Comparison of Hepatitis B Virus DNA, RNA, and Core Related Antigen as Predictors of Lamivudine Resistance in Patients with Chronic Hepatitis B

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The clinical usefulness of hepatitis B virus (HBV) DNA, RNA, and core related antigen (HBcrAg) assays for predicting the appearance of HBV DNA breakthrough was evaluated and compared in patients with chronic hepatitis B undergoing lamivudine therapy. Methods : Thirty six patients with chronic hepatitis B who received lamivudine therapy for more than 1 year were enrolled. HBV RNA was measured simultaneously with HBV DNA (HBV RNA/DNA) using a real-time detection polymerase chain reaction assay with a preceding step of reverse-transcription. HBV DNA was measured by an HBV AMPLICOR monitor kit. HBcrAg was measured using a chemiluminescence enzyme immunoassay. Results : Sixteen patients (44 %) developed HBV DNA breakthrough during the median observation period of 48.4 months (range 7.4-87.8 months). Afterwards, HBV DNA breakthrough was prospected using the three parameters taken 6 months after starting lamivudine therapy. The cut-off levels for predictions were determined by receiver operating characteristic curves, and were 2.6 log copies/ml for HBV DNA, 3.8 log U/ml for HBV RNA/DNA, and 4.0 log U/ml for HBcrAg. Sensitivity, specificity, and accuracy for predicting HBV DNA breakthrough were 25 %, 100 %, and 67 % respectively for HBV DNA. Similarly, they were 50 %, 90 %, and 72 % for HBV RNA/DNA, and 100 %, 40 %, and 67 % for HBcrAg. Conclusion : Our findings confirm that HBV DNA is useful for identifying patients who are at high risk for HBV breakthrough. HBcrAg is useful for isolating those who are at low risk, and HBV RNA/DNA showed predictive characteristics similar to HBV DNA with higher sensitivity and the highest accuracy. *Shinshu Med J 58 : 153—162, 2010*

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Key words : hepatitis B virus, HBV core related antigen, HBV RNA, lamivudine, breakthrough

I Introduction

Lamivudine (LAM), a nucleoside analogue, inhibits the replication of hepatitis B virus (HBV), reduces hepatitis, and improves histological findings of the liver during long-term therapy. Lamivudine treatment has also been reported to reduce the risk of complicating hepatocellular carcinoma[1)2)]. However, relapse of hepatitis due to the appearance of resistant viruses is a major drawback of lamivudine therapy[3)-5)]. Recently, new nucleoside and nucleotide analogues have been developed, such as adefovir dipiboxil and entecavir, which develop resistant viruses far less frequently than

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Abbreviations : HBV, hepatitis B virus ; HBcrAg, hepatitis B virus core related antigen ; HBV RNA/DNA, hepatitis B virus RNA and DNA ; LAM, lamivudine ; YMDD, tyrosine-methionine-aspartic acid-aspartic acid ; HBsAg, hepatitis B virus surface antigen ; HBeAg, hepatitis B virus e antigen ; HBeAb, anti-hepatitis B antibody ; RTD-PCR, real-time detection polymerase chain reaction assays ; ROC, receiver operating characteristic ; cccDNA, covalently closed circular DNA.

lamivudine, but these analogues still face the problem of resistant viruses[6)–8)].

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration[9)–12)], but can rapidly increase when tyrosine-methionine-aspartic acid-aspartic acid (YMDD) mutations induce lamivudine resistant strains[13)–16)]. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine, but monitoring these effects by HBV DNA level alone is not satisfactory because lamivudine resistance occurs even in patients who show undetectable levels of serum HBV DNA[13)17)18)].

We previously described a HBV core-related antigen (HBcrAg) assay[19)] which measures the total amount of protein encoded by the pre-core and core regions of the HBV genome, including core, e, and p22cr[20)] antigens. In those experiments, the serum level of HBcrAg was shown to be useful for predicting the occurrence of lamivudine resistance in a manner different from serum levels of HBV DNA[21)]. We also reported that serum HBV RNA is detectable in a form incorporated into virus particles in patients with hepatitis B undergoing lamivudine therapy, which could possibly represent a new viral marker of different significance than that of HBV DNA in lamivudine therapy[22)].

