

Original Article

Long-term outcomes of add-on adefovir dipivoxil therapy to ongoing lamivudine in patients with lamivudine-resistant chronic hepatitis B

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Aim: Add-on adefovir dipivoxil (ADV) therapy has been a standard rescue treatment for patients with lamivudine (LAM)-resistant chronic hepatitis B, but the overall benefits of long-term add-on ADV therapy are still limited. The aim of this study was to evaluate the long-term efficiency of add-on ADV treatment and to explore predictive factors associated with it.

Methods: A total of 158 patients with LAM-resistant chronic hepatitis B were included in this retrospective, multicenter, nationwide study in Japan. After confirming LAM resistance, ADV was added to LAM treatment. Three types of events were considered as outcomes: virological response, hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization. Virological response was defined as serum hepatitis B virus (HBV) DNA levels of less than 3 log copies/mL. Baseline factors contributing to these outcomes were examined by univariate and multivariate analyses.

Results: The median total duration of ADV treatment was 41 months (range, 6–84). The rate of virological response was

90.8% at 4 years of treatment; HBeAg clearance and ALT normalization were achieved by 34.0% and 82.7%, respectively, at the end of follow up. Each outcome had different predictive factors: baseline HBV DNA and albumin level were predictive factors for virological response, history of interferon therapy and ALT level for HBeAg clearance, and sex and baseline albumin level for ALT normalization.

Conclusion: Long-term add-on ADV treatment was highly effective in LAM-resistant chronic hepatitis B patients in terms of virological and biochemical responses. Lower HBV replication and lower albumin level at baseline led to better outcomes.

Key words: adefovir dipivoxil, alanine aminotransferase normalization, chronic hepatitis B, hepatitis B e antigen clearance, lamivudine resistance, virological response

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Received 29 January 2012; revision 11 April 2012; accepted 15 April 2012.

INTRODUCTION

CHRONIC HEPATITIS B (CHB) is an important cause of morbidity and mortality worldwide.^{1–3} The main goals of therapy in CHB patients are to prevent the development of liver failure, due to subsequent liver

cirrhosis, and the emergence of hepatocellular carcinoma (HCC). All of these are likely to be achieved by suppressing hepatitis B virus (HBV) replication, which thereby leads to remission of liver disease.⁴

Lamivudine (LAM) treatment has been used to prevent the progression of CHB and the development of HCC.⁵ LAM is an effective and well-tolerated treatment for patients with CHB, but it has the major limitation of drug-resistant mutants arising at a rate of 16–32% during the first year of treatment and increasing by 15% with each additional year of treatment.^{6–8} The widespread use of LAM monotherapy in CHB patients before introduction of entecavir, which is more potent, has progressively increased the numbers of patients with LAM-resistant HBV mutant strains.

Adefovir dipivoxil (ADV) has been reported to be effective in suppressing HBV replication and approved as a standard therapy in LAM-resistant patients.^{9,10} However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients are still limited. The aims of this study were to evaluate the long-term efficiency of ADV add-on treatment based on virological response (VR), hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization, and to explore the predictive factors associated with ADV add-on treatment.

METHODS

Patients

A TOTAL OF 158 patients (109 males and 49 females) were included in this retrospective study from 21 medical centers of the National Hospital Organization (NHO) in Japan. Both HBeAg positive and negative CHB patients were considered eligible if they had documented LAM resistance confirmed by detection of mutations in the YMDD motif of the reverse transcriptase gene of the virus (genotypic resistance), elevated serum HBV DNA levels (≥ 4 log copies/mL and/or >1 log copies/mL elevation from the LAM on-treatment nadir) and/or elevated serum ALT levels (>40 IU/L). Patients were excluded if they had decompensated liver cirrhosis, HCC at the initiation of ADV, or if they had co-infections (human immunodeficiency virus, hepatitis C virus) or other concomitant liver diseases such as autoimmune liver disease. Patients with no available clinical, biochemical, serological or virological data at baseline as well as every 6 months during treatment were also excluded.

