and was more advanced in group D than in group C (P = 0.008, $P < 0.001$, respectively).

Treatment regimen

All patients were treated with a weight-based, 1.5 μg/kg weekly dose of subcutaneous PEG-IFN α-2b (PegIntron, Schering-Plough, Osaka, Japan), in combination with RBV (Rebetol, Schering-Plough), which was given orally at a daily dose of 600-1000 mg based on body weight (600 mg for patients weighing less than 60 kg, 800 mg for those weighing 60-80 kg, and 1000 mg for those weighing 80 kg or over). The length of treatment was 48 wk for patients with HCV genotype 1 and 24 wk for patients with genotype 2. The above duration and dosage are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of RBV was required. Patients aged 65 years or older had a significantly higher frequency of RBV dose reduction during the treatment period than those aged less than 65 years old (HCV genotype 1: group A *vs* group B, 41.2% *vs* 49.0%, $P = 0.032$, genotype 2: group C *vs* group D, 28.6% *vs* 54.1%, $P < 0.001$). Some patients also had PEG-IFN α-2b-induced psychological adverse effects or a decrease in white blood cell and platelet counts. In such cases, a reduction in the dosage of PEG-IFN α-2b was required. Both PEG-IFN α-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, and 25×10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic disorders developed, continuation of treatment was judged not to be possible by the attending physician, or if the patient desired discontinuation of treatment.

Determination of baseline HCV RNA level and HCV genotype

The pretreatment, baseline, serum HCV RNA level was measured by a quantitative HCV RNA polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test v 2.0 using the 10-fold dilution method; Roche Diagnostics, Tokyo, Japan), which has a lower limit of quantitation of 5000 IU (13 500 copies)/mL (5 kIU/mL) and an outer limit of quantitation of 5 100 000 IU/mL (5100 kIU/mL). The HCV genotype was determined by type-specific primers of the core region of the HCV genome. The protocol for genotyping was carried out as previously described[9].

Efficacy of treatment

End of treatment (EOT) response and SVR were defined as serum HCV RNA undetectable at the end of treatment and at 24-wk follow-up after the end of treatment, respectively. EOT response and SVR were defined as non-detectable HCV-RNA as measured by qualitative COBAS Amplicor HCV Monitor Test v 2.0, with the results labeled as positive or negative. The lower limit of detection was 50 IU/mL (0.5 kIU/mL). The analysis of EOT and SVR was performed on an intention-to-treat basis.

Statistical analysis

Continuous data are expressed as mean ± SD. The statistics were carried out using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The χ^2 test, Fisher's exact test and Kruskal-Wallis test were used to determine the differences in baseline clinical characteristics, safety, efficacy of the combination therapy, adherence to the total dose, and the association between the adherence and SVR. Logistic regression analysis was used to identify the association between age and SVR. A $P < 0.05$ was considered significant.

RESULTS

EOT response rate by intention-to-treat analysis

Among patients with genotype 1, the EOT response rate was significantly higher in group A (497 of 685, 72.5%) than in group B (129 of 253, 45.0%) ($P < 0.001$). Among patients with genotype 2, there was no significant difference between groups C (239 of 252, 94.8%) and D (55 of 61, 90.1%).

SVR rate by intention-to-treat analysis

Of 1251 patients, 631 (50.4%) achieved SVR in the intention-to-treat analysis. The SVR rate was significantly higher for genotype 2 (249 of 313, 79.6%) than for genotype 1 patients (382 of 938, 40.7%) ($P < 0.001$). Among patients with genotype 1, the SVR rate was significantly higher in group A (324 of 685, 47.3%) than in group B (58 of 253, 22.9%) ($P < 0.001$). Among patients with genotype 2, SVR was also significantly higher in group C (209 of 252, 82.9%) than in group D (40 of 61, 65.6%) ($P = 0.004$). The rate of SVR was significantly higher for females (113 of 128, 88.3%) than for males (96 of 124, 77.4%) in group C only (Figure 1). Furthermore, we analyzed whether or not the SVR rate differed according to the age at which the combination treatment of PEG-IFN α-2b plus RBV was started. The results showed that the SVR rate decreased significantly with age for both genotype 1 and 2. SVR was achieved by 5.6%-26.3% of genotype 1 patients aged 70 years or older, and by 57.1%-100% of genotype 2 patients aged 70 years or older (Figure 2).

We previously reported a minimum acceptable dose of at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target dosage of RBV for the successful treatment of Japanese patients with genotype 1[8]. Therefore, we analyzed the SVR rates in patients with genotype 1 by the dosage they actually received during treatment (a total dose of at least 80% or more of PEG-IFN α-2b and 60% or more of RBV) (Table 3). The number who received at least this minimum acceptable dosage during treatment were 278 (40.6%) of 685 patients in group A and 62 (24.5%) of 253 in group B, significantly lower in group B than in group A ($P < 0.001$). Compared with patients who received less than the minimum acceptable dosage, in patients who received at least this minimum dosage, the SVR rates increased from 34.2% to 66.5% in group A patients and from 15.7% to 45.2%

Table 3 The comparison of the rate of sustained virological response of patients with genotype 1 receiving a dose of 80% or more of pegylated interferon α-2b plus 60% or more of ribavirin and the reduced dosage group: *n* (%)

	Male		Female		Total	
	n	SVR	*n*	SVR	*n*	SVR
Group A						
Minimum acceptable	168	116 (69.0)	110	69 (62.7)	278	185 (66.5)
Reduced	206	73 (35.4)	201	66 (32.8)	407	139 (34.2)
Total	374	189 (50.5)	311	135 (43.4)	685	324 (47.3)
Group B						
Minimum acceptable	31	15 (48.4)	31	13 (41.9)	62	28 (45.2)
Reduced	91	18 (19.8)	100	12 (12.0)	191	30 (15.7)
Total	122	33 (27.0)	131	25 (19.1)	253	58 (22.9)

Minimum acceptable: patients who received 80% or more of the target dose of pegylated interferon (IFN) α-2b and 60% or more of ribavirin (RBV). Reduced: Patients who received less than 80% of pegylated IFN α-2b and less than 60% of RBV. SVR: Sustained virological response.

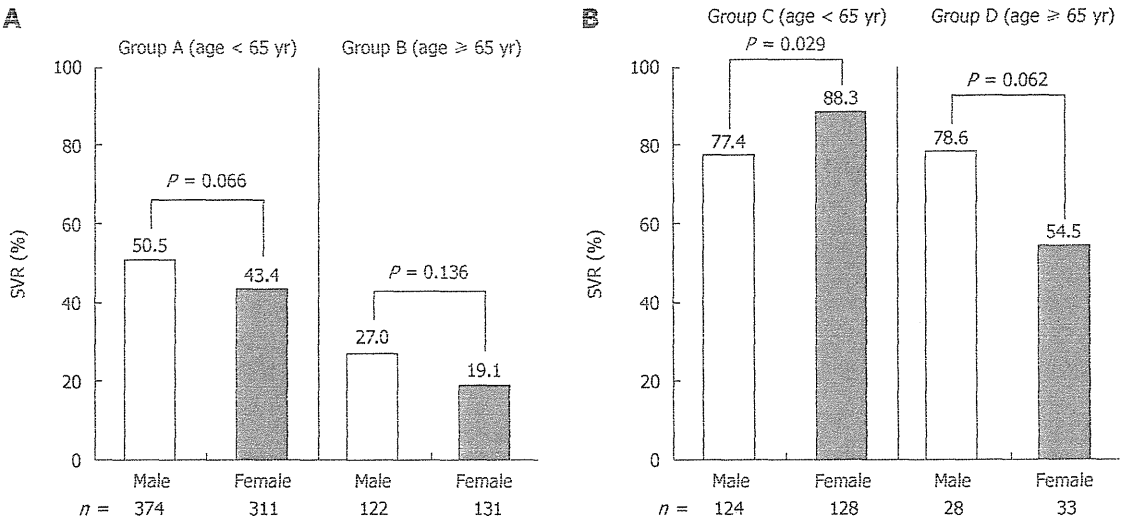

Figure 1 Virological response to the combination treatment by age and sex of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response.

($P < 0.001$) in group B patients. No significant difference between groups C and D was observed. On comparing patients whose platelet count was under 10×10^{10}/L, the SVR rate for genotype 1 was significantly lower in group B (2 of 36, 5.6%) than in group A (16 of 56, 28.6%) ($P < 0.001$). Among the patients with genotype 2, SVR was not significantly different between group C (9 of 16, 56.3%) and group D (2 of 7, 28.6%).

In a comparison of the SVR rate in patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between the genotypes (genotype 1: 118 of 310, 38.1% *vs* 264 of 628, 42.0%, genotype 2: 44 of 72, 61.1% *vs* 141 of 241, 58.5%). Furthermore, we compared the EOT response rate and SVR rate of cirrhosis patients whose liver fibrosis was F4, and found no significant difference between groups A (EOT: 16 of 30, 53.3%, SVR: 7 of 30, 23.3%) and B (EOT: 6 of 17, 35.3%, SVR: 2 of 17, 11.8%). In addition, no significant difference was found between groups C (EOT: 8 of 10, 80.0%, SVR: 6 of 10, 60.0%) and D (EOT: 9 of 12, 75.0%, SVR: 5 of 12, 41.7%).