Thus, in the present study, we sought to compare the clinical usefulness of using HBV DNA, HBV RNA, and HBcrAg to predict the occurrence of lamivudine resistance, as detected by HBV DNA breakthrough in patients with chronic hepatitis B receiving lamivudine therapy.

II Patients and Methods

A Patients

A total of 36 patients with chronic hepatitis B who started LAM therapy at Shinshu University Hospital between July 2002 and February 2004 were enrolled in this study. They consisted of 28 men and 8 women and had a median age of 55 years (range 29–80 years) at the commencement of LAM therapy. Chronic hepatitis B was defined as positive for HBV surface antigen (HBsAg) for more than 6 months with liver histological findings consistent with chronic hepatitis. All patients had elevations in serum alanine aminotransferase (ALT) levels, as well as detectable HBV DNA for at least 6 months. Immediately prior to LAM administration, 23 of the patients were positive for HBV e antigen (HBeAg) and 13 were positive for anti-HBV e antibody (HBeAb) and negative for HBeAg. The HBV genotype was C in all patients except three (two were genotype B and one was F). Patients received 100 mg doses of LAM daily for at least 12 months. No patient underwent treatment with any other anti-viral agent, such as interferon, before or during the present study, and all patients were negative for hepatitis C and human immunodeficiency virus antibodies. Written informed consent was obtained from each patient. This study was approved by the Ethics Committee of Shinshu University.

Serum samples were collected from the start of LAM therapy on a monthly basis and were stored frozen at −20 ℃ or below until assayed. The occurrence of LAM resistance was defined as a rapid increase in serum HBV DNA with the appearance of the YMDD mutations. Using these criteria, resistance appeared in 16 (44 %) of the 36 patients. The median period from the start of LAM therapy to the occurrence of resistance was 18.2 months, with a range of 7.4 to 57.7 months.

B Routine laboratory tests

Serological markers for HBV, including HBsAg, HBeAg, and HBeAb, were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami et al[23)]. The YMDD motif, a LAM-resistant mutation in the active site of HBV polymerase, was detected using an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan)[24)]. Serum concentration of HBV DNA was determined using an AMPLICOR HBV monitor kit (Roche, Tokyo, Japan), which had a

quantitative range of 2.6 to 7.6 log copies/ml.

C HBV core-related antigen assay

Serum HBcrAg was measured using a chemiluminescence enzyme immunoassay (CLEIA) as reported previously[25]. In brief, 100 μl aliquots of serum were mixed with pretreatment solution containing 15 % sodium dodecylsulfate. After incubation at 70 ℃ for 30 min, 50 μl pretreated serum was added to wells coated with monoclonal antibodies against denatured HBV core and e antigens (HB44, HB61, and HB114) and filled with 100 μl assay buffer. The mixture was then incubated for 2 hrs at room temperature. After washing with buffer, either alkaline phosphatase-labeled HB50 monoclonal antibody (specific for denatured HBV core antigen) or a mixture of HB91 and HB110 monoclonal antibodies (specific for denatured HBV core and e antigens) were added to wells and incubated for 1 hr at room temperature. After washing again, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and plates were incubated for 20 min more at room temperature. The relative chemiluminescence intensity was measured, and HBcrAg concentrations were read by comparison to a standard curve generated with recombinant pro-hepatitis B e antigen (amino acids -10 to 183 of the precore/core gene product). The concentration of HBcrAg was expressed as units/ml and the immunereactivity of recombinant pro-hepatitis B e antigen at 10 fg/ml was defined as 1 unit/ml. The lower detection limit of this assay was set at 2 log units/ml. Sera containing over 7 log units/ml of antigen were diluted 10 or 100 fold in normal human serum and measured again to obtain the end titer.

