3. Results

3.1. Study population

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

3.2. Virological and biochemical response

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

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

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

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

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

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

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

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

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

Table 2
Multivariate analysis of baseline factors associated with virological response

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

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

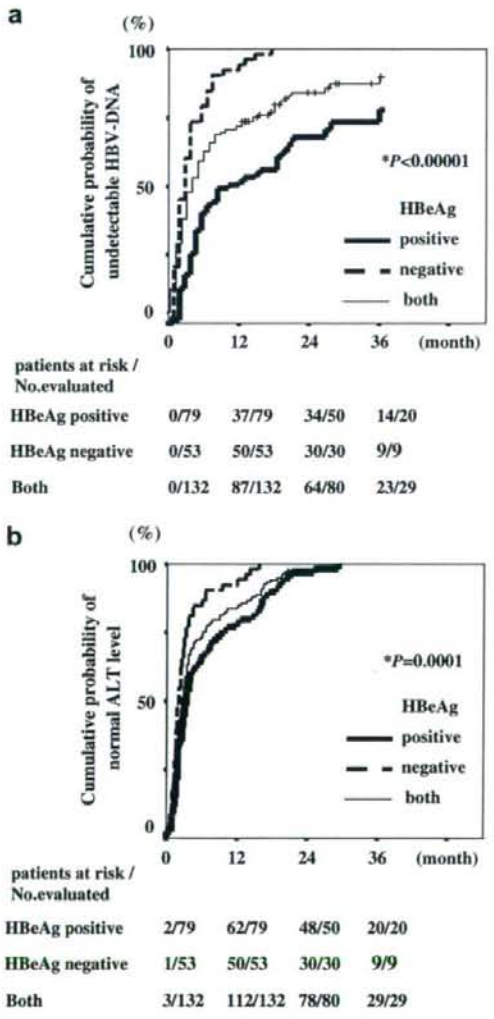

Fig. 1. (a) Cumulative probability of undetectable HBV-DNA during ADV + LAM combination therapy in patients with HBeAg-positive, -negative and both. (b) Cumulative probability of normal ALT during ADV + LAM combination therapy in patients HBeAg-positive, -negative and both. *P values between HBeAg-positive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

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

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

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

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

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

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

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

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

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

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

3.7. Clinical events

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

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

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

4. Discussion

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

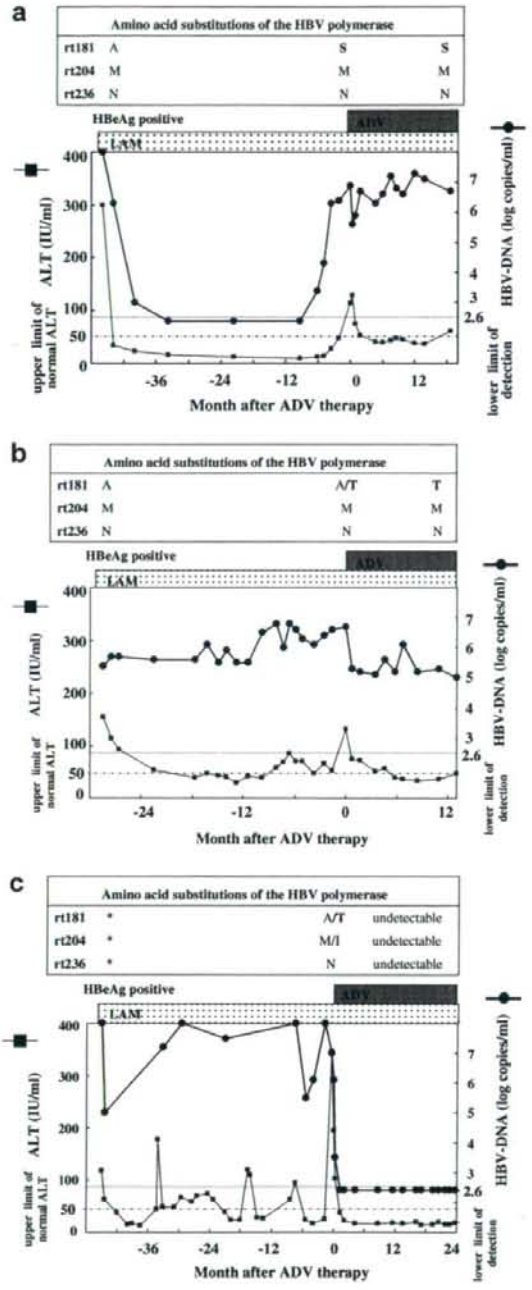

Fig. 2. Clinical course of three patients who showed emergence of ADV-resistant mutants at the commencement of ADV therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above each graph. (a) Clinical course of a patient who developed the rtA181S mutant. (b) Clinical course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

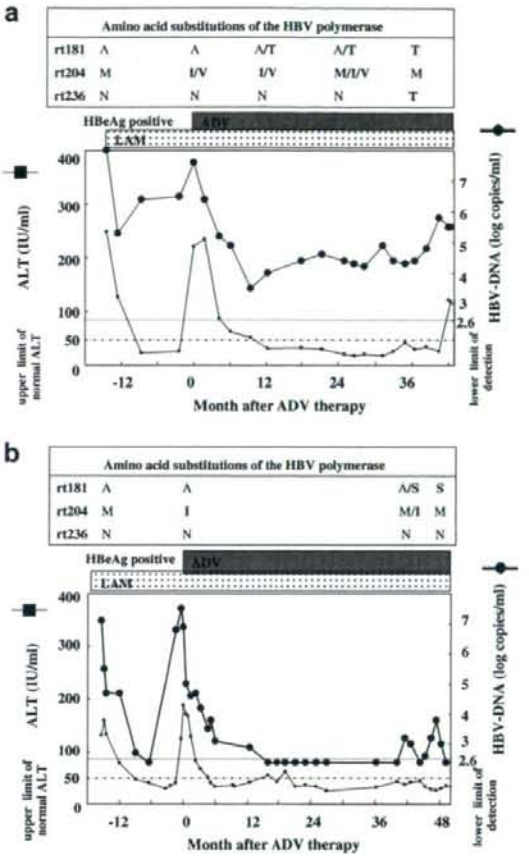

Fig. 3. Clinical course of two patients with LAM-resistant HBV who showed the emergence of an ADV-resistant mutant during combination therapy. Amino acid substitutions in the HBV polymerase are displayed in the panel above the graph. (a) Clinical course of a patient who developed the rtA181T + rtN236T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

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

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

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

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

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

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

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

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

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

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

We also demonstrated that the substitution at rt181 was associated with not only ADV resistance but also LAM resistance. At the commencement of ADV + LAM combination therapy, the substitutions at rtA181 as LAM resistance were identified in three patients (2.3%), who exhibited poor viral reduction

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

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

Acknowledgement

This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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Hepatocarcinogenesis Following HCV RNA Eradication by Interferon in Chronic Hepatitis Patients

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INTERNAL MEDICINE

Reprinted from Internal Medicine
Vol. 47, Pages 1637-1643
October 2008

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

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Abstract

Objective Interferon (IFN) therapy reduces the incidence of hepatocarcinogenesis in patients with hepatitis C viral (HCV) infection who achieve a sustained virological response (SVR). The aim of the present study was to determine the rate of hepatocarcinogenesis and the risk factor in sustained virological responders.

