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Original Article

Characteristics and prediction of hepatitis B e-antigen negative hepatitis following seroconversion in patients with chronic hepatitis B

Susumu Morita,[1]* Akihiro Matsumoto,[1]* Takeji Umemura,[1] Soichiro Shibata,[1] Nozomi Kamijo,[1] Yuki Ichikawa,[1] Takefumi Kimura,[1] Satoru Joshita,[1] Michiharu Komatsu,[1] Kaname Yoshizawa[1,2] and Eiji Tanaka[1]

[1]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, and* [2]*Department of Gastroenterology, National Hospital Organization Shinshu Ueda Medical Center, Ueda, Japan*

Aim: We analyzed the characteristics of alanine aminotransferase (ALT) abnormality after achieving hepatitis B e-antigen (HBeAg) seroconversion (SC) and other factors associated with the occurrence of HBeAg negative hepatitis.

Methods: We followed 36 patients with chronic hepatitis B from 3 years prior to at least 3 years after SC (mean, 11.6 years) and examined ALT, hepatitis B virus (HBV) DNA, HB surface antigen, HB core-related antigen (HBcrAg) levels and mutations related to HBeAg SC.

Results: ALT normalization (<31 IU/L for at least 1 year) was primarily observed until 2 years following SC, after which it became more infrequent. We next divided patients into abnormal (≥31 IU/L, $n = 20$) and normal (<31 IU/L, $n = 16$) groups based on integrated ALT level after the time point of 2 years from SC, and considered the former group as having HBeAg negative hepatitis in the present study. Although changes in median levels of ALT and HBcrAg differed significantly between the groups, multivariate analysis showed ALT normalization within 2 years after SC to be the only significant determining factor for this disease ($P = 0.001$). We then assessed the 19 patients whose ALT was normal at 2 years following SC, four of whom developed HBeAg negative hepatitis. Increased levels of HBV DNA ($P = 0.037$) and HBcrAg ($P = 0.033$) were significant factors of potential relevance.

Conclusion: ALT abnormality after 2 years of SC may be evaluated as HBeAg-negative hepatitis. ALT, HBV DNA and HBcrAg levels may be useful in predicting the outcome of patients who achieve HBeAg SC.

Key words: hepatitis B core-related antigen, hepatitis B virus, reactivation, seroconversion

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern with an estimated 350–400 million carriers worldwide. Whereas acute infection in adults is generally self-limiting, that during early childhood develops into persistent infection in most individuals, which can lead to chronic hepatitis and eventually liver cirrhosis and hepatocellular carcinoma (HCC).[1–3] The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT) and HBV DNA, hepatitis B e-antigen (HBeAg) status and estimated immunological status.[4] In the immune tolerance phase, HBeAg is positive, ALT level is normal, histological evidence of hepatitis is absent or minimal, and HBV DNA level is elevated. The chronic hepatitis B phase is characterized by raised ALT and HBV DNA levels. In this phase, the host's immune system initiates a response that results in active hepatitis. In patients who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion (SC) of HBeAg to HBe antibodies and the fall of HBV DNA level result in the disappearance of disease activity despite persisting hepatitis B surface antigen (HBsAg) and low HBV DNA level. The SC of

Correspondence: Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp
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HBeAg marks the transition from the hepatitis phase to the inactive carrier phase, which is generally thought to be a benign course for the HBV carrier, although hepatitis can sometimes reactivate spontaneously.[5]

Patients experiencing HBV reactivation undergo another transition characterized by increases in HBV DNA and ALT levels and disease activity without the reappearance of HBeAg. This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with moderate HBV DNA level occur in this phase.[6,7] It is thought that HBeAg negative chronic hepatitis B is caused by mutant strains of HBV that are unable to produce HBeAg[6,8] and tends to develop into cirrhosis and HCC more frequently than does HBeAg positive chronic hepatitis B.[9–13] Therefore, it is important to identify patients who are likely to develop HBeAg negative hepatitis after HBeAg SC from those who can maintain an inactive carrier phase. In the present study, we evaluated 36 patients with HBeAg SC to examine the effects of host factors and viral factors, including serum quantitative HBsAg, hepatitis B core-related antigen (HBcrAg), HBV DNA, PC (A1896) mutation and BCP mutations (T1762 and A1764) before, during and after SC.

METHODS

Patients

A TOTAL OF 36 patients with sustained HBeAg SC (24 men and 12 women; median age, 38 years [range, 23–65]) were enrolled in this study after meeting the following criteria: (i) follow ups for at least 3 years before and after HBeAg SC; and (ii) serum samples at several time points before, during and after SC available for testing. HBeAg SC was defined as seroclearance of HBeAg with the appearance of anti-HBe that was not followed by HBeAg reversion or loss of anti-HBe. All patients were seen at Shinshu University Hospital from 1985 to 2009. The median follow-up period after SC was 11.6 years (range, 3.2–26.0). HBsAg was confirmed to be positive on two or more occasions at least 6 months apart in all patients. No patients had other liver diseases, such as alcoholic or non-alcoholic fatty liver disease, autoimmune liver disease or drug-induced liver injury. Patients who were complicated with HCC or who showed signs of hepatic failure were excluded from the study. HBV genotype was C in all patients, who were also negative for antibodies to hepatitis C virus and HIV. Nucleoside/nucleotide analog (NUC) therapy was introduced in 14 patients after HBeAg SC on physicians' decision, and then follow up was stopped. No patient was treated with interferon during the study period. ALT, albumin, bilirubin, platelet and other relevant biochemical tests were performed using standard methods.[14] The integration value of ALT after SC was calculated using the method described by Kumada *et al.*[15] (median determination frequency, 4.7/year per person [range, 1.6–13.9]) because a previous study showed integration values to be more meaningful than arithmetic mean values in long-term follow-up cohorts.[16] As guidelines released by the Ministry of Health, Labor and Welfare of Japan advise consideration of antiviral therapy for patients with ALT levels of 31 IU/L or more,[17] an ALT integration value of less than 31 IU/L was defined as normal in this report. Serum samples were stored at −20°C until tested. Liver biopsies were performed by percutaneous sampling of the right lobe with a 14-G needle in eight patients with HBeAg negative hepatitis, as reported previously.[14] All biopsies were 1.5 cm or more in length. Liver histological findings were scored by the histology activity index of Knodell *et al.*[18] The protocol of this study was approved by the ethics committee of our university and was in accordance with the Declaration of Helsinki of 1975. Informed consent was obtained from each patient.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and anti-HBe, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Quantitative measurement of HBsAg was done using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan).[20] The assay had a quantitative range of −1.5 to 3.3 log IU/mL. Serum HBcrAg level was measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan) as described previously.[21] We expressed HBcrAg level in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL. End titers of HBsAg and HBcrAg were determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. HBV DNA level was measured using an Amplicor monitor assay with a dynamic range of 2.6–7.6 log copies/mL.[22] Six major genotypes (A–F) of HBV were determined using the method reported by Mizokami *et al.*,[23] in which the surface gene sequence amplified by polymerase chain reaction was analyzed by restriction fragment length polymorphism.