D HBV RNA/DNA

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) was used for isolation of HBV DNA and RNA from serum. Briefly, 200 μl of serum was added to 250 μL of freshly prepared working solu tion (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl [pH 4.4] and 20 % [vol/vol] Triton X-100) supplemented with 20 μg of poly (A) carrier RNA and 900 μg of Proteinase K. After incubation for 10 min at 72 ℃, 100 μl of isopropanol was added and the mixture was transferred into a High Pure filter tube fitted with a collection tube. The filter tube was centrifuged for 1 min at 8,000 rpm in a standard tabletop centrifuge at room temperature, then attached to a new collection tube. An inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl [pH 6.6] in ethanol) was added to the upper reservoir and the tube was centrifuged again for 1 min at 8,000 rpm. After being washed with 250 μl of wash buffer (20 mM NaCl, 2 mM Tris-HCl [pH 7.5] in ethanol), the filter was placed in a new collection tube and 50 μl of RNase-and DNase-free water was added to elute the DNA and RNA. After centrifugation for 1 min at 8,000 rpm, the eluted DNA and RNA was stored at −80 ℃. Synthesis of cDNA was performed at 42 ℃ for 30 min in a 20 μl reaction mixture containing 10 μL of the extracted DNA and RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM dNTP (1 mM each dATP, dGTP, dCTP and dTTP), 1 mM DTT, 100 nM reverse primer for the HBV surface gene (5'-GGTTGGTGAGTGATTGGAGGTT-3' ; nt. 345 to 324), 40 units of RNasin (TaKaRa, Kyoto, Japan), and 200 units of SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA). The reaction mixture was inactivated by heating to 70 ℃ for 15 min, then cooled to -80 ℃ until real-time detection polymerase chain reaction (RTD-PCR) assays. A 4 μl aliquot of DNA and cDNA solution was used for RTD-PCR, which was performed with the Light Cycler System (Roche Diagnostics) as reported previously[25]. The two primers and TaqMan probe used were designed from a region of the HBV surface gene : forward primer ; 5'-ACAACATCAG-GATTCCTAGGAC-3' (nt. 166 to 187), reverse primer as stated above (nt. 345 to 324), and TaqMan probe ; 5'-FAM-CAGAGTCTAGACTCGTGGTG-GACTTC-TAMRA-3' (nt. 244 to 269). An HBV genome (nt. 20 to 1805) subcloned into a pUC vector was used as an internal standard. The lower detection limit for the HBV RNA/DNA assay was set at 2.6 log copy/ml.

E Statistical analysis

The proportion of each clinical factor was

compared between the groups with or without HBV DNA breakthrough using the χ^2 and Fisher's exact probability tests, and group medians were compared using the Mann-Whitney's U test. Correlations between HBV RNA/DNA and HBV DNA or HBcrAg were tested using Spearman's analysis. The rates of HBV DNA breakthrough during LAM treatment between higher and lower level groups of HBV DNA, HBcrAg and HBV direct RT-PCR were analyzed using the Kaplan-Meier method, and the difference in incidences was assessed with the log-rank test. Receiver operating characteristic (ROC) curves were used to decide each cut-off point for predicting HBV DNA breakthrough. All tests were performed using the SPSS 10.0 J statistical software package (SPSS Inc., Chicago, IL). P values less than 0.05 were considered significant.

Ⅲ Results

A Comparison of characteristics between patients with and without HBV DNA breakthrough

HBV DNA breakthrough occurred in 16 (44.4 %) of 36 patients during the follow-up period. The cumulative HBV DNA breakthrough incidence in all patients was 8.3 % at 12 months, 27.8 % at 24 months, 33.5 % at 36 months, 40.2 % at 48 months, and 48.2 % at 60 months. The clinical characteristics at baseline in the 16 patients with HBV DNA breakthrough and 20 patients without are shown in Table 1. The median follow-up period did not differ, and no significant differences were observed in any other characteristics, including ALT, HBV DNA, HBcrAg, and HBV RNA/DNA.

