

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 <i>n</i> = 99 | HBeAg negative <i>n</i> = 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 (×10 <sup>4</sup> /mm <sup>3</sup> ) | 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 popula-

tion; 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,

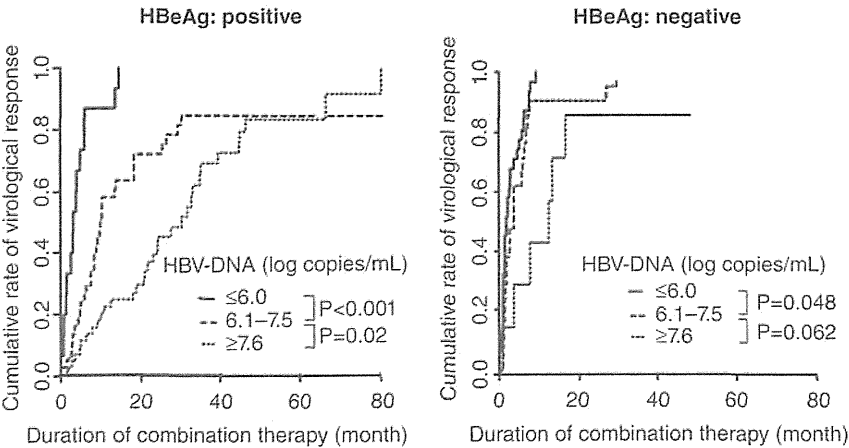


Figure 1 Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response 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<br><i>n</i> = 99 |                 |              |                 | HBeAg negative<br><i>n</i> = 59 |                 |
|---|---------------------------------|-----------------|--------------|-----------------|---------------------------------|-----------------|
|   | Univariate                      |                 | Multivariate |                 | Univariate                      |                 |
|   | HR                              | <i>P</i> -value | HR           | <i>P</i> -value | HR                              | <i>P</i> -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 (×10 <sup>4</sup> /mm <sup>3</sup> ) (<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, <i>n</i> = 99 |                 |              |                 | ALT normalization |                 |              |                 |
|---|---------------------------|-----------------|--------------|-----------------|-------------------|-----------------|--------------|-----------------|
|   | Univariate                |                 | Multivariate |                 | Univariate        |                 | Multivariate |                 |
|   | Odds ratio                | <i>P</i> -value | Odds ratio   | <i>P</i> -value | Odds ratio        | <i>P</i> -value | Odds ratio   | <i>P</i> -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 (×10 <sup>4</sup> /mm <sup>3</sup> ) (<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.<sup>11–17</sup> 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 treat-

ment. 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.<sup>10,18</sup> 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.<sup>19–21</sup> 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<sup>22</sup> as well as nucleos(t)ide analog therapy.<sup>23,24</sup> 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,<sup>25</sup> 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.

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## SUPPORTING INFORMATION

**A**DDITIONAL 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

**T**HE 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 Nakamura, Department of Gastroenterology, Kyushu Medical Center; Michiyasu Yagura, Department of Gastroenterology, Tokyo National Hospital; Hirotugu Takano, Department of Gastroenterology, Kure Medical Center; Takeaki Satoh, Center for Liver

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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.<sup>1–7</sup> Different genotypes are associated with distinct clinical manifestations, such as severity and progression of

liver disease, as well as response to antiviral treatments.<sup>8–10</sup> Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)<sup>11–13</sup>;

## Viral hepatitis

### 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)<sup>14 15</sup>; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).<sup>16</sup> Subgenotypes also influence the replication of HBV and clinical manifestation.<sup>15 17 18</sup>

According to a report from Japan in 2001,<sup>19</sup> 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.<sup>20 21</sup> 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.<sup>22 23</sup> 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.

