Table 2 Patient characteristics at the start of entacavir therapy in rtM204I alone and mix groups

	rtM204I	Mix (rtM204I + rtM204V)
Total number	7	11
Sex (female/male)	1/6	1/10
Age (years)†	37 (34–65)	39 (29–55)
Alanine aminotransferase (IU/L)†	119 (54–347)	112 (52–251)
Serum HBV-DNA‡ (Amplicor, log copy/mL)†	> 7.6 (6.2–>7.6)	> 7.6 (7.2–>7.6)
HBeAg (positive/negative)	3/4	10/1
HBV genotype (A/C)	0/7	1/9
Duration of lamivudine therapy (month)†	31 (19–47)	36 (10–48)

†Data are median (range).
‡HBV-DNA levels were measured by Amplicor HBV Monitor assay. HBV-DNA values below the lower limit of detection are listed as 2.6 log copy/mL and those over the upper limit of detection as 7.6 log copy/mL.
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

motif type was mixed. Therefore, none of the seven patients with both the rtM204I + rtM204V mixed-type and precore wild-type showed a negative result on the Amplicor HBV Monitor assay at 76 weeks.

Entecavir-resistant mutant during therapy

Analysis of the rt region sequences (amino acids 1–344) of HBV polymerase in one patient (no. 11) at 52 weeks showed a new substitution of rtS202G in addition to the lamivudine substitutions (rtL180M and rtM204V), which may indicate introduced ETV resistance.[19] Virological rebounds of rtM204V viral load of this patient were observed at 40 and 52 weeks (increase of 1.27 and 1.08 log copies from nadir by real-time PCR). There were no patients with virological rebounds except this patient (no. 11).

Changes in precore and core promoter sequences before and during therapy

Precore and core promoter sequences in 18 patients were analyzed during 1 year of treatment with ETV for lamivudine-breakthrough hepatitis. Precore sequences at baseline for lamivudine were the same as those at baseline for ETV in 10 of 18 patients (excluding one lacking lamivudine baseline data; Table 3). Analysis of serum samples obtained at ETV baseline revealed a precore stop codon mutation (*G1896A*) in nine of 18 patients. After the start of ETV, *G1896A* was replaced by wild-type virus in two patients (nos. 1 and 5) at 1 year. However, G1896 was replaced by *G1896A* in one patient (no. 11) with ETV resistance. Thus, *G1896A* was observed in five of 14 patients, excluding four PCR-negative patients, at 1 year.

Core promoter sequences at baseline for lamivudine therapy were the same as those at baseline for ETV in 15 of 18 patients (Table 3). Among the 18, 15 had core promoter mutations (A1762T and G1764A) in samples collected at ETV baseline. During treatment, core promoter mutations at baseline were similar to those at 1 year.

YMDD mutant type was changed after 1 year of ETV treatment in three patients (nos. 1, 7 and 13), with the baseline rtM204I + rtM204V mixed types replaced by the respective major YMDD mutant.

DISCUSSION

PATIENTS WHO RECEIVE lamivudine therapy may be treated for an extended period, increasing the probability of viral resistance and loss of clinical efficacy, involving an associated risk of increased viral replication, flares of ALT levels, and progression of liver disease.[5,8–10,28,29] ETV has previously been shown to be superior to lamivudine in the treatment of chronic HBV in nucleoside-naïve patients infected with wild-type HBV.[14] Moreover, a recent report showed that treatment with ETV at 0.5 or 1.0 mg daily was well tolerated, and resulted in significant reductions in HBV-DNA levels as well as normalization of ALT levels in HBeAg-positive and -negative lamivudine-refractory patients.[17] Although ETV in the recent report[17] was more effective at 1.0 than 0.5 mg, no differences between the two groups were seen in changes in viral load in the present study. Moreover, results for viral load changes by Amplicor HBV Monitor assay were the same in the present study (data not shown). This difference between these studies

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

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

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

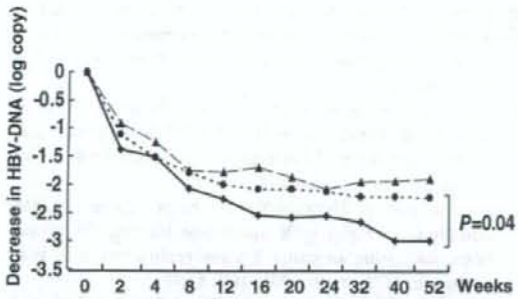

Figure 2 Mean log changes in the viral loads of each mutant of rtM204I alone and of rtM204I + rtM204V mixed type from baseline during the initial 52-week treatment with entacavir. HBV-DNA levels of rtM204I and rtM204V were measured by real-time polymerase chain reaction. HBV, hepatitis B virus. (◆),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

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

It has been reported that ETV is most effective against wild-type HBV (YMDD) and shows almost equally effective inhibition of the replication of rtM204I, rtL180M/rtM204V (rtM204V with rtL180M) and rtL180M/rtM204I (rtM204I with rtL180M) *in vitro*.[12,30] Supporting this, our study showed that rtM204I and rtM204V had similar sensitivity to ETV *in vivo*. In our study, however, rtM204I only or the presence of a precore mutation was more sensitive to ETV. These findings were seen mainly in patients without HBeAg. Moreover, rtM204I only or the presence of a precore mutation was also present in three HBeAg-positive patients (nos. 1, 3 and 5) whose HBV-DNA levels were negative on Amplicor HBV Monitor assay at 76 weeks. Our recent report also showed the greatest change in viral load was seen for rtM204I without HBeAg during adefovir dipivoxil (ADV) in addition to ongoing lamivudine therapy.[25] Considering these data, rtM204I virus is more sensitive to antiviral nucleoside analogs.

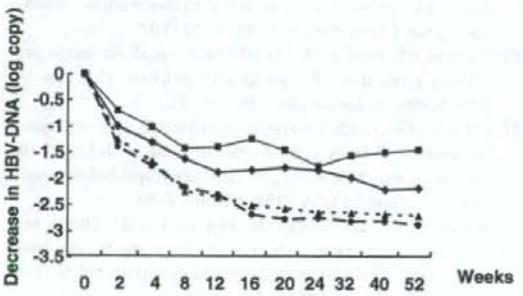

Figure 3 Mean log changes in the viral loads of rtM204I or rtM204V with or without *G1896A* from baseline during the initial 52-week treatment with entacavir. HBV-DNA levels of rtM204I and rtM204V were measured by real-time polymerase chain reaction. HBV, hepatitis B virus. (■), rtM204V without *G1896A*;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

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

In a recent investigation of lamivudine-resistant viruses, additional substitutions at rtT184, rtS202, or rtM250 were shown to further reduce ETV susceptibility.[19] The present and above studies add those at rtL180M and rtM204V to this list. One of our patients had rtS202G substitution in addition to rtL180M and rtM204V. Importantly, however, breakthrough hepatitis may occur even when the rate of substitutions (ETV-resistant) is not high.[19] Taken together, these findings suggest that ETV therapy may not be beneficial in

patients with either or both YVDD and no precore mutant.