Discontinuation of PEG-IFN α-2b plus RBV treatment and adverse effects

Of 1251 patients, 314 (25.1%) did not complete PEG-IFN α-2b plus RBV treatment due to adverse effects or other reasons. The discontinuation rate was significantly higher in patients with genotype 1 (273 of 938, 29.1%) than in those with genotype 2 (41 of 313, 13.1%) ($P < 0.001$) (Tables 4 and 5). Furthermore, the rate of discontinuation due to adverse effects was significantly higher in patients with genotype 1 (135 of 938, 14.4%) than in those with genotype 2 (23 of 313, 7.3%) ($P < 0.010$). The rates of discontinuation due to lack of treatment efficacy and for economic reasons (loss of job, inability to pay the medical costs) were also significantly higher in patients with genotype 1 (55 of 938, 5.9%, 15 of 938, 1.6%) than in those with genotype 2 (1 of 313, 0.3%, 0 of 938, 0%) ($P < 0.001$ and $P = 0.025$, respectively).

For genotype 1 patients, the discontinuation rate was significantly higher in group B (106 of 253, 42.9%) than in group A (167 of 685, 24.4%) ($P < 0.001$), and the rate of discontinuation due to adverse effects was also significantly

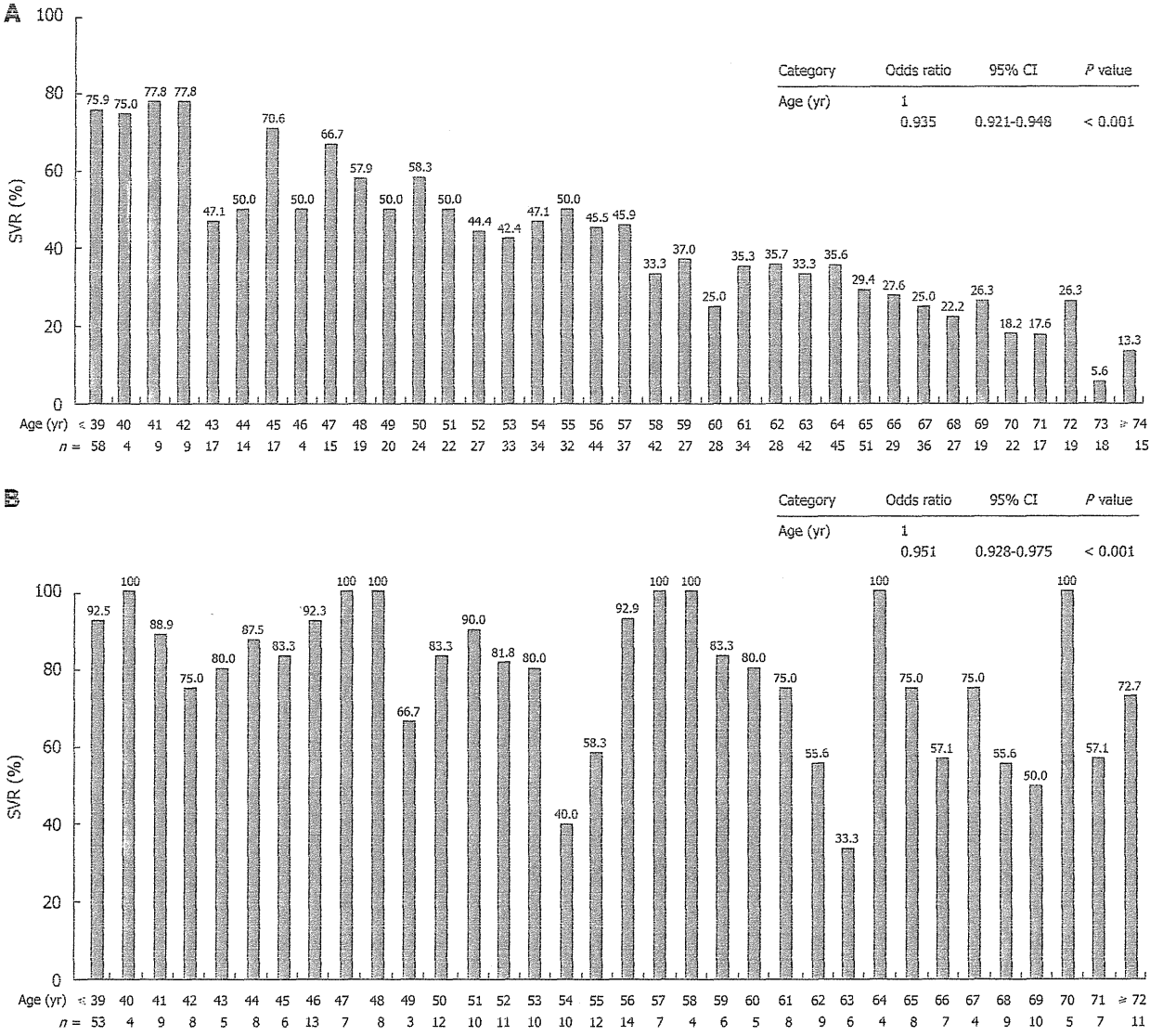

Figure 2 Virological response to the combination treatment by age of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response; CI: Confidence interval.

higher in group B (61 of 253, 24.1%) than in group A (74 of 685, 10.8%) ($P < 0.001$). General fatigue was the most frequent adverse effect, and was significantly more frequent in group B than in group A ($P < 0.001$). However, in these group 1 patients, RBV was reduced due to anemia in 12.5% (3 of 24) of group A and in 30.4% (7 of 23) of group B. Furthermore, rash and thrombocytopenia were significantly more frequent in group B than in group A ($P = 0.014$ and $P = 0.007$, respectively). In group A, depression was significantly more frequent in females than in males ($P = 0.012$). In genotype 2 patients, treatment discontinuation did not differ between group C (33 of 252, 13.1%) and group D (8 of 61, 13.1%), and the rate of discontinuation due to adverse effects did not differ between these groups (17 of 252, 6.7%, 6 of 61, 9.8%, respectively).

The mean time to discontinuation in group A (21.6 ± 11.9 wk) was not significantly different from group B (21.5 ± 12.6 wk), and the mean time in group C (11.0 ± 6.8 wk) was also not significantly different from group D (11.6 ± 6.0 wk). There was no significant difference between male and female patients in each group (male: 21.0 ± 12.4 *vs* female: 22.1 ± 11.8 in group 1, male: 11.3 ± 7.1 *vs* female: 10.9 ± 6.1 in group 2).

HCC was not seen in genotype 2 patients; only in patients with genotype 1 (29.5 ± 9.9 wk) and was more frequent in group B (5 of 253, 2.0%) than in group A (2 of 685, 0.3%) ($P = 0.008$).

DISCUSSION

In a large, national, multicenter Greek study involving 993 treated and 734 untreated patients with chronic hepatitis C, patients with cirrhosis, showed a protective effect of treatment even among those without SVR. For patients without cirrhosis, the beneficial effect of IFN α treatment was particularly evident in older patients; patients with the worst prognosis if left untreated. Therefore, IFN α-based treatment should be offered to older persons, as these are

Table 4 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 1 patients

	Group A (age < 65 yr)		Group B (age ≥ 65 yr)		Total
	Male (n = 374)	Female (n = 311)	Male (n = 122)	Female (n = 131)	
Discontinued number	101	66	52	54	273
Adverse effects	43	31	33	28	135
General fatigue	17	7	12	11	47
Depression	3	11	4	5	23
Appetite loss	1	0	1	0	2
Rash	3	2	3	4	12
Encephalopathy	1	0	0	0	1
Neutropenia	2	0	0	0	2
Anemia	3	2	4	1	10
Thrombocytopenia	1	0	3	1	5
Elevation of ALT	1	0	0	0	1
Hyperthyroidism	3	2	0	1	6
Hypothyroidism	0	1	0	0	1
Retinopathy	1	0	1	0	2
Interstitial pneumonia	2	0	1	1	4
Pulmonary disease (others)[1]	0	1	1	1	3
Psychoneurotic disorder[2]	2	0	2	0	4
Nervous disease[3]	1	1	0	1	3
Autoimmune disease[4]	0	2	0	1	3
Metabolic disease[5]	0	2	0	0	2
Digestive disorder[6]	2	0	1	1	4
Hepatocellular carcinoma	2	0	4	1	7
Malignancy (extra-liver)	0	1	1	0	2
No effect of treatment	22	18	7	8	55
Economic problem	9	3	0	3	15
Others[7]	25	13	7	14	59

[1]Includes pulmonary tuberculosis (n = 1), pneumonia (n = 1), tuberculous pleuritis (n = 1); [2]Includes psychiatric disorder (n = 2), disquiet (n = 1), insomnia (n = 1); [3]Includes nerve paralysis (n = 1), cerebral infarction (n = 1); [4]Includes rheumatoid arthritis (n = 2), myasthenia gravis (n = 1); [5]Includes diabetes mellitus (n = 1), hypertriglycemia (n = 1); [6]Includes cholecystitis (n = 3), pancreatitis (n = 1); [7]Includes 25, 13, 6 and 13 drop-outs from groups A, B, C and D, respectively: One for excessive alcohol consumption in group C and one was nursing in group D. ALT: Alanine aminotransferase.

the patients with the greatest potential benefit and may achieve SVR[16]. In Japan, the prevalence of chronic HCV infection increases with age, however, the optimal management of older patients has not yet been accurately defined. Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. In addition, the natural history of chronic hepatitis C in elderly patients is not accurately known, as the presence of comorbidity can affect illness progression and life expectancy. HCV became more prevalent in Japan decades before the United States[17]. Japanese patients with chronic hepatitis C treated with IFN are currently 10 to 15 years older than corresponding patients in the United States and European countries, where patients treated with antiviral treatment tend to average 45 years of age[18-20]. Therefore, our results can serve as a world-wide model for the treatment of older chronic hepatitis C patients.