The PC and BCP mutations of HBV were assessed as previously described. Briefly, the stop codon mutation in the PC region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Roche Diagnostics, Tokyo, Japan) with a sensitivity of 1000 copies/mL. The results were expressed as the percent mutation rate as defined by Aritomi *et al.*[24] The PC mutation was judged to exist when the mutation rate exceeded 50% in the present study because the mutation rate would increase to 100% once surpassing this value.[25] The BCP double mutation was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories) with a detection limit of 1000 copies/mL.[24] The BCP mutation was judged to exist for all classifications of mutant in the present study.

Statistical analysis

Clinical factors were compared between patients with and without HBeAg negative hepatitis after SC using the χ^2-test and Fisher's exact test, and group medians were compared using the Mann–Whitney *U*-test. Receiver-operator curves (ROC) with Youden's index were used to decide each cut-off point for predicting HBeAg negative hepatitis after SC. Differences between the analyzed groups were assessed using Kaplan–Meier analysis and the log-rank test. Sex, age at SC, HBcrAg level, ALT level, HBV DNA level, HBsAg level, PC mutation and BCP mutation were all suspected to be associated with ALT elevation after SC. Factors attaining a *P*-value of less than 20% in univariate analysis were used in multivariate analysis that employed a stepwise Cox proportional hazard model. These included level of serum albumin and platelet count at SC, levels of ALT at 0, 1, 2 and 3 years after SC, and levels of HBcrAg at 1, 2 and 3 years after SC. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan, Tokyo, Japan). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Baseline characteristics of patients

ALL 36 PATIENTS enrolled showed abnormal levels of ALT before SC, with the majority showing normalization around the time of SC. We defined ALT normalization as a decrease in ALT level to less than 31 IU/L for at least 1 year. The change in ratio of patients not achieving normalization over time revealed two distinct phases (Fig. 1): the first was a fast decline phase from 2 years before SC to 2 years afterwards, and the second was a slow decline phase from 2 years after SC to the end of follow up. Normalization of ALT during the fast phase was presumed to be associated with HBeAg SC, which was seen in 53% (19/36) of total patients. Based on this, we analyzed the risk factors associated with ALT abnormality after the time point of 2 years from SC by calculating integrated ALT levels (Fig. 2). We defined patients whose integrated ALT level exceeded 30 IU/L as having HBeAg negative hepatitis in the present study. Serum HBV DNA of over 4.0 log copies/mL was observed in all patients with HBeAg negative hepatitis.

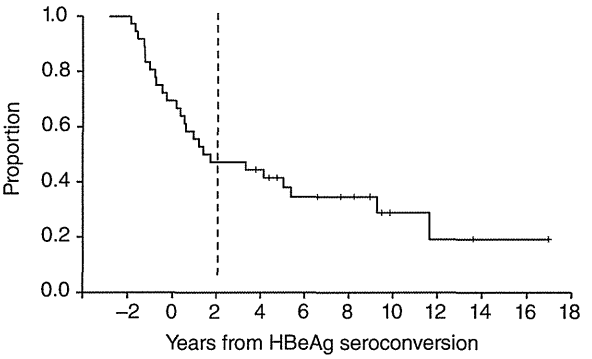

Figure 1 Changes in the proportion of patients with alanine aminotransferase (ALT) abnormality. ALT normalization was defined as ALT level decreasing to lower than 31 IU/L and maintained for at least 1 year. These data reveal two distinct time frames: a fast decline phase around the seroconversion (SC) period until 2 years afterwards, and a slow decline phase from 2 years after SC to the end of follow up. The vertical broken line at 2 years after SC indicates the borderline between the two phases. HBeAg, hepatitis B e-antigen.

Of the 36 patients enrolled, 20 (56%) developed HBeAg negative hepatitis and 16 (44%) did not. ALT normalization within 2 years after SC was significantly less frequent in patients with HBeAg negative hepatitis (Table 1). Median age, sex distribution and follow-up period did not differ between the two groups. Median albumin level tended to be lower in patients with HBeAg negative hepatitis, but only modestly. Eight of 20 HBeAg negative hepatitis patients underwent liver biopsy after SC. All had necroinflammatory activity. Initiation of NUC therapy was more common in the HBeAg negative hepatitis group.

Clinical and virological profiles

Changes in median levels of ALT, HBV DNA, HBsAg and HBcrAg during the course of SC have been compared between patients with and without HBeAg negative

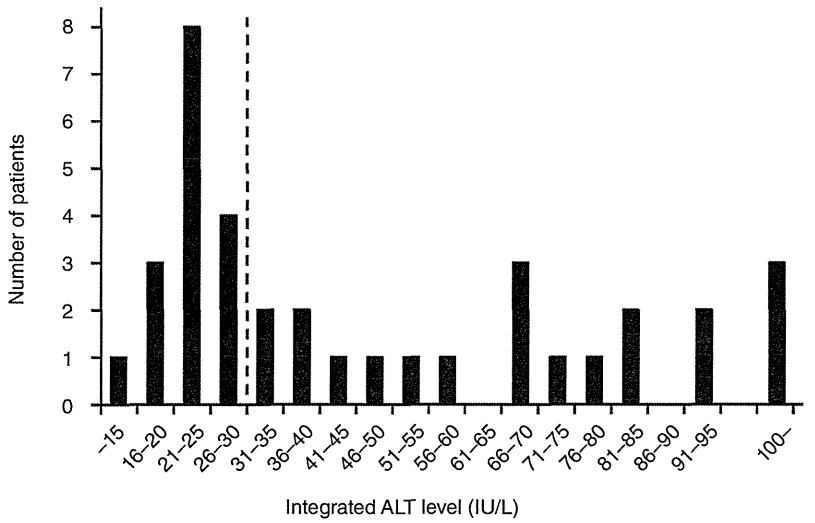

Figure 2 Distribution of integrated alanine aminotransferase (ALT) level from the time point of 2 years after seroconversion (SC) to the end of follow up.

hepatitis in Figure 3. We observed that median ALT level decreased around the time of SC in patients without HBeAg negative hepatitis, but did not in the other group. Overall, median ALT differed significantly between the two groups at the time of SC (43.0 vs 21.5 IU/L; $P = 0.009$) and at 1 (67.0 vs 15.0 IU/L; $P = 0.001$), 2 (52.0 vs 14.5 IU/L; $P < 0.001$) and 3 years (41.5 vs 15.0 IU/L; $P < 0.001$) afterwards (Fig. 3a). Median HBV DNA level decreased similarly in both groups around the time of SC (Fig. 3b). Median HBsAg level was unchanged or minimally decreased in both groups around the time of SC, but was significantly lower in patients with HBeAg negative hepatitis at 1 (3.9 vs 3.2 log IU/mL; $P = 0.025$) and 2 years (3.9 vs 3.2 log IU/mL; $P = 0.045$) before SC and at 2 years (3.7 vs 3.0 log IU/mL; $P = 0.023$) after SC (Fig. 3c). Median HBcrAg level decreased in both groups around the time of SC, but this decline was more gradual in patients with HBeAg negative hepatitis, becoming significantly higher at 1 (5.2 vs 3.9 log U/mL; $P = 0.011$), 2 (4.6 vs 3.5 log