Patient records were extracted from each institutional database. All data were labeled with their respective

institution and pooled. In total, 20 variables were examined to evaluate the long-term responses. The following variables were used as baseline factors: sex, HBeAg status, liver disease, age, body mass index, duration of LAM monotherapy, history of interferon (IFN) therapy, serum HBV DNA level, aspartate aminotransferase (AST), ALT, γ -glutamyl transpeptidase (γ -GTP), platelet (PLT) counts, and total bilirubin (T-Bil), albumin (Alb), prothrombin time (PT) and α -fetoprotein (AFP) levels. All were measured at the initiation of ADV therapy. For each variable, it was not used in the stepwise analysis if missing data accounted for more than 10% of the cases.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients and approval of this study was obtained from the NHO.

Statistical analysis

Three types of events were considered as outcomes: (i) VR; (ii) HBeAg clearance; and (iii) ALT normalization. VR was defined as serum HBV DNA levels of less than 3 log copies/mL by a quantitative real-time polymerase chain reaction assay, and ALT normalization was defined as a decrease in ALT levels to less than 31 IU/L during the on-treatment follow-up period. Baseline factors that could have an impact in the prediction of VR, HBeAg clearance as well as ALT normalization were investigated. The predictive value of several baseline parameters for VR was evaluated using time-to-event methods, because of the varying length of follow up. Time-to-event analysis was carried out using Kaplan–Meier estimates to draw cumulative incidence curves, compared by log-rank tests, as well as using univariate and multivariate Cox's proportional hazards models in combination with stepwise regression analysis. Factors contributing to HBeAg clearance and ALT normalization during ADV add-on therapy were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A stepwise variable selection procedure was used for variables that were at least marginally associated with the outcomes.

Covariates included in these analyses were binomial or continuous variables. Quartile analysis was initially performed separately for each continuous variable to make the decision regarding cut-off points. At first, we divided each continuous data into quarters to convert numerical values into four categorical values. Then, we estimated whether there was a regular trend among these four ordinal categorical data with outcome and selected a cut-off point among the 25th, 50th and 75th percentiles so that these variables could be appropriately

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

Baseline characteristics	HBeAg positive <i>n</i> = 99	HBeAg negative <i>n</i> = 59
Age (years)	51.6 (25.5–80.4)	59.3 (33.3–76.9)
Sex (male/female)	73/26	36/23
Liver disease (CH/cirrhosis)	79/20	38/21
Duration of LAM therapy (months)	29.8 (6.0–82.4)	39.3 (8.4–91.2)
History of IFN therapy (months)	39	15
HBV DNA (log copies/mL)	7.5 (2.1–7.6)	5.9 (2.1–7.6)
≤6	15	31
6–7.5	38	21
>7.5	46	7
Total bilirubin (mg/dL)	0.8 (0.3–5.2)	0.9 (0.41–3.7)
AST (IU/L)	60 (18–959)	60 (17–464)
ALT (IU/L)	80 (11–697)	86 (17–724)
γ-GTP (IU/L)	38 (12–325)	53 (10–740)
Albumin (g/dL)	4.3 (2.6–5.4)	4.3 (2.7–5.2)
Platelet count (×10 ⁴ /mm ³)	15.5 (3.7–50.0)	12.3 (1.7–33.2)

Continuous variables are expressed in median (range) and categorized variables in number.

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

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

RESULTS

IN THIS RETROSPECTIVE nationwide analysis of add-on ADV therapy in Japan, a total of 158 patients were enrolled from 2003–2010, consisting of 99 HBeAg positive and 59 HBeAg negative patients. Table 1 summarizes the baseline characteristics of the study popula-

tion; most were HBV genotype C. At the time of this analysis, the median total duration of ADV treatment was 41 months (range, 6–84), and the median time of LAM monotherapy, prior to initiation of ADV, was 34 months (range, 6–91).

VR

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

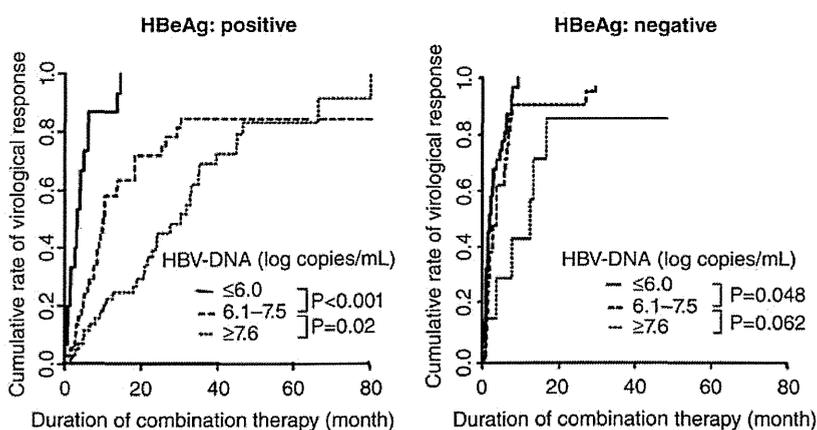


Figure 1 Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response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

