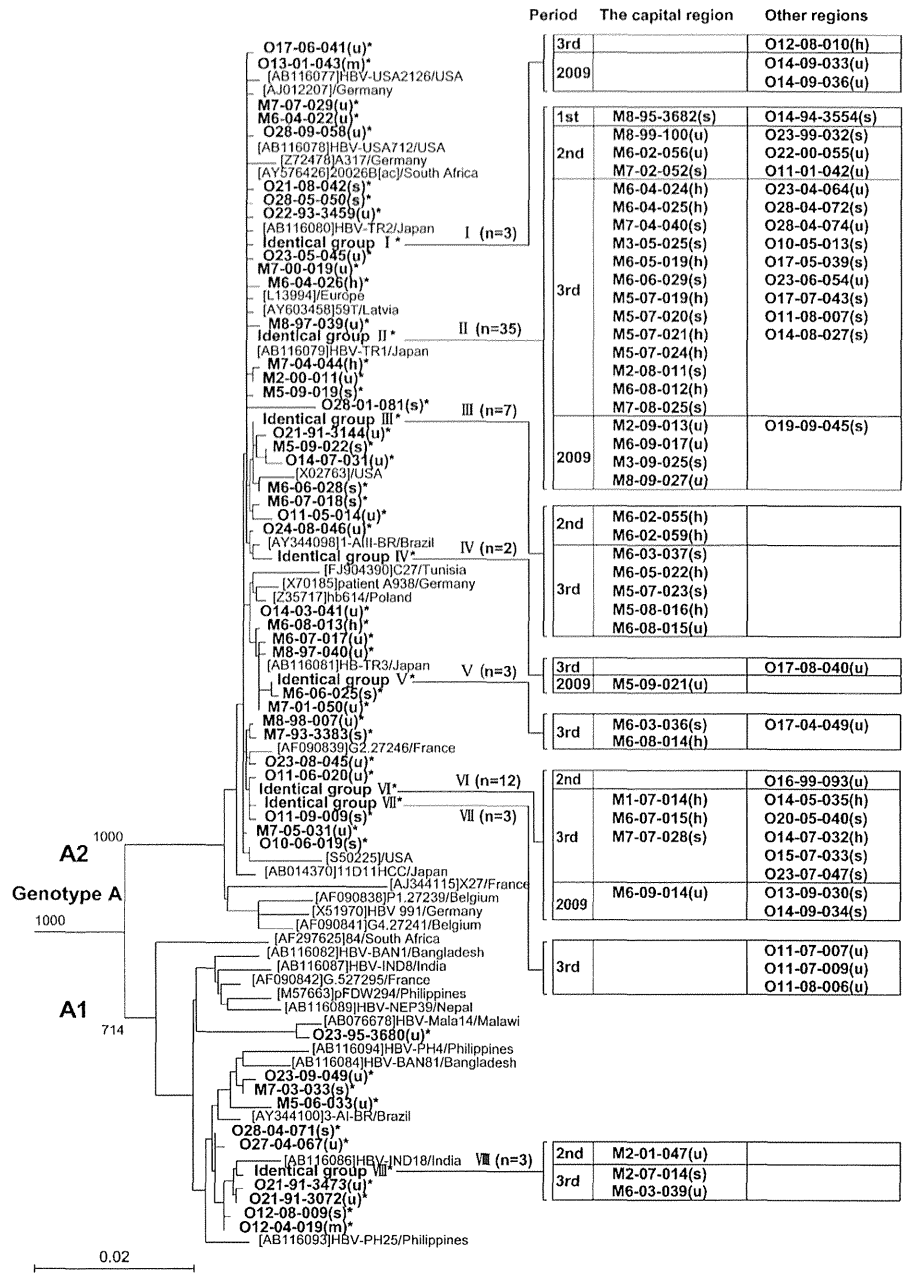

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1–M8 and those in other regions O9–O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991–2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1). The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

