Table 1 Patient baseline and demographic characteristics

	Total	Previous treatment		*P*-value*
		(−)	(+)	
Number of patients	76	46	30	
Age (years)	56.9 ± 11.3	54.9 ± 11.7	60.1 ± 10.1	0.049
Gender (male/female)	40/36	21/25	19/11	N.S.
Body mass index (kg/m^2)	23.0 ± 3.4	23.8 ± 3.6	21.9 ± 2.7	0.015
LDL cholesterol (mg/dL)	99.6 ± 26.7	102 ± 28.0	93.8 ± 23.7	N.S.
HCV RNA levels (log IU/mL)	6.3 ± 1.0	6.5 ± 0.6	6.0 ± 1.3	0.026
ALT (IU/L)	84.6 ± 65.8	93.3 ± 69.2	71.2 ± 59.0	N.S.
Gamma-glutamyl transferase (IU/L)	89.5 ± 108	82.5 ± 113	101 ± 101	N.S.
Alpha-fetoprotein (ng/mL)	19.3 ± 39.6	19.8 ± 49.6	18.9 ± 27.7	N.S.
Leukocyte count (/mm^3)	4800 ± 1660	5280 ± 1540	4060 ± 1600	0.0013
Hemoglobin (g/dL)	13.8 ± 1.9	14.3 ± 1.4	13.1 ± 2.3	0.0059
Platelet count (×10^4/mm^3)	15.4 ± 5.6	16.4 ± 5.2	13.9 ± 6.0	NS

Data are expressed as mean ± SD. *P-value indicates those between groups with and without pretreatment by Student's t-test or chi-square test. NS, not statistically significant; LDL, low-density lipoprotein; ALT, alanine aminotransferase.

treatment due to side effects. In the 30 previously treated patients, 7 (23.3%) had SVR, 3 (10.0%) relapsed, 16 (53.3%) did not respond, and 4 (13.3%) discontinued treatment due to side effects. It is well known that patient adherence to prescribed antiviral therapy beyond 12–24 weeks is advantageous for treatment response [12]. In the 46 treatment-naïve patients, 27 (58.6%) had ≥80% adherence to PEG-IFN and 18 (39.1%) had ≥80% adherence to RBV. In the 30 previously treated patients, 11 (36.6%) had ≥80% adherence to PEG-IFN and 9 (30.0%) had ≥80% adherence to RBV.

Proportions of patients with HCV RNA lower than the limits of detection and HCV RNA <1.7 log IU/mL over time in the treatment cohort

First, we compared the proportion of negative HCV RNA samples by COBAS TaqMan HCV test with that at <1.7 log IU/mL by COBAS TaqMan HCV test, since HCV RNA levels < 1.7 log IU/mL by COBAS TaqMan HCV test are considered undetectable by traditional methods (see 'Methods' section). The over-quantification problem of the older methods was demonstrated (Fig. 1a). Of 76 patients, at week 8, the differences between these numbers were significant (P = 0.013), but not at the other time points (Fig. 1a). In 40 males, the difference was not significant at any of the time points. In 36 female patients, the difference at only week 8 was significant (8/36 *vs* 18/36; P = 0.014). In 40 patients younger than 60 years, the difference at only week 8 was significant (13/40 *vs* 23/40; P = 0.024), but in the 36 older than 60, the difference was not significant at any of the time points. In the 46 treatment-naïve patients, the differences at week 8 were significant (16/46 *vs* 26/46; P = 0.036), but the difference was not significant at any time points in the 30 previously treated patients.

Prediction of SVR and non-SVR

The positive predictive value for SVR is shown in Fig. 1b. The positive predictive value for SVR based on undetectable HCV RNA by COBAS TaqMan HCV test over time was superior in the treatment cohort.

The negative predictive value for SVR is shown in Fig. 1c. The negative predictive values for SVR based on HCV RNA <1.7 log IU/mL by COBAS TaqMan HCV test were superior at any time point other than at week 16 (Fig. 1c).

In only two cases, out of 34 achieving SVR, HCV RNA could be detected by COBAS TaqMan HCV test at 12 weeks after starting treatment. One of them, a 63-year-old woman, was treatment-naïve and had 7.1 log IU/mL HCV RNA at pretreatment baseline. HCV RNA levels were 4.5, 1.2, 1.2, 0, 0, 0 and 0 log IU/mL, respectively, at 4, 8, 12, 16, 24, 36, and 48 weeks after treatment. She did not achieve EVR, but she reached SVR. The other, 52-year-old woman was treatment-naïve and had 7.1 log IU/mL HCV RNA at pretreatment baseline. HCV RNA levels were 5.4, 4.6, 3.7, 2.2, 0, 0 and 0 log IU/mL, respectively, at 4, 8, 12, 16, 24, 36, and 48 weeks after treatment. She did not achieve EVR, but she also reached SVR. On the other hand, in 7 cases obtaining EVR (all EVR were cEVR) by COBAS TaqMan HCV test, SVR was not obtained.

DISCUSSION

In this study, we focused on EVR and RVR evaluated by the COBAS TaqMan HCV test, as at this time we did not know

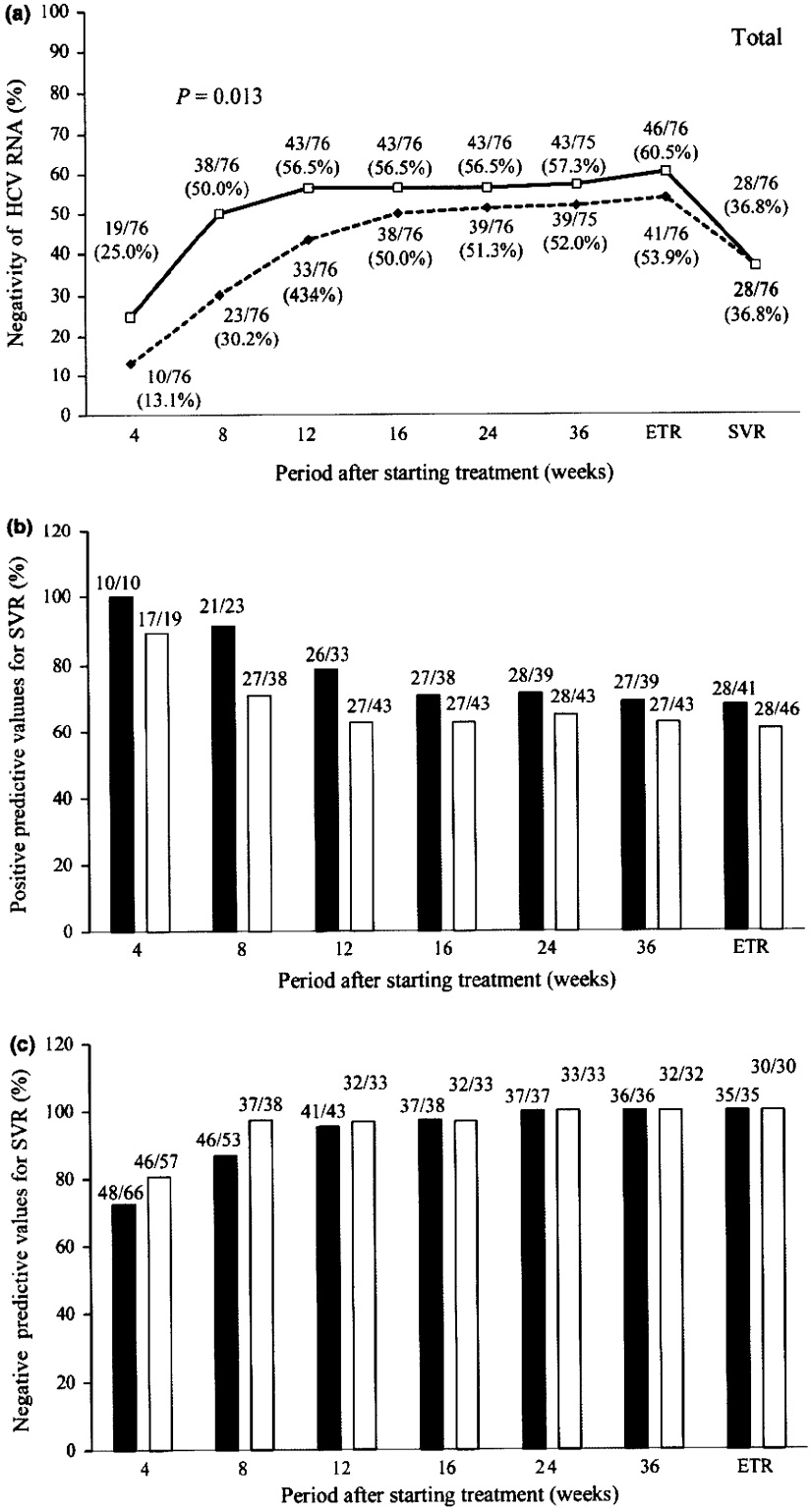

Fig. 1 (a) Proportions of patients with HCV RNA lower than the limits of detection (black diamonds) and HCV RNA <1.7 log IU/mL (white squares) by COBAS TaqMan HCV test over time in the treatment cohort. Total 76 patients were HCV RNA-positive at pretreatment baseline. (b, c) Predictive values for SVR and for non-SVR in patients with HCV RNA lower than the limits of detection (black columns) and HCV RNA <1.7 log IU/mL (white columns) by COBAS TaqMan HCV test over time in the treatment cohort. (b) Positive predictive values for SVR. (c) Negative predictive values for SVR. ETR, virological response at the end of treatment; SVR, undetectable serum HCV RNA at 24 weeks after the end of treatment; *P*-value, significant difference between two groups by chi-square test.