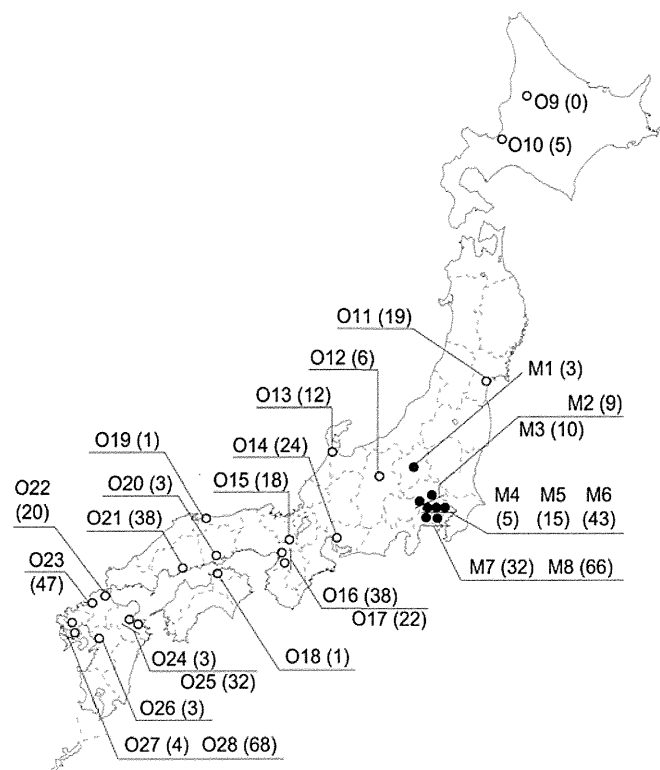
We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions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

## 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  $-20^{\circ}\text{C}$ .

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



**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, Sagami 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.

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)  $\leq 40\%$  and hepatic encephalopathy of grade  $\leq \text{I}$ . Fulminant hepatitis (FH) was diagnosed from PT  $\leq 40\%$  and hepatic encephalopathy of grade  $\geq \text{II}$ . 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



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.<sup>24</sup>

### 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.<sup>25</sup>

### 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  $\chi^2$  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 HBV 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,<sup>26 27</sup> 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 ( $9.6\pm7.6$  vs  $7.1\pm6.2$  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



Viral hepatitis

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 | 5/14 (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

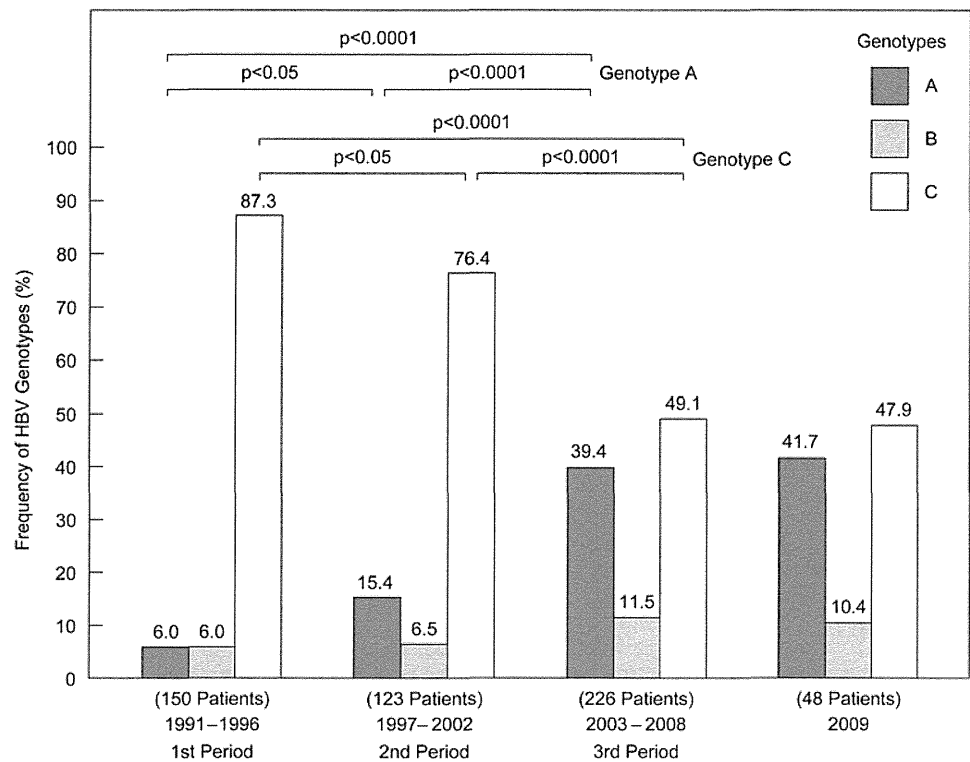
Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype 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.<sup>28 29</sup> 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<br>(1991–1996) | 2nd Period<br>(1997–2002) | 3rd Period<br>(2003–2008) | 2009          |
|----------------|--------------------|---------------------------|---------------------------|---------------------------|---------------|
| Capital region | 65/183 (35.5%)†*** | 2/42 (4.8%)‡* S***        | 12/41 (29.3%)†* S*        | 42/84 (50.0%)†*           | 9/16 (56.3%)  |
| Other regions  | 72/364 (19.8%)     | 7/108 (6.5%)S***          | 7/82 (8.5%)S***           | 47/142 (33.1%)            | 11/32 (34.4%) |
| Total          | 137/547 (25.0%)    | 9/150 (6.0%)‡* S***       | 19/123 (15.4%)S***        | 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.