Table 1 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis among total patients

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present ($n = 20$)	Absent ($n = 16$)	
Age at SC (years)†	40 (23–64)	38 (24–65)	0.504
Sex (male : female)	15:5	9:7	0.298
Follow-up period (years)†	10.6 (3.8–26.0)	12.4 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.1 (3.6–4.6)	4.3 (3.7–4.8)	0.030
Bilirubin (mg/dL)†	1.0 (0.4–2.6)	0.8 (0.5–1.3)	0.319
Platelets (/μL)†	13.9 (8.5–24.3)	18.1 (9.6–22.9)	0.187
ALT normalization within 2 years after SC‡	4 (20)	15 (94)	<0.001
Events during follow-up period			
Initiation of NUC therapy‡	12 (60)	2 (13)	0.006
Development of HCC‡	2 (10)	1 (6)	1.000

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

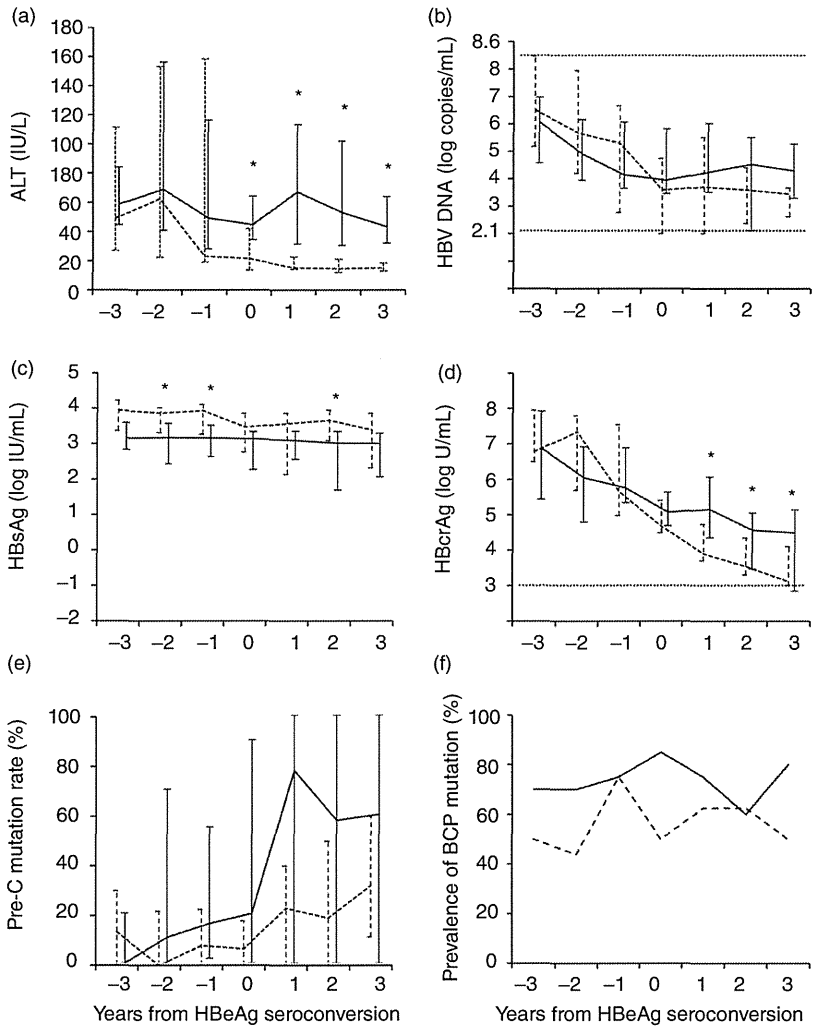

Figure 3 Changes in median levels of serum alanine aminotransferase (ALT) (a), hepatitis B virus (HBV) DNA (b), hepatitis B surface antigen (HBsAg) (c), hepatitis B core-related antigen (HBcrAg) (d) and PC mutation rate (e) are compared between patients with and without the occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis. A similar comparison is made for prevalence of patients with BCP mutations (f). Solid lines indicate patients with HBeAg negative hepatitis ($n = 20$) and broken lines indicate those without ($n = 16$). Data are shown as median values with 25% and 75% ranges at each point for (a–e). Horizontal broken lines in (b) and (d) indicate the upper and lower detection limits of the corresponding markers. *$P < 0.05$.

U/mL; $P = 0.041$) and 3 years (4.6 vs 3.1 log U/mL; $P = 0.016$) after SC (Fig. 3d). PC mutation rate increased similarly in both groups during the course of SC (Fig. 3e), and the prevalence of BCP mutation positive patients remained comparatively high in both groups throughout the study period (Fig. 3f).

All factors that were associated with the occurrence of HBeAg negative hepatitis were evaluated for independence by multivariate analysis. We found that only abnormal level of ALT (≥31 IU/L) at 2 years after SC (odds ratio, 42.0; 95% confidence interval, 4.3–405.4; $P = 0.001$) was an independent predictive factor. Therefore, we examined for factors associated with the occurrence of HBeAg negative hepatitis in the 19 patients whose ALT level had normalized by 2 years after SC. Four (21%) of these patients developed HBeAg negative hepatitis and the remaining 15 (79%) did not. We found no significant differences between the two groups with regard to age at SC, sex or laboratory data (Table 2). We next analyzed HBV DNA, HBsAg and HBcrAg levels at 2 years after SC to see if these factors could discriminate between patients with and without the development of HBeAg negative hepatitis. Cut-off values for each factor were determined by ROC analysis. As shown in Figure 4, serum levels of HBV DNA (7% vs 60%; $P = 0.037$) and HBcrAg (0% vs 44%; $P = 0.033$) were significant factors indicating susceptibility, but HBsAg was not.