whether there were discrepancies between undetectable RNA and <1.7 log IU/mL. We showed that the utilization of both undetectable RNA and values ≤1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test is useful and could predict SVR and non-SVR patients with greater accuracy. Our study also showed that the COBAS TaqMan HCV test is a more accurate method for evaluating EVR and RVR, although the negative predictive value was superior when ≤1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test was used.

Recently, there have been reports that in HCV genotype 2/3-infected patients with a very rapid viral response (vRVR), i.e. HCV RNA below 1000 IU/ml on day 7, treatment can be shortened to 12–16 weeks if no dose reduction has been made [13]. For genotype 1 patient with RVR, 24 weeks of treatment is recommended. For patients with cEVR, i.e. HCV RNA undetectable at week 12, 48 weeks of treatment is recommended, whereas 72 weeks of treatment should be considered for patients with partial EVR, i.e. at least a 2 log decrease in HCV RNA levels [8,14,15].

The COBAS TaqMan HCV test is useful for simultaneously analysing qualitative and quantitative HCV RNA or other viral RNA [5,16]. In the near future, we are expecting higher SVR rates in chronic hepatitis C patients treated with new drugs, such as NS3/4A protease inhibitors, NS5A inhibitors and others [4,17], and the COBAS TaqMan HCV test could be useful for selecting patients. The COBAS TaqMan HCV test assay may also be useful for shortening the treatment duration to 16/24 weeks [18]. In the near future, we will use direct antivirals with and later without IFN, HCV RNA testing will be done at weeks 1–4, and HCV RNA negativity will be the preferred parameter for determining the duration of therapy.

In conclusion, our study showed that the COBAS TaqMan HCV test was more sensitive than the heretofore-applied methods, and that it may provide a more accurate prediction of the treatment response, leading to a reduction in adverse events and treatment duration, and to the modification of antiviral regimens in at least some of the patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The requirement for a sufficient period of corticosteroid treatment in combination with nucleoside analogue for severe acute exacerbation of chronic hepatitis B

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Abstract
Background The prognosis of severe acute exacerbation of chronic hepatitis B is very poor if signs of liver failure appear. We have reported the efficacy of the early introduction of sufficient doses of corticosteroids (CSs) and nucleoside analogues (NAs), but the optimal period of immunosuppressive therapy has not been well demonstrated. In this study, we analyzed patients with severe acute exacerbation of chronic hepatitis B treated with CSs and NAs, prospectively, in order to clarify the factors affecting their outcome.
Methods Ten patients, admitted to our liver unit between 2000 and 2009, were defined as having severe exacerbation of chronic hepatitis B based on our uniform criteria, and were enrolled in this study. NAs and sufficient doses of CS were introduced as soon as possible after making the diagnosis of severe disease prospectively.
Results Seven of the 10 patients recovered. The absence of fulminant hepatitis on admission, the improvement of prothrombin time (PT) activity and the decline of hepatitis B virus (HBV) DNA during the first 2 and 4 weeks, respectively, were significant in the recovered patients, while the worsening of total bilirubin level during 4 weeks, especially between week 2 and week 4, was significant in those who died.
Conclusions In severe acute exacerbation of chronic hepatitis B, more than a few weeks of CS treatment in combination with an NA is required in the early stage, whereas a short period of conventional pulse therapy would be insufficient for treating this condition.

Keywords Chronic hepatitis B · Severe exacerbation · Corticosteroid · Nucleoside analogue

Abbreviations
HBV Hepatitis B virus
CS Corticosteroid
NA Nucleoside analogue

Introduction

Exacerbation of hepatitis B in chronic hepatitis B virus (HBV) carriers may occur spontaneously or in relation to cytotoxic or immunomodulatory therapy. A clinical picture ranging from anicteric hepatitis to severe exacerbation, sometimes fulminant liver failure, may develop, and it is associated with high mortality [1]. In a retrospective Japanese survey of HBV carriers with hematologic malignancies, a 53% incidence of severe hepatitis with a 24% mortality rate was reported in relation to chemotherapy [2]. For the treatment of patients with severe exacerbation, liver transplantation may be a viable option, although it is contraindicated in patients with underlying malignancies. However, the problem of the shortage of donor livers still remains in Japan. Thus, therapies other than transplantation must be further investigated.

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In HBV infection, liver injury is considered to be induced mainly by cytotoxic T-lymphocyte-mediated cytolytic pathways in HBV-infected hepatocytes [3], and it was suggested that treating chronic hepatitis B patients with corticosteroids (CSs) in order to inhibit an excessive immune response and prevent cytolysis of infected hepatocytes would be reasonable, if the HBV could be controlled [4]. However, the advantage of CSs in the treatment of chronic active hepatitis B was not confirmed by control studies, and their use for routine management has fallen out of favor [5–7], although those studies mainly dealt with cases of clinically "nonsevere" hepatitis.

As to the effects of CS treatment for "severe and potentially life-threatening" exacerbation of chronic hepatitis B, Lau et al. [8] reported that the reintroduction of long-term high-dose CS in the early phase of reactivation after the withdrawal of immunosuppressive therapy prevented both progressive clinical deterioration and the potential need for orthotopic liver transplantation.

Recently, nucleoside analogues (NAs) have been administered safely even in severe disease [9–13], and in our previous studies, we reported that the introduction of high-dose CS and NA could significantly reverse deterioration in patients with "clinically severe, life-threatening" exacerbation of chronic hepatitis B compared with historical controls, when used in the early stage of the illness [14, 15]. But the dose and the period of CS use have still to be clarified.

In this study, we analyzed patients with clinically severe exacerbation of chronic hepatitis B treated with the initiation of sufficient doses of CS and NA prospectively, in order to clarify the factors affecting the outcome and the optimal period of sufficient CS therapy required to suppress an excessive host immune response.

Patients and methods

Patients

Ten patients with severe acute exacerbation of chronic hepatitis B admitted to our liver unit (Chiba University Hospital and related hospitals) between 2000 and 2009 were studied. The diagnosis of a chronic hepatitis B viral carrier state was made based on either the positivity of hepatitis B surface antigen (HBsAg) for at least 6 months before entry or, in patients with follow-up periods less than 6 months before entry, it was based on the positivity of HBsAg, presence of anti-hepatitis B core antibody (HBcAb) at a high titer, and negativity or a low titer of IgM anti-hepatitis B core antibody (IgM-HBc). Patients fulfilling all the following three criteria during the course were defined as having severe exacerbation: prothrombin time (PT) activity less than 60% of normal control, total bilirubin (T-Bil) greater than 3.0 mg/dl, and alanine transaminase (ALT) greater than 300 IU/l during the course. Patients with PT activity less than 40% of control and hepatic encephalopathy were defined as having fulminant hepatitis. All patients were in poor general condition, including general malaise, fatigue, jaundice, edema, ascites, and encephalopathy. Histological examination was performed in the convalescent phase or after the death of patients. The work described in this manuscript has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of the ethics committee of our institutional review boards. Informed consent was obtained from all patients or appropriate family members.

All patients were negative for IgM anti-HAV antibody, anti-hepatitis D antibody, anti-HCV antibody, HCV RNA, IgM anti-Epstein–Barr virus antibody (IgM-EBV), IgM anti-herpes simplex virus antibody (IgM-HSV), IgM anti-cytomegalovirus antibody (IgM-CMV), anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1, and anti-mitochondrial antibody. Patients with histories of recent exposure to drugs and chemical agents as well as those with recent heavy alcohol intake were ruled out. One patient was HIV-positive but had no clinical evidence of acquired immune deficiency syndrome.