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

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

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

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

HBeAg clearance or HBeAg seroconversion

Among 99 HBeAg positive patients, HBeAg clearance and seroconversion were achieved by 17.1% and 11.0% at month 24, by 24.3% and 14.3% at month 36 of treatment, and by 34.0% and 16.0% by the end of follow up, respectively. Except for a history of IFN

therapy (OR = 2.46, 95% CI = 0.94–6.6, $P = 0.047$), none of the other baseline variables were significantly associated with HBeAg clearance, according to the results of the univariate logistic regression analysis. In multivariate analysis, serum ALT level and history of IFN therapy were independent predictive factors for HBeAg clearance (Table 3). No patient experienced a reappearance of HBeAg or reverse seroconversion to HBeAg positive status during this treatment.

Normalization of ALT levels

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

DISCUSSION

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

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

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

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

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

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

ment. This is the first report to show the significance of baseline Alb levels as we used a time-to-event method for large populations, which is a more powerful and informative method to assess the association of factors to time-to-event outcomes.

The rate of HBeAg clearance was 34% at the end of follow up, which was compatible with previous observations.^{10,18} According to the results of multivariate analysis, IFN history was the strongest predictor of HBeAg clearance. Of the 37 patients, 17 (46%) who had previously received IFN therapy achieved HBeAg loss, suggesting that previous IFN therapy might have some immune modulatory effect on the ongoing combination therapy. IFN-induced HBeAg loss has been reported to be durable after a follow-up period of 4–8 years.^{19–21} In addition, baseline ALT levels were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline ALT levels to be the strongest predictor of HBeAg seroconversion in response to IFN therapy²² as well as nucleos(t)ide analog therapy.^{23,24}

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

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

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

ACKNOWLEDGMENTS

THIS STUDY WAS supported in part by a Grant-in-Aid from the NHO (no. H21-NHO(Kan)-01). We express our deepest appreciation to the members of the Liver Disease Network Group of the NHO and to their institutions. For complete details, please see Appendix I.

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SUPPORTING INFORMATION

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

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

APPENDIX I

THE LIVER DISEASE Network Group of the National Hospital Organization consists of the following physicians and their institutions: Hiromi Ishibashi, Hiroshi Yatsuhashi, Department of Clinical Research Center, Nagasaki Medical Center; Makoto Nakamura, Department of Gastroenterology, Kyushu Medical Center; Michiyasu Yagura, Department of Gastroenterology, Tokyo National Hospital; Hirotugu Takano, Department of Gastroenterology, Kure Medical Center; Takeaki Satoh, Center for Liver

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Original Article

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

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

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

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

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

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

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

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Financial support

This research was supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.

Received 7 August 2011; revision 31 August 2011; accepted 5 September 2011.

INTRODUCTION

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

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

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

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

METHODS

Patients

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

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

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

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg¹⁹ was done using a chemiluminescence enzyme immunoassay

