47 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new invasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; 29: 1705–13.
48 Castera L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; 128: 343–50.
49 Saito H, Tada S, Nakamoto N *et al.* Efficacy of non-invasive elastometry on staging of hepatic fibrosis. *Hepatol Res* 2004; 29: 97–103.
50 Colletta C, Smime C, Fabris C *et al.* Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferase. *Hepatology* 2005; 42: 838–645.
51 Castera L, Foucher J, Bertet J, Couzigou P. FibroScan and FibroTest to assess liver fibrosis in HCV with normal aminotransferse. *Hepatology* 2006; 43: 373–4.
52 Alberti A. Towards more individualized management of hepatitis V virus patients with initially or persistently normal alanineaminotransferase levels. *J Hepatol* 2005; 42: 266–74.

Hepatology Research 2008; 38: 52–59 doi: 10.1111/j.1872-034X.2007.00205.x

Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

Naoki Hiramatsu,[1] Nao Kurashige,[1] Tsugiko Oze,[1] Tetsuo Takehara,[1] Shinji Tamura,[1] Akinori Kasahara,[1] Masahide Oshita,[2] Kazuhiro Katayama,[3] Harumasa Yoshihara,[4] Yasuharu Imai,[5] Michio Kato,[6] Sumio Kawata,[7] Hirohito Tsubouchi,[8] Hiromitsu Kumada,[9] Takeshi Okanoue,[10] Shinichi Kakumu[11] and Norio Hayashi[1]

[1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, [2]Osaka Police Hospital, Osaka, [3]Osaka Kouseinenkin Hospital, Osaka, [4]Osaka Rousai Hospital, Sakai, [5]Ikeda Municipal Hospital, Ikeda, [6]National Hospital Organization Osaka National Hospital, Osaka, [7]Department of Gastroenterology, Yamagata University School of Medicine, Yamagata, [8]Department of Digestive and Lifestyle Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, [9]Toranomon Hospital, Tokyo, [10]Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto and [11]Department of Internal Medicine, Division of Gastroenterology, Aichi Medical University School of Medicine, Aichi, Japan

Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naïve. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum $Hb \leq 8.5$ g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum $Hb \leq 8.5$ g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

Correspondence: Dr Naoki Hiramatsu, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. Email: hiramatsu@gh.med.osaka-u.ac.jp
Received 29 December 2006; revision 11 April 2007; accepted 25 May 2007.

of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm^3 and platelets ≥10^5/mm^3 within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Crnn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm^3)	5317 ± 1207
Neutrocytes (/mm^3)	2778 ± 902
Platelets (×10^4/mm^3)	17.4 ± 4.0
RBC (×10^4/mm^3)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 μg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = 32.3 × BW × (1 − 0.0094 × age) × (1 − 0.42 × sex)/Scr (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel-Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

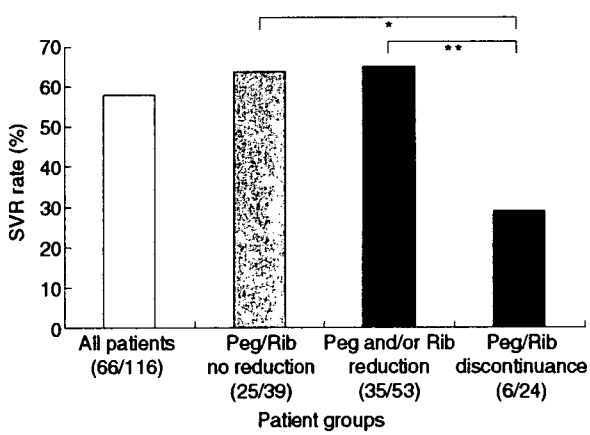

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (◫), patients without dose reduction; (■), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: *$P = 0.003$; **$P = 0.001$.

Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
20 ≤ CL/F (n = 12)	67% (8/12)	25% (3/12)	8% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	57% (13/23)	30% (7/23)	13% (3/23)	0
10 ≤ CL/F < 15 (n = 39)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

P = 0.031 (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
20 ≤ CL/F (n = 12)	92% (11/12)	12% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	83% (19/23)	17% (4/23)	0
10 ≤ CL/F < 15 (n = 39)	72% (28/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	50% (21/42)	43% (18/42)	7% (3/42)

P = 0.009 (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs (P = 0.003), and the group with reduction of PegIFN and/or ribavirin (P = 0.001).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the CL/F ≥ 20, 13% (3/23) for the 15 ≤ CL/F < 20, 23% (9/39) for the 10 ≤ CL/F < 15, and 26% (11/42) for the CL/F < 10 group. Ribavirin did not have to be discontinued due to severe anemia among patients with 15 ≤ CL/F, but did for the 18% (2/11) of those with CL/F < 10 and 22% (2/9) of those with 10 ≤ CL/F < 15. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of CL/F < 10, and 5% (2/39) of the group of 10 ≤ CL/F < 15. No patients of the group of CL/F ≥15 showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four-weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin (n = 53), 1.6 ± 1.2 g/dL among those with dose reduction (n = 39), and 1.8 ± 1.0 g/dL among

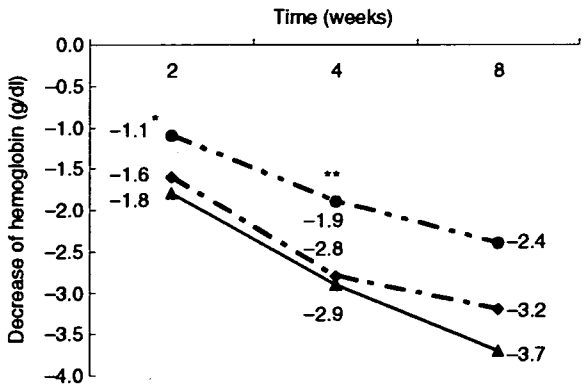

Figure 2 Course of ΔHb in the initial phase. (—··—), No reduction; (—·—·—), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel-Haenszel χ^2-test).

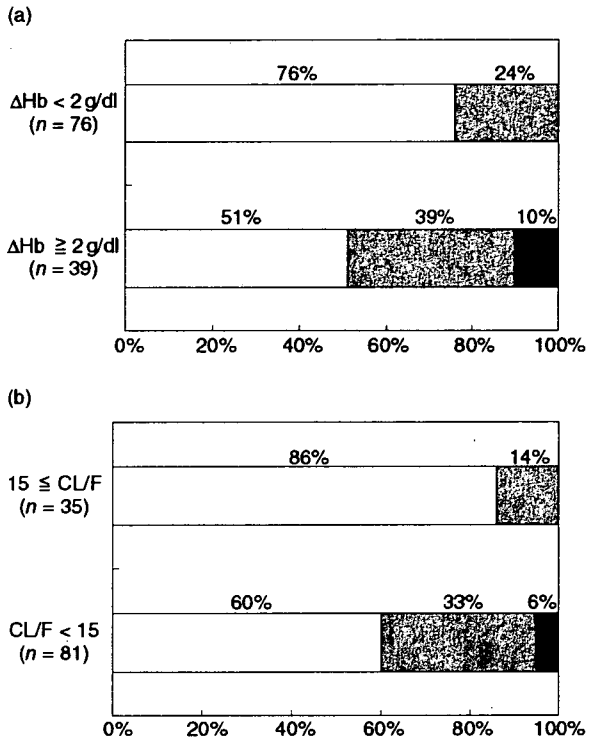

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (■), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dL decrease at two weeks from the baseline). $P = 0.009$ (Mantel-Haenszel χ^2-test). (b) according to CL/F levels. $P = 0.001$ (Mantel-Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients ($n = 5$) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL ($n = 76$)	ΔHb ≥ 2 g/dL ($n = 39$)
CL/F ≥ 15 ($n = 35$)	0/25	0/10
CL/F < 15 ($n = 80$)	0/51	4/29 (14%)