Treatment protocols

All patients treated were examined prospectively. Informed consent was obtained from the patients or appropriate family members. Patients were treated with the NA, lamivudine (LMV) before 2007 or with entecavir (ETV) after 2007, and CS. Early introduction of CS was defined as follows: 40 mg or more of prednisolone (PSL) daily was administered within 10 days after the diagnosis of severe disease, using the above-mentioned criteria. This dosage was maintained for a minimum of 4 days. When the patient showed a trend toward remission of PT, the dosage was reduced by 10 mg at least every 4 days and tapered off. Patients for whom more than 10 days had already passed after the diagnosis before they had been admitted to our unit were treated with delayed introduction of CS (delayed CS). Patients with marked prolongation of PT were treated with 1000 mg of methylprednisolone (MPSL) daily for 3 days followed by the same PSL therapy as that described above.

LMV was administered at a daily dose of 100–300 mg. ETV was administered at a daily dose of 0.5–1.0 mg. Patients were also treated with intravenous glycyrrhizin (stronger neominophagen C; SNMC), an aqueous extract of licorice root, at 60–100 ml/day this agent is reported to

have anti-inflammatory activity and has been used for the treatment of chronic viral hepatitis in Japan [16].

Serological markers

HBsAg, hepatitis B envelope antigen (HBeAg), anti-HBe antibody (HBeAb), HBcAb, IgM-HBc, IgM anti-HAV antibody, and anti-hepatitis D antibody were detected by commercial radioimmunoassay (Abbott Laboratories, Chicago, IL, USA), and HCV RNA was measured by nested reverse transcription polymerase chain reaction (RT-PCR) [17]. Second-generation anti-HCV antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). IgM-EBV, IgM-CMV, and IgM-HSV were examined by enzyme-linked immunosorbent assays. Antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal-1 antibody were examined by a fluorescent antibody method. HBV DNA level was measured by Amplicor HBV monitor (Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Differences in proportions among groups were compared by Fisher's exact probability test, Student's *t*-test, and Welch's *t*-test.

Results

Clinical features of severe chronic hepatitis B patients on admission

Of the 10 patients, 8 were men and 2 women. Mean age at the time of admission was 49.2 ± 11.2 years. Four patients had primary diseases and conditions (one non-Hodgkin's lymphoma, one rheumatoid arthritis after a curative operation for hepatoma, one HIV-positive without immunodeficiency, and one gastrointestinal stromal tumor), and 3 had been treated with immunosuppressive or cytotoxic drugs, suffering exacerbations after their withdrawal. Four patients were diagnosed with fulminant hepatitis on admission (Table 1).

Mean PT activity was 32 ± 10%, mean ALT 894 ± 596 IU/l, and mean T-Bil 12.4 ± 8.5 mg/dl. HBeAg/HBeAb status was +/− in 3, −/+ in 6, and +/+ in 1. Mean HBV DNA was 6.2 ± 1.6 logcopy/ml, and precore/core promoter mutation status is shown in Table 2. Mean alfa-fetoprotein (AFP) was 250.8 ± 293.1 ng/ml and mean hepatocyte growth factor (HGF) was 7.0 ± 10.6 ng/ml (Table 2).

Types of therapies

As the initial CS, 1000 mg of MPSL was introduced to 4 patients, 60 mg of PSL to 5, and 40 mg of PSL to one. The mean duration between the diagnosis of severe disease and introduction of CS was 6.4 ± 4.8 days, and the mean duration of CS therapy was 63.6 ± 56.5 days. Eight patients were treated with early CS and 2 with delayed CS. As the NA, LMV was introduced to 7 patients and ETV to 3. In the 4 patients with fulminant hepatitis, artificial liver support (plasma exchange and hemodiafiltration) was performed (Table 3).

Biochemical responses to therapy

Changes in PT activities, ALT levels, T-Bil levels, and HBV DNA levels after the introduction of combination therapy are shown in Fig. 1. Mean PT activity was 34 ± 10% before initiation of the combination therapy (week 0), 58 ± 23 at 2 weeks after starting (week 2), and 62 ± 30 at 4 weeks (week 4). The improvement in PT activity was significant between week 0 and 2, and between week 0 and 4 ($p = 0.01$ and $p = 0.02$, respectively)

Table 1 Clinical features of patients

Patient	Age (years)	Sex	Onset	Complication	History of immunosuppressive or cytotoxic therapy	Fulminant hepatitis on admission
1	43	M	2000		−	−
2	30	M	2002		−	−
3	35	M	2002		−	−
4	45	M	2003	HIV (+)	−	+
5	55	M	2004		−	+
6	63	F	2005		−	+
7	65	M	2006	Rheumatoid arthritis, post-operation for hepatoma	+	+
8	55	F	2007	Non-Hodgkin's lymphoma	+	−
9	51	M	2007		−	−
10	50	M	2009	Gastrointestinal stromal tumor	+	−

Table 2 Biochemical, virological and histological features of patients

Patient	PT (%)	ALT (IU/l)	T-Bil (mg/dl)	D-Bil (mg/dl)	α-Fetoprotein (ng/ml)	Hepatocyte growth factor (ng/ml)	Liver histology	HBeAg	HBeAb	HBV DNA (logcopy/ml)	Precore mutation	Core promoter mutation
1	28	1463	9.1	5.8	658.9	2.07	CH (F3, severe)	+	−	8.6	Wild	Mutant
2	26	1897	19.0	15.1	106.5	2.04	CH (F2, severe)	+	−	7.2	ND	ND
3	24	723	9.3	7.4	18.9	0.75	CH (F3, severe)	−	+	7.2	Mixed	Wild
4	32	278	10.6	4.5	672.8	33.69	ND	−	+	5.2	Mutant	Wild
5	32	129	12.7	7.6	103.0	3.68	ND	−	+	4.2	Mixed	Mutant
6	30	968	9.9	4.9	674.2	9.80	Massive necrosis	+	+	4.3	Mixed	Mutant
7	21	364	10.5	6.6	184.4	9.37	ND	−	+	5.8	Mixed	Mutant
8	57	1595	3.0	2.0	6.8	ND	ND	−	+	7.6	ND	ND
9	35	938	6.6	4.8	20.8	0.39	ND	+	−	7.5	Wild	Mutant
10	34	584	33.5	26.0	61.6	1.15	CH (F3, severe)	−	+	4.8	Mixed	Mutant

PT prothrombin time, *ALT* alanine transaminase, *T-Bil* total bilirubin, *D-Bil* direct bilirubin, *HBeAg* hepatitis B envelope antigen, *HBeAb* hepatitis B envelope antibody, *HBV* hepatitis B virus, *ND* not done, *CH* chronic hepatitis

(Fig. 1a). The mean ALT level was 1260 ± 539 IU/l at week 0, 101 ± 77 at week 2, and 50 ± 33 at week 4. The ALT levels fell in all patients during the treatment course, with the decline between weeks 0 and 2, and between weeks 0 and 4 being significant ($p < 0.001$) (Fig. 1b). The mean T-Bil level was 11.9 ± 9.1 mg/dl at week 0, 10.2 ± 8.2 at week 2, and 9.9 ± 9.5 at week 4. Changes in T-Bil levels were not significant in 4 weeks (Fig. 1c). HBV DNA was 6.4 ± 1.6 log copies/ml at week 0, 4.7 ± 1.4 at week 2, and 3.9 ± 1.4 at week 4. The differences were significant between weeks 0 and 2 and weeks 0 and 4 ($p = 0.02$ and $p = 0.001$, respectively) (Fig. 1d).

Comparison of backgrounds on admission between recovered patients and nonsurvivors

Differences in age, sex, a history of immunosuppressive or cytotoxic therapy, PT activity, ALT level, T-Bil level, HBV DNA level, HBeAg/HBeAb status, AFP level, and HGF level on admission were not significant between recovered patients and nonsurvivors. The presence of fulminant hepatitis on admission was significantly different between the two patient groups ($p = 0.03$).

Comparison of responses to therapy between recovered patients and nonsurvivors

The mean PT activity was 32 ± 5% at week 0, 63 ± 21 at week 2, and 67 ± 23 at week 4 in the recovered patients, and 30 ± 7%, 36 ± 4, and 27 ± 6 in the nonsurvivors. Improvement of PT activity was significant between weeks 0 and 2, and between weeks 0 and 4 ($p = 0.003$ and $p = 0.006$, respectively) in the recovered patients, but there was no significant difference in PT activity levels in the non-survivors.