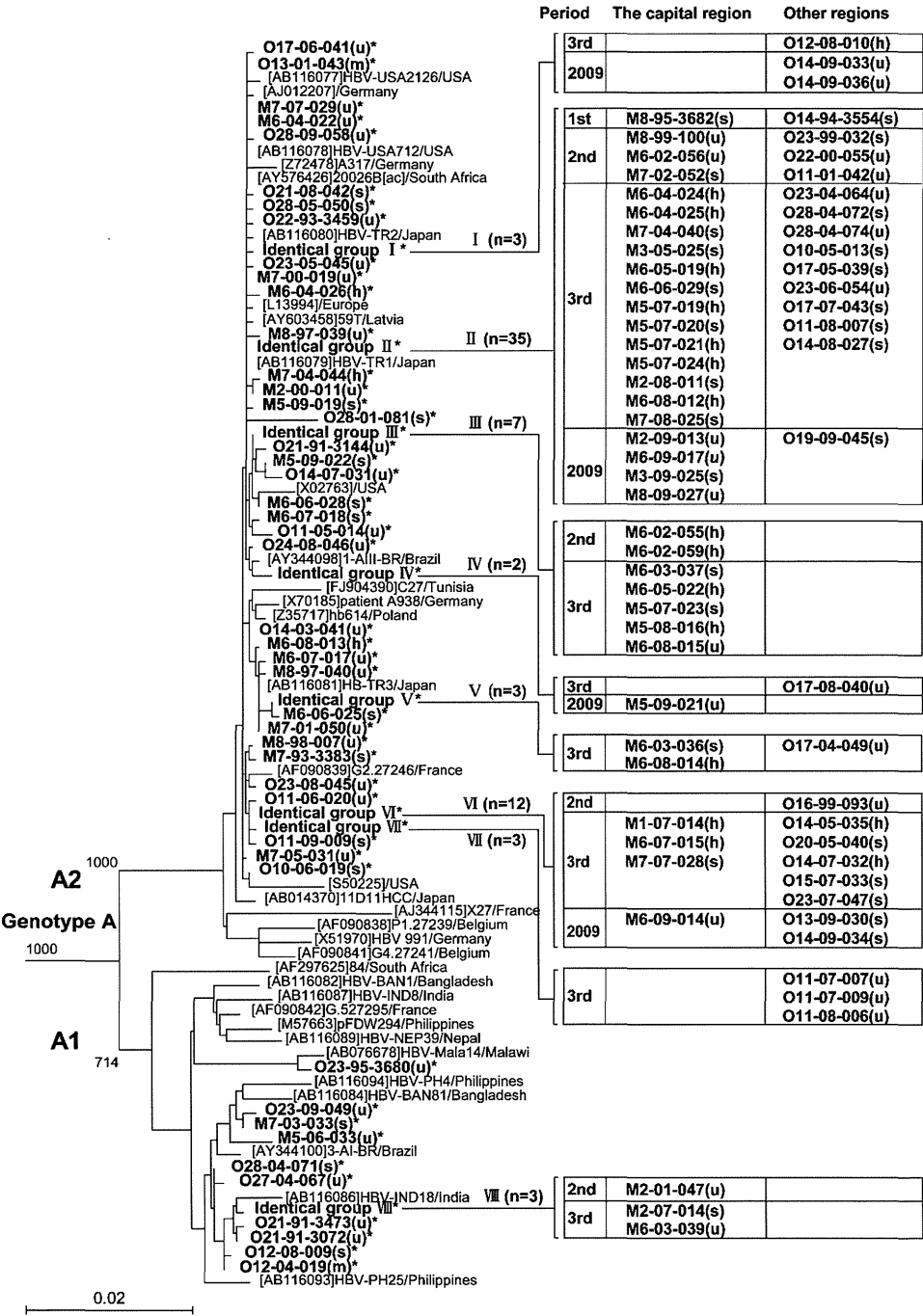
‡Significantly different compared with the 2nd period.

