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Review Article

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B

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Nucleoside/nucleotide analogs (NUC) can lead to rapid reduction in hepatitis B virus (HBV) DNA levels in blood and normalization of alanine aminotransferase levels in many patients. They also provide histological improvement which results in a reduction in liver carcinogenesis. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment. One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period. For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently. We performed research to investigate characteristics of the course after discontinuation of treatment and definition of hepatitis relapse and estimate the relapse rate. "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the study results. Because the guidelines are written in Japanese, we explain them in English as a review article.

Key words: discontinuation of treatment, hepatitis B virus cccDNA, hepatitis B, hepatitis relapse, nucleoside/nucleotide analog

INTRODUCTION

BECAUSE NUCLEOSIDE/NUCLEOTIDE analogs (NUC) that have been recently introduced to treat hepatitis B strongly inhibit proliferation of hepatitis B virus (HBV), they can lead to rapid reduction in HBV DNA levels in blood and normalization of alanine aminotransferase (ALT) levels in many patients.[1] They also provide histological improvement which results in a reduction in liver carcinogenesis[2,3] and can be administrated p.o. with few side-effects, so they are widely used in clinical practice. However, it is difficult to completely remove viruses even by NUC and there are some problems such as emergence of resistant strains and hepatitis relapse resulting from discontinuation of treatment.[4] One of the reasons for this is that NUC reduce the HBV DNA level in blood but have almost no effects on the HBV cccDNA level in hepatocyte nuclei, which are the origins of HBV replication, and HBV cccDNA remains for a long period.[5]

For treatment with NUC in patients with hepatitis B, it is considered that NUC should not be easily discontinued because discontinuation often results in hepatitis relapse. However, it has not been clearly revealed when and how hepatitis relapses occur after discontinuation. Although some patients do not experience hepatitis relapse after discontinuation of NUC, or experience only mild relapse and finally achieve a stable condition, it has not been established how to identify such patients efficiently.

We performed research funded by a Health and Labor Sciences Research Grant to investigate characteristics of the course after discontinuation of treatment, definition of hepatitis relapse and estimation of relapse rate.[6] "Guidelines for avoiding risks resulting from discontinuation of NUCs 2012" is summarized based on the

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study results.[7] The guidelines do not always recommend discontinuation of NUC. We determined them to be referred to if it is necessary to consider discontinuation of NUC due to various reasons.

SERUM MARKERS REFLECTING AMOUNT OF HBV CCCDNA IN HEPATOCYTES

THE REPLICATION PROCESS of HBV in hepatocytes is shown in Figure 1. HBV is an enveloped DNA virus containing a relaxed circular DNA genome converted into a cccDNA episome in the nucleus of infected cells.[8–11] These cccDNA molecules serve as transcriptional templates for production of viral RNA that encode both viral structural and non-structural proteins. Hepatitis B surface antigen (HBsAg) is translated from 2.1-kb and 2.4-kb mRNA. On the other hand, hepatitis B core antigen (HBcAg), p22cr antigen (p22crAg)[12] and hepatitis B e-antigen (HBeAg) are translated from 3.5-kb mRNA which also serves as pregenome RNA. HBeAg is secreted into the blood stream as a secretion protein, and p22crAg forms genome negative core particles. HBcAg forms nucleocapsid particles by incorporating pregenome RNA. Once the pregenome RNA is reverse transcribed to DNA, the particles are enveloped with lipid layer containing HBsAg and then secreted into blood stream as virions.[9,10] When the reverse transcriptation is inhibited by NUC, virus particles with RNA genome are secreted instead of those with DNA genome.[13,14]

Hepatitis B virus cccDNA is a stable molecule like chromosomal DNA which can be barely destroyed by DNase in natural conditions. Because NUC are inhibitors of reverse transcriptase, they have no direct effect on reducing intrahepatic cccDNA levels. Therefore, reactivation of HBV replication which originates from HBV cccDNA and incidental hepatitis relapse occurs when NUC are discontinued.

It is generally considered that HBV cccDNA levels in hepatocytes are well correlated with the proliferative potential of HBV;[5] serum markers reflecting the cccDNA level are suggested to be useful as clinical indicators. Serum level of HBV DNA correlates well with intrahepatic level of HBV cccDNA in the natural course but not under NUC treatment. NUC reduce serum level of HBV DNA rapidly by inhibiting the reverse transcription, but this inhibition does not reduce the cccDNA level.[5] On the other hand, serum levels of HBsAg and hepatitis B core-related antigen (HBcrAg) have been reported as markers reflecting cccDNA levels in hepatocytes even under NUC treatment.[15–18] HBcrAg assay measures all antigens coded by precore/core genome simultaneously which include HBcAg, HBeAg and p22crAg, and has been reported to be useful for predicting clinical outcomes of patients who were treated with NUC.[6,18–23] HBsAg level has received attention recently as a new marker and has been reported to be efficient in prediction of treatment effects by interferon and others.[15,16]

AIMS OF THESE GUIDELINES

THESE GUIDELINES AIM to identify patients with a higher possibility of successful discontinuation or patients who should continue treatments and avoid