The mean ALT level was 1068 ± 589 IU/l at week 0, 81 ± 31 at week 2, and 59 ± 39 at week 4 in the recovered patients, and 1387 ± 57 IU/l, 80 ± 78, and 30 ± 5 in the nonsurvivors. The ALT levels fell significantly between weeks 0 and 2, and between weeks 0 and 4 in both groups ($p < 0.005$).

The mean T-Bil level was 15.8 ± 9.8 mg/dl at week 0, 10.3 ± 10.0 at week 2, and 7.7 ± 8.4 at week 4 in the recovered patients, and 7.2 ± 3.8 mg/dl, 13.1 ± 2.2, and 21.1 ± 2.5 in the nonsurvivors. Changes in T-Bil levels were not significant during 4 weeks in the recovered patients, but the increases between weeks 0 and 4, and between weeks 2 and 4 were significant in the nonsurvivors ($p = 0.006$ and $p = 0.02$, respectively).

The mean HBV DNA was 6.7 ± 1.6 log copies/ml at week 0, 4.7 ± 1.7 at week 2, and 3.7 ± 1.5 at week 4 in the recovered patients, and 5.7 ± 1.8 log copies/ml, 4.8 ± 0.9, and 4.3 ± 1.5 in the nonsurvivors. HBV DNA

Table 3 Therapies and responses

Patient	Duration between diagnosis of severe disease and introduction of corticosteroid (days)	Corticosteroid therapy		Nucleoside analogue	Artificial liver support	Outcome
		Drug	Period (days)			
1	4	MPSL + PSL	63	Lamivudine	–	Recovery
2	3	PSL	183	Lamivudine	–	Recovery
3	3	PSL	150	Lamivudine	–	Recovery
4	3	MPSL + PSL	21	Lamivudine	PE + CHDF	Death
5	8	PSL	32	Lamivudine	PE + CHDF	Recovery
6	9	PSL	37	Lamivudine	PE + CHDF	Death
7	12	PSL	35	Lamivudine	PE + CHDF	Death
8	1	PSL	60	Entecavir	–	Recovery
9	5	MPSL + PSL	31	Entecavir	–	Recovery
10	16	MPSL + PSL	24	Entecavir	–	Recovery

PSL prednisolone, *MPSL* methylprednisolone, *PE* plasma exchange, *CHDF* continuous hemodiafiltration

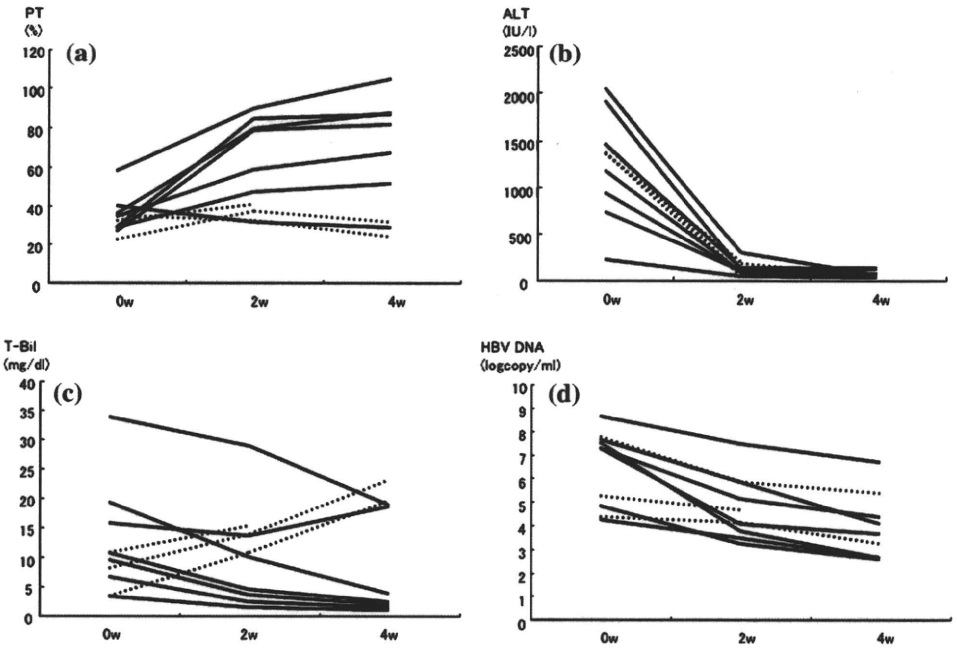

Fig. 1 Prothrombin time (*PT*) activities (**a**), alanine transaminase (*ALT*) levels (**b**), total bilirubin (*T-Bil*) levels (**c**), and hepatitis B virus (*HBV*) DNA levels (**d**) before and after treatment in 7 recovered patients and 3 nonsurvivors. *Solid and dashed lines* denote values for recovered and dead patients, respectively. *w*, Weeks

declined significantly between weeks 0 and 2, and between weeks 0 and 4 in the recovered patients ($p = 0.04$ and $p = 0.003$, respectively). In contrast, the decline of the HBV load was not significant in the nonsurvivors.

The mean duration between the diagnosis of severe disease and the introduction of CS was 5.7 ± 5.0 days in the recovered patients, and 8.0 ± 4.6 days in the nonsurvivors. The difference between them was not significant. The mean duration of CS therapy was 77.6 ± 63.2 days in the recovered patients.

Histological examination

Liver histology in 5 patients showed massive necrosis in one nonsurvivor and chronic hepatitis in 4 recovered patients (F2, severe in 1 and F3, severe in 3) (Table 2).

Long-term outcomes of recovered patients

In the 7 recovered patients, LMV was introduced to 4 and ETV to 3, and adefovir was not introduced at all. CS doses were tapered to cessation in all patients.

Discussion

The prognosis of severe acute exacerbation of chronic hepatitis B is very poor if signs of liver failure appear. This is recognized everywhere around the world [1, 18, 19]. The survival rate of patients with fulminant liver failure in HBV carriers was less than 20% without liver transplantation in a Japanese nationwide survey between 1998 and 2003 [18]. No effective therapy other than liver transplantation is

established for severe acute exacerbation of chronic hepatitis B. Therefore, establishing other effective therapies is urgently required for such patients. This has been an important clinical problem in Japan, where a serious shortage of donor livers still remains.

As mentioned above, in our previous study, we reported that the introduction of high-dose CS could reverse deterioration in patients with "clinically severe, life-threatening" exacerbation of chronic hepatitis B, when used in the early stage of the illness [14].

Recently, NAs, which are strongly active against HBV by interfering with HBV reverse transcriptase activity, have been administered in patients with chronic hepatitis B, and dramatic biochemical and histological improvements were achieved. It was proven that NAs could be administered safely even in severe disease [9–13], but the mortality is still high in patients with liver failure. Tsubota et al. [20] reported that LMV monotherapy offered no significant advantage over conventional supportive treatment for rapid progression to hepatic failure, nor did the therapy offer improvement and prolongation of short-term survival in patients with spontaneous severe acute exacerbation of chronic hepatitis B, but they noted that the combination of any effective therapeutic strategies with LMV should be aggressively instituted. Chien et al. [21] reported that LMV failed to prevent death in patients with severe acute exacerbation if it was administered after the serum bilirubin level rose above 20 mg/dl.

HBV DNA is reduced rapidly with the administration of NAs, but improvement in liver function and liver regeneration, is delayed by a few weeks to a few months [10, 14, 15]. During this time-lag phase, excessive immunological reaction may continue, liver cell injury may progress, and liver regeneration may be impaired. If effective therapeutic approaches were to be available in this phase, they would certainly be beneficial for these patients. CS therapy would be a candidate, as it inhibits excessive immune response and prevents cytolysis of infected hepatocytes. Therefore, we defined the criteria of severe disease in 1997, as described above, and after 1997 we treated patients with severe disease with the early initiation of sufficient doses of CS prospectively, and we used a combination of early and sufficient doses of CS and NA after 1999. In our previous studies, we described the significant effect of the combination therapy of CS and NA compared with historical controls [14, 15].

However, CS has not been used for the treatment of chronic hepatitis B because it might enhance the replication of HBV through a steroid-responsive element in the HBV genome [22]. Gregory et al. [23] reported that CS would likely have proved beneficial if treatment had been started "much earlier" in the course of the illness, while CS was equally ineffective in the treatment of severe viral hepatitis by a double-blind, randomized trial. On the other hand, Hansson et al. [24] reported that, in the treatment of fulminant hepatitis B patients with foscarnet, clinical recovery was related to the influence on the immune system rather than an influence on the reduction of HBV DNA.

