Table 3 Serial precore and core promoter sequences of patients treated with lamivudine and entecavir

Patient	Genotype	Lamivudine					Entecavir									
		Baseline					Baseline					1 year				
		eAg	YMDD Motif	Precore nt 1896	CP nt		YMDD eAg	Motif	Precore nt 1896	CP nt		YMDD eAg	Motif	Precore nt 1896	CP nt	
					1762	1764				1762	1764				1762	1764
1	C	+	M	G	A/T	G/A	+	V+I	G/A	T	A	+	V	G	T	A
2	C	+	M	G	T	A	+	V+I	G	T	A	+	V+I	G	T	A
3	C	+	M	G	T	A	+	I	G	T	A	+	N	N	N	N
4	C	+	M	G/A	A	G	+	V+I	A	A	G	+	V+I	A	A	G
5	C	ND	ND	ND	ND	ND	+	I+V	G/A	T	A	+	I+V	G	A	A
6	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	G	T	A
7	A	+	M	G	D	D	+	V+I	G	D	D	+	V	G	T	A
8	C	+	M	G	T	A	+	I	G	T	A	+	I	G	T	A
9	C	+	M	G/A	D	D	+	I+V	A	A/T	G/A	+	I+V	A	T	A
10	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	G	T	A
11	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	G	T	A
12	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	A	T	A
13	C	+	M	G/A	T	A	+	I+V	G	T	A	–	I+V	G	T	A
14	C	+	M	G/A	A	A	–	I	A	A	A	–	I	A	A/T	A
15	C	–	M	G/A	T	A	–	I	A	T	A	–	I	A	T	A
16	C	–	M	A	T	A	–	I	A	T	A	–	N	N	N	N
17	C	–	M	G/A	T	A	–	I	A	T	A	–	N	N	N	N
18	C	–	M	A	T	A	–	I	A	T	A	–	N	N	N	N

Baseline, time at the beginning of therapy; CP, core promoter; D, deletion; eAg, HBeAg; ND, not done; N, polymerase chain reaction-negative; YMDD motif: M, YMDD; I, rtM204I; V, rtM204V; I+V, mixed-type (rtM204I and rtM204V), major type is listed first.

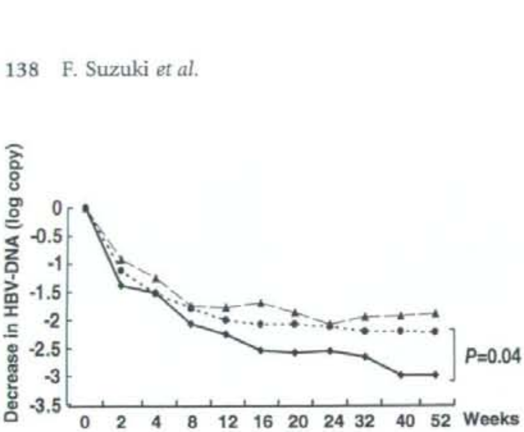

Figure 2 Mean log changes in the viral loads of each mutant of rtM204I alone and of rtM204I + rtM204V mixed type from baseline during the initial 52-week treatment with entacavir. HBV-DNA levels of rtM204I and rtM204V were measured by real-time polymerase chain reaction. HBV, hepatitis B virus. (◆), rtM204I in rtM204I alone; (●), rtM204I in mixed type; (▲), rtM204V in mixed type.

may have resulted from differences in race or HBV genotype (the major genotype is C in Japan). Few reports of ETV therapy against lamivudine-resistant HBV genotype C infection have appeared, and further study is necessary.

It has been reported that ETV is most effective against wild-type HBV (YMDD) and shows almost equally effective inhibition of the replication of rtM204I, rtL180M/rtM204V (rtM204V with rtL180M) and rtL180M/rtM204I (rtM204I with rtL180M) *in vitro*.[12,30] Supporting

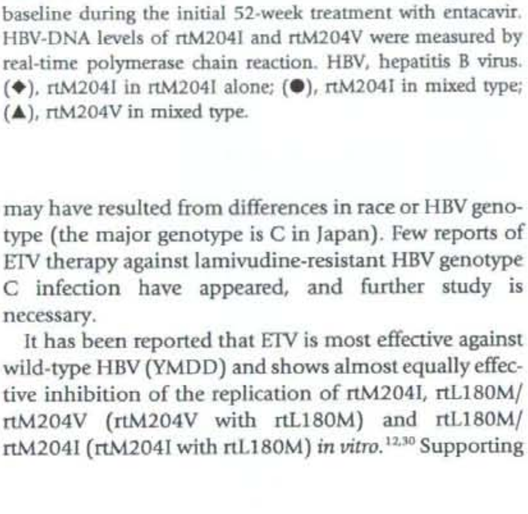

Figure 3 Mean log changes in the viral loads of rtM204I or rtM204V with or without *G1896A* from baseline during the initial 52-week treatment with entacavir. HBV-DNA levels of rtM204I and rtM204V were measured by real-time polymerase chain reaction. HBV, hepatitis B virus. (■), rtM204V without *G1896A*; (◆), rtM204I without *G1896A*; (▲), rtM204V with *G1896A*; (●), rtM204I with *G1896A*.

this, our study showed that rtM204I and rtM204V had similar sensitivity to ETV *in vivo*. In our study, however, rtM204I only or the presence of a precore mutation was more sensitive to ETV. These findings were seen mainly in patients without HBeAg. Moreover, rtM204I only or the presence of a precore mutation was also present in three HBeAg-positive patients (nos. 1, 3 and 5) whose HBV-DNA levels were negative on Amplicor HBV Monitor assay at 76 weeks. Our recent report also showed the greatest change in viral load was seen for rtM204I without HBeAg during adefovir dipivoxil (ADV) in addition to ongoing lamivudine therapy.[25] Considering these data, rtM204I virus is more sensitive to antiviral nucleoside analogs.

During lamivudine therapy, precore mutants tended to be replaced by wild-type virus at 1 year, and this was unrelated to the emergence of YMDD motif mutations.[23,24] Patients who showed mutations in the YMDD motif during long-term lamivudine therapy, in contrast, exhibited the reappearance of precore mutants.[24] Although the number of patients was small, ETV therapy in the present study also appeared to result in the preferential selection of wild-type virus. This finding was also seen from changes in the viral load of rtM204I and rtM204V with *G1896A* (Table 3). This finding suggests that antiviral nucleoside analogs such as lamivudine and ETV selectively suppress precore mutants over wild-type virus. In contrast, we did not see this replacement by wild-type at 1 year for core promoter mutations. Our results thus conflict with those of two previous studies, which showed that core promoter mutations during lamivudine therapy also tended to be replaced by wild-type virus at 1 year[23,24] and, more recently, that three of five seroconverters of HBeAg harbored core promoter mutations at baseline that were progressively replaced by the wild-type genome during ADV monotherapy.[31] The reason for this apparent discrepancy is unclear. In any case, our present study indicated that, compared to initial lamivudine therapy or ADV monotherapy, ETV may be less effective against core promoter mutants than wild-type virus.

In a recent investigation of lamivudine-resistant viruses, additional substitutions at rtT184, rtS202, or rtM250 were shown to further reduce ETV susceptibility.[19] The present and above studies add those at rtL180M and rtM204V to this list. One of our patients had rtS202G substitution in addition to rtL180M and rtM204V. Importantly, however, breakthrough hepatitis may occur even when the rate of substitutions (ETV-resistant) is not high.[19] Taken together, these findings suggest that ETV therapy may not be beneficial in patients with either or both YVDD and no precore mutant.

In conclusion, we analyzed changes in viral loads of rtM204I and rtM204V during ETV therapy for lamivudine-resistant virus. Results showed that rtM204I and rtM204V had similar sensitivity to ETV. However, rtM204I only or the existence of a precore mutation conferred greater sensitivity to ETV. Moreover, antiviral nucleoside analogs such as lamivudine and ETV selectively tended to suppress precore mutants over wild-type virus. Further studies of virological changes and clinical efficacy during longer ETV therapy for lamivudine-resistant virus are necessary.

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HEPATOLOGY

Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: Randomized controlled trial in Japanese patients

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Abstract

Background and Aim: Entecavir is a potent inhibitor of both wild-type and lamivudine-resistant hepatitis B virus (HBV) with proven clinical efficacy. We conducted a randomized, double-blind, multicenter study in Japan (ETV-052) evaluating the efficacy and safety of two doses of entecavir in adult patients with lamivudine-refractory chronic hepatitis B infection.

Methods: Eighty-four patients with chronic hepatitis B who were refractory to lamivudine therapy were switched from lamivudine to daily oral doses of 0.5 mg entecavir (41 patients) or 1 mg entecavir (43 patients) for 52 weeks.