Table 2 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis in 19 patients whose ALT levels were normal at 2 years after SC

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present ($n = 4$)	Absent ($n = 15$)	
Age at SC (years)†	41 (30–43)	37 (23–65)	0.549
Sex (male : female)	2:2	8:7	1.000
Follow-up period (years)†	9.1 (8.3–14.1)	12.2 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.3 (3.8–4.3)	4.3 (3.7–4.7)	0.364
Bilirubin (mg/dL)†	1.0 (1.0–1.3)	0.8 (0.5–1.3)	0.083
Platelets (/μL)†	14.9 (13.3–16.4)	16.9 (9.6–22.5)	0.667
Events during follow-up period			
Initiation of NUC therapy‡	3 (75)	2 (13)	0.037
Development of HCC‡	1 (25)	1 (7)	0.386

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

DISCUSSION

ALTHOUGH ACTIVE HEPATITIS usually subsides following HBeAg SC, it recurs in a considerable proportion of patients several years afterwards. Hsu *et al.*[5] followed 283 patients with HBeAg SC for a median follow-up period of 8.6 years and observed that ALT elevation of over twice the upper limit of normal occurred in 94 patients (33%). Of these, 68 (72%) were considered to have HBeAg negative hepatitis B because HBV DNA was detectable without the reappearance of HBeAg at the time of ALT elevation. HBeAg negative hepatitis is a major health concern because its occurrence is closely associated with progression to cirrhosis and development of HCC,[9–12] and thus prediction of its onset is important. Hsu *et al.*[5] found that patients with more frequent acute exacerbations of hepatitis before HBeAg SC and those with cirrhosis at the time of HBeAg SC had a higher risk of developing HBeAg negative hepatitis. Although significant, these factors were insufficient to accurately predict the occurrence of the disease.[26–30] Therefore, we analyzed several additional factors, including HBV DNA, HBsAg and HBcrAg levels, as well as viral mutations that halt HBeAg production.

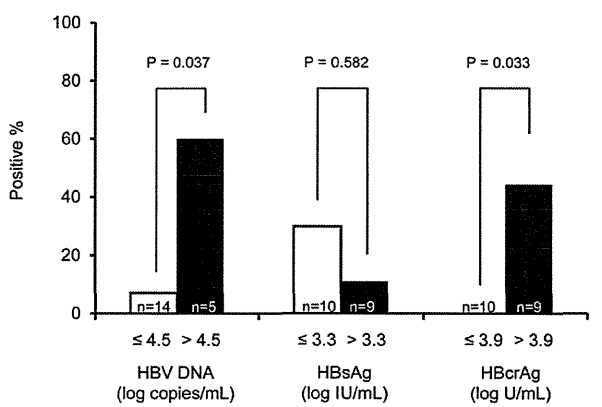

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver-operator curve analysis. HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

In the present study, we found that the majority of patients with HBeAg SC achieved normalization of ALT within 2 years following SC, after which such normalization became relatively rare. Abnormal ALT was determined using the distribution of integrated ALT level from 2 years after SC to the end of follow up, which clearly showed the existence of two groups. We defined patients with an abnormal integrated level of ALT as having HBeAg negative hepatitis because this abnormality tended to persist and was preceded by HBV DNA elevation. Our result also conferred the important realization that ALT abnormality within 2 years after SC may not necessarily indicate the occurrence of HBeAg negative hepatitis, which has a poor prognosis. NUC

therapy was not available for patients with chronic hepatitis B in Japan when our subjects began follow up. Hence, the natural history of SC has been evaluated in this cohort. Follow up stopped in this study when NUC therapy was commenced. Currently, we perform NUC therapy on patients with HBe negative hepatitis based on age and ALT activity, as advised by the Ministry of Health, Labor and Welfare.[17]

Many host and viral factors were also analyzed to predict the occurrence of HBeAg negative hepatitis in the current study. Host factors, including age and sex, did not differ between the groups with and without HBeAg negative hepatitis, but changes in median ALT level around SC clearly differed between the two groups. Specifically, ALT level did not decrease even after SC in patients with HBeAg negative hepatitis, while it normalized during the SC period in those without. Viral factors were analyzed at several time points around SC. Among them, median HBcrAg level clearly differed between the groups; HBcrAg showed a steep decrease around the SC period in patients without HBeAg negative hepatitis, while it exhibited a significantly slower decline in those with. Similarly to earlier reports, median levels of HBV DNA and HBsAg showed some differences between the two groups, but these were not remarkable when analyzed chronologically. Negative results were also seen in the analyses of PC and BCP mutations. Multivariate analysis showed that abnormal ALT level at 2 years after SC was the only significant factor to predict the occurrence of HBeAg negative hepatitis among the factors analyzed. Because patients with normal ALT had maintained that level for at least 1 year, this result may indicate that continuous normalization of ALT is rare in patients with HBeAg negative hepatitis after SC and that ALT abnormality is associated with higher levels of HBcrAg and HBV DNA.

Because ALT level was closely related to the occurrence of HBeAg negative hepatitis, we next analyzed for predictive factors in patients whose ALT level was normal (<31 IU/L) at 2 years after SC. We observed that increased HBV DNA and HBcrAg levels at 2 years after SC were significant factors for predicting the occurrence of HBeAg negative hepatitis, but that HBsAg level was not. Single or combined monitoring use of HBV DNA and HBcrAg levels may therefore be useful to predict the recurrence of hepatitis in patients whose ALT level normalizes following HBeAg SC. However, further studies are required to verify this in the clinical setting.

Whereas HBsAg is a serum marker commonly used for the diagnosis of HBV infection, HBcrAg assays measure serum levels of HBc, HBe and the 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[31] Because the latter assay measures all antigens transcribed from the precore/core gene, it is regarded as core-related.[21] It has been suggested that viral antigen levels, including those of HBsAg and HBcrAg, are differently associated with HBV activity from HBV DNA and ALT levels, and thus are useful for predicting the future activity of hepatitis B. For example, HBcrAg level was seen to predict hepatitis relapse after discontinuation of NUC therapy,[32,33] and HBsAg level has been reportedly associated with the response to pegylated interferon therapy differently from HBV DNA.[34,35] Both antigen levels are believed to be related to intracellular levels of HBV cccDNA. However, it is possible that levels of HBsAg and HBcrAg have different roles in monitoring viral activity because the transcription of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.[1] The serum level of HBcrAg was more useful than that of HBsAg to predict the occurrence of HBeAg negative hepatitis in the present study. This difference may be attributed to the fact that the production of all antigens that constitute HBcrAg is regulated by the same system as that of HBeAg, while the production of HBsAg is not.

Lastly, it is reasonable to presume that the PC and BCP mutations which halt HBeAg production are associated with integrated values of ALT elevation because the disease is essentially caused by HBV containing these mutations.[8,10] However, the prevalence of either mutation did not differ between the groups at any time point during the study. Our results showed that almost all patients had PC and/or BCP mutations, especially after SC, and implied that the existence of these mutations alone was not sufficient for developing ALT elevation. HBV genotype is also closely associated with HBeAg SC,[36] but we could not include genotype as a factor because our entire cohort was genotype C.

A recent review by Papatheodoridis *et al.*[37] showed that histologically significant liver disease is rare in HBeAg negative patients with persistently normal ALT based on stringent criteria and serum HBV DNA of 20 000 IU/mL or less. They suggest that such individuals can be considered as true inactive HBV carriers, who require continued follow up rather than liver biopsy or immediate therapy. On the contrary, liver biopsy samples obtained from eight of our patients with HBeAg negative hepatitis having elevated ALT levels after SC revealed necroinflammatory activity. Hence, it remains controversial if histological findings are important for diagnosis of HBeAg negative hepatitis.