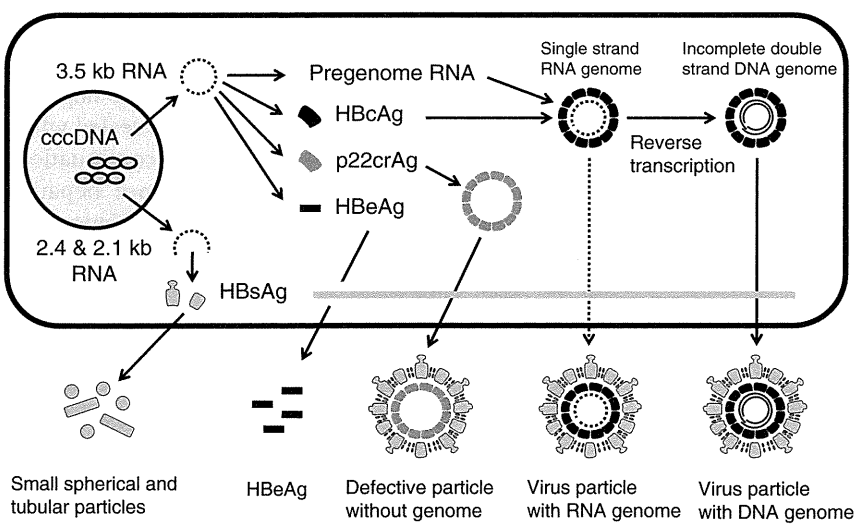

Figure 1 Replication process of hepatitis B virus (HBV) which originates from HBV cccDNA molecules pooled in nucleus of hepatocyte. HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; p22crAg, p22cr antigen.

risks resulting from discontinuation of NUC as much as possible by establishing indicators for follow up after discontinuation (Appendix 1-I). Successful discontinuation in the guidelines is defined as final achievement of the inactive carrier state with ALT level of less than 30 IU/L and HBV DNA level in blood of less than 4.0 log copies/mL. These criteria were defined in compliance with the guidelines for treatment of chronic hepatitis B in Japan.[24] It is known that patients in the inactive carrier state show no progression of hepatic diseases and a reduction in the carcinogenic rate[25,26] and the criteria are considered to be appropriate.

REQUIREMENTS TO AVOID RISK OF DEVELOPING SEVERE HEPATITIS RESULTING FROM RELAPSE

WE ARE CURRENTLY unable to predict hepatitis relapse after discontinuation of NUC with sufficient accuracy. Therefore, we reviewed the risk of developing severe hepatitis and established requirements to prevent severe hepatitis (Appendix 1-II).[27] The presence of understanding the risks of hepatitis relapse and severe hepatitis by both doctors and patients as well as the availability of a follow-up system after discontinuation and appropriate treatment for relapse are the basic essential requirements. Considering that patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis can easily develop severe hepatitis and have higher risks of carcinogenesis in the future, we determined that those patients should not easily discontinue NUC.

ASSESSMENT OF PROLIFERATIVE POTENTIAL OF HBV AND CONDITIONS TO REDUCE THE RELAPSE RISK

IT HAS BEEN experienced that patients with insufficient reduction of HBV DNA level or with HBeAg positive at the time of discontinuation of NUC can develop hepatitis relapse at higher rates after discontinuation. The tendency was also confirmed scientifically in our study.[6] The cut-off value of HBV DNA level to predict hepatitis relapse was 3.0 log copies/mL by receiver operating characteristic (ROC) analysis. Almost all patients with higher HBV DNA levels or were HBeAg positive relapsed within a year while nearly 30% of patients with HBV DNA levels less than 3.0 log copies/mL and without HBeAg were in a stable condition for a long period (Fig. 2). Based on these results, we included sufficient reduction in HBV DNA levels and

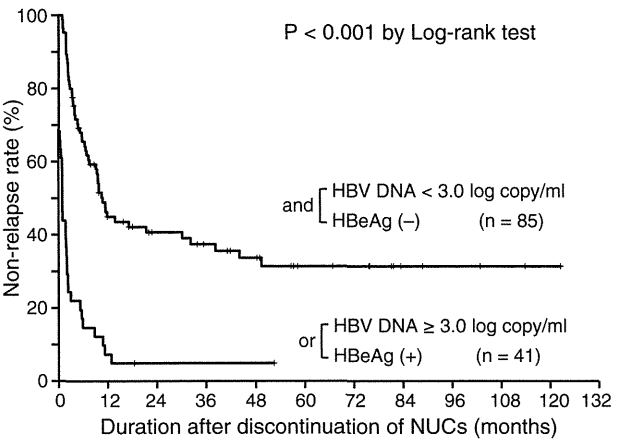

Figure 2 Comparison of non-relapse rates using Kaplan–Meier method between 41 patients with serum hepatitis B virus (HBV) DNA not lower than 3.0 log copies/mL or with hepatitis B e-antigen (HBeAg) and 85 patients with serum HBV DNA lower than 3.0 log copies and without HBeAg at the time of nucleoside/nucleotide analog (NUC) discontinuation.

HBeAg negativity in requirements for discontinuation. We determined the reference range of sufficient reduction in HBV DNA levels in the actual guidelines not to be less than 3.0 log copies/mL but to be negative by real-time polymerase chain reaction (PCR) in consideration of safety.