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

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Substitution of Amino Acid 70 in the Hepatitis C Virus Core Region of Genotype 1b Is an Important Predictor of Elevated Alpha-Fetoprotein in Patients Without Hepatocellular Carcinoma

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Previous studies identified amino acid (aa) substitutions of the hepatitis C virus core region of genotype 1b (HCV-1b core region) and elevated serum alpha-fetoprotein (AFP) levels as predictors of poor virologic response to pegylated interferon (PEG-IFN) plus ribavirin (RBV), and also as risk factors for hepatocarcinogenesis. The present study evaluated the impact of aa substitutions of HCV-1b core region on AFP, as a surrogate marker of hepatocarcinogenesis, on AFP levels in 569 Japanese patients with HCV-1b but without HCC, and investigated the predictive factors of elevated AFP ($\geq$11 μg/L). High AFP levels were detected in 27.4% of the patients. The rate of hepatocarcinogenesis in a group of 109 patients who received IFN monotherapy and followed-up for 15 years, was significantly higher in patients with abnormal than normal AFP. Multivariate analysis of 569 patients identified fibrosis stage (*F*3,4), aspartate aminotransferase ($\geq$76 IU/L), substitution of aa 70 (glutamine or histidine), and platelet count ($<15.0 \times 10^4/\mu l$) as significant determinants of elevated AFP. In 49 patients with abnormal AFP levels and substitutions at aa 70 who were treated with PEG-IFN + RBV, the rate of normalization of AFP was significantly lower in non-virological responders (28.6%) than in transient (71.4%) and sustained (100%) virological responders. The results indicated that substitution of aa 70 of HCV-1b core region is an important predictor of elevated AFP in non-HCC patients, and that eradication of the mutant virus normalizes AFP. The results highlight the importance of eradication of mutant type virus of aa 70 for reducing the risk of hepatocarcinogenesis.
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KEY WORDS: HCV; core region; genotype; AFP; hepatocellular carcinoma; glutamine; histidine

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy can achieve a high sustained virological response, although patients with non-virological response who remain HCV-RNA-positive at the completion of treatment are also encountered [Akuta et al., 2005, 2006, 2007a,b,c]. Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b (HCV-1b core region) and elevated alpha-fetoprotein (AFP) levels were predictors of poor virological response to PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors and surrogate markers of hepatocarcinogenesis [Ikeda et al., 2006; Akuta et al., 2007d].

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The use of elevated AFP as a predictor of early hepatocarcinogenesis in non-HCC patients might be clinically useful. AFP is a fetal glycoprotein produced by the yolk sac and fetal liver [Bergstrand and Czar, 1956], and has been used widely as a serum marker for the diagnosis of HCC [Sato et al., 1993; Johnson, 2001]. Furthermore, elevated serum AFP is also associated with various chronic liver diseases and hepatic regeneration [Kew et al., 1973; Silver et al., 1974; Elftherious et al., 1977; Alpert and Feller, 1978]. Although a mild rise in serum AFP is commonly seen in chronic HCV-infected patients, its clinicopathological significance remains to be defined. Previous studies indicated that high serum AFP levels correlated with fibrosis stages 3 and 4 [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2002, 2004], levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [Chu et al., 2001; Stein and Myaing, 2002; Hu et al., 2004], prothrombin time [Hu et al., 2004], and HCV-1b [Chu et al., 2001], in chronic HCV-infected patients. However, it is not clear whether mild elevation of AFP in the absence of HCC is associated with eventual development of HCC in HCV-infected patients. Furthermore, the impact of viral factors, such as aa substitutions of HCV-1b core region, on elevated AFP is still unclear.

The aims of the present study conducted in HCC-free Japanese patients infected with HCV-1b, were the following. (1) To evaluate the impact of elevated AFP, especially mild elevation of AFP, on hepatocarcinogenesis in IFN-treated patients without HCC during a long-term (15 years) follow-up period. (2) To identify the impact of aa substitutions in the core region on AFP levels in such patients, and determine the predictive factors for elevated AFP. (3) To investigate the normalization rates of AFP levels after eradication of HCV-RNA by PEG-IFN plus RBV combination therapy.

PATIENTS AND METHODS

Study Population

At Toranomon Hospital, Tokyo, Japan, 2,841 HCV-infected Japanese patients were recruited consecutively into the study protocol of IFN monotherapy between February 1987 and August 2007, and 929 HCV-infected Japanese patients were consecutively recruited into the study protocol of the combination therapy with PEG-IFNα-2b plus RBV between December 2001 and August 2007. Among these, 569 patients were selected in the present retrospective study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positive for HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor™, Roche Diagnostics, Indianapolis, IN). (2) They were naive to antiviral treatment. (3) They were infected with HCV-1b alone. (4) AFP levels were measured frequently, and substitutions of aa 70 or 91 in the HCV core region (HCV mutant-70 and HCV mutant-91, respectively) were determined at the commencement of the first course of antiviral treatment. (5) They were free of HCC based on clinical examination, laboratory tests, and imaging studies at baseline. (6) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (7) All were free of coinfection with human immunodeficiency virus. (8) None had other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital. Table I summarizes the profiles and laboratory data of the 569 patients at the commencement of antiviral treatment. They included 347 males and 222 females, aged 18–77 years (median, 55 years). Of the total group of 569 patients, 229 received IFN monotherapy, while 340 were treated with PEG-IFN plus RBV combination therapy. Among the patients who received IFN monotherapy, 109 patients started the monotherapy between February 1987 and August 1992, received at least two courses of such therapy, and were followed-up for 15 years. They were evaluated for the rate of development of HCC, associated with a rise in AFP level relative to that measured before the first course IFN monotherapy (baseline). At baseline, the latter group consisted of 80 males and 29 females, aged 22–69 with a median age of 46 years. The numbers of patients with fibrosis stages 1, 2, 3, and 4 were 57, 37, 14, and 1, respectively. The median AST and ALT levels were 85 IU/L (range, 27–400 IU/L) and 138 IU/L (range, 50–594 IU/L), respectively. The median platelet count was $17.0 \times 10^4/\mu l$ (range, 9.8×10^4 to $31.2 \times 10^4/\mu l$). The median viremia level was 5.8 Mequiv./ml (range, <0.5–46.5 Mequiv./ml). The median AFP level was 5 μg/L (range, 2–239 μg/L). The median follow-up time was 16.0 years (range, 0.1–20.3 years). With regard to

TABLE I. Profile and Laboratory Data of 569 Patients Infected with HCV Genotype 1b

Number of patients	569
Sex (male/female)	347/222
Age (years)*	55 (18–77)
Serum aspartate aminotransferase (IU/L)*	59 (17–400)
Serum alanine aminotransferase (IU/L)*	84 (15–594)
Platelet count ($\times 10^4/\mu l$)*	16.1 (3.8–40.2)
Serum alpha-fetoprotein (μg/L)*	6 (2–459)
Fibrosis stage (F1/F2/F3/F4/ND)	227/132/76/17/117
Level of viremia (high titer/low titer)**	522/47
Amino acid substitutions in core region***	
aa 70 (wild/mutant)	340/229
aa 91 (wild/mutant)	341/228
Treatment	
IFN monotherapy/PEG-IFN plus RBV	229/340

Data are number of patients, except those denoted by *, which represent the median (range) values. (**) Level of viremia was evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml). (***) The presence of arginine at aa 70 was evaluated as wild type, while other patterns (glutamine/histidine) as mutant type. The presence of leucine at aa 91 was evaluated as wild type, while other patterns (methionine) as mutant type.
Normal reference ranges: 11–38 IU/L for aspartate aminotransferase; 6–50 IU/L for alanine aminotransferase (IU/L); ≤10 μg/L for alpha-fetoprotein. ND: not done; IFN: interferon; PEG-IFN: pegylated interferon; RBV: ribavirin.

the protocol of IFN monotherapy, 68 (62.4%) patients received IFN-α alone; 36 (33.0%) patients received IFN-β alone; while the remaining 5 (4.6%) patients received a combination of IFN-α and IFN-β. The median IFN dose per day of 6 million units (MU, range; 1–10 MU) was administered. IFN monotherapy included initial aggressive induction therapy, consisting of every day within the first 8 weeks of commencement of therapy, followed subsequently by three times per week.

On the other hand, 340 patients received PEG-IFNα-2b combination therapy at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.0 mg/kg (range, 3.4–14.2 mg/kg) daily for a median duration of 48 weeks (range, 9–112 weeks).

In this study, patients who were HCV-RNA-negative by qualitative PCR analysis at 24 weeks after the completion of therapy, were defined as sustained virological responders. On the other hand, patients who were HCV-RNA-negative by qualitative PCR analysis at the completion of 24-week treatment but became HCV-RNA-positive after the 24-week therapy, were defined as transient virological responders. Patients who remained HCV-RNA-positive by quantitative and/or qualitative PCR analyses at the completion and after treatment, were defined as non-virological responders.

