depended on the fact that, in the present study, treatment was begun as soon as possible, and some patients may have had a milder grade of acute exacerbation of chronic hepatitis B than those in the previous report [4]. We believe that patients with acute exacerbation of chronic hepatitis B need to be subjected to treatment as promptly as possible.

The major routes of HBV infection in our country have been mother-to-child transmission and blood transfusion. However, cases with HBV transmitted through sexual contact are increasing, especially among HIV-1-seropositive patients [26]. One should bear in mind that knowledge about interactions between ETV and anti-HIV nucleoside analogues is limited [27]. Because long-term use of LAM induces LAM-resistant mutants [28], we can only use LAM for short-term treatment of patients with acute exacerbation of chronic hepatitis B. On the other hand, the present study also revealed that patients receiving ETV did not need to change drugs.

Recently, there have been several reports that reactivation of HBV is a fatal complication following systemic chemotherapy or other immunosuppressive therapy including rituximab and steroid therapies mainly in HBsAg-positive and -negative lymphoma patients. It is important to enable early diagnosis of HBV reactivation as well as initiation of antiviral therapy [29, 30].

In conclusion, ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should start to treat these patients with NUCs as soon as possible.

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ABBREVIATIONS

ETV: Entecavir; HIV: Human immunodeficiency virus; IVR: Initial virological response; LAM: Lamivudine; NUC: Nucleoside analogue.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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Clinical importance of serum hepatitis B surface antigen levels in chronic hepatitis B

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SUMMARY. Quantitative serology for hepatitis B surface antigen (HBsAg) is a new candidate marker for prediction of clinical outcome. The aim of this study was to investigate the clinical significance of quantifying HBsAg in patients with hepatitis B virus (HBV) infection. A total of 424 patients who tested positive for HBsAg and were referred to Chiba University Hospital between January 1985 and April 2008 were included in the study, and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen (HBeAg), alanine aminotransferase level (ALT), HBV DNA level, number of platelets and development of hepatocellular carcinoma. Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method. The study group consisted of 239 men and 185 women, and their average age was 40.6 ± 14.0 years. HBsAg showed a positive correlation with HBV DNA level (Pearson's product moment correlation, $r = 0.586$, $P < 0.001$) and a weak inverse correlation with age ($r = 0.3325$, $P < 0.001$). A control study, matched with age and sex, was performed between two groups with and without HBeAg seroconversion during follow-up period. Compared with the age and sex-matched controls, the change in HBsAg levels per year showed a significant decrease 2 years before seroconversion (paired *t*-test, $P < 0.05$). The serial measurement of quantitative HBsAg level has the possibility of predicting the occurrence of HBeAg seroconversion.

Keywords: chronic hepatitis B, HBeAg seroconversion, HBs antigen quantification.

INTRODUCTION

An estimated 350 million persons worldwide are chronically infected with HBV [1]. Chronic infection with HBV can progress to cirrhosis, liver failure and hepatocellular carcinoma (HCC), and is a major cause of mortality worldwide [2,3]. Loss of hepatitis B e antigen (HBeAg), accompanied by seroconversion to anti-HBe antibody, usually results in normalized serum alanine aminotransferase (ALT) and decreased HBV DNA levels, and may lead to improved hepatic necroinflammation and confer a better clinical outcome [4–6]. In a recent study of the natural history of chronic hepatitis B (CHB) in 3233 Asian patients, the median age of HBeAg seroconversion was 35 years [7]. HBeAg seroconversion may occur spontaneously at a rate of 5–10% per year [8]. Thus, in clinical practice, HBeAg seroconversion is recognized as a successful serologic response to the treatment of HBeAg-positive CHB.

Determining an accurate prognosis for HBV carriers, based on clinical presentation, is important for clinical management of the disease. Various studies have been performed to distinguish the positive and negative prognostic factors for HBV carriers. The level of HBV DNA, evaluated by TaqMan® PCR method, is an important predictor of clinical outcome in patients with HBV infection [9], but its efficacy is limited [10]. Therefore, we need another marker for predicting the clinical outcome of HBV carriers. Recently, quantitative serology for hepatitis B surface antigen (HBsAg) has been developed as one of the promising candidates. Chan *et al.* [11] found that peginterferon (PegIFN) alfa-2a provided a significant reduction in HBsAg level in the sera of patients with HBeAg-positive CHB. Moreover, HBsAg decline was significantly associated with HBeAg seroconversion

Abbreviations: ALT, serum alanine aminotransferase; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CI, confidence interval; CLEIA, chemiluminescent enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HBcrAg, hepatitis B virus core-related antigens; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV DNA, hepatitis B virus deoxyribo nucleic acid; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IU, international units; LC, log copies; NA, nucleoside/nucleotide analogues; OR, odds ratio; PCR, polymerase chain reaction; PegIFN, peginterferon; PLTs, number of platelets; SD, standard deviation.

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1 year post-treatment, and on-treatment HBsAg levels could be used as an early predictor of durable off-treatment response to PegIFN-based therapy in the individual patient. Recently, Chan *et al.* [12] reported about HBsAg reduction and the fluctuation in titre before and after HBeAg seroconversion of untreated patients.

In this study, based on a cohort of patients with CHB with long-term follow-up, we investigated the HBsAg levels at various stages of CHB. We also aimed to investigate the value of quantitative HBsAg for predicting clinical outcomes in HBeAg-positive CHB patients. Our results clarified the importance of evaluating serum HBsAg levels in patients with CHB.

MATERIAL AND METHODS

Patients

This was a retrospective analysis. Between January 1985 and April 2008, all patients visiting the Chiba University Hospital and who were HBsAg-positive carriers (n = 676) were approached for participation in the study. This study was reviewed and approved by the institutional review board of Chiba University School of Medicine. The patients' consent was obtained for the storage and use of serum. Patients who were positive for hepatitis C virus antibody and those who had another potential cause of chronic liver diseases (autoimmune hepatitis and primary biliary cirrhosis) were excluded from the study. Those patients with <1 year of observation or who had been given antiviral drugs (lamivudine or entecavir) at entry also were excluded from the analysis. As a result, 424 patients were selected for further analysis. To clarify the relationship between the level of HBsAg and other factors, HBV DNA, alanine aminotransferase (ALT) and the number of platelets (PLTs) were analyzed. In addition, we analyzed whether the level of HBsAg was related to the occurrence of HCC. The serum samples from the patients were stored at −20 °C, and the oldest sample obtained from each patient was used to define the level of HBV DNA and HBsAg at entry.

Laboratory assays

Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay (CLEIA) method and the HISCL-2000i (Sysmex Corporation, Kobe, Japan). HBeAg and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL, USA). Anti-HCV was detected by ELISA (Ortho Diagnostics, Tokyo, Japan). The serum HBV DNA level was quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a linear range of quantification of 2.6–7.6 log copies (LC) per mL. The six major genotypes of HBV (A–F) were determined by ELISA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). HBV serum core–related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan).

Serial changes in HBsAg levels during long-term follow-up of HBeAg-positive patients

To observe the serial changes in the HBsAg levels in HBeAg-positive patients, we extracted the HBeAg-positive patients at the beginning of the observation period. Among 424 HBsAg-positive patients, 183 were HBeAg positive. To clarify the long natural history of HBV carriers, we excluded those who could not be followed for more than 5 years. Finally, 120 patients who could be followed for more than 5 years were enrolled and their HBsAg levels were evaluated every year with an error of <2 months.