SSignificantly different compared with the 3rd period.

\* $p<0.05$ , \*\*\* $p<0.0001$ .

Viral hepatitis

**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 09–028 (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.



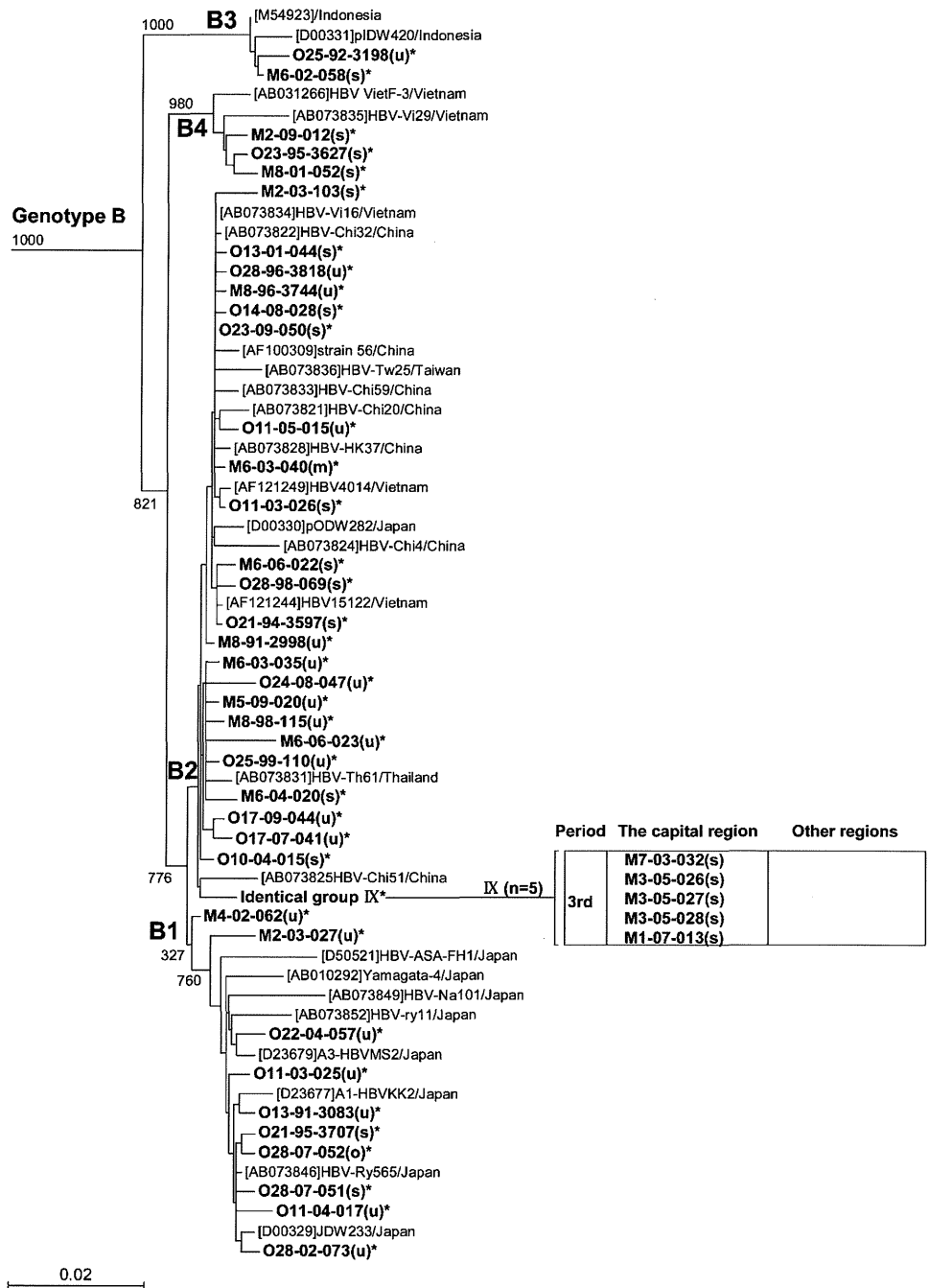
estimated one million HBV carriers in Japan at present.<sup>30</sup> Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and 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.<sup>31 32</sup>