Results: The proportions of patients achieving the primary end-point ($\geq 2\ \log_{10}$ reduction in HBV-DNA from baseline by polymerase chain reaction assay or undetectable HBV-DNA levels [<400 copies/mL] at week 48) were 90% and 93% for entecavir 0.5 mg and 1 mg, respectively, with 33% of patients in each dosing group achieving <400 copies/mL. The mean reduction in HBV-DNA from baseline was 3.58 and 3.75 $\log_{10}$ copies/mL for entecavir 0.5 mg and 1 mg, respectively. High proportions of patients achieved alanine aminotransferase normalization at week 48 (0.5 mg 86%, 1 mg 78%). Histological improvement was observed in most patients (0.5 mg 52%, 1 mg 60%). Virological breakthrough (increase in HBV-DNA of $\geq 1\ \log_{10}$ copies/mL from nadir) was observed in one patient but was not associated with selection of entecavir-associated resistance substitutions. Entecavir was well tolerated, with no patients discontinuing study drug due to adverse events.

Conclusions: These findings indicate that entecavir is safe and effective for the treatment of Japanese adults with lamivudine-refractory chronic hepatitis B.

Introduction

Chronic hepatitis B is a life-threatening disease, often called the 'silent killer', which eventually leads to liver cirrhosis, decompensated hepatic disease, or hepatocellular carcinoma in 20–40% of patients.[1] Prevalence is high in the Asia–Pacific region; of the 350 million patients with chronic hepatitis B worldwide, approximately 75% live in Asia.[2] Since the introduction of mass vaccination in Japan, hepatitis B virus (HBV) infection rates have dropped significantly (0.8% in 2000); however, 38% of these individuals are chronically infected and the disease burden is still high.[2] Recent studies indicate that elevated levels of HBV-DNA are associated with the development of cirrhosis and hepatocellular carcinoma and that the risk of complications and disease progression can be reduced by suppressing HBV-DNA replication with antiviral therapy.[3–5] However, the emergence of resistance and the consequent re-elevation in viral loads negates the benefit of therapy[6] and alternative treatment options are required for further benefit.

Lamivudine was the first nucleoside analog developed for the treatment of chronic hepatitis B and was approved by the FDA in 1998. Since then, this drug has been widely used because of its efficacy during short-term dosing;[5,7] however, relapse of hepatitis associated with the emergence of lamivudine-resistant viral strains has proved to limit its long-term efficacy.[6,8,9] The incidence of resistance has been reported to be approximately 20% annually.[6,8,9]

Lamivudine-resistant viruses carry substitutions in the reverse transcriptase region of HBV polymerase, usually a methionine to valine or isoleucine at amino acid position 204 (rtM204I/V) in the tyrosine–methionine–aspartate–aspartate (YMDD) motif which is often accompanied by a compensatory substitution at position 180 (rtL180M). Lamivudine resistance leads to loss of virological suppression and increases in serum alanine aminotransferase (ALT) levels, which may be followed by hepatitis progression and, in patients with cirrhosis, by hepatic decompensation, hepatocellular carcinoma, and removal from liver transplant lists.[10,11] Continued lamivudine treatment is of no clinical benefit in these patients, so alternative treatments are needed.[6] Adefovir dipivoxil has demonstrated activity against lamivudine-resistant virus, with increasing evidence that adding adefovir to continued lamivudine is associated with lower adefovir resistance rates.[12,13]

Entecavir is a deoxyguanosine analog that has recently been approved in many countries worldwide, including Japan. Entecavir exhibits potent antiviral activity, with a 50% effective concentration more than 300-fold greater than that of lamivudine *in vitro*.[14,15] In multinational phase II and III clinical trials, entecavir has been shown to be well tolerated and effective for the treatment of chronic hepatitis B in lamivudine-refractory patients at a dose of 1 mg daily.[14,16,17] We report here the results of a phase II clinical trial assessing the efficacy and safety of 52 weeks of entecavir treatment in Japanese patients with lamivudine-refractory chronic hepatitis B.

Methods

Patients

The present study included men and women, aged 20–75 years, with chronic hepatitis B infection and evidence of active viral replication (HBV-DNA levels $>10^5$ copies/mL) despite ongoing lamivudine therapy, with a history of at least 24 weeks of lamivudine therapy or documented evidence of infection with HBV carrying lamivudine-associated substitutions. Patients were required to have elevated ALT levels (1.3 to 10× the upper limit of normal [ULN]), compensated liver disease (total bilirubin ≤2.5 mg/dL, prothrombin time ≤3 s longer than normal control value or international normalized ratio ≤1.5, albumin ≥3.0 g/dL, and no current evidence or history of variceal bleeding, hepatic encephalopathy, or ascites requiring diuretics or paracentesis).

Exclusion criteria included the following: coinfection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus; other forms of liver disease; therapy with any anti-HBV drug other than lamivudine within 24 weeks prior to randomization; and more than 12 weeks of therapy with a nucleoside or nucleotide analog with activity against HBV.

Written informed consent was obtained from all subjects, and the study was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice Guidelines, and Articles/Notifications of the Ministry of Health and Labor in Japan.

Study design

This was a multicenter, randomized, double-blind study carried out at 16 sites in Japan. Eligible subjects were randomly assigned to receive either entecavir 0.5 mg daily ($n = 41$) or entecavir 1 mg daily ($n = 43$) for 52 weeks. Patients continued their lamivudine therapy until the start of entecavir treatment. Patients who completed 52 weeks of dosing could enroll in an open-label entecavir rollover study. Patients not entering the rollover study were to be followed up for 24 weeks post-dosing and could receive alternative anti-HBV therapy.

Study assessments and end-points

Response to therapy was assessed at Week 52, based on results obtained at Week 48. Patients were seen for scheduled visits at baseline (Day 1), Weeks 2 and 4, and every 4 weeks until Week 52 or the end of dosing. HBV-DNA levels were determined by Roche Amplicor™ polymerase chain reaction (PCR) assay (lower limit of detection 400 copies/mL)[18] at baseline and at weeks 4, 8, 12, 24, 36, and 48. Histological examinations were carried out for all patients via liver biopsy at baseline and at Week 48. Biopsies were evaluated by a Biopsy Reading Committee blinded to treatment and sequence, and were scored according to the Knodell histological activity index (HAI) and fibrosis scores,[19] and the corresponding New Inuyama scores.[20]

The primary efficacy end-point was the proportion of subjects achieving a reduction in HBV-DNA by PCR assay of at least 2 $\log_{10}$ copies/mL from baseline or to <400 copies/mL at Week 48. Secondary end-points included the mean change from baseline in HBV-DNA and the proportions of patients achieving HBV-DNA <400 copies/mL; ALT <1.25× ULN; hepatitis B e antigen (HBeAg) loss and HBeAg seroconversion (loss of HBeAg and appearance of antibody to HBeAg [anti-HBe]) among patients who were HBeAg positive at baseline; and complete response (defined as HBV-DNA <400 copies/mL by PCR assay and ALT <1.25× ULN and being HBeAg negative if they were HBeAg positive at baseline) at Week 48. Histological improvement was defined as a ≥2-point decrease in Knodell necroinflammatory score and no worsening of fibrosis (i.e. ≥1-point increase in Knodell fibrosis score). Liver biopsies were also evaluated using the New Inuyama classification system.

Safety information was obtained from all patients who received at least one dose of study drug. Safety end-points included adverse events, laboratory abnormalities, and discontinuation of study drug due to adverse events or laboratory abnormalities. The date of onset, measures taken, causes, and outcome of all adverse drug reactions were investigated and recorded. Causal relationships with the study drug were assessed by the physician. The proportion of patients who experienced an ALT flare (defined as an on-treatment ALT measurement >2× baseline and >10× ULN) was also determined.

Resistance assessment

Genotypic analysis to detect substitutions in the HBV polymerase at residues rt180, 184, 202, 204, and 250 was carried out on HBV-DNA serum samples collected at Week 24 (or last available on-therapy sample for patients who discontinued prematurely) for all patients who experienced a ≥1 $\log_{10}$ copies/mL increase in HBV-DNA from the nadir. Genotypic resistance analysis of HBV-DNA polymerase was performed at a central laboratory (SRL, Inc., Tokyo, Japan). HBV-DNA was extracted and codons 1 to 344 of the reverse transcriptase encoding region were PCR amplified and sequenced at a central laboratory. We also investigated lamivudine resistance-associated substitutions (at rt204) using a PCR-enzyme-linked minisequence assay (Medical & Biological Laboratories Co., Aichi, Japan) in all patients at baseline and at Week 48.