Patients and Method The study subjects were 1,193 patients with HCV-related chronic liver disease and IFN- or IFN plus ribavirin-induced SVR. The age, male/female ratio, and liver fibrosis stage [(F0-F3)/LC] were 15-83 years, 808/385, and 1106/41, respectively. Patients were followed-up for 8.3 years (range, 0 to 19.0 years) and the incidence of hepatocellular carcinoma was recorded.

Results Hepatocellular carcinogenesis was detected in 23 patients during the follow-up. The crude rates of hepatocarcinogenesis at 5, 10, and 15 years were 1.5%, 2.4% and 4.1%, respectively. Multivariate analysis identified cirrhosis, male sex and age older than 50 years as determinants of hepatocarcinogenesis with hazard ratios of 12.9 ($p<0.001$), 6.45 ($p=0.012$), and 20.2 ($p=0.004$), respectively.

Conclusion Long-term follow-up of patients with chronic HCV infection is necessary even in those who show SVR, especially in male elderly patients with cirrhosis.

Key words: hepatitis C virus, hepatocellular carcinoma, chronic hepatitis, sustained virological response, cox proportional hazard model

(Inter Med 47: 1637-1643, 2008)
(DOI: 10.2169/internalmedicine.47.1087)

Introduction

Interferon (IFN) is effective in eliminating HCV and reducing serum alanine aminotransferase (ALT) in some patients with chronic hepatitis C viral (HCV) infection (1-3). A reduction in the incidence of hepatocellular carcinoma (HCC) in patients with HCV-associated hepatitis and cirrhosis treated with IFN has been reported by many investigators (4-14). The previous study (14) suggested that fluctuations and persistently high levels of ALT in patients with chronic HCV infection enhances the carcinogenic process. From the viewpoint of liver carcinogenesis, IFN plays a suppressive action on the development of HCC through reduction or complete remission of inflammatory activity. Multivariate analysis has indicated that IFN lowers the carcinogenesis rate in those patients who show IFN-induced reduction in ALT levels (15). In patients with IFN-induced normalization of ALT levels, the groups at high risk for carcinogenesis were older, male, and a more advanced histologic stage (16). Patients who show elimination of HCV RNA are considered to exhibit normalization of ALT levels. Therefore, the incidence of carcinogenesis is assumed to be lower in patients with sustained virological response (SVR) than in those who show biochemical response (BR) and no response (NR) to IFN therapy. SVR was defined as persistent disappearance of HCV RNA after therapy, BR as normal ALT values without elimination of HCV RNA for at least 6 months after therapy, and NR as persistently abnormal or only transient normalization of ALT for less than 6 months. However, he-

Department of Hepatology, Toranomon Hospital, Tokyo
Received for publication March 6, 2008; Accepted for publication May 21, 2008
Correspondence to Dr. Miharu Hirakawa, ZXC00701@nifty.ne.jp

patocarcinogenesis still occurs in patients with SVR (17-31). In the studies of Toyoda et al (17) and Ikeda et al (18), the risk factors for carcinogenesis were not discussed due to few sustained virological responders with carcinogenesis. Tokita et al (19) and Kobayashi et al (20) indicated that the risk factors of hepatocarcinogenesis after elimination of HCV RNA are severe fibrosis, male sex, and regular consumption of moderate amounts of alcohol, and old age at the start of IFN treatment. Their hazard ratios could not be estimated because of the relatively small number of patients with SVR. Ikeda et al (21) indicated the hazard ratios of risk factors; older age, increased aspartate aminotransferase (AST), and decreased platelet count. However, the study population was restricted to patients who received IFN monotherapy and it did not include patients who received either pegylated interferon (PEG IFN) or combination therapy of IFN and ribavirin.

The aims of this study were to estimate the rate of hepatocellular carcinogenesis in patients with chronic HCV infection who show SVR to IFN monotherapy or combination therapy of IFN and ribavirin and to determine the risk factors that affect carcinogenesis rate in such patients using multivariate analysis.

Patients and Methods

Study population

In this retrospective cohort study, all patients with chronic HCV infection who started IFN therapy between February 1987 and July 2006 in the Department of Hepatology, Toranomon Hospital were analyzed in the database. Prior to IFN therapy, they were positive for anti-HCV (second- or third generation anti-HCV, enzyme-linked immunosorbent assay, Dainabot, Osaka, Japan) and HCV RNA. Anti-HCV was assayed using stored frozen sera at -80°C. HCV RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc., Belleville, NJ) or the branched DNA probe assay (b DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan). The medical records of 1,193 patients with HCV infection, who had achieved HCV RNA elimination after IFN therapy or the combination therapy of IFN and ribavirin were obtained. The sera of all patients were negative for hepatitis B surface antigen (HBsAg; radioimmunoassay, Austria, Abbott Laboratories, Detroit, MI). The study protocol was approved by the Human Ethics Review Committee of Toranomon hospital.

Clinical background and laboratory data

The background of 1,193 patients who achieved SVR is shown in Table 1. They included 809 men and 384 women, who were 15 to 83 years old with a median age of 50 years at the commencement of therapy. HCV genotype was analyzed by the immunoserological typing method using a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan).