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

REFERENCES

1 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; 27: 1394–402.
2 Imai Y, Kasahara A, Tanaka H *et al.* Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response. *J Gastroenterol* 2004; 39: 1069–77.
3 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41: 17–27.
4 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426–32.
5 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485–92.
6 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–65.
7 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
8 Hiramatsu N, Kasahara A, Nakanishi F *et al.* The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan. *Hepatol Res* 2004; 29: 142–7.
9 Bruno S, Camma C, Di Marco V *et al.* Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J Hepatol* 2004; 41: 474–81.
10 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–55.
11 Berg T, Von Wagner M, Nasser S *et al.* Extended treatment duration for Hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; 130: 1086–97.
12 Lodato F, Azzaroli F, Brillanti S *et al.* Higher doses of peginterferon alpha-2b administered twice weekly improve sustained virological response in difficult-to-treat patients with chronic hepatitis C: results of a pilot randomized study. *J Viral Hepat* 2005; 12: 536–42.
13 Lindahl K, Stahle L, Bruchfeld A, Schvarcz R. High-dose ribavirin in combination with standard dose peginterferon for treatment of patients with chronic hepatitis C. *Hepatology* 2005; 41: 275–9.
14 Bodenheimer HC Jr, Lindsay KL, Davis GL *et al.* Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. *Hepatology* 1997; 26: 473–7.
15 De Franceschi L, Fattovich G, Turrini F *et al.* Hemolytic anemia induced by ribavirin therapy in patients with chronic hepatitis C virus infection: role of membrane oxidative damage. *Hepatology* 2000; 31: 997–1004.
16 Van Vlierbergh H, Delanghe JR, De Vos M, Leroux-Roel G. Factors influencing ribavirin-induced hemolysis. *J Hepatol* 2001; 34: 911–16.
17 Tappero G, Ballare M, Farina M, Negro F. Severe anemia following combined alpha-interferon/ribavirin therapy of chronic hepatitis C. *J Hepatol* 1998; 29: 1033–4.
18 Afdhal NH, Dieterich DT, Pockros PJ *et al.* Epoetin alfa maintains ribavirin dose in HCV-infected patients: a prospective, double-blind, randomized controlled study. *Gastroenterology* 2004; 126: 1302–11.
19 Pockros PJ, Shiffman ML, Schiff ER *et al.* Epoetin alfa improves quality of life in anemic HCV-infected patients receiving combination therapy. *Hepatology* 2004; 40: 1450–8.
20 Dieterich DT, Wasserman R, Brau N *et al.* Once-weekly epoetin alfa improves anemia and facilitates maintenance

of ribavirin dosing in hepatitis C virus-infected patients receiving ribavirin plus interferon alfa. *Am J Gastroenterol* 2003; **98**: 2491–9.

21 Lindahl K, Schvarcz R, Bruchfeld A, Stahle L. Evidence that plasma concentration rather than dose per kilogram body weight predicts ribavirin-induced anaemia. *J Viral Hepat* 2004; **11**: 84–7.

22 Jen JF, Glue P, Gupta S, Zambas D, Hajian G. Population pharmacokinetic and pharmacodynamic analysis of ribavirin in patients with chronic hepatitis C. *Ther Drug Monit* 2000; **22**: 555–65.

23 Kamar N, Chatelut E, Manolis E, Lafont T, Izopet J, Rostaing L. Ribavirin pharmacokinetics in renal and liver transplant patients: evidence that it depends on renal function. *Am J Kidney Dis* 2004; **43**: 140–6.

24 Karino Y, Kato T, Arakawa T *et al.* Total clearance (CL/F) of ribavirin is the factor most influencing the incidence of hemolytic anemia during IFN plus ribavirin therapy. *Hepatology* 2004; **40** (Suppl 1): 358.

25 Oze T, Hiramatsu N, Kurashige N *et al.* Early decline of hemoglobin correlates with progression of ribavirin-induced hemolytic anemia during interferon plus ribavirin combination therapy in patients with chronic hepatitis C. *J Gastroenterol* 2006; **41**: 862–72.

26 Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147–67.

27 Hiramatsu N, Oze T, Tsuda N *et al.* Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy? *Hepatol Res* 2006; **35**: 185–9.