Statistical analysis

Analyses of efficacy end-points were based on treated subjects. For binary end-points, subjects with missing Week 48 measurements were treated as missing (non-completer = missing analysis). Ninety-five percent confidence intervals (CI) were calculated for proportions of patients achieving each end-point. Parameters represented by continuous variables were summarized by the mean and standard error. Wilcoxon signed-rank test was carried out for comparison of New Inuyama scores in baseline and post-treatment samples.

Results

Study population

Of the 115 patients enrolled and screened, 84 were randomized and received at least one dose of study treatment. Forty-one patients received entecavir 0.5 mg and 43 received entecavir 1 mg daily (Table 1). One patient in each group withdrew consent before completing 48 weeks of treatment. All remaining patients completed 52 weeks of dosing (Table 1) and then entered a rollover study. The two treatment arms were well balanced at baseline for demographic and disease characteristics (Table 1). Most patients were male (85%) and all were Japanese, with a mean age of 44.1 years in the entecavir 0.5 mg group and 42.3 years in the entecavir 1 mg group. The majority of patients were HBeAg positive (73% entecavir 0.5 mg, 77% entecavir 1 mg) and were infected with HBV genotype C (90.0% entecavir 0.5 mg, 97.6% entecavir 1 mg). Mean HBV-DNA at baseline was 7.72 $\log_{10}$ copies/mL in the 0.5 mg group and 7.59 $\log_{10}$ copies/mL in the 1 mg group. Virus from all patients carried YMDD substitutions at rt204. No patients had previously received interferon therapy.

Table 1 Disposition and baseline characteristics of lamivudine-refractory chronic hepatitis B patients treated with entecavir

	Entecavir 0.5 mg	Entecavir 1 mg
Randomized	41	43
Treated	41	43
Completed 52 weeks of dosing	40	42
Male, *n* (%)	35 (85)	37 (86)
Age (years), mean ± SD	44.1 ± 10.5	42.3 ± 7.3
Weight (kg), mean ± SD	67.6 ± 10.7	65.7 ± 11.2
Ethnicity		
Japanese, *n* (%)	40 (100)	42 (100)
HBV-DNA, mean ± SD		
$\log_{10}$ copies/mL by PCR	7.72 ± 0.77	7.59 ± 1.07
HBeAg positive, *n* (%)	30 (73)	33 (77)
ALT (IU/L), mean ± SD	134.5 ± 119.8	132.9 ± 102.1
Knodell HAI score		
Mean ± SE (*n*)	7.2 ± 0.6 (26)	6.5 ± 0.5 (35)
YMDD mutation present	41 (100)	43 (100)
HBV genotype, *n* (%)		
C	36 (90.0)	41 (97.6)
A	1 (2.5)	0
B	1 (2.5)	1 (2.4)
Others (D, E, F, AD, DE)	2 (5.0)	0

ALT, alanine aminotransferase; HAI, histological activity index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; YMDD, tyrosine–methionine–aspartate–aspartate motif.

Virological response

A reduction in HBV-DNA by 2 $\log_{10}$ copies/mL or to below the limit of detection (400 copies/mL) at Week 48 (the primary end-point) was achieved by 90% (95% CI 76.3, 97.2) of patients in the entecavir 0.5 mg group and 93% (95% CI 80.5, 98.5) of patients in the entecavir 1 mg group (Table 2). In the entecavir 1 mg group, this end-point was achieved by 74% of patients by Week 4 and by more than 90% of patients by week 8 (Fig. 1). In the entecavir 0.5 mg treatment group, 24 weeks of treatment were required for 90% of patients to achieve the end-point. Similarly, a higher proportion of patients in the 1 mg group than in the 0.5 mg group achieved undetectable levels of HBV-DNA (<400 copies/mL) at Week 24 (19% [8/42 patients] *vs* 13% [5/40 patients] respectively), but the proportions of patients achieving this end-point were similar by Week 48 (33% [14/42 patients] *vs* 33% [13/40 patients] respectively).

Entecavir treatment resulted in a rapid decline in HBV-DNA levels in both treatment groups (Fig. 2), achieving a mean change from baseline at Week 24 of -3.20 ± 0.18 $\log_{10}$ copies/mL for the 0.5 mg group and -3.44 ± 0.18 $\log_{10}$ copies/mL for the 1 mg group (Table 2). Further reductions were observed between Weeks 24 and 48, with the mean change from baseline reaching -3.58 ± 0.21 $\log_{10}$ copies/mL and -3.75 ± 0.19 $\log_{10}$ copies/mL in the entecavir 0.5 and 1 mg groups, respectively (Fig. 2).

Biochemical response

At baseline, approximately 90% of patients exhibited ALT levels ≥1.25× ULN. The proportion of subjects whose ALT levels had normalized (<1.25× ULN: WHO toxicity grade 0) at Week 24 was 76% (28/37 patients) for the 0.5 mg group and 73% (27/37 patients) for the 1 mg group. At Week 48, the proportions increased to 86% (32/37 patients) for the 0.5 mg group and 78% (29/37 patients) for the 1 mg group (Table 2). The differences between the two groups were not significant at either time point ($P = 0.422$).

Serological response

Among HBeAg-positive patients at baseline, the proportion of patients who achieved HBeAg seroconversion (loss of HBeAg and appearance of anti-HBe) was higher in the 1 mg group at Week 24 (9% [3/33 patients] compared with 0% [0/29 patients] for the 0.5 mg group) but was similar between groups at Week 48; 17% (5/29 patients) for the 0.5 mg group and 15% (5/33 patients) for the 1 mg group ($P = 1.000$; Table 2).

Table 2 Virological, biochemical, and histological responses at Weeks 24 and 48

	Entecavir 0.5 mg ($n = 40$)	Entecavir 1 mg ($n = 42$)
HBV-DNA by Roche Amplicor™ PCR assay		
Reduction in HBV-DNA >2.0 $\log_{10}$ copies/mL or undetectable at Week 48 (primary study end-point), *n* (%)	36 (90)	39 (93)
Reduction from baseline ($\log_{10}$ copies/mL), mean ± SE		
At Week 24	−3.20 ± 0.18	−3.44 ± 0.18
At Week 48	−3.58 ± 0.21	−3.75 ± 0.19
HBV-DNA <400 copies/mL, *n* (%)		
At Week 24	5 (13)	8 (19)
At Week 48	13 (33)	14 (33)
Normalization of ALT levels[†]		
At Week 24, *n*/*n* with abnormal baseline (%)	28/37 (76)	27/37 (73)
At Week 48, *n*/*n* with abnormal baseline (%)	32/37 (86)	29/37 (78)
HBeAg seroconversion[‡]		
At Week 24, *n*/HBeAg positive *n* at baseline (%)	0/29 (0)	3/33 (9)
At Week 48, *n*/HBeAg positive *n* at baseline (%)	5/29 (17)	5/33 (15)
Complete response[§] at Week 48, *n* (%)	6 (15)	6 (14)
Histological improvement		
Histological improvement,[¶] *n* (%)	12/23[††] (52)	21/35[††] (60)
Knodell HAI scores, reduction from baseline at Week 48, mean ± SE	−3.3 ± 0.6*	−3.6 ± 0.5*
Improvement of grading score in Inuyama classification, *n* (%)	16/26 (62)*	19/35 (54)*

* $P < 0.0001$ compared with baseline, Wilcoxon signed-rank test.
[†]WHO grade 0, ALT <1.25× ULN.
[‡]Seroconversion was defined as the acquisition of antibodies to HBeAg.
[§]Complete response was defined as undetectable HBV-DNA (<400 copies/mL), HBeAg negative, and normal ALT.
[¶]≥2-point decrease in Knodell necroinflammatory score from baseline with no worsening of the Knodell fibrosis score.
[††]Patients with evaluable baseline and Week 42 history.
ALT, alanine aminotransferase; HAI, histological activity index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; ULN, upper limit of normal.

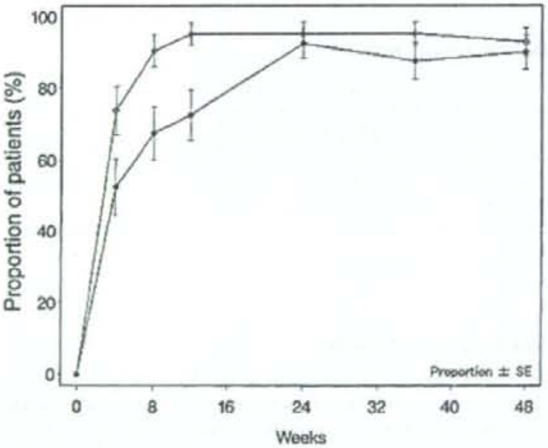

Figure 1 Proportion of patients (%) who achieved a reduction in hepatitis B virus (HBV)-DNA ≥2.0 $\log_{10}$ copies/mL or <400 copies/mL in the entecavir 1 mg (○) and 0.5 mg (●) groups.