Table 1. Patients' Profiles, Virological, Histological Characteristics of the Patients Prior to Their Interferon (IFN) Therapy and Protocol of IFN Therapy

Number of patients	1193
Sex (M / F)	809 / 384
Age (years) *	50 (15-83)
Observation period (year) *	8.3 (0.0-19.0)
HCV genotype	
Genotype 1a, 1b	494 (41.4%)
Genotype 2a, 2b	670 (56.2%)
Genotype 1+2	5 (0.4%)
Genotype 3	1 (0.1%)
Undetermined	23 (1.9%)
Histological stage of hepatitis	
F0 (no fibrosis)	7 (0.6%)
F1 (slight fibrosis)	738 (61.9%)
F2 (moderate fibrosis)	289(24.2%)
F3 (severe fibrosis)	72 (6.0%)
F4 (cirrhosis)	41 (3.4%)
Not examined	46 (3.9%)
IFN therapy	
Monotherapy	1032 (86.5%)
Combination therapy with ribavirin	161 (13.5%)
Type of IFN	
IFN-α	850 (71.2%)
PEG IFN-α	46 (3.9%)
IFN-β	251 (21.0%)
IFN-α/PEG IFN-α+IFN-β	47 (3.9%)

IFN: interferon, PEG IFN: pegylated interferon

*Data are median (minimum, maximum) values.

The HCV genotype was 1 (genotype 1a and 1b) in 494 patients, 2 (genotype 2a and 2b) in 670 patients, 1 plus 2 in 5 patients, 3 in 1 patient. Before treatment, 1,131 patients underwent liver biopsy with or without peritoneoscopy to assess the staging of liver fibrosis and the grade of inflammatory activity based on the classification of Desmet (32). Staging of liver fibrosis was defined as F0 (no fibrosis), F1 (fibrosis portal expansion), F2 (bridging fibrosis), F3 (bridging fibrosis with architectural distortion) and F4 (cirrhosis). Additionally, 16 patients were diagnosed as cirrhosis by peritoneoscopy without biopsy, laboratory values or clinical features: 1147 patients were diagnosed with chronic hepatitis (n=1,106) and cirrhosis (n=41) (F0/F1/F2/F3/F4=7/738/289/72/41).

Treatment protocol

IFN was performed once in 973 patients and more times of therapy in 220 patients (twice/three times/four times/five times/six times=166/38/13/2/1). IFN and ribavirin combination therapy was used to eliminate HCV RNA for 161 patients, while IFN monotherapy eliminated HCV RNA for the other 1,032 patients. The type of IFN was IFN-α (natural or recombinant)/PEG IFN-α in 896 patients (75.1%); IFN-α in 850 patients (71.2%), PEG IFN-α in 46 patients (3.9%), IFN-β (natural) in 251 patients (21.0%) and IFN-α or PEG IFN-α and IFN-β in 47 patients (3.9%).

A total of 613 patients (51.4%) received 3 to 9 million units of IFN everyday for 8 weeks followed by twice or three times a week for 1 to 305 weeks (for 16 to 22 weeks in 75% of patients), 304 patients (25.5%) received 3 to 9 million units of IFN everyday for 1-5 weeks followed by three times a week, 5 patients (0.4%) for 12 weeks and one patient (0.1%) for 24 weeks followed by intermittent administration. A total of 124 patients (10.4%) underwent short therapy with IFN everyday for 4-8 weeks, 2 patients (0.2%) for 10-12 weeks, 18 patients (1.5%) for 18-24 weeks. 2 patients (0.2%) had a prolonged administration of IFN for 11 and 13 months. And 63 patients (5.3%) underwent intermittent administration of three times a week for 4weeks to 70 months. This protocol is one of the low-dose intermittent IFN therapies. A total of 48 patients (4.0%) underwent 50-180 μg of PEG IFN once a week: 8 patients for 24 weeks and 40 patients for 48 weeks.

Follow-up and diagnosis of hepatocellular carcinoma

Almost all patients were followed-up every week or biweekly during IFN monotherapy. This included hematological, biochemical, and virological tests. Patients treated with pegylated IFN were also checked every week or biweekly. After the completion of treatment, monthly follow-up was continued until the virological response could be determined. When SVR was confirmed, imaging studies were conducted once or twice per year in the majority of patients; these included computed tomography (CT) or ultrasonography (US), except those patients who were lost to follow-up. Angiography was performed only when HCC was highly suspected on CT or US. The presence of a characteristic hypervascular nodule on angiography was considered a specific finding for HCC, and histological confirmation was usually not required in the majority of such cases. The clinical trends of tumor markers were also taken into account. When angiography could not be performed, the hepatic mass was considered HCC when CT showed a hypervascular mass and the tumor marker level was elevated. No fine needle biopsy or histopathological examination was performed before treatment.

The date of the last follow-up in this study was March 1, 2007. The median observation period of the entire group was 8.3 years with a range of 0.0 to 19.0 years. As for patients of the combination therapy, the median follow-up period was 3.2 years with a range of 0.0 to 7.5 years.

Statistical analysis

Non-parametric procedures were employed for the analysis of background clinicopathological parameters, including Mann-Whitney U-test. The rate of hepatocarcinogenesis was calculated for the period between the end of IFN therapy and appearance of HCC, using Kaplan-Meier technique (33). Differences in carcinogenesis curves were tested using the log-rank test. Independent factors associated with the appearance of HCC were studied using stepwise Cox regression analysis (34). The following seven variables were analyzed for potential covariates for liver carcinogenesis; age, sex, fibrotic stage of hepatitis at the initiation of the IFN therapy, HCV genotype, use of ribavirin (monotherapy or combination therapy), type of IFN (α or β, and number of treatments. Factors found significant were entered into a multivariate Cox proportional hazard model. A P-value less than 0.05 was considered significant. Data were analyzed using the SPSS software ver. 11.0.1J (SPSS Inc., Chicago, IL).

Crude rates of hepatocarcinogenesis

During a median observation period of 8.3 years with a range of 0.0 to 19.0 years, HCC was diagnosed in 23 (1.9%) of the 1,193 patients. The median interval between the end of therapy and detection of HCC was 3.1 years (range, 0-12.9 years).

The characteristics of HCC patients are shown in Table 2. Patients who developed HCC before the initiation of IFN therapy were excluded. Four patients (Nos. 2, 5, 15 and 22) developed HCC before the diagnosis of SVR but after the elimination of HCV RNA. The surgically resected liver tissue was also examined by the PCR method in 4 cases (Nos. 1, 6, 13 and 14), which showed no HCV RNA.