This study has the main limitations of a retrospective design and a small cohort size. However, our findings from careful extended follow up indicate that ALT abnormality after 2 years from SC can be considered to be HBeAg negative hepatitis, and that HBcrAg and HBV DNA levels may be useful for predicting the long-term outcome of patients who achieve HBeAg SC and ALT normalization.

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Research Paper

Efficacy of Lamivudine or Entecavir against Virological Rebound after Achieving HBV DNA Negativity in Chronic Hepatitis B Patients

Tomoo Miyauchi[1,*], Tatsuo Kanda[1,*,✉], Masami Shinozaki[2], Hidehiro Kamezaki[1], Shuang Wu[1], Shingo Nakamoto[1], Kazuki Kato[3], Makoto Arai[1], Shigeru Mikami[4], Nobuyuki Sugiura[5], Michio Kimura[3], Nobuaki Goto[2], Fumio Imazeki[1,6], and Osamu Yokosuka[1]

1. Department of Gastroenterology and Nephrology, Chiba University, Graduate School of Medicine, Chiba (260-8677), Japan.
2. Department of Gastroenterology, Numazu City Hospital, Numazu (410-0302), Japan.
3. Department of Medicine, Social Insurance Funabashi Central Hospital, Funabashi (273-8556), Japan.
4. Department of Gastroenterology, Kikkoman General Hospital, Noda (278-0005), Japan.
5. Department of Gastroenterology, National Hospital Organization Chiba Medical Center, Chiba (260-8606), Japan.
6. Safety and Health Organization, Chiba University, Chiba (263-8522), Japan.

*These authors contributed equally.

✉ Corresponding author: Tatsuo Kanda, M.D., Associate Professor, Department of Gastroenterology and Nephrology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba (260-8677), Japan. Tel: +81-43-226-2086, Fax: +81-43-226-2088; Email: kan-dat-cib@umin.ac.jp.

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Abstract

Nucleos(t)ide analogues (NAs) lead to viral suppression and undetectable hepatitis B virus (HBV) DNA in some individuals infected with HBV, but the rate of virological rebound has been unknown in such patients. We examined the prevalence of virological rebound of HBV DNA among NA-treated patients with undetectable HBV DNA. We retrospectively analyzed 303 consecutive patients [158 entecavir (ETV)- and 145 lamivudine (LAM)-treated] who achieved HBV DNA negativity, defined as HBV DNA < 3.7 log IU/mL for at least 3 months. They were followed up and their features, including their rates of viral breakthrough, were determined. Viral rebound after HBV DNA negativity was not observed in the ETV-group. Viral rebound after HBV DNA negativity occurred in 38.7% of 62 HBe antigen-positive patients in the LAM-group. On multivariate analysis, age was an independent factor for viral breakthrough among these patients (P = 0.035). Viral rebound after HBV DNA negativity occurred in 29.1% of 79 HBe antigen-negative patients in the LAM-group. Differently from LAM, ETV could inhibit HBV replication once HBV DNA negativity was achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical practice.

Key words: Entecavir, HBeAg, HBV DNA, Lamivudine, Virological rebound.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem and one of the risk factors for the development of hepatocellular carcinoma (HCC) worldwide [1,2]. Chronic HBV infection has been linked epidemiologically to the development of HCC for more than 30 years [3]. To date, the mechanism of HBV-related hepatocarcinogenesis is not clear. Although effective vaccine exists for preventing HBV

infection [4], acute liver failure due to HBV or acute exacerbation of chronic hepatitis B is also a life-threatening disease [5,6].

Positivity for hepatitis B e antigen (HBeAg), which in serum indicates active viral replication in hepatocytes, is associated with an increased risk of HCC [7]. Chronic HBV carriers with high-titer viremia are also at increased risk for HCC [8]. The risk for cirrhosis and that for HCC increase significantly with increasing HBV DNA levels [9, 10]. Thus, it cannot be overstated that HBV DNA should be directly suppressed to prevent the development of HCC.

There are several nucleos(t)ide analogues (NAs) for the treatment of chronic hepatitis B [11]. Currently, the Japanese national health insurance system approves lamivudine (LAM) and entecavir (ETV) as first-line therapy for treatment-naïve patients with chronic hepatitis B, although some patients are treated with standard interferon-alfa or peginterferon-alfa-2a [6,12]. In general, LAM, the first oral NA available for the treatment of chronic hepatitis B, is associated with high rates of drug-resistance, with ~76% after 8 years of treatment [13,14]. ETV is found to be superior to LAM from the point of view that ETV is stronger than LAM and that resistance to ETV is rare, about 1.2% after 5 years of ETV treatment [14,15].

The aim of this study was to determine the efficacy and the rates of virological rebound after achieving HBV DNA negativity in the use of ETV or LAM in clinical practice. Our study showed that ETV could inhibit HBV replication if HBV DNA negativity had been achieved, but LAM was unable to inhibit HBV replication even if HBV negativity was achieved in the early phase.

MATERIALS AND METHODS

Patients and Study Design

This was a retrospective analysis comparing the rates of virological rebound in patients treated with ETV versus those in patients treated with LAM. A total of 303 patients were examined from Chiba University Hospital, Chiba, Japan, and 4 affiliated hospitals between the period of January 2000 and December 2011. NAs-naïve chronic hepatitis B patients daily receiving 0.5 mg of ETV (ETV group, N=158) or receiving 100 mg of LAM (LAM group, N=145) with undetectable HBV DNA (< 3.7 log IU/mL) for three months were enrolled. Some of the included patients had been previously reported [12, 16]. All patients had serum hepatitis B surface antigen (HBsAg) detectable for at least 6 months, regardless of their HBeAg status. They were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine (No. 977).

Definition of Virological Rebound of HBV

We defined virological rebound as $\geq$ 3.7 log IU/mL for at least 3 months after achieving undetectable HBV DNA.

Monitoring of HBV DNA, Serum Liver Function Tests and Hematological Tests

The primary outcome of this study was the virological rebound. Patients were followed up at least every 3 months to examine physical status and to monitor liver biochemistry and virology. 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) [17]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [18]. HBV DNA was measured by transcription-mediated amplification (TMA) assay, COBAS Amplicor HBV Monitor assay, or COBAS TaqMan (Roche Diagnostics, Branchburg, NJ, USA). The clinical efficacy of NAs was assessed as the proportion of patients achieving HBV DNA negativity, defined as an HBV DNA level of < 3.7 log IU/mL.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Differences were evaluated by Student's *t*-test, chi-square test, or Fisher's exact test. $P < 0.05$ was considered statistically significant. Variables with $P < 0.05$ at univariate analysis were retained for multivariate logistic-regression analysis. For all tests, two-sided P-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel-statistics program for Windows, version 7 (SSRI, Tokyo, Japan).