Statistical analysis

The baseline data are presented as mean ± SD or median and range. The difference in the values of clinical parameters between the two groups was analyzed by paired t-test, unpaired t-test, Welch t-test and chi-square test. Pearson's product moment correlation coefficient analysis was used for statistical analyses, as appropriate, with the statistical program SPSS 16.1 (SPSS Inc., Chicago, IL, USA); a P value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics and the relationship between HBsAg quantification and other clinical markers

The baseline clinical and virological characteristics of the 424 HBsAg-positive carriers are shown in Table 1. First, we investigated the relationship between HBsAg and other virological and clinical markers. The relationships of HBsAg (log IU/mL) with age, gender, HBV genotype and HBeAg status are illustrated in Fig. 1. Gender was not associated with HBsAg titre (Fig. 1a). In contrast, the level of HBsAg in the patients with HBV genotype C differed significantly from those with genotype B ($P < 0.05$, unpaired t-test) (Fig. 1b). The average of HBsAg titre was significantly higher in HBeAg-positive patients compared with those who were HBeAg negative, with statistical difference (unpaired t-test, $P < 0.05$, Fig. 1c). HBsAg showed a significant positive correlation with the HBV DNA level (Pearson's product moment correlation, $r = 0.586$, $P < 0.001$, Fig. 2a), and a weak and inverse correlation between HBsAg and age is also shown (Fig. 2b). In contrast, HBsAg did not show a good correlation with ALT level or PLTs (Figs 2c,d). Next, we used the Cox proportional hazards model to investigate whether HBsAg could be a predictive marker for the occurrence of HCC. Screening for the detection of HCC was performed based on the typical findings of abdominal ultrasonography, dynamic computed tomog-

Table 1 Baseline characteristics of HBsAg-positive patients

Parameters	Total patients	HBeAg-positive patients*
Total patients	424	120
Gender (male/female)	239/185	68/52
Age (average ± SD) (years)	40.6 ± 14.0	34.3 ± 13.1
HBeAg status (positive/negative)	183/241	120/0
HBV DNA level (average ± SD) (log copies/mL)	5.5 ± 1.9	7.1 ± 1.2
ALT level (average ± SD) (IU/L)	70.4 ± 79.3	93.2 ± 96.2
PLT number (average ± SD) ($\times 10^4$ number/μL)	20.8 ± 6.6	20.2 ± 6.2
Follow-up (average ± SD) (years)	5.4 ± 5.1	10.0 ± 5.5
Genotype A/B/C/D/not determined	6/30/250/0/138	2/6/110/2
HBsAg level (average ± SD) (log IU/mL)	3.42 ± 1.15	4.02 ± 0.98
Antiviral drugs	48	34
HCC occurrence	18	4

ALT, alanine aminotransferase; PLT, the number of platelets; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; *120 patients of HBeAg-positive patients were followed for more than 5 years.

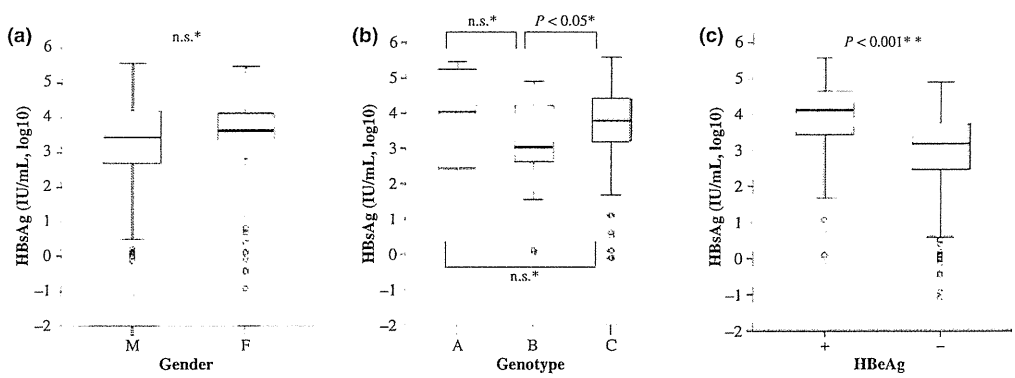

Fig. 1 The association between HBsAg level (log IU/mL) and (a) gender, (b) HBV genotype and (c) HBeAg status. There was no significant difference between HBsAg and gender ($P = 0.146$, unpaired *t*-test). In contrast, compared with genotype B, the level of HBsAg in the patients with HBV genotype C was significantly different ($P < 0.05$, unpaired *t*-test). There was a significant difference according to the positive or negative status of HBeAg ($P < 0.001$, unpaired *t*-test). HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

raphy, angiography and/or magnetic resonance imaging. For all of the patients who were suspected as HCC by image analysis, the diagnosis of HCC was confirmed by pathological analysis. Univariate analysis revealed that age [compared with young patients: odds ratio (OR) = 1.10, 95% confidence interval (CI) = 1.01–1.11], number of PLTs (compared with patients of low PLTs: OR = 0.98, 95% CI = 0.97–0.99) and HBV DNA level (compared with patients of low HBV DNA levels: OR = 1.32, 95% CI = 1.05–1.67) at baseline were predictive factors for HCC occurrence, not HBsAg titre (compared with patients of low HBsAg levels: OR = 0.79, 95% CI = 0.56–1.10). Multivariate analysis revealed that age (compared with young patients: OR = 1.07, 95% CI = 1.03–1.11) and number of PLTs (compared with patients of low PLTs: OR = 0.99, 95% CI = 0.98–0.99) at baseline were predictive factors for HCC occurrence.

The effect of serial change of HBsAg in HBeAg-positive HBV carriers

The baseline clinical characteristics of 120 HBeAg-positive carriers are shown in Table 1, and the level of HBsAg were

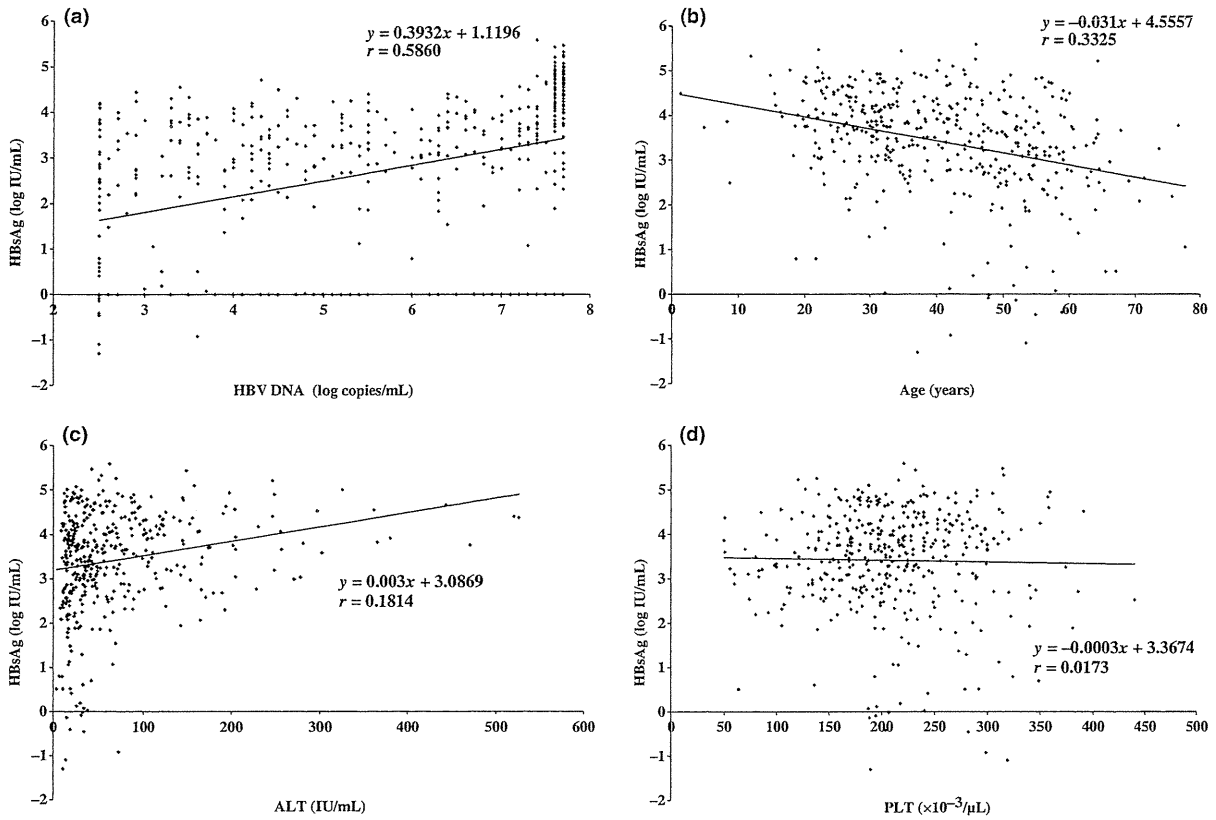

Fig. 2 Correlation between serum HBsAg levels and other clinical markers. (a) HBV DNA levels (Pearson's product moment correlation coefficient analysis; $r = 0.586$, $P < 0.001$), (b) age ($r = 0.333$, $P < 0.001$), (c) serum ALT levels ($r = 0.181$, $P < 0.001$), (d) the number of platelets ($r = 0.017$, $P = 0.347$). HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