HCC patients included 21 men and 2 women; the median age at the start of IFN therapy and at diagnosis of HCC was 58 (range, 50-70) and 62 (51-76), respectively. The HCV genotype was 1 in 9 patients and 2 in 14 patients. Chronic hepatitis was diagnosed in 14 patients (F1/F2/F3=2/12/0) and cirrhosis in 9, at the time of initiation of IFN therapy. The type of therapy for hepatitis was IFN monotherapy in 22 patients and the combination therapy in 1. The number of HCC tumors was one in 18 patients, two in 3 patients and more than two in 2 patients. A typical hypervascular mass on angiography or perfusion defect on CT during arterial portography (CT-AP) was noted in 20 patients. Angiography could not be performed in the other three patients; they had a hypo-enhanced, iso enhanced, and hyper-enhanced tumor on CT, respectively. Treatment was radical in 21 patients; including hepatectomy in 17 and percutaneous locoregional therapy in 4 patients. At the time of surgical resection, the fibrosis staging was histopathologically examined in 16 patients. Twelve patients was diagnosed as hepatitis (F1/F1-2/F2/F3=2/1/6/3) and 4 as cirrhosis. In

Table 2. Carcinogenesis after HCV RNA Elimination

No	Gender	Age at the start of IFN	Age at the carcinogenesis	Genotype	Type of IFN	Fibrosis staging before IFN Tx	Interval between the end of IFN Tx and carcinogenesis, yr	Number of Tumor	Tumor size, mm	Treatment for HCC	Fibrosis staging at the time of carcinogenesis	Differentiation of HCC
1	M	50	51	1b	α	F2	1.0	1	15	Hepatectomy	F3	Moderate
2	M	52	54	2a	α	F1	0.6	1	18	Hepatectomy	F2	Well
3	F	54	59	2a	α	F2	3.5	1	17	Hepatectomy	F1-2	Moderate
4	M	55	60	1b	α	F2	3.7	1	16	Hepatectomy	F2	Moderate
5	M	55	56	1b	Peg α+Rib	F2	0.0	1	21	RFA	-	-
6	M	55	57	2a	α2a	F4	1.9	1	19	Hepatectomy	F4	Moderate
7	M	57	67	2	α	F2	8.9	1	47	Hepatectomy	F1	Moderate
8	M	55	59	2a	α2a	F4	3.1	1	18	Hepatectomy	F4	Moderate
9	M	55	62	1b	β	F4	6.5	1	16	Hepatectomy	F4	Poor
10	M	57	58	1b	α	F2	0.9	1	16	Hepatectomy	F2	Moderate
11	M	57	59	1b	α	F2	1.2	1	20	Hepatectomy	F2	Moderate
12	M	58	66	2a	α2b	F1	8.7	1	26	Hepatectomy	F1	Well
13	M	58	62	1b	β	F2	3.9	1	30	Hepatectomy	F2	poor>moderate
14	M	59	69	2b	α	F2	9.1	1	21	Hepatectomy	F2	Moderate
15	M	59	61	1b	α	F4	0.1	1	30	Hepatectomy	F3	poor>moderate
16	M	61	63	2	α	F2	1.8	4+LN meta	23	Hepatectomy+MCT	F3	moderate>well
17	M	62	65	2a	β	F4	2.4	2	20,20	RFA	-	-
18	M	62	75	2a	α2a	F4	12.9	1	23	Hepatectomy	F4	Moderate
19	M	63	66	2a	β	F2	3.6	Uncountable	Diffuse	No treatment	-	
20	M	65	71	2a	α	F2	5.0	2	12, 8	Hepatectomy	-	Necrosis
21	F	66	68	2a	α	F4	0.9	1	13	RFA	-	-
22	M	69	72	1b	β	F4	0.4	2	13, 13	Hepatectomy	-	moderate, poor
23	M	70	76	2a	β	F4	5.4	1	10	RFA+PMCT	-	-

IFN: interferon, Tx: therapy, Peg: pegylated interferon, Rib: ribavirin, LN meta: lymph node metastasis,
RFA: radiofrequency ablation, MCT: microwave coagulation therapy, PMCT: percutaneous microwave coagulation therapy.

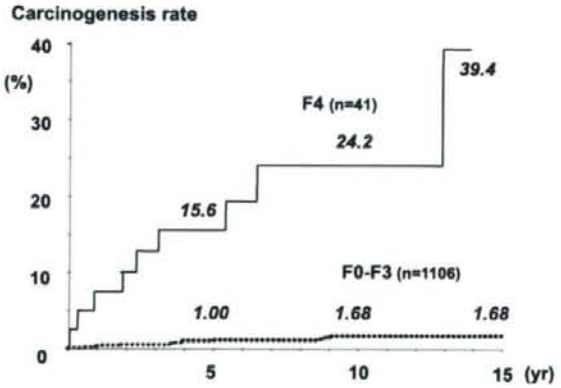

Figure 1. **Rates of hepatocarcinogenesis in 41 patients with cirrhosis (F4) and 1,106 patients with liver fibrosis stage F0-F3.**

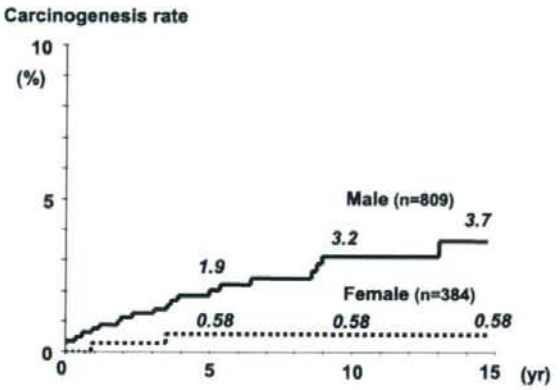

Figure 2. **Rates of hepatocarcinogenesis in 809 male patients and 384 female patients.**

comparison with the staging at the initiation of IFN therapy, 3 cases showed improvement in the fibrosis, 10 showed no change, and 3 showed progression.

The crude rates of hepatocarcinogenesis in the SVR patients were 1.5%, 2.4% and 2.7% at the end of the 5th year, 10th year and 15th year, respectively.

Determinants of hepatocarcinogenesis

The rate of carcinogenesis was significantly higher in 41 patients with cirrhosis (F4) than in 1,106 patients with liver fibrosis of F0-F3 ($p<0.0001$, Fig. 1). The respective cumulative HCC development rates in patients with cirrhosis at 5, 10, and 15 years after SVR were 15.6%, 24.2% and 39.4%. On the other hand, the respective rates for patients with F0-F3 were 1.00%, 1.68% and 1.68% at 5, 10, and 15 years after SVR. When patients were divided into two groups with F2-4 and with F0-1, rates of the former group were 4.16%, 6.52% and 7.58%, while those of latter group were 0.13%, 0.40% and 0.40%. The incidence rates of HCC increases with the fibrotic stage; those with F1, F2 and F4 were 0.14%, 3.43% and 15.6% at 5 years, 0.40%, 5.22% and 24.2% at 10 years, and 0.40%, 5.22% and 39.4% at 15 years, respectively.