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,<sup>33</sup> 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<sup>21 34</sup>; it is more common for genotype A than other genotypes.<sup>22 35 36</sup> 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.<sup>37</sup>

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.<sup>38</sup>

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.<sup>26</sup> 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.<sup>39</sup>

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

## Viral hepatitis

Yotsuyanagi *et al*<sup>23</sup> reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*<sup>41</sup> 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.<sup>42</sup> 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.<sup>41</sup>

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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## Original Article

# Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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**Aim:** The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

**Methods:** A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

**Results:** Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log

copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

**Conclusions:** It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

**Key words:** discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

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## INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.<sup>1–3</sup> Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide



analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).<sup>4</sup> NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,<sup>5–7</sup> and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;<sup>8</sup> drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.<sup>9,10</sup>

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,<sup>11–13</sup> but relapse after discontinuation is not rare.<sup>14–17</sup> Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,<sup>9,15</sup> reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

## METHODS

### Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the

following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,<sup>18</sup> NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at  $-20^{\circ}\text{C}$  or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

### Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg<sup>19</sup> was done using a chemiluminescence enzyme immunoassay

(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of  $-1.5$  to  $3.3$  log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),<sup>20</sup> which had a quantitative range of  $2.6$  to  $7.6$  log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)<sup>21</sup> with a quantitative range of  $2.1$  to  $9.0$  log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*<sup>22</sup>

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.<sup>23,24</sup> Briefly,  $150$   $\mu$ L of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at  $10$  fg/mL was defined as  $1$  U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at  $3.0$  to  $6.8$  log U/mL.

### Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's  $\chi^2$  tests

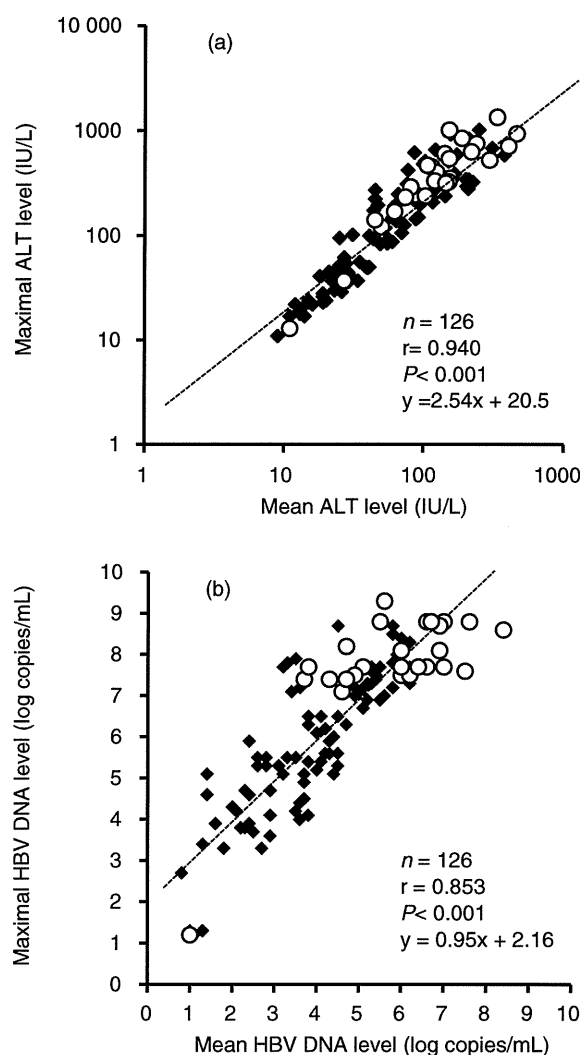
were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann-Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a *P*-value  $< 0.2$  in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P*-values of less than  $0.05$  were considered to be statistically significant.