evaluated every year. The annualized rate of occurrence of HCC was 0.41% and one patient died of HCC and liver failure, although the death caused from liver failure without HCC was not observed. Seroconversion of HBeAg occurred during the follow-up of 61 patients (average age 32.8 ± 12.5 years). Antiviral drugs were used in 34 patients during follow-up. Of the 120 HBe-positive patients, 34 did not show HBeAg seroconversion and were not given antiviral drugs. Although HBsAg in these patients tended to decrease gradually year-by-year, there was a significant difference only after 5, 9 and 10 years from entry (Mann–Whitney *U* test, $P < 0.05$) (Fig. 3a). After the start of antiviral drugs, the level of HBsAg showed a significant decrease with statistical difference (paired *t*-test, $P = 0.035$) (Fig. 3b). Interestingly, in the patients in whom HBeAg seroconversion occurred during the natural course, the changes in HBsAg levels per year showed a significant decrease 2 years before seroconversion compared with the previous year (paired *t*-test, $P < 0.05$) (Fig. 3c). In addition, the levels of HBsAg showed a significant decrease after HBeAg seroconversion (paired *t*-test, $P = 0.035$).

The serial change in HBsAg levels before and after HBeAg seroconversion compared with the age- and sex-matched controls

Seroconversion of HBeAg has been reported to be influenced by gender [13], and in addition, from our analysis, the levels of HBsAg showed a gradual decrease. Therefore, we performed a control study, matched with age and sex, between two groups with and without HBeAg seroconversion during follow-up period. We extracted the patients who were matched for age and sex and compared 18 who did not show seroconversion through the course to 21 who showed seroconversion spontaneously, without treatment with a nucleotide analogue or interferon (IFN). A significant difference was not found in clinical background in this control study (Table 2). The changes in HBsAg levels in the groups with and without HBeAg seroconversion are shown in Fig. 4a. The level of HBsAg in the two groups gradually decreased over time, but the decline of HBsAg in the patients without HBeAg seroconversion was not significant over the course of a year. On the contrary, in the patients in whom

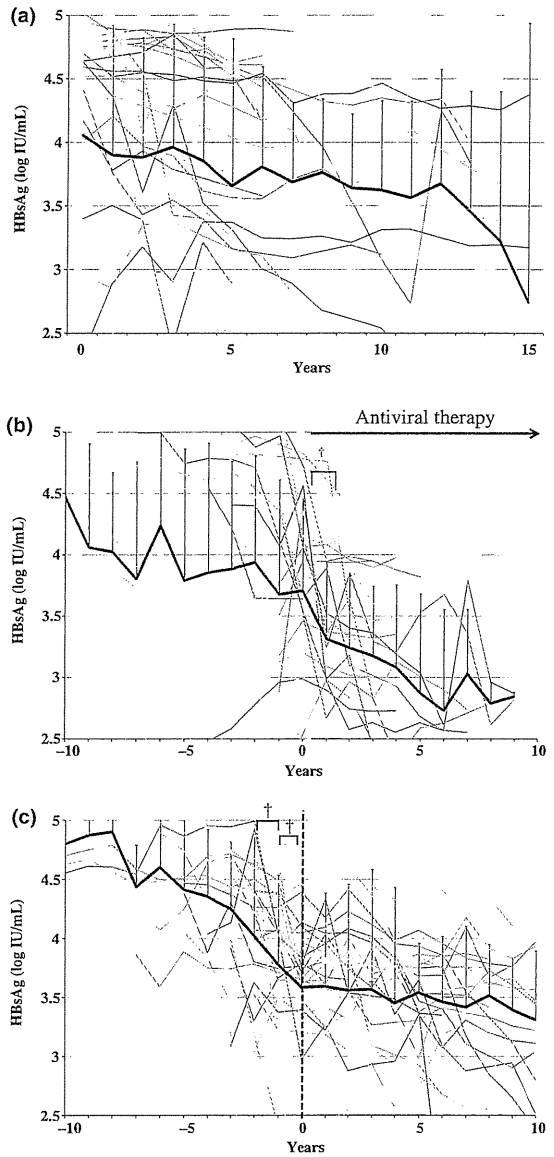

Fig. 3 The serial change of HBsAg level in HBeAg-positive patients with (a) no HBeAg seroconversion and no use of antiviral drugs (n = 34), (b) the use of antiviral drugs (n = 32), (c) HBeAg seroconversion during the follow-up period (n = 35). (a) Compared with the level at entry, a continuous decrease was not observed, although there was a significant difference only after 5, 9 and 10 years from entry ($P < 0.05$, Mann–Whitney U test). (b) The level of HBsAg showed a statistically significant decrease after commencement of antiviral therapy (†$P < 0.05$, paired t-test). (c) HBsAg showed a significant decrease at 2 years before seroconversion (†$P < 0.05$, paired t-test). HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

HBeAg seroconversion occurred during the natural course, the changes in HBsAg levels per year showed a significant decrease 2 years before seroconversion compared with the previous year (paired t-test, $P < 0.05$) (Fig. 4a). Next, we compared the difference in HBsAg levels between the two groups. There was a significant difference between the two groups 1 year before, on and after HBeAg seroconversion (unpaired t-test, $P < 0.05$). The HBeAg titre did not differ significantly between the two groups before seroconversion (Fig. 4b). The levels of HBcrAg showed an obvious decrease after HBeAg seroconversion, but before this, there was no significant decrease in the patients with or without HBeAg seroconversion (Fig. 4c).

DISCUSSION

The natural history of CHB is typically regarded as consisting of some phases that have been classified mainly by serum ALT levels, HBeAg and HBsAg serostatus, and HBV DNA levels. The understanding of the natural history of CHB has been facilitated by the improved sensitivity of serological and virological makers. HBsAg was the first HBV-encoded protein to be discovered [14]. Detection of HBsAg in serum is the fundamental diagnostic marker of HBV infection. HBsAg is a component of the Dane particle, which contains the viral genome, and subviral particles, but the mechanisms that regulate the production of HBsAg, particularly the subviral particles, are largely unclear [15]. Excess HBsAg may serve as a possible mechanism for evading the host immune responses, in that anti-HBs antibodies provide protective immunity [16]. One of our aims was to determine the change of HBsAg levels during the natural history of infection. Thompson *et al.* [17] reported that the level of HBsAg was related to the HBeAg status, as seen here. Some studies reported that positive correlations have been observed between the level of HBsAg and serum HBV DNA [18,19], again as seen here, but another study reported no such correlation [20]. Regarding the relationship with age, Kohmoto *et al.* [19] reported that the level of HBsAg was negatively correlated with the patient's age. We also found a weak and negative correlation between the levels of HBsAg and age, but in the analysis only of HBeAg-positive patients who did not show HBeAg seroconversion and who were not treated with antiviral drugs during follow-up period, the serial change of HBsAg levels showed no obvious decrease. Thus, the patients' age might have a direct effect on the level of HBsAg, but clinical events such as HBeAg seroconversion or the treatment of antiviral drugs might have a greater impact. Some studies reported that the level of HBsAg showed the difference among HBV genotypes [20,21]. In fact, we showed that the level of HBsAg in the patients with HBV genotype B was less than genotype C, but a limitation of this study was that most HBV carriers in our analysis were infected with genotype C of HBV. Therefore, we could not clarify the difference of HBsAg level among genotypes during HBeAg seroconversion.