Factors relating to hepatitis relapse after discontinuation were analyzed in the population except for patients who were obviously predicted to relapse after discontinuation, or those with HBV DNA levels of not less than 3.0 log copies/mL or were HBeAg positive. The following factors were calculated to be significant: duration of treatment period of NUC; HBsAg levels at the time of discontinuation; and HBcrAg levels at the time of discontinuation. Because the cut-off value in duration of treatment period was calculated as 16 months, we overestimated and established that NUC should be discontinued more than 2 years after the initial administration in the guidelines.[6]

Two cut-off values were suggested from the results of the ROC analysis for the HBsAg and HBcrAg levels at the time of discontinuation (Fig. 3): 1.9 and 2.9 log IU/mL for the HBsAg level and 3.0 and 4.0 log U/mL for the HBcrAg level, respectively. Based on this, HBsAg and HBcrAg levels were scored as shown in Appendix 1-III and three groups – low-risk, medium-risk and high-risk – were determined. The percentage of prediction success was 80–90% in the low-risk group, approximately 50% in the medium-risk group and 10–20% in the high-risk

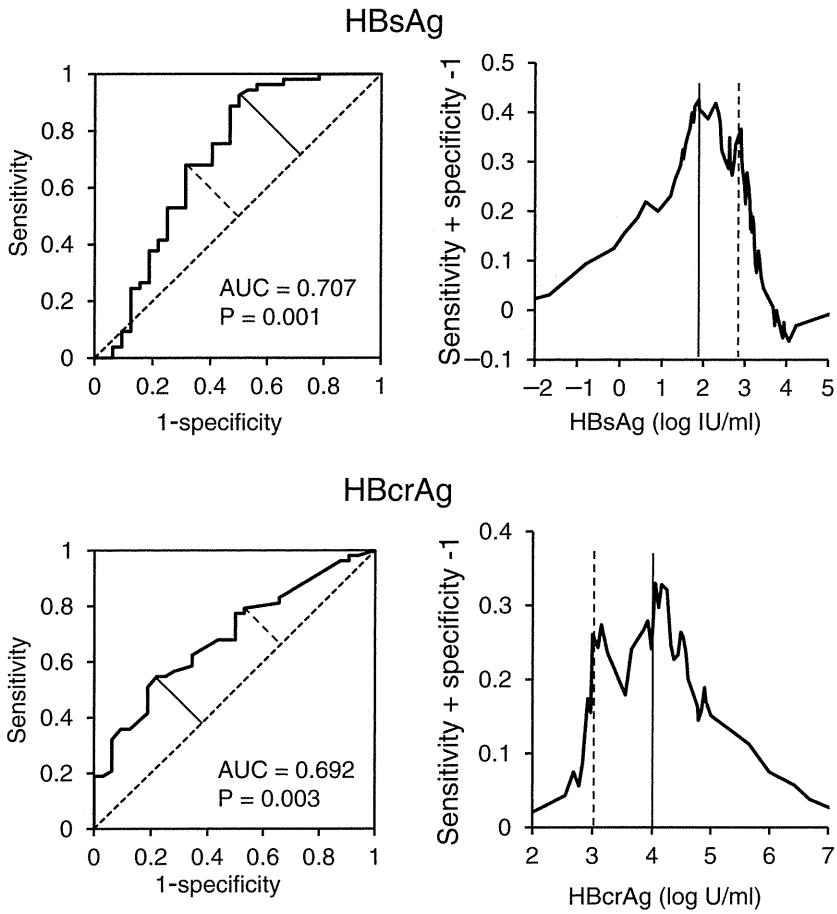

Figure 3 Receiver operating characteristic (ROC) analysis of hepatitis B surface antigen (HBsAg) and HB core-related antigen (HBcrAg) levels to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg levels. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points. AUC, area under the ROC.

group (Fig. 4). In further investigation of factors relating to hepatitis relapse in each group, no factors were newly found in the low- and medium-risk groups but age was a significant factor in the high-risk group. Although the percentage of prediction success rate is low in the high-risk group (10–20%), it resulted in slightly higher rates of 30–40% with those patients younger than 35 years old.[6] It was interesting to find that the combination of HBsAg and HBcrAg levels were useful in preparing these guidelines for discontinuation. Because productions of HBsAg and HBcrAg are regulated by different promoter and enhance systems of HBV genome, their clinical values vary.

FOLLOW-UP METHOD AFTER DISCONTINUATION AND CONDITIONS FOR RETREATMENT

FOLLOW-UP AFTER DISCONTINUATION of NUC includes periodical measurement of HBV DNA levels (real-time PCR) and ALT levels. This study revealed that

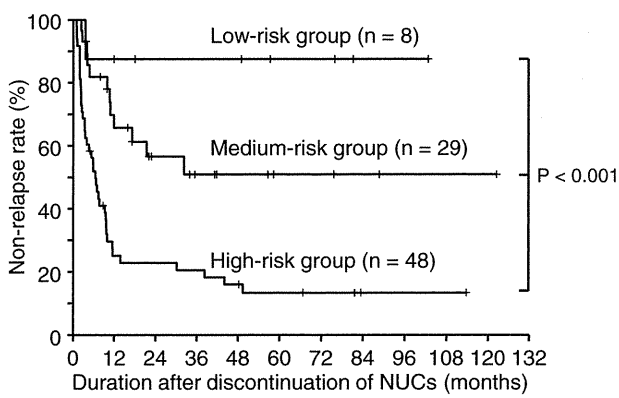

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen and HB core-related antigen levels at the time of nucleoside/nucleotide analog (NUC) discontinuation.