In our previous study, none of the patients treated with high doses of CS monotherapy showed increased HBV replication during short-term observation periods [14]. In another study, HBV DNA decreased significantly during the first 4-week period with a combination therapy of CS and NA [15]. In these studies, we used a historical control instead of a randomized controlled group, because ethical issues prevent a randomized control study in such life-threatened patients. Therefore, we believe that CS is not contraindicated for the treatment of a specific population of chronic hepatitis B patients.

In the present study, the differences in background parameters on admission, including age, PT activity, T-Bil level, HBV DNA level, AFP level, and HGF level were not significant between the recovered patients and the nonsurvivors. The difference in mean duration between the diagnosis of severe disease and the introduction of CS was also not significant between the two groups. These findings contradicted our expectations that liver function deterioration would be more advanced, liver regeneration would be more impaired, and the timing of introduction of CS would be later in the nonsurvivors than in the recovered patients. The only difference found between the groups on admission was in the presence of fulminant hepatitis.

Regarding the relationship between responses to the therapy and clinical outcomes, improvement of PT activity and decline of HBV DNA between weeks 0 and 2, and between weeks 0 and 4 were found in the recovered patients, and significant improvements of ALT levels between weeks 0 and 2 and between weeks 0 and 4 were found in both groups. The improvement of T-Bil level was not significant during this period in the recovered patients, while the increases of T-Bil level between weeks 0 and 4, and between weeks 2 and 4 were significant in the nonsurvivors.

Taken together, the findings of the present study indicate that patients with fulminant hepatitis on admission, no improvement of PT activity during the first 2 weeks of combined CS and NA treatment, and worsening of T-Bil level during 4 weeks of treatment, especially worsening between week 2 and week 4, could not possibly be salvaged by the combination therapy of CS and NA and would urgently need liver transplantation. This timing of the assessment would be sufficiently early to avoid infectious complications.

We were able to shorten the CS treatment period while monitoring the viral load after we were able to use an NA in combination with the CS from 1999, and recently, the period has become shortened, to 20–30 days.

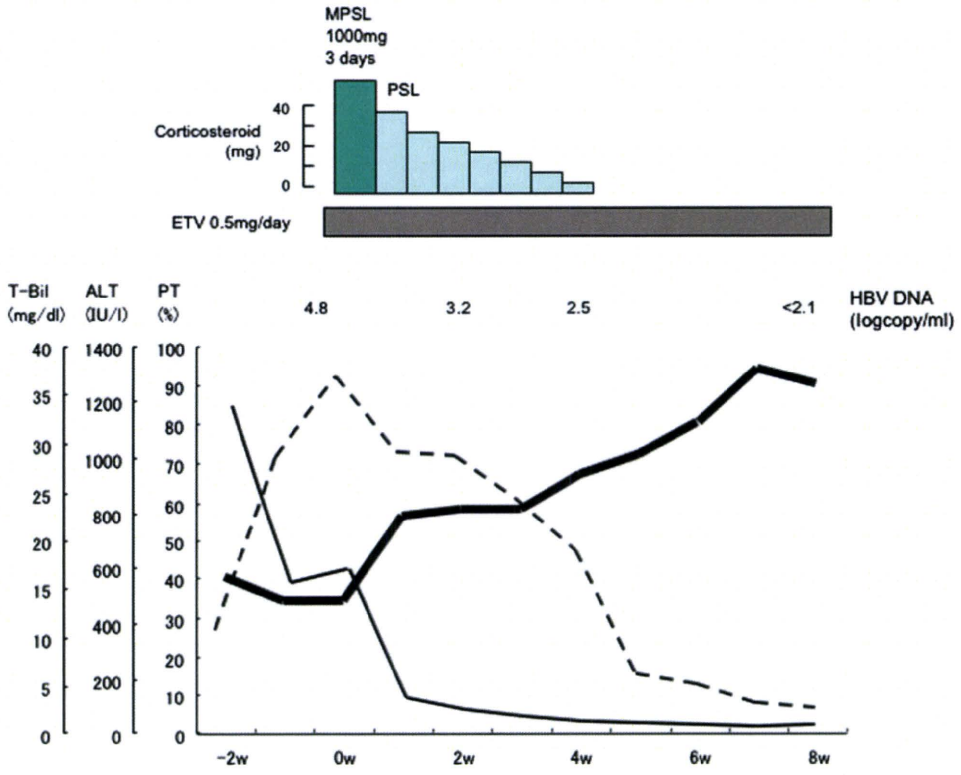

Fig. 2 Clinical course of a 50-year-old male patient. He was an asymptomatic carrier of HBV with hepatitis B envelope antibody (HBeAb). He was treated with surgery and imatinib for gastrointestinal stromal tumor. After 6 months of imatinib treatment, he had severe exacerbation of chronic hepatitis B and was transferred to our unit. Entecavir (*ETV*) and corticosteroid pulse (methylprednisolone; *MPSL*) was administered the day after admission, and he responded to the therapy. *Thick solid, thin solid, and dashed lines* denote PT, ALT, and T-Bil, respectively

In our previous studies, none of the patients with delayed CS of more than 10 days after the diagnosis of severe disease recovered, with/without antiviral drugs including NA being implemented. This might have been because large numbers of hepatocytes were likely already destroyed and inhibition of the inflammatory reaction might not have been effective when the start of the treatment was delayed beyond 10 days. In the present study, one patient (patient 10 in Table 1) with a high T-Bil level of more than 30 mg/dl and prolonged PT activity recovered with ETV and delayed CS introduced 16 days after diagnosis (Fig. 2). It was not clear why this patient recovered regardless of such advanced disease, and a greater number of such patients should be studied.

Two patients (patients 4 and 6) died even with early CS and NA. The timing of diagnosing severe disease was delayed in these patients, although CS and NA were started within 10 days after this diagnosis. This emphasizes the necessity for even earlier diagnosis of severe disease.

Recently, Matsumoto et al. [25] reported 2 patients with severe exacerbation of chronic hepatitis B with coagulopathy who were treated with a combination of ETV and early-phase CS, based on our previous report. Although one patient met our criteria and the other did not, without jaundice, and the durations from clinical onset to the administration of the combination therapy were not described, both patients recovered successfully. This case report supports our previous studies [14, 15]. Matsumoto et al. [25] stopped CS after HBV DNA became undetectable, and as a result, the periods of immunosuppression were sufficient, at 10 and 12 weeks, respectively. We suppose that the periods could be shortened as described above in order to avoid infectious complications. HBV DNA levels decreased in both patients during the clinical course in spite of using CS, which was in accordance with our present and previous studies.

In summary, our study indicates that more than a few weeks of CS treatment in combination with an NA is required in the early stage of severe acute exacerbation of chronic hepatitis B, whereas a short period of conventional pulse therapy would be insufficient for this condition. However, the number of patients in our study was small and further studies are necessary.

Acknowledgments We are indebted to all our colleagues at the liver units of our hospitals who cared for the patients described herein.

Conflict of interest statement No conflicts of interest exist.

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ORIGINAL ARTICLE

Initial Virological Response and Viral Mutation with Adefovir Dipivoxil Added to Ongoing Lamivudine Therapy in Lamivudine-Resistant Chronic Hepatitis B

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Abstract
Background Although adefovir dipivoxil (ADV) has been used for antiviral treatment of lamivudine (LAM)-resistant chronic hepatitis B (CHB) patients, the long-term efficacy of this treatment is not well understood. Initial virological response (IVR) has been reported to be an important factor in relation to the development of ADV-resistance.
Aims We therefore examined the factors associated with IVR and ADV mutation in these patients.
Methods Forty-nine LAM-resistant CHB patients with ADV add-on LAM therapy, 47% of whom were hepatitis B e-antigen (HBeAg)-positive with median treatment duration of 23 months, were enrolled in this study. Patients were classified into IVR and non-IVR groups on the basis of viral suppression status. Mutational analysis of the HBV polymerase/reverse transcriptase (rt) domain was performed by PCR-direct sequencing.
Results Serum HBV DNA was undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84% of patients at 24, 48, and 96 weeks, respectively, after ADV add-on LAM therapy. IVR was achieved in 82% of patients, and ALT normalized at week 24 in 90% of IVR and 78% of non-IVR patients. The lower pretreatment HBV DNA level and virus-containing mutations other than double mutation of rtL180M + rtM204V were significantly associated with IVR ($P = 0.002$ and $P = 0.014$, respectively). ADV-resistant mutations in the RT motif, reported previously, were not detected.
Conclusion IVR is useful for predicting the antiviral efficacy of ADV and LAM combination therapy in LAM-resistant CHB.