It has been well documented that the combination therapy of PEG-IFN α-2b plus RBV is more effective than previous IFN monotherapy in chronic hepatitis C patients[7,8]. There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1, which revealed low rates of SVR (31.1%-51.9%)[21-24]. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. Because the present study was a large multicenter design, it is useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients. The present study confirmed the results of our previous study which showed that the SVR rate was significantly higher for genotype 2 than for genotype 1 patients[8]. Another important result was that the ability to take at least a minimum acceptable dosage during treatment increased the SVR rate by about three times in older patients with genotype 1. This result also confirmed previous studies which indicated the importance of giving at least the minimum acceptable treatment dosage in patients infected with HCV genotype 1, especially older patients[23,24].

Secondly, we compared discontinuation of treatment by genotype and sex. In genotype 1 patients, adverse effects were seen more often in older than in younger patients. This was the most important reason why the rate of treatment discontinuation was higher in older than in younger patients, and affected the outcome of PEG-IFN α-2b plus RBV combination therapy. General fatigue was the most common adverse effect in older patients. Because older patients often have impaired renal function, they have increased blood levels of RBV[25,26]. They are also inclined to be anemic and to have general fatigue. However, only a small number of older patients in the present study had reduced RBV due to anemia. Therefore, general fatigue is probably a direct adverse effect of PEG-IFN α-2b. We previously reported that herbal medicine

Table 5 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 2 patients

	Group C (age < 65 yr)		Group D (age ≥ 65 yr)		Total
	Male (n = 124)	Female (n = 128)	Male (n = 28)	Female (n = 33)	
Discontinued number	18	15	4	4	41
Adverse effects	6	11	3	3	23
General fatigue	1	3	1	0	5
Depression	0	2	0	0	2
Appetite loss	0	0	0	0	0
Rash	2	1	0	2	5
Encephalopathy	0	0	0	1	1
Neutropenia	0	2	0	0	2
Anemia	0	0	2	0	2
Thrombocytopenia	2	0	0	0	2
Elevation of ALT	0	0	0	0	0
Hyperthyroidism	0	1	0	0	1
Hypothyroidism	0	1	0	0	1
Retinopathy	0	0	0	0	0
Interstitial pneumonia	0	0	0	0	0
Pulmonary disease(others)	0	0	0	0	0
Psychoneurotic disorder	0	0	0	0	0
Nervous disease[1]	1	1	0	0	2
Autoimmune disease	0	0	0	0	0
Metabolic disease	0	0	0	0	0
Digestive disorder	0	0	0	0	0
Hepatocellular carcinoma	0	0	0	0	0
Malignancy (extra-liver)	1	0	0	0	1
No effect of treatment	1	0	0	0	1
Economic problem	0	0	0	0	0
Others[2]	10	4	1	1	16

[1]Includes nerve paralysis (n = 1), tetany (n = 1); [2]All patients were drop out. ALT: Alanine aminotransferase.

relieved the adverse effects of IFN, including general fatigue[27]. Herbal medicine may be useful for mitigating general fatigue during PEG-IFN α-2b plus RBV combination treatment, especially in older patients.

The rate of discontinuation was lower in patients with genotype 2 than in patients with genotype 1, and there was no difference between the older and the younger patients with genotype 2. These results are possibly a consequence of the shorter term of treatment in genotype 2 and the many genotype 1 patients who discontinued due to lack of efficacy.

Two of the characteristics of older patients in the present study were that both hemoglobin and platelet count were significantly lower than in younger patients. The SVR rate was significantly lower when the platelet count was less than 10×10^{10}/L. Furthermore, the older genotype 1 patients were often forced to discontinue treatment due to thrombocytopenia and the occurrence of HCC. These findings appear to result from advanced liver fibrosis in older chronic hepatitis C patients. Therefore, the possibility of HCC during long-term IFN treatment in older patients must be considered.

We previously reported that older female patients had a low response to IFN-α monotherapy[9], and other investigators have reported that older female patients have a poor response to PEG-IFN α-2b plus RBV[22,28]. Although our data showed that sex was not related to SVR, the reason for this finding was not fully elucidated. In any case, studies have conclusively shown that it is important to begin treatment with PEG-IFN α-2b plus RBV combination therapy as soon as possible. Our data suggest that age may be a more important factor than sex for increasing the rate of SVR. Resistance to treatment in older patients may be due to IFN-immunomodulation, advanced liver fibrosis, or reduced dosage.

To maximize adherence to the optimal treatment regimen, the treatment schedule can be modified or other therapeutic modalities added, such as hematopoietic growth factors[29] or the new thrombopoietin-receptor agonist, eltrombopag, for the antiviral treatment of older patients with chronic hepatitis C[30]. A further individualized treatment protocol based on viral kinetics might be more practical[31].

In conclusion, PEG-IFN α-2b plus RBV treatment was effective in the treatment of older chronic hepatitis C patients when they received at least the minimum acceptable treatment dosage. However, there were frequent adverse effects and treatment discontinuation. It is necessary to control for adverse effects that might interrupt treatment and to begin this combination therapy as soon as possible, especially in older patients.

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COMMENTS

Background

Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. However, there is little data concerning the response and safety of combination treatment for a large number of older patients with chronic hepatitis C virus infection. Therefore, in an attempt to ameliorate these problems, the authors decided to treat older patients with pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Research frontiers

The combination treatment of PEG-IFN α-2b plus RBV improved the sustained virological response rate in chronic hepatitis C patients. However, the current issue is whether or not to treat older patients because of low response and high dropout rate.

Innovations and breakthroughs

There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. This study is very useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients, because of its large scale, multicenter design.

Applications

The study demonstrated that PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum required treatment dosage. Furthermore, this study suggested that the combination treatment and beginning this therapy as soon as possible are important, especially in older patients.

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

REFERENCES

1 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52

2 **Hayashi J**, Furusyo N, Ariyama I, Sawayama Y, Etoh Y, Kashiwagi S. A relationship between the evolution of hepatitis C virus variants, liver damage, and hepatocellular carcinoma in patients with hepatitis C viremia. *J Infect Dis* 2000; **181**: 1523-1527

3 **Hayashi J**, Ohmiya M, Kishihara Y, Tani Y, Kinukawa N, Ikematsu H, Kashiwagi S. A statistical analysis of predictive factors of response to human lymphoblastoid interferon in patients with chronic hepatitis C. *Am J Gastroenterol* 1994; **89**: 2151-2156

4 **Furusyo N**, Hayashi J, Ohmiya M, Sawayama Y, Kawakami Y, Ariyama I, Kinukawa N, Kashiwagi S. Differences between interferon-alpha and -beta treatment for patients with chronic hepatitis C virus infection. *Dig Dis Sci* 1999; **44**: 608-617

5 **Furusyo N**, Hayashi J, Ueno K, Sawayama Y, Kawakami Y, Kishihara Y, Kashiwagi S. Human lymphoblastoid interferon treatment for patients with hepatitis C virus-related cirrhosis. *Clin Ther* 1997; **19**: 1352-1367

6 **Kashiwagi K**, Furusyo N, Kubo N, Nakashima H, Nomura H, Kashiwagi S, Hayashi J. A prospective comparison of the effect of interferon-alpha and interferon-beta treatment in patients with chronic hepatitis C on the incidence of hepatocellular carcinoma development. *J Infect Chemother* 2003; **9**: 333-340

7 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

8 **Furusyo N**, Kajiwara E, Takahashi K, Nomura H, Tanabe Y, Masumoto A, Maruyama T, Nakamuta M, Enjoji M, Azuma K, Shimono J, Sakai H, Shimoda S, Hayashi J. Association between the treatment length and cumulative dose of pegylated interferon alpha-2b plus ribavirin and their effectiveness as a combination treatment for Japanese chronic hepatitis C patients: project of the Kyushu University Liver Disease Study Group. *J Gastroenterol Hepatol* 2008; **23**: 1094-1104

9 **Hayashi J**, Kishihara Y, Ueno K, Yamaji K, Kawakami Y, Furusyo N, Sawayama Y, Kashiwagi S. Age-related response to interferon alfa treatment in women vs men with chronic hepatitis C virus infection. *Arch Intern Med* 1998; **158**: 177-181

10 **Iwasaki Y**, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, Shimoe T, Takaguchi K, Hashimoto N, Kobatake T, Tomita M, Kawaguchi M, Kobashi H, Sakaguchi K, Shiratori Y. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology* 2006; **43**: 54-63

11 **Arase Y**, Suzuki F, Suzuki Y, Akuta N, Kawamura Y, Kobayashi M, Hosaka T, Sezaki H, Yatsuji H, Kobayashi M, Ikeda K, Kumada H. Side effects of combination therapy of peginterferon and ribavirin for chronic hepatitis-C. *Intern Med* 2007; **46**: 1827-1832

12 **Arase Y**, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Akuta N, Someya T, Koyama R, Hosaka T, Sezaki H, Kobayashi M, Kumada H. Long-term outcome after interferon therapy in elderly patients with chronic hepatitis C. *Intervirology* 2007; **50**: 16-23

13 **Ikeda K**, Arase Y, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Akuta N, Kobayashi M, Saitoh S, Suzuki F, Suzuki Y, Kumada H. Necessities of interferon therapy in elderly patients with chronic hepatitis C. *Am J Med* 2009; **122**: 479-486