Laboratory Investigations

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for AST, ALT, and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, CA) or quantitative PCR assay (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after the antiviral therapy. The lower limits of these assays were 0.5 Meq/ml (10^6 genomic equivalents per milliliter) by branched DNA assay, or 5 KIU/ml by quantitative PCR assay. Samples with undetectable levels by these quantitative assays (<0.5 Meq/ml, or <5 KIU/ml) were checked also by HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor™, Roche) during and after treatment especially, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml. In this study, levels of viremia were evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml).

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo). The biopsy material was fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid–Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis and liver cirrhosis were diagnosed based on histological assessment according to the scoring system of Desmet et al. [1994].

Detection of Amino Acid Substitutions in Core Region

Okamoto et al. [2007] developed a simple PCR method for detecting substitutions of aa 70 or aa 91 in HCV-1b core region using mutation-specific primer, as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70: arginine, aa 91: leucine) and mutant HCV-1b (aa 70: glutamine/histidine, aa 91: methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/ml using quantitative assay with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche), the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases. Mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J (accession no. D90208) was considered a prototype and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples [Kato et al., 1990; Akuta et al., 2005]. In the present study, PCR using primers specific for substitutions of aa 70 or aa 91 was performed in samples collected from 454 patients [Okamoto et al., 2007]; the remaining 115 patients were analyzed by direct sequencing [Akuta et al., 2005, 2006].

Diagnosis of Hepatocellular Carcinoma

Patients were examined for HCC by abdominal ultrasonography every 3–6 months. If HCC was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy, were used to confirm the diagnosis.

Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test. Multiple comparisons were conducted by the Bonferroni test. The cumulative rate of hepatocarcinogenesis was calculated using the Kaplan–Meier technique; differences between carcinogenesis curves between groups were tested using the log-rank test. Statistical analyses of the rate of hepatocarcinogenesis according to

groups were calculated using the period from start of the first course of IFN monotherapy. Univariate and multivariate logistic regression analyses were used to determine the independent predictive factors of elevated AFP. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with elevated AFP included the following pretreatment variables: sex, age, AST, ALT, platelets, pathological staging, viremia level, and aa substitutions in the core region. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Cumulative Rate of Hepatocarcinogenesis According to AFP Levels

Of the 229 patients who received IFN monotherapy, 109 could be evaluated for the rate of development of HCC based on AFP levels measured at the start of the first course IFN monotherapy (baseline), during a follow-up period of 15 years. All 109 patients received two or more courses of IFN monotherapy; 66 patients received two courses of IFN (including 16 patients who achieved sustained virological response), 35 patients received three courses (including 4 patients who achieved sustained virological response), 7 patients received four courses (including 1 patient who achieved sustained virological response), and one patient received six courses (did not achieve sustained virological response). Thus, 21 of 109 patients achieved sustained virological response after multicourses of IFN monotherapy. For those who received 1, 2, 3, 4, 5, and 6 courses of IFN monotherapy, the median total duration of IFN therapy was 23.9 weeks (range, 0.9–134.7 weeks), 24.0 (range, 1.3–313.7), 25.1 (range, 3.1–193.1), 40.3 (range, 21.0–86.3), 23.6, and 67.9, respectively, and the median total dose of IFN was 526 MU (range, 22–1393 MU), 589 (range, 57–4005), 501 (range, 28–3477), 536 (range, 363–1553), 708, and 1200, respectively. The median cumulative total duration and cumulative total dose, which represented the cumulative total duration and total dose of every course of every patient were 57.7 weeks (range, 14.0–467.6 weeks) and 1380 MU (range, 521–4805 MU), respectively. The median period during which no IFN was administered was 3.7 years (range, 0.1–7.0 years). Finally, the median dose of IFN per week was 22.5 MU (range, 3.7–43.9).

During the follow-up, 8.6% (7 of 81 patients), 20.0% (3 of 15), and 38.5% (5 of 13) developed HCC in patients with AFP levels below 1 (≤10 μg/L), from 1 to 2 (11–20 μg/L), and above twice (≥21 μg/L) the upper limit of normal (ULN), respectively. In patients with AFP levels below 1, from 1 to 2, and above 2 times the ULN, the cumulative hepatocarcinogenesis rates were 0, 7.1, 0% at the end of 5 years; 3.1, 23.4, 37.5% at the end of 10 years; and 14.5, 23.4, 58.3% at the end of 15 years, respectively. The rates were significantly different among the three groups ($P < 0.001$; log-rank test) (Fig. 1). Especially, the rate of hepatocarcinogenesis in patients with normal AFP levels was significantly lower than in those with AFP levels above twice ULN ($P < 0.001$), and tended to be lower than in those with AFP levels from 1 to 2 times ULN ($P = 0.070$). The rate of hepatocarcinogenesis in patients with AFP levels above twice ULN was not significantly higher than in those with AFP levels from 1 to 2 times ULN. Thus, the rate of hepatocarcinogenesis was significantly higher in patients with abnormal AFP levels than in those with normal AFP levels ($P < 0.001$).

Predictive Factors of Elevated AFP in Univariate and Multivariate Analyses

The virological, clinical, and biochemical features of the whole population sample of 569 patients were analyzed to determine factors that could predict elevated AFP (≥11 μg/L). Elevated AFP was detected in 156 of 569 (27.4%) patients. Univariate analysis identified seven parameters that influenced significantly high AFP level. These included age (≥45 years, $P = 0.001$), AST (≥76 IU/L, $P < 0.001$), ALT (≥100 IU/L, $P < 0.001$), platelets ($<15.0 \times 10^4$/μl, $P < 0.001$), stage of fibrosis ($F3,4$, $P < 0.001$), and aa substitutions of the core region (mutant type of aa 70, $P < 0.001$, and aa 91, $P = 0.035$). Multivariate analysis identified four parameters that independently influenced high AFP level, including stage of fibrosis ($F3,4$, $P < 0.001$), AST (≥76 IU/L, $P < 0.001$), substitution of aa 70 (mutant type, $P < 0.001$), and platelet count ($<15.0 \times 10^4$/μl, $P = 0.019$) (Table IIA).

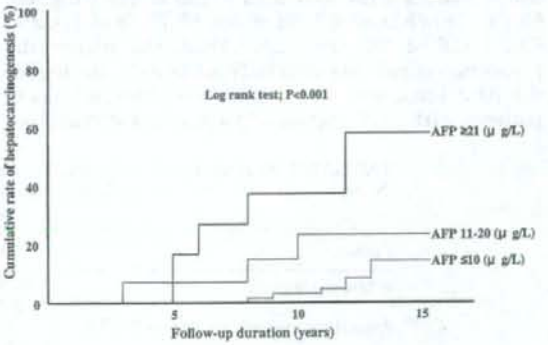

Fig. 1. Cumulative rate of hepatocarcinogenesis according to AFP levels at the start of first course IFN monotherapy. The rate of hepatocarcinogenesis in patients with normal AFP levels (≤10 μg/L) was significantly lower than in those with AFP levels above twice the upper limit of normal (≥21 μg/L) ($P < 0.001$), and tended to be lower than in those with AFP levels from 1 to 2 times the upper limit of normal (11–20 μg/L) ($P = 0.070$). The rate of hepatocarcinogenesis in patients with abnormal AFP levels (≥11 μg/L) was significantly higher than in those with normal AFP levels ($P < 0.001$).

TABLE IIA. Factors Associated with Elevated Serum AFP Levels (≥11 μg/L) in Patients Infected with HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Fibrosis stage	1: *F*1,2	1	
	2: *F*3,4	5.014 (2.746–9.153)	<0.001
Aspartate aminotransferase (IU/L)	1: <76	1	
	2: ≥76	4.592 (2.707–7.789)	<0.001
Substitution of aa 70	1: wild type	1	
	2: mutant type	2.618 (1.561–4.391)	<0.001
Platelet count ($\times 10^4/\mu l$)	1: ≥15.0	1	
	2: <15.0	1.912 (1.111–3.289)	0.019

*The presence of arginine at aa 70 was evaluated as wild type, while other patterns (glutamine/histidine) as mutant type. Normal reference ranges: ≤10 μg/L for alpha-fetoprotein.