Amino Acid Substitutions in the Hepatitis C Virus Core Region are the Important Predictor of Hepatocarcinogenesis

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

We showed previously that amino acid (aa) substitutions in hepatitis C virus core region (HCV-CR) are negative predictors of virologic response to pegylated interferon (IFN) plus ribavirin therapy. HCV-CR induces hepatocellular carcinoma in transgenic mice, but the clinical impact is still unclear. To evaluate the impact of aa substitutions in HCV-CR on hepatocarcinogenesis, we performed a follow-up study on 313 noncirrhotic consecutive naïve patients infected with HCV genotype 1b who received IFN monotherapy. The median follow-up was 14.7 years. A sustained virologic response (SVR) after the first IFN was achieved by 65 patients (20.8%) (group A). Of 248 patients (79.2%) of non-SVR after first IFN, 112 (35.8%) did not receive additional IFN (group B), and the remaining 136 (43.5%) received multicourse IFN monotherapy (group C). As a whole, cumulative hepatocarcinogenesis rates in double wild-type (arginine at aa 70/ leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type. Multivariate analyses identified 3 parameters (fibrosis stage 3, nondouble wild-type of HCV-CR, and group B) that tended to or significantly influenced hepatocarcinogenesis independently. With regard to hepatocarcinogenesis rates in group C according to HCV-CR and the mean alanine aminotransferase (ALT) during IFN-free period, significantly higher rates were noted in patients of nondouble wild-type with ALT levels of more than 1.5 times the upper limit of normal (25.7%) compared with the others (2.4%). *Conclusion:* Amino acid substitutions in the HCV-CR are the important predictor of hepatocarcinogenesis. In multicourse IFN therapy to nondouble wild-type, we emphasize the importance of reducing the risk of hepatocarcinogenesis by mean ALT during an IFN-free period below 1.5 times the upper limit of normal. (HEPATOLOGY 2007;46: 1357-1364.)

Hepatitis C virus usually causes chronic infection, which can result in chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1-5] In patients with chronic HCV, treatment with IFN can induce viral clearance and marked biochemical and histological improvement.[6,7]

For chronic HCV infection, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy is an expensive treatment modality that is accompanied by severe side effects and high sustained virological response (SVR). Patients who do not achieve SVR need to be identified before the start of combination therapy to avoid unnecessary side effects and high costs. Thus, safer IFN monotherapy should be considered to reduce the risk of hepatocarcinogenesis in patients unsuitable for PEG-IFN plus RBV therapy. We studied previous determinants of response to PEG-IFN plus RBV in patients with high titers of HCV genotype 1b (≥100 KIU/mL), which is dominant in Japan. Our results identified substitution of amino acids (aa) 70 and/or 91 in the HCV core region (HCV-CR) as an independent and significant negative predictor associated with virological response.[8-10] Furthermore, we reported that multicourse IFN monotherapy reduces the risk of hepatocarcinogenesis and increases survival even if patients fail to achieve SVR after a single-course IFN, and

Abbreviations: aa, amino acid(s); HCV-CR, hepatitis C virus core region; MU, million units; PEG-IFN, peginterferon; RBV, ribavirin; SVR, sustained virologic response.

From the [1]Department of Hepatology and [2]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan.

Received March 21, 2007; accepted May 25, 2007.

Supported in part by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare, Japan.

Address reprints requests to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp; fax: (81) 44-860-1623.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21836

Potential conflict of interest: Nothing to report.

that low ALT levels during an IFN-free period is associated with lower rates of hepatocarcinogenesis.[11] Hence, multicourse IFN monotherapy might be expected to reduce the risk of hepatocarcinogenesis in patients who have negative predictors for PEG-IFN plus RBV.

Despite numerous lines of epidemiological evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC.[12] It has become evident that HCV-CR has oncogenic potential through the use of transgenic mice, but the clinical impact of HCV-CR on hepatocarcinogenesis is still unclear.[13] Whether substitution of aa 70 and/or 91 in HCV-CR as a predictor of virological response for PEG-IFN plus RBV therapy also affects hepatocarcinogenesis awaits further investigation.

The present study included 313 consecutive naïve cases infected with HCV genotype 1b in whom 15 years had elapsed since induction of IFN monotherapy. The aims of the study were: (1) to evaluate the clinical impact of aa substitutions in the HCV-CR on hepatocarcinogenesis; (2) to analyze the predictive factors associated with hepatocarcinogenesis in patients who received IFN monotherapy; and (3) to evaluate the long-term efficacy of multicourse IFN monotherapy on hepatocarcinogenesis as examined through analysis of the outcomes of single and multicourses of IFN.