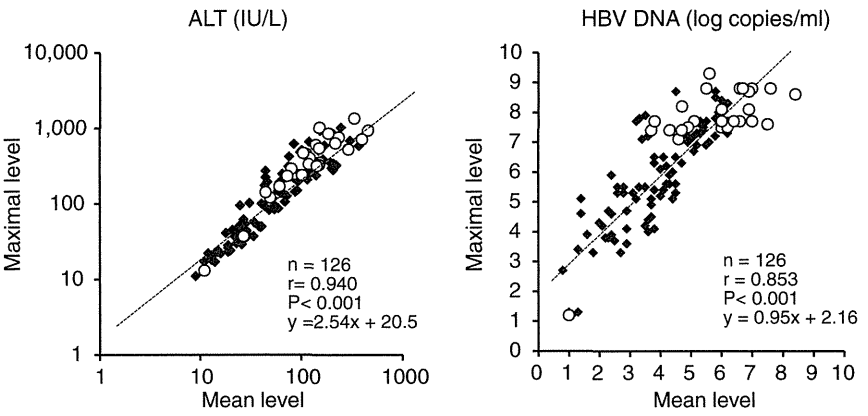

Figure 5 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (left) and hepatitis B virus (HBV) DNA (right) after discontinuation of nucleoside/nucleotide analog (NUC). Open circles indicate patients with detectable hepatitis B e-antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

relapse after discontinuation occurs mostly within 1 year, gradually decreases after 1 year and rarely occurs after the first 3 years of discontinuation.[6] Therefore, we determined it necessary to pay attention especially to relapse immediately after discontinuation. In particular, we determined that it is desirable to follow up patients by blood tests at every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.

One of the important points is what the definition of hepatitis relapse is and how to follow up after discontinuation. Transient abnormalities in the ALT level or the HBV DNA level may be observed in approximately two-thirds patients who would finally achieve the inactive carrier state. Therefore, even if the ALT or HBV DNA levels show mild elevations, it is possible to follow up without retreatment. However, no criteria have been identified about when to discontinue follow up and start retreatment. We assessed the transitions of ALT levels and HBV DNA levels after discontinuation of NUC by the mean and maximum values to identify the criteria. From this assessment, a strong correlation was shown between the mean and the maximum value in both (Fig. 5).[6] Results of the ROC analysis revealed that the mean ALT of 30 IU/L corresponded to the maximum ALT of 79 IU/L and the mean HBV DNA of 4.0 log copies/mL corresponded to the maximum HBV DNA of 5.7 log copies/mL. Patients with ALT values of not less than 80 IU/L after discontinuation are highly likely to show a mean value of more than 30 IU/L and not assumed to finally meet the criteria for successful discontinuation. Similarly, patients with HBV DNA value of not less than 5.8 log copies/mL after discontinuation are most likely to show a mean value of more than 4.0 log copies/mL and not assumed to meet the criteria for successful discontinuation. Based on these results, we established the condition that patients with ALT value of not less than 80 IU/L or HBV DNA level of not less than 5.8 log copies/mL are less likely to finally achieve the inactive carrier state and should be considered for retreatment with NUC. It is considered that NUC can be discontinued more efficiently and specifically in this condition. Physicians can use more severe criteria at their own discretion in consideration of safety. Less strict criteria also can be used, but it is recommended that the treatment should be done under a certain policy and do not follow the treatment without any aims.

KEY POINTS AND FUTURE ISSUES

THIS MAY BE the first guideline for discontinuation of NUC. Most of the data used in this guideline are retrospective and some points remain unsolved. Over 90% of the patients enrolled had genotype C and over 90% of cases were treated with lamivudine until discontinuation.[6] Therefore, key points and future issues are summarized in Appendix 1-V. This guideline provides information to support physicians to decide NUC discontinuation timing but physicians should actually consider for each patient whether NUC can be discontinued or not because long-term prognosis after NUC discontinuation is not yet clear enough and patients' wishes and physicians' decision need to be prioritized. When NUC cannot be successfully discontinued, one of the options is re-administration of NUC. However, it has not been investigated whether re-administration of NUC results in the emergence and development of resistant strains. Further, it is not resolved which NUC should be given when re-administration is required. The consent of patients will be necessary on these points.

One of the issues to be investigated in the future is to improve accuracy in predicting hepatitis relapse after discontinuation. Investigations on the following approaches are suggested: higher sensitivity HBV DNA, HBV RNA,[13,14] HBV genotypes and HBV genetic mutations. Because these guidelines were prepared based on retrospective studies, it is necessary to validate them with prospective studies. In addition, how to actively discontinue NUC by sequential treatment with interferon also should be included as an important issue to be investigated.

Three kinds of NUC are available now in Japan. Lamivudine was the first NUC introduced into Japan in 2000. Adefovir dipivoxil is used mainly for patients with lamivudine resistance. Entecavir is now recommended as the first-choice NUC. Over 10 years have passed since the first NUC became available in Japan and this is the first full-scale guideline for NUC discontinuation. Although this guideline may not be completely sufficient and needs further investigations, this is the first step leading to a better one in the future.