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

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

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

Statistical analyses

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

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

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

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

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

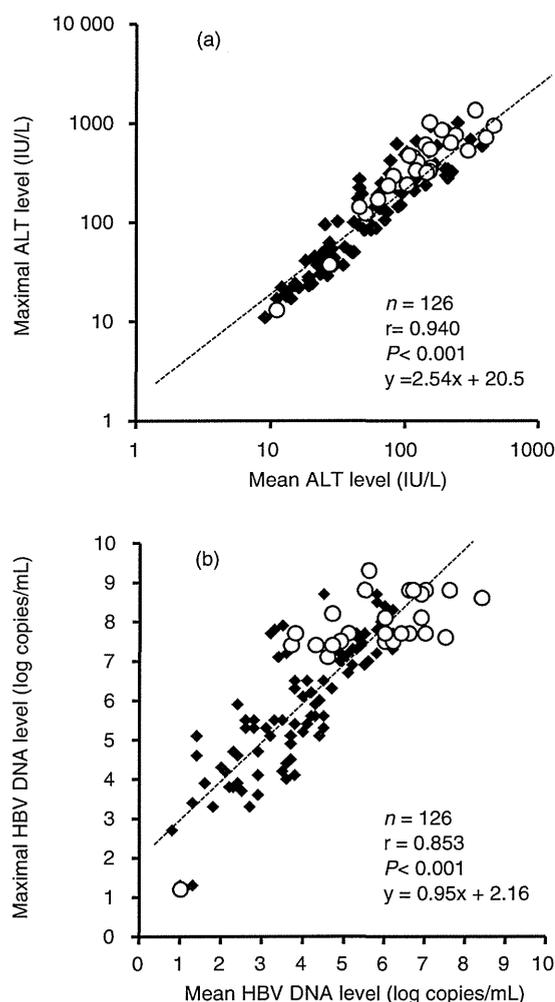


Figure 1 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without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

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

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

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

Elimination of cases likely to show relapse of hepatitis

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

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

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

Factors associated with relapse of hepatitis after discontinuation of NAs

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

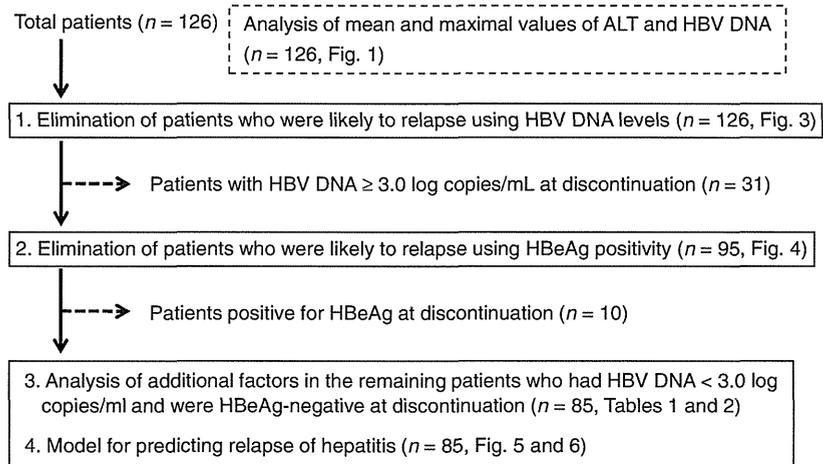


Figure 2 The progression of analyses in the present study and population structure of each analysis.

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

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

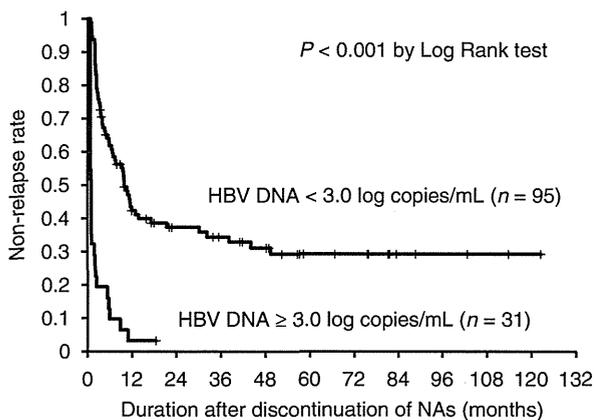


Figure 3 Comparison of non-relapse rates using the Kaplan–Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

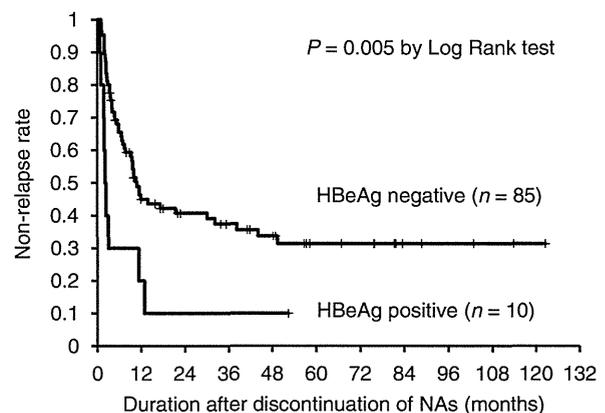


Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

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

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

†Data are expressed as the median (range)