The rate of hepatocarcinogenesis among 809 male patients was significantly higher than among 384 female patients ($p=0.018$, Fig. 2); the respective rates at 5, 10 and 15 years were 1.87%, 3.18% and 3.67% for males and 0.58%, 0.58% and 0.58% for females.

The rate of hepatocarcinogenesis among 570 patients aged >50 years was greater than among 623 patients aged <51 years at the start of IFN therapy ($p<0.0001$, Fig. 3); the respective rates at 5, 10 and 15 years for the former group were 2.92%, 4.93% and 5.81%, compared with 0.16%, 0.16% and 0.16% for the later.

Multivariate analysis identified three factors to be associated with the rate of development of HCC: sex, age at start IFN treatment, and fibrotic stage in the liver tissue. Multi-

variate analysis was performed using non-time dependent proportional hazard analysis. Fibrotic stage, sex, and age were identified as significant independent factors that influenced the rate of future hepatocarcinogenesis (Table 3). Cirrhosis (F4) was associated with a higher risk of hepatocarcinogenesis with a hazard ratio of 12.9 (95% confidence interval, 5.5-30.6, p<0.001) compared with F1-3 stage. Similarly, male sex (6.45, p=0.012) and older age than 50 years (20.2, p=0.004) were associated with a higher risk. Serological grouping of HCV, type of therapy (monotherapy or combination therapy), type of IFN of the final therapy, and number of therapies did not significantly influence the rate of hepatocarcinogenesis. When the patients were divided into two groups with F0-1 and with F2-4, hazard ratios of F2-4, male and older age were 13.4 (3.1-57.8, p<0.0001), 7.00 (1.63-29.99, p=0.0009) and 17.6 (2.3-131.6, p=0.005). When the patients were divided into two groups with F0-2 and F3-4, hazard ratios of F3-4, male and older age were 5.8 (2.5-13.8, p<0.0001), 6.78 (1.58-29.07, p=0.01) and 22.9 (3.0-172.2, p=0.002).

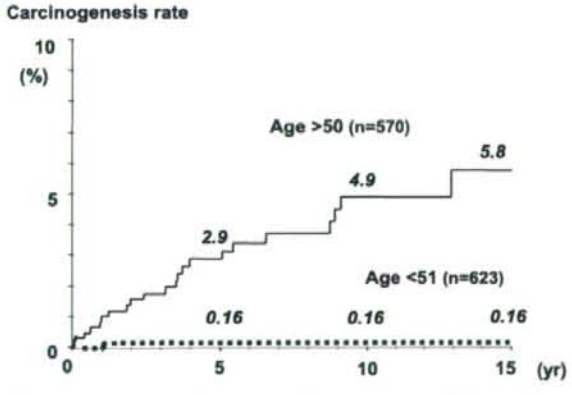

Figure 3. **Rates of hepatocarcinogenesis in 570 patients older than 50 and 623 patients younger than 51 years.**

Discussion

Epidemiological data on the rate of development of HCC in patients with chronic hepatitis (35) and those with cirrhosis (36) indicate that the life expectancy of patients with HCV-related chronic liver disease is significantly influenced by the development of HCC. Up to 75% of patients with HCV infection and cirrhosis eventually develop HCC (15). IFN can be considered to have anti-carcinogenic properties through its anti-inflammatory action, since several studies have already described that the cancer suppressive activity of IFN in those patients who show HCV RNA eradication was similar to that of patients with ALT normalization without HCV RNA elimination (BR) (15, 37-40). After excluding patients with cirrhosis, the previous report (40) showed that the rate of carcinogenesis was lower in patients with SVR than in those with BR because HCV-elimination does not result in re-elevation and exacerbation of ALT. As a follow-up to the above studies, the rate of hepatocarcinogenesis in SVR patients with either chronic hepatitis or cirrhosis was estimated in the present study.

In spite of the anti-carcinogenic effect of SVR, 23 cases developed HCC following elimination of HCV RNA among 1,193 patients. The median interval between the end of IFN therapy and carcinogenesis is 3.1 years with a range of 0.0 to 12.9 years. Among 23 cases, 22 patients had regular examinations of at least once a year, and 21 of them received radical treatment such as hepatectomy or radiofrequency ablation. The high rate of radical treatment was probably due to the preserved liver function after HCV RNA elimination.

HCCs in six cases that were detected in the year after the end of the interferon therapy could have been already present before elimination of HCV RNA. Even when we exclude these cases, multivariate analysis identified the same factors such as higher histological stage, male sex and age older age as determinants of hepatocarcinogenesis. The haz-

Table 3. **Factors Associated with Hepatocarcinogenesis in Sustained Virological Responders with Chronic HCV Infection**

Factors	Category	Hazard ratio	95% confidence interval	p
Fibrotic stage	1: F0-3	1		
	2:F4	12.9	(5.5-30.6)	<0.001
Gender	1: women	1		
	2: men	6.45	(1.51-27.64)	0.012
Age (years)	1: <51	1		
	2: >50	20.2	(2.7-152.9)	0.004

ard ratios of cirrhosis, male sex and age older than 50 years were 10.9 (4.0-29.8, p<0.001), 10.4 (1.4-78.2, p=0.024) and 17.0 (2.2-130.7, p=0.006), respectively.

The rates of hepatocarcinogenesis in patients with histological stage F0-F3 were 1.00%, 1.68%, 1.68% at 5, 10, and 15 years after SVR, respectively. These rates were about 20% less than the rates reported previously for patients with chronic hepatitis; 4.8%, 13.6%, and 26.0%, respectively (35). The rates of hepatocarcinogenesis in patients with cirrhosis were 15.6%, 24.2%, and 39.4% at 5, 10, and 15 years, respectively, which were about 65% less than the rates reported previously for patients with cirrhosis; 21.5%, 53.2% and 75.2%, respectively (36). These results indicate that IFN has a more marked effect in reducing the rate of hepatocarcinogenesis in patients with F0-F3 than in those with cirrhosis. Furthermore, the difference in the rate of hepatocarcinogenesis in patients who show SVR and those with chronic HCV-infected patients increases with time, since the likelihood of development of HCC before elimination of HCV RNA decreases as time passes after IFN therapy.