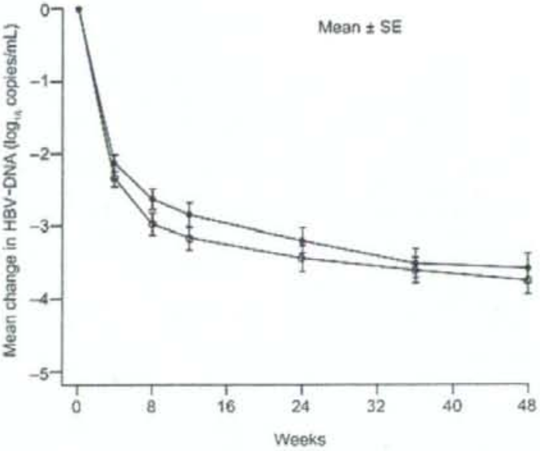

Figure 2 Mean change from baseline in hepatitis B virus (HBV)-DNA ($\log_{10}$ copies/mL) by polymerase chain reaction assay through 48 weeks in patients treated with entecavir 1 mg (○) and 0.5 mg (●).

Table 3 Summary of adverse events reported during treatment periods

	No. subjects (%)	
	Entecavir 0.5 mg ($n = 41$)	Entecavir 1 mg ($n = 43$)
Any adverse event	41 (100)	43 (100)
Clinical adverse event	37 (90)	40 (93)
Laboratory adverse event	38 (93)	38 (88)
Most frequent clinical adverse events†		
Nasopharyngitis	15 (37)	21 (49)
Headache	9 (22)	13 (30)
Upper respiratory tract infection	7 (17)	1 (2)
Malaise	6 (14.6)	9 (21)
Any severe (grade 3–4) adverse events‡ (%)	6 (15)§	5 (12)¶
Any serious adverse events‡ (%)	1 (2)	3 (7)
Discontinuations due to adverse events‡ (%)	0	0
Death (%)	0	0
ALT flare††	0	3 (7)

†Occurring in at least 15% of patients.
‡Including laboratory abnormalities.
§Eight events occurred in six subjects.
¶Nine events occurred in five subjects.
††ALT flare defined as ALT >2× baseline and 10× ULN. All ALT flares were associated with ≥2 $\log_{10}$ reduction in HBV-DNA.
ALT, alanine aminotransferase; HBV, hepatitis B virus; ULN, upper limit of normal.

Complete response

The proportion of patients who achieved a complete response (HBV-DNA <400 copies/mL by PCR assay and ALT <1.25× ULN and a HBeAg-negative status if they were HBeAg positive at baseline) at Week 48 was similar between groups; 15% (6/40 patients) for the 0.5 mg group and 14% (6/42 patients) for the 1 mg group ($P = 0.855$; Table 2). The durability of responses off treatment in these patients was not assessed, because all patients were enrolled into an entecavir open-label rollover protocol.

Histological response

Paired baseline and Week 48 liver biopsies were available for 26 patients in the 0.5 mg group and 35 patients in the 1 mg group (evaluable biopsy pairs were available in 23 patients in the 0.5 mg group and 35 patients in the 1 mg group). Among patients with evaluable biopsy pairs, histological improvement, defined as a ≥2-point decrease in Knodell HAI and no worsening of fibrosis at Week 48, was seen in 52% (12/23) of patients in the 0.5 mg group and in 60% (21/35) of patients in the 1 mg group (Table 2). Knodell HAI scores were significantly reduced in both treatment groups, dropping by a mean of 3.3 points for the 0.5 mg group and 3.6 points for the 1 mg group ($P < 0.001$ compared with baseline for both groups). Grading of necroinflammation according to the New Inuyama classification was also significantly improved over 48 weeks of treatment in both groups ($P < 0.0001$ compared with baseline for both groups), for 62% (16/26) of patients in the 0.5 mg group and 54% (19/35) of patients in the 1 mg group. No patients exhibited any worsening of grading. There were no significant changes in fibrosis from baseline in either group, as assessed by Knodell fibrosis scores (improvement, no change, and worsening in four, 14, and five patients in the 0.5 mg group and four, 28, and three patients in the 1 mg group, respectively) and staging of New Inuyama classification (improvement, no change, and worsening in seven, 10, and six patients in the 0.5 mg group and six, 26, and three patients in the 1 mg group, respectively).

Resistance analysis

At baseline, rtM204I/V, which is associated with lamivudine resistance, was detected in virus samples from all patients. During the treatment period, only one patient, in the 1 mg group, exhibited increases in HBV-DNA level by 1 $\log_{10}$ copies/mL or more from the nadir (virological breakthrough). There was no emergence of novel amino acid substitutions conferring resistance to entecavir in this patient through to week 48.

A sensitive PCR-based method was used to examine the evolution of amino acid substitutions associated with lamivudine resistance during entecavir treatment in all patients. At week 48, results were available for 39 patients in the 0.5 mg group and 42 patients in the 1 mg group. Substitutions that reverted the YMDD mutants back to wild-type (rtI204M or rtM/I204M) were observed in three of 42 patients in the 1 mg group, whereas no patient in the 0.5 mg group reverted to wild-type at week 48.

Safety

The frequencies of adverse events were similar between treatment groups (Table 3). The majority of adverse events were mild or moderate (grade 1–2) and resolved on treatment in 94% of patients. The most frequent adverse events in both groups were nasopharyngitis, headache, upper respiratory tract infection, nausea, and malaise (Table 3). Serious adverse events included cholecystitis in the 0.5 mg group, and colon polyp, meniscus impairment, and elevation of serum ALT levels in the 1 mg group. In all cases, patients recovered without discontinuation of study drug. No deaths were noted during the trial. ALT flare (ALT levels >2-fold of baseline levels and >10× ULN) occurred in three patients, all in the 1 mg group (Table 3). In each of these patients, the ALT flare was transient and was associated with declining HBV-DNA.

Discussion

In multinational clinical trials, entecavir has demonstrated potent inhibition of viral replication in lamivudine-refractory patients.[14,16] The results of the present study confirm the efficacy of entecavir in Japanese patients with more than 90% of patients in both treatment groups achieving the primary end-point (a reduction in HBV-DNA from baseline of ≥2 $\log_{10}$ copies/mL or to <400 copies/mL by PCR assay at week 48). Although there was no significant difference between the two groups at Week 48, the time course for reduction in HBV-DNA was more rapid with the 1 mg dose than with the 0.5 mg dose, and more than 90% of subjects in the 1 mg group had achieved the primary end-point by Week 8, whereas this proportion of responders achieved the primary end-point in the 0.5 mg group by Week 24. As entecavir's genetic barrier to resistance is reduced in the presence of lamivudine resistance-associated mutations, these results stress the importance of rapidly suppressing viral replication to avoid development of an entecavir-resistant virus.

The reduction in mean HBV-DNA seen in these entecavir-treated patients is consistent with that observed in a phase III clinical trial of entecavir in lamivudine-refractory patients (ETV-026)[16] and a phase II clinical trial of entecavir in Chinese lamivudine-refractory patients (ETV-056).[17] These two studies recruited patients with higher baseline viral load than our study of Japanese patients and therefore the observed change in viral load following treatment is more pronounced.

Although the cut-offs for HBV-DNA undetectability and ALT normalization were slightly higher in the present study (<400 copies/mL and <1.25× ULN) compared with ETV-026 and ETV-056 (<300 copies/mL and ≤1× ULN), the proportions of patients achieving the secondary end-points were consistent among treatment groups in this study and those observed in the global and Japanese programs. At the time this study was designed, the WHO toxicity scale was used to define ALT normalization.