In this study, a high HBsAg level was not related to the high incidence of HCC. In contrast, age, PLTs and the HBV

Table 2 Clinical characteristics of the patients with age- and sex-matched controls

Parameters	Seroconversion (+)	Seroconversion (−)	*P*
Patient numbers	21	18	
Gender (male/female)	8/13	11/7	n.s.
Median Age (years)	30.0 (24–55)	31.0 (18–46)	n.s.
HBV Genotype (A/B/C)	1/3/17	0/0/26	n.s.
HBV DNA level (average ± SD) (log copies/mL)	7.4 ± 0.6	7.4 ± 0.5	n.s.
HBsAg level (average ± SD) (log IU/mL)	4.70 ± 4.79	4.70 ± 4.54	n.s.

HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

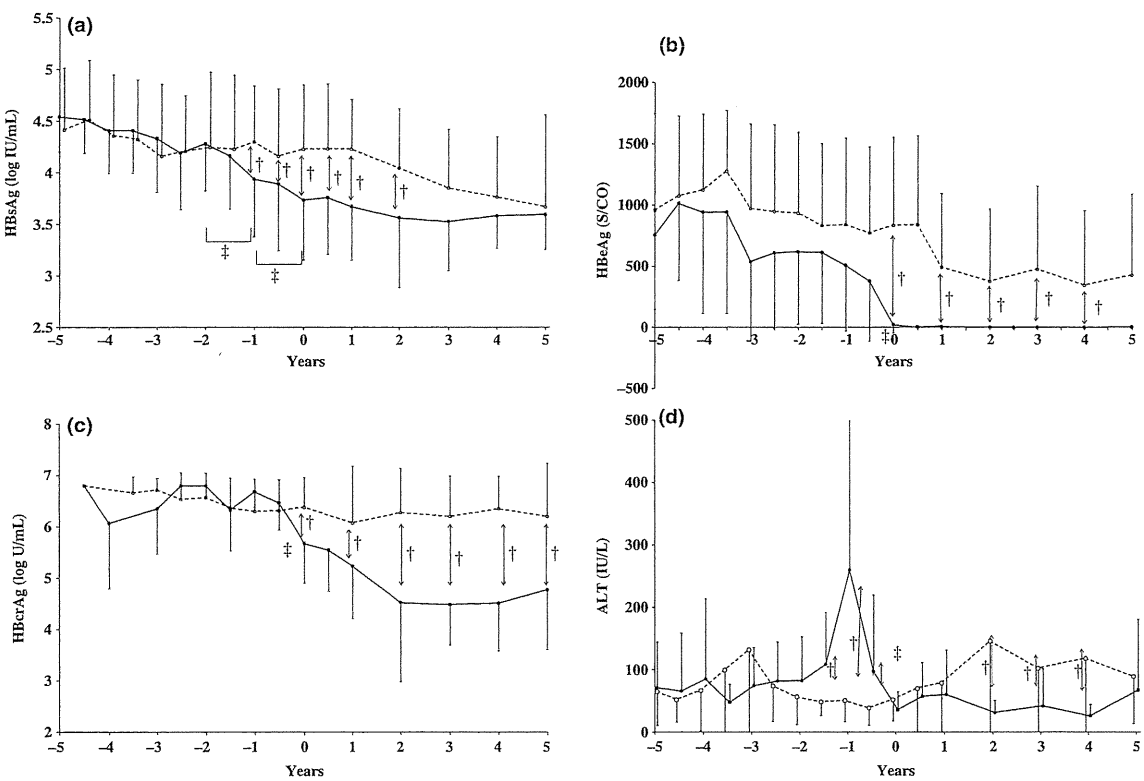

Fig. 4 Comparison of serial changes in (a) HBsAg, (b) HBeAg, (c) HBcrAg level and (d) ALT during 10 years around HBeAg seroconversion with those of age-and sex-matched controls without seroconversion. The group with HBeAg seroconversion (n = 21) is shown with closed circles and that without seroconversion (n = 18) with open circles. The time point of seroconversion was designated as 0 year. (a) There was no significant difference between the two groups until 2 years before seroconversion, but 1 year before, on and after HBeAg seroconversion, there were significant differences between the two groups (†P < 0.05, unpaired t-test). In the patients with seroconversion, HBsAg showed a significant decrease 2 years before seroconversion (‡P < 0.05, paired t-test). (b) The HBeAg level differed significantly between the two groups after HBeAg seroconversion (†P < 0.05, unpaired t-test, ‡P < 0.05, paired t-test). (c) The core-related antigen of HBV (HBcrAg) level showed a significant difference between the two groups after seroconversion (†P < 0.05, unpaired t-test, ‡P < 0.05, paired t-test). (d) The level of ALT in the patients with HBeAg seroconversion showed a significant increase half a year before HBeAg seroconversion. The ALT level differed significantly between the two groups before and after HBeAg seroconversion († P < 0.05, unpaired t-test, ‡P < 0.05, paired t-test). HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

DNA level at baseline showed a strong relation with tumour development, as we have reported previously [22], although HBsAg seroclearance at age <50 years was reported to be associated with a lower risk for the development of HCC [23]; that is, there was a difference in clinical outcome between the low level of HBsAg and its seroclearance. Therefore, the final goal of therapy for HBV carriers might be set as HBsAg seroclearance.

It is well known that seroconversion of HBeAg to its antibody is associated with a decrease in serum HBV DNA to low or undetectable levels, clinical remission and an improvement in hepatic inflammatory activity. Some studies reported that high pretreatment ALT levels and low serum HBV DNA levels were independently associated with an increased rate of seroconversion after treatment with either IFN [24–26] or nucleoside/nucleotide analogues (NA) [27]. Further analysis also showed that factors such as viral genotype [28], quantitative HBeAg [29] and active histological disease [30] also may be important predictors of seroconversion after treatment with IFN or NA [31]. The quantitative monitoring of HBsAg titre also has been suggested as a predictor of treatment response, especially for IFN-based therapies in chronic HBV infection [32]. In our study, HBsAg decreased significantly, compared with a group without seroconversion, for 2 years prior to seroconversion. In the age-and sex-controlled study, we analyzed the relationship between the level of HBsAg at 140 time points and the occurrence within 2 years of HBeAg seroconversion. In 10 of 19 patients (52.6%), when the level of HBsAg showed more than 50% decrease compared with the previous year, HBeAg seroconversion occurred within 2 years, which differed significantly (chi-square test, $P = 0.003$). Thus, this study suggested that quantitative measurement of the HBsAg titre might clinically be useful and that it becomes possible to build a treatment strategy by predicting whether seroconversion will occur. The reason why the level of HBsAg decreased before HBeAg seroconversion with preceding the decrease of HBeAg titre or HBcrAg level remains unclear. The current findings speculated us that it was most likely due to the integration of HBV into the host genome that potentially provides a separate template for the production of HBsAg or the cytokine-dependent modification of viral replication pathways [21].

In a recent report, there was no obvious decline in HBsAg at the time of HBeAg seroconversion, compared with the decline of HBV DNA, from the evaluation of HBsAg only at two points before seroconversion [12]. By the evaluation of serial changes in HBsAg levels before seroconversion, in addition to the difference of HBV genotype or race, our study might show differences from that finding.

HBV covalently closed circular DNA (cccDNA) is important for virus replication and impacts on clinical outcome [33], and HBsAg has been evaluated recently as a surrogate marker of cccDNA [34,35]. Because liver biopsy was not a routine procedure, we did not measure cccDNA directly in liver. Further studies are required to clarify the precise significance of HBsAg levels because a direct association between HBV cccDNA levels in liver and HBsAg levels in serum remains to be shown.

In conclusion, the titre of HBsAg is a new marker related to HBV replication and its serial measurement possibly may be a predictive factor for HBeAg seroconversion.