14 **Furusyo N**, Katoh M, Tanabe Y, Kajiwara E, Maruyama T, Shimono J, Sakai H, Nakamuta M, Nomura H, Masumoto A, Shimoda S, Takahashi K, Azuma K, Hayashi J. Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: a project of the Kyushu University Liver Disease Study Group. *World J Gastroenterol* 2006; **12**: 784-790

15 **Matsuo S**, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A. Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 2009; **53**: 982-992

16 **Manesis EK**, Papatheodoridis GV, Touloumi G, Karafoulidou A, Ketikoglou J, Kitis GE, Antoniou A, Kanatakis S, Koutsounas SJ, Vafiadis I. Natural course of treated and untreated chronic HCV infection: results of the nationwide Hepnet. Greece cohort study. *Aliment Pharmacol Ther* 2009; **29**: 1121-1130

17 **Tanaka Y**, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ. Inaugural Article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA* 2002; **99**: 15584-15589

18 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499

19 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492

20 **Poynard T**, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426-1432

21 **Antonucci G**, Longo MA, Angeletti C, Vairo F, Oliva A, Comandini UV, Tocci G, Boumis E, Noto P, Solmone MC, Capobianchi MR, Girardi E. The effect of age on response to therapy with peginterferon alpha plus ribavirin in a cohort of patients with chronic HCV hepatitis including subjects older than 65 yr. *Am J Gastroenterol* 2007; **102**: 1383-1391

22 **Thabut D**, Le Calvez S, Thibault V, Massard J, Munteanu M, Di Martino V, Ratziu V, Poynard T. Hepatitis C in 6,865 patients 65 yr or older: a severe and neglected curable disease? *Am J Gastroenterol* 2006; **101**: 1260-1267
23 **Honda T**, Katano Y, Shimizu J, Ishizu Y, Doizaki M, Hayashi K, Ishigami M, Itoh A, Hirooka Y, Nakano I, Urano F, Yoshioka K, Toyoda H, Kumada T, Goto H. Efficacy of peginterferon-alpha-2b plus ribavirin in patients aged 65 years and older with chronic hepatitis C. *Liver Int* 2010; **30**: 527-537
24 **Huang CF**, Yang JF, Dai CY, Huang JF, Hou NJ, Hsieh MY, Lin ZY, Chen SC, Hsieh MY, Wang LY, Chang WY, Chuang WL, Yu ML. Efficacy and safety of pegylated interferon combined with ribavirin for the treatment of older patients with chronic hepatitis C. *J Infect Dis* 2010; **201**: 751-759
25 **Paroni R**, Del Puppo M, Borghi C, Sirtori CR, Galli Kienle M. Pharmacokinetics of ribavirin and urinary excretion of the major metabolite 1,2,4-triazole-3-carboxamide in normal volunteers. *Int J Clin Pharmacol Ther Toxicol* 1989; **27**: 302-307
26 **Bruchfeld A**, Lindahl K, Schvarcz R, Ståhle L. Dosage of ribavirin in patients with hepatitis C should be based on renal function: a population pharmacokinetic analysis. *Ther Drug Monit* 2002; **24**: 701-708
27 **Kainuma M**, Hayashi J, Sakai S, Imai K, Mantani N, Kohta K, Mitsuma T, Shimada Y, Kashiwagi S, Terasawa K. The efficacy of herbal medicine (kampo) in reducing the adverse effects of IFN-beta in chronic hepatitis C. *Am J Chin Med* 2002; **30**: 355-367
28 **Sezaki H**, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Miyakawa Y, Kumada H. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig Dis Sci* 2009; **54**: 1317-1324
29 **Afdhal NH**, Dieterich DT, Pockros PJ, Schiff ER, Shiffman ML, Sulkowski MS, Wright T, Younossi Z, Goon BL, Tang KL, Bowers PJ. Epoetin alfa maintains ribavirin dose in HCV-infected patients: a prospective, double-blind, randomized controlled study. *Gastroenterology* 2004; **126**: 1302-1311
30 **McHutchison JG**, Dusheiko G, Shiffman ML, Rodriguez-Torres M, Sigal S, Bourliere M, Berg T, Gordon SC, Campbell FM, Theodore D, Blackman N, Jenkins J, Afdhal NH. Eltrombopag for thrombocytopenia in patients with cirrhosis associated with hepatitis C. *N Engl J Med* 2007; **357**: 2227-2236
31 **Berg T**, Weich V, Teuber G, Klinker H, Möller B, Rasenack J, Hinrichsen H, Gerlach T, Spengler U, Buggisch P, Balk H, Zankel M, Neumann K, Sarrazin C, Zeuzem S. Individualized treatment strategy according to early viral kinetics in hepatitis C virus type 1-infected patients. *Hepatology* 2009; **50**: 369-377

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Four-year study of lamivudine and adefovir combination therapy in lamivudine-resistant hepatitis B patients: influence of hepatitis B virus genotype and resistance mutation pattern

J. Inoue, Y. Ueno, Y. Wakui, H. Niitsuma, K. Fukushima, Y. Yamagiwa, M. Shiina, Y. Kondo, E. Kakazu, K. Tamai, N. Obara, T. Iwasaki and T. Shimosegawa *Division of Gastroenterology, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan*

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SUMMARY. To investigate the efficacy of long-term lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy in 3TC-resistant chronic hepatitis B virus (HBV) infected patients, we analysed 28 3TC-resistant patients treated with the combination therapy during 47 months (range, 9–75). At 12, 24, 36, and 48 months, the rates of virological response with undetectable HBV DNA (≤2.6 log copies/mL) were 56, 80, 86, and 92%, respectively. Among 17 hepatitis B e antigen (HBeAg)-positive patients, HBeAg disappeared in 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. When HBV genotypes were compared, patients with genotype B achieved virological response significantly more rapidly than those with genotype C ($P = 0.0496$). One patient developed virological breakthrough after 54 months, and sequence analysis of HBV obtained from the patient was performed. An rtA200V mutation was present in the majority of HBV clones, in addition to the 3TC-resistant mutations of rtL180M+M204V. The rtN236T ADV-resistant mutation was observed in only 25% clones. *In vitro* analysis showed that the rtA200V mutation recovered the impaired replication capacity of the clone with the rtL180M+M204V mutations and induced resistance to ADV. Moreover, rtT184S and rtS202C, which are known entecavir-resistant mutations, emerged in some rtL180M+M204V clones without rtA200V or rtN236T. In conclusion, 3TC+ADV combination therapy was effective for most 3TC-resistant patients, especially with genotype B HBV, but the risk of emergence of multiple drug-resistant strains with long-term therapy should be considered. The mutation rtA200V with rtL180M+M204V may be sufficient for failure of 3TC+ADV therapy.

Keywords: chronic hepatitis B, drug resistance, HBV, rtA200V.

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infection, and chronic hepatitis often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome, and nucleoside or nucleotide analogues inhibit HBV replication by interfering with reverse transcriptase/DNA polymerase of the virus [2]. Although therapy with these drugs results in virological, biochemical, and histological improvement in most patients [3], the effect is often transient because of the emergence of drug-resistant HBV mutants [4].

Lamivudine (3TC), a nucleoside analogue of L-deoxycytidine, is associated with highly frequent emergence of drug-resistant mutants: the cumulative rate is about 20% per year [5,6]. Mutations that result in the replacement of methionine at amino acid 204 to valine or isoleucine (rtM204V/I) within the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the reverse transcriptase (RT) region of HBV polymerase are found in most of the 3TC-resistant isolates [7]. Compensatory mutations rtV173L and rtL180M, which restore the replication capacity of the YMDD mutant *in vitro*, are observed frequently together with the YMDD mutation [8,9]. Adefovir dipivoxil (ADV) is a phosphonate nucleotide analogue of adenosine monophosphate, and ADV-resistance rates are lower than those of 3TC [10]. Two mutations, rtA181V/T and rtN236T, are associated with resistance to ADV [11–14], and the cumulative 5-year occurrence of genotypic resistance is reported to be 29% [15]. *In vitro* studies showed that these mutations confer a weaker

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; RT, reverse transcriptase; TDF, tenofovir disoproxil fumarate.

Correspondence: Yoshiyuki Ueno, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo, Aoba-ku, Sendai 980-8574, Japan. E-mail: yueno@mail.tains.tohoku.ac.jp

decrease in the susceptibility to ADV, in comparison with the greater decrease in 3TC susceptibility because of the YMDD mutant [11,16]. This finding may explain the lower rate of the emergence of ADV resistance.

Although the number of approved drugs has increased in recent years, the treatment of chronic HBV infection remains a clinical challenge. Especially, how to manage drug-resistant patients including 3TC-resistant patients is a major problem. Continuation of 3TC monotherapy or retreatment with 3TC after its temporary discontinuation is ineffective options for 3TC-resistant patients [17]: the lack of any further benefit and the possibility of rapid re-emergence of resistant HBV have been reported [18]. Against 3TC-resistant HBV, ADV and entecavir (ETV) have a suppressive effect *in vivo* and *in vitro* [19–21]. Combination therapy of ADV and 3TC is effective for 3TC-refractory patients and has a low frequency of viral breakthrough [22]: the 3-year cumulative rate of *de novo* resistant mutants was 4% with no development of viral breakthrough in 3TC-resistant patients. However, further longer-term efficacy of the combination therapy remains unknown. ETV is a potent drug with infrequent development of resistance for treatment-naïve patients [23]. ETV monotherapy was shown to be effective during the first year of therapy in 3TC-resistant patients [20], but pre-existing 3TC-resistant mutants are favourable for the emergence of ETV resistance [21], and a comparatively high rate of the emergence of ETV-resistant strains has been reported in long-term studies [23]. Therefore, ETV monotherapy seems to be a less attractive option for the long-term treatment of 3TC-resistant patients.