Keywords Chronic hepatitis B · Adefovir dipivoxil · Lamivudine · Initial virological response · Mutation

Abbreviations

ADV	Adefovir dipivoxil
ALT	Alanine aminotransferase
CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IVR	Initial virological response
LAM	Lamivudine
rt	Reverse transcriptase

Introduction

Because of the frequent development of life-threatening sequelae, for example liver cirrhosis and hepatocellular carcinoma (HCC), chronic hepatitis B (CHB) infection is a major public health problem worldwide, affecting over 350 million people [1], especially in Asia and Africa [2–4]. The levels of circulating hepatitis B virus (HBV) DNA reflect the status of HBV replication in the liver and are thought to be related to future incidence of cirrhosis, HCC [2, 5–8], and HCC-related mortality [9]. Therefore, complete and sustained suppression of viral replication is the most important objective of treatment of chronic HBV infection. Long-term administration of nucleos(t)ide analogues may

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prevent these complications. Lamivudine (LAM) has been used as first-choice therapy for CHB patients, regardless of HBeAg status, because of its potency, safety profile, and relatively low cost [10]. However, the efficacy of long-term therapy with LAM is compromised by viral resistance; the annualized incidence rate of LAM-resistant mutations was 22% [11] and reached 71% in year 4 [12].

Adefovir dipivoxil, an oral pro-drug of adefovir (ADV), is a synthetic adenine nucleotide analogue that has been shown to be effective in suppression of HBV DNA, HBeAg seroconversion, alanine aminotransferase (ALT) normalization, and histological improvement, regardless of HBeAg status [13–15]. The drug has been shown to have antiviral activity against not only wild-type HBV [13, 14] but also LAM-resistant HBV mutants both in vitro and in vivo [16, 17]. In contrast with LAM therapy, the benefit of ADV therapy is the delayed and infrequent selection of drug-resistant viruses [14, 18–20]. The cumulative incidence of an ADV-resistant mutation emerging in nucleos(t)ide treatment-naïve CHB patients at 48, 96, 144, 192, and 240 weeks was 0, 0.8–3, 11, 18%, and up to 29%, respectively [13, 21–25].

The antiviral activity of ADV has been reported to be lower in LAM-resistant CHB patients than in treatment-naïve patients [26–28]. However, the factors associated with antiviral efficacy of ADV are still not well understood.

We have previously studied the association between lamivudine sensitivity and amino acid substitutions in the reverse transcriptase (RT) region of HBV polymerase and found that sequence analysis of the RT domain is useful for predicting sensitivity to LAM therapy [29].

In this study we assessed the long-term efficacy of ADV add-on therapy for CHB patients with LAM-resistance, analyzed the relationship between amino acid substitution in the RT domain and sensitivity to ADV add-on LAM therapy for LAM-resistant CHB patients, and determined the risk factors associated with the initial virological response (IVR).

Materials and Methods

Patients

CHB patients ($n = 49$) who received 10 mg daily of ADV as add-on therapy to ongoing LAM (100 mg daily) after the emergence of LAM resistance were enrolled at Chiba University Hospital between 2004 and 2009. All patients were negative for hepatitis C, hepatitis D, and human immunodeficiency virus antibodies. Sera obtained from patients at the commencement of ADV add-on LAM therapy were stored at −20°C until analysis. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological Examination

HBsAg, HBeAg, and anti-HBe antibody were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL, USA). HBV genotype was determined from patients' sera by ELISA (HBV Genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan) based on the method described by Usuda et al. [30]. Serum HBV DNA levels were monitored every four weeks using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 log copies/mL.

Viral Genome Sequencing

Pretreatment sera were obtained from 31 patients and nucleotide sequences could be analyzed in 22 patients. Sequence analysis for detection of HBV-DNA mutations in serum samples in the non-IVR group was performed after 24, 48, and 96 weeks of treatment. To amplify the region encompassing the polymerase reverse transcriptase (RT) domain, DNA extracted from 200 μL serum was used as a template and long-range PCR and nested PCR were performed in a 50-μL reaction using LA Taq polymerase (TaKaRa Bio, Kyoto, Japan) under the following conditions: 5-min activation at 94°C, 35 cycles or 30 cycles with denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 68°C for 90 s and 1 min in the first and second round, respectively. The last cycle was followed by a final extension at 72°C for 7 min. An 862 base-pair fragment (nt 242-1103) containing the polymerase RT domain was amplified. The primers for the first round of PCR were 5′-CCT CAG GCT CAG GGC ATA-3′ (sense, nt 3082-3099) and 5′-GAC GGG ACG TAG ACA AAG G-3′ (antisense, nt 1436-1418). The primers for the second round of PCR were 5′-CAG AGT CTA GAC TCG TGG-3′ (sense, nt 242-258) and 5′-GGC GAG AAA GTG AAA GCC-3′ (antisense, nt 1103-1086). The PCR product was sequenced using the primers: 5′-TGG CTC AGT TTA CTA GTG CC -3′ (nt 668-687), 5′-GGC ACT AGT AAA CTG AGC CA-3′ (nt 687-668), and the primers for the second round of PCR. The amino acid sequence of each protein was deduced from the nucleotide sequence. The HBV genotype was also confirmed on the basis of the viral sequence data obtained.

Definition of Initial Virological Response and Undetectable HBV DNA

An initial virological response (IVR) was defined as HBV DNA < 4 $\log_{10}$ copies/mL after treatment for 24 weeks [26]. HBV DNA < 2.6 $\log_{10}$ copies/mL was regarded as "serum HBV DNA undetectable".

Statistical Analysis

Categorical variables between groups were compared by use of Fisher's exact test. The Mann–Whitney U test was used for assessing the association between baseline factors and the occurrence of IVR. Results were considered statistically significant at $P < 0.05$.

Results

Clinical and Biochemical Data of the Patients

A total of 49 patients were included in this analysis. Thirty-six (71%) were men, the median age when ADV was added to LAM treatment was 55 years (range: 35–71 years), and 24 patients (47%) were HBeAg-positive. Pretreatment ALT levels ranged from 14 to 1495 IU/L (median: 129 IU/L), and the median pretreatment HBV DNA level was 6.9 $\log_{10}$ copies/mL (range: 2.8–8.8 $\log_{10}$ copies/mL). The median duration of treatment with LAM was 25.5 months (range: 3–78 months). The median duration of combination treatment with ADV and LAM was 29 months (range: 8–63 months) (Table 1).The median duration of treatment with LAM was 26 months (range: 3–78 months) and 23 months (range: 12–50 months) in the IVR and non-IVR groups, respectively (P = N.S.).

Frequency of Undetectable HBV DNA Levels

In all patients, sequential monitoring revealed that 24, 48, and 96 weeks after addition of ADV to ongoing LAM therapy serum HBV DNA levels were undetectable (<2.6 $\log_{10}$ copies/mL) in 67, 82, and 84%, respectively,

Table 1 Clinical and biochemical data of patients infected with hepatitis B virus

Number of patients	49
Median age, years (range)	55 (35–71)
Male sex, number (%) of patients	36 (71%)
HBeAg positive, number (%) of patients	24 (47%)
Median pretreatment ALT level, IU/L (range)	129 (14–1495)
Median pretreatment HBV DNA level, $\log_{10}$ copies/mL (range)	6.9 (2.8–8.8)
Median duration of LAM therapy, months (range)	26 (3–78)
Median duration of ADV therapy, months (range)	29 (8–63)