The entire population sample was also analyzed to determine factors that could predict elevated AFP above twice ULN (≥21 μg/L); which was noted in 75 of 569 (13.2%) patients. Univariate analysis identified seven parameters that significantly influenced elevated AFP above twice ULN. These included age (≥45 years, $P = 0.015$), AST (≥76 IU/L, $P < 0.001$), ALT (≥100 IU/L, $P < 0.001$), platelet count ($<15.0 \times 10^4/\mu l$, $P < 0.001$), stage of fibrosis (*F*3,4, $P < 0.001$), and aa substitutions of the core region (mutant type of aa 70, $P < 0.001$, and aa 91, $P = 0.008$). Multivariate analysis identified four parameters that influenced independently elevated AFP above twice ULN, including stage of fibrosis (*F*3,4, $P < 0.001$), AST (≥76 IU/L, $P < 0.001$), and aa substitutions of the core region (HCV mutant-91, $P = 0.029$, and -70, $P = 0.056$) (Table IIB).

AFP Levels and aa Substitutions of Core Region

The entire population sample was also analyzed to determine the relationship between aa substitutions of the core region and AFP levels. The proportions of patients with HCV mutant-70 among those with AFP levels below 1, from 1 to 2, from 2 to 4, from 4 to 8, and above 8 times ULN were 33.4% (138 of 413 patients), 53.1% (43 of 81), 60.0% (24 of 40), 66.7% (8 of 12), and 69.6% (16 of 23) (Fig. 2A). Thus, the higher the proportion of patients with HCV mutant-70, the higher the AFP level, and significantly lower proportions of patients with HCV mutant-70 were noted among those with normal AFP levels (33.4%) than those with AFP levels from 1 to 2 times (53.1%) ($P = 0.001$) and above twice ULN (64.0%) ($P < 0.001$).

The proportions of patients with HCV mutant-91 among those with AFP levels below 1, from 1 to 2, from 2 to 4, from 4 to 8, and above 8 times ULN were 37.3% (154 of 413 patients), 40.7% (33 of 81), 67.5% (27 of 40), 25.0% (3 of 12), and 47.8% (11 of 23) (Fig. 2B). Thus, a higher frequency of HCV mutant-91 did not correlate with high AFP levels. In particular, significantly higher proportion of patients with HCV mutant-91 were noted among those with AFP levels from 2 to 4 times ULN (67.5%) than in those with AFP levels below 2 times (37.9%, $P < 0.001$) and above 4 times (40.0%, $P = 0.021$).

Normalization Rates of AFP Levels Based on Eradication of HCV-RNA With PEG-IFN Plus RBV Combination Therapy

Finally, the proportion of patients who showed normalization of AFP after commencement of PEG-IFNα-2b plus RBV combination therapy was determined in those at high risk for hepatocarcinogenesis, who had abnormal AFP levels (>10 IU/L) and HCV mutant-70 at baseline. Of the 340 patients, 49 had both abnormal AFP level and HCV mutant-70 at baseline. Of these, 14.3% (7 of 49 patients) could achieve sustained virological response, 28.6% (14 of 49) showed transient virological response, and 57.1% (28 of 49) had non-virological response. Table III summarizes the characteristics of

TABLE IIB. Factors Associated with Elevated Serum AFP Above Twice the Upper Limit of Normal (≥21 μg/L) in Patients Infected with HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Fibrosis stage	1: *F*1,2	1	
	2: *F*3,4	6.875 (3.485–13.56)	<0.001
Aspartate aminotransferase (IU/L)	1: <76	1	
	2: ≥76	6.144 (3.088–12.23)	<0.001
Substitution of aa 91	1: wild type	1	
	2: mutant type	2.101 (1.077–4.099)	0.029
Substitution of aa 70	1: wild type	1	
	2: mutant type	1.914 (0.984–3.722)	0.056

*The presence of arginine at aa 70 was evaluated as wild type, and other patterns (glutamine/histidine) as mutant type. The presence of leucine at aa 91 was evaluated as wild type, and other pattern (methionine) as mutant type. Normal reference ranges: ≤10 μg/L for alpha-fetoprotein.

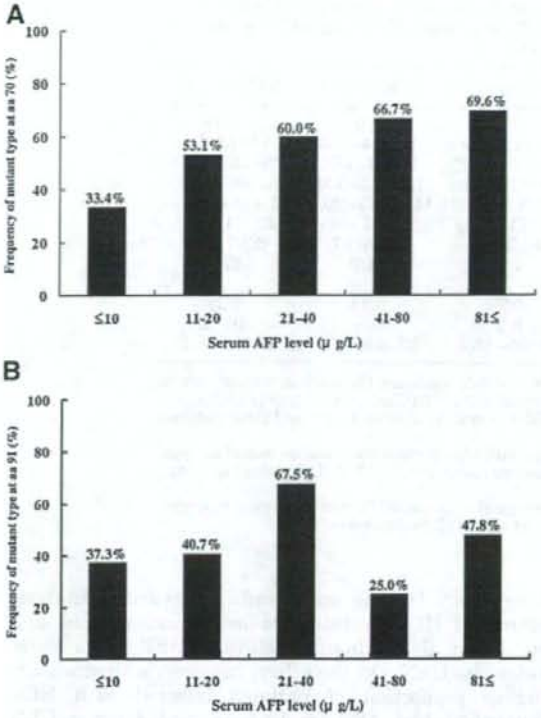

Fig. 2. **A**: Frequency of mutation in aa at position 70 of the HCV-1b core region according to serum AFP levels. Higher frequencies of the mutation correlated with higher serum AFP levels. Significantly lower frequencies of the mutant type were noted in patients with normal AFP levels (≤10 μg/L) than in those with levels from 1 to 2 times (11–20 μg/L, $P = 0.001$) and above twice the upper limit of normal (≥21 μg/L, $P < 0.001$), respectively. **B**: Frequency of mutation in aa at position 91 of the HCV-1b core region according to serum AFP levels. Higher frequencies of the mutation did not correlate with higher AFP levels. Significantly higher frequencies of the mutant type were noted in patients with AFP levels from 2 to 4 times the upper limit of normal (21–40 μg/L) than in those with levels below 2 times (≤20 μg/L, $P < 0.001$) and above 4 times (≥41 μg/L, $P = 0.021$).

these 49 patients at the commencement of combination therapy, according to treatment efficacy. The duration of treatment of non-virological responders was significantly shorter than that of sustained- ($P < 0.001$; Bonferroni test) and transient-virological responders ($P = 0.011$; Bonferroni test). Furthermore, AST levels of non-virological responders were significantly lower than those of sustained virological responders ($P = 0.049$; Bonferroni test). However, there were no significant differences in other patient characteristics at the commencement of treatment among the three groups.

The proportions of patients who showed normalization of AFP at the completion of treatment were 71.4% (5 of 7), 71.4% (10 of 14), and 53.6% (15 of 28) for the sustained-, transient-, and non-virological responders, respectively. There were no significant differences in the normalization rates at the completion of treatment among the three groups (Bonferroni test). However, the proportions of patients who showed normalization of AFP at 24 weeks after completion of treatment were 100% (7 of 7), 71.4% (10 of 14), and 28.6% (8 of 28) in the sustained-, transient-, and non-virological responders, respectively. The normalization rate in non-virological responders was significantly lower than in sustained- ($P = 0.001$; Bonferroni test) and transient virological responders ($P = 0.012$; Bonferroni test) (Fig. 3).