RESULTS

A total 303 patients were recruited into either the ETV group (n = 158) or the LAM group (n = 145), with a follow-up period of 33.7 $\pm$ 11.3 months (28.6 $\pm$ 11.3 months or 39.3 $\pm$ 31.4 months, respectively). Baseline demographic and laboratory data are summarized in Table 1. There were no differences in age, gender, HBV DNA, alanine aminotransferase (ALT) levels, ultrasound findings/presence of cirrhosis, and periods from the initial administration of ETV or LAM to

undetectable HBV DNA, between the ETV and LAM groups, although the proportion of HBeAg-positive patients in the ETV group (55%) tended to be higher than that in the LAM group (44%).

Virological Rebound

The patient flow and outcome are summarized in Figure 1. We excluded 9 patients, whose HBeAg status at baseline was unknown, from this analysis. When comparing the baseline characteristics of patients according to HBeAg status, HBeAg-positive patients were younger, had higher ALT levels and HBV DNA levels, and less cirrhotic findings by ultrasound than HBeAg-negative patients (Table 2). The period from the initial administration of ETV or LAM to the determination of undetectable HBV DNA in the HBeAg-negative group tended to be shorter than that in the HBeAg-positive group (Table 2).

In the ETV group, none of the patients had virological rebound during the follow-up periods. In the LAM group, 24 and 23 patients of 62 HBeAg-positive and 79 HBeAg-negative patients at baseline, respectively, developed evidence of virological rebound. In the 24 HBeAg-positive patients at baseline with virological rebound, 9, 8, 3, 1, 2, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$, and details unknown, respectively. In the 23 HBeAg-negative patients at baseline with virological rebound, 10, 8, 3, 0, 1, and 1 had virological rebound at ≤ 1, $1 \sim \leq 2$, $2 \sim \leq 3$, $3 \sim \leq 4$, $4 \sim \leq 5$ and details unknown, respectively. Baseline characteristics of patients treated with ETV or LAM according to HBeAg status are shown in Table 3. In the ETV group, the period from the initial administration of ETV to the determination of undetectable HBV DNA in the HBeAg-negative group was the same as that in the HBeAg-positive group (Table 3). In the LAM group, the period from the initial administration of LAM to undetectable HBV DNA in the HBeAg-negative group was shorter than that in the HBeAg-positive group (Table 3). In the HBeAg-positive patients, the period from the initial administration to undetectable HBV DNA in the ETV group was shorter than that in the LAM group (Table 3).

Predictors of Virological Rebound in Patients treated with LAM

To clarify the predictors of virological rebound in patients treated with LAM, we compared the pretreatment factors between patients with and without virological rebound according to HBeAg status (Table 4A & 4B). Univariative analysis showed that age, HBV DNA, ALT levels and the period from the initial administration of LAM to the determination of undetectable HBV DNA in HBeAg-positive patients contributed to the occurrence of virological rebound (Table 4A). Factors significantly associated with virological rebound in HBeAg-positive patients treated with LAM by univariate analysis were also analyzed by multivariate logistic regression analysis. Virological rebound was attained independently of age in HBeAg-positive patients treated with LAM (Table 4C). In HBeAg-negative patients, no significant factors contributing to virological rebound could be found (Table 4B).

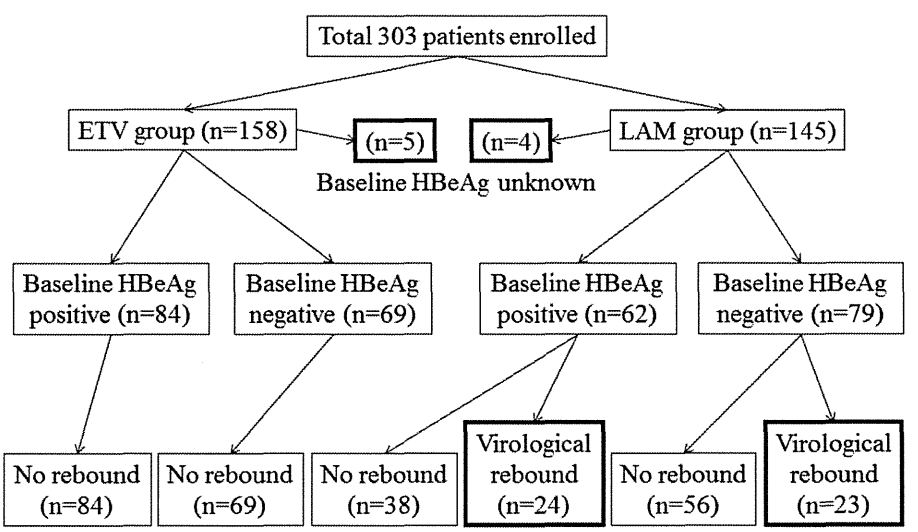

Figure 1. Study design and patient flow for both groups.

Table 1. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM).

	Total	ETV group	LAM group	*P*-values
Number	303	158	145	
Age (years)	51 ± 12	51 ± 12	50 ± 12	*N.S.*
Gender (male)	205	101	104	*N.S.*
HBeAg (+)	146	84	62	*0.079*
HBV DNA (log IU/mL)	6.5 ± 1.5	6.6 ± 1.7	6.4 ± 1.3	*N.S.*
ALT (IU/L)	203 ± 280	187 ± 290	220 ± 266	*N.S.*
US: Cirrhosis (+)	113	56	57	*N.S.*
Periods to undetectable HBV DNA (months)	10.0 ± 18.2	8.5 ± 11.9	11.8 ± 23.3	*N.S.*

Data are expressed as mean ± SD. ETV group, patients receiving 0.5 mg of ETV daily; LAM group, patients receiving 100 mg of LAM daily; *P*-values, *P*-values between ETV and LAM groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 2. Baseline characteristics of patients according to HBeAg status.

HBeAg	Positive group	Negative group	*P*-values
Number	146	148	
Age (years)	46 ± 12	55 ± 11	*< 0.001*
Gender (male)	101	97	*N.S.*
HBV DNA (log IU/mL)	7.2 ± 1.1	5.8 ± 1.4	*< 0.001*
ALT (IU/L)	257 ± 332	156 ± 211	*0.002*
US: Cirrhosis (+)	41	70	*< 0.001*
Periods to undetectable HBV DNA (months)	11.0 ± 18.1	7.4 ± 14.4	*0.063*

Data are expressed as mean ± SD. *P*-values, *P*-values between HBeAg-positive and HBeAg-negative groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 3. Baseline characteristics of patients treated with entecavir (ETV) or lamivudine (LAM) according to HBeAg status.