B Changes in serum HBV DNA, HBcrAg and HBV RNA/DNA

Changes in serum levels of HBV DNA, HBcrAg, and HBV RNA/DNA from baseline to 6 months of lamivudine therapy are shown in Fig. 1. HBV DNA decreased rapidly and became undetectable within 6 months in all except 4 patients with HBV DNA breakthrough. HBcrAg decreased more slowly than HBV DNA, and became undetectable only in 2 (6 %) of the 36 patients at 6 months. HBV RNA/DNA decreased faster than HBcrAg, but slower than HBV DNA, and became undetectable at 6 months in 15 (42 %) of the 36 patients.

Although HBV RNA/DNA was significantly correlated with both HBV DNA (Fig. 2A) and HBcrAg (Fig. 2B) at the start of lamivudine therapy, this association was lost at 6 months because over 90 % of patients became undetectable for HBV DNA (Fig. 2C). On the other hand, HBV RNA/DNA retained its correlation with HBcrAg at 6 months (Fig. 2D). Although significant, the HBcrAg to HBV RNA/DNA ratio tended to be lower when compared to baseline.

C Prediction of occurrence of lamivudine resistance

The occurrence of lamivudine resistance was next prospected using the levels of HBV DNA, HBcrAg, and HBV RNA/DNA measured at 6

Table 1 Baseline characteristics of patients with and without HBV DNA breakthrough during lamivudine therapy

	with breakthrough	without breakthrough	p
Number	16	20	
Age (y.o.)[a]	56.0 (29.5－64.0)	53.6 (41.0－79.5)	0.660
Gender (male/female)	13/3	15/5	1.000
Follow-up period (months)[a]	53.2 (21.5－84.0)	67.5 (45.8－89.5)	0.421
HBeAg (+/－)	10/6	9/11	0.508
HBeAb (+/－)	6/10	11/9	0.508
ALT (IU/l)[a]	60 (22－499)	119 (20－1816)	0.156
HBV DNA (log copy/ml)[a]	6.7 (4.8－＞7.6)	6.1 (3.9－＞7.6)	0.338
HBcrAg (log U/ml)[a]	5.9 (4.3－8.2)	5.8 (2.9－8.7)	0.683
HBV RNA/DNA (log U/ml)[a]	6.2 (4.8－8.3)	6.3 (4.4－8.8)	0.916

[a] Data are expressed as median (range)