Although some studies have reported that elderly male patients with severe fibrotic stage could be at a high risk for hepatocarcinogenesis even when they show SVR, the hazard ratios in such patients have not been reported probably because of shorter follow-up period and the relatively small number of patients. In this study, the follow-up period was longer than that of previous studies, allowing meaningful multivariate analysis (e.g., Cox hazards model). The results of such analysis showed that the risk of carcinogenesis increases with the histologic stage of the liver, age and male sex. This finding was similar to that reported in a study of untreated patients (41, 42) or IFN-treated hepatitis patients with the histological stage of F0-F3 (15).

Treatment of patients with chronic HCV infection using PEG IFN-α and ribavirin resulted in persistently negative tests for serum HCV RNA in 40-50% of patients with HCV genotype 1 and 75-80% with HCV genotype 2 or 3. The present study also showed that neither type of IFN (α or β) nor the use of ribavirin altered the rate of carcinogenesis. Further studies are needed with a longer follow-up period since the follow-up period of patients treated with the combination therapy ranged from only 0 to 7.5 years (median 3.2years) and was shorter than that of patients who received IFN monotherapy.

In conclusion, the results emphasize the importance of long-term follow-up of patients with chronic HCV infection, even those who show SVR to IFN therapy, especially male elderly patients with severe fibrosis of the liver.

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INTERNAL MEDICINE

ORIGINAL ARTICLE

Natural Human Interferon β Plus Ribavirin Combination Therapy in Japanese Patients Infected with Hepatitis C Virus and a High Viral Load

Yoshio Katamura[1], Fumitaka Suzuki[1], Norio Akuta[1], Hitomi Sezaki[1], Hiromi Yatsuji[1], Norihiro Nomura[1], Yusuke Kawamura[1], Tetsuya Hosaka[1], Masahiro Kobayashi[1], Yoshiyuki Suzuki[1], Satoshi Saito[1], Yasuji Arase[1], Kenji Ikeda[1], Mariko Kobayashi[2] and Hiromitsu Kumada[1]

Abstract

Objective The aim of this pilot study was to determine the safety and efficacy of natural human interferon β (nIFNβ) plus ribavirin (RBV) in patients with chronic hepatitis C who did not respond to pegylated interferon alpha (PEG-IFN), with special emphasis on the incidence of mental disorders or refusal for fear of adverse effects.

Methods We studied 19 patients with HCV genotype 1b, 2a or 2b and a high viral load, including 8 patients with mental disorders. They were treated with nIFNβ-RBV. Eleven patients with HCV genotype 1b of these patients were treated with nIFNβ-RBV for 48 weeks (group A), and compared with 22 matched controls treated with PEG-IFN plus RBV for 48 weeks (group B). The other 8 patients with HCV genotype 2 were treated with nIFNβ-RBV for 24 weeks.

Results Six of 8 patients with mental disorders and 9 of 11 patients without mental disorders completed nIFNβ-RBV therapy; 1 patient with mental disorder dropped out due to exacerbation of depression, and 3 patients suspended the therapy due to insufficient response. The sustained virological response (SVR) was 27% (3/11) in group A and 41% (9/22) in group B ($p = 0.70$). During treatment, platelet count increased in group A but not in group B. SVR was 88% (7/8) in patients of genotype 2 and high viral load treated with nIFNβ plus RBV.

Conclusion nIFNβ-RBV therapy offers sufficient safety and efficacy for patients with mental disorders, and thus could represent an excellent second-line therapy for subpopulations that are not suitable for PEG-IFN-RBV.

Key words: hepatitis C virus, interferon β, ribavirin, depression

(Inter Med 47: 1827-1834, 2008)
(DOI: 10.2169/internalmedicine.47.1436)

Introduction

Pegylated interferon α (PEG-IFN) plus ribavirin (RBV) is the first line treatment for patients infected with hepatitis C virus (HCV) genotype 1, and high viral load, and can achieve sustained virological response (SVR) in 41-47% of these patients (1-3). However, such treatment causes adverse effects in some patients, such as mental disorders, apathy and laboratory abnormalities. Previous studies indicated that 10-16% of patients treated with PEG-IFN plus RBV for 48 weeks discontinued the therapy due to adverse effects, especially depression (1-3). Individuals with depression or previous history of interferon (IFN) α-induced mental disorders

[1]Department of Hepatology, Toranomon Hospital, Tokyo and [2]Research Institute for Hepatology, Toranomon Hospital, Tokyo
Received for publication June 21, 2008; Accepted for publication July 15, 2008
Correspondence to Dr. Fumitaka Suzuki, fumitakas@toranomon.gr.jp

are not suitable candidates for IFNα therapy (4). Moreover, several patients reject the therapy for fear of depression arising as a side effect. IFNβ, a type I IFN that binds to the same cell surface receptor as IFNα, triggers distinct biological responses and elicits distinct patterns of gene expression within the same target cells (5-8). Three forms of human IFNβ are available (9): *1)* Natural human IFNβ (nIFNβ) is produced by human fibroblasts. *2)* Recombinant human IFNβ-1a (rhIFNβ-1a) is procured by mammalian cells and is identical to nIFNβ. *3)* Recombinant human IFNβ (rhIFNβ-1b) is produced by *Escherichia coli* in which cysteine at position 17 is substituted by serine. Previous reports showed that IFNβ has sufficient tolerability (10). Other reports indicated that IFNβ is effective in HCV eradication, although it seems that IFNβ monotherapy does not result in a satisfactory outcome in patients with HCV, particularly those infected with genotype 1 and have a high viral load (11, 12). Recent randomized trials demonstrated the efficacy of rhIFNβ-1a plus RBV (13, 14). However, there is little information regarding nIFNβ plus RBV (15, 16). Furthermore, the safety of IFNβ for patients with mental disorders has not been reported.

There is evidence to suggest that monitoring of early viral kinetics is useful for earlier identification of the likelihood of response to IFN therapy (17). Correlation viral kinetics and therapeutic outcome of PEG-IFN plus RBV has been investigated (18, 19), but not that of IFNβ plus RBV.

The present pilot study included 19 patients who had not received PEG-IFN or IFNα for their mental disorders or refused PEG-IFN for fear of adverse effects. The objective of this study was to assess the safety and efficacy of nIFNβ plus RBV in Japanese patients infected with HCV genotype 1b or 2 and high viral load. In addition, we assessed viral kinetics in patients infected with HCV genotype 1b with high viral load treated with nIFNβ plus RBV.