	ETV group		LAM group	
HBeAg	Positive	Negative	Positive	Negative
Number	84	69	62	79
Age (years)	48 ± 12	56 ± 11*	44 ± 11 ##	54 ± 11**
Gender (male)	53	45	48	52**
HBV DNA (log IU/mL)	7.5 ± 1.1	5.7 ± 1.5*	6.9 ± 1.1$	5.9 ± 1.3**
ALT (IU/L)	219 ± 325	159 ± 246	309 ± 334	154 ± 174**
US: Cirrhosis (+)	25	29	16	41
Periods to undetectable HBV DNA (months)	8.3 ± 10.5	7.3 ± 11.0	15.0 ± 24.7$$	7.5 ± 16.9#

Data are expressed as mean ± SD. HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *$P < 0.001$, compared to HBeAg-positive of ETV group; **$P < 0.001$ and #$P = 0.034$, compared to HBeAg-positive of LAM group; ##$P = 0.041$, $P = 0.001$ and $$P = 0.027$, compared to HBeAg-positive of ETV group.

Table 4A. Predictors of virological rebound in patients treated with lamivudine (LAM). (A) Comparison of HBeAg-positive patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	38	23	
Age (years)	42 ± 11	49 ± 11	*0.019*
Gender (male)	30	17	*N.S.*
HBV DNA (log IU/mL)	6.9 ± 1.2	6.8 ± 0.9	*N.S.*
ALT (IU/L)	379 ± 377	196 ± 205	*0.037*
US: Cirrhosis (+)	7	9	*N.S.*
Periods to undetectable HBV DNA (months)	20.6 ± 29.1	4.1 ± 3.1	0.009

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4B. (B) Comparison of HBeAg-negative patients with or without virological rebound by univariate analysis.

Virological rebound	No	Yes	*P-values*
Number	56	22	
Age (years)	54 ± 11	54 ± 10	*N.S.*
Gender (male)	40	12	*N.S.*
HBV DNA (log IU/mL)	5.9 ± 1.4	5.9 ± 1.0	*N.S.*
ALT (IU/L)	163 ± 179	137 ± 163	*N.S.*
US: Cirrhosis (+)	30	11	*N.S.*
Periods to undetectable HBV DNA (months)	7.3 ± 14.8	3.1 ± 2.1	*N.S.*

Data are expressed as mean ± SD. *P*-values, *P*-values between patients with or without virological rebound groups; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; US, ultrasound findings; *N.S.*, no statistically significant difference.

Table 4C. (C) Factor associated with virological rebound among HBeAg-positive patients treated with LAM by multivariate analysis.

Factor	Category	Odds ratio	95% CI	*P-value*
Age ≤ 44.5 (years)	(+/-)	0.222	0.0547-0.9023	0.0354

DISCUSSION

To date, there is not much data regarding virological rebound after achieving HBV DNA negativity in the use of ETV or LAM. A recent report supported the merit of the change from LAM to ETV [14]. This study concluded that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM.

The present study revealed that ETV could suppress HBV replication after achieving HBV DNA negativity, although additional longer follow-up studies will be needed. On the other hand, LAM could not suppress HBV replication even after achieving HBV DNA negativity (Figure 1), although most cases with virological rebound were observed within 2 years of the start of LAM medication. We could not check the emergence of YMDD motif mutations [19] in all of the cases because the present study was performed as part of regular clinical practice. Of 2 of the HBeAg-positive patients at baseline with virological rebound, one showed YVDD motif (50%). In 4 of the HBeAg-negative patients at baseline with virological rebound, one YVDD motif (25%) and three YIDD motifs (75%) were seen. Virological rebound may not mean the emergence of NA-resistance mutations [12].

We do not know the reason why virological rebound was attained independently of age in HBeAg-positive patients treated with LAM. HBeAg to anti-HBe antibody seroconversions were found in 20 and 11 patients with and without virological rebound, that is, the HBeAg to anti-HBe antibody seroconversion rates were similar in the two groups (data not shown), although the number of study patients seemed small in the present study. Further studies might be needed. In any event, it might be important to consider the LAM-to-ETV switch in HBeAg-positive patients treated with LAM, although some of our patients in the LAM group remained HBV-negative throughout the observation period.

In the present study, 95.3% (122 of 128), 82.3% (14 of 17) and 89.2% (25 of 28) had an adherence rate >90% [16] in ETV-treated, LAM-treated with virological rebound and LAM-treated patients without virological rebound, respectively. These results supported our previous study that viral breakthrough associated with poor adherence could be a more important issue in the treatment with especially stronger NAs, such as ETV [12,16], although we cannot ensure durable HBV negativity after NAs are discontinued. We and others reported that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection [16,20,21]. Of interest, the virological rebound with the use of LAM seemed unrelated to the HBeAg status, suggesting that it was dependent on resistant mutation.

Recently, other effective antiviral therapies such as peginterferon [22,23] and tenofovir [24,25] were reported to be useful for the control of HBV infection. These drugs might also be candidates for treating virological rebound. Fung et al. [14] reported that prior optimal viral suppression with ETV did not confer any significant advantage for patients who switched to LAM. Our results also supported the previous studies that ETV was much more efficient than LAM [26-29]. In conclusion, ETV could inhibit HBV replication if HBV DNA negativity had been achieved. In contrast, LAM could not inhibit HBV replication even if HBV negativity was achieved in the early phase. Attention should be paid to these features in clinical

practice.

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CONFLICT OF INTEREST

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ABBREVIATIONS

ALT: alanine aminotransferase; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LAM: lamivudine; NA: nucleos(t)ide analogue.

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Hepatology Research

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Original Article

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

Yoshihito Uchida, Jun-ichi Kouyama, Kayoko Naiki, Kayoko Sugawara, Mie Inao, Nobuaki Nakayama and Satoshi Mochida

Department of Gastroenterology and Hepatology, Faculty of Medicine, Saitama Medical University, Moroyamacho, Japan

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

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

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

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

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

INTRODUCTION

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

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

Correspondence: Dr Satoshi Mochida, Department of Gastroenterology and Hepatology, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan. Email: smochida@saitama-med.ac.jp
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showing nucleotide differences of more than 8% from each other.[10] The frequency of each genotype among isolates from patients with HBV infection differs depending on the geographic area of the world;[11] genotype A HBV strains prevail in Africa, Europe and India, while genotype B and C strains are frequent in Asia, and genotype E strains in sub-Saharan Africa. On the other hand, genotype D strains are distributed all over the world, and genotype F and H strains are found exclusively in Central and South America. It has been demonstrated that the clinical features of patients with HBV infection, including their responses to antiviral therapies, differ depending on the genotype of the viral strain causing the infection,[12] suggesting that identification of the HBV genotype causing the infection, in addition to determination of the serum HBV DNA levels and mutation profile of the viral genome is crucial to establish the therapeutic strategy in patients with both acute and chronic liver diseases caused by HBV.