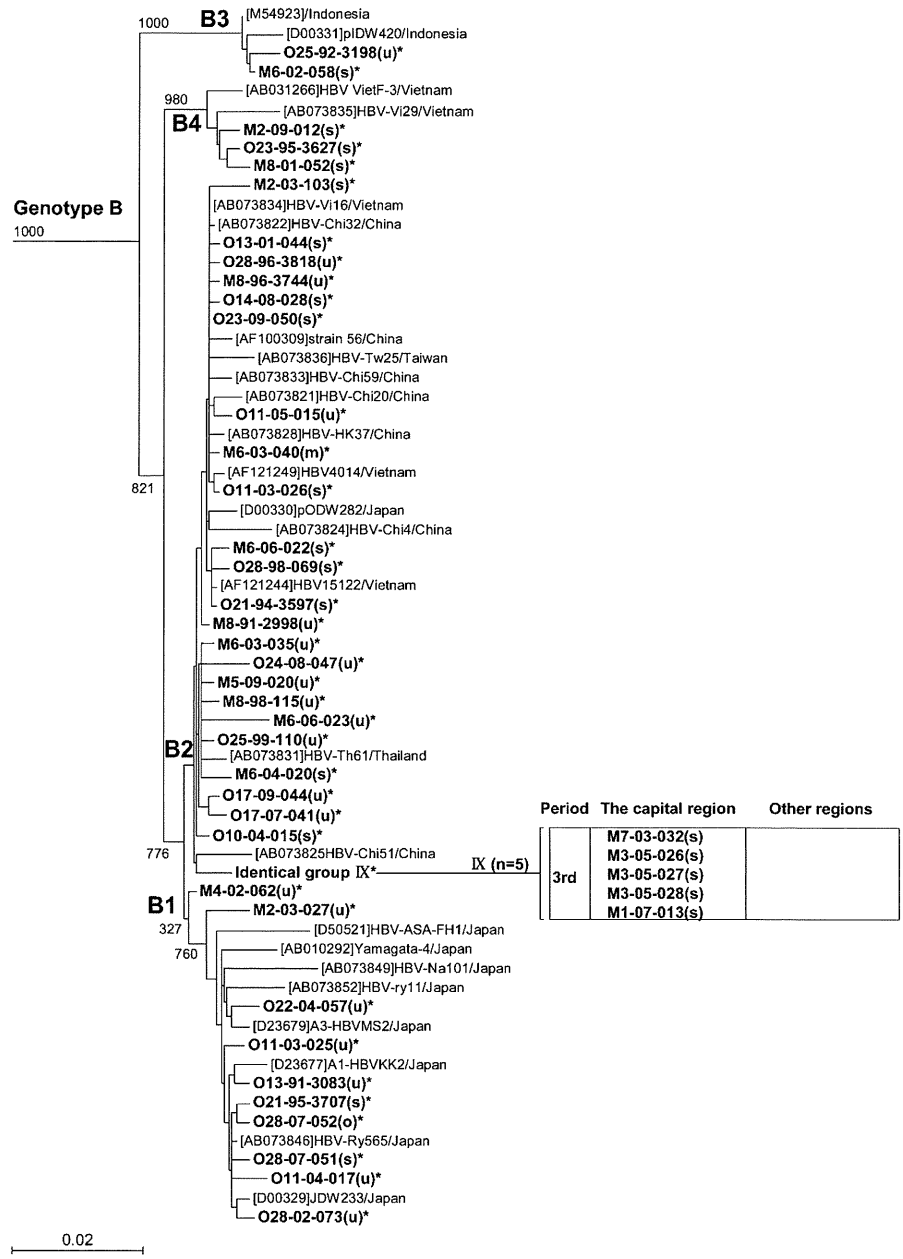

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.

those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20 21 40] as well as around Aichi located in the middle of Mainland Japan.[22]

Yotsuyanagi *et al*[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5–6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European–American type (A2), and the remaining 11.4% possessed the Asian–African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2–B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5–6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

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Ethics approval Approved by the ethics committee of each institution.