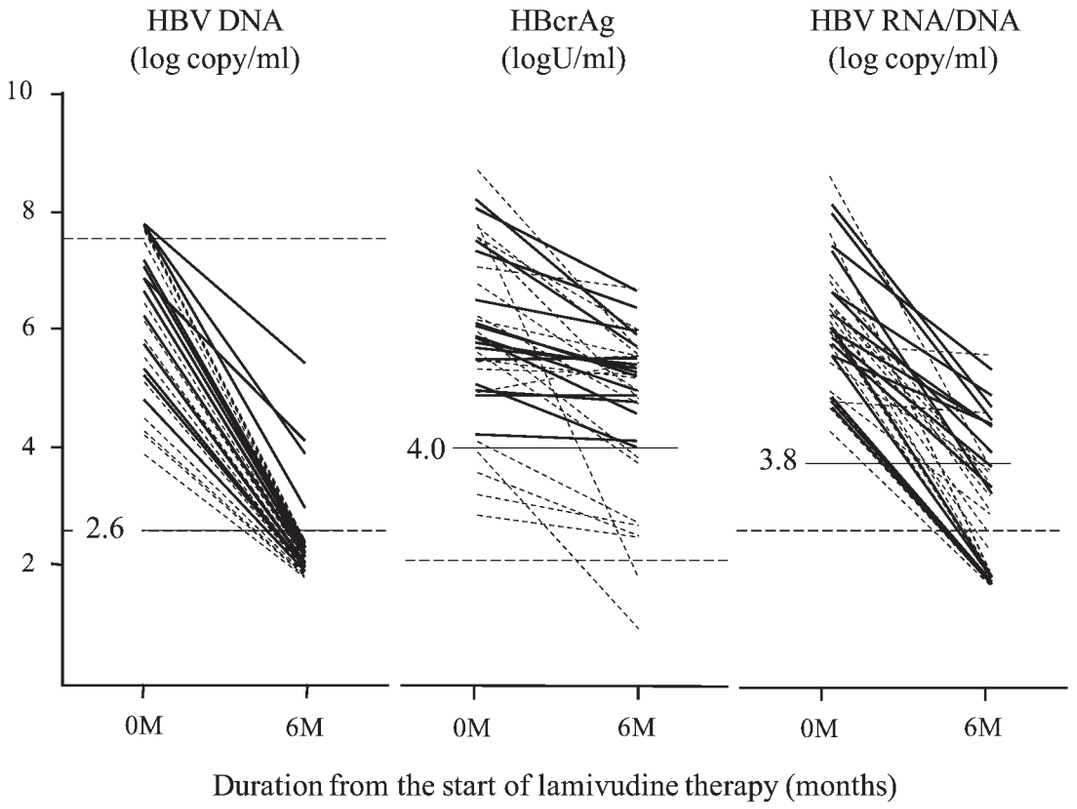

Fig. 1 Changes in serum levels of HBV DNA, HBcrAg, and HBV RNA/DNA from baseline to month 6. The solid line indicates patients with HBV DNA breakthrough during the observation period, and the broken line indicates patients without. The horizontal broken line indicates the lower detection limit of each assay. Cut-off values for predicting lamivudine resistance were 2.6 log copies/ml in HBV DNA (the same as the lower detection limit), 4.0 log U/ml in HBcrAg (solid line) and 3.8 log copies/ml in HBV RNA/DNA (solid line). The upper detection limit of HBV DNA is also shown by a horizontal broken line at 7.6 log copy/ml because 4 patients showed levels higher than the upper detection limit at the baseline.

months after starting lamivudine therapy. The cut-off values of HBV DNA (2.6 log copies/ml), HBcrAg (4.0 log U/ml), and HBV RNA/DNA (3.8 log copies/ml) for prediction of resistance was determined by ROC analysis. The sensitivity, specificity, and accuracy for predicting breakthrough were 25 %, 100 %, and 67 % respectively for HBV DNA. Similarly, they were 100 %, 40 %, and 67 % for HBcrAg, and 50 %, 90 %, and 72 % for HBV RNA/DNA. The positive predictive values for predicting HBV DNA breakthrough by HBV DNA, HBcrAg, and HBV RNA/DNA combined was 100 %, 57.1 %, and 80.0 % respectively. Similarly, the negative predictive values were 63.6 %, 100 %, and 62.5 %.

The cumulative occurrence of HBV DNA breakthrough was compared using a log-rank test between two groups of patients divided by the cut-off values of HBV DNA ($P<0.0003$), HBcrAg ($P=0.0088$), and HBV RNA/DNA ($P=0.0011$) (Fig. 3). All 4 patients whose HBV DNA levels were higher than the cut-off showed lamivudine resistance within 24 months of the start of therapy. None of the 9 patients whose HBcrAg levels were less than the cut-off showed lamivudine resistance during the follow-up period. HBV RNA/DNA showed an intermediate character between HBV DNA and HBcrAg in predicting the occurrence of HBV DNA breakthrough.