DISCUSSION

Elevated AFP in HCV-infected patients without HCC might be useful early predictor of hepatocarcinogenesis, but there is little evidence that mild elevation of AFP in such patients is associated with eventual development of HCC. Ikeda et al. [2006] reported that AFP level above twice ULN was an independent and significant determinant of hepatocarcinogenesis in patients with HCV-related cirrhosis. The present study of HCV-infected patients treated with IFN and followed for up to 15 years also showed that the rate of hepatocarcinogenesis was significantly higher in patients with abnormal AFP levels than in those with normal levels. In particular, the rate of hepatocarcinogenesis in patients with normal AFP levels was significantly lower than in those with levels above twice the ULN, and tended to be lower than in those with levels from 1 to 2 times ULN (i.e., mild elevation of AFP). To our knowledge, the present study is the first to report the hepatocarcinogenesis rates according to AFP levels in HCV-infected patients followed over a 15-year period, including mild elevation of AFP in patients without HCC.

Despite numerous epidemiologic studies linking HCV infection and the development of HCC, it remains controversial whether HCV itself plays direct or indirect role in the pathogenesis of HCC [Koike, 2005]. Studies using transgenic mice concluded that the HCV core region can potentially cause HCC [Moriya et al., 1998], but the clinical impact of HCV core region on hepatocarcinogenesis is still not clear. Previous studies identified substitutions in aa 70 and/or 91 in the HCV-1b core region and elevated AFP levels as predictors of poor virological response to PEG-IFN plus RBV [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also as risk factors for hepatocarcinogenesis [Ikeda et al., 2006; Akuta et al., 2007d]. It is speculated that cases resistant to treatment might ultimately develop HCC. The present study indicated that mutation in aa 70 in the core region predicted elevation of AFP in HCV-infected non-HCC patients. These results support the oncogenic potential of the HCV core region and clinically link mutations in this region to HCC.

Previous reports identified PA28γ-dependent pathway as a mechanism of HCV-associated hepatocarcinogenesis. Moriishi et al. demonstrated that knockout of the PA28γ gene induced accumulation of HCV core protein in nuclei of hepatocytes of HCV core gene transgenic mice and disrupted the development of both hepatic steatosis and HCC [Moriishi et al., 2003, 2007]. Furthermore, HCV core protein also enhanced the

TABLE III. Patient Characteristics at Commencement of Combination Therapy of Pegylated Interferon α-2b Plus Ribavirin, of 49 Patients with Abnormal AFP Levels and Mutant Type of aa 70

	SVR (n = 7)	TVR (n = 14)	NVR (n = 28)
Sex (male/female)	3/4	9/5	12/16
Age (years)*	58 (43–64)	56 (34–63)	57 (43–66)
Serum aspartate aminotransferase (IU/L)*	83 (37–324)[a]	84 (34–266)	76 (28–135)
Serum alanine aminotransferase (IU/L)*	99 (41–344)	126 (42–504)	82 (37–218)
Platelet count ($\times 10^4/\mu l$)*	11.6 (8.0–19.3)	14.1 (7.5–20.6)	12.4 (6.6–27.3)
Serum alpha-fetoprotein (μg/L)*	17 (11–161)	21 (11–38)	22 (11–427)
Fibrosis stage (F1/F2/F3/F4/ND)	0/3/2/0/2	2/0/5/0/7	6/3/7/2/10
Level of viremia (high titer/low titer)**	7/0	14/0	27/1
Amino acid substitutions in core region***			
aa 70 (wild/mutant)	0/7	0/14	0/28
aa 91 (wild/mutant)	5/2	6/8	16/12
Treatment duration (weeks)	75 (60–85)[b]	53 (46–77)[c]	47 (12–112)

Data are number of patients, except those denoted by *, which represent the median (range) values. (**) Level of viremia was evaluated as high titer (≥1.0 Meq/ml, or ≥100 KIU/ml) and low titer (<1.0 Meq/ml, or <100 KIU/ml). (***) The presence of arginine at aa 70 was evaluated as wild type, and other patterns (glutamine/histidine) as mutant type.
The presence of leucine at aa 91 was evaluated as wild type, and other pattern (methionine) as mutant type.
Normal reference ranges: 11–38 IU/L for aspartate aminotransferase; 6–50 IU/L for alanine aminotransferase (IU/L); ≤10 μg/L for alpha-fetoprotein.
SVR: sustained virological response; TVR: transient virological response; NVR: non-virological response; ND: not done. [a]$P = 0.049$, [b]$P < 0.001$, [c]$P = 0.011$, compared with NVR by Bonferroni test.

binding of liver X receptor α (LXRα)/retinoid X receptor α (RXRα) to the LXR-response element in the presence of PA28γ [Moriishi et al., 2007]. Thus, PA28γ could play a crucial role in the development of HCV-associated steatogenesis and hepatocarcinogenesis. Further studies are necessary to link the results of animal studies and the clinical impact of aa substitutions in HCV core region on hepatocarcinogenesis.

Chu et al. [2001] indicated that elevation of AFP in the absence of HCC might be associated with HCV-1b infection, and that such rise could correlate with more severe hepatic necroinflammation and fibrosis/cirrhosis and higher viremia levels. The results of the present study indicated that patients infected with HCV mutation-70 had elevated serum AFP levels, although the relation between HCV mutation-91 and AFP was not very clear. On the one hand, multivariate analysis identified HCV mutation-91 as an independent and significant determinant of elevated AFP levels above twice the ULN. On the other; however, a significantly higher proportion of patients infected with HCV mutant-91 had AFP levels from 2 to 4 times ULN compared to those with levels below 2 times and levels above 4 times, i.e., there was no relation between the frequency of HCV mutant-91 and serum AFP levels. Further large-scale studies should be performed to investigate the relationship between HCV mutant-91 and elevated AFP.

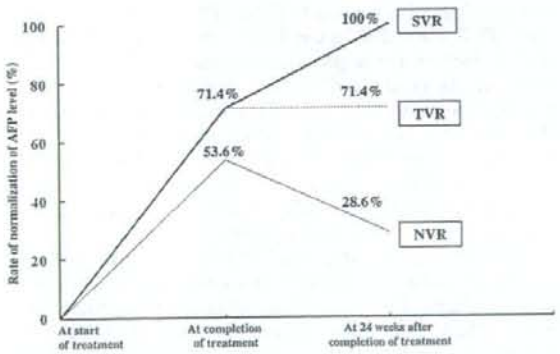

Fig. 3. Normalization rates of AFP levels at and 24 weeks after completion of treatment in sustained virological responders (SVR), transient virological responders (TVR), and non-virological responders (NVR).

Previous studies reported that IFN monotherapy [Arase et al., 2007] and IFN plus RBV combination therapy [Yu et al., 2006; Chen et al., 2007] results in reduction of AFP levels and the likelihood of hepatocarcinogenesis. In the present study, viral eradication (sustained virological response) in patients who received PEG-IFN plus RBV combination therapy was associated with normalization of AFP in patients at high risk for hepatocarcinogenesis (i.e., those with abnormal AFP levels and HCV mutant-70). These results emphasize that the risk of hepatocarcinogenesis could be reduced by eradication of HCV mutant-70. The results also showed that the proportion of patients with normalization of AFP levels was significantly higher in transient virological responders than in non-virological responders, suggesting that transient virological response could also result in the suppression of hepatocarcinogenesis, even when a sustained virological response is not achieved. In Japan, only 3 years had elapsed since the introduction of PEG-IFNα-2b plus RBV combination therapy into the Japanese Government Health Insurance system, and accordingly, the long-term effects of this combination therapy on hepatocarcinogenesis could not be evaluated in the present study. Further studies

that include patients treated with not only IFN but also PEG-IFN plus RBV, should be performed in the future.

In conclusion, the results of the present study indicated that substitution of aa at position 70 of the HCV-1b core region can predict elevation of serum AFP levels in non-HCC patients, and that eradication of the mutant virus seems to induce normalization of AFP. This finding highlights the importance of eradication of this mutant virus in reducing the risk of hepatocarcinogenesis. The limitations of the present study were that it did not investigate other genotypes apart from HCV-1b, the geographic diversities of HCV-1b core region (distribution of wild or mutant type), and the study of other races apart from Asians in Japan. Further prospective studies, matched for HCV genotype, aa substitutions of the core region, and race, of a large group of patients are required to determine the meaning of elevated AFP in non-HCC patients.