Several previous reports have described the differences in the responses to antiviral therapy between HBV genotypes. A case–control study of 3TC treatment for genotypes B and C showed that the responses were not different, but the emergence of the YMDD mutation was more frequent in genotype C [24]. It was also reported that the YMDD mutation and breakthrough hepatitis developed more often in patients with genotype A than in patients with genotype B or C [25]. However, the impact of the genotype on the efficacy to ADV is uncertain.

Here, we studied the long-term efficacy of 28 3TC-resistant patients treated with the combination of 3TC and ADV and compared the response between HBV genotypes. Sequence analysis of HBV from a patient with resistance to the combination therapy was performed, and *in vitro* drug susceptibility of the mutant HBV clones was assessed to clarify the mechanism of the emergence of resistance.

MATERIALS AND METHODS

Patients

A total of 28 consecutive Japanese patients with chronic HBV infection who were treated with 3TC+ADV at Tohoku University Hospital from June 2003 to August 2009 for more than 6 months were enrolled in this study. All patients developed virological breakthrough during 3TC monotherapy, and ADV was added in. Virological breakthrough was defined as an increase in the serum HBV DNA level of ≥1 log copies/mL, which was determined using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan), at two or more consecutive examinations in comparison with the lowest level after treatment. To evaluate renal function, the estimated glomerular filtration rate (eGFR) level using the Cockcroft-Gault formula [(140 - age) × (weight in kilograms) × (0.85 if female)/(72 × serum creatinine)] [26] was calculated. No patients were infected with HCV, nor had a history of other liver diseases. The patients were evaluated for the rate of virological response (undetectable HBV DNA: <2.6 log copies/mL), biochemical response [alanine aminotransferase (ALT) normalization: ≤35 IU/L], hepatitis B e antigen (HBeAg) loss, and virological breakthrough.

Antiviral treatment

Adefovir dipivoxil was administered at a dosage of 10 mg/day in all but one patient in addition to 3TC at a dosage of 100 mg/day. One patient received 10 mg of ADV on alternate days and 50 mg/day of 3TC daily because of reduced eGFR at the start of treatment. This occurred when the eGFR level dropped to <50 mL/min.

Determination of HBV genotype

The HBV genotype was determined as described previously [27] with minor modifications. Briefly, total DNA was extracted from 50 μL of serum sample by QIAamp Blood Mini kit (QIAGEN GmbH, Hilden, Germany) and subjected to nested polymerase chain reaction (PCR) with high fidelity polymerase (PrimeSTAR HS DNA polymerase; TaKaRa Bio Inc., Shiga, Japan), to amplify a 396-nt sequence in the S gene. The amplification products were sequenced on both strands directly using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac (Version 12.2.7; Genetyx Corp., Tokyo, Japan). The genotype of HBV was determined by phylogenetic analysis with HBV isolates whose genotype was known.

Sequencing analysis of HBV reverse transcriptase region

Total DNA extracted from 50 μL of serum sample was subjected to nested PCR to amplify the 1148-nt sequence [nt 52 to 1199, the nucleotide numbers are in accordance with a genotype C HBV isolate of 3,215 nt (AB033550)] including the RT region of HBV polymerase. The first-round PCR was carried out with primers B026 [5′-TCA TCC WCA GGC CAT GCA GTG GA-3′ (W = A or T)] and B025 (5′-CTA GGA GTT CCG CAG TAT GGA TCG-3′), and the second round with

primers B011 [5′-YTT YCC TGC TGG TGG CTC CAG TTC-3′ (Y = C or T)] and B024 (5′-GGG GTT GCG TCA GCA AAC ACT TG-3′). The amplification products were sequenced on both strands directly or after cloning into pUC118. Sequencing analysis after cloning was performed at nt 497-1161.

Construction of plasmid

A cloned mutant sequence including the RT region from a sample obtained after the development of 3TC and ADV resistance was digested with BlnI (TaKaRa Bio Inc.) and EcoT22I (TaKaRa Bio Inc.). The digested fragment (nt 179-1068) was ligated into the BlnI-EcoT22I site of pBFH2R, which contained a 1.3-fold HBV genome [28]. Quick Change II-E Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce nucleotide substitutions into the plasmid. Each mutation found in the RT region, rtL180M [C to A at nt 667 (C667A)], rtT184S (A679T), rtA200V (C728T), rtS202C (A733T), rtM204V (A739G), and rtN236T (A836C), was converted into the wild type or another mutant nucleotide. To construct plasmids with combined nucleotide substitutions, these converted plasmids were used next as templates. As a result, variant constructs harbouring rtM204I, rtL180M+M204V, rtL180M+T184S+M204V, rtL180M+A200V+M204V, rtL180M+S202C+M204V, rtL180M+M204V+N236T, and rtL180M+A200V+M204V+N236T were composed, and all constructs were sequenced to confirm the nucleotide substitutions.

Cell culture and transfection

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum at 37 °C and 5% CO_2. Cells were seeded in 24-well plates at 1.25×10^5 cells/well. On the next day, 375 ng of plasmid DNA were transfected into these cells using TransIT LT-1 Transfection Reagent (Mirus, Madison, WI, USA), and cells were washed twice with phosphate-buffered saline after 4 h. Five hundred microliter of the medium and various amounts of adefovir (Toronto Research Chemicals Inc., Ontario, Canada) were added, and the culture supernatant was collected 4 days later. Experiments were performed at least in triplicate.

Real-time PCR and determination of IC_{50}

HBV DNA in the culture supernatant was quantified by real-time PCR as described previously [28] to determine the 50% inhibitory concentration (IC50) for ADV of each mutant HBV clone. Briefly, to digest the input plasmid DNA in the culture supernatant, 5 μL of the supernatant were treated with 5 units of DNase I (TaKaRa Bio Inc.) at 37 °C for 2 h, and the reaction was stopped with EDTA. Then, total DNA was extracted with a QIAamp DNA Blood Mini kit, and 10 μL of 200 μL DNA solution were subjected to real-time PCR using a LightCycler (Roche Diagnostics). Dose–response curves were plotted to determine the ADV IC50.

Statistical analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and Mann–Whitney *U* test for comparison of continuous variables between two groups. The cumulative rate of undetectable HBV DNA or ALT normalization was calculated using the Kaplan–Meier method, and differences between the curves were tested using Log-rank test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Study profile

The demographic and clinical profiles of the 28 patients [20 men and 8 women, median age 53.5 years (range 18–72)] at commencement of 3TC+ADV therapy are shown in Table 1. One (3.6%), 7 (25.0%), and 19 (67.9%) patients had HBV of genotypes A, B, and C, respectively. Eight (28.6%) patients had cirrhosis, 7 (25.0%) had HCC, and 17 (60.7%) patients were HBeAg positive. The mutations of the YMDD motif were determined by direct sequencing, and the YIDD, YVDD, and YIDD+YVDD mixed pattern were found in 14 (50%), 11 (39%), and 2 (7%)of the patients, respectively. Only one (4%) patient had no mutation in the YMDD motif. There were no significant differences in the profiles between patients with genotype B and those with genotype C.

Response to lamivudine and adefovir dipivoxil combination therapy

The 3TC-resistant patients treated with the combination therapy were followed up for a median of 47 months (range, 9–75). All patients continued to be treated with 3TC and ADV until virological breakthrough. The 6-, 12-, 24-, 36-, and 48-month rates of virological response with HBV DNA ≤ 2.6 log copies/mL were 39, 56, 80, 86, and 92%, respectively (Table 2). The ALT normalization rates were 57% at 6 months, 70% at 12 months, 84% at 24 months, 82% at 36 months, and 77% at 48 months. When compared between genotype B and C, the results of patients with genotype B tended to be favourable for both virological and biochemical response (Figs 1a,b). The cumulative probability of undetectable HBV DNA was significantly higher in genotype B than in genotype C ($P = 0.0496$), whereas there was no significant difference in that of ALT normalization. Notably, patients with genotype B achieved early virological response (HBV DNA < 2.6 log copies/mL at 6 months) significantly more frequently than those with genotype C

Table 1 Demographic and clinical characteristics of the 28 lamivudine-resistant patients at the start of adefovir addition to the treatment

	Overall (n = 28)*	Genotype B (n = 7)	Genotype C (n = 20)
Age (years), median (range)	53.5 (18–72)	51.0 (18–72)	53.5 (35–68)
Male patients, no. (%)	20 (71.4)	5 (71.4)	14 (70.0)
Patients with cirrhosis, no. (%)	8 (28.6)	1 (14.3)	7 (35.0)
Patients with HCC, no. (%)	7 (25.0)	0 (0)	7 (35.0)
HBeAg positive, no. (%)	17 (60.7)	3 (42.9)	13 (65.0)
HBV DNA (log copies/mL), median (range)	7.6 (4.3 to >7.6)	7.2 (5.3 to >7.6)	7.6 (4.3 to >7.6)
Patients with rtM204 mutation (M:I:V:I/V, no.)	1:14:11:2	1:3:2:1	0:11:8:1
ALT (IU/L), median (range)	86.5 (29–1027)	314.0 (47–760)	78.5 (29–1027)
T. Bil (mg/dL), median (range)	1.1 (0.5–4.5)	1.1 (0.5–1.5)	1.1 (0.5–4.5)
Albumin (g/dL), median (range)	4.1 (2.7–4.8)	4.2 (3.8–4.8)	4.0 (2.7–4.6)
Serum creatinine (mg/dL), median (range)	0.7 (0.4–1.2)	0.7 (0.6–1.2)	0.7 (0.4–1.2)
Prior lamivudine therapy (month), median (range)	28.6 (2–76)	36.5 (2–76)	28.6 (5–65)

HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; T. Bil, total bilirubin. *One patient had genotype A HBV.