ACKNOWLEDGMENTS

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APPENDIX

Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs 2012

I. Aims of these guidelines

IN TREATMENT WITH nucleoside/nucleotide analogs (NUC) in patients with chronic hepatitis B, it is an important treatment goal to aim at drug-free status by discontinuation of NUC. However, discontinuation of NUC often results in hepatitis relapse which may become severe. Sufficient consideration must be given to the risk in case of discontinuation.

Hepatitis B surface antigen (HBsAg) negativity is the goal of treatment with NUC, but it cannot be always achieved easily. Therefore, discontinuation may be considered even if HBsAg remains positive. These guidelines aim to discontinue NUC in such conditions and finally achieve the inactive carrier state (alanine aminotransferase [ALT] <30 IU/L and hepatitis B virus [HBV] DNA level in blood <4.0 log copies/mL).

It is currently unknown which of the two options for NUC, discontinuation or continuation, is effective on life prognosis or liver carcinogenesis. We established these guidelines to be referred in case of considering discontinuation due to various reasons. We aimed to identify patients with a high possibility of successful discontinuation or patients who should inversely continue the treatment and establish indicators for follow up after discontinuation to avoid risks resulting from discontinuation of NUC as much as possible.

II. Requirements to avoid risk of developing severe hepatitis resulting from relapse

The following requirements are determined for discontinuation to previously assume and avoid the risk of developing severe hepatitis.

1. Both the doctor and the patient fully understand the risk of a high frequency of hepatitis relapse that may become severe.
2. It is possible to follow up as well as to treat appropriately in case of relapse. (Involvement of a specialist is recommended.)
3. The patient has mild hepatic fibrosis with good hepatic functional reserve and will not easily develop severe hepatitis in relapse. (NUC should not be discontinued in patients with hepatic cirrhosis or chronic hepatitis with progressed fibrosis similar to cirrhosis.)

III. Assessment of proliferative potential of HBV and conditions to reduce the relapse risk

1. Requirements for discontinuation of nucleoside/nucleotide analogs.
   Almost all patients with high proliferative potential of HBV will relapse after discontinuation. It is essential not to discontinue NUC in these patients and the requirements were determined as follows: (i) HBV DNA level in blood is negative (real-time PCR) at the time of discontinuation; and (ii) hepatitis B e-antigen (HBeAg) level in blood is negative at the time of discontinuation.
2. Condition for duration of treatment period of NUC.
   Because short-term treatment with NUC can easily result in relapse, it is recommended to meet the following condition: more than 2 years after the initial administration of NUC.
3. Assessment of relapse risk by scoring of viral antigen levels.
   For the patients who meet the requirements for discontinuation (HBV DNA negative and HBeAg negative at the time of discontinuation), the HBsAg level and the HBcrAg level at the time of discontinuation can be scored to predict the relapse risk by the following three groups based on the total score. This risk prediction aims to determine whether NUC should be discontinued or not by reference to it to reduce the relapse risk.

HBsAg levels at the time of discontinuation	Scores	Hepatitis B core-related antigen (HBcrAg) levels at the time of discontinuation	Scores
<1.9 log IU/mL (<80 IU/mL)	0	<3.0 log U/mL	0
1.9–2.9 log IU/mL (80–800 IU/mL)	1	3.0–4.0 log U/mL	1
≥2.9 log IU/mL (≥800 IU/mL)	2	≥4.0 log U/mL	2

Relapse risk	Total scores	Percentage of prediction success	Assessment
Low-risk group	0	80–90%	Discontinuation can be considered. It is essential to pay attention to relapse because some patients of low risk may develop hepatitis relapse.
Medium-risk group	1–2	~50%	Discontinuation can be considered depending on the situation. Further consideration is needed about conditions and the way to discontinue in the future.
High-risk group	3–4	10–20%	Continuous treatment is recommended. However, patients under 35 years old show a relatively higher rate of successful discontinuation of 30–40%.

IV. Follow-up method after discontinuation and conditions for retreatment

1. HBV DNA levels (real-time PCR) and ALT levels must be periodically measured after discontinuation of NUC to pay attention to HBV proliferation and hepatitis relapse resulting from proliferation.
2. Relapse after discontinuation is mostly observed within 1 year and then gradually decreases. It is rare to relapse after the first 3 years. Therefore, it is necessary to pay attention to relapse immediately after discontinuation. In particular, patients should be followed up by blood tests every 2 weeks up to 16 weeks after discontinuation and every 4 weeks after 16 weeks.
3. Transient abnormalities in ALT levels or HBV DNA levels may be observed in approximately two-thirds of patients who successfully discontinued NUC and would finally achieve the inactive carrier state. Therefore, even if the ALT level or the HBV DNA level shows mild elevations, it is possible to keep following up without retreatment. However, patients who meet the following condition are less likely to finally achieve the inactive carrier state and should be considered for NUC retreatment.