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ORIGINAL ARTICLE

Emergence of entecavir-resistant mutations in nucleos(t)ide-naive Japanese patients infected with hepatitis B virus: Virological breakthrough is also dependent on adherence to medication

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Abstract

Objective. Currently, five nucleos(t)ide analogues (NUCs) are available for the treatment of chronic hepatitis B in the world. We examined the prevalence of hepatitis B virus (HBV) DNA and alanine aminotransferase normalization in patients receiving entecavir (ETV) and the frequency of ETV-resistant mutations during an approximately 27-month use of ETV in chronic hepatitis B patients in an urban hospital in Japan. ***Materials and methods.*** A retrospective analysis of 81 NUC-naive chronic hepatitis B patients who received 0.5 mg of ETV daily was performed. HBV DNA was measured and sequence analysis of HBV DNA was performed in virological breakthrough patients. ***Results.*** Hepatitis B e antigen (HBeAg)-positive patients with HBV DNA 5.0–7.0 log IU/mL group and all HBeAg-negative patients achieved serum HBV DNA negativity by 12 months. Four patients experienced virological breakthrough during ETV therapy. Two patients had no genotypic mutations, and medical interviews revealed that they had poor adherence to ETV. ***Conclusions.*** We found that some of the HBV virological breakthroughs during ETV treatment were related to poor adherence to medication, highlighting that clinicians should pay attention to the emergence of resistant mutants as well as adherence to ETV.

Key Words: *Adherence, entecavir, HBV, resistant mutants, virological breakthrough*

Introduction

Two billion people have been exposed to hepatitis B virus (HBV), and 350–400 million people remain chronically infected worldwide. In Japan, the prevalence of HBV carriers is estimated at ~1% of the population, but HBV is one of the major health issues because it leads to acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1–5].

Recent studies have shown that the serum HBV DNA level is one of the most potent risk factors for the development of cirrhosis or HCC, and it seems that suppressing the serum HBV viral load is essential for improving the prognosis of HBV carriers [6,7]. Currently, there are five approved nucleos(t)ide analogues (NUCs) for the treatment of chronic hepatitis B [8]. At present, the Japanese national health insurance system approves entecavir (ETV) as the first-line therapy for chronic hepatitis B, although some patients are treated with standard interferon-alfa. ETV is an NUC belonging to a new subgroup, cyclopentane [9], and has been shown to be highly effective in suppressing HBV replication to an undetectable level and normalizing alanine aminotransferase (ALT), although NUCs do not eradicate the virus. Most patients therefore require long durations of treatment, but prolonged treatment is associated with increasing rates of drug resistance. There was

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also a report that the 3-year cumulative probability of resistance was 1.7% for 0.5 mg/day ETV therapy in NUC-naive Japanese patients [10].

In the present study, we examined the prevalence of HBV DNA and ALT normalization in patients receiving ETV as well as the frequency of ETV-resistant mutations during an approximately 27-month use of ETV in chronic hepatitis B patients in an urban hospital in Japan. We found a relationship between some of the HBV virological breakthroughs during ETV treatment with poor adherence to medication, and clinicians need to focus on the possible emergence of resistant mutants as well as the adherence to ETV.

Patients and methods

Patients

A retrospective analysis of NUC-naive chronic hepatitis B patients ($n = 81$) receiving 0.5 mg of ETV daily at Chiba University Hospital between May 2003 and December 2009 was performed. The patients were divided into three groups based on their HBV DNA level just before starting ETV according to the Japanese Ministry of Health, Labor and Welfare Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B [11]: HBV DNA <5.0 log IU/mL (n = 14, 17.3%), 5.0–7.0 log IU/mL (n = 30, 37.0%), and >7.0 log IU/mL (n = 37, 45.7%) (Table I). All patients had serum hepatitis B surface antigen (HBsAg) detectable for at least 6 months, regardless of their hepatitis B e antigen (HBeAg) status. They were negative for hepatitis C virus and HIV antibodies. They were followed up at least every 3 months to examine physical status and monitor liver biochemistry and virology. Adherence to ETV was assessed during each visit to the clinic. This study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine.

Serological examination

All clinical laboratory tests including hematological data, biochemical data, and HBV serologies were performed at the Central Laboratory of Chiba University Hospital. HBsAg, HBeAg, and anti-HBe antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [12]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al. [13]. HBV DNA was measured by Roche Amplicor™ PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan). The clinical efficacy of ETV was assessed as the proportion of patients achieving HBV DNA negativity, which is defined as an HBV DNA level of <2.6 log IU/mL and that of patients achieving ALT normalization (normal range: 8–42 IU/L). Using generally available biological parameters, the aspartate aminotransferase (AST) to platelets ratio index (APRI), and serum liver fibrosis score, was calculated according to the following formula: AST/35 × 100/platelet count [14,15].

Sequence analysis of HBV DNA

Sera obtained from patients were stored at -20°C until analysis. HBV polymerase/reverse transcriptase (RT) substitutions were analyzed for all patients who had experienced virological breakthrough (>1 log IU/mL

Table I. Baseline characteristics of patients.

	HBV DNA (log IU/mL)				HBeAg	
	Total	<5.0	5.0–7.0	>7.0	Positive	Negative
Number of cases	81	14	30	37	40	41
Age (years)	49.7 ± 12.2	55.7 ± 13.1[a]	50.9 ± 11.3	46.4 ± 11.8[a]	44.7 ± 10.3[e]	54.5 ± 12.0[e]
Gender (male/female)	55/26	7/7	22/8	26/11	28/12	27/14
HBeAg (+/-)	40/41	0/14[b]	11/19[b]	29/8[b]		
Genotype (B/C/N.D.)	2/33/46	0/2/12	2/14/14	0/17/20	1/23/16	1/10/30
ALT (IU/L)	169 ± 186	85.1 ± 115	185 ± 192	189 ± 197	179 ± 190	159 ± 184
AST (IU/L)	108 ± 113	62.6 ± 87.5	128 ± 149	109 ± 81.1	104 ± 83.6	111 ± 136
Platelets ($\times 10^4/mm^3$)	16.2 ± 8.2	21.4 ± 15.5[c]	14.9 ± 5.0[c]	15.5 ± 5.9	15.7 ± 5.5	16.8 ± 10.2
APRI (<0.50/0.50–1.50/>1.50)	14/35/32	7/5/2[d]	4/11/15[d]	3/19/15[d]	2/24/14[f]	12/11/18[f]

Abbreviations: ALT = alanine aminotransferase; APRI = AST to platelets ratio index; AST = aspartate aminotransferase; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; N.D = not determined; SD = standard deviation. APRI, AST/35 × 100/platelet count [15];
Data are expressed as mean ± SD. [a]$p = 0.036$ between <5.0 group and >7.0 group; [b]$p < 0.001$ among three groups; [c]$p = 0.041$ between <5.0 group and 5.0–7.0 group; [d]$p = 0.0049$ among three groups; [e]$p < 0.001$ between HBeAg-positive and HBeAg-negative groups; [f]$p = 0.0020$ between HBeAg-positive and HBeAg-negative groups.

increase in serum HBV DNA level from nadir) using ETV on-treatment sera. Briefly, HBV DNA was extracted from 100 μL of sera using SepaGene (Sanko Junyaku, Tokyo, Japan). Nested PCR was performed using LA Taq polymerase (Takara Bio, Otsu, Shiga, Japan) under the following conditions: 5-min denaturation at 94°C, 35 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 1.5 min [5]. An 862 base-pair fragment (nt 242–1103) containing the polymerase RT domain was amplified on PCR Thermal Cycler Dice Model TP600 (Takara Bio). The primers for the second round of PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242–258) and 5′-GGC GAG AAA GTG AAAGCC-3′ (antisense, nt 1103–1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTAGTG CC -3′ (nt 668–687), 5′-GGC ACT AGT AAA CTGAGC CA-3′ (nt 687–668), and the primers for the second round of PCR. To prepare the sequence template, PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA, USA) and then sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were analyzed using Applied Biosystems 3730 × 1 (Life Technologies) [16].