ALT, alanine aminotransferase; HBV, hepatitis B virus; ADV, adefovir dipivoxil

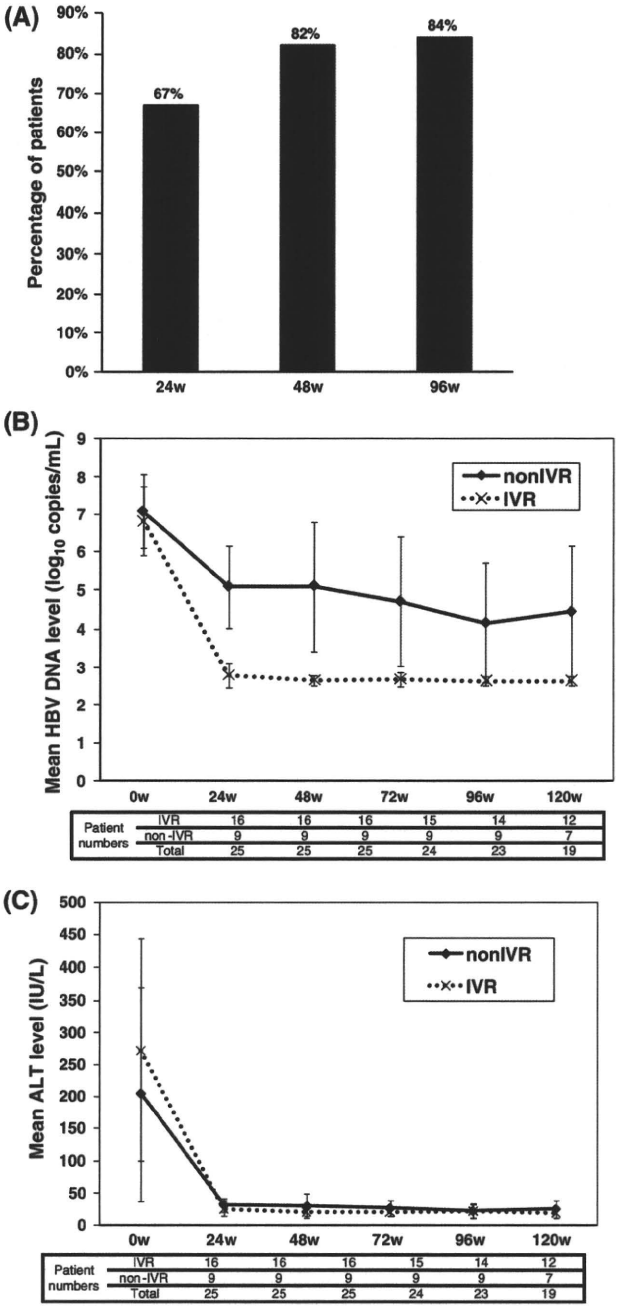

Fig. 1 **a** Percentages of patients with reduction in HBV DNA by <2.6 $\log_{10}$ copies/mL 24, 48, and 96 weeks after ADV add-on LAM. **b**, **c** Sequential HBV DNA and ALT levels according to initial virological response (IVR). Patients who achieved IVR are represented by the *dashed line*; those who did not are represented by the *solid line*. Data represent mean ± SD. An HBV DNA level below 2.6 $\log_{10}$ copies/mL was regarded as being approximately equal to 2.6 $\log_{10}$ copies/mL. The numbers of IVR and non-IVR patients, and total patient numbers, at each time point are also shown in the table

(Fig. 1a). Among the 24 HBeAg-positive patients, HBeAg seroconversion was observed to be 5 and 16% after 48 and 96 weeks, respectively.

Comparison of Characteristics Between the IVR and Non-IVR Groups

According to the IVR definition, patients were classified into two groups, an IVR group and a non-IVR group, for further analyses (Table 2). IVR was achieved in 82% of the 49 patients. As expected, patients who achieved IVR had a more marked drop in HBV DNA levels during the first 24 weeks and this reduction lasted throughout the follow-up period (Fig. 1b). The rates of ALT normalization at week 24 were 90 and 78% in the IVR and non-IVR groups, respectively (Fig. 1c).

Comparison of patient characteristics in the IVR and non-IVR groups showed that the HBV DNA level at baseline was lower in the IVR group than in the non-IVR group ($P = 0.002$). The non-IVR group had a high percentage of HBeAg-positive patients at baseline compared with the IVR group (78% vs. 42%, $P = 0.054$). More women than men achieved IVR (Table 2, $P = 0.036$). There were no significant differences between the baseline characteristics age, body mass index, or baseline serum ALT levels of the two groups. Genotype was determined in 27 patients. There were 25 with genotype C and 2 with genotype A; genotype C was detected in 16/18 of the IVR group and in 9/9 of the non-IVR group (P = N.S.).

Amino Acid Sequences of RT Motif Domains Between the IVR and Non-IVR Groups

The polymerase RT domains were sequenced to investigate the relationship with sensitivity to ADV add-on LAM therapy. We compared the deduced amino acid sequences of A, B, C, D, and E domains of the RT motif between the two groups (13 and 9 patients in the IVR and non-IVR groups, respectively), but found neither ADV-resistant mutations nor any significant differences, except for the substitutions at rtL180 and rtM204. Double mutation (rtL180M + M204V/I) or single mutation (rtM204I, rtM204V) was observed among the patients studied (Fig. 2). However, the HBV-containing double mutant (rtL180M + M204V) was observed more frequently in the non-IVR group than in the IVR group (78% vs. 28%, $P = 0.014$).

Characteristics of Patients with Lasting High HBV DNA Levels

Most of the patients achieved undetectable levels of HBV DNA after 48 weeks of ADV add-on LAM therapy, but six patients in the non-IVR group were found to have sustained high HBV DNA levels (>5 $\log_{10}$ copies/mL) beyond 48 weeks of ADV add-on LAM treatment. Two of these patients (n-IVR 5 and n-IVR 9) developed virological breakthrough (VBT, elevation of >1 $\log_{10}$ copies/mL from nadir). VBT occurred at weeks 68 and 48 in patients n-IVR 5 and n-IVR 9, respectively. To investigate the additional amino acid substitution in the RT domain during treatment of these patients we analyzed the amino acid sequences at several time points during combination therapy. Four cases were found to be infected with HBV carrying the rtL180M + M204V double mutation at the commencement of ADV add-on LAM therapy (Table 3). Sequential analysis of RT mutations of the four patients is shown in Fig. 3. Because no additional amino acid substitutions, including ADV-resistant mutations, were detected in any samples tested, an alternate mechanism is likely to be responsible for the insufficient response of these patients to therapy.

Discussions

In LAM therapy for patients with chronic HBV infection, emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. For this reason, LAM has been replaced

Table 2 Comparison of patient characteristics between IVR and non-IVR groups at enrollment

	IVR group ($n = 40$)	Non-IVR group ($n = 9$)	P value
Median age, years (range)	54 (35–71)	50 (38–67)	0.588[b]
Male/female	26/14	9/0	0.036[a]
Median body mass index, kg/m^2 (range)	22.0 (17.5–27.9)	22.8 (19.4–25.2)	0.795[b]
HBeAg positive rates	42%	78%	0.054[a]
Median ALT level, IU/L (range)	117 (14–1495)	199 (35–710)	0.439[b]
Median HBV DNA level, $\log_{10}$ copies/mL (range)	6.8 (2.8–8.7)	8.0 (7.1–8.8)	0.002[b]
rtL180M + M204V	28%	78%	0.014[a]

ALT, alanine aminotransferase

[a] Fisher's exact test

[b] Mann–Whitney U test

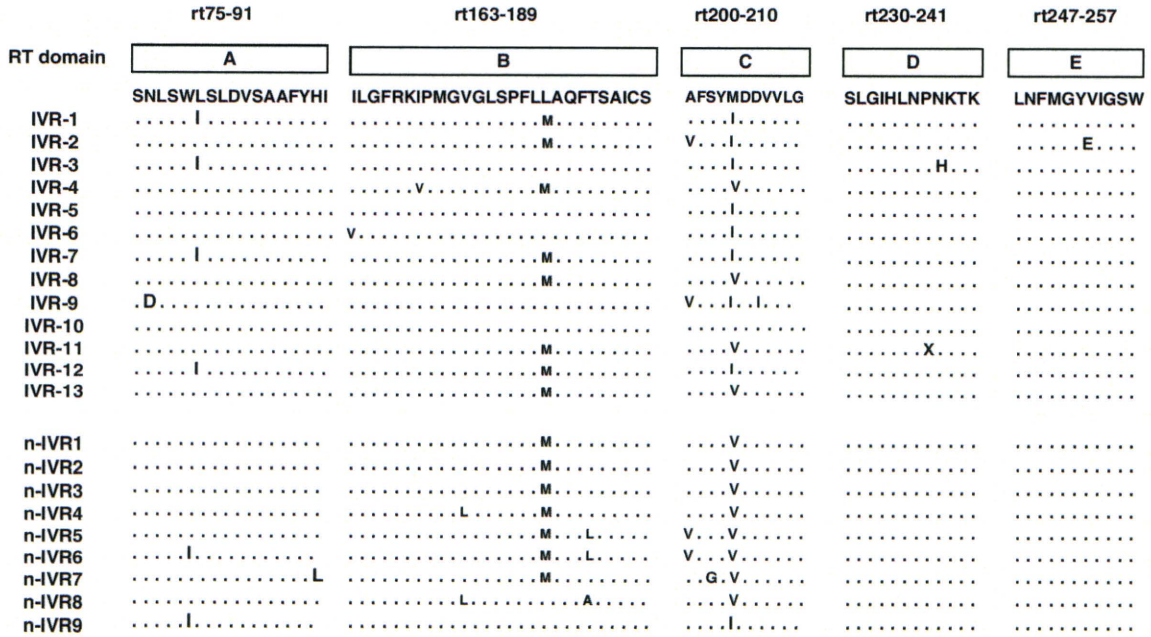

Fig. 2 Amino acid sequences of A, B, C, D, E domains of the RT motif are shown for the initial virological response (IVR) group and the non-IVR group. Double mutation of rtL180M + rtM204V is predominant in the non-IVR group compared with the IVR group (78% vs. 28%, $P = 0.014$)