Contributors YT, HY and HI designed data collection tools, monitored data collection for the whole study, wrote the statistical analysis plan, cleaned and analysed the data. YT, HY and YM drafted and revised the paper. HY, NM, MN, EM, TK, YW, TM, MS, TH, TS, YM, TK, MT, HK, HO, SH and SA collaborated in data and sample collection.

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Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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Research Article

An Increased Ratio of Glycated Albumin to HbA1c Is Associated with the Degree of Liver Fibrosis in Hepatitis B Virus-Positive Patients

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Background. In hepatitis B virus- (HBV-) positive patients, the relationship between the metabolic variables and histological degree of liver fibrosis has been poorly investigated. *Methods.* A total of 176 HBV-positive patients were assessed in whom the ratios of glycated albumin-to-glycated hemoglobin (GA/HbA1c) were calculated in order to investigate the relationship with the degree of liver fibrosis. *Results.* The GA/HbA1c ratio increased in association with the severity of fibrosis (METAVIR scores: F0-1: 2.61 ± 0.24, F2: 2.65 ± 0.24, F3: 2.74 ± 0.38, and F4: 2.91 ± 0.63). The GA/HbA1c ratios were inversely correlated with four variables of liver function: the prothrombin time (PT) percentage ($P < 0.0001$), platelet count ($P < 0.0001$), albumin value ($P < 0.0001$), and cholinesterase value ($P < 0.0001$). The GA/HbA1c ratio was positively correlated with two well-known markers of liver fibrosis, FIB-4 ($P < 0.0001$) and the AST-to-platelet ratio index (APRI) ($P < 0.0001$). Furthermore, the GA/HbA1c showed better correlations with two variables of liver function (PT percentage and cholinesterase value) than did FIB-4 and with all four variables than did the APRI. *Conclusion.* The GA/HbA1c ratio is associated with the degree of liver fibrosis in HBV-positive patients.

1. Introduction

In patients with chronic liver disease (CLD), liver biopsy is the gold standard method to evaluate the degree of liver fibrosis [1]. However, a liver biopsy is a costly and invasive technique associated with a risk of complications. In addition, there can be sampling errors, because only 1/50,000 of the organ is used for the analysis [1]. Furthermore, it has been reported that there are inter- and intraobserver discrepancies of 10% to 20% for biopsy samples [2, 3]. Therefore, many noninvasive markers of fibrosis available via laboratory tests have been reported, and hepatitis C virus- (HCV-) positive patients have provided a good research base in this context.

It is known that significant differences are observed between HCV-positive patients and hepatitis B virus- (HBV-) positive patients, not only in the etiology, but also in terms of many other clinical parameters, including the natural history of the disease, the laboratory parameters, and the liver histology [4, 5]. However, the number of reports regarding fibrosis markers for HBV-positive patients is much lower than that for HCV-positive patients. In particular, there have been few reports about the relationship between the metabolic parameters and histological degree of liver fibrosis in HBV-positive patients, despite the fact that the liver functions as an important metabolic organ.

The values of glycated proteins reflect the plasma glucose level, and glycated hemoglobin (HbA1c) is commonly used as a reliable index of glycemic control in diabetic patients [6, 7]. The turnover period of hemoglobin in erythrocytes is about four months, and the HbA1c level therefore reflects the plasma glucose levels for the past few months [8]. Glycated albumin (GA) is another marker of the glycemic control during the past few weeks, because the turnover of albumin is about three weeks [9, 10]. In patients with CLD, hypersplenism abbreviates the lifespan of erythrocytes, leading to lower HbA1c values relative to the degree of glycemia. In contrast, the GA levels in CLD patients are higher than those estimated based on the levels of glycemia, because the turnover of serum albumin in CLD patients is increased as a result of the compensation for the decreased albumin production in the liver [11]. Since the HbA1c shows lower values and the GA shows higher values in CLD patients, the GA/HbA1c ratio is predicted to be high in patients with CLD. Indeed, the GA/HbA1c ratio has been reported to be associated with the histological stage of liver fibrosis and portal hypertension in HCV-positive CLD and nonalcoholic steatohepatitis [12–15]. In the present study, we investigated the GA/HbA1c ratio in HBV-positive patients and its correlation with liver fibrosis.