Patients and Methods

Study population

Nineteen HCV-infected Japanese patients were enrolled in this trial between 2001 and 2006 at Toranomon Hospital, Tokyo. The enrollment criteria were HCV genotype 1b, 2a or 2b confirmed by polymerase chain reaction (PCR) ; serum HCV RNA levels >100,000 international units (IU)/mL on quantitative PCR assay (defined as "high" viral load, Amplicor HCV Monitor version 2.0, Roche Diagnostics, Tokyo, Japan) ; No treatment with corticosteroids, immunosuppressants, or antiviral agents within 6 months prior to this trial; negativity for hepatitis B surface antigen (HBsAg), as determined by radioimmunoassay; hemoglobin concentration >12.0 g/dL; neutrophil count >1,500/μL; platelet count >60,000/μL; serum creatinine <1.5 times above the upper limit of normal; and body weight between 40 and 100 kg. The exclusion criteria were liver cancer or severe liver failure; pregnant or breastfeeding women; past history of hypersensitivity reactions to IFN or ribavirin. Psychiatric exclusion criteria included preexisting severe depression and suicidal ideation and/or attempt.

Eleven patients with HCV genotype 1b treated with nIFNβ plus RBV were defined as group A. To compare the clinical efficacy of the treatment, we retrospectively selected 22 patients treated with PEG-IFN plus RBV, matched 1:2 with patients of group A for genotype, sex, age, and response to previous IFNα or IFNα plus RBV (control group; group B). Patients of group B were selected from among 407 patients of Toranomon Hospital.

Study protocol

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each subject. Treatment was provided for 48 weeks to HCV genotype 1b, and for 24 weeks to HCV genotype 2a or 2b, with subsequent 24-week follow-up period.

nIFNβ group: 11 patients with HCV genotype 1b (group A), were treated with nIFNβ (Feron, Toray Industries Inc., Tokyo) intravenously at a dose of 6 million units (MU) daily for 2-8 weeks, followed by three times a week for 40-46 weeks (total 48 weeks). In group A, nIFNβ was administrated daily for 2 weeks to 6 patients, for 4 weeks to 1 patient and for 8 weeks to 4 patients. Eight patients with HCV genotype 2 were treated with nIFNβ intravenously at a dose of 6 MU daily for 2-8 weeks and then three times a week for 16-22 weeks (total 24 weeks).

Control group: 22 patients with HCV genotype 1b (group B) were treated with PEG-IFNα2b (Schering-Plough, Osaka, Japan) subcutaneously at a dose of 1.5 μg/kg weekly for 48 weeks.

Each patient was treated with oral RBV (Schering-Plough) at a total dose of 600-1,000 mg twice daily for 48 weeks for HCV genotype 1b and for 24 weeks for HCV genotype 2. The dose was adjusted according to body weight (600 mg for patients weighing ≤60 kg, 800 mg for those between 60 and 80 kg, and 1,000 mg for patients weighing between 80 and 100 kg). Both nIFNβ or PEG-IFNα2b and RBV were concurrently initiated.

Serum samples were collected from the patients at 0, 2 days, and 2, 4, 12 weeks, at the end of therapy, and 24 weeks after the end of therapy. HCV RNA in serum was quantified at each point by a quantitative reverse-transcription polymerase chain reaction (PCR) assay (Cobas Amplicor HCV Monitor version 2.0 using the 10-fold dilution method, Roche) with a low detection limit of 5 KIU/mL. When HCV RNA was undetectable by quantitative PCR assay, it was assessed using qualitative detection assay (Amplicor HCV, Roche) with a low detection limit of 50 IU/mL. End-of-treatment response (ETR) was defined as no detectable serum HCV RNA at the end of treatment. SVR was defined as no detectable serum HCV RNA at 24 weeks after the end of treatment.

Table 1. **Clinical Characteristics of Chronic Hepatitis C Patients with High Viral Load Treated with Combination Therapy of nIFNβ Plus Ribavirin**

n	19
Age (years)*	60 (33-73)
Gender (male/female)	9/10
Leukocytes (/μL)*	4600 (2300-7500)
hemoglobin (g/dL)*	14.3 (10.5-15.9)
Platelets ($\times 10^3$/μL)*	19 (6.8-25.4)
Alanine aminotransferase (IU/L)*	60 (24-726)
Genotype (1b/2a/2b)	11/4/4
HCV-RNA (KIU/mL)*	1100 (400-5000)
Histology (F: 1/2/3/4/ND)	3/6/1/1/8
Interferon: naive/retreatment	9/10
Previous IFN therapy	
Virological response : yes/no	6/4
Monotherapy / combination therapy	9/1

*Data represent the median (range) values.

nIFNβ, natural human interferon β; IFN, interferon

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

Dose reduction of IFN and RBV

nIFNβ was reduced from 6 MU to 3 MU when neutrophil count decreased to <750/μL or platelet count to <30,000/μL. Furthermore, the dose of PEG-IFNα2b was reduced from 1.5 to 1.0-0.5 μg/kg/week if neutrophil count decreased to <750/μL or platelet count to <80,000/μL. RBV was reduced in a stepwise fashion by 200 mg/day when hemoglobin concentration decreased to 10 g/dL. Further dose reduction or discontinuation of these drugs was applied for ongoing hematological adverse effects or other unendurable adverse effects such as mental disorder, flu-like syndrome, and gastrointestinal symptoms.

Statistical analysis

Treatment outcome was analyzed on intention-to-treat basis. Mann-Whitney U test or Fisher's exact probability test was used for comparison between groups. All p values for statistical tests were two-tailed and those <0.05 were considered to denote a significant difference. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Clinical background

The nIFNβ group consisted of 19 patients; 5 (26%) patients developed depression or had a history of depression, 3 (16%) had a history of IFNα-induced depression, 7 (37%) refused PEG-IFN for fear of adverse effects such as depression, 3 (16%) were older than 65 years, and 1 (5%) suffered from severe fatigue associated with previous IFNα therapy.