Table 3 Pretreatment characteristics of patients with sustained elevation of HBV DNA levels after 48 weeks of ADV treatment

	n-IVR 1	n-IVR 3	n-IVR 5	n-IVR 7	n-IVR 8	n-IVR 9
Age (years)	61	41	44	42	51	64
Gender	Male	Male	Male	Male	Male	Male
HBeAg	Positive	Positive	Positive	Negative	Positive	Positive
HBV-DNA level (log_{10} copies/mL)	8	7.5	7.3	7.1	8	8
ALT level (IU/L)	358	389	35	416	85	56
Mutation in the RT region	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rt180M + rtM204V	rtM204V	rtM204I
Virological breakthrough	Negative	Negative	Positive	Negative	Negative	Positive

RT, reverse transcriptase

by newly developed nucleos(t)ide analogues, for example ADV and entecavir (ETV), for treatment of chronic hepatitis B. ETV has been reported to be more effective at reducing HBV DNA, and induces the drug-resistant mutant virus less frequently than LAM in nucleos(t)ide-naïve patients [24, 31].

The IVR, which was recently defined as HBV DNA < 4 log_{10} copies/mL after 24 weeks on treatment [26], was reported to be associated with the antiviral efficacy of ADV and the emergence of an ADV-resistant mutation in LAM-resistant CHB [32–34]. Several previous studies have suggested that lower pretreatment HBV DNA levels, higher pretreatment ALT, HBeAg negativity, and the presence of liver cirrhosis were associated with the virological response. In agreement with a previous report [33], this study showed that patients without IVR exhibited higher baseline HBV DNA levels than patients with IVR (8.0% vs. 6.8%, $P = 0.002$). Other studies have identified HBV virological rebounds during LAM or ADV treatment in the absence of mutation associated with drug resistance [22, 35]. The possibility of patient dosing adherence may be one of the factors leading to non-IVR.

The analyses of the amino acid sequence of the RT motif at the commencement of ADV add-on therapy revealed that it was difficult to achieve optimum viral suppression in patients who were infected with the virus carrying the rtL180M + M204V double mutation compared with other mutational patterns, for example the rtL180M + rtM204I double mutation, or rtM204V and rtM204I single mutations. Because the number of samples

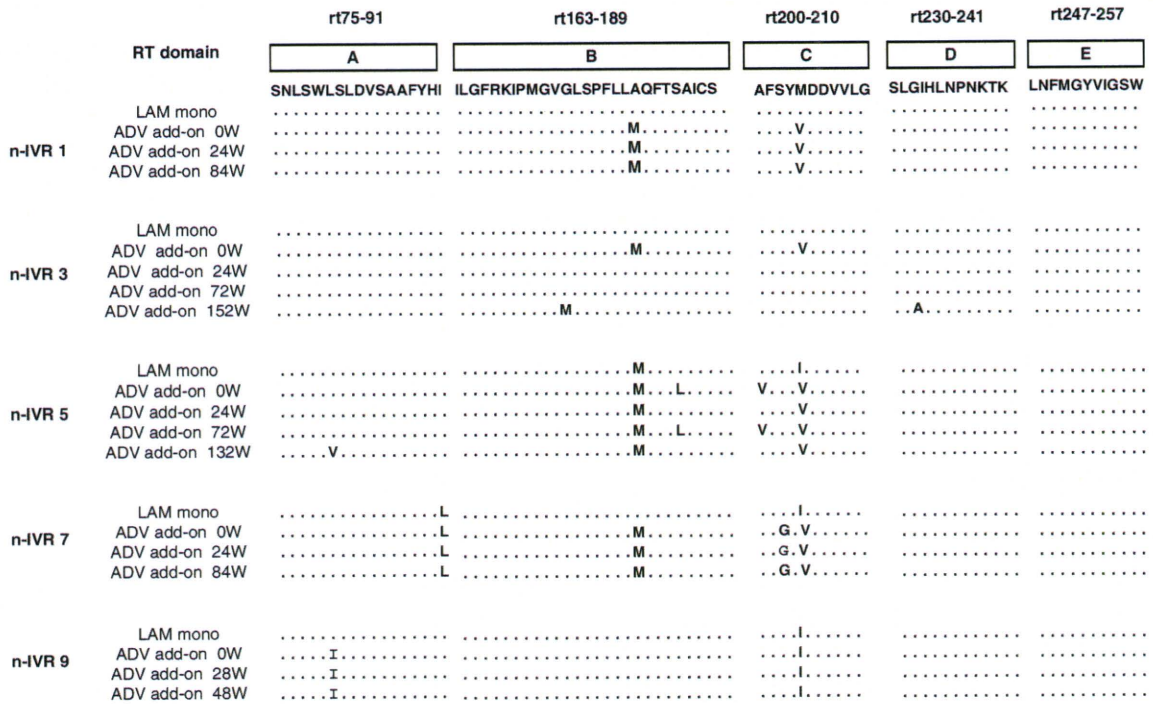

Fig. 3 Amino acid sequences of five representative patients in the non-IVR group are shown. Emergence of the rtL180M + M204V double mutation was observed in four of five patients from the commencement of ADV add-on LAM combination therapy

detected containing these mutations was small, and the alleged association was negative, further study will be needed to confirm this result.

Suzuki et al. [36] reported that the rtM204I mutant was associated with an earlier virological response as compared with the rtM204V mutant, and virological suppression of the mutation rtL180M was linked to that of rtM204I or rtM204V [36]. Furthermore, Suzuki et al. [36] showed that when viral loads of both mutants (rtM204V and rtM204I) were similar at the commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. In our study, six patients in the non-IVR group had sustained elevation of HBV DNA levels (>5 $\log_{10}$ copies/mL), yet endured ADV add-on LAM co-administration for more than 48 weeks, and four of the six patients had mutant virus carrying the rtL180M + M204V double mutation (Table 3).

Cha et al. [37] assessed the patterns of LAM-resistant mutations and the effect of such mutations on virological response to ADV monotherapy in LAM-resistant CHB. They established the mutational patterns, for example rtM204V ± rtL180M ± rtV173L, rtM204I ± rtL180M, rtM204I ± rtL80I, compared the IVR status with these mutations, and found that the antiviral effect of ADV did not differ significantly among these patterns. Lada et al. [38] studied the susceptibility of LAM-resistant HBV to ADV in vitro. They reported that in samples with triple LAM resistance-associated amino acid changes rtV173L + L180M + M204V, HBV DNA reduction at week 48 was lower than for samples which had only the rtL180M + M204V mutations. In our study, rtV173L was observed only in the non-IVR group but the incidence did not differ significantly between groups. Our results are partially discordant with these previous studies, and differences between the studies, for example the additional mutations and use of ADV monotherapy, may be a possible explanation for the different outcomes.

In a randomized controlled study of ADV therapy in 42 patients who had genotypic LAM resistance with virological and clinical breakthrough, Rapti et al. [39] found that ADV resistance was not detected in the 28 patients undergoing ADV add-on LAM combination therapy but was detected in three patients (21%) upon viral/biochemical breakthrough after switching to ADV monotherapy. In our study, most of the patients treated with ADV add-on LAM therapy exhibited sustained viral suppression, except for two patients who had emergent virological breakthroughs. The sequencing analyses, however, demonstrated no ADV-resistant mutations (rtN236T, rtA181V/T, and rtI233V), suggesting the other mechanisms, for example viral mutation in the remaining part of the sequences or host factors, may be responsible for the reduced efficacy of the combination therapy in these two patients.

In conclusion, ADV add-on LAM therapy for LAM-resistant CHB patients was effective in suppressing viral replication and normalizing ALT levels. However, in cases

with high pre-treatment HBV DNA levels and the rtL180M + rtM204V double mutation, the antiviral effect of ADV is likely to be weak. Careful monitoring for the emergence of ADV-resistant mutation during prolonged treatment is critical.

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