‡Data are expressed as a positive number (%)

ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

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

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

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

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

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

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

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

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

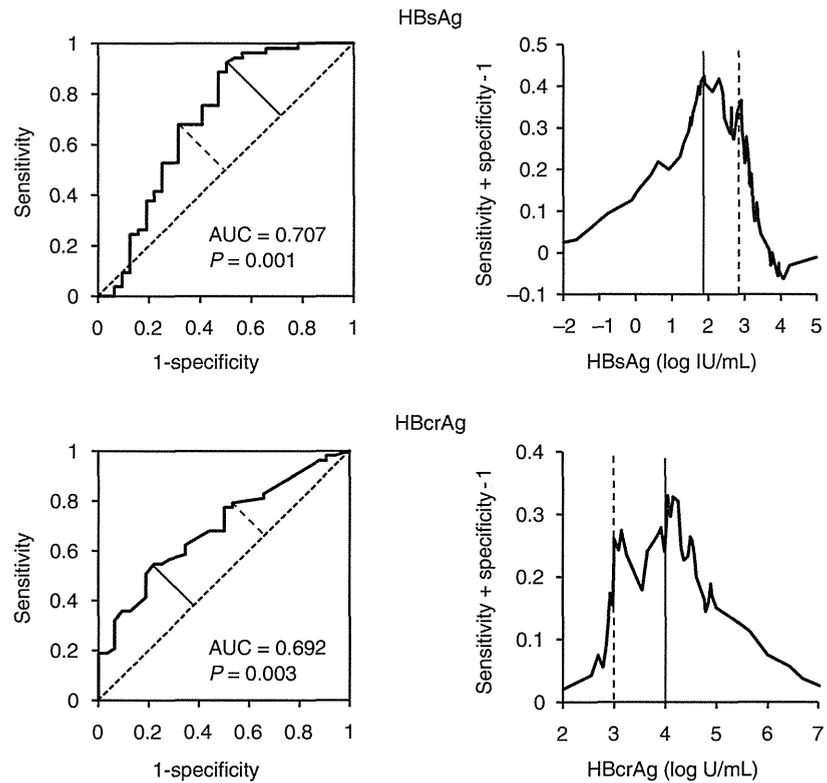


Figure 5 Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short 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.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan–Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in

group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard

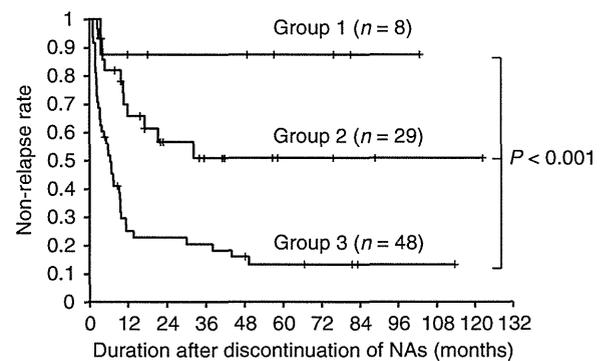


Figure 6 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

ratio = 5.25, range 2.37–11.65, $P < 0.001$). No significant factors were associated with relapse in group 2 patients.

DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.²⁵ Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.¹⁴ Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).^{26–28} Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.⁹ Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.^{1,2,18,29} Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,¹⁸ and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.^{30,31} We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discontinuation.

Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.³² Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.³³ However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.³⁴ To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.^{28,35,36} The HBcAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.³⁷ Serum HBcAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,^{24,34,38} and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.^{39,40} It is possible that levels of HBsAg and HBcAg have different roles in

monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.³ Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in

all groups, similarly to a report by Liu *et al.*¹⁴ Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported in part by a research grant from the Ministry of Health, Labor and Welfare of Japan.

We thank Ms. Hiroe Banno for her secretarial assistance and thank Ms. Nozomi Kamijo and Ms. Etsuko Iigahama for their technical assistance. We also thank Mr Trevor Ralph for his English editorial assistance.

