patients should be completed to assess the antiviral efficacy and deliberate clinical application of ETV therapy for HBV/HIV coinfection.

Both adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) have recently been shown to effectively inhibit HBV replication in patients with HBV/HIV coinfection, irrespective of LAM resistance.[19,20] ADV exerts only anti-HBV activity and is available for patients with HBV/HIV coinfection who have no need for HAART or who are receiving a stable HAART regimen. In contrast, TDF can be used as a component of HAART because of its valuable antiviral activity against both HBV and HIV. Accordingly, ADV and TDF are currently useful drugs for patients with HBV/HIV coinfection and may be subsequent therapeutic options for the patient reported in this study.

Our patient was found to be infected with HBV of genotype H, a globally rare genotype. To date, the full-length sequences of eight genotype H HBV strains have been reported from the USA, Nicaragua and Japan (see Fig. 2). Of them, one strain has been obtained from a Japanese patient with chronic HBV monoinfection who underwent ETV therapy as a naïve patient and showed ETV resistance later.[21] The relevance of the genotype frequency to the therapeutic efficacy of ETV should be studied extensively in HBV-infected patients treated with ETV.

In Japan, genotypes B and C are prevalent in chronic HBV carriers who acquire the infection mainly through the mother-to-child transmission route. In contrast, the foreign HBV strains other than genotypes B and C have been shown to be involved in a considerable proportion of patients with acute HBV infection.[22] Infection of such foreign types of HBV possibly occurs through sexual contacts in Japan. In our patient with HBV/HIV coinfection who had genotype H HBV of foreign origin, it is speculated that acute HBV infection occurring 3 years before his first visit led to the transition to chronicity. The time of HIV infection cannot be defined due to the lack of HIV-RNA testing during the period of acute HBV infection. The possibility of simultaneous infection with HBV and HIV cannot be excluded, despite the negative result of anti-HIV at that time, because the test may have taken place during the immunological window period of HIV infection.

In summary, we have introduced a patient with HBV/HIV coinfection who underwent ETV therapy in addition to the HAART regimen and showed ETV resistance in the early phase of therapy. Our finding suggests that, in ETV therapy for patients with HBV/HIV infection, great care should be taken against the emergence of ETV-resistant HBV, especially in patients with pre-existing LAM-resistant HBV.

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Original Article

Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine

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Aim: Lamivudine (LAM) has been widely used to treat chronic hepatitis B (CHB) patients, but the emergence of a LAM-resistant virus greatly limits its therapeutic efficacy. In this study, we tried to identify factors affecting the emergence of a LAM-resistant virus in CHB patients treated with LAM.

Methods: The subjects were 190 CHB patients in continuous LAM therapy (139 males, mean age 50 years, 87 HBeAg-positive). The mean duration of follow-up was 39 months (range 12–104). The initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) at 6 months.

Results: IVR was positive in 86% of the patients. The cumulative emergence rates of LAM-resistant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years. In univariate analysis, factors contributing to the emergence of LAM-resistant virus were baseline HBV DNA > 6.5 logcopies/mL ($P = 0.0044$), HBeAg-positivity ($P = 0.0062$), IBR ($P = 0.01$) and IVR ($P < 0.0001$). The cumulative emergence rates of LAM-resistant virus in IVR-positive and -negative patients were 4% and 41% at 1 year, and 41% and 79% at 3 years. In multivariate analysis, only IVR was an independent factor affecting the emergence of LAM-resistant virus ($P < 0.0001$).

Conclusion: IVR is a useful factor for predicting the emergence of LAM-resistant virus in CHB patients treated with LAM. For IVR-negative patients, therapeutic options other than LAM monotherapy should be used because of the high incidence of the emergence of LAM-resistant virus.

Key words: chronic hepatitis B, initial viral response, lamivudine monotherapy, lamivudine-resistant virus

INTRODUCTION

MORE THAN 350 million people are chronically infected with hepatitis B virus (HBV) worldwide.[1] Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Nucleos(t)ide analogs are widely used to suppress HBV replication and the progression of HBV-related liver diseases. Lamivudine (LAM), the first approved nucleoside analog for chronic HBV infection, has been shown to suppress viral replication and disease activity.[2] In addition, LAM therapy has recently been reported to reduce the incidence of HCC, the risk of major complications and to improve survival.[3,4] However, the relatively high incidence of LAM resistance is a serious problem in the case of LAM therapy for chronic HBV infection. The emergence of LAM-resistant HBV is linked to the reappearance of active viral replication, followed by the worsening of liver disease.

LAM-resistant HBV is based on point mutation within the YMDD motif of the reverse transcriptase domain of

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HBV (YMDD mutation).[5,6] The emergence rates of the mutant virus have been reported to be 24% at 1 year and 70% at 4 years from the start of treatment.[7]

Recent work has shown that newly developed nucleos(t)ide analogs, such as adefovir dipivoxil (ADV) and entecavir (ETV), are also useful agents for controlling patients with chronic HBV infection.[8–11] In particular, the drug-resistant mutant virus has been reported to appear less frequently in cases of treatment with ADV and ETV than with LAM.[12,13] For this reason, LAM has been replaced by ADV and ETV for the treatment of chronic hepatitis B. However, there are still a considerable number of patients with chronic HBV infection who are already on continuous LAM therapy. Thus, further clarification is needed of what factors influence the emergence of the LAM-resistant HBV in LAM treatment for chronic HBV infection.

For a more precise evaluation, we investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus in patients with chronic hepatitis B treated with LAM.

METHODS

Patients and treatment

THIS STUDY WAS conducted at nine institutions in the Osaka area of Japan (Osaka Police Hospital, Osaka Minami Medical Center, Osaka Kouseinenkin Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda City Hospital, Osaka National Hospital, Otemae Hospital and Osaka University Hospital). The subjects were 190 consecutive patients with chronic hepatitis B who underwent continuous LAM therapy for more than 12 months. All patients tested positive for hepatitis B surface antigen (HBsAg) or had detectable levels of HBV DNA in their sera by the polymerase chain reaction (PCR)-based method (for 100 patients)[14] or the transcription-mediated amplification (TMA) method (for 90 patients).[15] Exclusion criteria were patients with antihepatitis C antibody, antihuman immunodeficiency virus antibody and other forms of liver diseases (alcoholic liver disease, drug-induced liver disease and autoimmune hepatitis). Forty-one (22%) patients had previously received interferon (IFN)-α therapy for 24 weeks.

All patients were treated with 100 mg of LAM daily. After the beginning of the therapy, liver function tests and HBV DNA were measured every other month for the first 6 months and every two months thereafter. HBeAg and anti-HBe were tested every 6 months. In 33 patients (18%), combination therapy with IFN was carried out for the initial 6 months. Three or six megaunits of natural IFN-á were administered daily for the first 2 weeks and three times a week thereafter, followed by LAM monotherapy. The mean follow-up period of the 190 patients was 39 (range 12–104) months. The LAM-resistant YMDD mutant virus was detected by the PCR-enzyme-linked minisequence (ELMA) assay[16] when the virological or biochemical breakthrough was observed. The YMDD mutant virus was found in 86 (45%) patients during follow-up. Fifty-eight of these patients underwent ADV therapy in addition to ongoing LAM treatment and were excluded from the follow-up when ADV administration began. In this study, the initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) after 6 months of therapy.

Table 1 Patient characteristics

Gender (male/female)	139/51
Age (years)	50 ± 11
Chronic hepatitis/liver cirrhosis	113/77
Hepatocellular carcinoma	14 (7%)
AST (IU/L)	122 ± 157
AST (IU/L)	177 ± 236
ALT (≤1/1–2/2–5/>5 × ULN)	22/53/65/50
Platelet (10^4/mm^3)	12.6 ± 5.1
Prothrombin time (%)	71.5 ± 16.6
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)
HBeAg (positive/negative)	87/103
Combination with interferon	33 (17%)
Duration of treatment (months)	38.9 ± 17.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ULM, upper limit normal.

The patients' clinical characteristics are shown in Table 1. There were 139 males and 51 females, ranging in age from 25 to 75 (mean 50) years. Of them, 113 (59%) patients were diagnosed as having chronic hepatitis and the remaining 77 patients (41%) as having cirrhosis according to liver histology and/or the imaging procedure. HCC was developed in 14 (7%) patients. The aspartate aminotransferase (AST) at baseline was 122 ± 157 IU/l, and the ALT at baseline was 177 ± 236 IU/L. Abnormal ALT was observed in 168 (88%) patients. Eighty-seven patients (46%) tested positive for HBeAg. The median HBV DNA at baseline was 6.5 (range 3.0 to 7.6<) logcopies/mL.

HBV testing

HBsAg, hepatitis B e antigen (HBeAg) and antihepatitis B e antibody (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay.

The HBV DNA level was measured by the PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan)[14] or the TMA method (TMA-HPA; Fujirebio, Tokyo, Japan),[15] which have lower detection limits of 2.6 and 3.7 logcopies/mL, respectively. The LAM-resistant YMDD mutant virus was examined by the PCR–ELMA method.[16]

Statistical analysis

Comparisons of categorical and continuous variables between groups were done by the χ^2-test, Student's *t*-test and Mann–Whitney's *U*-test. The cumulative emergence rates of LAM-resistant virus were evaluated with the Kaplan–Meier's curve and the differences between groups were analyzed by the log–rank test. For multivariate analysis to investigate factors affecting the cumulative emergence rate of LAM-resistant virus, Cox proportional hazard regression analysis was carried out. A *P*-value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Therapeutic efficacy and the emergence of LAM-resistant mutant virus

AMONG THE 190 patients with chronic hepatitis B who underwent continuous LAM therapy, reduction of HBV DNA to less than 4 logcopies/mL was observed in 86% (163/190) at 6 months, 89% (151/170) at 1 year, 88% (83/94) at 2 years and 89% (48/54) at 3 years of the treatment. Normalization of ALT was achieved by 77% (146/190) at 6 months, 83% (141/170) at 1 year, 81% (76/94) at 2 years and 83% (45/54) at 3 years. Among the 87 HBeAg-positive patients, HBeAg was cleared in 22% (19/86) at 6 months, 26% (21/80) at 1 year, 22% (11/50) at 2 years and 43% (16/37) at 3 years. As for the virological and biochemical response at 6 months of therapy, 163 (86%) of the patients achieved IVR, whereas IBR was seen in 146 (77%) of patients.

When the various patient characteristics were compared between IVR-positive and -negative patients (Table 2), HBV DNA at baseline tended to be lower in patients showing IVR (median 6.5 [range 3.0 to 7.6<] logcopies/mL) than in those who did not show IVR (median 7.3 [range 4.3 to 7.6<] logcopies/mL) ($P < 0.0001$). IVR-negative patients had higher HBeAg positivity at baseline than IVR-positive patients (81% vs 40%, $P = 0.01$). As for the emergence of LAM-resistant mutant virus during follow-up, it was detected more frequently in IVR-negative patients (21/27, 78%) than in IVR-positive patients (65/163, 40%) ($P = 0.002$).

Among the 190 patients examined in this study, the emergence of LAM-resistant YMDD mutant virus occurred in 86 (45%) patients during follow-up. The cumulative probabilities of the emergence of the YMDD mutant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years.

Factors affecting the emergence of LAM-resistant mutant virus

Factors affecting the cumulative probability of the emergence of the YMDD mutant virus were investigated using

Table 2 Comparison of patient characteristics between IVR-positive and -negative patients

	IVR ($n = 163$)	Non-IVR ($n = 27$)	*P*-value
Gender (male/female)	118/45	21/6	NS
Age (years)	50 ± 11	48 ± 12	NS
Chronic hepatitis/liver cirrhosis	91/72	22/5	NS
Hepatocellular carcinoma	13 (8.0%)	1 (4%)	NS
AST (IU/L)	131 ± 167	69 ± 34	NS
ALT (IU/L)	190 ± 252	100 ± 55	NS
ALT (≤1/1–2/2–5/>5 × ULN)	21/43/52/47	1/10/13/3	NS
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)	7.3 (4.3–7.6<)	<0.0001
HBeAg (positive/negative)	65/98	22/5	0.01
Combination with interferon	27 (17%)	6 (22%)	NS
Emergence of LAM-resistant viruses	65 (40%)	21 (78%)	0.002
Duration of treatment (months)	39.2 ± 17.2	37.3 ± 19.1	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IVR, initial viral response; LAM, lamivudine; NS, not significant; ULN, upper limit normal.

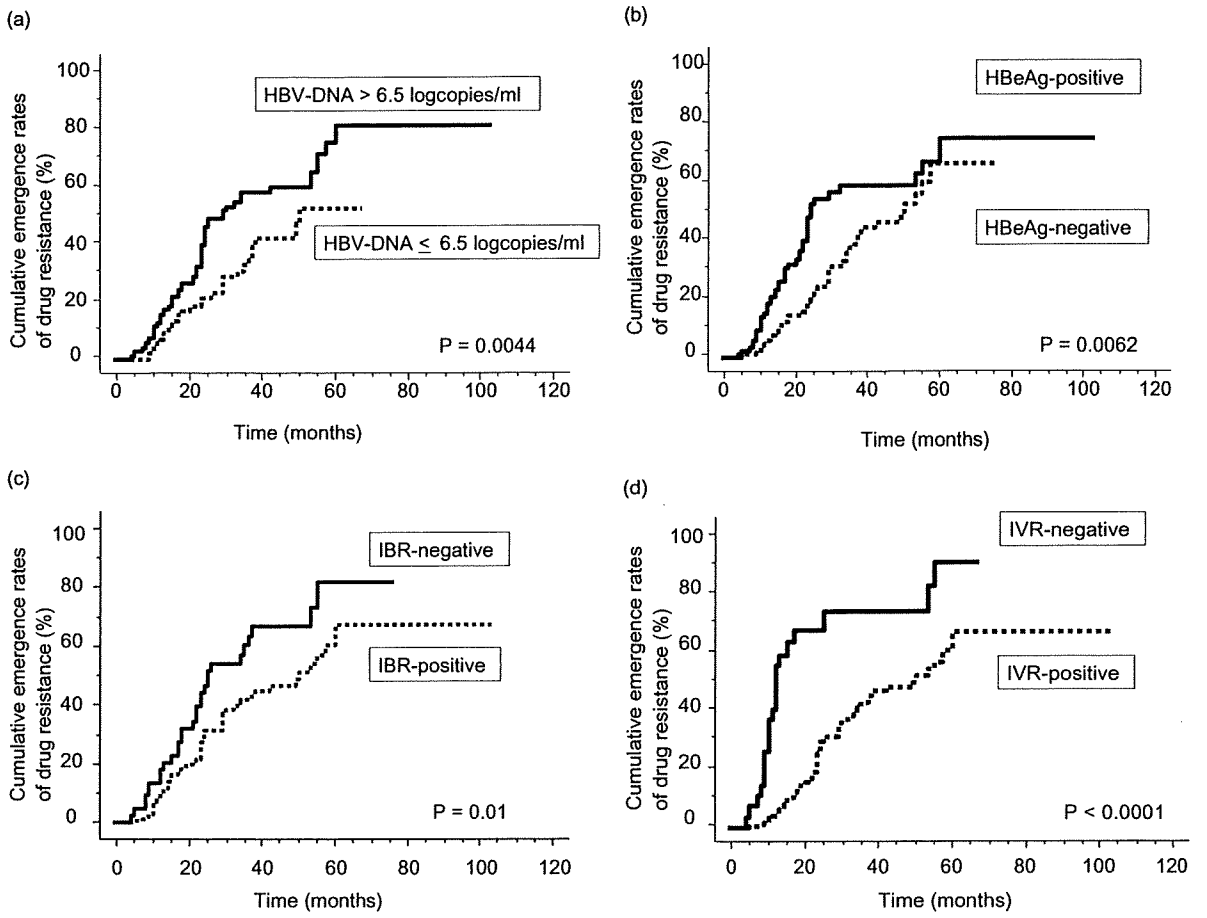

Figure 1 Cumulative emergence rate of lamivudine (LAM)-resistant virus in patients with chronic hepatitis B virus (HBV) infection treated with LAM according to: (a) HBV DNA at baseline; (b) hepatitis B e antigen (HBeAg) status; (c) the presence or absence of initial biochemical response (IBR); and (d) the presence or absence of initial viral response (IVR).

both univariate and multivariate analyses. Nine baseline and on-treatment factors – gender, age, liver disease (chronic hepatitis or cirrhosis), ALT at baseline, HBeAg positivity, HBV DNA at baseline, combination therapy with IFN-α, presence of IBR and presence of IVR – were examined. The cumulative emergence of LAM-resistant virus was significantly higher in patients with baseline HBV DNA > 6.5 logcopies/mL than in those with HBV DNA ≤ 6.5 logcopies/mL ($P = 0.0044$) (Fig. 1a). HBeAg-positive patients revealed a significantly higher emergence rate of the LAM-resistant virus than HBeAg-negative patients ($P = 0.0062$) (Fig. 1b). A significant difference was also seen in the cumulative emergence of the YMDD mutant virus between IBR-positive and -negative patients ($P = 0.01$) (Fig. 1c). Furthermore, the cumulative emergence of LAM-resistant mutant virus was much higher in the IVR-negative patients than in the IVR-positive patients ($P < 0.0001$) (Fig. 1d). The cumulative emergence rates of LAM-resistant virus in the IVR-positive and -negative patients were 4% and 41% at 1 year, 25% and 69% at 2 years, and 41% and 79% at 3 years, respectively. Gender, age, liver disease, ALT at baseline and combination therapy of IFN-α did not show a significant relation with the emergence of the YMDD mutant virus. When factors influencing the higher cumulative emergence of LAM-resistant virus were searched for by multivariate analysis, only the absence of IVR was selected as a significant independent factor ($P < 0.001$) (Table 3), with high HBV DNA, HBeAg positivity and the absence of IBR not being selected.

Table 3 Factors associate with emergence of LAM-resistant virus determined by multivariate analysis

	Hazard ratio	95% confidence interval	*P*-value
Gender			
0: male	1	0.497–1.455	0.55
1: female	1.176		
Age			
0: ≤50	1	0.640–1.700	0.87
1: >50	0.959		
Chronic hepatitis/liver cirrhosis			
0: CH	1	0.656–1.740	0.79
1: LC	0.935		
Pretreatment ALT (IU/L)			
0: ≤200	1	0.605–1.818	0.87
1: >200	0.953		
HBV DNA (logcopies/mL)			
0: ≤6.5	1	0.394–1.125	0.13
1: >6.5	1.502		
HBeAg			
0: negative	1	0.499–1.337	0.42
1: positive	1.225		
Combination therapy with interferon			
0: no	1	0.410–1.303	0.29
1: yes	1.368		
IBR			
0: positive	1	0.483–1.312	0.37
1: negative	1.256		
IVR			
0: positive	1	0.159–0.536	<0.001
1: negative	3.425		

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IBR, initial biochemical response; IVR, initial viral response; LAM, lamivudine.

DISCUSSION

IN LAM THERAPY for patients with chronic HBV infection, the emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. To resolve this, detailed studies are needed to identify factors related to the emergence of the YMDD mutant virus. To date, a few investigators have suggested male gender, advanced age, high baseline ALT, the presence of severe acute exacerbation of the liver disease, high baseline HBV DNA and HBeAg-positivity as possible predictors of the emergence of LAM-resistant virus.[7,17,18] Lower body surface area was also reported as a significant factor for virological and biochemical therapeutic effect.[19] In the present study, we studied 190 patients with chronic hepatitis B treated with LAM and investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus. Univariate analysis revealed that two baseline factors, high HBV DNA and HBeAg positivity, had a relation to the high incidence of the YMDD mutant virus, which is consistent with previous reports.[7,17,18] In addition, two on-treatment factors, IBR and IVR, were found to be correlated with the emergence of LAM resistance. Patients who did not show IVR had a 3.4-fold higher incidence of the emergence of the YMDD mutant virus than those who did show IVR. This agrees with a previous report that the HBV DNA level after 6 months of therapy may be a determinant for subsequent occurrence of a LAM-resistant mutant virus.[20] Multivariate analysis showed that only the absence of IVR was a significant factor contributing to the emergence of LAM-resistant virus. Baseline HBV DNA and HBeAg status were not selected as significant factors by multivariate analysis probably because of the tendency for higher HBV DNA and high frequency of HBeAg positivity in IVR-negative patients compared with IVR-positive patients. It is particularly interesting that the absence of IVR, rather than other baseline and on-treatment factors, was a powerful independent pre-

dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

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MAJOR ARTICLE

Supportive Role Played by Precore and PreS2 Genomic Changes in the Establishment of Lamivudine-Resistant Hepatitis B Virus

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Background. Hepatitis B virus (HBV) establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation. However, both lamivudine-resistant viruses with and those without rtL180M can exist in clinical settings. To elucidate the differences between viruses with and those without rtL180M, we conducted full-length sequencing analysis of HBV derived from patients with type B chronic hepatitis showing lamivudine resistance.

Methods. The full-length HBV DNA sequences derived from 44 patients showing lamivudine resistance were determined by polymerase chain reaction direct sequencing. Viral replicative competence was examined by in vitro transfection analysis using various HBV-expressing plasmids.

Results. Throughout the HBV genome, a precore-defective A1896 mutation and a short deletion in the preS2 gene were detected more frequently in viruses without rtL180M than in those with it (64% vs. 17% [$P < .005$] and 50% vs. 10% [$P < .01$], respectively). In vitro transfection analysis revealed that the level of reduction in intracellular viral replication caused by the introduction of lamivudine resistance–associated mutations was lower in precore-defective and preS2-deleted viruses than in wild-type virus.

Conclusions. Both the precore-defective mutation and the preS2 deletion may play a supportive role in the replication of lamivudine-resistant HBV, which may be a reason for there being no need for the compensatory rtL180M mutation in lamivudine-resistant HBV possessing the precore and preS2 genomic changes.

Therapeutic concepts for hepatitis B virus (HBV) infection have been strikingly modified by the introduction of nucleos(t)ide analogues. Nucleos(t)ide analogues, such as lamivudine, adefovir dipivoxil, entecavir, tenofovir disoproxil fumarate, emtricitabine, telbivudine, and clevudine, have been shown to lead to suppression of viral replication and improvement of liver diseases in chronic HBV infection [1–10]. However, the effectiveness of nucleos(t)ide analogues is debilitated by the emergence of drug-resistant mutant virus. Treatment with lamivudine has been shown to lead to a higher rate of emergence of drug-resistant virus than with other newly developed nucleos(t)ide analogues, such as adefovir dipivoxil and entecavir [11–14]. The incidence of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of therapy [11].

Lamivudine resistance is known to be caused by a point mutation within the reverse transcriptase (rt) domain of the HBV polymerase gene, either rtM204V or rtM204I [15–17]. In addition, an rtL180M mutation has been shown to be frequently found together with the rtM204V/I mutation associated with lamivudine resistance [15–17]. Previous studies using in vitro transfection with the HBV-expressing plasmid have demonstrated that the rtM204V/I mutation principally confers lamivudine resistance but results in a decrease in viral replicative activity [18, 19]. It has also been shown that

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the rtL180M mutation has no relevance to lamivudine resistance in itself but restores the reduced replicative activity caused by the lamivudine resistance–associated rtM204V/I mutation [20]. In light of these findings, the rtL180M mutation has been recognized as being compensatory for the support of replication in lamivudine-resistant HBV. Among patients with type B chronic hepatitis (CH-B) showing lamivudine resistance, almost all rtM204V mutations have been detected in conjunction with the rtL180M mutation, and rtM204I mutations have been found either in isolation or together with the rtL180M mutation [15–17]. Thus, the compensatory rtL180M mutation is not always necessary for generating replicative-competent lamivudine-resistant HBV in the clinical setting. Virus without the rtL180M mutation is speculated to possess specific features in the genome that support viral replicative activity, compared with virus with the mutation. However, differences between lamivudine-resistant viruses with and those without the rtL180M mutation have not been elucidated.

To clarify this, we determined the nucleotide sequences of full-length HBV DNA in 44 patients with CH-B who showed lamivudine resistance, by means of the direct sequencing method. Differences in the whole HBV genome were comprehensively investigated in relation to the presence or absence of the rtL180M mutation.

METHODS

Patients. The subjects were 44 consecutive patients with CH-B (37 males and 7 females) who received lamivudine therapy and became refractory to it at Osaka University Hospital and National Hospital Organization Osaka National Hospital. At the beginning of therapy, all patients tested positive for hepatitis B surface antigen (HBsAg) and were positive for HBV DNA by a branched DNA assay (Quantiplex HBV DNA; Chiron) or a polymerase chain reaction (PCR)–based assay (Amplicor HB Monitor; Roche Diagnostics). All patients were negative for antibodies to hepatitis C virus and HIV; none showed evidence of alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury. Eight patients (18%) had previously received interferon (IFN) therapy.

All patients were treated with 100 mg of lamivudine daily, and liver function tests and monitoring of HBV markers were conducted during follow-up. In 16 patients (36%), natural IFN-α therapy (Sumiferon; Sumitomo Pharmaceuticals) was administered in combination with lamivudine for the initial 24 weeks (total dose, 432 million units). For all 44 patients, the lamivudine-resistant rtM204V/I mutation was detected by a PCR enzyme-linked minisequence assay (Sumitomo Metal Industries) [21] after an initial reduction and subsequent increase in HBV DNA during therapy. All serum samples for sequencing analysis of full-length HBV DNA were collected after the emergence of the lamivudine-resistant mutant virus and were stored at −80°C until use. The serum sampling points ranged from 0.8 to 5.5 years (median, 2.7 years) after the commencement of lamivudine therapy. In addition, pairwise serum samples obtained before therapy were used to determine the nucleotide sequences in portions of HBV DNA as baseline controls for 23 patients (52%).

Patient characteristics at the point of analysis were as follows. Age ranged from 25 to 74 years (median, 51 years). Hepatitis B e antigen (HBeAg) was found in 31 patients (70%), and antibody to HBeAg developed in all 13 HBeAg-negative patients (30%). Serum HBV DNA levels ranged from 3.5 to >7.6 $\log_{10}$ copies/mL (median, 7.2 $\log_{10}$ copies/mL). Serum alanine aminotransferase (ALT) levels ranged from 11 to 393 IU/L (median, 66 IU/L). Chronic hepatitis was diagnosed in 34 patients (77%), cirrhosis in 6 (14%), and hepatocellular carcinoma in 4 (9%), on the basis of liver biopsy and/or abdominal imaging procedures. Informed consent was obtained from all patients.

Genomic analysis of full-length HBV nucleotide sequences. From the serum sample, full-length HBV DNA was amplified by PCR and directly sequenced as described elsewhere [22]. The full-length HBV DNA sequences derived from the 44 patients with lamivudine-resistant CH-B (GenBank accession numbers AB367392–AB367435) were aligned together with the 12 representative HBV strains of various genotypes by means of CLUSTALW software. Phylogenetic tree analysis was then conducted [23, 24].

Plasmid and transfection. The HBV-expressing plasmid pHBC was derived from the genotype C2 HBV strain adr4 (GenBank accession number X01587) [25]. pHBC was constructed by inserting the 1.2-fold HBV genome into pBluescriptIISK$^+$. pHBC-PC and pHBC-ΔPS2, which were generated by site-directed mutagenesis, possessed the precore-defective A1986 mutation and the short deletion of 45 bp (nt 11–55) within the preS2 gene. Further site-directed mutagenesis was done to introduce rtM204V and rtL180M, rtM204I and rtL180M, or rtM204I alone into pHBC, pHBC-PC, and pHBC-ΔPS2. pCMV-SEAP was a secreted alkaline phosphatase–expressing plasmid.

For transfection, 3×10^5 Huh7 cells were seeded on a 35-mm-diameter culture dish and transfected with 1 μg of various HBV-expressing plasmids and 0.06 μg of pCMV-SEAP, using FuGENE6 reagent (Roche Diagnostics). On day 5, the culture supernatant and cell lysate were collected. Transfection efficiency was evaluated by measuring the secreted alkaline phosphatase activity.

Detection of viral progeny DNA and antigen in HBV-expressing cells. For detection of intracellular HBV DNA, cells were lysed in buffer containing 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, and 1% Nonidet-P40. After a 15-min incubation on ice, nuclei were removed by brief centrifugation. Then, the sample was incubated at 37°C for 30 min in the presence of 0.1 mg/mL DNaseI and 10 mmol/L $MgCl_2$. After the reaction was stopped by adding EDTA, the sample was subjected to over-

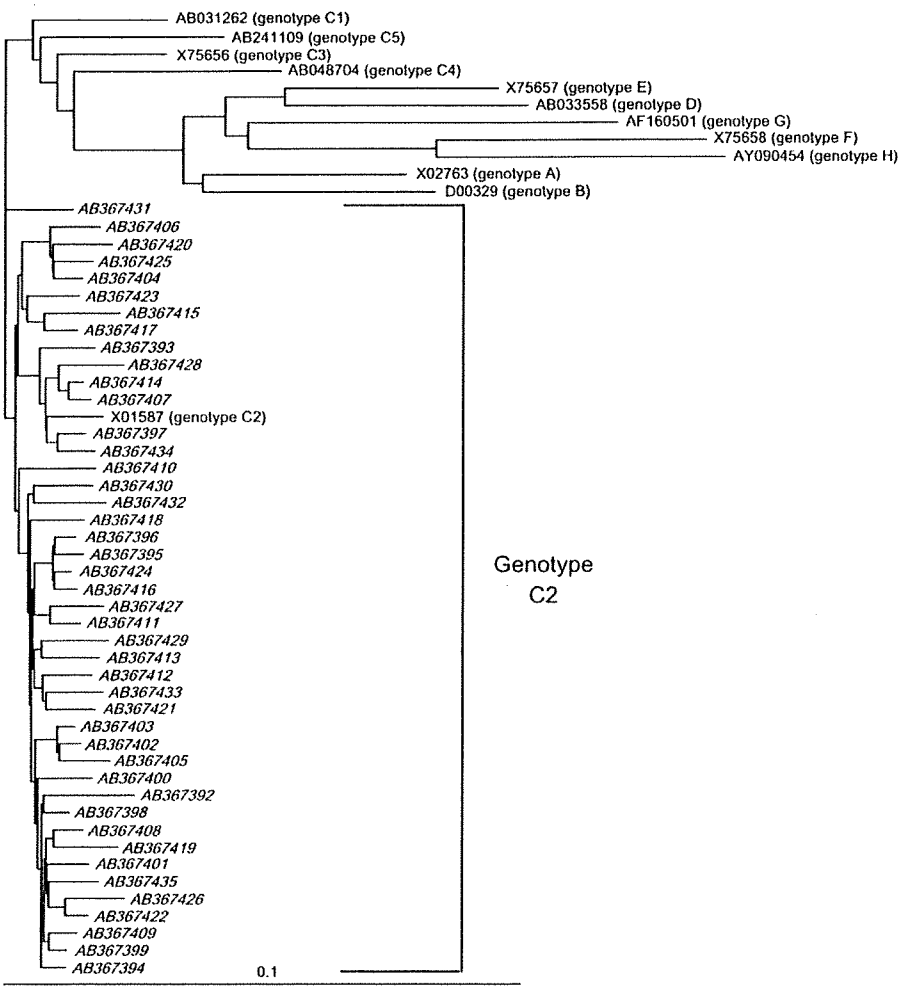

Figure 1. Phylogenetic tree analysis including 44 lamivudine-resistant hepatitis B virus (HBV) strains obtained in the present study and 12 representative HBV strains of various genotypes. All HBV strains are represented as GenBank accession nos., and the 44 lamivudine-resistant HBV strains are indicated by italics.

night incubation at 37°C in buffer containing 1% sodium dodecyl sulfate and 0.5 mg/mL proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA sample was subjected to Southern blot analysis to detect HBV DNA, using a nonradioactive detection system (Alkphos Direct; GE Healthcare Life Sciences). Finally, the signals were analyzed quantitatively using image analyzing software (ImageJ; version 1.38). To detect extracellular HBV DNA, the transfection was scaled up to the 60-mm-diameter culture dish. After clarification by centrifugation at 8300 *g* for 30 min, the culture medium (3 mL) was centrifuged through a 20% sucrose cushion at 192,000 *g* for 4 h, using the Beckmann SW55Ti rotor. Then, DNA was extracted from the pellet and subjected to Southern blot analysis as described above. HBsAg and HBeAg in the culture medium were measured by chemiluminescent immunoassay.

Statistical analysis. Statistical analysis was performed by the χ^2 test, Fisher's exact test, and the Mann-Whitney *U* test. The results for the in vitro transfection study were examined by 1-way analysis of variance, and pairwise comparison was done by Fisher's protected least significant difference test. $P < .05$ was considered to indicate statistical significance.

RESULTS

Patient clinical characteristics and lamivudine resistance–associated mutations. All 44 HBV strains obtained from the patients with lamivudine-resistant CH-B comprised 3161–3230 nt in length and belonged to genotype C2, the most prevalent type in Japan (figure 1). As for lamivudine resistance–associated mutations in these strains, the rtM204V mutation was observed in 16 strains (36%), whereas the remaining 28 strains (64%) had the rtM204I mutation. The compensatory rtL180M mutation was found in 30 strains (68%). All 16 strains with rtM204V and 14 (50%) of the 28 strains with rtM204I possessed the rtL180M

Table 1. Clinical features of patients with lamivudine-resistant type B chronic hepatitis, according to the mutational status of rt180 and rt204.

Clinical feature	rt180 status			rt204 status		
	rtL180M positive (n = 30)	rtL180M negative (n = 14)	P	rtM204V (n = 16)	rtM204I (n = 28)	P
Age, years	48 (25–74)	55 (27–71)	NS	48 (25–74)	51 (27–71)	NS
Sex, M/F, no.	25/5	12/2	NS	13/3	24/4	NS
Liver disease, chronic hepatitis/cirrhosis/ hepatocellular carcinoma, no.	26/3/1	8/4/2	NS	13/2/1	21/5/2	NS
ALT level, IU/L	66 (11–331)	67 (25–393)	NS	85 (17–261)	54 (11–393)	NS
HBeAg, positive/negative, no.	23/7	8/6	NS	13/3	18/10	NS
HBV DNA level, $\log_{10}$ copies/mL	7.5 (3.5 to >7.6)	7.1 (3.6 to >7.6)	NS	7.5 (3.8 to >7.6)	7.1 (3.5 to >7.6)	NS
Previous IFN therapy, no. (%)	6 (20)	2 (14)	NS	3 (19)	5 (18)	NS
Combination therapy with IFN, no. (%)	10 (33)	6 (43)	NS	4 (25)	12 (43)	NS
Duration of lamivudine administration until point of analysis, years	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS

NOTE. Data are median (range) values, unless otherwise indicated. HBeAg, hepatitis B e antigen; IFN, interferon; NS, not significant.

mutation, in agreement with previous reports with respect to the emergence pattern of the rtM204V/I and rtL180M mutations [15–17].

Various patient clinical characteristics were first correlated with the presence or absence of the rtL180M mutation or with the alternative of the rtM204V or rtM204I mutation in our 44 patients with CH-B (table 1). No differences were observed between patients with and those without the rtL180M mutation with respect to age, sex ratio, disease severity, ALT level, HBeAg positivity, serum HBV DNA level, frequency of previous IFN therapy, frequency of combination therapy with IFN, and total duration of lamivudine administration until the point of analysis. Also, there were no significant differences concerning these 9 characteristics between patients with virus having the rtM204V mutation and those with virus having the rtM204I mutation.

Genomic changes throughout the HBV genome associated with lamivudine resistance–associated mutations. Next, the genomic changes, which were significantly correlated with the occurrence of rtL180M or the preference for rtM204V or rtM204I, were investigated for the 44 HBV strains derived from the patients. As shown in table 2, 8 mutations and 1 deletion were identified as viral genomic changes significantly associated with the presence or absence of rtL180M. Among them, the A1896 mutation, which forms the in-frame stop codon in the

Table 2. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with and those without the rtL180M mutation.

Viral genomic changes	Consensus nucleotide[a]	Amino acid substitution	rtL180M, no. (%)		P
			Positive (n = 30)	Negative (n = 14)	
Mutation					
A373	C	Pol-L428M (rtL82M)	0 (0)	3 (21)	<.05
T619	C	None	0 (0)	3 (21)	<.05
G739	T	Pol-M550V (rtM204V), surface-I95R	16 (53)	0 (0)	<.001
T/C/A741	G	Pol-M550I (rtM204I), surface-W96L/S/stop	14 (47)	14 (100)	<.001
A1896	G	Precore-W28stop	5 (17)	9 (64)	<.005
T2102	C	None	0 (0)	3 (21)	<.05
A/G2660	C	Pol-N118K	0 (0)	3 (21)	<.05
A2860	T	PreS1-S5T, pol-V184D[b]	0 (0)	4 (29)	<.01
Deletion					
6–54-bp deletion within nt 1–55	...	Truncation of 2–18 amino acids in preS2[c]	3 (10)	7 (50)	<.01

[a] Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].
[b] One patient had the pol-V184Q amino acid substitution due to a mutation in the adjacent nucleotide position.
[c] Detailed patterns of the preS2 deletion are shown in figure 2.

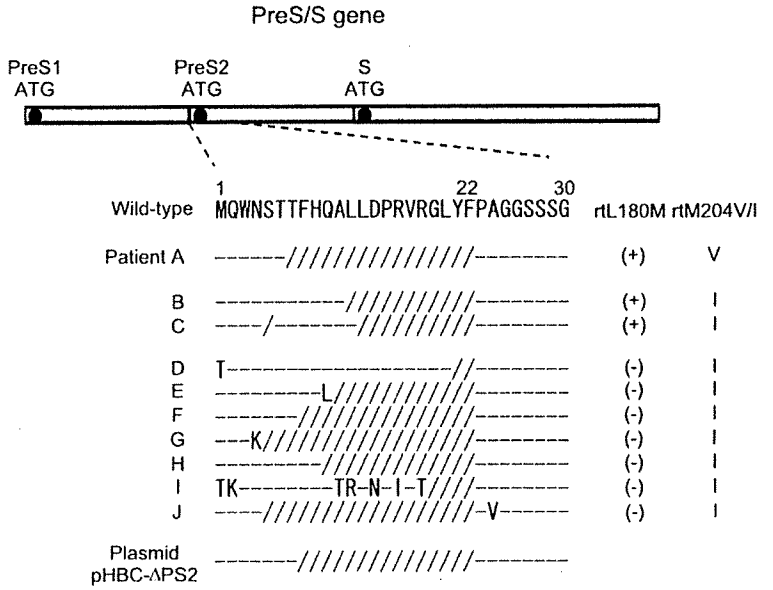

Figure 2. Patterns of the short deletion in the preS2 gene observed in lamivudine-resistant hepatitis B virus (HBV) strains. Ten of the 44 patients (patients A–J) had virus with the deletion in the preS2 gene of various patterns. The top sequence represents the amino acid sequence of the genotype C2 HBV DNA strain adr4 [25] as a representative strain. As for sequences derived from the patients, residues identical to the top sequence are indicated by dashes, whereas deletions of amino acid residues are shown by slashes. All deletions were found within the codon positions 5–22 of the preS2 gene. The bottom sequence represents the deletion pattern of the plasmid (pHBC-ΔPS2) used for in vitro transfection analysis (see figure 3), which expresses HBV DNA with the short deletion in the preS2 gene.

precore gene and results in the disability of HBeAg synthesis [26, 27], was found more frequently in viral strains without rt180M than in those with it (64% vs. 17%; $P < .005$). Viral strains lacking rtL180M possessed the short deletion in the preS2 gene more frequently than those with rtL180M (50% vs. 10%; $P < .01$). The lengths of the deletion ranged from 12 to 54 bp, and all deletions were located within codon positions 5 to 22 of the preS2 gene (figure 2). Significant differences were also seen in the occurrences of 5 additional mutations—A373, T619, T2102, A/G2660, and A2860—between strains with and those without rtL180M. The detection rate of these 5 mutations was generally low among the lamivudine-resistant HBV strains obtained in this study. The G739 and T/C/A741 mutations are the causes of the rtM204V and rtM204I amino acid changes, and the occurrences of these mutations differed between viral strains with and those without rtL180M ($P < .001$), as described above.

Throughout the HBV genome, 5 mutations were significantly associated with the preference for the rtM204V or rtM204I mutation in the 44 lamivudine-resistant HBV strains (table 3). Of them, 3 mutations—C565, A853, and C1568—were found more frequently in strains with rtM204V than in those with rtM204I, but the frequencies of these mutations were considerably low in our lamivudine-resistant HBV strains. The occurrence of the A667 mutation, which accounts for rtL180M, was

Table 3. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with the rtM204V and rtM204I mutations.

Mutation	Consensus nucleotide[a]	Amino acid substitution	No. (%) rtM204V (n = 16)	No. (%) rtM204I (n = 28)	P
C565	T	None	4 (25)	0 (0)	<.05
T/C646	A	Pol-V519L (rtV173L)	5 (31)	0 (0)	<.005
A667	T	Pol-L528M (rtL180M)	16 (100)	14 (50)	<.001
A853	C	None	3 (19)	0 (0)	<.05
C1568	T	Pol-L826P	3 (19)	0 (0)	<.05

[a] Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

Table 4. Changing pattern of the precore defective A1896 mutation and short deletion in the preS2 gene from the pretreatment baseline to development of lamivudine resistance in relation to the presence or absence of the rtL180M mutation.

Type of mutation	Pattern of mutation		rtL180M, no.	
	Before therapy	After therapy[a]	Positive (n = 15)	Negative (n = 8)
Precore-defective A1896 mutation	−	−	8	2
	+	+	4	4
	−	+	1	2
	+	−	2	0
Short deletion in the preS2 gene	−	−	12	5
	+	+	0	1
	−	+	2	2
	+	−	1	0

[a] After development of lamivudine-resistant mutant virus.

higher in viral strains with rtM204V than in those with rtM204I ($P < .001$), as shown above. The T/C646 mutation, which causes the rtV173L change, was detected in 5 strains (31%) with rtM204V, compared with none of those with rtM204I ($P < .005$). It has been reported that the rtV173L mutation was detected together with the rtM204V and rtL180M mutations and was considered to be associated with lamivudine resistance [17, 28]. Our finding concerning the rtV173L mutation agreed with those of previous reports.

According to these observations, the relevance of the precore-defective A1896 mutation and the preS2 deletion to the absence of rtL180M was the most distinctive feature of the lamivudine-resistant HBV strains on screening of the whole genome. We therefore directed our attention to these precore and preS2 genomic changes and further investigated their role in the establishment of lamivudine-resistant virus.

Serial changes in the precore mutation and the preS2 deletion in lamivudine-resistant virus before and after lamivudine therapy. Serial changes in the precore-defective A1896 mutation, the short deletion in the *preS2* gene, and the drug resistance–associated rtM204V/I, rtL180M, and rtV173L mutations were investigated in the 23 (52%) of 44 patients with CH-B whose serum samples obtained before lamivudine therapy were available (table 4). Of the 11 patients with virus having the precore-defective mutation after the development of lamivudine resistance, 8 had virus that already possessed the mutation before therapy. Thus, the precore-defective mutation was generally a preexisting genomic change in most patients showing lamivudine resistance. On the other hand, of the 5 patients with virus that had the deletion in the preS2 gene after the development of drug resistance, 4 had virus that did not possess the deletion before therapy. The frequent detection of the preS2 deletion in lamivudine-resistant virus compared with virus before therapy indicates that this deletion may be coselected with drug resistance–associated mutations during the establishment of lamivudine-resistant mutant virus. As for the lamivudine-resistant rtM204V/I, rtL180M, and rtV173L mutations, they were not detected in any of the 23 viruses before lamivudine therapy, as expected.

Effect of the precore mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV in vitro. We further conducted in vitro transfection analysis to explore the influence of the precore-defective mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV. Three plasmids that expressed wild-type virus, precore-defective virus, and virus with the preS2 deletion were prepared. Next, plasmids with rtM204V plus L180M, rtM204I plus L180M, and rtM204I alone were synthesized in each of the 3 HBV-expressing backbone constructs. The level of intracellular HBV DNA was examined in cells transfected with these HBV-expressing plasmids. As shown in figure 3*A* and 3*B*, the introduction of lamivudine resistance–associated mutations into the virus with the wild-type backbone led to a decrease in viral replication (lanes 1–4). In addition, the replicative competence of the drug-resistant virus lacking rtL180M tended to be lower than that of the virus having rtL180M, although the difference was not statistically significant. As for the precore-defective virus, its replicative activity at baseline was higher than that of the wild-type virus (lanes 1 and 5). The decline in HBV replication due to the insertion of drug resistance–associated mutations was also observed for the virus with the precore-defective backbone. However, unlike for the virus with the wild-type backbone, the replicative activity of the precore-defective virus with lamivudine-resistant mutations was maintained at a considerable level (lanes 5–8). As for the virus with the preS2-deleted backbone, a reduction in viral replication due to the introduction of lamivudine resistance–associated mutations was also seen, but the degree of the reduction was not as great as that in the wild-type virus (lanes 9–12). Thus, both the precore-defective mutation and the preS2 deletion possessed activity supporting the viral replicative competence of lamivudine-resistant HBV, although the activity with the preS2 deletion was not as strong as that with the precore-defective mutation. The

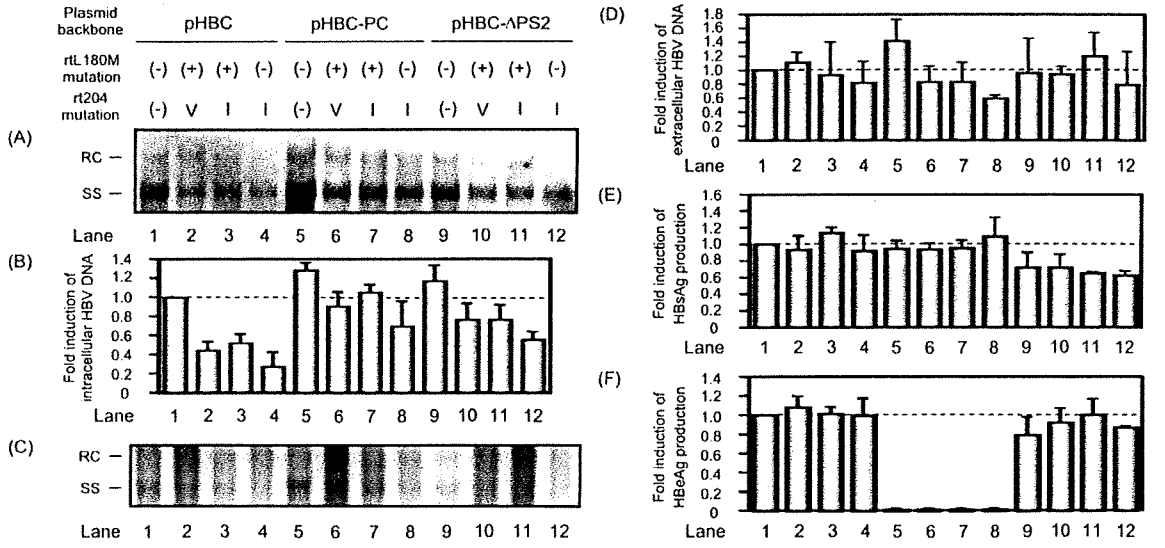

Figure 3. Levels of intracellular and extracellular progeny viral DNA and viral antigen production in cultured cells transfected with wild-type, precore-defective, or preS2-deleted hepatitis B virus (HBV)–expressing plasmids with or without lamivudine resistance-associated mutations. *A,* Representative result of Southern blot analysis to detect the intracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *B,* Quantitative analysis of the level of intracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lane 1 vs. 2–4, 1 vs. 5, 2 vs. 6 and 10, 3 vs. 7 and 11, 4 vs. 8 and 12, 5 vs. 6 and 8, and 9 vs. 10–12. *C,* Representative result of Southern blot analysis to detect extracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *D,* Quantitative analysis of the level of extracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 4 times, and results are shown as mean ± SD values. A statistically significant difference was not observed by 1-way analysis of variance. *E,* Levels of hepatitis B surface antigen (HBsAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBsAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 5 vs. 9, 3 and 7 vs. 11, and 4 and 8 vs. 12. *F,* Levels of hepatitis B e antigen (HBeAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBeAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 9 vs. 5, 2 and 10 vs. 6, 3 and 11 vs. 7, and 4 and 12 vs. 8. RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA.

tendency appeared to be more evident in the drug-resistant virus without the rtL180M mutation. This may be a reason for the compensatory rtL180M mutation not being necessary during the establishment of lamivudine resistance in the HBV strain having the precore and preS2 genomic changes.

When the level of extracellular HBV DNA was examined in cells transfected with various HBV-expressing plasmids (figure 3*C* and 3*D*), no significant differences were observed among wild-type, precore-defective, and preS2-deleted viruses with respect to the reduction of viral secretion caused by the introduction of the lamivudine resistance–associated mutation. The discrepant results between the intracellular and extracellular viral DNA levels likely occurred because the extracellular viral DNA assay was less sensitive to minute changes in viral replication than the intracellular viral DNA assay.

As for the levels of production of HBsAg and HBeAg, the virus with the preS2-deleted backbone produced less HBsAg than did the viruses with the wild-type and precore-defective backbones (figure 3*E*). The wild-type and preS2-deleted viruses secreted HBeAg, whereas the precore-defective virus did not (figure 3*F*). The lamivudine resistance-associated mutations did not affect the production levels of HBV antigens.

DISCUSSION

HBV establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation [15–20]. The present study aimed to investigate the genomewide peculiarity of lamivudine-resistant HBV. In particular, we elucidated the differences between viruses with and those without the compensatory rtL180M mutation. For this purpose, we conducted full-length sequencing analysis of lamivudine-resistant viruses derived from patients with CH-B by means of the PCR direct sequencing method. In some patients, the results were also confirmed by the PCR-subcloning method (data not shown). As a result, the precore-defective

A1896 mutation and the short deletion in the preS2 gene were identified as genomic changes significantly associated with the occurrence of the rtL180M mutation. These 2 viral genomic changes were found to be highly relevant to the observation that the rtL180M mutation was not needed for the establishment of the lamivudine-resistant mutant virus. This suggests that the precore-defective mutation and the preS2 deletion may function as surrogates for the compensatory rtL180M mutation and assist replication of lamivudine-resistant HBV. In the serial analysis of the mutations examined before and after lamivudine therapy, the preS2 deletion tended to be coselected with the drug resistance–associated mutation after therapy, although this tendency was not seen in the case of the precore-defective mutation. This also indicates that the preS2 deletion may have some advantage for establishment of lamivudine-resistant HBV.

We further conducted in vitro transfection analysis to verify the possible supportive role played by the precore and preS2 genomic changes in replication of lamivudine-resistant virus. The intracellular viral DNA was measured as a marker of viral replicative competence. In the wild-type virus, lamivudine resistance–associated mutations reduced viral replicative competence, and the rtL180M mutation compensated for viral replication to a certain degree. This agreed with previous findings of some other investigators [18–20]. On the other hand, the reduction in the viral replication level caused by the lamivudine-resistant mutations was lower in the precore-defective and preS2-deleted viruses than in the wild-type virus. Even the lamivudine-resistant virus without the rtL180M mutation maintained a substantial level of replicative activity in the viruses with precore and preS2 genomic changes. Thus, our results contribute evidence for a supportive role of both precore and preS2 genomic changes in the replicative competence of lamivudine-resistant HBV. This tendency was not evident in the case of the extracellular viral DNA assay, which may have been due to this assay's lower ability to detect slight changes in viral replicative activity.

As for the functional role played by the precore-defective A1896 mutation in the replication competence of lamivudine-resistant HBV, enhanced replicative activity of virus with lamivudine resistance caused by introduction of the precore-defective mutation has been reported for the recombinant HBV–expressing baculovirus system using the genotype D HBV strain [29]. Another previous in vitro transfection analysis using the genotype A HBV strain revealed that experimental insertion of the precore-defective mutation together with the T1858 mutation compensated for the replication competence of the virus possessing lamivudine-resistant mutations [30]. Our experimental result using the genotype C2 HBV strain is consistent with these previous findings. In addition, we showed in the present study that the preS2 deletion may also play a supportive role in the replication yield of lamivudine-resistant HBV, although the enhancement of viral replication caused by the preS2 deletion was not as strong as that caused by the precore-defective mutation.

It remains unclear why the precore-defective mutation leads to an increase in the viral replication of drug-resistant HBV. Previous in vitro transfection analyses have shown that the precore-defective mutation had no influence on viral replicative competence [29–31]. However, in our transfection analysis using the genotype C2 HBV strain, the replicative competence of the precore-defective virus tended to be higher than that of the wild-type virus, even when viruses without the lamivudine resistance–associated mutations were compared. It has recently been shown that the precore-defective mutation caused an elevation in viral replication in the particular HBV strain of genotype B1 [32]. According to this, the precore-defective mutation may in some way enhance HBV replication irrespective of the lamivudine resistance.

As for the involvement of the preS2 deletion in the replicative advantage of lamivudine-resistant HBV, the deletion results in truncation of the polymerase protein as well as the surface protein. Such truncation of the polymerase protein may increase the enzymatic activity and replication capacity of drug-resistant virus. As another possibility, the surface protein with the preS2 deletion may link to incomplete envelopment and subsequent intracellular accumulation of immature viral particles, resulting in an elevated intracellular HBV DNA level. However, this is improbable, because viral envelopment and secretion may be achieved efficiently in preS2-deleted virus as well as wild-type and precore-defective viruses, as was shown in the extracellular viral DNA assay.

In summary, our findings indicate that a precore-defective A1896 mutation and a short deletion in the preS2 gene may support viral replicative activity and substitute for the compensatory rtL180M mutation. Both the precore-defective mutation and the preS2 deletion have been shown to be frequently found during chronic HBV infection [26, 27, 33]. It is noteworthy that such naturally occurring frequent genomic changes in HBV significantly affect the establishment of drug-resistant viral strains. The lamivudine-resistant rtM204V/I mutation has also been reported to be completely or partially involved in resistance to other nucleos(t)ide analogues (emtricitabine, telbivudine, entecavir, and clevudine) [8, 9, 14, 34]. Our findings reveal novel aspects about the establishment of drug-resistant virus possessing the rtM204V/I and rtL180M mutations during the antiviral treatment of patients with CH-B.

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Mutations Associated With the Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Patients Resistant to Lamivudine With Type B Chronic Hepatitis

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Factors influencing the therapeutic efficacy of adefovir dipivoxil added to continuing lamivudine have not been elucidated in lamivudine-resistant patients with type B chronic hepatitis. The viral mutations influencing the efficacy of treatment with adefovir dipivoxil were investigated by sequencing analysis of the whole virus genome. Thirty patients resistant to lamivudine receiving adefovir dipivoxil therapy added to lamivudine were studied. From serum samples obtained before the administration of adefovir dipivoxil, full-length viral DNA sequences were determined by PCR-direct sequencing. Susceptibility of the virus to adefovir was examined further using in vitro transfection analysis. By screening the whole viral genome, the presence of two mutations, a T-to-C/G/A mutation at nt1753 (V1753) and an A-to-C mutation at nt2189 (C2189), correlated with the higher incidence of sustained viral DNA clearance during therapy ($P < 0.005$ and $P < 0.05$). In multivariate analysis, the V1753 ($P = 0.001$) and the C2189 ($P = 0.007$) mutations, and elevated transaminase ($P = 0.011$) and low viral load ($P = 0.008$) at the baseline were selected as significant independent factors associated with improved antiviral efficacy. In vitro transfection analysis showed no differences in susceptibility to adefovir among wild-type virus and C1753 and C2189 mutant viruses, suggesting that the virus possessing these mutations may be eradicated more efficiently than the wild-type virus by treatment regardless of a direct antiviral effect of adefovir. ***J. Med. Virol. 81:798–806, 2009.***

KEY WORDS: antiviral therapy; hepatitis B virus; mutation

INTRODUCTION

Treatment of patients with chronic hepatitis B virus (HBV) infection is aimed at suppressing continuously viral replication thereby preventing progression of liver disease. Lamivudine has been shown to achieve reduction of HBV DNA and histological improvement in the liver in most patients with type B chronic hepatitis [Lai et al., 1998; Dienstag et al., 1999]. However, its long-term administration often causes the emergence of drug-resistant virus, resulting in loss of antiviral activity. The frequency of lamivudine resistance has been reported to be 24% after 1 year and 70% after 4 years of therapy [Lai et al., 2003]. This resistance is conferred by an rtM204V/I mutation occurring within the reverse transcription domain of the HBV polymerase gene [Liaw et al., 2000; Leung et al., 2001; Lai et al., 2003]. The rtL180M mutation is also combined frequently with rtM204V/I [Lai et al., 2003].

Adefovir dipivoxil has been shown to result in significant virological and histological improvement in both nucleoside-naïve and lamivudine-resistant patients with type B chronic hepatitis [Hadziyannis et al., 2003; Marcellin et al., 2003; Perrillo et al., 2004; Peters et al., 2004]. The incidence of adefovir dipivoxil resistance in nucleoside-naïve patients has been reported to be 6% after 3 years and 29% after 5 years of therapy [Hadziyannis et al., 2005, 2006], which is less frequent than that occurring with lamivudine therapy

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alone. As for lamivudine-resistant patients, adefovir dipivoxil resistance has been observed in 18% of patients after 1 year after the change from lamivudine to adefovir dipivoxil [Lee et al., 2006]. Two mutations, rtA181V/T and rtN236T, have been shown to confer resistance to adefovir dipivoxil [Hadziyannis et al., 2005, 2006; Lee et al., 2006]. In the case of adefovir dipivoxil administration added to lamivudine therapy, mutant strains resistant to both drugs develop rarely, although there have been reports of a few patients with resistance to both [Villet et al., 2006; Karatayli et al., 2007].

Thus far, factors affecting the efficacy of adefovir dipivoxil added to lamivudine treatment have not been clarified fully in lamivudine-resistant patients with type B chronic hepatitis. In the present study, sequencing analysis of full-length HBV DNA was undertaken in lamivudine-resistant patients with type B chronic hepatitis, who received adefovir dipivoxil added to the continuing lamivudine treatment, and attempts were made to identify the viral mutations associated significantly with therapeutic efficacy.

PATIENTS AND METHODS

Patients and Treatment

Thirty consecutive patients with type B chronic hepatitis at Osaka University Hospital or the National Hospital Organization Osaka National Hospital participated in this study. These patients were also subjects in a previous study that investigated the correlation of viral genomic changes with occurrences of lamivudine-resistant rtM204V/I and rtL180M mutations [Ohkawa et al., 2008]. All 30 patients had been treated with 100 mg/day of lamivudine and had shown lamivudine resistance with the detection of lamivudine-resistant mutant virus. The total duration of the preceding lamivudine therapy ranged from 20 to 60 (median 35) months. All the patients received 10 mg/day of adefovir dipivoxil continuously in addition to lamivudine. Liver function tests and HBV markers were measured every month for the initial 6 months and every 2 months thereafter. The follow-up period of adefovir dipivoxil therapy ranged from 6 to 40 (median 19) months.

Clinical Features of the Patients

The clinical and virological features of the patients at the commencement of adefovir dipivoxil administration are shown in Table I. The 26 males and 4 females, aged 25–71 (median 49) years, all had hepatitis B surface antigen (HBsAg). Hepatitis B e antigen (HBeAg) was found in 21 (70%) patients. HBV DNA ranged from 4.9 to >7.6 (median 7.2) $\log_{10}$ copies/ml, and alanine aminotransferase (ALT) ranged from 16 to 455 (median 108) IU/L. With respect to liver diseases, 23 (77%) were diagnosed with chronic hepatitis, 3 (10%) with cirrhosis and 4 (13%) with hepatocellular carcinoma (HCC) according to a liver biopsy and/or imaging procedures. None of the patients had evidences of hepatitis C virus- or human immunodeficiency virus-related diseases, alcoholic liver disease, autoimmune hepatitis and drug-induced liver injury. Serum samples for sequencing analysis were collected within 2 months before the commencement of adefovir dipivoxil administration and stored at −80°C. Pairwise serum samples obtained before lamivudine therapy were also collected and used for analysis in 14 of the 30 patients with type B chronic hepatitis. Informed consent was obtained from all patients.

Measurement of HBV Markers

HBsAg, HBeAg and antibody to HBeAg (anti-HBe) were measured by enzyme immunoassay. Serum HBV DNA was quantitated by a PCR-based assay (Amplicor HB Monitor, Roche Diagnostics Co. Ltd, Tokyo, Japan) having a lower detection limit of 2.6 $\log_{10}$ copies/ml.

Sequencing of Full-Length HBV DNA

The full-length HBV DNA was amplified by PCR and subjected to direct sequencing analysis as described elsewhere [Kanada et al., 2007]. In 25 of the 30 patients with type B chronic hepatitis, sequence data of full-length HBV DNA, that had been determined in a previous study [Ohkawa et al., 2008], were also used in this study. In the remaining five patients, HBV DNA sequences were updated using serum samples obtained just before the commencement of adefovir dipivoxil administration. The Genbank accession numbers of the nucleotide sequences in HBV strains determined in this study are shown in Table I.

Plasmid and Transfection

The HBV-expressing plasmid pHBC carried approximately 1.2 times the genomic length of HBV adr4 strain of genotype C (Genbank accession no. X01587) [Fujiyama et al., 1983]. pHBC-C1753 and pHBC-C2189, which had the C1753 and C2189 mutations in their inserted HBV sequences, were generated by site-directed mutagenesis. pCMV-SEAP was the expression plasmid of a secreted alkaline phosphatase.

Huh7 cells (3×10^5 cells) were seeded on a 35-mm-diameter culture dish and transfected with 1 μg of HBV-expressing plasmid and 0.06 μg of pCMV-SEAP using the FuGENE6 reagent (Roche Diagnostics Co. Ltd). After overnight culture, the cells were treated with 1 or 10 μM of lamivudine (GlaxoSmithKline Co. Ltd, Tokyo, Japan), 1 or 10 μM of adefovir (Toronto Research Chemicals, Inc., North York, Canada), 10 μM of lamivudine plus 10 μM of adefovir, or left untreated. The cells were harvested 3 days after transfection. The culture supernatant was used for measurement of alkaline phosphatase activity to evaluate the efficiency of transfection.

Detection of HBV DNA Replicative Intermediate

For detection of the HBV DNA replicative intermediate, the cells were lysed with buffer containing