Patients and Methods

Patients. Among 573 consecutive HCV-infected patients in whom IFN monotherapy was induced between February 1987 and August 1992 at Toranomon Hospital, 313 were selected in the present study based on the following criteria: (1) patients naïve to IFN monotherapy; (2) patients infected with HCV genotype 1b alone; (3) patients with chronic hepatitis, without cirrhosis or HCC, as confirmed via biopsy examination within 6 months of enrollment; (4) patients not treated with IFN plus RBV combination therapy during follow-up time; (5) patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emeryville, CA), and positive for HCV RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor, Roche Diagnostic Systems, CA); (6) patients free of coinfection with human immunodeficiency virus; (7) patients not treated with antiviral or immunosuppressive agents within 6 months of enrollment; (8) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake); (9) patients free of other types of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease; (10) patients without or with well-controlled diabetes; and (11) patients who consented to the study.

With regard to the clinical features of 313 patients at the start of the first course of IFN monotherapy, there were 223 men and 90 women aged 15-66 years with a median age of 47 years. The numbers of patients with fibrosis stages 1, 2, and 3 were 179, 107, and 27, respectively. The median ALT level was 138 IU/L (range, 24-636 IU/L), and the median platelet count was $17.4 \times 10^4/\mu L$ (range, 8.9×10^4-$39.2 \times 10^4/\mu L$). The median viremia level was 4.0 Meq/mL (range, <0.5-67.0 Meq/mL). The median follow-up time was 14.7 years (range, 0.1-20.1 years).

Furthermore, at the first course of IFN monotherapy, 222 patients (70.9%) received IFN-α alone; 83 patients (26.5%) received IFN-β alone; and the remaining 8 patients (2.6%) received a combination of IFN-α and IFN-β. A median IFN dose per day of 6 million units (MU) (range, 1-10 MU) was administered. As a whole, a median total dose of IFN of 525 MU (range, 22-3,696 MU) was administered during a median period of 23.9 weeks (range, 0.6 to 205.4 weeks). Patients mainly received IFN monotherapy, including initial aggressive induction therapy (every day within 8 weeks, followed by 3 times per week).

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

Methods. The primary measure of efficacy of treatment was sustained virological response (SVR), defined as negative HCV RNA via qualitative analysis with PCR at 24 weeks after cessation of IFN therapy. Patients who achieved SVR after the first course of IFN monotherapy were classified as group A. Patients who did not achieve SVR after the first course of IFN monotherapy were classified into 2 groups based on whether they received other courses of IFN monotherapy. Patients who did not receive further courses of IFN monotherapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression, and cardiopulmonary disease during and after the first course of IFN, or the lower levels of ALT, were classified as group B. Patients who received 2 or more courses of IFN monotherapy were classified as group C.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. HCV genotype was determined via PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[14] In all cases, HCV-RNA viremia level was measured by branched DNA assay version 2.0 (Chiron Corp.) at commence-

ment of therapy using frozen samples, and the results were expressed as 10^6 genomic equivalents per milliliter (Meq/mL). The lower limit of the assay was 0.5 Meq/mL. Samples with undetectable levels using this quantitative assay (<0.5 Meq/mL) were also evaluated via HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor) during and after therapy especially, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/mL.

Detection of Amino Acid Substitutions in Core Region. We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in HCV-CR of genotype 1b 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 (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 version 2.0 using the 10-fold dilution method), the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases.[15] In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double wild-type, while the other patterns were nondouble wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype, and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples.[8,16] In this study, the PCR genotyping could be performed in 232 patients; the remaining 81 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

Liver Histopathological Examination. 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), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H. K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al.[17]

Follow-Up. Clinical and laboratory assessments were performed at least once every month before, during, and after 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 before, during, and after treatment, and were also analyzed for ALT levels and HCV-RNA levels at various time points.

Follow-up time represented the time from the start of the first course of IFN treatment until death or until the last visit.

Diagnosis of HCC. Patients were examined for HCC via abdominal ultrasonography every 3-6 months. If HCC was suspected based on ultrasonographic results, additional procedures such as CT, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy (if necessary), were used to confirm the diagnosis.