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010 for Windows™ 7. Continuous variables were expressed as mean ± standard deviation and were compared by one-factor analysis of variance. Categorical variables were compared by Chi-square test. Baseline was taken as the date when the first dose of ETV was taken. Statistical significance was considered at $p < 0.05$.

Results

Baseline characteristics of the patients

Eighty-one patients (67.9% male) were included in this study. The group with HBV DNA >7.0 log IU/mL was younger than that with HBV DNA <5.0 log IU/mL ($p = 0.036$) (Table I). Treatment duration was not significantly different among these three groups (21.4 ± 9.8, 27.8 ± 16.3, and 33.1 ± 24.6 months; $p = 0.16$). The status of HBeAg differed significantly among these three groups ($p < 0.001$). There was a statistically significant difference in platelet counts between the HBV DNA <5.0 log IU/mL and 5.0–7.0 log IU/mL groups ($p = 0.041$). This suggested that patients with an HBV DNA level <5.0 log IU/mL were likely not to progress to liver fibrosis because APRI tended to be lower and HBeAg was negative. To compare HBeAg-positive and HBeAg-negative cases, the patients were divided into two groups based on their HBeAg status just before starting ETV. The HBeAg-positive cases were younger than the HBeAg-negative cases ($p < 0.001$).

Virological response

The numbers (proportions) of patients achieving serum HBV DNA negativity at 3, 6, 12, 18, 24, and 36 months, respectively, in the three groups are shown in Figure 1. At 3 months, HBV DNA negativity of the HBV DNA <5.0 log IU/mL group was higher than that of the HBV DNA 5.0–7.0 log IU/mL group ($p = 0.0040$). At 3 and 6 months, HBV DNA negativity of the HBV DNA <5.0 log IU/mL group was higher than that of the HBV DNA >7.0 log IU/mL group ($p < 0.001$ and $p = 0.018$, respectively) (Figure 1A). At 12 and 24 months, HBV DNA negativity of the HBV DNA 5.0–7.0 log IU/mL group was higher than that of the HBV DNA >7.0 log IU/mL group ($p = 0.034$ and 0.035, respectively).

The patients with HBV DNA <5.0 log IU/mL all achieved serum HBV DNA negativity throughout the duration. The patients with HBV DNA 5.0–7.0 log IU/mL all achieved serum HBV DNA negativity by 12 months after starting treatment. Only one patient in this group experienced an increase in HBV DNA, at 29 months, but the duration of serum HBV DNA detectability was only 4 months, when he had poor adherence. From then, after discussions with his physician, he understood the importance of taking ETV for suppression of HBV replication and strictly adhered to the treatment schedule, and HBV DNA became undetectable again (Patient 3 in Table II). On the other hand, in the HBV DNA >7.0 log IU/mL group, the proportions of patients achieving serum HBV DNA negativity were 84.8%, 88.0%, 76.2%, and 69.2% at 12, 18, 24, and 36 months, respectively. In three patients of this group, HBV DNA increased at 28, 26, and 12 months, respectively (Patients 1, 2, and 4 in Table II). When we investigated the negativity of HBV DNA with or without HBeAg at baseline, HBeAg-positive patients with HBV DNA 5.0–7.0 log IU/mL group (Figure 1B) and all HBeAg-negative patients achieved serum HBV DNA negativity by 12 months (Figure 1C).

The proportions of patients achieving serum HBeAg negativity in HBeAg-positive patients with HBV DNA 5.0–7.0 log IU/mL group were 0%

(0/11), 9.1% (1/11), 9.1% (1/11), 22.2% (2/9), 20% (1/5), and 25% (1/4) at 3, 6, 12, 18, 24, and 36 months, respectively. On the other hand, in the HBV DNA >7.0 log IU/mL HBeAg positive-group, the proportions of patients achieving serum HBeAg negativity were 5.1% (2/28), 11.1% (3/27), 23.1% (6/26), 30.0% (6/20), 38.9% (7/18), and 60.0% (6/10) at 3, 6, 12, 18, 24, and 36 months, respectively.

Biochemical response

At 3 months, the proportion of ALT normalization of the HBV DNA 5.0–7.0 log IU/mL group was higher than that of the HBV DNA >7.0 log IU/mL group (24/28 vs. 20/35, $p = 0.014$). When we investigated the normalization of ALT with or without HBeAg at baseline, HBeAg-positive patients seemed

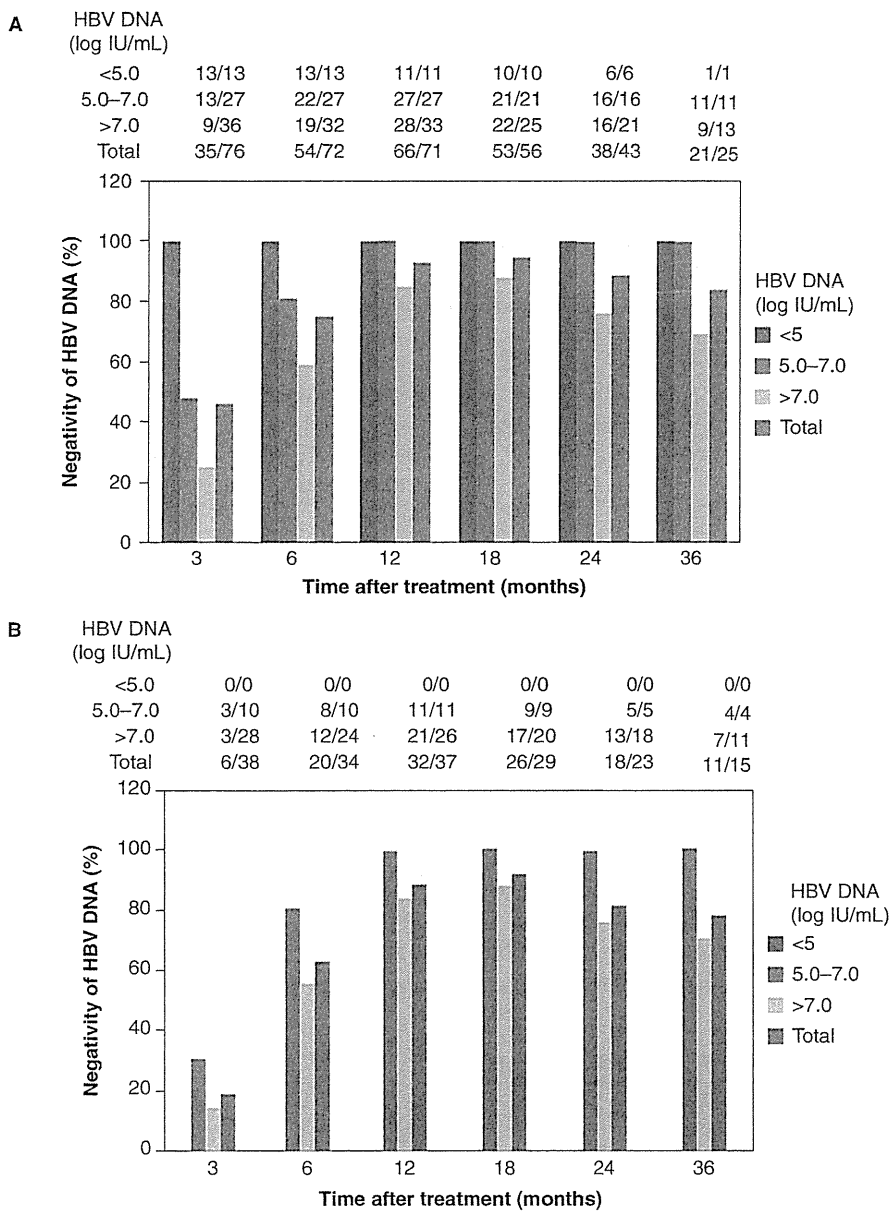

Figure 1. Negativity of HBV DNA (%) during ETV treatment. (A) A total of 81 patients (violet bars) were included in this study. Patients were divided into three groups: HBV DNA <5.0 log IU/mL group ($n = 14$, 17.3%) (blue bars), 5.0–7.0 log IU /mL group ($n = 30$, 37.0%) (red bars), and >7.0 log IU/mL group ($n = 37$, 45.7%) (green bars). (B) HBeAg-positive patients ($n = 40$). (C) HBeAg-negative patients ($n = 41$). ETV = entecavir; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus.