2. Materials and Methods

2.1. Patients. We studied a total of 173 HBV-positive patients who had undergone percutaneous liver biopsies between January 2008 and March 2010 at our institution. This study was retrospective and consecutively included all patients who fulfilled the following conditions: (1) HBV infection diagnosed by positive HBsAg status for at least six months. (2) Blood samples, including samples for an analysis of the GA and HbA1c levels, were obtained on the same day as the liver biopsies. Patients with the following conditions were excluded from the study: the presence of other liver diseases, hepatocellular carcinoma, immunosuppressive therapy, HCV coinfection, and insufficient liver tissue for the staging of fibrosis (a minimum of 15 mm of liver tissue with five or more portal tracts was required for diagnosis). The present study did not include patients whose GA/HbA1c ratios could have been influenced by poorly controlled diabetes.

The characteristics of the study population are summarized in Table 1. The study conformed to the ethical guidelines of the Declaration of Helsinki, and written informed consent regarding the liver biopsy and use of clinical data was obtained from all patients on admission. This study was approved by the ethics committee of the institutional review board.

2.2. Laboratory Data and Liver Biopsy. The HbA1c was measured by high-performance liquid chromatography, with calibration using Japan Diabetes Society (JDS) Lot 2 [15, 16]. The value for HbA1c (%) was estimated as a NGSP equivalent value (%) calculated using the following formula: in the range of JDS values ≤4.9%: NGSP (%) = JDS (%) + 0.3% and in the range of JDS 5.0–9.9%: NGSP (%) = JDS (%) + 0.4% [17]. Routine laboratory studies, including platelet counts, the prothrombin time (PT) percentage, and liver function tests (ALT, AST, alkaline phosphatase, albumin, and cholinesterase), were also performed.

Table 1: The characteristics of the 173 hepatitis B virus- (HBV-) positive patients.

Age (years)	46 (25–79)
Gender (male/female)	96/77
AST (IU/L)	27 (11–269)
ALT (IU/L)	28 (7–680)
γ-GTP (IU/L)	25 (7–349)
ALP (IU/L)	203 (71–835)
Total bilirubin (mg/dL)	0.8 (0.1–2.3)
Albumin (g/dL)	3.90 ± 0.40
Hemoglobin (g/dL)	13.5 ± 3.8
Platelets ($\times 10^3/\mu$L)	178 ± 72
PT (%)	89.8 ± 12.3
Diabetes mellitus (present/absent)	6/167
Glucose (mg/dL)	91.3 ± 13.9
Triglyceride (mg/dL)	99.0 ± 45.5
Total cholesterol (mg/dL)	177 ± 32
Body mass index	22.9 ± 4.1
HBV-DNA (log copies/mL)	3.7 (n.d–over 9.1)*
HBe antigen (positive/negative)	59/114
Treatment with NAs (present/absent)	67/106
Histological stage of liver fibrosis (F0-1/F2/F3/F4)	94/38/28/13

n.d: not detectable; NAs: nucleoside/nucleotide analogues.
*HBV-DNA ranged from undetectable level in patients under treatment of NAs to over measurable level (9.1 log copies/mL) in patients without treatment.