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

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

METHODS

Patients and experimental designs

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

DNA extraction and direct nucleotide sequencing of the HBV strains

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

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

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

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

Whole-genome cloning of HBV strains

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

SimPlot analysis and construction of the phylogenetic tree

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

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

RESULTS

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

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

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

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

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

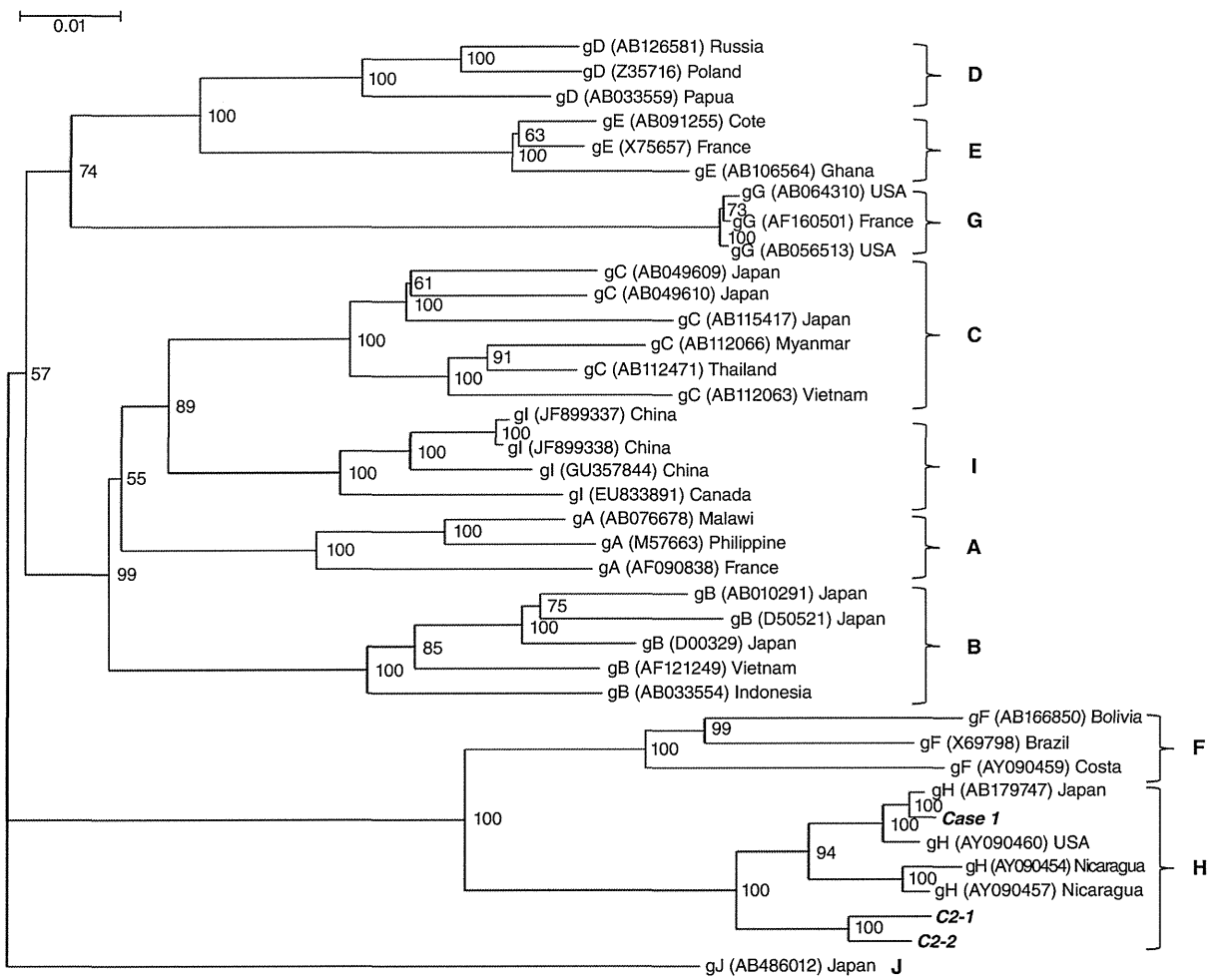

Figure 1 A phylogenetic tree constructed based on the full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 and case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides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

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

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

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

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

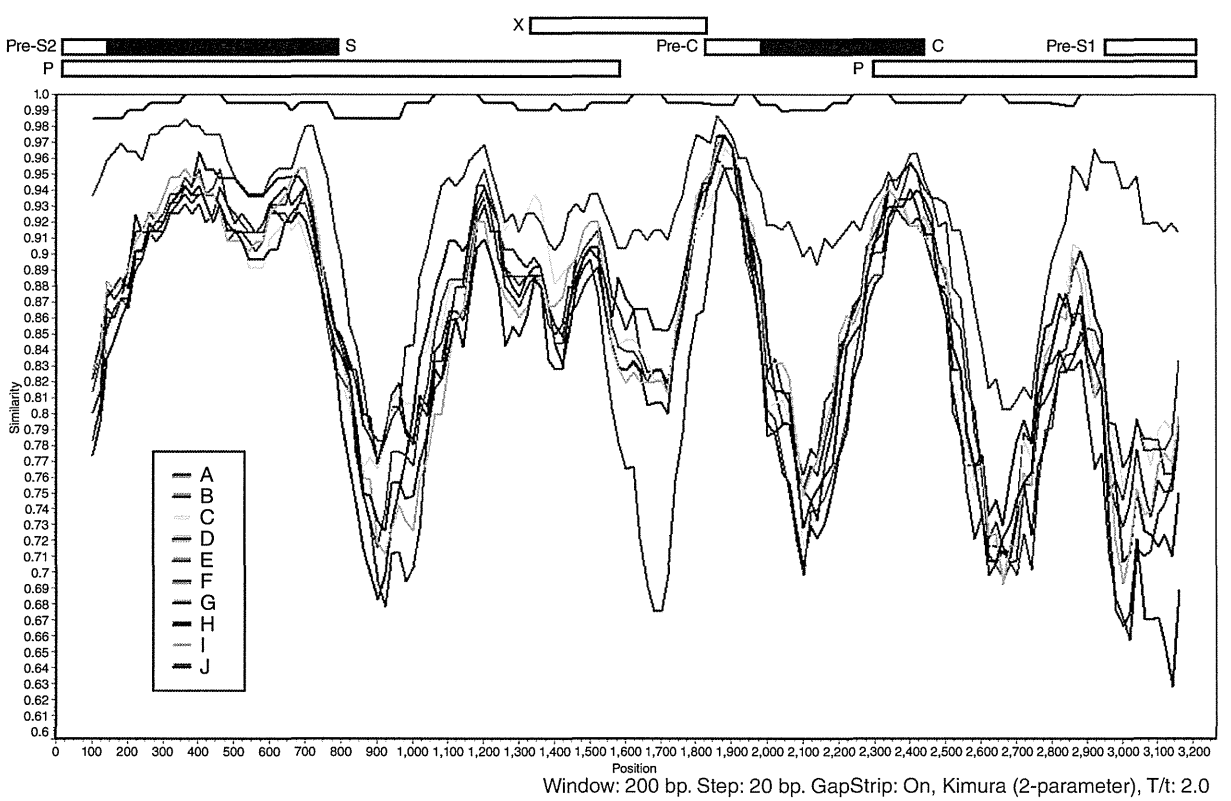

Figure 2 Nucleotide similarity comparison of a full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size and gap-stripped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