In all patients who developed depression during treatment or had previous history of depression, depression was diagnosed by psychiatrists at our hospital. Table 1 shows the clinical features of 19 patients treated with nIFNβ plus RBV. Ten (53%) patients had been treated with IFN previously. Among the retreatment patients, 4 (40%) were nonresponders to previous IFN therapy. Table 2 shows the clinical features of 11 patients with HCV genotype 1 and high viral load treated with IFNβ plus RBV (case; group A) and 22 patients treated with PEG-IFN (control; group B) groups. There were no significant differences between the two groups in HCV-RNA level, fibrosis score, and laboratory pa-

Table 2. **Comparison of Clinical Features of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin**

	Group A (IFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
n	11	22	
Age (years)*	57 (36-67)	54 (29-67)	matched
Sex (male/female)	7/4	14/8	matched
Interferon: naive/retreatment	4/7	8/14	matched
Previous IFN therapy			
Virological response: yes/no	4/3	8/6	matched
Monotherapy/IFN+RBV	6/1	12/2	matched
HCV-RNA (KIU/mL)*	1300 (530-3400)	2200 (370-4500)	0.26
Leukocytes (/μL)*	4700 (2300-6000)	4350 (2800-7100)	0.65
Hemoglobin (g/dL)*	14.7 (12.8-15.9)	14.5 (12.8-16.4)	0.88
Platelets ($\times 10^3$/μL)*	18.8 (9-24.9)	15.6 (9-25)	0.47
Alanine aminotransferase (IU/L)*	60 (40-156)	69 (28-237)	0.29
γ-glutamyl transpeptidase (IU/L)*	43 (15-106)	43 (20-244)	0.38
LDL-C (mg/dL)*	105 (52-162)	109 (50-162)	0.70
ICG-R(15) (%)*	11 (5-16)	12 (8-45)	0.052
RBV dose/body weight (mg/kg)*	11.8 (11.1-13.3)	11.1 (2.7-14)	0.063
Histology (F: 1/2/3/4/ND)	2/3/1/1/4	11/5/4/0/2	0.23

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α;

*Data represent the median (range) values.

IFN, interferon; RBV, ribavirin; LDL-C, low density lipoprotein cholesterol

ICG-R(15), indocyanine green retention rate at 15 minutes

Liver fibrosis classified as: F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; ND, not done.

rameters shown in Table 2.

Safety profile of nIFNβ plus RBV therapy

Table 3 shows the clinical features of 8 patients (genotype 1b; n=5, genotype 2; n=3) who developed mental disorders. One patient (12.5%) received antidepressant, and 2 patients (25%) received anti-anxiety drugs at the start of the treatment. During the therapy, those 3 patients did not need additional drugs. On the other hand, 1 patient (12.5%) received anti-anxiety drugs but such treatment was discontinued due to exacerbation of depression at 32 weeks after the start of therapy, and 1 patient (12.5%) received antidepressant during the therapy. The remaining 3 patients (37.5%) did not need anti-anxiety drugs/antidepressants. Among the remaining 11 patients with no history of mental disorder, 1 patient (9%) received anti-anxiety drugs during the therapy.

nIFNβ dose reduction was necessary in 1 (5.3%) patient due to the development of neutropenia. RBV dose reduction was applied in 9 (47%) patients, due to anemia (n=8) and extensive skin eruption (n=1). Therapy was suspended in 3 (16%) patients due to insufficient response to the therapy.

In the control group (n=22), treatment was discontinued in 2 (9%) patients due to adverse effects (fatigue and depression). Eight (36%) patients required PEG-IFN dose re-

Table 3. **Clinical Features of Patients with Mental Disorders Treated with nIFNβ Plus Ribavirin**

Patient No.	Gender	Age	Genotype	Mental disorder	Prescription at start of therapy	Prescription during therapy	nIFNβ plus ribavirin	Therapy duration (W)	Virological response
1	F	60	1b	depression	none	Anti-anxiety drug	Dropped out due to depression	32	NR
2	M	46	1b	depression	antidepressant	keeping on	Dropped out due to insufficient response	24	NR
3	M	49	1b	depression	none	antidepressant	completed	48	NR
4	M	40	1b	IFN-Induced	none	none	completed	48	SVR
5	M	60	1b	IFN-Induced	none	none	completed	48	NR
6	F	57	2a	depression	antianxiety drug	keeping on	completed	24	SVR
7	F	63	2a	IFN-Induced	antianxiety drug	keeping on	completed	24	SVR
8	F	60	2b	depression	none	none	completed	24	SVR

nIFNβ, natural human interferon β; IFN, interferon; SVR, sustained viral response; NR, non-responder

Table 4. **Virological Response of Patients with Genotype 1b and High Viral Load Treated with nIFNβ Plus Ribavirin and PEG-IFN Plus Ribavirin**

	Group A (nIFNβ+RBV)	Group B (PEG-IFN+RBV)	p value
End-of-treatment response	5/11 (45%)	15/22 (68%)	0.270
Sustained virological response	3/11 (27%)	9/22 (41%)	0.703

nIFNβ, natural human interferon β; PEG-IFN, pegylated interferon α; RBV, ribavirin

duction due to fatigue (n=2), dizziness (n=1), neutropenia (n=4) and thrombocytopenia (n=1). RBV dose reduction was applied in 12 (55%) patients due to anemia. Therapy was suspended in 2 (9%) patients at 24 weeks after commencement due to insufficient response.

Efficacy of IFNβ plus RBV therapy

Table 4 shows the ETR and SVR rates of groups A and B. ETR and SVR were achieved in 45% (5/11) and 27% (3/11) patients of group A (nIFNβ plus RBV) and in 68% (15/22) and 41% (9/22) patients of group B (PEG-IFN plus RBV), respectively. Differences between groups A and B were not significant ($p = 0.27$ and 0.70, respectively). In patients of genotype 2 and high viral load, ETR and SVR to nIFNβ plus RBV therapy were achieved in 88% (7/8) and 88% (7/8) patients, respectively.

Profile of leukocyte count, hemoglobin concentration and platelet count

Figure 1 shows profile of leukocyte count, hemoglobin concentration, platelet count of groups A and B. These parameters were assessed at week 2, week 4, and every 4 weeks until week 48. In groups A and B, the average leukocyte count, hemoglobin concentration and platelet count at baseline were 4,540/mm^3 and 4,590/mm^3, 14.4 g/dL and 14.6 g/dL, 17.4×10^4/mm^3 and 16.1×10^4/mm^3, respectively. There were no significant differences in leukocyte count and hemoglobin concentration between groups A and B at each time point. However, the dynamics of platelet count was different between the two groups. In group A, the platelet count decreased to 15.1×10^4/mm^3 at week 2, but increased above baseline after week 4. In groups A and B, the platelet count was significantly different at week 4, 8, 12, 16, 20, 24, 40, 44 and 48, respectively.