Emergence of entecavir-resistant HBV in nucleoside-naive patients is rare due to entecavir's ability to suppress viral load to undetectable levels and its high genetic barrier.[21] Amino acid substitutions rtL180M and rtM204I (associated with resistance to lamivudine) reduce *in vitro* viral susceptibility to entecavir by approximately eightfold.[21] For resistance to entecavir to occur, additional amino acid substitutions are required at either rtT184, rtS202, or rtM250.[16,21] In the present study, virological breakthrough (HBV-DNA increase of ≥1 $\log_{10}$ copies/mL from nadir) was observed in one patient in the 1 mg group (at week 44). DNA sequencing of the polymerase gene in this patient revealed the presence of amino acid substitutions rtL180M and rtM204V at baseline and at week 52; there was no change in the pattern of these amino acid substitutions and none of the additional amino acid substitutions which are required for entecavir resistance (at positions rtT184, rtS202, or rtM250) were seen to emerge. ALT levels were within the normal range by the end of treatment (week 52) in this patient. In study ETV-026, rates of entecavir resistance associated with virological breakthrough were 1% and 9% after 1 and 2 years, respectively.[22] Furthermore, cumulative 4-year resistance rates in lamivudine-refractory patients of approximately 40% have been reported[23] and 29% (12/42) of Japanese patients who were treated with entecavir 1 mg through 3 years had evidence of substitutions associated with entecavir resistance[24] indicating that longer term follow up in the patients described here is necessary.

Previous reports indicate that entecavir treatment is well tolerated and associated with a low incidence of on-treatment ALT flare. The most frequent adverse events in these studies were upper respiratory tract infection, headache, fatigue, cough, nausea, and nasopharyngitis.[16,25,26] We observed a similar pattern of adverse events in our clinical trial. The incidences of serious adverse events, grade 3–4 elevations of ALT, and ALT flares were also comparable to rates previously reported in multinational clinical studies.[16,25,26] Moreover, there were no discontinuations of entecavir therapy due to adverse events and no deaths. These results confirm the safety and tolerability of entecavir in lamivudine-refractory Japanese patients.

ALT flares represent a particular safety problem in patients with hepatitis B, potentially leading to decompensated hepatic disease. In this clinical trial, there was no washout period between the end of lamivudine treatment and the initiation of entecavir therapy, and no overlap. Consistent with that seen in the previous clinical studies,[14,25,26] the incidence of ALT flare was low in this clinical trial. The three cases of ALT flare that did occur (all in the 1 mg group) were not associated with hepatic decompensation. All three ALT flares occurred within 8 weeks of initiating entecavir treatment, were associated with at least a 2 $\log_{10}$ reduction in HBV-DNA levels, and resolved on continued entecavir treatment. There was no re-elevation of ALT observed. This may be attributable to the transient reconstitution of the host's immune response as a result of the inhibition of viral replication.[14,25] Our results confirm that it is not necessary to overlap lamivudine therapy with entecavir during the transition from lamivudine to entecavir therapy.[14,25] It should be noted that this study did not include patients with decompensated liver disease and that entecavir's safety profile in this group of patients has yet to be established.

The results of this clinical trial demonstrate the antiviral efficacy of entecavir, at 0.5 mg and 1 mg, in Japanese patients with lamivudine-refractory chronic hepatitis B. Entecavir was generally well tolerated in this population, indicating that there are no specific safety concerns for the treatment of Japanese patients with this agent. The efficacy and resistance profiles of 0.5 mg and 1 mg doses at 48 weeks were similar; however, the rapid suppression of viral load seen with the 1 mg dose is likely to contribute to a more robust long-term resistance profile than with the 0.5 mg dose. For this reason, a daily dose of 1 mg entecavir is recommended for treatment of lamivudine-refractory Japanese patients.

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Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: Two-year follow-up☆

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Background/Aims: **We studied the long-term efficacy (median follow-up of 28 months) of adefovir (ADV) in combination with lamivudine (LAM) in 132 LAM-resistant Japanese patients with chronic genotype C-dominant hepatitis B virus (HBV) infection.**

Methods: **The viral response (undetectable HBV-DNA by PCR assay) and the predictor of viral response were evaluated. The emergence of ADV-resistant mutants was investigated during the combination therapy.**

Results: **The cumulative probability of viral response was 69% at 12 months, and 81% at 24 months. Multivariate analysis identified baseline HBe antigen status ($P = 0.0001$), aspartate aminotransferase level (AST) ($P = 0.001$) and HBV-DNA level ($P = 0.002$) as determinants of viral response to treatment. At the beginning of ADV therapy, substitutions at rtA181 (rtA181T and rtA181S) were identified in 3 patients (2.3%). In the remaining 129 patients, the rtM204 mutants were identified at baseline, and two (1.6%) of the 129 patients developed new ADV-resistant mutants; one was rtA181S and another was rtA181T plus rtN236T mutation.**

Conclusions: **Adefovir and lamivudine combination therapy effectively suppressed viral replication and maintained the efficacy well in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants is rare, at least over a period of 2 years, in patients with combination therapy.**

Keywords: **Adefovir dipivoxil; Lamivudine-resistant mutant; Hepatitis B virus; rtA181T; rtN236T; Combination therapy**

1. Introduction

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma [1,2]. To date, interferon and three nucleoside and nucleotide analogues (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection in Japan, while telbivudine is licensed in Europe and North America [3,4]. Nucleoside and nucleotide analogues

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Abbreviations: LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

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suppress HBV replication in most patients and improve transaminase levels and liver histology [5–7]. However, prolonged therapy results in the emergence of drug-resistant mutants.

The rate of emergence of drug-resistant mutants is higher in patients treated with LAM than ADV and ETV, and the emergence of such mutants is followed by increases in viral load and re-elevation of transaminase levels [8–10]. Most LAM-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [11,12]. Both experimental and clinical studies have shown that ADV and ETV could suppress not only wild-type but also LAM-resistant strains and have been confirmed as salvage therapy for LAM-refractory patients [13,14]. However, a few studies have already reported the emergence of resistant mutants to these drugs. ADV-resistant mutations are more common in LAM-resistant patients than in treatment-naïve patients during ADV monotherapy, and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [15,16]. However, a recent study reported that LAM-resistant HBeAg-negative patients treated with combination therapy of ADV with LAM did not develop resistance to ADV over a period of 3 years and the rate of undetectable HBV-DNA in combination therapy was higher than in the ADV monotherapy [14].

Recently, we reported the efficacy of ADV plus LAM combination therapy in patients with LAM-resistant chronic HBV infection [17]. However, the number of patients was limited and the virological analysis was inadequate in that study. In the present study, we analyzed the efficacy of ADV plus LAM combination therapy in 132 LAM-resistant patients with chronic hepatitis B over a period of 2 years. We also investigated the emergence of ADV-resistant mutants before and during the combination therapy.

2. Patients and methods

2.1. Patients

A total of 132 consecutive adult Japanese patients with chronic HBV infection were treated with adefovir dipivoxil at Toranomon Hospital, Tokyo, Japan, in addition to ongoing LAM treatment for more than 52 weeks starting in 2002. Enrolment in this study and the start of ADV treatment were determined by the following criteria: (1) Increase in serum HBV-DNA levels of ⩾1 log copies/ml during LAM treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy. (2) Detection of mutations of the YMDD motif and/or other mutations related to LAM resistance before the start of ADV treatment, as diagnosed by the PCR-based method described later and/or direct sequence analysis. (3) No history of treatment with other nucleoside analogues such as famciclovir and entecavir. The exclusion criteria were as follows: (1) Serum creatinine levels ⩾1.5 mg/dl. (2) Patients coinfected with hepatitis C, delta viruses, or HIV. (3) History of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

2.2. Methods

Patients received a 10-mg once-daily dose of oral ADV, in addition to ongoing LAM treatment (100 mg/day). Blood samples were obtained once every month during the ADV + LAM combination therapy, and analyzed for virological markers, biochemical markers, together with liver function tests, renal function tests, and complete blood cell counts. The primary efficacy measures were undetectable HBV-DNA level by PCR assay (<2.6 log copies/ml) and normalization of ALT level (<50 IU/ml); the secondary efficacy measure was HBeAg seroconversion. The rate of each measure was evaluated 6, 12, 18 and 24 months after the start of ADV + LAM treatment.

2.3. Analysis of virological markers

HBsAg, HBeAg and antibody against HBeAg (anti-HBe) were determined by commercially available radioimmunoassay systems (Abbott Japan, Tokyo, Japan). HBV-DNA serum level was determined by using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). The HBV genotype was determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Tokyo) based on the method of Usuda et al. [18].

2.4. Detection of antiviral-resistant mutations

Substitution at rtM204 of the YMDD motif was identified at baseline by using the Enzyme-Linked Mini-sequence Assay with a commercial assay kit (PCR-ELMA; Genome Science). HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo) and dissolved in 20 μl H_2O. Detection of substitutions at rtA181 and rtN236 was achieved by PCR with restriction fragment length polymorphism (RFLP). For this purpose, HBV-DNA extracted from serum samples was amplified by PCR using primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCACTG AACAAATGGCACTAGTAAGCTGA -3′ for rtA181, and 5′-CCA CTTTTCTTTTGTCTTTGGGTATACATTTAA-3′ and 5′-GATCG GCAGAGGAGCCACAA -3′ for rtN236. The PCR products were digested with five units of restriction enzyme *EspI* for rtA181, DraI for rtN236 and subjected to electrophoresis in 3.5% agarose gel. With regard to the sensitivity of the RFLP assay, when the mutant was mixed with 10-fold the amount of wild-type, the mutant (⩾10^2 copies/ml) could be detected. The nucleotide and amino acid substitutions of the detected mutant samples were confirmed by direct sequence analysis.