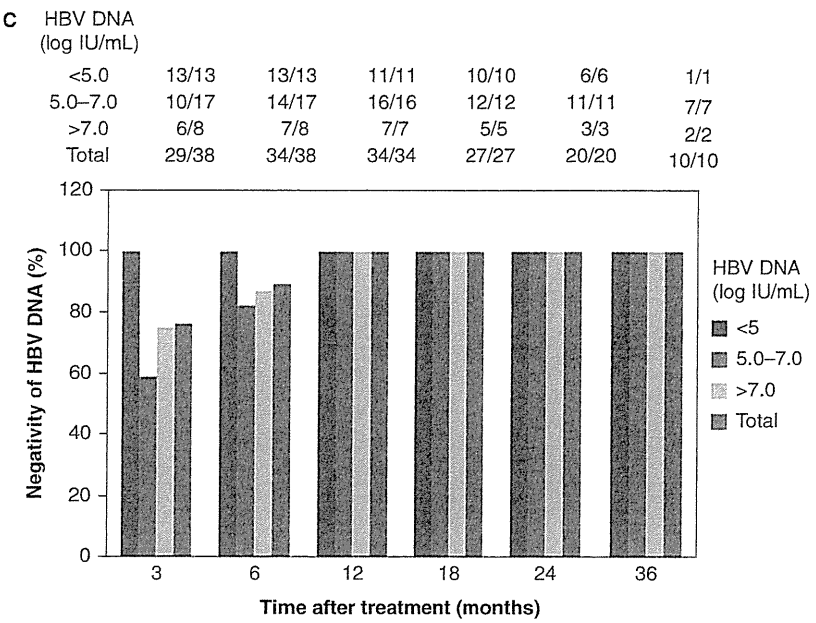

Figure 1. (*Continued*).

to have a slower response to ETV than HBeAg-negative patients (data not shown).

Sequence analysis

Four patients, one with HBV DNA 5.0–7.0 log IU/mL and three with HBV DNA >7.0 log IU/mL, experienced virological breakthrough during ETV therapy and were all analyzed to clarify whether genotypic mutations were acquired or not (Table II). Two of the three patients with HBV DNA >7.0 log IU/mL acquired genotypic mutations resistant to ETV. One had rtS202G (Patient 1 in Table III) and the other rtT184A (Patient 2 in Table III), both accompanied by lamivudine (3TC)-resistant substitutions (rtL180M and rtM204V). The other two patients had no genotypic mutations (Patients 3 and 4 in Table III). The physicians who had seen these patients at the time of virological breakthrough performed medical interviews to ask about their adherence, adding the information to their medical charts, and their poor adherence to ETV was revealed.

Discussion

In the present study, an approximately 27-month ETV treatment for NUC-naive patients resulted in an optimized outcome, in line with previous reports [10,17]. Early on-treatment virological response leads to optimized long-term outcome. We found two HBeAg-positive patients with ETV-resistant mutations. One had rtS202G (Patient 1 in Table III) and the other rtT184A (Patient 2 in Table III), accompanied by 3TC-resistant substitutions (rtL180M and rtM204V) [17–19]. These two patients discontinued ETV and then received a combination therapy of 100 mg 3TC and 10 mg adefovir-dipivoxil daily, as previously reported [20]. As ETV-resistance can be

Table II. Characteristics of patients with HBV virological breakthrough.

Patient	Age (years)/gender	HBV genotype	Baseline HBeAg	Baseline HBV DNA (log IU/mL)	Baseline ALT (IU/L)	Duration of treatment before VB (months)	Adherence to ETV
1	49/M	C	+	7.3	107	28	Good
2	57/M	C	+	>7.6	55	26	Good
3	38/M	C	+	6.9	59	29	Poor
4	46/F	C	+	>7.6	85	12	Poor

Abbreviations: ALT = alanine aminotransferase; ETV = entecavir; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; VB = virological breakthrough.

Table III. Amino acid mutations of HBV DNA polymerase sequences in patients with virological breakthrough.

Amino acid No	180	184	202	204	250
Wild sequences	L	T	S	M	M
Patient 1	**M**	T	**G**	**V**	M
2	**M**	**A**	S	**V**	M
3	L	T	S	M	M
4	L	T	S	M	M

Abbreviations: HBV = hepatitis B virus; **Bold**, amino acid mutations. Numbers of top line indicate amino acid positions [5].

relatively easily diagnosed, it is important to perform both the HBV DNA and the resistance tests. The mechanism by which ETV-resistant substitutions can induce virological breakthrough during ETV therapy is largely known [21]. We also found two patients with poor adherence to ETV. Multiple drug-resistant strains were also reported, and so monitoring NUC-resistant mutations is essential. In such a case, we have to pay attention to patients with poor adherence as well [19].

In Japan, HBV genotype C is predominant. HBV genotype C is reported to be associated with delayed HBe seroconversion, more advanced liver disease, and increased probability of HCC development [22,23]. No statistical difference was observed in response to ETV among patients with different genotypes [24], although HBV genotype A and B patients were reported to respond to standard interferon-alfa better than HBV genotype C and D patients [23]. In this study, HBeAg-negative patients achieved serum HBV DNA negativity by 12 months. On the other hand, HBeAg-positive patients tended to have poor response to ETV. One HBeAg-negative patient of the total 81 patients became negative for HBsAg (data not shown). These observations might suggest important pathogenic differences in HBV genotypes.

It is well known that non-adherence results in non-control of other common diseases such as diabetes mellitus [25] and hypertension [26]. Poor medication adherence among HIV-infected adults leads to neuropsychological dysfunction [27] as well as increase of HIV RNA [28]. Recently, Ha et al. [29] also reported that medication non-adherence was likely to be a more important contributor to treatment failure than antiviral resistance, especially with new anti-HBV agents such as ETV and tenofovir. Although the number of patients in our study is small, our results highlight the importance of making efforts to ensure medication adherence for HBV-positive patients and providing support to improve poor adherence to control HBV replication.

In the present study, ETV resulted in less ETV-resistant mutations in NUC-naive Japanese patients than in previous reports with other drugs such as 3TC [5,18]. In conclusion, attention should be paid to patients with poor adherence as well as emerging ETV-resistant mutations in HBV during ETV-treatment.

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Original Article

Recommendation of lamivudine-to-entecavir switching treatment in chronic hepatitis B responders: Randomized controlled trial

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Aim: In the 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan), lamivudine (LAM)-continuous treatment was recommended in patients treated with LAM for more than 3 years who maintained hepatitis B virus (HBV) DNA less than 2.6 log copies/mL, because in these patients LAM resistance might exist and switching treatment to entecavir (ETV) might cause ETV resistance. However, there was no evidence on whether switching treatment to ETV- or LAM-continuous treatment was better in those patients. In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment.

Methods: Twenty-seven patients treated with LAM for more than 3 years whose HBV DNA levels were less than 2.6 log copies/mL were enrolled and randomly divided into two groups, LAM-continued group or switching to ETV group. Then, we examined incidence of virological breakthrough (VBT) and breakthrough hepatitis (BTH) in each group.

Results: There was no BTH in any of the patients. VBT was observed in six patients of the LAM group (6/15, 40%), and no patient of the ETV group (0/11, 0%) ($P = 0.02$). The differences of the proportion of cumulated VBT using a log–rank test with Kaplan–Meier analysis were significant between the LAM and ETV groups ($P = 0.025$).

Conclusion: In patients treated with LAM for more than 3 years maintaining HBV DNA less than 2.6 log copies/mL, switching treatment to ETV is recommended at least during the 2 years' follow-up period.