Table 2 Virological and biochemical response to lamivudine and adefovir combination therapy during a median of 47 months

	Months of treatment						
Response	0 (n = 28)	6 (n = 28)	12 (n = 27)	24 (n = 25)	36 (n = 22)	48 (n = 13)	60 (n = 7)
HBV DNA < 2.6	0 (0)	11 (39.3)	15 (55.6)	20 (80.0)	19 (86.4)	12 (92.3)	6 (85.7)
HBV DNA 2.6 to <5.0	1 (3.6)	15 (53.6)	11 (40.7)	5 (20.0)	3 (13.6)	1 (7.7)	1 (14.3)
HBV DNA ≥ 5.0	27 (96.4)	2 (7.1)	1 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)
ALT normalization*	NA	16 (57.1)	19 (70.4)	21 (84.0)	18 (81.8)	10 (76.9)	6 (85.7)
HBeAg disappearance†	NA	1/17 (5.9)	4/17 (23.5)	4/16 (25.0)	8/13 (61.5)	7/8 (87.5)	4/5 (80.0)
Virological breakthrough	NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14.3)

Values are shown as numbers of patients followed by percentages in parentheses. NA, not applicable. *ALT ≤ 35 IU/L. †Values are shown as numbers of patients/total followed by percentages in parentheses.

[5/7 (71%) vs. 5/20 (25%), P = 0.0427]. Although the status of HBeAg at the start of ADV seemed to influence the response, the difference was not significant (Figs 1c,d). Among 17 HBeAg-positive patients, HBeAg disappeared in 6% at 6 months, 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. There was no patient with hepatitis B surface antigen (HBsAg) loss during follow-up in this study.

Three of 22 patients who were treated for more than 36 months did not achieve virological response. One of them developed virological breakthrough after 54 months of combination therapy. The other patients had 2.8 and 3.5 log copies/mL of serum HBV DNA at 36 months of therapy but did not develop breakthrough. None of the patients experienced biochemical breakthrough. One patient with HCC died of HCC progression at 9 months after ADV. None of the 21 patients without HCC at the start of ADV developed HCC during follow-up.

The renal toxicity with a ≥0.3 mg/dL increase in serum creatinine level was observed in five of the 28 patients. Two of them had a ≥0.5 mg/dL increase: the serum creatinine levels were increased from 0.8 to 1.4 mg/dL after 31 months in a patient, and from 0.9 to 1.7 mg/dL after 34 months in another patient. As their eGFR levels were lowered to 39 and 29 mL/min, the dosage of ADV was reduced to alternate-day administration. After the reduction of ADV, their serum creatinine and eGFR recovered.

Profile of a patient with lamivudine and adefovir dipivoxil resistance

He was a 53-year-old Japanese man with HBeAg-positive liver cirrhosis at the start of 3TC monotherapy in April 2002. The genotype of HBV was found to be genotype C. His clinical course is shown in Fig. 2. He developed breakthrough hepatitis with serum HBV DNA of >7.6 log copies/mL and alanine aminotransferase (ALT) of 236 IU/L in March 2003. ADV was added to the ongoing 3TC therapy in June 2003, and HBV DNA was gradually reduced reaching <2.6 log copies/mL 3 years later. However, virological

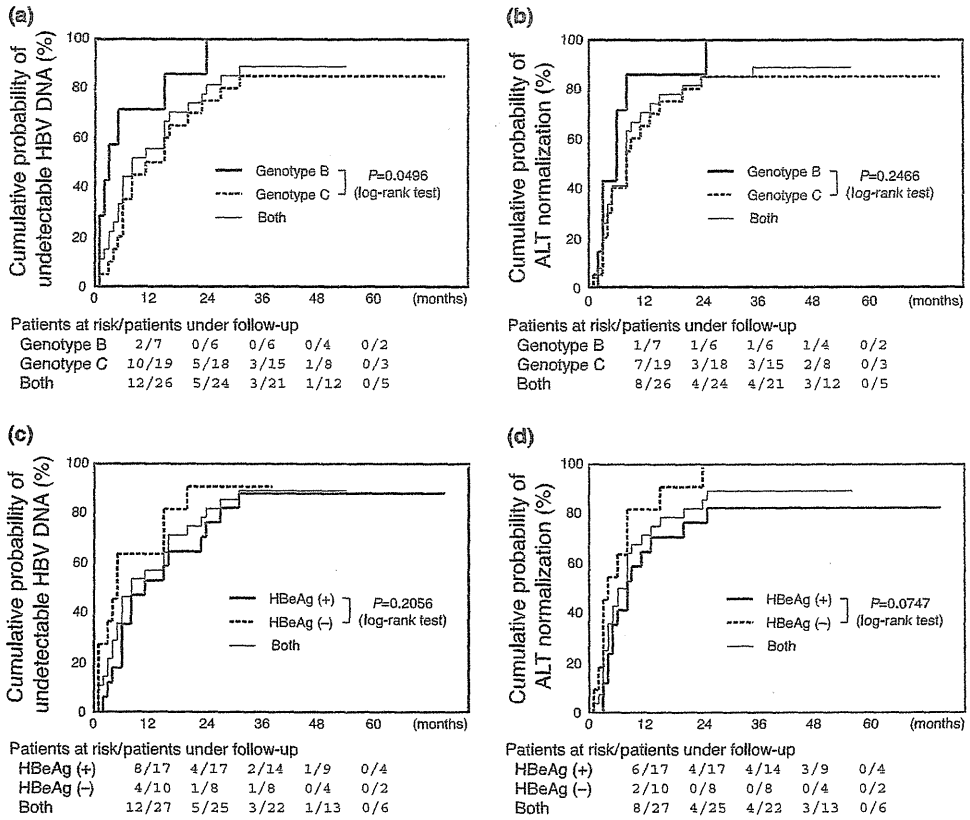


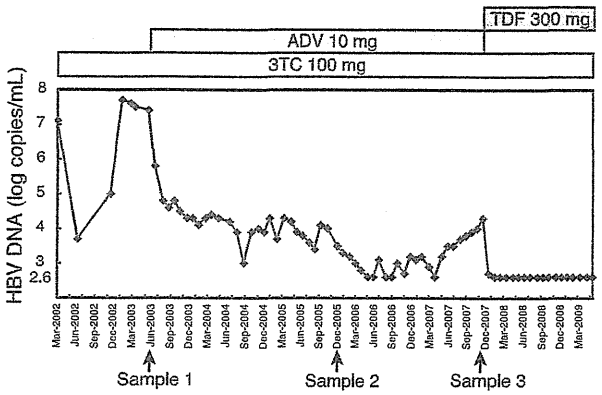
Fig. 1 Cumulative probability of virological or biochemical response during lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy. (a) Cumulative probability of undetectable HBV DNA (<2.6 log copies/mL) in patients with genotype B and those with genotype C. (b) Cumulative probability of ALT normalization (≤35 IU/L) in patients with genotype B and those with genotype C. (c) Cumulative probability of undetectable HBV DNA in HBeAg-positive patients and HBeAg-negative patients. (d) Cumulative probability of ALT normalization in HBeAg-positive patients and HBeAg-negative patients.

breakthrough was observed at 4 years after starting ADV, and his HBV DNA reached 4.3 log copies/mL in December 2007. Because his liver was cirrhotic and the hepatic functional reserve was impaired, combination therapy of tenofovir disoproxil fumarate (TDF) and 3TC was started before ALT flair. Two months later, his HBV DNA was suppressed to <2.6 log copies/mL, and viral breakthrough has not been observed to date (20 months later).

Mutations found in the HBV reverse transcriptase region of the lamivudine and adefovir dipivoxil-resistant patient

To investigate the mutations responsible for the viral breakthrough during the 3TC and ADV combination therapy, nucleotide sequences of the HBV RT region of the patient were compared between 3 time points: at the beginning of ADV treatment, at 30 months after ADV therapy, and at the time of viral breakthrough (54 months after ADV therapy). Direct sequencing analysis showed 10 amino acid changes during the clinical course (Fig. 2). The 3TC-resistant mutation of rtM204I changed to rtM204V after ADV treatment. Along with the change, the mixed mutation of rtL180L/M changed to rtL180M, which was reported to emerge with rtM204V during 3TC therapy [9]. The rtN236T mutation, which is a known ADV-resistance mutation [11], emerged as a mixed mutation with wild type (rtN236N/T) after viral breakthrough. Notably, rtA200V, which has never been reported as an ADV-resistant mutation, emerged also after viral breakthrough as a mixed mutation (rtA200V/A). Meanwhile, no specific mutation was found in the 2 patients without virological breakthrough who did not achieve virological response after 3 years of the combination therapy.

Clonal analysis was performed to examine the significance of these mutations of the RT region (Table S1). Several minor mutations were found during the 3TC and ADV therapy. After viral breakthrough, rtA200V was found in 63% of the clones, while rtN236T was found in only 25% of the clones. Therefore, rtA200V seemed to be responsible for the treatment failure of ADV. Moreover, rtT184S and S202C, which were reported as ETV resistance-associated mutations [29], were found as a minor population.