In the present study, the values of two biomarkers associated with the progression of liver fibrosis (FIB-4 and the APRI, the AST-to-platelet count ratio index) were calculated, because these markers were previously shown to be associated with the progression of liver fibrosis [18–20]. The FIB-4 and APRI values were calculated based on formulas developed by Vallet-Pichard et al. [21] and Wai et al. [22], respectively: FIB-4 = Age [years] × AST [U/L]/(platelets [10^9/L] × (ALT [U/L])$^{1/2}$), in which the age of the patient is the age at the time of liver biopsy and APRI = 100 × (AST level/upper limit of normal)/platelets [10^9/L].

Liver biopsy examinations were carried out according to the standard techniques. All liver samples were evaluated by well-trained pathologists at our institute, with an evaluation of the fibrosis stage and activity grade. Fibrosis was staged on a scale of F0–F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; F4, liver cirrhosis) according to the METAVIR scoring system [23]. The histological findings of the biopsy tissues were also routinely evaluated in our department. All authors participated in the conferences about the histological findings, and the final results were confirmed by two authors (H. Enomoto and H. Imanishi) who received training for histological studies.

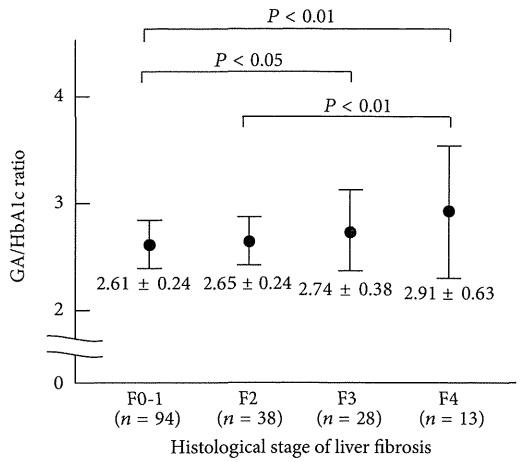

FIGURE 1: GA/HbA1c ratios in relation to the METAVIR fibrosis scores among the HBV-positive patients. The GA/HbA1c ratio increased in association with the stage of liver fibrosis. There were significant differences between the F0-F1 versus F3, F0-F1 versus F4, and F2 versus F4 groups.

2.3. Statistical Analysis. In the present study, we investigated whether the GA/HbA1c ratio is associated with the degree of liver fibrosis in HBV-positive patients. The data for the comparisons among the groups "F0-1 versus F2 versus F3 versus F4" was analyzed by a nonrepeated measurements ANOVA, and statistical significance was consequently evaluated with the Bonferroni correction. The relationships between the GA/HbA1c ratio and other variables, including the FIB-4 and APRI, were evaluated with Spearman's correlation coefficient. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. The GA/HbA1c Ratio Increases with the Histological Stage of HBV-Positive Patients. A total of 173 HBV-positive patients were included in this study. The characteristics of the study population are summarized in Table 1. The population consisted of 96 (55.5%) male patients and 77 (44.5%) female patients, and the age of patients ranged from 25 to 79 years old (median 46 years old). As shown in Figure 1, the mean value of the GA/HbA1c ratio increased in association with the histological stage of liver fibrosis in the HBV-positive patients.

3.2. The GA/HbA1c Ratio Is Associated with the Laboratory Parameters in HBV-Positive Patients. We next investigated whether the GA/HbA1c ratio was related to the laboratory parameters of the liver function, including the PT (%), platelet count, albumin level, and cholinesterase level. Table 2 shows that there is a significant reciprocal correlation of the GA/HbA1c ratio with the PT (%) ($R = -0.396$, $P < 0.0001$) and platelet count ($R = -0.421$, $P < 0.0001$) in HBV-positive patients. The GA/HbA1c ratio was also inversely correlated with the serum albumin level ($R = -0.332$, $P < 0.0001$) and the cholinesterase level ($R = -0.411$, $P < 0.0001$).

TABLE 2: The correlations of the three biomarkers with liver function parameters.