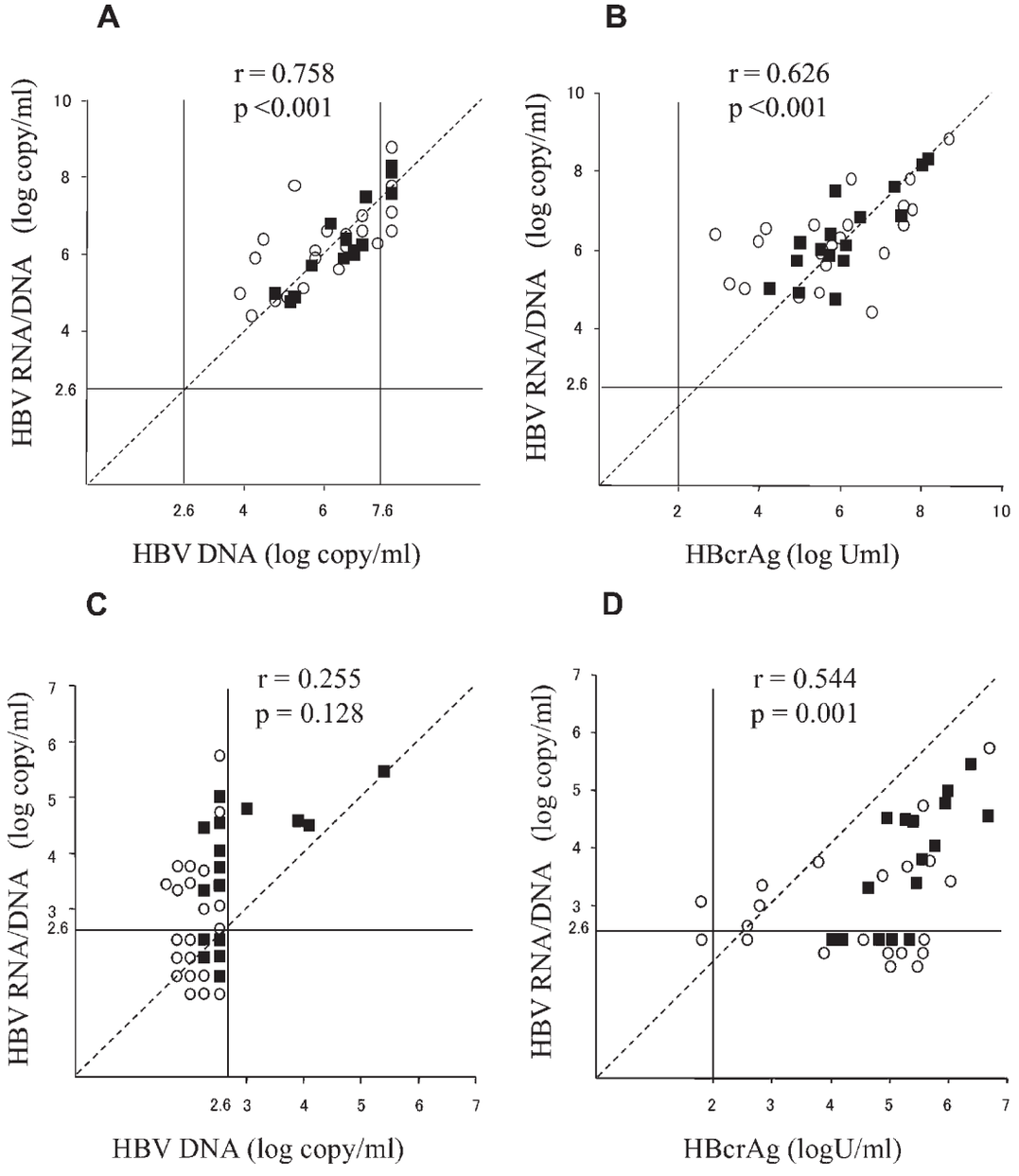

Fig. 2 Correlation between the levels of the three parameters at baseline and at 6 months after starting lamivudine administration: (A) between HBV DNA and HBV RNA/DNA at baseline, (B) between HBcrAg and HBV RNA/DNA at baseline, (C) between HBV DNA and HBV RNA/DNA at 6 months, and (D) between HBcrAg and HBV RNA/DNA at 6 months. Closed squares indicate patients with breakthrough during the observation period, and open circles indicate patients without.