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Hepatitis B Virus e Antigen Physically Associates With Receptor-Interacting Serine/Threonine Protein Kinase 2 and Regulates *IL-6* Gene Expression

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We previously reported that hepatitis B virus (HBV) e antigen (HBeAg) inhibits production of interleukin 6 by suppressing NF- κ B activation. NF- κ B is known to be activated through receptor-interacting serine/threonine protein kinase 2 (RIPK2), and we examined the mechanisms of interleukin 6 regulation by HBeAg. HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks nucleotide-binding oligomerization domain-containing protein 1 ligand-induced NF- κ B activation in HepG2 cells. Our findings identified novel molecular mechanisms whereby HBeAg modulates intracellular signaling pathways by targeting RIPK2, supporting the concept that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Hepatitis B virus (HBV) nucleoprotein exists in 2 forms [1, 2]. Nucleocapsid, designated HBV core antigen (HBcAg), is an intracellular, 21-kDa protein that self-assembles into particles that encapsidate viral genome and polymerase and is essential for function and maturation of virion. HBV also secretes a nonparticle second form of the nucleoprotein, designated

precore or HBV e antigen (HBeAg) [1, 2]. Precore and core proteins are translated from 2 RNA species that have different 5' initiation sites. Precore messenger RNA (mRNA) encodes a hydrophobic signal sequence that directs precore protein to the endoplasmic reticulum, where it undergoes N- and C-terminal cleavage within the secretory pathway and is secreted as an 18-kDa monomeric protein [3–5].

Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 are cytosolic pattern-recognition receptors involved in the sensing of bacterial peptidoglycan subcomponents [6]. NOD1 and NOD2 stimulation activates NF- κ B through receptor-interacting serine/threonine protein kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK), a caspase-recruitment domain-containing kinase. RIPK2 is also involved in Toll-like receptor (TLR)-signaling pathway and plays an important role in the production of inflammatory cytokines through NF- κ B activation [6, 7].

We previously reported that HBeAg inhibits the production of interleukin 6 (IL-6) through suppression of NF- κ B activation [4]. In the present study, we investigated the molecular mechanism of HBeAg functions for the requirement of RIPK2 in NF- κ B transcriptional regulation.

METHODS

Cell Culture and Plasmids

HepG2, Huh7, HT1080, COS7, and HEK293T cells were used in the present study. Stable cell lines were obtained as previously described [4]. Briefly, HepG2, Huh7, and HT1080 were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(-) in Effectene (Qiagen). After G418 screening, HBeAg-positive and -negative HepG2/Huh7/HT1080 cell lines were collected for further analysis [4]. The plasmid pCXN2-HBeAg(+), which can produce both HBeAg and core peptides, and the plasmid pCXN2-HBeAg(-), which can produce only core peptides, were obtained as described previously [4]. pNF- κ B-luc, which expresses luciferase upon promoter activation by NF- κ B, was purchased from Stratagene [4]. pGFP-human RIPK2 (kindly provided by Prof John C. Reed, Sanford-Burnham Institute for Medical Research) can express GFP-human RIP2^{WT} [8].

HepG2 cells were transfected with plasmid control-small hairpin RNA (shRNA) or with RIPK2-shRNA (Santa Cruz). After puromycin screening, individual colonies were picked up and examined for expression of endogenous RIPK2, and clones HepG2-shC and HepG2-shRIPK2-3 were selected for subsequent studies.

Received 1 January 2012; accepted 3 February 2012; electronically published 21 May 2012.

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The Journal of Infectious Diseases 2012;206:415–20

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DOI: 10.1093/infdis/jis363

Luciferase Assays and Treatment of Cells With NOD Ligands

Around 1.0×10^5 HepG2 and Huh7 cells were plated in 6-well plates (Iwaki Glass, Tokyo, Japan) for 24 hours and transfected with 0.4 μg of pNF- κB -luc. For luciferase assay of NF- κB activation, cells were treated for 4 hours with or without NOD1 ligand (C12-iEDAP, 2.5 $\mu\text{g}/\text{mL}$) and NOD2 ligand (muramyl dipeptide [MDP], 10 $\mu\text{g}/\text{mL}$) (InvivoGen) at 44 hours after transfection [9]. After 48 hours, cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined as described previously [4].

RNA Extraction, Complementary DNA (cDNA) Synthesis, Real-Time Polymerase Chain Reaction (PCR) Analysis, and PCR Array

Total RNA was isolated by RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Qiagen) [4]. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems). Gene expression profiling of 84 TLR-related genes was performed using RT² profiler PCR arrays (Qiagen) in accordance with the manufacturer's instructions [4].