	Correlation coefficient		
	FIB-4	APRI	GA/HbA1c
Prothrombin time (%)	−0.362	−0.284	−0.396
Platelet count	−0.532	−0.372	−0.421
Albumin value	−0.372	−0.301	−0.332
Cholinesterase value	−0.344	−0.315	−0.411

GA/HbA1c: glycated albumin- (GA-) to-glycated hemoglobin (HbA1c) ratio.
APRI: AST-to-platelet ratio index.

These findings showed that the GA/HbA1c ratio increased in association with changes in the levels of markers related to liver fibrosis.

3.3. The GA/HbA1c Ratio and Fibrosis-Related Markers in HBV-Positive Patients. Since we found that the GA/HbA1c ratio was associated with the stage of liver fibrosis in the HBV-positive patients, we therefore investigated the relationships of the GA/HbA1c ratio with two previously established fibrosis-related markers, FIB-4 and APRI. As shown in Figure 2, the GA/HbA1c ratio was significantly correlated with the FIB-4 ($R = 0.598$, $P < 0.0001$) and APRI ($R = 0.505$, $P < 0.0001$). We examined the correlations of these biomarkers with four parameters of liver function (PT percentage, albumin value, platelet count, and cholinesterase value) and found that the GA/HbA1c ratio showed better correlations than the FIB-4 value for two parameters (PT percentage and cholinesterase value). In addition, the correlations of the GA/HbA1c ratio with the findings of liver function tests were higher than those of the APRI for all four parameters (Table 2).

4. Discussion

Liver biopsy is the gold standard method for histologically assessing liver fibrosis. However, a liver biopsy is an invasive procedure carrying a small risk of severe complications. In addition to the FIB-4 and APRI, noninvasive biomarkers, such as the FibroTest score [24], Forns score [25], Hepascore [26], FibroMeter [27], FibroIndex [28], and Lok index [29], were previously reported to be associated with the liver fibrosis. In the present study, we showed that the GA/HbA1c ratio is associated with the histological stage of liver fibrosis in HBV-positive patients (Figure 1). We also showed that the GA/HbA1c ratio was significantly related to the laboratory variables of liver function (Table 2). Among the previously reported biomarkers for liver fibrosis, the FIB-4 and APRI are simple and useful markers that can be measured using routinely available clinical parameters without any specialized equipment. We found that the GA/HbA1c ratio significantly correlated with these well-established markers in HBV-positive patients (Figure 2). These findings suggest that there is a strong relationship between the GA/HbA1c ratio and the levels of fibrosis-related markers in HBV-positive patients.

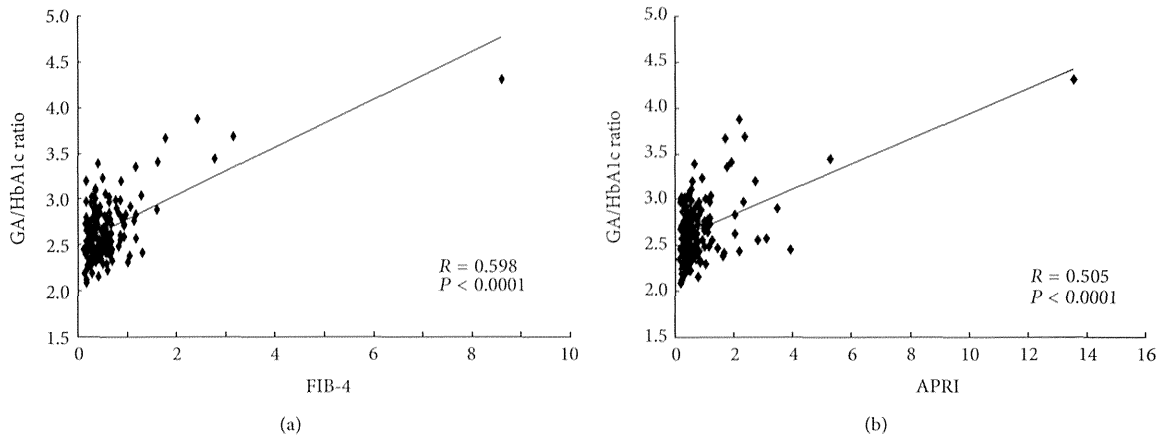

FIGURE 2: The correlation of the GA/HbA1c ratio with other fibrosis-related biomarkers. The GA/HbA1c ratio was correlated with the FIB-4 and APRI. APRI: AST-to-platelet ratio index.