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Efficacy of Low-Dose Intermittent Interferon-Alpha Monotherapy in Patients Infected With Hepatitis C Virus Genotype 1b Who Were Predicted or Failed to Respond to Pegylated Interferon Plus Ribavirin Combination Therapy

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The efficacy of interferon (IFN) monotherapy for non-responders to pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy is still unclear. To evaluate the impact of IFN monotherapy on biochemical response, 200 consecutive patients infected with HCV genotype 1b, who received low-dose intermittent IFN-alpha monotherapy, were investigated. A median IFN dose per day of 3 million units was administered during a median period of 74 weeks. As a whole, the ALT normalization rates were 50.5, 65.9, 58.4, and 61.7% at 4, 12, 24, and 48 weeks, respectively. In 40 patients, who had abnormal AFP levels at the start of treatment, 52.5% achieved normalization of AFP within 48 weeks. Multivariate analysis identified indocyanine green retention rate at 15 min as the parameter that influenced significantly and independently ALT normalization. ALT normalization rates of patients who were predicted to be poor responders to PEG-IFN plus RBV combination therapy (but not substitutions of amino acid 70 and/or 91 in the HCV core region, female sex, and lower levels of low-density lipoprotein cholesterol) were similar to others. Furthermore, the ALT normalization rates in non-responders to combination therapy were 29.2, 60.9, 60.0, and 40.0% at 4, 12, 24, and 48 weeks, respectively. The results suggest that low-dose intermittent IFN monotherapy is an efficacious therapeutic regimen for patients unsuitable for PEG-IFN plus RBV, including non-responders, because it can lead to ALT normalization and thus a reduced risk of hepatocarcinogenesis. ***J. Med. Virol. 80:1363–1369, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HCV; interferon; ribavirin; ALT; hepatocellular carcinoma; core region; AFP; low-density lipoprotein cholesterol

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. Treatment of HCV-chronic hepatitis with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989].

Pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy for chronic HCV infection is expensive and associated with severe side effects but treated patients show a high-sustained virological response. Patients who do not achieve sustained virological response need to be identified before the start of combination therapy, in order to avoid unnecessary side effects and high costs. Thus, the safer IFN monotherapy should be selected as the therapeutic regimen for patients unsuitable for PEG-IFN plus RBV therapy. In a series of papers, Akuta et al. [2005a, 2006, 2007a,b,c] studied determinants of the response to PEG-IFN plus RBV in patients with high titers of genotype 1b (≥100 kiloIU [KIU]/ml), which is

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dominant in Japan. They identified substitutions of amino acid (aa) 70 and/or 91 in the HCV core region, female sex, and low levels of low-density lipoprotein cholesterol as independent and significant pretreatment negative predictors associated with virological response. Furthermore, previous studies reported that low-dose intermittent IFN monotherapy, as a treatment strategy, induces biochemical response [i.e., normalization of alanine aminotransferase (ALT) and alpha-fetoprotein (AFP) levels] and reduces the risk of hepatocarcinogenesis, even if patients failed to achieve sustained virological response [Arase et al., 2001, 2007; Nomura et al., 2007; McHutchison et al., 2008]. Hence, low-dose intermittent IFN monotherapy might be beneficial therapeutically in reducing the risk of hepatocarcinogenesis in patients who are predicted to be non-responsive to PEG-IFN plus RBV.

The present study included 200 consecutive patients infected with HCV genotype 1b, who were treated by self-injection of low-dose intermittent natural IFN-alpha. The aims of the study were the following. (1) To investigate the normalization rates of alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels within 48 weeks after the commencement of treatment. (2) To examine the predictive factors associated with ALT normalization. (3) To evaluate the efficacy of IFN monotherapy in patients with predictors of poor response to IFN plus RBV combination therapy. (4) To evaluate the efficacy of IFN monotherapy for non-responders to IFN plus RBV combination therapy.

PATIENTS AND METHODS

Patients

Among 252 consecutive HCV-infected patients who started IFN monotherapy between April 2005 and July 2007 at Toranomon Hospital, 200 were selected in the present study based on the following criteria. (1) Patients treated by self-injection of natural IFN-alpha (Sumiferon®; Sumitomo Pharmaceutical Co., Osaka, Japan). (2) Patients infected with HCV genotype 1b alone. (3) Patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, CA), and positive for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Pleasanton, CA). (4) Patients who have not been treated with antiviral or immunosuppressive agents, except for IFN plus RBV combination therapy, within 6 months of enrolment. (5) Patients free of HCC. (6) Patients free of coinfection with human immunodeficiency virus. (7) Lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake). (8) Patients free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease, and (9) patients who consented to the study.

With regard to the clinical features of 200 patients at the start of IFN monotherapy, there were 103 men and 97 women, aged 27–77 with a median age of 62 years. The median ALT level was 80 IU/L (range, 6–487 IU/L), and the median platelet count was $13.0 \times 10^4/mm^3$ (range, 3.8×10^4–$28.0 \times 10^4/mm^3$). The median viremia level was 1,200 KIU/ml (range, 5–>5,000 KIU/ml) (Table I). Furthermore, 162 of the 200 patients (81%) received IFN-alpha monotherapy by three times per week; the remaining 38 patients (19%) received IFN-alpha monotherapy that included an initial daily administration in the first 8 weeks, followed by three times per week. A median IFN dose per day of 3 million units (MU, range; 3–6 MU) was administered during a median period of 74 weeks (range; 2–118 weeks). Of the 200 patients, 40 had not achieved sustained virological response with prior therapy of IFN plus RBV, and especially 27 patients of them had been treated with adequate combination therapy for at least 24 weeks (median, 43 weeks; range, 24–73 weeks).

Efficient treatment represented normalization of ALT levels (normal reference ranges: 6–50 IU/L) and AFP levels (normal reference ranges: ≤20 μg/L) during and at the end of 48-week treatment protocol.

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Laboratory Investigations

Blood samples were obtained at least once every month from the commencement of treatment, and were tested for ALT and AFP levels. The serum samples were frozen at −80°C within 4 hr of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Indianapolis, IN) at the commencement of treatment. The lower detection limit of the assay was 5 KIU/ml.

Detection of Amino Acid Substitutions in Core Region

With use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined, and it was compared with the consensus sequence constructed on 50 clinical samples [Akuta et al., 2005a] for detecting substitutions at aa 70 of arginine (wild) or glutamine/histidine (mutant) and aa 91 of leucine (wild) or methionine (mutant). In the present study, aa substitutions of the core region were analyzed by direct sequencing [Akuta et al., 2005a, 2006]. The PCR genotyping could be performed in 193 patients; the remaining seven patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

Histopathological Examination of the Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman

TABLE I. Patient Profile and Laboratory Data at Commencement of Interferon Monotherapy in 200 Patients Infected With HCV Genotype 1b