Condition to consider retreatment with NUC
ALT ≥80 IU/L or HBV DNA ≥5.8 log copies/mL after discontinuation

V. Key points and future issues

1. The status differs in each patient. Objectives and significance also differ by patient. Thus, doctors must determine whether NUC should be discontinued or not in consideration of those conditions. In case of considering discontinuation, it is recommended to consult with a specialist of hepatic diseases.
2. In case of retreatment with NUC due to hepatitis relapse after discontinuation, it is unknown whether it results in higher emergence of strains resistant to NUC or not compared with patients without discontinuation.
3. Because HBV carriers rarely experience hepatitis relapse even in the inactive carrier state (HBV DNA <4.0 log copy/mL and ALT <30 IU/L), they must be followed up after successful discontinuation. Liver carcinogenesis also requires follow up.
4. The followings are included in future issues; improvement of accuracy in the criteria for discontinuation of NUC; investigation of the criteria used in these guidelines in a prospective study; and investigation of the way to actively discontinue NUC using sequential treatment with interferon.

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Original Article

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

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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

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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.

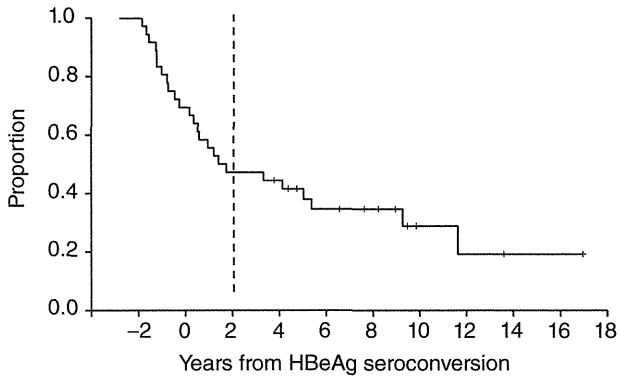

Figure 1 Changes in the proportion of patients with alanine aminotransferase (ALT) abnormality. ALT normalization was defined as ALT level decreasing to lower than 31 IU/L and maintained for at least 1 year. These data reveal two distinct time frames: a fast decline phase around the seroconversion (SC) 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

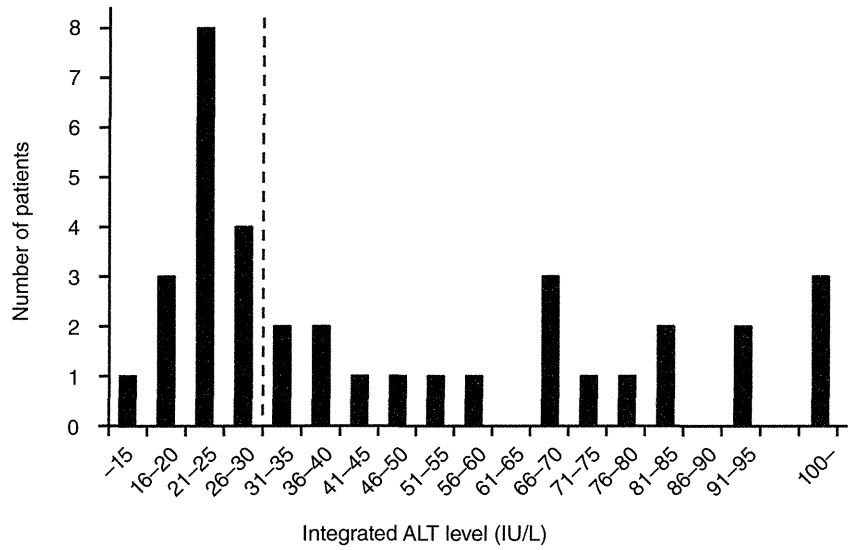

Figure 2 Distribution of integrated alanine aminotransferase (ALT) level from the time point of 2 years after seroconversion (SC) to the end of follow up.

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.