When we examined the four variables of liver function, the correlation coefficients of the GA/HbA1c ratio were higher than those of the FIB-4 for two of the variables. In addition, the GA/HbA1c ratio was better correlated with all four variables examined than was the APRI (Table 2). It has been reported that the etiology of CLD influences the performance of liver fibrosis biomarkers. Unlike that observed in HCV-positive patients, noninvasive biomarkers are sometimes reported to not provide a correct evaluation of the degree of liver fibrosis in HBV-positive patients [30–32]. One major reason could be that the previously established biomarkers are obtained using calculations which include the AST and/or ALT. HBV infections sometimes show an acute liver inflammatory phase, and the AST and ALT values can therefore change from a mildly elevated level to an extremely high level in the same patient depending on the time when the patient is evaluated. In the present study, we included all HBV-positive patients without setting an upper limit for the AST and ALT levels, and some patients with remarkably elevated AST and ALT levels showed very high FIB-4 and APRI indices. The APRI is calculated using only the AST value and platelet count, while the FIB-4 calculation includes both AST and ALT values. Therefore, the acute elevation of AST and ALT in HBV-positive patients should more severely affect the value of the APRI than the FIB-4, although the ALT value was used as the $(ALT)^{1/2}$ for the FIB-4 calculation. The advantage of using the GA/HbA1c ration may therefore depend on the instability of AST and ALT values in HBV-positive patients, because the GA/HbA1c ration is calculated using only the values of two glycated proteins and is independent of the AST and ALT values.

Since the liver plays a central role in metabolism, the progression of liver disease should lead to changes in metabolic parameters. However, most of the established biomarkers for liver fibrosis depend on only nonmetabolic parameters, such as the values of AST, ALT, and the platelet count. Recently, some groups, including our group, reported that the GA/HbA1c ratio was associated with the degree of liver fibrosis in various types of CLD, such as HCV-related CLD and nonalcoholic steatohepatitis [12–15]. Furthermore, we have reported that the amino acid imbalance was associated with the degree of liver fibrosis and the severity of esophageal varices in HCV-positive patients, thus suggesting that metabolism-related parameters could be potential biomarkers for the severity of CLD [33].

We herein demonstrated that the GA/HbA1c ratio increased in association with the stage of liver fibrosis in HBV-positive patients; however, the differences among the fibrosis stages were relatively small (Figure 1). Therefore, the GA/HbA1c ratio alone is not an ideal biomarker to evaluate liver fibrosis, although its correlations with the liver functional tests were as good as the previously reported well-established markers, the FIB-4 and APRI (Table 2). In addition, the present study was a simple descriptive study and did not have a prospective or longitudinal design. Therefore, we cannot draw any conclusions regarding the relationships with the progression of liver fibrosis or clinical outcomes. Recently, there was a report that it was possible to predict portal hypertension using three metabolic parameters [34]. A new biomarker based on a combination of metabolic parameters that includes the GA/HbA1c ratio would be useful for evaluating liver fibrosis in HBV-positive patients.

5. Conclusion

In conclusion, we herein demonstrated that the GA/HbA1c ratio increases in association with the stage of liver fibrosis and is correlated with the levels of markers related to liver fibrosis in HBV-positive patients.

Abbreviations

GA: Glycated albumin
CLD: Chronic liver disease
HCV: Hepatitis C virus
HBV: Hepatitis B virus
PT: Prothrombin time.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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