Demographic data	
Number of patients	200
Sex (M/F)	103/97
Age (years)*	62 (27–77)
History of blood transfusion	81 (40.5%)
Family history of liver disease	58 (29.0%)
Body mass index (kg/m^2)*	22.8 (15.6–32.9)
Laboratory data*	
Serum aspartate aminotransferase (IU/L)	69 (18–756)
Serum alanine aminotransferase (IU/L)	80 (6–487)
Serum albumin (g/dl)	3.7 (2.6–4.4)
Gamma-glutamyl transpeptidase (IU/L)	49 (11–368)
Leukocyte count ($/mm^3$)	4,000 (1,700–8,100)
Hemoglobin (g/dl)	13.9 (8.9–17.3)
Platelet count ($\times 10^4/mm^3$)	13.0 (3.8–28.0)
Indocyanine green retention rate at 15 min (%)	20 (4–62)
Serum iron (μg/dl)	146 (37–322)
Serum ferritin (μg/L)	136 (<10–1,308)
Creatinine clearance (ml/min)	99 (13–167)
Level of viremia (KIU/ml)	1,200 (5–>5,000)
Alpha-fetoprotein (μg/L)	9 (2–398)
Total cholesterol (mg/dl)	165 (15–296)
High-density lipoprotein cholesterol (mg/dl)	45 (21–80)
Low-density lipoprotein cholesterol (mg/dl)	96 (43–237)
Triglycerides (mg/dl)	93 (46–228)
Uric acid (mg/dl)	5.4 (2.8–9.4)
Fasting blood sugar (mg/dl)	97 (67–228)
Histological findings	
Stage of fibrosis (F1/F2/F3/F4/ND)	45/42/35/19/59
Hepatocyte steatosis (none to mild/moderate to severe/ND)	90/24/86
Treatment	
Interferon dose (million units/day)	3 (3–6)
Presence of initial daily interferon administration	38 (19.0%)
Amino acid substitutions in the core region[a]	
aa 70 (wild/non-wild/ND)	118/72/3
aa 91 (wild/non-wild/ND)	124/69/0
aa 70 and aa 91 (double wild/non-double wild/ND)	76/115/2

Data are number and percentage of patients, except those denoted by *, which represent the median (range) values.
Two patterns of mutant and competitive are indicated as non-wild. The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns were non-double wild-type. ND, not determined.
[a]Amino acid substitutions were evaluated in 193 patient using pretreatment sera by direct sequencing.

needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (HK) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994].

Follow-Up

Clinical and laboratory assessments were performed at least once every month from the commencement of treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month from the commencement of treatment, and were also analyzed for levels of ALT and AFP at various time points. Follow-up time represented the time from the start of treatment until the stop of treatment, or until the last visit.

Statistical Analysis

Analysis of efficacy of treatment was performed on an intention to treat basis. The χ^2 test, Fisher's exact probability test, and Mann–Whitney's *U*-test were used to compare the background characteristics between groups. The cumulative ALT normalization rates were calculated using the Kaplan–Meier technique; differences between the curves were tested using the log-rank test. Statistical analyses of ALT normalization according to groups were calculated using the period from the commencement of IFN monotherapy. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with ALT normalization within 48 weeks after the commencement of treatment. The odds ratios and 95% confidence intervals (95%CI) were also calculated. Potential predictive factors associated with ALT normalization

included the following 29 variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, AST, ALT, albumin, γGTP, leukocyte count, hemoglobin, platelet count, indocyanine green retention rate at 15 min, serum iron, serum ferritin, creatinine clearance, level of viremia, AFP, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting blood sugar, fibrosis stage, hepatocyte steatosis, IFN dose per day, and presence of initial daily IFN administration. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into a multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS, Inc., Chicago, IL). All P values of less than 0.05 by the two-tailed test were considered significant.

RESULTS

Efficacy of IFN Monotherapy

The rates of ALT normalization in the intention to treat population analysis were evaluated at 0, 4, 12, 24, and 48 weeks after commencement of treatment. As a whole, the rates were 18.0% (36/200), 50.5% (94/186), 65.9% (116/176), 58.4% (90/154), and 61.7% (71/115), respectively. Thus, ALT normalization rates favorably exceeded 50% at 4 weeks. Furthermore, in 40 patients with abnormal AFP levels (≥21 μg/L) at the commencement of treatment, AFP levels of 92.5% (37/40) decreased and those of 7.5% (3/40) increased within 48 weeks. Especially, 52.5% (21/40) achieved normalization of AFP within 48 weeks. These results indicate that low-dose intermittent IFN monotherapy achieved favorable biochemical response.

Predictive Factors Associated With ALT Normalization by Univariate and Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict ALT normalization within 48 weeks after the commencement of treatment. Univariate analysis identified nine parameters that tended to or significantly correlated with ALT normalization. These included AST ($P = 0.007$), ALT ($P = 0.009$), γGTP ($P = 0.075$), platelets ($P = 0.093$), fibrosis stage ($P = 0.066$), indocyanine green retention rate at 15 min ($P = 0.026$), serum iron ($P = 0.003$), high-density lipoprotein cholesterol ($P = 0.067$), and AFP ($P = 0.046$). These factors were entered into multivariate analysis, which then identified indocyanine green retention rate at 15 min ($P = 0.027$) as the parameter that influenced significantly and independently ALT normalization (Table II).

Efficacy of IFN Monotherapy in Patients With Predictors of Poor Response to PEG-IFN Plus RBV Combination Therapy

Figure 1 shows the prevalence with respect to ALT normalization rates in patients with predictors of poor response to PEG-IFN plus RBV combination therapy.

TABLE II. Factors Associated With ALT Normalization During Interferon Monotherapy, Identified by Univariate and Multivariate Analysis

		Univariate Cox proportional hazard model		Multivariate Cox proportional hazard model	
Factor	Category	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Aspartate aminotransferase (IU/L)	1:<70	1		—	—
	2:≥70	0.589 (0.400–0.867)	0.007	—	
Alanine aminotransferase (IU/L)	1:<75	1		—	—
	2:≥75	0.588 (0.395–0.875)	0.009	—	
γ-Glutamyl transpeptidase (IU/L)	1:<50	1		—	—
	2:≥50	0.636 (0.386–1.047)	0.075	—	
Platelets ($\times 10^4/mm^3$)	1:<15.0	1		—	—
	2:≥15.0	1.397 (0.946–2.064)	0.093	—	
Fibrosis stage	1:1.2	1		—	—
	2:3.4	0.627 (0.381–1.031)	0.066	—	
Indocyanine green retention rate at 15 min (%)	1:<20	1		1	—
	2:≥20	0.557 (0.333–0.932)	0.026	0.503 (0.274–0.925)	0.027
Serum iron (μg/dl)	1:<150	1		—	—
	2:≥150	0.522 (0.342–0.797)	0.003	—	
High-density lipoprotein cholesterol (mg/dl)	1:<45	1		—	—
	2:≥45	1.468 (0.973–2.215)	0.067	—	
Alpha-fetoprotein (μg/L)	1:<10	1		—	—
	2:≥10	0.662 (0.441–0.992)	0.046	—	

Only variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate and multivariate Cox proportional hazard model are shown.
95% CI, 95% confidence interval.

According to the substitutions of core aa 70 and aa 91, the ALT normalization rates were 15.7% (18/115) versus 22.4% (17/76) at 0 week, 42.6% (46/108) versus 61.4% (43/70) at 4 weeks, 62.1% (64/103) versus 69.7% (46/66) at 12 weeks, 59.3% (54/91) versus 56.1% (32/57) at 24 weeks, and 58.5% (38/65) versus 63% (29/46) at 48 weeks for non-double wild-type and double wild-type, respectively [not significantly different, except for 4 weeks ($P = 0.021$), Fig. 1A].

According to sex, the ALT normalization rates were 22.7% (22/97) versus 13.6% (14/103) at 0 week, 50.6% (44/87) versus 50.5% (50/99) at 4 weeks, 66.3% (53/80) versus 65.6% (63/96) at 12 weeks, 62.7% (42/67) versus 55.2% (48/87) at 24 weeks, and 67.3% (33/49) versus 57.6% (38/66) at 48 weeks for female and male, respectively (not significant, Fig. 1B).

According to the levels of low-density lipoprotein cholesterol, the ALT normalization rates were 15.5% (9/58) versus 20.0% (23/115) at 0 week, 37.0% (20/54) versus 54.1% (59/109) at 4 weeks, 58.8% (30/51) versus 66% (68/103) at 12 weeks, 58.5% (24/41) versus 53.8% (49/91) at 24 weeks, and 66.7% (22/33) versus 57.4% (39/68) at 48 weeks for low levels (<86 mg/dl) and high levels (≥86 mg/dl), respectively [not significant, except for 4 weeks ($P = 0.047$), Fig. 1C].