2.5. Statistical analysis

All data were analyzed using the statistical package SPSS II (version 10.0, SPSS Inc, Chicago, IL). Non-parametric tests including the chi-squared test, Fisher's exact probability test, and the Mann–Whitney *U*-test were used to compare the background characteristics and efficacy. The cumulative rate of undetectability of HBV-DNA and HBeAg loss was calculated using the Kaplan–Meier method and differences between the curves were tested using the log-rank test. Univariate analyses were conducted using logistic regression analysis. All factors found to be at least marginally associated ($P < 0.15$) were entered into multivariate analysis using a stepwise Cox regression analysis. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Study population

The clinical and virological profiles of the 132 patients at the start of ADV + LAM treatment are shown in Table 1. At the commencement of ADV + LAM treatment, 41 patients (31.1%) had cirrhosis, and 79 patients (59.8%) were positive for HBeAg. Six of the 132 patients were treated with ADV at the time of virological breakthrough and the remaining 126 patients were treated at the time of breakthrough hepatitis.

3.2. Virological and biochemical response

The cumulative rates of undetectable serum HBV-DNA levels (<2.6 log copies/ml) were 56% at the end of 6 months, 69% at 12 months, 81% at 24 months and 87% at 36 months. The cumulative rates of normalized serum ALT levels were 73% at the end of 6 months, 85% at 12 months and 99% at 24 months. Of the 79 HBeAg-positive patients, the cumulative rates of HBeAg loss were 10% at 6 months, 16% at 12 months, 34% at 24 months and 39% at 36 months. The cumulative rates of HBeAg seroconversion were 7.5% at 6 months, 13% at 12 months, 24% at 24 months and 32% at 36 months.

3.3. Baseline parameters associated with virological response as determined by univariate and multivariate analyses

Univariate analysis identified six baseline parameters that influenced the undetectability of serum HBV-DNA during therapy: HBeAg status (negative; $P < 0.00001$), HBV-DNA (<7 log copies/ml; $P < 0.00001$), AST (>150 IU/L; $P < 0.00001$), ALT (>200 IU/L; $P = 0.0074$), fibrosis (liver cirrhosis; $P = 0.0057$) and T-Bil (>1 mg/dl; $P = 0.0535$). No association with other factors was noted: patient age, sex, serum albumin, serum creatinine, platelet count, YMDD mutant status and HBV genotype.

Multivariate analysis that included the above variables identified four parameters that independently influenced the virologic response: HBeAg status ($P = 0.0001$), AST ($P = 0.001$), HBV-DNA ($P = 0.002$), and fibrosis ($P = 0.015$) (Table 2). These results confirmed that HBeAg status is the most influential factor of undetectability of HBV-DNA. The time to undetectable HBV-DNA was significantly shorter in HBeAg-negative than in-positive patients ($P = 0.00001$). The time to normalization of ALT level was also shorter in HBeAg-negative than in-positive patients (Fig. 1a and b). The rates of undetectable HBV-DNA in the HBeAg-negative group were 94% at the end of 12 months and 100% at 24 months. On the other hand, the undetectability rates of HBV-DNA in the HBeAg-positive group were 47% at the end of 12 months, 68% at 24 months and 78% at 36 months (Fig. 1, Table 3). Therefore, we thought that it was important to investigate the predictive factor(s) of virologic response in HBeAg-positive patients. There were 21 non-responders (HBV-DNA ⩾4.5 log copies/ml at 6 months of ADV + LAM), whose HBV-DNA level were all over 7 log copies/ml. Therefore we selected the responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) with high levels HBV-DNA (⩾7 log copies/ml) at baseline and we found 15 patients who fulfilled the criteria. The 36 HBeAg-positive patients with high levels HBV-DNA underwent sequence analysis of the RT lesion in the polymerase gene. However, there were no differences in the RT lesion; i.e., rtH55, rtL80, rtV173, rtM180, rtI233, and rtN337, between responders (HBV-DNA <2.6 log copies/ml at 6 months of ADV + LAM) and non-responders (HBV-DNA >4.5 log copies/ml at 6 months of ADV + LAM).

Table 1
Baseline characteristics at commencement of adefovir dipivoxil ($n = 132$)

Age (years)*	47 (26–73)
Gender (Male:Female)	105:27
Prior LAM therapy (month)*	31 (8–110)
ADV treatment duration (month)*	28 (12–50)
Presence of cirrhosis (%)	41/132 (31.1)
HBV genotype (A:B:C:D)	7:5:119:1
HBeAg-positive (%)	79/132 (59.8)
HBV-DNA (log copies/ml)*	7.3 (3.3–>7.6)
rtM204 mutant (%)	130/132 (98.4)
I:V:I + V#	69:28:33
AST (IU/L)*	132 (31–1413)
ALT (IU/L)*	132 (24–1563)
T-Bil (mg/dl)*	0.8 (0.6–6.0)
Albumin (g/dl)*	3.9 (2.8–4.7)
Serum creatinine (mg/dl)*	0.8 (0.4–1.3)

* Data are median values (range).
\# I → YIDD, V → YVDD, I + V → YIDD + YVDD mix.

3.4. Genotypic analysis of ADV- and LAM-resistant mutants

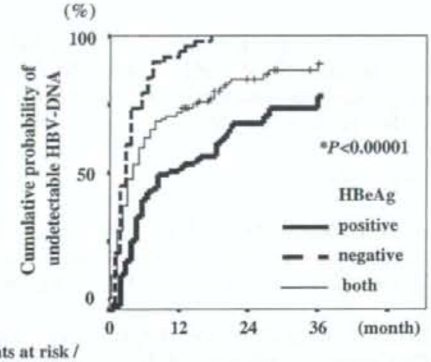
Genotypic resistance to ADV was looked for in PCR positive (HBV-DNA ⩾ 2.6 log copies/ml) samples. Number of samples tested at baseline, 1 year and 2 years were 131 of 132 samples, 45 of 45 samples, 16 of 16 samples, respectively. The substitutions at rtA181 and rtN236 were assessed annually by RFLP method and direct sequence. At baseline, substitutions at rtA181 were identified in 3 patients (2.3%), whose genotypes were rtA181T without substitution at rt204, rtA181S without substitution at rt204 and rtA181T plus rtM204I double mutation (Fig. 2). On the other hand, substitution at rt236 was not identified at the start of ADV. In

Table 2
Multivariate analysis of baseline factors associated with virological response

Factors	Category	Hazard ratio	95% CI	P
HBeAg status	1: negative	1		
	2: positive	0.380	0.242–0.595	0.0001
AST (IU/L)	1: <150	1		
	2: ⩾150	2.115	1.357–3.296	0.001
HBV-DNA (log copies/ml)	1: <7	1		
	2: ⩾7	0.532	0.353–0.797	0.002
Cirrhosis	1: no cirrhosis	1		
	2: cirrhosis	1.683	1.107–2.559	0.015

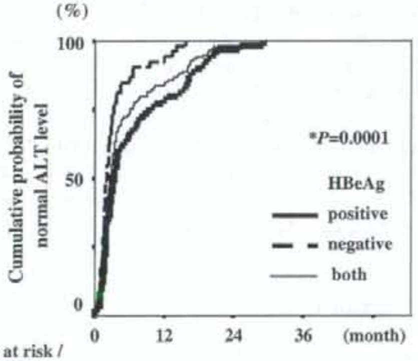
Note. Virological response: undetectable serum HBV-DNA by amplicor monitor assay (<2.6 log copies/ml).

a



patients at risk / No.evaluated				
HBeAg positive	0/79	37/79	34/50	14/20
HBeAg negative	0/53	50/53	30/30	9/9
Both	0/132	87/132	64/80	23/29

b



patients at risk / No.evaluated				
HBeAg positive	2/79	62/79	48/50	20/20
HBeAg negative	1/53	50/53	30/30	9/9
Both	3/132	112/132	78/80	29/29

Fig. 1. (a) Cumulative probability of undetectable HBV-DNA during ADV + LAM combination therapy in patients with HBeAg-positive, -negative and both. (b) Cumulative probability of normal ALT during ADV + LAM combination therapy in patients HBeAg-positive, -negative and both. *P values between HBeAg-positive and -negative groups.

the remaining 129 patients, rtM204 mutations without substitutions at rt181 and rt236 were identified. Following ADV + LAM combination therapy, new ADV-resistant strains were identified in two patients (1.6%); one had rtA181S and the other had rtA181T plus rtN236T double mutation; they were the only two patients (among the 129 patients) who showed virological rebound during ADV + LAM therapy (Fig. 3). The cumulative rate of ADV-R was calculated every year; 1% of the first year, 1% of the second year, 1% of the third year and 8% of the fourth year. However, long follow-up studies of larger population samples are needed for a more accurate evaluation of the cumulative rate.