Ⅳ Discussion

HBV is an enveloped DNA virus containing a relaxed circular DNA genome that is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells which serves as a transcriptional template for the production of viral RNA. Reverse transcription of pregenomic RNA and second-strand DNA synthesis then occurs in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine inhibits reverse transcription of pregenomic RNA, it directly suppresses production of HBV virions, and serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the amount of cccDNA decreases quite slowly

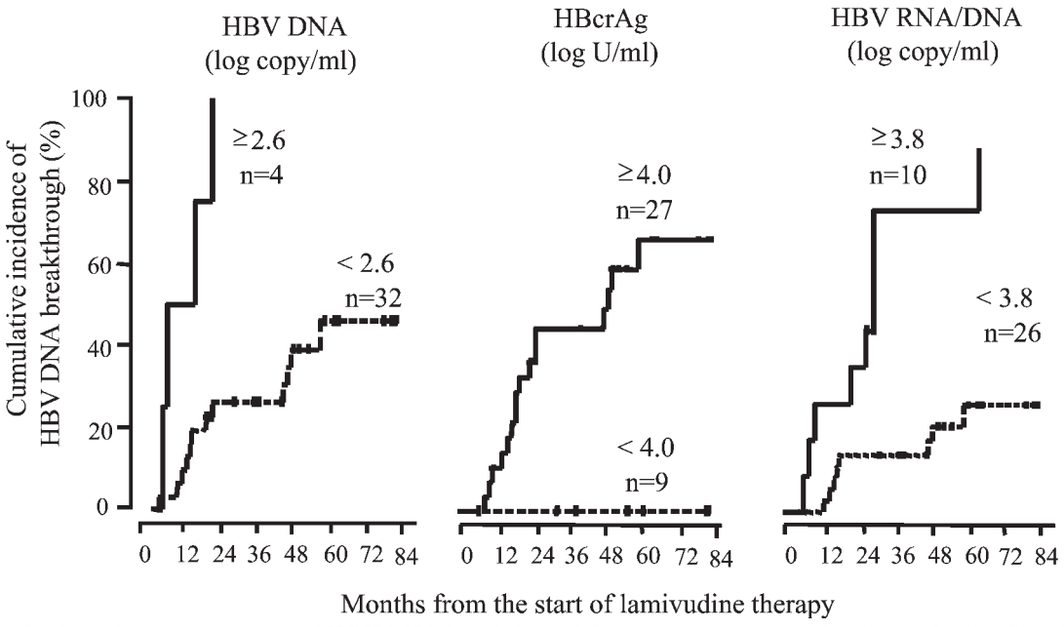

Fig. 3 Cumulative occurrence of HBV DNA breakthrough between two groups of patients classified by the selected cut-off values of HBV DNA (2.6 log copy/ml), HBcrAg (4.0 log U/ml), and HBV RNA/DNA (3.8 copy/ml).

after commencement of nucleoside analogues. Intrahepatic HBV cccDNA has been reported to be superior to serum HBV DNA in predicting virologic response to nucleoside/nucleotide analogue therapies, such as lamivudine[26)–30)]. However, the measurement of cccDNA seems ill-suited for clinical use because it requires a liver biopsy and complicated measurements. The HBcrAg assay developed by our research group has been shown to be useful for identifying patients who are at low risk of developing lamivudine resistance during therapy[21)], as well as those who are at low risk of hepatitis reactivation after cessation of lamivudine administration[31)32)]. The serum HBcrAg levels were well correlated with intrahepatic cccDNA level after HBV DNA became undetectable during anti-viral therapy[33)]. Here, serum HBcrAg may reflect the intrahepatic cccDNA better than serum HBV DNA under lamivudine therapy since lamivudine inhibits the synthesis of HBV DNA from pregenomic RNA transcribed from cccDNA, but does not inhibit synthesis of viral proteins which are translated from viral mRNA directly.