## RESULTS

### Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below  $4.0$  log copies/mL and ALT below  $30$  IU/L according to the Japanese guidelines for the treatment of hepatitis B.<sup>18</sup> However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total,  $26$  ( $76\%$ ) of  $34$  patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured  $11.0$  times per year on average during the first year and  $4.1$  times per year on average thereafter.

The mean values of HBV DNA were significantly ( $P < 0.001$ ) correlated with maximal values with a correlation coefficient of  $0.853$ . Similarly, the mean values of ALT were significantly ( $P < 0.001$ ) correlated with maximal values with a correlation coefficient of  $0.940$  (Fig. 1). The mean HBV DNA value of  $4.0$  log copies/mL corresponded to a maximal HBV DNA value of  $5.7$  by ROC analysis (AUC =  $0.930$ ,  $P < 0.001$ ), and the mean ALT value of  $30$  IU/L corresponded to a maximal ALT value of  $79$  IU/L (AUC =  $0.988$ ,  $P < 0.001$ ). These results suggested that patients having serum HBV DNA higher



**Figure 1** Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

than 5.7 log copies/mL during the follow-up period after NA discontinuation were not likely to achieve the HBV DNA criterion of a successful discontinuation of below 4.0 log copies/mL. Similarly, it could be inferred that patients reaching ALT levels higher than 79 IU/L would also not likely achieve the ALT criterion of a successful discontinuation of below 30 IU/L.

Based on our findings, we judged that a relapse of hepatitis B occurred when serum ALT exceeded 79 IU/L or when serum HBV DNA exceeded 5.7 log copies/mL

following NA discontinuation. Accordingly, 92 (73%) of the 126 patients enrolled in the present study showed a relapse. We set the follow-up period as discontinuation to relapse for relapse patients and as discontinuation to the last recorded examination for patients without relapse. Whereas re-administration of NAs due to relapse was commenced in 70% of relapse patients in the follow-up period, none was performed in non-relapse patients during that time.

### Elimination of cases likely to show relapse of hepatitis

As it is generally believed that patients who are positive for HBeAg and/or have a higher level of HBV DNA at discontinuation of NAs are likely to relapse, these factors were assessed first. The progression of analyses in the present study and the population structure of each analysis are shown in Figure 2.

The non-relapse rate was compared using the Kaplan–Meier method between 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL and 95 patients with levels lower than 3.0 log copies/mL when NAs were discontinued (Fig. 3). The revised cut-off value of 3.0 log copies/mL was determined by ROC analysis ( $AUC = 0.709$ ,  $P < 0.001$ ). Thirty (97%) of 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL relapsed within one year of discontinuation. On the other hand, approximately 30% of patients with levels lower than 3.0 log copies/mL showed prolonged non-relapse. Thus, the 31 patients with high HBV DNA at the time of discontinuation were eliminated from the following analyses.

In the remaining 95 patients, the non-relapse rate was compared using the Kaplan–Meier method between 10 patients with detectable HBeAg and 85 patients without HBeAg when NAs were discontinued (Fig. 4). Ninety percent of patients with HBeAg experienced relapse within one year, which was significantly ( $P = 0.005$ ) higher than in cases without HBeAg. In patients without HBeAg, the non-relapse rate decreased rapidly during the first year to approximately 45%, and then decreased relatively slowly over the following 3 years to nearly 30%. It is noteworthy that this subgroup did not relapse afterwards. Since the relapse rate was high among patients with detectable HBeAg, they were excluded from the following analyses as well.

### Factors associated with relapse of hepatitis after discontinuation of NAs

Additional factors associated with relapse of hepatitis were analyzed in the remaining 85 patients who were