The above results indicate that in low-dose intermittent IFN monotherapy, ALT normalization rates of patients with predictors of poor response were not different from those without such predictors.

Efficacy of IFN Monotherapy in Non-Responders to IFN Plus RBV Combination Therapy

The rates of ALT normalization were evaluated in 27 patients who had received prior therapy with adequate IFN plus RBV for at least 24 weeks but showed no sustained virological response. The rates were 14.8% (4/27), 29.2% (7/24), 60.9% (14/23), 60% (12/20), and 40.0% (4/10) at 0, 4, 12, 24, and 48 weeks, respectively. Thus, ALT normalization rates favorably exceeded 60% at 12 weeks. These results indicate that non-responders to IFN plus RBV treated again with low-dose intermittent IFN monotherapy can achieve a favorable biochemical response.

DISCUSSION

For chronic HCV infection, PEG-IFN plus RBV combination therapy is expensive, associated with severe side effects but results in a high-sustained virological response. However, it is desirable to identify those patients who do not achieve sustained virological response before the start of the combination therapy in order to free them of unnecessary side effects and high costs. One alternative option for such patients is IFN

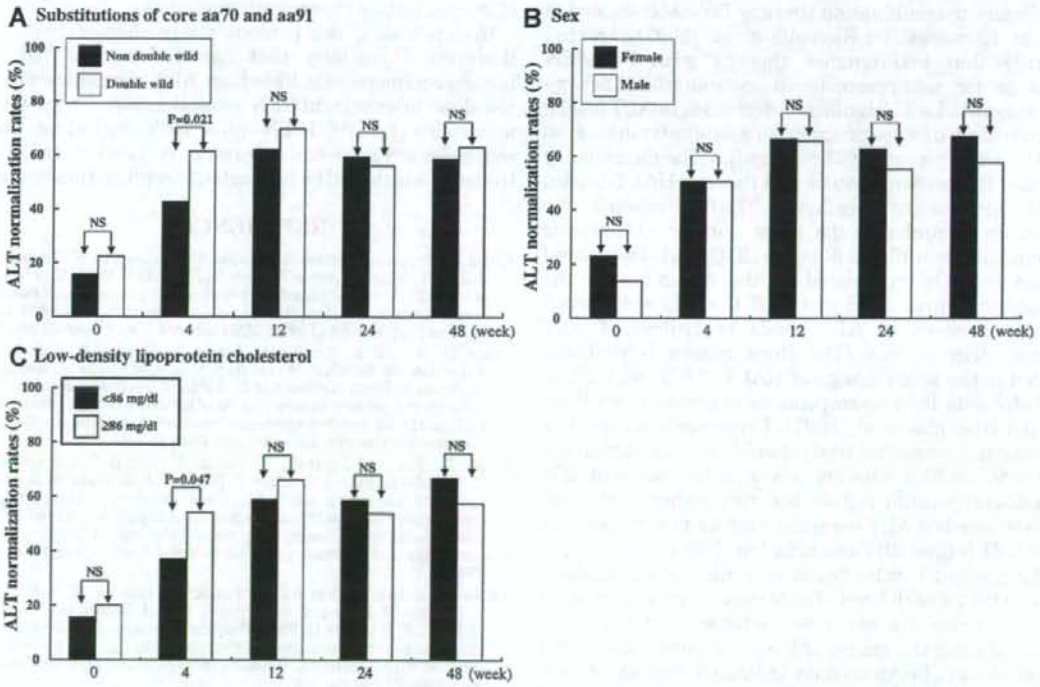

Fig. 1. The ALT normalization rates at 0, 4, 12, 24, and 48 weeks after commencement of IFN monotherapy, according to (A) substitutions of core aa 70 and aa 91, (B) sex, and (C) the levels of low-density lipoprotein cholesterol.

monotherapy, which could reduce the risk of hepatocarcinogenesis [Arase et al., 2001, 2007; Akuta et al., 2005a,b; Donlin et al., 2007; Nomura et al., 2007; McHutchison et al., 2008].

The efficacy of IFN monotherapy in patients who are predicted to respond poorly to IFN plus RBV combination therapy is still unknown. Previous results identified substitutions of aa 70 and/or 91 in the core region, female sex, and lower levels of low-density lipoprotein cholesterol as independent and significant pretreatment negative predictors associated with virological response [Akuta et al., 2005a, 2006, 2007a,b,c]. These studies also showed that substitutions of aa 70 and/or 91 are risk factors for hepatocarcinogenesis in the same patients [Akuta et al., 2007d]. The present study of low-dose intermittent IFN monotherapy showed that ALT normalization rates of patients who were predicted to have a poor response to the combination therapy were not different from others. It is important to achieve a better biochemical response regardless of core aa substitutions as risk factor of hepatocarcinogenesis. Thus, a low-dose intermittent IFN monotherapy for patients predicted to fail to respond to PEG-IFN plus RBV is an efficacious therapeutic regimen for normalization of ALT and thus reduction of risk of hepatocarcinogenesis.

The efficacy of IFN monotherapy for non-responders to IFN plus RBV combination therapy is still controversial. In the present study of low-dose intermittent IFN monotherapy, ALT normalization rates in non-responders to combination therapy favorably exceeded 60% at 12 weeks. Di Bisceglie et al. [2007] reported recently that maintenance therapy with PEG-IFN alpha-2a for non-responder to combination therapy was associated with significant decreases in ALT levels, but the rate of disease progression was similar in treated (34.1%) and untreated (33.8%) groups. The discrepancy between the present results and those of HALT-C trial may be due to one or more factors. The first reason for the difference is probably the large number of cirrhotic patients (about 40% of all) in HALT-C trial. The second reason is probably related to the difference in the efficacy measures used in HALT-C trial, which evaluated decreases in ALT levels regardless of ALT normalization or not. The third reason is probably related to the study design of HALT-C trial with PEG-IFN alpha-2a for non-responders to combination therapy [Di Bisceglie et al., 2007]. Large-scale prospective randomized controlled trials should be conducted in the future to confirm whether low-dose intermittent IFN monotherapy could reduce the risk of hepatocarcinogenesis based on ALT normalization for non-responders to PEG-IFN plus RBV combination therapy.

The present results based on multivariate analysis showed that a high level of indocyanine green retention rate at 15 min is a negative predictor of ALT normalization during the course of low-dose intermittent IFN monotherapy. Previous data indicated that absence of advanced liver fibrosis is a positive predictor of sustained virological response to IFN monotherapy and IFN plus RBV combination therapy [Jouet et al., 1994; Poynard et al., 2000; Bruno et al., 2004]. Akuta et al. [2005a, 2007a] also showed previously that high levels of indocyanine green retention rate at 15 min or low levels of serum albumin are associated with advanced liver fibrosis, and that they are independent and significant negative predictors of the virological response to PEG-IFN plus RBV. To our knowledge, this is the first report that describes the relationship between indocyanine green retention rate at 15 min level and biochemical response during IFN monotherapy. Di Bisceglie et al. [2007] recently reported that maintenance therapy with PEG-IFN alpha-2a failed to halt liver disease progression in patients with advanced hepatic fibrosis. Further studies of large number of patients are required to investigate the relationship between severity of histopathological liver changes and biochemical response during low-dose intermittent IFN monotherapy.

One limitation of the present study was that viral factors other than the core region of HCV genome were not examined, such as the interferon sensitivity determining region (ISDR) and the interferon/ribavirin resistance determining region (IRRDR) of NS5A region [Enomoto et al., 1995, 1996; El-Shamy et al., 2007]. Biochemical response during low-dose intermittent IFN monotherapy seems to be based on a dynamic tripartite interaction of virus, host, and treatment regimen. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

In conclusion, the present study showed that one therapeutic regimen that can reduce the risk of hepatocarcinogenesis based on ALT normalization is low-dose intermittent IFN monotherapy, for patients unsuitable for PEG-IFN plus RBV including non-responders. Large-scale prospective randomized controlled trials should be conducted to confirm this finding.

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