During combination therapy, 105 patients achieved virological response. Ninety-eight of 105 (93.3 %) patients maintained virological response. Only one patient was included according to our definition of virological breakthrough that was defined as increase in serum HBV-DNA levels of ⩾1 log copies/ml (3.6 log copies/ml) during combination therapy and also developed rtA181S mutation (Fig. 3b). However, the remaining 6 patients showed fluctuated HBV-DNA level of between <2.6 and 3.1 log copies/ml transiently, whose genotypes were wild-type at rtA181 and rtN236 during treatment.

3.5. Clinical course of patients who had developed rtA181 mutations at the start of ADV + LAM combination therapy

Three patients developed substitutions at rtA181 associated with LAM resistance. All patients were HBeAg-positive at the start of LAM. As shown in Fig. 2, two of the three patients developed rtA181T and rtA181S without YMDD mutation and the viral load did not respond sufficiently to ADV therapy. The patient with rtA181S continued to show HBV-DNA >7 log copies/ml after 2 years of ADV + LAM treatment (Fig. 2a). Subsequently, the patient was changed to 0.5 mg of ETV, which resulted in 2 log copies/ml reduction in viral load and improvement of ALT. The other patient developed rtA181T mutation mixed with wild strain (Fig. 2b). At the end of 6-month

Table 3
Undetectable rate of HBV-DNA by Amplicor monitor assay in HBeAg-negative and -positive patients

HBV-DNA (log copies/ml)	Baseline	6 months	12 months	18 months	24 months
HBeAg-negative					
<2.6	0 (0%)	40 (75%)	50 (94%)	47 (100%)	30 (100%)
2.6–<4.5	3 (6%)	13 (25%)	3 (6%)	0 (0%)	0 (0%)
≥4.5	50 (94%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total, *n* (%)	53 (100%)	53 (100%)	53 (100%)	47 (100%)	30 (100%)
HBeAg-positive					
<2.6	0 (0%)	32 (40%)	37 (47%)	34 (54%)	34 (68%)
2.6–<4.5	3 (4%)	26 (33%)	29 (37%)	21 (35%)	15 (30%)
≥4.5	76 (96%)	21 (27%)	13 (16%)	8 (13%)	1 (2%)
Total, *n* (%)	79 (100%)	79 (100%)	79 (100%)	63 (100%)	50 (100%)

ADV + LAM therapy, the HBV-DNA level diminished by 1.5 log copies/ml and ALT level improved to the normal range. At that time, only the mutant strain (rtA181T) was detected, suggesting that the viral reduction was due to the suppression of wild-type HBV strain. The HBV-DNA level was persistently above 5 log copies/ml even at the end of 1 year of ADV + LAM therapy. On the other hand, in the patients with rtA181T + rtM204I mutation, viral load rapidly decreased to the undetectable HBV-DNA level at the end of 6 months of ADV + LAM combination therapy (Fig. 2c).

3.6. Clinical course and clonal analysis in patients who developed ADV-related mutation during combination therapy

Fig. 3 shows the clinical course of patients with ADV-resistant mutants. The first ADV-resistant HBV strain was isolated from a 32-year-old Japanese man with genotype C (Fig. 3a). At 15 months after the start of LAM, viral and biochemical breakthroughs were observed. To suppress the viral HBV-DNA, ADV was added to LAM therapy. The mutant strain with rtA181T associated with ADV resistance appeared at 6 months of ADV + LAM therapy, while another rtN236T mutation appeared at 3 years of ADV therapy. Moreover, breakthrough hepatitis was observed after 3.5 years of ADV + LAM therapy (Table 4). Interestingly, the rtA181T at the end of 6 months of ADV therapy, due to single nucleotide substitution (TGG to TGA), resulted in early termination of overlapping HBs gene by creating a stop codon. On the other hand, at the end of 3 years of ADV therapy, all rtA181T mutant strains changed to double nucleotide substitutions (TGG to TTA), which induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L) developed.

Another mutant strain was detected in a 38-year-old Japanese man with genotype C-HBV infection (Fig. 3b). Following 46 months of ADV + LAM therapy when the viral load was increased, the rtA181S mutant strain without YMDD mutation was detected; however, the viral load diminished naturally to an undetectable level in a few months.

3.7. Clinical events

After the addition of ADV, 4 of the 132 (3%) patients elevated in serum creatinine >0.5 mg/dl above baseline and their ADV dose was reduced to 10 mg every other day.

Eight patients developed hepatocellular carcinoma (HCC) before the addition of ADV. After the addition of ADV, four patients developed HCC. Three of the four patients (75%) had cirrhosis at the start of ADV. The median duration from the start of ADV to the development of HCC was 14 months (range, 6–26 months). At the diagnosis of HCC, 3 of the 4 patients (75%) had undetectable HBV-DNA.

Of the 41 patients with cirrhosis, 5 patients had ascites and/or pleural effusion at the start of ADV. In 4 of the 5 patients, the fluid level diminished and disappeared during combination therapy. Only one patient with HCC showed worsened liver failure and died 22 months later. All patients without HCC and decompensation at the start of ADV therapy did not develop liver decompensation during follow-up.

4. Discussion

The efficacy of ADV combined with LAM has been reported in some studies; however, the rate of HBV-DNA undetectability under combination therapy was found to be the same as in patients treated with ADV alone [14,19]. We investigated whether combination therapy is characterized by a low risk of ADV resistance. In this study, we studied the long-term efficacy of ADV when added to LAM in 132 patients with chronic hepatitis B who developed LAM resistance. The results demonstrated that combination therapy

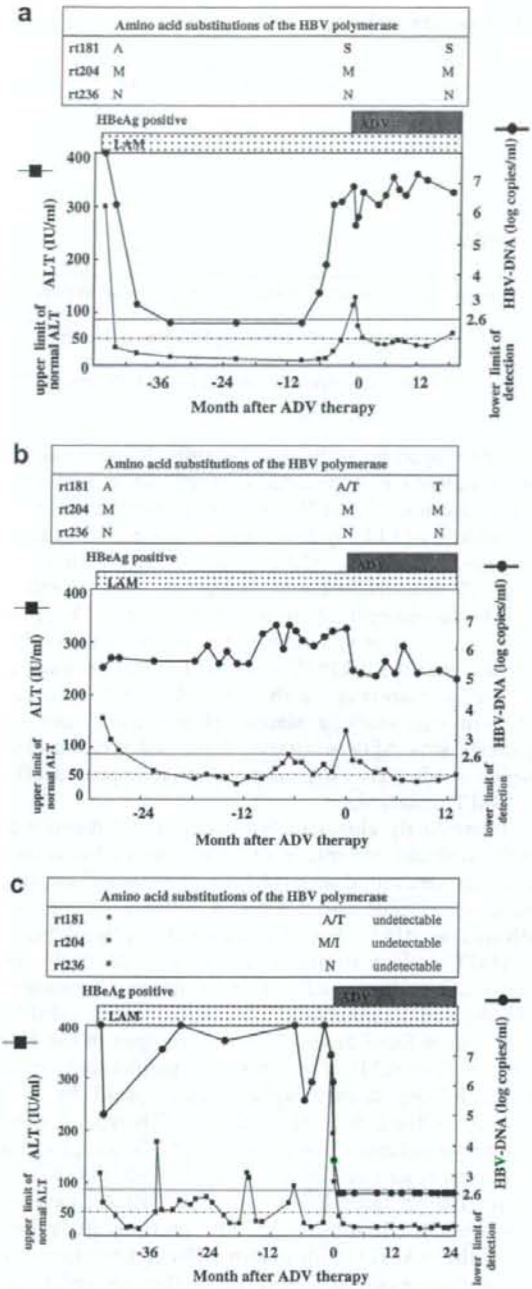


Fig. 2. Clinical course of three patients who showed emergence of ADV-resistant mutants at the commencement of ADV therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above each graph. (a) Clinical course of a patient who developed the rtA181S mutant. (b) Clinical course of a patient who developed the rtA181T mutant. (c) Clinical course of a patient who developed the rtA181T with rtm204I mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase; *no data.