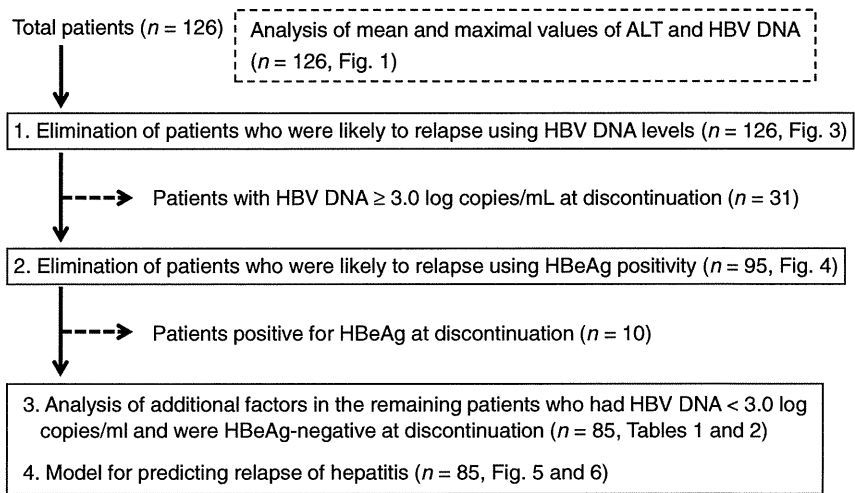


Figure 2 The progression of analyses in the present study and population structure of each analysis.

both negative for HBeAg and whose serum HBV DNA was lower than 3.0 log copies/mL at NA cessation. Table 1 shows the comparison of clinical and virological backgrounds between the 53 relapse and 32 non-relapse patients using univariate analysis. Age and gender distributions were similar between the groups. Approximately 75% of the 85 patients had HBV genotype C, but the distribution of genotypes did not differ between the groups. Approximately 90% of patients were being treated with LVD alone at the time of discontinuation, compared with 6% of patients being given ETV. The median duration of NA treatment was about two times longer in patients without relapse. Levels of both HBsAg

and HBcrAg were significantly lower in non-relapse patients than in relapse patients at the time of NA discontinuation. The difference between serum HBsAg was also significant at the initiation of NAs, but not that of HBcrAg. As only patients with HBV DNA lower than 3.0 log copies/mL were analyzed, the majority of these cases showed levels below the 2.6 log copies/mL lower detection limit of the Amplicor assay at NA discontinuation. We therefore also tested HBV DNA with a TaqMan assay, which had a higher sensitivity than the Amplicor assay, in 43 patients whose serum samples were available. The prevalence of patients having a negative detection signal did not differ between the two groups. The number of

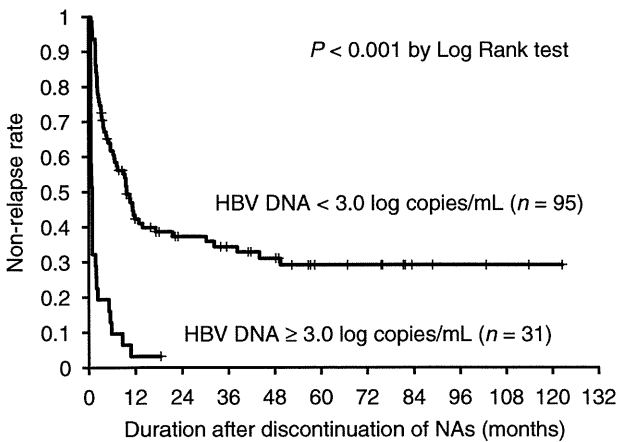


Figure 3 Comparison of non-relapse rates using the Kaplan-Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

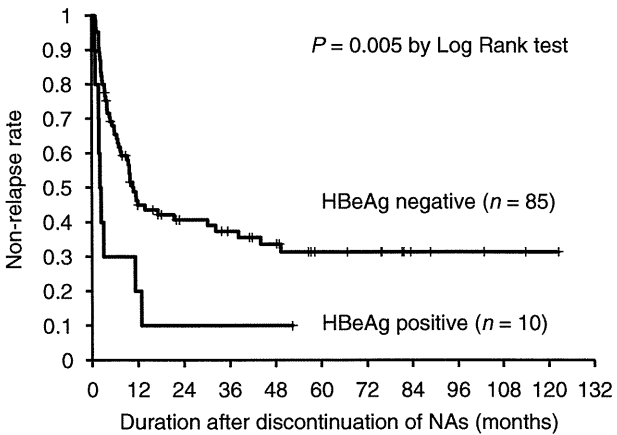


Figure 4 Comparison of non-relapse rates using the Kaplan-Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