Key words: chronic hepatitis B, entecavir, lamivudine, lamivudine resistance, randomized controlled trial, switching treatment

INTRODUCTION

OVER THE PAST two decades, treatment of chronic hepatitis B (CHB) has greatly improved with the availability of nucleos(t)ide analogs (NA), including lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine, clevudine and tenofovir. NA target

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the reverse transcriptase of hepatitis B virus (HBV), and are highly effective in suppressing HBV replication and clinical progression to liver cirrhosis and hepatocellular carcinoma in CHB patients.[1–4]

Lamivudine, ADV and ETV are commonly available in Japan. LAM, the first approved NA, has been shown to provide benefit for CHB patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology.[5,6] However, a serious problem of LAM is the high incidence of drug resistance during long-term treatment. The detection rate of LAM resistance has been reported to be 24% at 1 year and 70% after 5 years of treatment.[7–10] Even when the HBV DNA level was maintained at less than 2.6 log copies/mL, the accumulated incidence of LAM resistance reached 65% in patients treated with LAM for a long period (3 to ~10 years).[11] LAM resistance is caused by amino acid substitution(s) at rtM204V/I within the reverse transcriptase domain of the HBV polymerase gene.[12–14] The emergence of a LAM-resistant strain leads to virological breakthrough (VBT) and breakthrough hepatitis (BTH).

Recently, ETV has been demonstrated to exert antiviral efficacy in both NA-naïve and LAM-resistant CHB patients.[15–17] The frequency of ETV resistance has been reported to be 1.2% after 5 years of treatment in NA-naïve CHB patients.[18,19] On the other hand, in switching treatment to ETV for LAM-resistant CHB patients, the cumulative probability of ETV resistance increases.[17,20] After 5 years of treatment, 51% of LAM-refractory patients treated with ETV showed genotypic ETV resistance.[21]

The 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on LAM therapy are summarized in Table 1.[22] Regardless of duration of LAM administration, in cases where HBV DNA is more than 2.6 log copies/mL with BTH, ADV add-on treatment was recommended. In patients treated with LAM for less than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, switching to ETV was recommended. On the other hand, in patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, LAM-continuous treatment was recommended because in these patients LAM resistance might exist, and switching treatment to ETV might cause ETV resistance. However, there is insufficient evidence on whether switching treatment to ETV- or LAM-continuous treatment is better for CHB patients treated with LAM for more than 3 years with HBV DNA of less than 2.6 log copies/mL.

In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment in CHB patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL.

METHODS

Patients

A TOTAL OF 27 CHB patients (mean age 55 ± 9 years, 17 men) from 11 institutions all over Japan (Hokkaido University Hospital, Tohoku University Hospital, Akita City Hospital, Kuramitsu Clinic, Juntendo University Hospital, Chukyo Hospital, Nagoya City University Hospital, Okayama University Hospital, Kawasaki Medical University Hospital, Ehime University Hospital, Shin-Kokura Hospital) were enrolled from April 2008. All the patients were followed at least 6 months after they were diagnosed with CHB. Their characteristics are shown in Table 2. They were treated with LAM (100 mg/day) for more than 3 years (median 50 months, range 36–106 months). Before starting LAM administration, all patients were positive for hepatitis B surface antigen (HBsAg) in serum, abnormal for ALT, detectable for HBV DNA, and were not

Table 1 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on lamivudine treatment

Duration of lamivudine treatment HBV DNA		<3 years	≥3 years
<2.6 log copies/mL, persistently		May be switched to ETV 0.5 mg/day	LAM 100 mg/day
≥2.6 log copies/mL	No BTH†	May be switched to ETV 0.5 mg/day	LAM 100 mg/day
	With BTH	Add on ADV 10 mg/day	Add on ADV 10 mg/day

†After checking for absence of LAM resistance.
ADV, adefovir; BTH, breakthrough hepatitis; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine.

Table 2 Characteristics of LAM continuous group and ETV switch group at baseline

	LAM ($n = 15$)	ETV ($n = 11$)	*P*-value
Male	10	6	NS
Age	53 ± 7	57 ± 7	NS
Duration of LAM administration (month)	59 ± 23	55 ± 18	NS
ALT (IU/L)	33 ± 29	28 ± 22	NS
HbeAg positive	1	1	NS

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; LAM, lamivudine; NS, not significant.

infected with hepatitis C virus and HIV. Patients diagnosed with alcoholism, primary biliary cirrhosis or autoimmune hepatitis were excluded.

Study design

The overview of this study design is shown in Figure 1. Twenty-seven patients treated with LAM for more than 3 years were enrolled, who showed HBV DNA of less than 2.6 log copies/mL at entry. They were randomly divided into two groups by each institution, the LAM-continued group (LAM group) or switching to the ETV group (ETV group). The primary end-points were the incidences of VBT and BTH in each group. VBT was defined as having more than 1 log copies/mL increase of HBV DNA level from the nadir on at least two occasions after initial virological response. BTH was defined as showing abnormal ALT level due to LAM or ETV resistance. All subjects were monitored at least every 3-month intervals. At every visit, routine examination with biochemical (ALT, bilirubin, albumin) and virological (HBV DNA level, hepatitis B e-antigen [HBeAg], anti-HBe) assessments took place. The mean follow-up period was 24 ± 3 months.

This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) on 4 April 2008 as "A randomized trial of lamivudine continuous therapy and entecavir switching therapy for chronic hepatitis B patients treated with lamivudine monotherapy" (no. UMIN000001120).

The study protocol conformed to the Declaration of Helsinki, and was approved by the Committee for Ethics of Medical Experiments on Human Subjects of all the institutions, and written informed consent was obtained from every participant.

Serological and virological markers of HBV

Hepatitis B surface antigen, antibody against HBsAg (anti-HBs), HBeAg and antibody against HBeAg (anti-HBe) were determined using commercially available enzyme immunoassays. HBV DNA was determined by an Amplicor HBV Monitor (Roche Molecular Systems, Branchburg, NJ, USA; detection limit 2.6 log copies/mL)

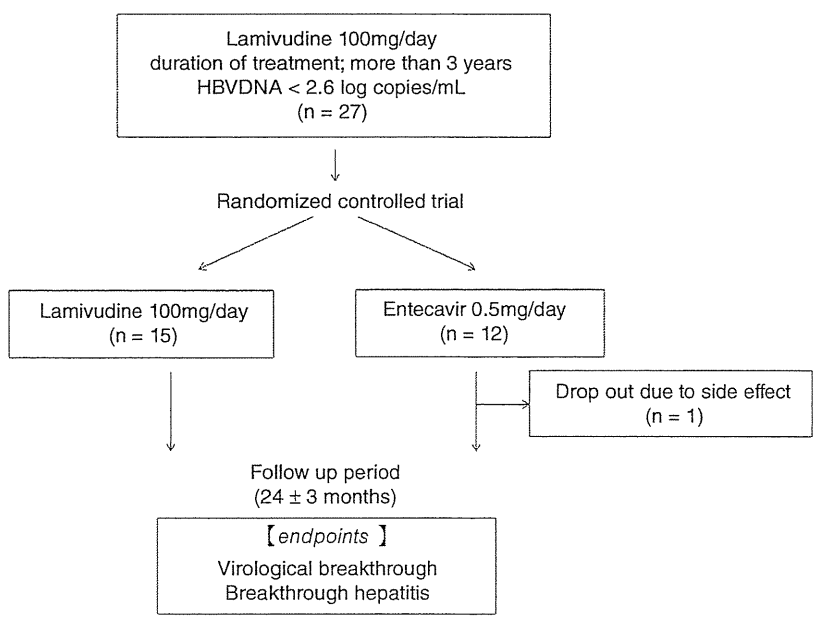

Figure 1 Overview of this study design. Twenty-seven patients treated with lamivudine for more than 3 years whose hepatitis B virus (HBV) DNA was maintained at <2.6 log copies/mL were enrolled. They were randomly divided into two groups by each institution, lamivudine-continued group or switching to entecavir group. We examined the incidence of virological breakthrough and breakthrough hepatitis in each group.