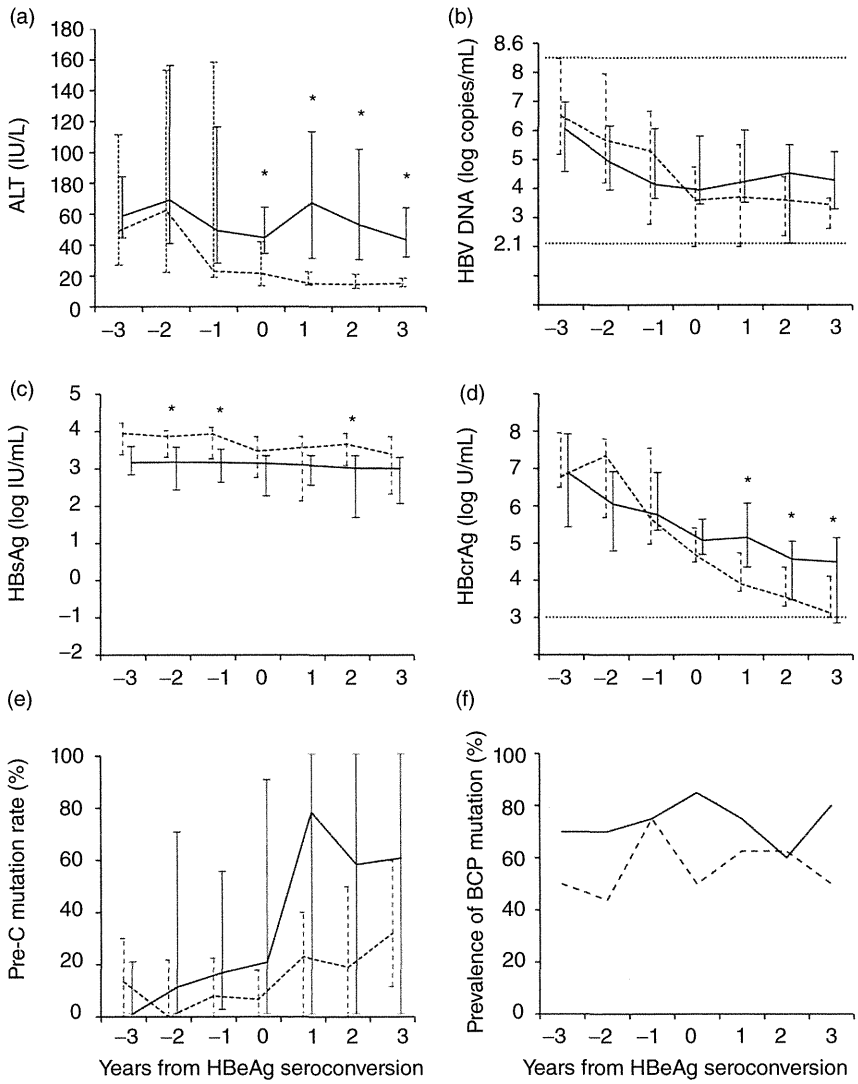

Figure 3 Changes in median levels of serum alanine aminotransferase (ALT) (a), hepatitis B virus (HBV) DNA (b), hepatitis B surface antigen (HBsAg) (c), hepatitis B core-related antigen (HBcrAg) (d) and PC mutation rate (e) are compared between patients with and without the occurrence of hepatitis B 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.

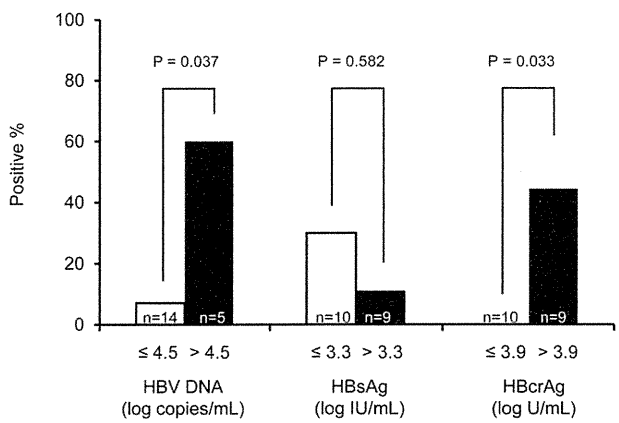

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver–operator curve analysis. HBcrAg, hepatitis B core-related 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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Impact of Peginterferon Alpha-2b and Entecavir Hydrate Combination Therapy on Persistent Viral Suppression in Patients with Chronic Hepatitis B

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The ideal approach to treat chronic hepatitis B remains controversial. This pilot study aimed to evaluate the effectiveness of peginterferon (PEG-IFN) α-2b and entecavir hydrate (ETV) as a combination therapy for patients with chronic hepatitis B, particularly in the context of virological response and the reduction of intrahepatic covalently closed circular DNA (cccDNA). A total of 17 patients with hepatitis B virus (HBV) genotype C were enrolled in this study. All subjects were treated with this combination therapy for 48 weeks and observed for an additional 24 weeks. All patients underwent liver biopsy before and after the therapy period. Changes in cccDNA levels and liver histology were monitored between biopsies. Among the 11 patients who exhibited pretherapy hepatitis B e antigen (HBeAg), 8 (73%) showed evidence of HBeAg seroconversion by the end of the follow-up period. Serum HBV DNA levels decreased by 5.2 and 3.3 log copies/ml (mean) by the end of the therapy and follow-up periods, respectively. In addition, intrahepatic cccDNA decreased significantly to 1.4 log copies/μg (mean) by the end of the therapy period. Among the 11 patients who did not experience viral relapse, only 2 (18%) exhibited high levels of cccDNA (>4.5 log copies/μg) by the end of the treatment period. In contrast, all relapsed subjects exhibited significantly higher levels of cccDNA than subjects who did not relapse ($P = 0.027$). The combination regimen is a promising approach to treat chronic hepatitis B and may achieve significant reduction in serum HBV DNA and intrahepatic cccDNA. ***J. Med. Virol. 85:987–995, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B virus; peginterferon α-2b; entecavir hydrate; combination therapy; covalently closed circular DNA

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) occurs commonly and is associated with increased risk of cirrhosis and the development of hepatocellular carcinoma [Lai et al., 2003]. This type of hepatitis is a worldwide health problem, but achievement of sustained suppression of HBV replication by conventional antiviral agents is sometimes difficult because of the unique nature of HBV replication. For example, after it infects hepatocytes, linear HBV DNA transforms into covalently closed circular DNA (cccDNA), which represents the intracellular HBV template [Newbold et al., 1995; Arase et al., 2002]. Various nucleotide analogues, such as lamivudine (LVD) [Dienstag et al., 1995; Lai et al., 1998; Leung

Additional supporting information may be found in the online version of this article.