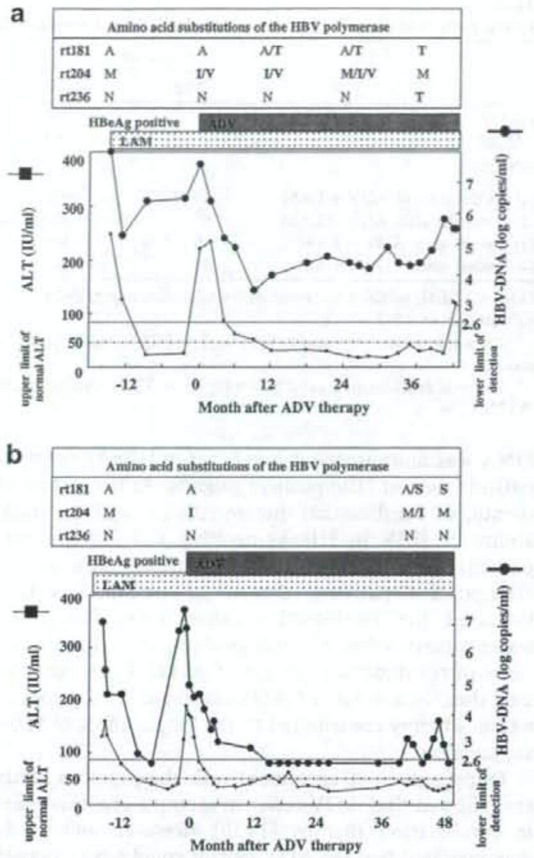


Fig. 3. Clinical course of two patients with LAM-resistant HBV who showed the emergence of an ADV-resistant mutant during combination therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above the graph. (a) Clinical course of a patient who developed the rtA181T + rtN236T mutant. (b) Clinical course of a patient who developed the rtA181S mutant. LAM, lamivudine; ADV, adefovir; rt, reverse transcriptase.

rapidly and consistently suppressed the HBV-DNA. Moreover, we demonstrated that the emergence of ADV-resistant mutants was rare during the combination therapy for up to 3 years. However, our virological analysis showed that substitutions at rt181, which were associated with both LAM and ADV resistance, need to be evaluated during combination therapy.

Multivariate analysis in this study revealed that the baseline HBeAg status, AST level and HBV-DNA level influenced the cumulative probability of undetectability of serum HBV-DNA. A number of previous studies also identified almost the same predictors of virological response during ADV alone or combination therapy [20,21]. In particular, the undetectable rate of HBV-

Table 4
Clonal analysis of samples from the patient who developed resistance to ADV + LAM combination therapy

	Relative rate (%) of clones (No. of clones/total)				
	Wild	rtM204I/V	rtA181T(1)	rtA181T(2)	rtA181T + rtN236T
rtA181	–	–	T(HBsAgstop)*	T(sW172L)#	T(sW172L)#
rtM204	–	I/ V	–	–	–
rtN236	–	–	–	–	N
(1) At the start of ADV + LAM	40 (4/10)	60 (6/10)	0	0	0
(2) 6 months after ADV + LAM	0	59 (13/22)	41 (9/22)	0	0
(3) 2 years after ADV + LAM	16 (4/25)	36 (9/25)	36 (9/25)	12 (3/25)	0
(4) 3 years after ADV + LAM	0	0	0	0	100 (20/20)

Note. rtM204I, methionine to isoleucine substitution at rt204; rtM204V, methionine to valine substitution at rt204; rtA181T, alanine to threonine substitution at rt181.

* The single nucleotide substitution (TGG to TGA) resulted in rtA181T mutation and early termination of overlapping HBs gene by creating a stop codon.

\# The double nucleotide substitution (TGG to TTA) resulted in amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L).

DNA was more frequent and faster in HBeAg-negative patients than in HBe-positive patients. At the end of 12 months of combination therapy, the rates of undetectability of HBV in HBeAg-negative and HBe-positive patients were 94% and 47%, respectively. However, in HBe-positive patients, the longer treatment course increased the virological response more frequently. Some patients achieved virological response after 2 more years of combination therapy (Fig. 1a). It was considered that the low risk of ADV resistance during combination therapy contributed to the longer effect of HBV suppression.

Our result is in agreement with the previous study that showed that ADV-resistant mutants are infrequent in combination therapy [14,19]; however, our study demonstrated that the ADV mutant could have emerged during combination therapy. We identified the emergence of rtA181T/S and/or rtN236T mutation in two of the 129 patients with YMDD mutant as an ADV-resistant strain during the ADV + LAM combination therapy. To our knowledge, this is the first report of emergence of ADV-resistant mutant followed by breakthrough hepatitis during combination therapy as shown in Fig. 3a. A previous open-label study in HBeAg-negative LAM-resistant patients demonstrated that combination therapy did not result in the development of resistance to ADV over a period of 3 years, in contrast to ADV monotherapy that was associated with the development of such resistance in 21% of the patients after the first year [14]. Although another recent study reported the appearance of ADV-resistant mutants in three patients during combination therapy, they were all initially switched to ADV monotherapy and later changed to the combination therapy after several months [20]. Another recent study reported an emergence rate of ADV resistance during ADV + LAM of 1% at 1 year and 4% at 3 years; however, no virological rebound was noted [21]. The patients in our study continued to show a viral load of up to 5.8 log copies/ml and developed breakthrough hepatitis. Some studies of ADV monotherapy reported that the rise in ALT after the emergence of rtA181 and/or rtN236 mutant is mild to moderate [9,14,24]. Several *in vitro* studies including our previous study [22–25] demonstrated that the rtA181T and rtN236T mutant leads to a minor reduction in the susceptibility to both LAM and ADV. However, one study of 998 naïve patients treated with ADV showed that the rtA181V + rtN236T mutation was significantly associated with virological breakthrough [26]. In our study, a similar phenomenon emerged; patients with ADV resistance developed breakthrough hepatitis after rtN236T mutation that appeared after rtA181T mutation.

Interestingly, clonal analyses of HBV in patients with ADV-resistant mutants in this study showed that such mutants were mixed with rtA181T mutants without substitutions at rt204, and rtM204I/V mutants without substitution at rt181. Moreover, we identified two types of rtA181T mutant strains; one was a single nucleotide substitution that induced prematurely terminated HBsAg and the other was a double nucleotide substitution that induced amino acid substitutions in the HBs antigen. The rtA181T mutant with prematurely terminated HBsAg cannot replicate and spread by itself because of the lack of HBs antigen. This type of strain is thought to replicate *in vivo* supplied HBs antigen from wild-type strains as helpers. Thus, the mutants changed themselves to the HBV with mature HBsAg by additional nucleotide substitution. Our previous study identified the rtA181T with mature HBsAg first; however, the mutant emerged during LAM therapy and it did not show a stepwise process [25].

We also demonstrated that the substitution at rt181 was associated with not only ADV resistance but also LAM resistance. At the commencement of ADV + LAM combination therapy, the substitutions at rtA181 as LAM resistance were identified in three patients (2.3%), who exhibited poor viral reduction during the combination therapy. Of note, the rtA181S mutation is a novel LAM-resistant strain that has never been reported. There are a few reports of the rtA181 mutation associated with LAM resistance. A recent study reported the presence of rtA181T mutants in 3 of 57 (5.3%) LAM-resistant patients [15] and another study showed that 6 of 145 (4%) LAM-resistant patients developed rtA181T/V mutation [21]. If ADV therapy produces insufficient reduction of LAM-resistant HBV, it is important to suspect the emergence of ADV-related mutant at the commencement of ADV therapy and plan a new treatment strategy. However, there is no consensus at present on the management of patients with ADV + LAM-resistant mutant. Entecavir was the only agent reported to be effective both *in vitro* and *in vivo*. In our study, the patient with rtA181S mutation was switched to entecavir therapy; however, this did not produce a sufficient reduction in the viral load. On the other hand, recent studies reported the efficacy of tenofovir for patients with LAM-resistant mutants [27,28]. Further studies are needed to clear this issue.

In conclusion, ADV in combination with LAM effectively suppressed viral replication and was efficacious in LAM-resistant patients with chronic HBV infection. Genotypic analysis indicated that the emergence of ADV-resistant mutants was rare in patients on ADV + LAM combination therapy at least for 2 years. However, virological analysis showed that the substitution at rt181, which was associated with both LAM and ADV resistance, was needed for careful monitoring before and during combination therapy.

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