Gene expression was normalized to 2 internal controls (GAPDH and/or β -actin) to determine the fold-change in gene expression between the test sample (HBeAg-positive HepG2/Huh7/HT1080) and the control sample (HBeAg-negative HepG2/Huh7/HT1080) by the $2^{-\Delta\Delta\text{CT}}$ (comparative cycle threshold) method [4]. Three sets of real-time PCR arrays were performed. Some results of HepG2 cells were previously reported [4].

Coimmunoprecipitation

Cells were cotransfected with 2.5 μg pCXN2-HBeAg(+) or 2.5 μg pCXN2-HBeAg(-), as well as with 2.5 μg pGFP-human RIPK2, and cell lysates were prepared after 48 hours, using lysis buffer containing a cocktail of protease inhibitors. Cell lysates were incubated with anti-GFP rabbit polyclonal antibody (Santa Cruz) or anti-HBe mouse monoclonal antibody (Institute of Immunology, Tokyo, Japan) for 3 hours at 4°C, followed by overnight incubation with protein G-Sepharose beads (Santa Cruz). Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed by incubating the membrane for 1 hour with anti-HBe antibody. Proteins were detected by enhanced chemiluminescence (GE Healthcare), using an image analyzer (LAS-4000, Fuji Film). The membrane was reprobed with a monoclonal antibody to GFP or RIPK2 (Cell Signaling).

Transfection of pGFP-Human RIPK2 and Confocal Microscopy

Formaldehyde (3.7%)-fixed cells were incubated with anti-HBe antibody and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 555 conjugate, Cell Signaling).

Cells were mounted for confocal microscopy (ECLIPSE TE 2000-U, Nikon). Whenever necessary, images were merged digitally to monitor colocalization. Cotransfection of 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(-) with 0.3 μg pGFP-human RIPK2 into the cells was performed. After 48 hours, intracellular localization of RIPK2 was visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6

Cell culture fluid was analyzed for IL-6 by ELISA (KOMA-BIOTECH, Seoul, Korea), in accordance with the manufacturer's protocol [4].

Small Interfering RNA (siRNA) Transfection and Wound-Healing Assay

Control siRNA (siC) and siRNA specific for RIPK2 (siRIPK2) were purchased from Thermo Fisher Scientific. Cells were transfected with siRNA by electroporation. After 48 hours, cells were treated with 10 ng/mL tumor necrosis factor α (TNF- α) (Wako Pure Chemical, Osaka, Japan), while the wound-healing (ie, scratch) assay was performed using a p-200 pipette tip to induce RIPK2 [10]. Up to 12 hours after scratching, the cells were observed by microscopy. Cell migration was measured using Scion Images (SAS). Migration by siC-transfected cells was set at 1.

Statistical Analysis

Results are expressed as mean values \pm SD. The Student *t* test was used to determine statistical significance.

RESULTS

HBeAg Downregulates RIPK2 Expression

To explore the effect of HBeAg on TLR-related gene expression, we generated HepG2, Huh7, and HT1080 cell lines that stably expressed HBV core region with or without precore region. HT1080, a primate fibrosarcoma cell line, is useful for the study of interferon signaling. HBeAg and HBV core-related antigen (HBcrAg) levels of these cell lines demonstrated that expression of HBV core region without HBV precore region did not allow HBeAg secretion by cells (data are cited elsewhere [4] or not shown). First, we performed real-time RT-PCR analysis of these cell lines, using focused gene arrays (Figure 1A). We observed that, in 3 cell lines, 5 genes (*RIPK2*, *TLR9*, *TNF*, *CD180*, and *IL1A*) were downregulated ≥ 1.3 -fold in HBeAg-positive cells than in HBeAg-negative cells. We chose to focus our investigation on RIPK2 because HBeAg inhibits the production of IL-6 through the suppression of NF- κB activation [4], and NF- κB is known to be activated through RIPK2 [4]. RIPK2 expression was >100 -, 1.41-, and 1.45-fold lower in HBeAg-positive HepG2, Huh7, and HT1080 cells, respectively, compared with their HBeAg-negative counterparts