Conflicts of interest: The authors declare no conflicts of interest.

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et al., 2001], adefovir dipivoxil (ADV) [Hadziyannis et al., 2003; Marcellin et al., 2003], entecavir (ETV) [Chang et al., 2006; Lai et al., 2006], and tenofovir disoproxil fumarate have been approved as treatments to suppress HBV replication. However, the mechanism of action of nucleotide analogues is limited to reverse transcription and does not decrease the quantity of cccDNA; the cessation of this type of treatment frequently results in viral relapse. In addition, long-term use of nucleotide analogues is hampered by considerable emergence of resistant mutants [Yuen et al., 2001, 2007; Lok et al., 2003].

On the other hand, peginterferon (PEG-IFN) is known to reduce the quantity of cccDNA, presumably by inducing cytotoxic T lymphocytes (CTL), which destroy infected hepatocytes [Wursthorn et al., 2006]. Despite its rapid anti-viral effects, PEG-IFN monotherapy alone is less effective than nucleotide analogues [Wu et al., 1990]. From this perspective, a combination approach of immune modification (PEG-IFN) and blockade of reverse-transcription (nucleotide analogues) conceivably may compensate for the antiviral shortcomings inherent to each as a monotherapy, and thus, appears promising for achieving long-term suppression of viral replication that continues after the completion of antiviral therapy. However, a relatively low amount of data has been generated on the combined use of PEG-IFN and nucleotide analogues to treat chronic hepatitis B.

The present study evaluated prospectively the effectiveness of combined PEG-IFN α-2b and ETV treatment in patients with chronic hepatitis B. ETV was selected among several nucleotide analogue options because it exerts the strongest antiviral activity and has the lowest incidence of resistant mutation [Chang et al., 2006; Lai et al., 2006]. A systematic and comprehensive analysis was conducted to establish an HBV profile based on several related markers, which included serial measurements of HBeAg, anti-HBe antibody, serum HBV DNA and RNA, and intrahepatic cccDNA, and histological evaluations throughout the clinical course. This report offers profound insight on the antiviral impact of PEG-IFN and ETV combination therapy in patients with chronic hepatitis B.

METHODS

Patient Characteristics and Study Design

A total of 17 patients with chronic hepatitis B received combination therapy of PEG-IFN α-2b (PegIntron, Schering-Plough; Kenilworth, NJ) and ETV (Baraclude, Bristol-Myers Squibb; Princeton, NJ) between February 2008 and April 2010 in Kinki University Hospital or Osaka Red Cross Hospital. All patients were serum-positive for hepatitis B surface antigen (HBsAg) for at least 6 months. Additional inclusion criteria included serum HBV DNA levels greater than 5 log copies/ml at a measurement obtained 4 weeks before the first biopsy, serum alanine aminotransferase (ALT) levels greater than 31 IU/ml, and no treatment with nucleic acid analogues or IFN within 3 years prior to study initiation. Subjects with hepatitis C virus, hepatitis D virus, human immunodeficiency virus, a history of hepatocellular carcinoma, autoimmune hepatitis, primary biliary cirrhosis, or decompensated cirrhosis were excluded from the study. Patient characteristics are listed in Table I.

After patients provided informed consent, both drugs were administered throughout the 48-week treatment phase. Treatment consisted of daily doses of oral ETV (0.5 mg) and weekly subcutaneous injection of PEG-IFN α-2b (1.5 μg/kg body weight). PEG-IFN α-2b was selected for the IFN component of therapy because its dosing strategy is adjusted for body weight. For histological analysis and assessment of intrahepatic viral DNA, liver biopsy samples were obtained before and after the 48-week treatment period. All biopsies were performed percutaneously. The 48-week treatment phase was followed by a 24-week treatment-free phase. The protocol included ETV monotherapy after the 24-week follow-up phase for subjects who had relapsed after they received the full combination treatment of PEG-IFN α-2b and ETV. The schematic representation of schedule is shown in Supplementary Figure 1. The Medical Ethics Committee of Kinki University School of Medicine and Osaka Red Cross Hospital approved this study.

Response to Therapy

The virological response to combination therapy was defined as a decrease in serum HBV DNA

TABLE I. Characteristics of the Patients at Baseline

	HBeAg-positive (n = 11)	HBeAg-negative (n = 6)	Overall (n = 17)
Age (year, mean ± SD)	45 ± 12	50 ± 11	47 ± 12
Gender (male, no.; %)	9 (82)	4 (67)	13 (76)
Serum HBV DNA (log copies/ml, mean ± SD)	7.8 ± 1.3	6.8 ± 1.3	7.5 ± 1.4
ALT (IU/l, mean ± SD)	191 ± 161	93 ± 77	157 ± 143
Necroinflammation score (mean ± SD)	5.9 ± 2.3	5.5 ± 3.5	5.8 ± 2.7
No. of cases with F score >3 (%)	9 (82)	3 (50)	12 (71)
cccDNA (log copies/μg, mean ± SD)	5.8 ± 1.1	4.8 ± 0.5	5.4 ± 1.0

All patients analyzed were Asian with HBV of genotype C.
SD, standard deviation; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; IU, international unit.