RT mutation	L80	P109	D134	R153	L180	A200	M204	V214	L229	N236
Sample 1	I/L	P/Q	G/D	R/Q	L/M	–	I	–	L/W	–
Sample 2	–	–	–	–	M	–	V	A	–	–
Sample 3	–	–	G/D	–	M	V/A	V	–	–	N/T

Fig. 2 Clinical course of a lamivudine (3TC)-resistant patient who developed virological breakthrough during 3TC and adefovir dipivoxil (ADV) combination therapy, and changes of amino acids in the reverse transcriptase (RT) region detected by direct sequencing analysis. After breakthrough, therapy was switched to 3TC plus tenofovir disoproxil fumarate (TDF) combination. The arrows indicate the time point when serum samples were obtained for sequencing analysis. Sample 1, 2, and 3 were obtained at the start of ADV, 30 months after ADV, and 54 months after ADV, respectively.

To investigate further the mutant populations, the combinations of these mutations and 3TC-resistant mutations were analysed (Fig. 3). At 30 months after ADV therapy, 100% of clones had mutations rtL180M+M204V. Subsequently, the mutations of rtT184S, A200V, S202C, and N236T emerged in the rtL180M+M204V clones after viral breakthrough. Of note, rtN236T was not found in clones without rtA200V.

Replication capacity and drug susceptibility of HBV mutants

We analysed the replication capacity of HBV clones with combined mutations as shown in Fig. 3. A clone with rtL180M+M204V+N236T mutations, which was not found in the patient, was also included for comparison. Consistent with a previous report [30], 3TC-resistant mutations of rtM204I or rtL180M+M204V lowered the replication capacity significantly in comparison with the wild-type clone (Table 3). From additional mutations to rtL180M+M204V found in the patient, only rtA200V restored the impaired replication capacity significantly. The ETV-resistant mutation of rtT184S and rtS202C did not seem to have such an effect. The ADV-resistant mutation, rtN236T, lowered the replication capacity further, and rtA200V did not restore the lowered capacity caused by rtN236T.

The 7 HBV clones with mutations in the RT region were analysed for their susceptibility to ADV. The IC_{50} of each clone is shown in Table 3. The clones with the 3TC-resistant mutations of rtM204I or rtL180M+M204V showed moderate resistance to ADV. In comparison with the clone with rtL180M+M204V, clones with additional mutations of rtT184S, A200V, or S202C showed significantly higher resistance to ADV. An additional mutation of rtN236T led to much greater resistance to ADV. Taking into account the results from the clonal analysis of serum samples and the replication capacity of each clone, rtA200V may be responsible for the treatment failure of 3TC+ADV therapy when it presents with 3TC-resistant mutations such as rtL180M+M204V. The mutations of rtT184S or S202C with rtL180M+M204V also confer ADV resistance, but the clones

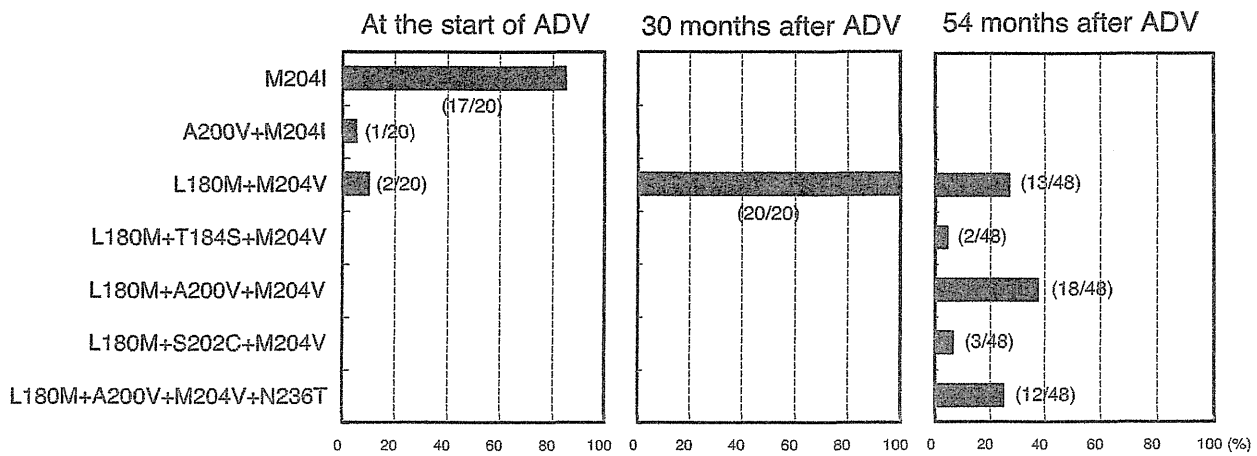


Fig. 3 Clonal analysis of HBV obtained from the patient with 3TC and ADV resistance. The serum samples were collected at the time points indicated in Fig. 2. The percentages (no. of clones/total in parentheses) of the clones with the combined mutations in the RT region are shown.

Table 3 Replication capacity and susceptibility to adefovir of the HBV mutants

HBV mutants	HBV DNA ($\times 10^7$ log copies/mL)*	Fold replication†	IC_{50} (μM)*	Fold resistance†
Wild type	13.60 ± 3.50	1	0.42 ± 0.06	1
M204I	2.17 ± 0.38	0.16	0.87 ± 0.2	2.07
L180M+M204V	4.38 ± 0.77	0.32	0.73 ± 0.06	1.74
L180M+T184S+M204V	5.98 ± 0.80	0.44	0.91 ± 0.04	2.17‡
L180M+A200V+M204V	8.90 ± 0.56	0.65‡	1.09 ± 0.12	2.60‡
L180M+S202C+M204V	4.86 ± 0.19	0.36	2.19 ± 0.63	5.21‡
L180M+M204V+N236T	0.88 ± 0.68	0.07‡	>10	>25
L180M+A200V+M204V+N236T	0.54 ± 0.38	0.04‡	>10	>25

*Values are expressed as means ± SD of experiments performed in triplicate. †(Mean value of the mutant)/(mean value of the wild type). ‡$P < 0.05$ in comparison with the clone with rtL180M+M204V.

with these mutations were not major, because they had no effect in enhancing the replication capacity of HBV.

DISCUSSION

As clinical and histological improvement accompanies reductions in HBV replication, therapies that reduce HBV replication are expected to limit the progression of liver disease and improve the natural history of chronic HBV infection [10]. Currently, the management of hepatitis B patients with drug resistance is one of the major problems in clinical practice for hepatitis B. A substantial part of 3TC-treated patients has mutant HBV with the YMDD mutation, and several clinical trials to treat 3TC-resistant hepatitis B have been performed. It has been reported first that with ADV alone and in combination with 3TC, the viral and biochemical responses were the same for 3TC-resistant patients in a 1 year study [31]. However, several studies of longer term treatment have shown that adding ADV was superior to switching to ADV monotherapy for patients with 3TC resistance [32–34]. In this study, we demonstrated that the add-on ADV therapy for 3TC-resistant hepatitis B patients effectively suppressed serum HBV DNA for a median of 47 months. Moreover, the biochemical response of ALT normalization was achieved in 77% patients and HBeAg loss in 88% of the HBeAg-positive patients at 48 months. The undetectability of HBV DNA was assessed by the Amplicor HBV monitor test, but recently, this can be assessed by a more sensitive real-time assay such as the Cobas TaqMan HBV test (Roche Diagnostics). The treatment duration to achieve HBV DNA undetectability might be longer if a more sensitive assay was used.

The influence of HBV genotype on the response or resistance to ADV has not been clarified, whereas the efficacy to 3TC was reported to be different between HBV genotypes [24,25]. This study showed that the virological response to 3TC+ADV was significantly earlier in genotype B than in C. However, there were several limitations of the results: the patients with genotype B were fewer, and no multivariate analysis was performed. In addition, all patients with HCC were genotype C, and ALT levels of genotype B tended to be higher, although there were no significant differences. The effect of genotype on the response to 3TC±ADV should be confirmed in larger studies. The baseline HBeAg status in 3TC+ADV combination therapy in 3TC-resistant patients was reported to influence the viral response: HBeAg-negative patients showed better virological and biological response [35]. In this study, the same tendency was observed, but the difference was not significant.

Initial virological suppression by ADV monotherapy was reported to be a good prognostic factor for the treatment of both naïve patients [36] and 3TC-resistant patients [37]. Taking into account the results of this study and previous reports, it is suggested that patients with genotype B HBV might develop resistance to 3TC+ADV less frequently than those with genotype C. In fact, the 3TC+ADV-resistant patient in this study was infected with genotype C HBV. Because the development of resistance to 3TC+ADV combination therapy is rare [22,35], it is difficult to evaluate whether the early virological response or genotype B is associated with the lower frequency of resistance to 3TC+ADV combination therapy. Further long-term study is needed to clarify this issue.

Although the emergence of resistance in this study was rare during the combination therapy as previously reported [22,35], one patient developed virological breakthrough after 4.5 years. We identified a characteristic mutation pattern of HBV in this patient. The mutation of rtA200V rescued the *in vitro* replication capacity that was impaired by rtL180M+M204V and reduced the susceptibility to ADV. In previous reports, rtA200V emerged as an additional mutation with the 3TC-resistant mutation in patients under 3TC monotherapy [38,39]. The effect of this mutation is not as strong as the effect of rtM204I/V±L180M on 3TC susceptibility *in vitro*, which showed >1000-fold resistance [40]. However, the clinical dose of ADV is comparatively low

because of renal toxicity [41], and the weakly resistant profile *in vitro* can explain the great clinical impact. Villet *et al.* reported that rtA200V was observed in a patient with 3TC monotherapy, and it was no longer detected after the combination therapy with ADV and 3TC [39]. The difference of results between the previous study and our study may be because of the emergence of mutations with a potent effect on ADV resistance, such as rtV173L and rtA181V, in the previous study. Because these mutations may have a greater effect on ADV resistance than rtA200V, the HBV clones with rtA200V seemed to disappear in the previous study case.