**Table 1** Comparison of clinical and virological backgrounds between patients with and without relapse of hepatitis at initiation and discontinuation of nucleos(t)ide analogs (NAs)

| Background  | Non-relapse patients<br>(n = 32) | Relapse patients<br>(n = 53) | P-value |
|---|----------------------------------|------------------------------|---------|
| At initiation of NAs                                    |                                  |                              |         |
| Age (years)†  | 47 (17–75)                       | 48 (26–74)                   | >0.2    |
| Gender (M : F)  | 23:9                             | 32:21                        | >0.2    |
| ALT (IU/L)†   | 183 (9–1182)                     | 187 (20–2052)                | >0.2    |
| Genotype (A : B : C : UD)                               | 1:2:21:8                         | 0:3:44:6                     | 0.193   |
| HBeAg (positive)‡                                       | 11 (34%)                         | 16 (30%)                     | >0.2    |
| HBV DNA   |                                  |                              |         |
| Amplicor assay (log copies/mL)†                         | 6.2 (<2.6–>7.6)                  | 6.5 (<2.6–>7.6)              | 0.099   |
| HBsAg (log IU/mL)†                                      | 2.7 (0.1–4.3)                    | 3.3 (1.6–3.9)                | 0.018   |
| HBcrAg (log U/mL)†                                      | 5.2 (<3.0–>6.8)                  | 5.6 (<3.0–>6.8)              | >0.2    |
| At discontinuation of NAs                               |                                  |                              |         |
| Age (years)†  | 50 (21–78)                       | 49 (26–79)                   | >0.2    |
| NAs (LVD : LVD+ADV : ETV : ADV)                         | 28:1:3:0                         | 50:0:2:1                     | >0.2    |
| Duration of NA treatment (months)†                      | 36 (4–129)                       | 17 (4–84)                    | 0.007   |
| Follow-up period after discontinuation of NAs (months)† | 45 (6–123)                       | 12 (1–111)                   | 0.002   |
| ALT (IU/L)†   | 16 (7–38)                        | 20 (9–65)                    | 0.002   |
| HBV DNA   |                                  |                              |         |
| Amplicor assay (log copies/mL)†                         | <2.6 (<2.6–2.9)                  | <2.6 (<2.6–2.9)              | >0.2    |
| TaqMan assay (negative signal)‡                         | 5 (23%)<br>(n = 22)              | 3 (14%)<br>(n = 21)          | >0.2    |
| TaqMan assay (negative or positive signal)‡             | 13 (59%)<br>(n = 22)             | 13 (62%)<br>(n = 21)         | >0.2    |
| HBsAg (log IU/mL)†                                      | 2.0 (<–1.5–4.3)                  | 3.1 (0.6–4.0)                | 0.001   |
| HBcrAg (log IU/mL)†                                     | 3.4 (<3.0–4.9)                   | 4.3 (<3.0–>6.8)              | 0.003   |

†Data are expressed as the median (range)  
‡Data are expressed as a positive number (%)  
ADV; adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

patients with a negative detection signal or a positive signal also did not vary significantly. The follow-up period after discontinuation of NAs was significantly shorter in patients with relapse than in those without because formal follow-up ended once patients relapsed. The median period of follow-up was 45 months in patients without relapse.

Multivariate analyses revealed that a shorter duration of NA treatment and higher levels of HBsAg and HBcrAg at discontinuation were significantly associated with the occurrence of hepatitis relapse (Table 2). The cut-off

values that showed the highest significance by ROC analysis were 1.9 log IU/mL for HBsAg (AUC = 0.707, *P* = 0.001), 4.0 log U/mL for HBcrAg (AUC = 0.692, *P* = 0.003), and 16 months (AUC = 0.674, *P* = 0.007) for treatment duration.

**Model for predicting relapse of hepatitis using levels of HBsAg and HBcrAg**

The existence of a second cut-off value was suggested by ROC analysis for both of HBsAg (2.9 log IU/mL) and HBcrAg (3.0 log IU/mL) to discriminate between

**Table 2** Multivariate analysis of factors associated with relapse of hepatitis after discontinuation of nucleos(t)ide analogs (NAs)

| Factor                                   | Hazard ratio | 95%CI      | P-value |
|--|--------------|------------|---------|
| HBsAg at discontinuation ≥ 1.9 log IU/mL | 5.21         | 1.87–14.55 | 0.002   |
| HBcrAg at discontinuation ≥ 4.0 log U/mL | 2.20         | 1.25–3.87  | 0.006   |
| Duration of NA treatment ≥ 16 months     | 0.54         | 0.31–0.93  | 0.027   |

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen.