with those given ≥10 mg/kg/day of the mean ribavirin dose in this study [26.9% (49/182) *vs* 12.4% (26/209), $P < 0.001$] (data not shown). It seems possible to start ribavirin at a lower dose and increase it by degrees with monitoring of Hb level during treatment of patients with mild anaemia or ischemic heart disease, because the ribavirin dose appears to affect the viral relapse as the total dose over 48 weeks, not during the first 12 weeks.

In conclusion, our results have demonstrated that Peg-IFN α-2b is dose-dependently correlated with c-EVR and maintaining as high a drug dose of Peg-IFN α-2b as possible (≥1.2 μg/kg/week) during the first 12 weeks can yield higher c-EVR rates, leading to better treatment outcomes for patients with CH-C genotype 1.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B

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Abstract
Purpose The antiviral effect of adefovir dipivoxil (ADV) added to ongoing lamivudine (LAM) treatment for LAM-resistant chronic hepatitis B (CHB) differs among patients. We investigated clinical factors affecting the response to ADV therapy in LAM-resistant CHB.
Methods The subjects were 75 LAM-resistant CHB patients treated with ADV in addition to LAM. Virological response (VR) was defined as HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the start of ADV therapy. Clinical factors contributing to VR were examined by univariate and multivariate analyses.
Results Lower HBV DNA at baseline and negative hepatitis B e antigen (HBeAg) were significant factors affecting VR in univariate analysis. In multivariate analysis, lower HBV DNA at baseline ($P = 0.005$), negative HBeAg ($P = 0.009$), and higher ALT ($P = 0.036$) were significant independent factors contributing to VR. In HBeAg-positive patients, HBV DNA clearance was more frequently observed during ADV therapy in patients with baseline HBV DNA ≤7.0 logcopies/ml than in those with baseline HBV DNA >7.0 logcopies/ml. By contrast, the link of lower HBV DNA at baseline to better therapeutic response was not evident in HBeAg-negative patients.

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Conclusion In ADV therapy added to ongoing LAM treatment for LAM-resistant CHB, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. Addition of ADV should be done promptly before marked increase in HBV DNA, especially in CHB patients showing LAM resistance positive for HBeAg.

Keywords Adefovir dipivoxil · Lamivudine resistance · Chronic hepatitis B

Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. Chronic HBV infection can cause liver cirrhosis and hepatocellular carcinoma (HCC), resulting in hepatic disease-related deaths of 500,000 to 1.2 million persons [2, 3]. To prevent disease progression and improve the prognosis of patients with chronic HBV infection, HBV DNA replication must be continuously suppressed as much as possible by antiviral therapy. For this purpose, nucleos(t)ide analogs are currently used for a wide range of patients with chronic HBV infection because of their strong antiviral activities and fewer side effects.

Lamivudine (LAM) is the first approved nucleos(t)ide analog for chronic hepatitis B (CHB) patients, but the increasing incidence of LAM resistance during long-term LAM therapy is a serious problem. The emergence rate of the LAM-resistant virus has been reported to be 24% at 1 year and 70% at 4 years of treatment [4]. Almost all LAM resistance is caused by rtM204V/I mutation occurring in the reverse transcriptase domain of the HBV polymerase gene [5].

To counteract this resistance, adefovir dipivoxil (ADV) was considered as it exerts antiviral effects not only on nucleos(t)ide analog-naïve CHB patients but also on LAM-resistant ones [6–9]. ADV-resistant mutation has been reported to be detected in 11% of patients at 3 years and 29% at 5 years for nucleos(t)ide analog-naïve CHB patients [10]. ADV resistance results from rtA181V/T and/or rtN236T mutation [10]. Either switching from LAM to ADV or adding ADV to LAM has been shown to be effective for LAM-resistant CHB patients. In the case of switching from LAM to ADV, ADV resistance has been reported to appear in 18% of patients at 1 year, which is more frequent than in the case of ADV monotherapy for nucleos(t)ide analog-naïve patients [11]. On the other hand, in the case of ADV administration in addition to LAM, the emergence of resistant virus for both LAM and ADV has been reported to be rare for at least 3 years of treatment [12]. Therefore, ADV therapy added to ongoing LAM treatment is currently accepted as the main therapeutic regimen for LAM-resistant CHB patients rather than a switch from LAM to ADV. However, the antiviral effect of ADV therapy in addition to LAM treatment differs among patients with LAM-resistant CHB.

In this study, we investigated clinical factors influencing the therapeutic efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients.

Patients and methods

Patients

The participating centers were 12 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, NTT Nishinihon Osaka Hospital, Higashiosaka City General Hospital, Suita Municipal Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital, and Osaka University Hospital). The subjects were 75 consecutive CHB patients showing LAM resistance. Before the preceding LAM therapy, they all had had hepatitis B surface antigen (HBsAg) for more than 6 months and levels of HBV DNA detectable by the polymerase chain reaction (PCR) method [13]. None of them tested positive for hepatitis C virus antibody or human immunodeficiency virus antibody, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

Anti-HBV treatment

All patients were administered 100 mg of LAM daily. Thirteen (17%) patients had had a history of interferon (IFN) therapy. LAM resistance was judged by detection of rtM204V/I mutation (for 37 patients) or by the existence of virological breakthrough (for 38 patients). Virological breakthrough was defined as the reappearance of detectable HBV DNA of more than 1 log increase in HBV DNA from the nadir on repeated occasions. The median duration of the preceding LAM therapy was 38 (range, 11–83) months. After the emergence of LAM resistance, all patients received 10 mg of ADV daily in addition to ongoing LAM therapy. After the commencement of ADV therapy, liver function and HBV DNA tests were conducted monthly for the first 6 months and every 2 months thereafter. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked every 2 months. The median follow-up duration of ADV therapy was 22 (range 12–51) months. HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the beginning of ADV therapy was defined as a virological response (VR).

Baseline characteristics of the patients

The baseline characteristics of the patients at the commencement of ADV therapy were as follows. They were 59 males and 16 females, with a median age of 54 (range 27–79) years. Forty-one (55%) tested positive for HBeAg, and anti-HBe developed in 34 patients. The virus was genotyped for 13 patients, all of whom were infected with HBV of genotype C. The HBV DNA ranged from 3.1 to >7.6 (median 7.1) logcopies/ml, and the median ALT level ranged from 15 to 500 (median 105) IU/L. The median levels of total bilirubin and albumin were 0.8 (range 0.4–3.9) mg/dl and 3.9 (range 2.1–4.8) g/dl, respectively. The median platelet counts were 11.7 (range 3.5–25.5) $\times$ $10^4/mm^3$ Of the 75 patients, 27 (36%) showed features of cirrhosis by liver biopsy and/or imaging procedures. Five patients (7%) developed HCC as detected by imaging modalities.

HBV testings

HBsAg, HBeAg, and anti-HBe were examined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics, Tokyo, Japan) [13], with a lower detection limit of 2.6 logcopies/ml. The LAM-resistant rtM204V/I mutation was examined by PCR-enzyme-linked minisequence assay [14]. HBV genotype was determined based on PCR-direct sequencing of portions of core and polymerase genes. The primers used for this study were BF1s (5′-TTT TTC ACC TCT GCC TAA TCA-3′, nt 1821–1841), BR3 (5′-TTC CCG AGA TTG AGA TCT TC-3′, nt 2440–2421), BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369), and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176) [15, 16].

Statistical analysis

Group comparisons were carried out by the chi-square test, Student's t test and Mann–Whitney's U test. Independent factors contributing to VR during ADV therapy added to ongoing LAM treatment were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A P-value of less than 0.05 (two-tailed) was considered to indicate a significant difference. All statistical analyses were performed using the SPSS version 15.0J software (SPSS, Chicago, IL).

Results

Virological and biochemical response to ADV therapy added to ongoing LAM in CHB patients showing LAM resistance

Of the 75 CHB patients showing LAM resistance who underwent ADV therapy added to ongoing LAM treatment, HBV DNA clearance was achieved in 29 (39%) of 75 at 6 months, 35 (47%) of 75 at 12 months, and 34 (72%) of 47 at 24 months. Among the HBeAg-positive patients, HBeAg loss was observed in 8 (20%) of 41 at 6 months, 7 (18%) of 39 at 12 months, and 6 (22%) of 27 at 24 months. As for the biochemical response, ALT normalization ($\leq$40 IU/l) was seen in 57 (76%) of 75 at 6 months, 56 (75%) of 75 at 12 months, and 40 (85%) of 47 at 24 months of treatment.

Pretreatment clinical factors associated with therapeutic response to ADV in addition to LAM treatment

We first investigated pretreatment clinical factors associated with the therapeutic efficacy of ADV added to ongoing LAM treatment by univariate analysis. The baseline characteristics of patients at the beginning of ADV therapy in addition to LAM in the presence or absence of VR are shown in Table 1. Patients showing VR had significantly lower HBV DNA at baseline than patients who did not achieve VR [median 6.3 (range 3.1 to >7.6) vs. 7.3

Table 1 Patient clinical characteristics at the beginning of ADV therapy in addition to LAM in LAM-resistant CHB patients in the presence or absence of virological response (VR)

Clinical characteristics	VR (n = 35)	Non-VR (n = 40)	P value
Gender (male/female)	26/9	33/7	0.386
Age (years)	52 (28–67)	55 (27–79)	0.896
Duration of prior LAM therapy (months)	38 (12–83)	37 (13–64)	0.856
Positive HBeAg	12 (34%)	29 (73%)	0.001
HBV DNA (logcopies/ml)	6.3 (3.1 to >7.6)	7.3 (3.9 to >7.6)	0.002
ALT (IU/l)	106 (16–500)	75 (15–455)	0.136
Total bilirubin (mg/dl)	0.9 (0.4–3.9)	0.7 (0.4–3.9)	0.664
Albumin (g/dl)	4.0 (2.4–4.8)	3.8 (2.1–4.6)	0.351
Platelet count ($\times 10^4/mm^3$)	12.2 (4.8–24.1)	11.5 (3.5–25.5)	0.854
Liver disease (chronic hepatitis/cirrhosis)	20/15	28/12	0.247
Presence of HCC (%)	2 (6%)	3 (8%)	0.757

Continuous variables are expressed as median (range)

Table 2 Baseline factors affecting virological response (logistic regression analysis, stepwise method)

Factors	Category	Odds ratio	95% CI	*P*
Gender	Male/female			NS
Age (years)	By 1 year			NS
Duration of prior LAM therapy (months)	By 1 month			NS
HBeAg	Negative/positive	5.766	1.855–36.62	0.009
HBV DNA (logcopies/ml)	By 1 logcopy/ml	2.362	1.335–5.178	0.005
ALT (IU/l)	By 1 IU/l	1.006	1.000–1.011	0.036
Total bilirubin (mg/dl)	By 1 mg/dl			NS
Albumin (g/dl)	By 1 g/dl			NS
Platelet count ($\times 10^4$/mm^3)	By 1 $\times 10^4$/mm^3			NS
Liver disease	Chronic hepatitis/cirrhosis			NS
Presense of HCC (%)	No/yes			NS

CI Confidence interval, *NS* not significant

(range 3.9 to >7.6), $P = 0.002$]. HBeAg was detected in only 12 (34%) of 35 patients with VR, compared with 29 (73%) of 40 patients without VR ($P = 0.001$). Gender ratio, age, duration of preceding LAM therapy, ALT, total bilirubin, albumin, platelet counts, disease severity, and presence of HCC did not differ between VR and non-VR patients.

Factors affecting the therapeutic response to ADV therapy in addition to ongoing LAM were also evaluated by multivariate analysis (Table 2). Eleven pretreatment clinical factors were applied to the analysis as variables. Two factors, lower baseline HBV DNA ($P = 0.005$, odds ratio: 2.362, 95% confidence interval: 1.335–5.178) and negative HBeAg ($P = 0.009$, odds ratio: 5.766, 95% confidence interval: 1.855–36.62), were selected as significant independent factors affecting VR, as was the case for univariate analysis. In addition, higher baseline ALT was also chosen as a significant independent factor ($P = 0.036$, odds ratio 1.006, 95% confidence interval: 1.000–1.011). As for the biochemical response to ADV therapy added to LAM, no pretreatment clinical factors showed a significant relationship with the occurrence of ALT normalization in our 75 LAM-resistant CHB patients.

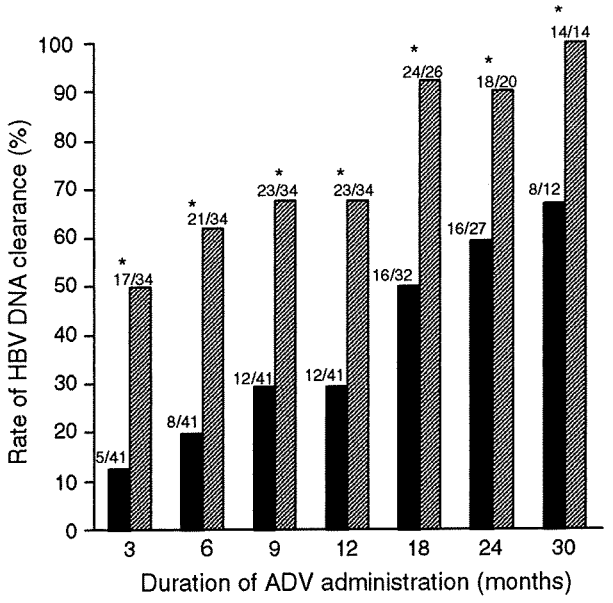

Fig. 1 Rates of HBV DNA clearance in CHB patients positive or negative for HBeAg during ADV therapy in addition to LAM. $*P < 0.05$ between HBeAg-positive and HBeAg-negative patients. *Solid bars* HBeAg-positive patients, *hatched bars* HBeAg-negative patients

HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment according to HBeAg status

Next, we investigated HBV DNA clearance during ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients positive or negative for HBeAg (Fig. 1). In HBeAg-positive patients, HBV DNA was cleared in 8 (20%) of 41 at 6 months, 12 (29%) of 41 at 12 months, and 16 (59%) of 27 at 24 months. On the other hand, HBV DNA clearance was seen in 21 (62%) of 34 at 6 months, 23 (68%) of 34 at 12 months, and 18 (90%) of 20 at 24 months in HBeAg-negative patients. A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was observed between HBeAg-positive and HBeAg-negative patients at 3, 6, 9, 12, 18, 24, and 30 months of treatment. Thus, patients negative for HBeAg tended to respond to ADV therapy added to ongoing LAM treatment better than those positive for it in LAM-resistant CHB.

HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in relation to HBeAg status and baseline HBV DNA

We examined HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in HBeAg-positive and

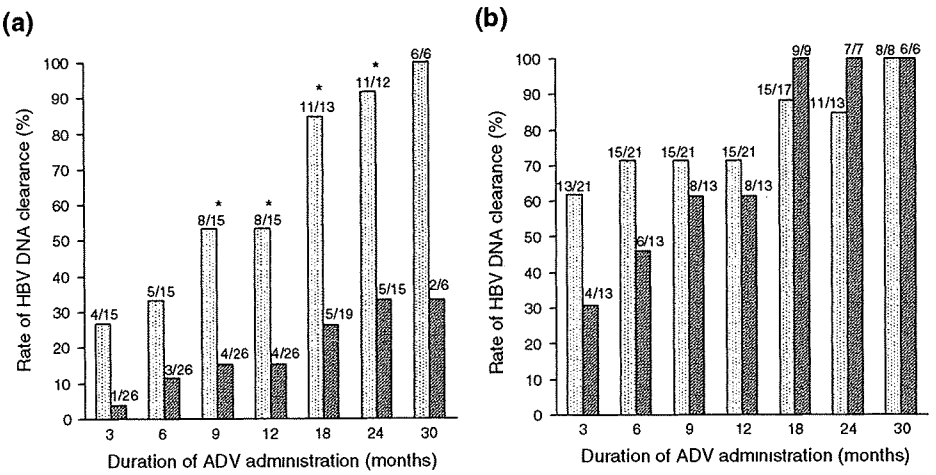

Fig. 2 Rates of HBV DNA clearance during ADV therapy in addition to LAM according to HBV DNA at baseline in **a** HBeAg-positive CHB patients and **b** HBeAg-negative CHB patients. *$P < 0.05$ between patients with low (≤7.0 logcopies/ml) and high (>7.0 logcopies/ml) HBV DNA. *Dotted bars* Patients with HBV DNA ≤7.0 logcopies/ml at baseline, *hatched bars* patients with HBV DNA >7.0 logcopies/ml at baseline

HBeAg-negative CHB patients in relation to baseline HBV DNA. In the case of HBeAg-positive CHB patients (Fig. 2a), the rates of HBV DNA clearance were 33% (5/15) at 6 months, 53% (8/15) at 12 months, and 92% (11/12) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). By contrast, the frequencies of HBV DNA clearance were only in 12% (3/26) at 6 months, 15% (4/26) at 12 months, and 33% (5/15) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was observed between patients with low and high viremia at 9, 12, 18, and 24 months of treatment. In the case of HBeAg-negative patients (Fig. 2b), the rates of HBV DNA clearance were 71% (15/21) at 6 months, 71% (15/21) at 12 months, and 85% (11/13) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). The frequencies of HBV DNA clearance were 46% (6/13) at 6 months, 62% (8/13) at 12 months, and 100% (7/7) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). No significant differences were observed in the frequency of HBV DNA clearance between patients with low and high viremia. According to these findings, the relevance of lower baseline HBV DNA for achieving a better antiviral effect was evident only in HBeAg-positive patients, but not in HBeAg-negative ones in ADV therapy added to LAM treatment for LAM-resistant CHB.

Discussion

This study investigated factors affecting the antiviral efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients. Therapeutic efficacy was assessed as the presence or absence of VR. Both univariate and multivariate analyses revealed that lower baseline HBV DNA and negative HBeAg were strong factors associated with a better therapeutic response. Another significant factor revealed by multivariate analysis was high ALT, although it was weaker than the other two factors. In previous investigations, female gender, lower baseline HBV DNA, negative HBeAg, higher ALT, and genotype D rather than A have been reported to contribute to better VRs to ADV therapy in nucleos(t)ide-naïve and LAM-resistant CHB patients [17–21]. Our results agreed partially with them. The present study, as well as previous studies [18, 19], also revealed that a high baseline ALT may be a determining factor for a better response to ADV therapy in addition to LAM treatment in LAM-resistant CHB. This may be because the host immune response against viral antigens induced by active breakthrough hepatitis has a favorable antiviral effect during ADV therapy. In this study, however, a low baseline viremic level was shown to be a stronger factor than high baseline ALT. The baseline ALT level was the third factor contributing to VR. Therefore, in LAM-resistant CHB, ADV administration should be started before the flare-up of ALT elevation, especially in patients with severe liver disease such as cirrhosis.

In LAM-resistant patients, the HBV DNA level is low during the initial phase, but increases with time, leading to the onset of breakthrough hepatitis. Thus, in ADV therapy added to LAM treatment for LAM-resistant-CHB, the baseline HBV DNA level varies with the observation period after the emergence of LAM resistance. A previous report on Italian HBeAg-negative CHB patients showing LAM resistance revealed that patients with low viremia and normal ALT tended to respond to ADV therapy in addition to LAM treatment better than those with high viremia and abnormal ALT [17]. In the present study conducted in Japan, a genotype C-endemic area, such a close relationship between lower baseline HBV DNA and better therapeutic response was remarkable in

HBeAg-positive patients but not in HBeAg-negative ones. Our finding suggests that, in LAM-resistant CHB, ADV should be added before the HBV DNA begins to increase markedly, especially in HBeAg-positive patients.

In this study, none of the 75 patients showed virological breakthrough after the beginning of ADV administration. All displayed more than 1 log reduction of HBV DNA at 12 months of ADV treatment. This indicates that our patients may not have produced viruses resistant to both LAM and ADV. The emergence of resistant viruses has been reported to be rare in combination therapy using LAM and ADV for LAM-resistant CHB patients, although recent studies have found the existence of a virus resistant to both drugs [22, 23]. The rtA181V/T/S mutation has been reported to confer cross resistance to LAM and ADV [22, 23]. In ADV monotherapy for nucleos(t)ide analog-naïve CHB patients, the absence of HBV DNA reduction to <4 logcopies/ml at 24 weeks of treatment has been reported to be related to the higher emergence of a ADV-resistant virus [24], as is the case in LAM monotherapy [25]. In ADV therapy added to LAM treatment in LAM-resistant CHB patients, the poor response during the initial phase may lead to the development of virus resistance to LAM and ADV as well. From this point of view, the addition of ADV to ongoing LAM treatment before the elevation of HBV DNA may be beneficial in LAM-resistant CHB patients to avoid the development of a multi-drug-resistant virus. Recently, some investigators have reported that tenofovir disoproxil fumarate is effective against a virus resistant to both LAM and ADV [22, 23], but it has not yet been approved for clinical use.

Our results conclusively showed that, with ADV therapy added to LAM treatment for LAM-resistant CHB patients, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. After the emergence of LAM resistance, ADV should be added before the marked elevation of HBV DNA in order to attain better antiviral efficacy, especially in HBeAg-positive patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Lamivudine-to-entecavir switching treatment in type B chronic hepatitis patients without evidence of lamivudine resistance

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Abstract
Purpose A considerable number of chronic hepatitis B (CH-B) patients remain under continuous lamivudine treatment, although switching treatment to entecavir could be beneficial. We investigated the antiviral efficacy of switching treatment to entecavir in CH-B patients without apparent evidence of lamivudine resistance during the preceding lamivudine treatment.
Methods Forty-four CH-B patients, who underwent lamivudine treatment for more than 6 months and showed no evidence of lamivudine resistance, switched to entecavir. Serial changes in hepatitis B virus (HBV) DNA were correlated with the patients' baseline HBV DNA at the commencement of entecavir administration. The entecavir-resistant substitution was examined by PCR-direct sequencing. The median follow-up period of entecavir treatment was 20 (10–23) months.
Results All 31 patients with baseline HBV DNA <2.6 logcopies/ml maintained HBV DNA-negative status during entecavir treatment. Of seven patients having HBV DNA of 2.6–<4.0 logcopies/ml, all achieved undetectable HBV DNA at the end of follow-up. As for six patients having HBV DNA ≥4.0 logcopies/ml, three patients achieved undetectable HBV DNA, whereas virological breakthrough was observed in one patient at month 15. An entecavir-resistant virus having rtM204V, rtL180M and rtS202G substitutions was detected in this patient.
Conclusions The lamivudine-to-entecavir switching treatment may be generally recommendable in CH-B patients without evidence of lamivudine resistance during

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the preceding lamivudine treatment. However, great care should be taken with respect to the emergence of entecavir-resistance, especially in patients who do not respond well to the preceding lamivudine treatment.

Keywords Chronic hepatitis B · Lamivudine resistance · Entecavir-resistance

Introduction

Nucleos(t)ide analogs have been accepted as useful agents for suppressing hepatitis B virus (HBV) replication and disease progression in patients with type B chronic hepatitis (CH-B). Lamivudine, the first approved nucleoside analog, has been shown to provide short-term benefit for CH-B patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology [1, 2]. However, a serious shortcoming of lamivudine is the high incidence of drug resistance during long-term treatment. The detection rate of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of treatment [3]. Lamivudine resistance is caused by an rtM204V/I substitution within the reverse transcriptase domain of HBV polymerase gene [4–6]. An rtL180M substitution frequently emerges as a "replication-compensatory" one with the "resistance-causative" rtM204V/I substitution [4–7]. The emergence of lamivudine-resistant mutant HBV leads to the elevation of HBV DNA ("virological breakthrough") and the subsequent increase of ALT ("breakthrough hepatitis"), resulting in disease progression. Adefovir dipivoxil and tenofovir disoproxil fumarate have been shown to be effective in both nucleos(t)ide analog-naïve and lamivudine-resistant CH-B patients [8–13].

Recently, entecavir has been demonstrated to exert antiviral efficacy in both nucleos(t)ide analog-naïve and lamivudine-refractory CH-B patients [14–16]. The frequency of entecavir-resistance has been reported to be less than 1% at 4 years of treatment in nucleos(t)ide analog-naïve CH-B patients [17]. On the other hand, in switching treatment to entecavir for lamivudine-refractory CH-B patients, most of whom developed lamivudine resistance during the preceding lamivudine therapy, the cumulative probability of entecavir-resistance has been reported to be no less than 40% at 4 years of treatment [17]. Entecavir-resistance has been shown to be established by amino acid substitution(s) at rt184, rt202 and/or rt250 along with the lamivudine-resistant rtM204V and rtL180M substitutions [18]. In the case of nucleos(t)ide analog-naïve patients, the requirement of at least three amino acid substitutions serves as a high genetic barrier to entecavir-resistance. By contrast, in the case of lamivudine-resistant patients, a lower genetic barrier results in higher incidence of entecavir-resistance because two amino acid substitutions, rtM204V and rtL180M, already exist from the preceding lamivudine treatment. The reduced susceptibility to entecavir of the lamivudine-resistant virus compared with the wild-type virus is also a reason for the higher emergence rate of entecavir-resistance in lamivudine-resistant patients than in nucleos(t)ide analog-naïve ones [19].

Although lamivudine is not currently recommended as a first-line drug for nucleos(t)ide analog-naïve CH-B, a considerable number of CH-B patients are under continuous treatment with lamivudine. In these patients, the switch to entecavir treatment could be advantageous over continuation of lamivudine treatment by offering stronger antiviral efficacy and less chance of drug resistance. With respect to the manner of emergence of entecavir-resistance, switching a patient's treatment may be more appropriate before the appearance of lamivudine resistance than after its development. However, the usefulness of lamivudine-to-entecavir switching treatment has not been assessed in CH-B patients without apparent evidence of lamivudine resistance.

This led us to investigate the antiviral efficacy and emergence of entecavir-resistance in CH-B patients who showed no evidence of lamivudine resistance during the preceding lamivudine treatment and underwent the switching treatment to entecavir.

Patients and methods

Patients

This study included 44 consecutive CH-B patients from 10 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, Suita Municipal Hospital, Yao Municipal Hospital, Osaka Rousai Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital and Osaka University Hospital) who underwent continuous lamivudine treatment (100 mg/day) for more than 6 months and showed no apparent evidence of lamivudine resistance. Before starting the preceding lamivudine treatment, all patients had abnormal ALT, positive hepatitis B surface antigen (HBsAg) and a detectable level of HBV DNA according to PCR-based assay (Amplicor HB Monitor, Roche Diagnostics) or branched DNA assay (Quantiplex HBV DNA, Chiron). None of them showed evidence of dual infection with hepatitis C virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis and drug-induced liver injury. The total duration of the preceding lamivudine treatment ranged from 6 to 73 (median, 14)

months. The absence of lamivudine resistance was defined by no detection of the rtM204V/I substitution as measured by the PCR–enzyme linked minisequence assay (ELMA) (Sumitomo Metal Industries) [20] for 33 patients, or by the lack of virological breakthrough as judged by more than 1 log increment in HBV DNA from the nadir for the remaining 11 patients. All of the 44 patients switched to 0.5 mg/day of entecavir administration. After the beginning of entecavir treatment, liver function tests and HBV markers were measured at 1- to 2-month intervals. When virological breakthrough was observed during follow-up, entecavir-resistance-associated mutations were examined by means of a PCR-direct sequencing method. The follow-up period of entecavir treatment ranged from 10 to 23 (median 20) months.

Baseline characteristics of the patients

At the commencement of switching treatment to entecavir, the 28 males and 16 females were aged 33–79 (median 59) years. Seventeen patients (39%) tested positive for hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) developed in all of the 27 HBeAg-negative patients. Among the 27 HBeAg-negative patients, four achieved HBeAg clearance during the preceding lamivudine treatment. HBV DNA at baseline varied among patients from <2.6 to 5.2 logcopies/ml. The baseline ALT ranged from 11 to 78 (median 25) IU/l. Regarding the liver diseases of the patients, 27 (61%) showed features of chronic hepatitis, 11 (25%) of liver cirrhosis and six (14%) of hepatocellular carcinoma (HCC) according to liver biopsy and/or abdominal imaging procedures. HBV genotype was examined for 14 patients, and all of them had HBV genotype C, the most predominant genotype in Japan. Informed consent was obtained from all patients.

Serological and virological markers of HBV

HBsAg, HBeAg and anti-HBe were determined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics) whose lower detection limit is 2.6 logcopies/ml. Lamivudine-resistant rtM204V/I substitution was examined by the PCR–ELMA method (Sumitomo Metal Industries) (20), which is capable of detecting the mutant virus in a mixed viral population if it is present at more than 10% of the total population. The entecavir-resistance-associated substitutions and HBV genotype were determined by a PCR-direct sequencing method. As for oligonucleotide primers for PCR reaction, the outer primer sets were BF5 (5′-AAG AGA CAG TCA TCC TCA GG-3′, nt 3183–3202) and BR1s (5′-AAA AAG TTG CAT GGT GCT GG-3′, nt 1825–1806), and the inner primer sets were BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176). After DNA extraction, the DNA sample was subjected to the PCR reaction for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) using the inner primer set, followed by a final extension at 72°C for 10 min. If amplification was not successful by the single PCR reaction, the nested PCR was conducted; the first round PCR was done using the outer primer sets for 35 cycles, and the aliquot of the product was used for the second round PCR for 30 cycles using inner primer sets. All sequencing reactions of the PCR products were carried out using the BigDye Terminater Ver. 3.1 Cycle Sequencing Kit, and 3100 or 3730 Genetic Analyzer (Applied Biosystems), which allowed determination of the amino acid sequences of rt85-344. For determining the HBV genotype, nucleotide sequences obtained in each of the patients were aligned along with representative HBV strains of genotype A–H, and a phylogenetic tree was constructed in the homepage of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Statistical analysis

Statistical analysis for group comparison was performed by Fisher's exact probability test and Mann–Whitney's non-parametric U test using the SPSS version 15.0J software (SPSS Inc, Chicago, IL). A p value of less than <.05 was considered to be significant.

Results

Classification of patients who underwent lamivudine-to-entecavir switching treatment according to baseline HBV DNA

The 44 CH-B patients who underwent the switching treatment from lamivudine to entecavir were first classified according to their baseline HBV DNA at the commencement of entecavir administration. HBV DNA was not detectable (<2.6 logcopies/ml) in 31 patients (70%) at baseline. Seven patients (16%) had baseline HBV DNA of 2.6–<4.0 logcopies/ml. In the remaining six patients (14%), the baseline HBV DNA was ≥4.0 logcopies/ml. When patient clinical characteristics were compared among the three patient groups (Table 1), nine (29%) of the 31 patients with baseline HBV DNA <2.6 copies/ml tested positive for HBeAg at the commencement of switching treatment to entecavir, compared with five of the six (83%) patients with baseline HBV DNA ≥4.0 copies/ml ($p < .05$). Gender ratio, age, ALT at baseline, liver disease, duration of the preceding lamivudine treatment and

Table 1 Patient clinical characteristics and the therapeutic efficacy in 44 CH-B patients in relation to their baseline HBV DNA

	Baseline HBV DNA		
	<2.6 logcopies/ml ($n = 31$)	2.6–<4.0 logcopies/ml ($n = 7$)	≥4.0 logcopies/ml ($n = 6$)
At the commencement of switching treatment to entecavir			
Gender (male/female)	19/12	5/2	4/2
Age (years)	60 (35–79)[a]	65 (41–69)	55 (33–65)
HBeAg (positive/negative)	9/22	3/4	5/1[b]
HBV DNA (logcopies/ml)	<2.6	3.1 (2.6–3.6)[c]	4.6 (4.0–5.2)[c,d]
rtM204V/I mutation (absence/NT)	23/8	5/2	5/1
ALT (IU/l)	25 (11–64)	31 (13–46)	20 (17–78)
Chronic hepatitis/cirrhosis/HCC	19/7/5	4/2/1	4/2/0
Follow-up period of entecavir treatment (months)	19 (10–23)	19 (10–22)	20 (16–22)
The rate of undetectable HBV DNA level during follow-up	31 (100%)	7 (100%)	3 (50%)[c]
Emergence of entecavir-resistance during follow-up	0 (0%)	0 (0%)	1 (17%)
At the commencement of preceding lamivudine treatment			
HBeAg (positive/negative)	12/19	4/3	5/1
HBV DNA (logcopies/ml)	6.5 (4.3–7.6‹)	6.6 (6.2–7.6‹)	7.6‹ (5.9–7.6‹)
Duration of preceding lamivudine treatment (months)	15 (6–73)	10 (7–42)	9 (8–32)

NT not tested

[a] Values are expressed as median (range)

[b] $p < .05$ versus baseline HBV DNA <2.6 logcopies/ml group

[c] $p < .01$ versus baseline HBV DNA <2.6 logcopies/ml group

[d] $p < .01$ versus baseline HBV DNA of 2.6–<4.0 logcopies/ml group

follow-up period of entecavir treatment did not differ among the three groups. Also, there was no significant difference in HBV DNA and the frequency of positive HBeAg at the commencement of preceding lamivudine treatment among them.

Antiviral efficacy and drug resistance in lamivudine-to-entecavir switching treatment in relation to baseline HBV DNA

Next, we investigated serial changes in HBV DNA after the switch from lamivudine to entecavir treatment in CH-B patients in relation to the baseline HBV DNA. All 31 patients with baseline HBV DNA <2.6 logcopies/ml maintained undetectable HBV DNA during the follow-up period of entecavir treatment. Figure 1 shows the longitudinal evaluation of HBV DNA during the switching treatment to entecavir in patients with a detectable level of baseline HBV DNA. In patients having baseline HBV DNA of 2.6–<4.0 logcopies/ml (Fig. 1a), all of the seven patients achieved sustained undetectable HBV DNA during follow-up, although HBV DNA was transiently detected in one patient. As for patients having baseline HBV DNA ≥4.0 logcopies/ml (Fig. 1b), three (50%) of the six patients achieved sustained undetectable HBV DNA during follow-up. In two patients, HBV DNA was not cleared entirely, but declined to 2.9 and 2.7 logcopies/ml at month 18, respectively. In sequencing analysis at that time, the former patient had the lamivudine-resistant rtM204I substitution, although it was not detected by the PCR–ELMA assay at the start of entecavir treatment. The latter patient had no drug resistance-associated substitutions. In the sixth patient, HBV DNA decreased initially, but virological breakthrough was seen at month 15. The entecavir-resistant virus was detected after virological breakthrough. The detailed disease course of the entecavir-resistant patient is described below. As for the relationship of baseline HBV DNA to the frequency of undetectable HBV DNA, HBV DNA was cleared more frequently in patients with baseline HBV DNA <2.6 logcopies/ml than in those with baseline HBV DNA ≥4.0 logcopies/ml (100 vs. 50%, $p < .01$) (Table 1).

Serial changes in ALT during lamivudine-to-entecavir switching treatment were further examined. Among the 31 patients with baseline HBV DNA <2.6 logcopies/ml, the baseline ALT was within the normal range (≤40 IU/l) in 27 patients, 24 of whom showed sustained ALT normalization during follow-up. In the remaining three patients, ALT became slightly abnormal (≤60 IU/l) during follow-up. As for four patients with abnormal baseline ALT, the level was normalized in three, whereas a slight elevation of ALT (≤60 IU/l) continued in one during follow-up.

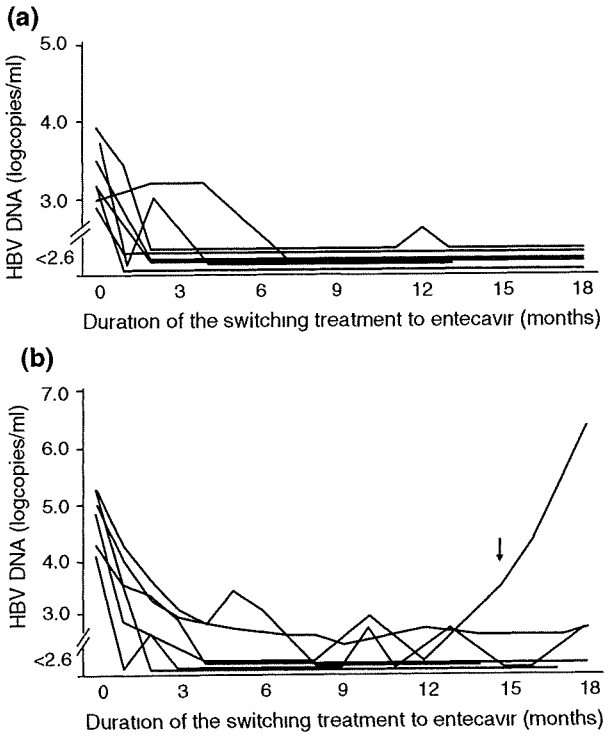

Fig. 1 Changes in HBV DNA after commencement of switching treatment from lamivudine to entecavir in CH-B patients with baseline HBV of (**a**) 2.6–<4.0 logcopies/ml and (**b**) ≥4.0 logcopies/ml. The *black arrow* indicates the time point of virological breakthrough

Among the 13 patients having a detectable level of baseline HBV DNA, five patients (three with baseline HBV DNA of 2.6–<4.0 logcopies/ml and two with baseline HBV DNA ≥4.0 logcopies/ml) had abnormal ALT at baseline but showed ALT normalization during follow-up. In the remaining eight patients, ALT continued to be normal from the beginning of entecavir treatment.

Disease course of the CH-B patients showing entecavir-resistance during lamivudine-to-entecavir switching treatment

The disease course of the entecavir-resistant patient is shown in Fig. 2. This patient was a 33-year-old HBeAg-positive male, whose liver biopsy showed features of chronic hepatitis. He underwent the preceding lamivudine treatment for 8 months. HBV DNA decreased from >7.6 to 4.6 logcopies/ml, and ALT was normalized during the lamivudine therapy. The rtM204V/I substitution was not detected before the switch to entecavir treatment by the PCR–ELMA analysis. After the commencement of entecavir treatment, HBV DNA was cleared at month 5. However, virological breakthrough was seen at month 15, and HBV DNA was further increased to 6.1 logcopies/ml at month 18. The sequencing analysis at month 18 revealed the rtM204V, rtL180M and rtS202G substitutions. Two additional substitutions, rtL267M and rtQ316H, were also found, when the amino acid sequences were compared with three representative genotype C HBV isolates (Genbank accession nos. V00867, X01587 and D00630) [21–23]. Breakthrough hepatitis was not evident after the emergence of entecavir-resistant mutant virus. The sequencing analysis also revealed that he was infected with HBV of genotype C.

Discussion

Entecavir treatment has been shown to exhibit more powerful antiviral efficacy and less frequent drug resistance than lamivudine treatment in nucleos(t)ide analog-naïve CH-B patients [14, 15, 17]. Entecavir is also effective in patients showing lamivudine resistance during the preceding lamivudine treatment, but its efficacy is limited due to the higher incidence of entecavir-resistance, compared with nucleos(t)ide analog-naïve ones [16, 17]. This is because entecavir-resistance is established based on two lamivudine-resistant substitutions, rtM204V and rtL180M, and additional mutation(s) occurring at rt184, rt202 and/or rt250 [18]. A considerable number of CH-B patients remain under continuous lamivudine treatment, while the lamivudine-to-entecavir switching treatment could yield a practical benefit. The switching treatment may be more promising for patients before the appearance of lamivudine resistance than after its development. In the present study, we investigated the efficacy of lamivudine-to-entecavir switching treatment in CH-B patients without apparent evidence of lamivudine resistance during the preceding lamivudine treatment.

We evaluated the antiviral efficacy of the switching treatment to entecavir in relation to the baseline HBV DNA at the commencement of the entecavir administration. In all patients having baseline HBV DNA <2.6 logcopies/ml, who revealed a good response to the preceding lamivudine treatment, HBV DNA continued to be undetectable during the switching treatment to entecavir. Also, all patients having baseline HBV DNA of 2.6–<4.0 logcopies/ml achieved sustained undetectable HBV DNA during the follow-up period of entecavir treatment. Among six patients having baseline HBV DNA ≥4.0 logcopies/ml, who did not respond well to the preceding lamivudine treatment, HBV DNA was cleared in three during follow-up. Its reduction by up to 3.0 logcopies/ml was seen in two additional cases without emergence of the entecavir-resistant virus. Thus, the antiviral efficacy of the lamivudine-to-entecavir switching treatment was exhibited in almost all CH-B patients in parallel with that of the preceding

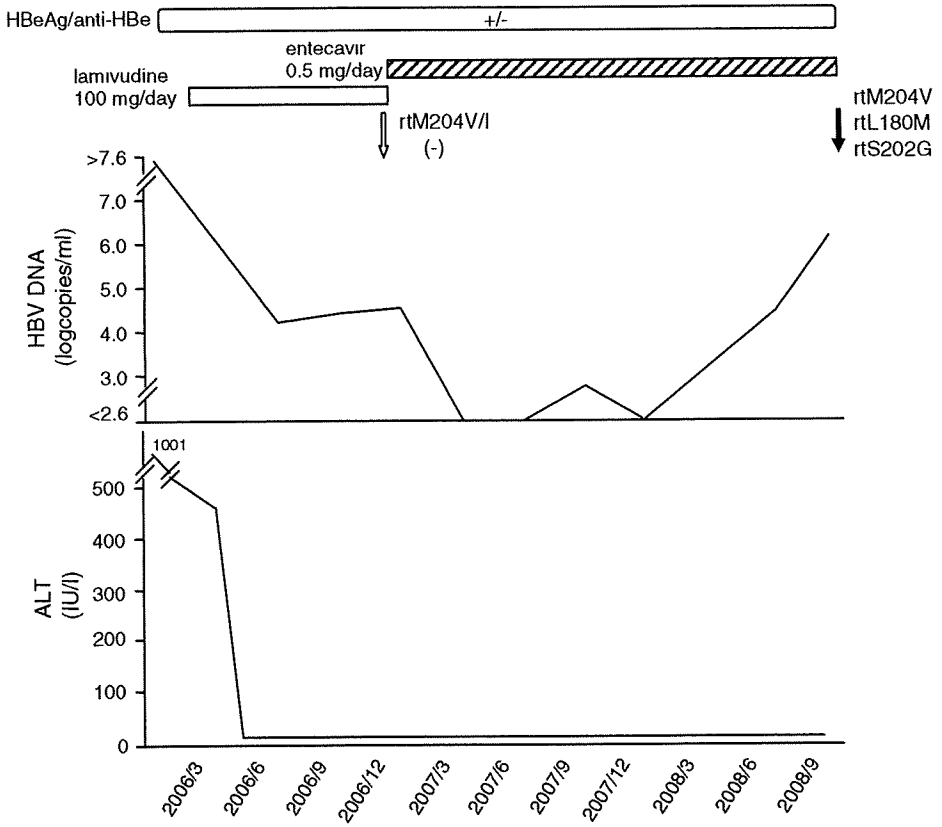

Fig. 2 Disease course of the CH-B patient showing entecavir-resistance during switching treatment to entecavir. The *white arrow* indicates the time point of the PCR–ELMA assay to detect rtM204V/I mutation, whereas the *black arrow* indicates the time point of the PCR-direct sequencing analysis

lamivudine treatment. In addition, the switching treatment to entecavir tended to yield a greater decrease in HBV DNA than the preceding lamivudine treatment. These results indicate that the switch from lamivudine to entecavir may be generally recommendable compared with continuation of lamivudine administration in CH-B patients without evidence of lamivudine resistance.

In this study, one of the six patients having baseline HBV DNA ≥4.0 logcopies/ml showed entecavir-resistance during the switching treatment to entecavir. It was probably due to the existence of an extremely small amount of lamivudine-resistant virus mixed with a predominant wild-type virus, which could not be detected by the sensitive PCR–ELMA assay at the start of the switch to entecavir treatment. It is speculated that, during entecavir treatment, the lamivudine-resistant virus having rtM204V and rtL180M substitutions may become predominant with time, followed by the establishment of entecavir-resistant virus via the additional rtS202G substitution. Compared to the low incidence of drug resistance in entecavir treatment for nucleos(t)ide analog-naïve CH-B patients [17], the entecavir-resistance may occur more frequently in the lamivudine-to-entecavir switching treatment for patients without evidence of lamivudine resistance. In particular, patients who do not achieve a good response to the preceding lamivudine treatment are speculated to have a higher risk for the development of entecavir-resistance in the switching treatment to entecavir, although it should be verified by further studies.

In conclusion, in CH-B patients receiving the continuous lamivudine treatment, it may be recommendable to switch to entecavir treatment before the appearance of lamivudine resistance. It may contribute to reducing the subsequent emergence of drug resistance. However, great care should be taken with respect to the emergence of entecavir-resistant virus after the switch to entecavir treatment, especially in patients who do not respond well to the preceding lamivudine treatment. Our retrospective study with a small number of patients and a short duration of follow-up cannot draw a definite conclusion but still provides some information about the clinical possibilities of the lamivudine-to-entecavir switching treatment. Further detailed investigation with a larger number of patients and a longer follow-up period may offer better understanding.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Abstract
Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.
Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.
Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.
Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/mm^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load ($\geq$100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for $\leq$60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for $>$ 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years ≥ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%≤ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	