Maturation of the HBV genome occurs in nucleocapsids. Viral polymerase initiates encapsidation by binding to the encapsidation signal, epsilon, and a secondary structure on the pregenomic RNA, which is then complexed with core proteins to form nucleocapsids. The polymerase-epsilon interaction is also the first step in initiating reverse transcription of pregenomic RNA to yield the negative DNA strand of the viral genome[29)32)]. Therefore, we can hypothesize that HBV particles containing pregenomic HBV RNA are produced rather than those containing HBV DNA during lamivudine therapy. Lamivudine inhibits reverse transcription of pregenomic RNA, suggesting that HBV particles containing HBV RNA are produced and may account for the majority of HBV particles[22)]. Accordingly, it is also possible that serum HBV RNA reflects intrahepatic cccDNA and is useful for predicting the occurrence of lamivudine resistance. Recently, Hatakeyama et al. reported that serum HBV RNA was a predictor of early emergence of the YMDD mutant in patients treated with lamivudine[34)]. In a previous study, we demonstrated a method to measure serum HBV RNA only by eliminating HBV DNA. However, this method is prohibitively complicated for testing many samples because it includes a digestion step with DNAase.

As such, HBV DNA and RNA were measured simultaneously in the present study by eliminating the digestion step. Because the proportion of HBV DNA to HBV RNA at 6 months was quite low, we believe that the possibility of HBV RNA as a predictor for lamivudine resistance could be tested.

This study compared the abilities of HBV DNA, HBV RNA/DNA, and HBcrAg for predicting occurrence of lamivudine resistance. Lamivudine resistance was monitored by HBV DNA breakthrough because it is more sensitive than hepatitis breakthrough in detecting resistance. The specificity of HBV DNA breakthrough was confirmed by the existence of YMDD mutations. HBV DNA appears useful for detecting patients who are at high risk of developing lamivudine resistance, but not for selecting patients who are at low risk because the positive predictive value was as high as 100 % and sensitivity as low as 25 %. On the contrary, HBcrAg was useful for identifying patients who are at low risk of lamivudine resistance, but not for patients who are at high risk since the negative predictive value was as high as 100 % and specificity as low as 40 %. HBV DNA breakthrough did not occur until 60 months from the start of lamivudine therapy in 9 patients whose HBcrAg was less than 4.0 log U/ml at 6 months of therapy.

The ability of HBV RNA/DNA for predicting the occurrence of lamivudine resistance landed between those of HBV DNA and HBcrAg ; both the positive (80 %) and negative (63 %) predictive values were intermediate. The accuracy (72 %) of HBV RNA/DNA was highest among the three parameters. Thus, HBV RNA/DNA is presumed to have the predictive characteristics of both HBV DNA and HBcrAg, with the additional feature of including a wider range of patients.

Lamivudine has already been eliminated from first line therapy in naive chronic hepatitis B patients due to a higher incidence of developing resistant mutations than new antiviral agents, such as adefovir dipivoxil and entecavir. However, a considerable number of patients who began lamivudine administration in the past still take this treatment, so the present study may be valuable to such patients when they consider changing therapies in the future. For example, lamivudine patients who show low levels of HBV RNA/DNA or HBcrAg do not necessarily need to change their therapy to a new antiviral regimen. Additionally, since the main mechanisms of suppressing HBV replication are similar among lamivudine, entecavir, and adefovir dipivoxil, it is possible that HBV DNA, HBV RNA/DNA, and HBcrAg are useful for monitoring the antiviral effects of these drugs as well. However, further studies are required to determine whether these three assays are indeed applicable to antiviral agents other than lamivudine.

In conclusion, monitoring HBV DNA is useful for identifying patients with chronic hepatitis B under lamivudine therapy who are at high risk of lamivudine resistance, and measurement of HBcrAg is useful for isolating those who are at low risk of HBV DNA breakthrough. The predictive characteristics of HBV RNA/DNA are similar to that of HBV DNA with higher sensitivity, and show the highest accuracy.

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