The known ADV-resistant mutation of rtN236T was found in only 25% clones, exclusively with rtA200V. This may indicate that rtN236T appeared after the emergence of rtA200V. In the active replication of the clones with rtA200V, which restored the replication capacity and enhanced ADV resistance, other mutations including rtN236T might occur more readily.

The rtA200V mutation is the result of nucleotide substitution C728T. This change in the overlapping S region results in an amino acid substitution affecting HBsAg: Leu to Phe at aa192 (sL192F). There is a possibility that sL192F may affect the replication capacity of HBV, but the actual mechanism is unknown.

Interestingly, the ETV-resistant mutations of rtT184S and rtS202C were also detected during 3TC+ADV combination therapy by clonal analysis. These mutations confer ETV resistance in the presence of the 3TC-resistant mutations of rtM204I/V±L180M [21]. This study showed that these mutations also have an ADV-resistance profile. These mutations may not cause viral breakthrough, because the population of these mutants in the patient was minor (4% and 6%, respectively), and their replication capacity was lower than that with rtA200V *in vitro*. The emergence of these mutations suggested that long-term 3TC+ADV therapy has the possibility of leading to multiple drug resistance including ETV resistance.

The combination therapy of 3TC and ADV is very effective with little frequency of viral breakthrough for 3TC-refractory patients. However, some patients do not achieve complete viral suppression of serum HBV DNA to under 2.6 log copies/mL. It was considered that the incomplete suppression of viral replication might favour further selection of drug-resistant mutants [42]. Although there have been a few reports of cases that showed resistance to 3TC+ADV therapy to date, the number of resistant cases will increase along with the increase in cases with long-term therapy. The 3TC- and ADV-resistant patient in this study was treated with 3TC and TDF after the virological breakthrough, and HBV DNA was promptly suppressed. Although TDF was reported to show cross-resistance with ADV *in vitro* [16,40,43], there are several reports that showed the effectiveness of TDF for ADV-refractory patients [44–46]. It is thought that the potency of TDF might result from its higher clinical dose compared to that of ADV [47].

In conclusion, this study showed that the combination therapy of 3TC and ADV effectively suppressed HBV replication in 3TC-resistant patients with chronic HBV infection for 4 years. Especially, patients with genotype B achieved earlier virological response than those with genotype C. However, one of the 28 patients developed virological breakthrough during the combination therapy over 4 years, and the HBV mutation of rtA200V, in addition to 3TC-resistant mutations, was demonstrated to contribute to the ADV resistance. Moreover, ETV-resistant mutations emerged coincidentally in minor HBV clones. The risk of emergence of multiple drug-resistant mutant should be considered in cases with long-term therapy with nucleos(t)ide analogues, especially when serum HBV DNA cannot be suppressed completely. Potent antiviral agents should be administered in such cases to prevent the emergence of multiple drug-resistant HBV mutants that are difficult to treat.

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REFERENCES

1 Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; 350: 1118–1129.
2 Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. *Proc Natl Acad Sci U S A* 1991; 88: 8495–8499.
3 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–68.
4 Ling R, Mutimer D, Ahmed M *et al.* Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; 24: 711–713.
5 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; 36: 687–696.
6 Zoulim F. Hepatitis B virus resistance to antivirals: clinical implications and management. *J Hepatol* 2003; 39(Suppl. 1): S133–S138.
7 Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology* 1996; 24: 714–717.
8 Delaney IV WE, Yang H, Westland CE *et al.* The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 2003; 77: 11833–11841.

9 Ono SK, Kato N, Shiratori Y *et al.* The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 2001; 107: 449–455.
10 Dienstag JL. Hepatitis B virus infection. *N Engl J Med* 2008; 359: 1486–1500.
11 Angus P, Vaughan R, Xiong S *et al.* Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 2003; 125: 292–297.
12 Villeneuve JP, Durantel D, Durantel S *et al.* Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. *J Hepatol* 2003; 39: 1085–1089.
13 Fung SK, Andreone P, Han SH *et al.* Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. *J Hepatol* 2005; 43: 937–943.
14 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. *N Engl J Med* 2005; 352: 2673–2681.
15 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; 131: 1743–1751.
16 Brunelle MN, Jacquard AC, Pichoud C *et al.* Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. *Hepatology* 2005; 41: 1391–1398.
17 Papatheodoridis GV, Manolakopoulos S, Dusheiko G, Archimandritis AJ. Therapeutic strategies in the management of patients with chronic hepatitis B virus infection. *Lancet Infect Dis* 2008; 8: 167–178.
18 Lau DT, Khokhar MF, Doo E *et al.* Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; 32: 828–834.
19 Westland CE, Yang H, Delaney IV WE *et al.* Activity of adefovir dipivoxil against all patterns of lamivudine-resistant hepatitis B viruses in patients. *J Viral Hepat* 2005; 12: 67–73.
20 Chang TT, Gish RG, Hadziyannis SJ *et al.* A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; 129: 1198–1209.
21 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004; 48: 3498–3507.
22 Lampertico P, Vigano M, Manenti E, Iavarone M, Sablon E, Colombo M. Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; 133: 1445–1451.
23 Tenney DJ, Rose RE, Baldick CJ *et al.* Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. *Hepatology* 2009; 49: 1503–1514.
24 Orito E, Fujiwara K, Tanaka Y *et al.* A case-control study of response to lamivudine therapy for 2 years in Japanese and Chinese patients chronically infected with hepatitis B virus of genotypes Bj, Ba and C. *Hepatol Res* 2006; 35: 127–134.
25 Kobayashi M, Suzuki F, Akuta N *et al.* Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. *J Med Virol* 2006; 78: 1276–1283.
26 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; 130: 461–470.
27 Takahashi M, Nishizawa T, Gotanda Y *et al.* High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. *Clin Diagn Lab Immunol* 2004; 11: 392–398.
28 Inoue J, Ueno Y, Nagasaki F *et al.* Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis. *Virology* 2009; 395: 202–209.
29 Tenney DJ, Rose RE, Baldick CJ *et al.* Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. *Antimicrob Agents Chemother* 2007; 51: 902–911.
30 Melegari M, Scaglioni PP, Wands JR. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. *Hepatology* 1998; 27: 628–633.
31 Peters MG, Hann HW, Martin P *et al.* Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 2004; 126: 91–101.
32 Gaia S, Barbon V, Smedile A *et al.* Lamivudine-resistant chronic hepatitis B: an observational study on adefovir in monotherapy or in combination with lamivudine. *J Hepatol* 2008; 48: 540–547.
33 Fung SK, Chae HB, Fontana RJ *et al.* Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol* 2006; 44: 283–290.
34 Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology* 2007; 45: 307–313.
35 Yatsuji H, Suzuki F, Sezaki H *et al.* Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; 48: 923–931.
36 Gallego A, Sheldon J, Garcia-Samaniego J *et al.* Evaluation of initial virological response to adefovir and development of adefovir-resistant mutations in patients with chronic hepatitis B. *J Viral Hepat* 2008; 15: 392–398.
37 Chan HL, Wong VW, Tse CH *et al.* Early virological suppression is associated with good maintained response to adefovir dipivoxil in lamivudine resistant chronic hepatitis B. *Aliment Pharmacol Ther* 2007; 25: 891–898.
38 Zollner B, Petersen J, Puchhammer-Stockl E *et al.* Viral features of lamivudine resistant hepatitis B genotypes A and D. *Hepatology* 2004; 39: 42–50.
39 Villet S, Pichoud C, Villeneuve JP, Trepo C, Zoulim F. Selection of a multiple drug-resistant hepatitis B virus strain in a liver-transplanted patient. *Gastroenterology* 2006; 131: 1253–1261.

40 Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol* 2006; 44: 593–606.
41 Marcellin P, Chang TT, Lim SG *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; 348: 808–816.
42 Santantonio T, Fasano M, Durantel S *et al.* Adefovir dipivoxil resistance patterns in patients with lamivudine-resistant chronic hepatitis B. *Antivir Ther* 2009; 14: 557–565.
43 Qi X, Xiong S, Yang H, Miller M, Delaney WE. In vitro susceptibility of adefovir-associated hepatitis B virus polymerase mutations to other antiviral agents. *Antivir Ther* 2007; 12: 355–362.
44 Tan J, Degertekin B, Wong SN, Husain M, Oberhelman K, Lok AS. Tenofovir monotherapy is effective in hepatitis B patients with antiviral treatment failure to adefovir in the absence of adefovir-resistant mutations. *J Hepatol* 2008; 48: 391–398.
45 van Bommel F, Zollner B, Sarrazin C *et al.* Tenofovir for patients with lamivudine-resistant hepatitis B virus (HBV) infection and high HBV DNA level during adefovir therapy. *Hepatology* 2006; 44: 318–325.
46 Choe WH, Kwon SY, Kim BK *et al.* Tenofovir plus lamivudine as rescue therapy for adefovir-resistant chronic hepatitis B in hepatitis B e antigen-positive patients with liver cirrhosis. *Liver Int* 2008; 28: 814–820.
47 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–539.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Clonal analysis of HBV RT region of samples from the patient with lamivudine and adefovir resistance.

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Ⅲ. 平成23年度 研究成果の刊行に関する一覧表