Statistical Analysis. The χ^2 test, Fisher exact probability test, and Mann-Whitney U test were used to compare background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. Cumulative hepatocarcinogeneses were calculated using the Kaplan-Meier technique; differences between survival curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis according to groups were calculated using the period from start of the first course of IFN monotherapy. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. We also calculated the OR and 95% CI. Potential predictive factors associated with hepatocarcinogenesis included the following 11 variables: age, sex, histological stage, viremia level, serum AST, serum ALT, platelet count, aa substitutions in HCV-CR, total IFN dose, total IFN duration, and group of treatment. Each variable was transformed into categorical data consisting of 2 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 tested using the 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 2-tailed test were considered significant.

Results

Efficacy of IFN Monotherapy. 65 patients (20.8%) achieved SVR after the first course of IFN monotherapy (group A). Of 248 (79.2%) non-SVR patients after the first course of IFN, 112 (35.8%) did not receive a second course of IFN monotherapy (group B), while the remain-

Table 1. Patient Characteristics at Start of First Course of IFN Monotherapy

	Group A (n = 65)	Group B (n = 112)	Group C (n = 136)
Sex (male/female)	45/20	75/37	103/33
Age (years)*	44 (15-64)†	51 (23-66)	45 (22-63)‡
Viremia level (Meq/mL)*	0.6 (<0.5-45.0)	5.9 (<0.5-67.0)§	5.3 (<0.5-57.0)‖
Fibrosis stage (F1/F2/F3)	49/14/2	54/50/8¶	76/43/17#
AST (IU/L)*	83 (16-198)	74 (22-398)	75 (24-400)
ALT (IU/L)*	153 (24-416)	120 (38-636)	138 (50-594)
Platelet count ($\times 10^4/\mu L$)*	18.7 (9.7-31.0)	17.1 (9.7-39.2)	17.0 (8.9-31.2)
Core region (double wild/nondouble wild/ND)**	10/15/5	31/44/7	41/71/8

*Median †$P = 0.009$, ‡$P = 0.007$ compared with group B via Bonferroni test. §$P < 0.0001$, ‖$P < 0.0001$, ¶$P = 0.006$, #$P = 0.009$, compared with group A via Bonferroni test.

** Amino acid substitutions were evaluated in pretreatment serum samples of 232 patients via PCR with mutation-specific primers. Two patterns of mutant and competitive were labeled as nonwild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, while the other patterns were considered nondouble wild-type.

Abbreviation: ND, not determined.

ing 136 (43.5%) received 2 or more courses of IFN monotherapy (group C). Of 136 patients in group C, 80 patients received 2 courses of IFN (21 of whom achieved SVR), 44 patients received 3 courses (6 of whom achieved SVR), 11 patients received 4 courses (2 of whom achieved SVR), and 1 patient received 6 courses (and did not achieve SVR). Thus, 29 patients in group C achieved SVR after multiple courses of IFN monotherapy.

In groups A and B, the median total duration of IFN was 24.1 weeks (range, 4.0-205.4 weeks) and 23.7 weeks (range, 2.9-75.1 weeks). The median total dose of IFN was 528 MU (range, 43-3,696 MU) and 498 MU (range, 72-870 MU). In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in group C, the median total durations of IFN were 23.9 weeks (range, 0.6-136.4 weeks), 24.0 weeks (range, 1.3-313.7 weeks), 25.3 weeks (range, 3.1-198.1 weeks), 40.4 weeks (range, 21.0-86.3 weeks), 23.6 weeks, and 67.9 weeks, respectively. In the first, second, third, fourth, fifth, and sixth courses of IFN monotherapy in group C, the median total doses of IFN were 525 MU (range, 22-2,312 MU), 558 MU (range, 57-4005 MU), 522 MU (range, 28-3,477 MU), 565 MU (range, 363-1,080 MU), 708 MU, and 1,200 MU, respectively. The median cumulative total durations and cumulative total doses, which represented the cumulative total duration and total dose of every course of every patient of group C, were 65.6 weeks (range, 8.4-474.4 weeks) and 1,388 MU (range, 354-4,805 MU), respectively. The median periods free of IFN in group C were 3.6 years (range, 0.1-7.3 years). In conclusion, the median dose of IFN per week in group A, B, and C were 21.8 MU/week (range, 6.7-42.0 MU/week), 22.0 MU/week (range, 4.5-42.0 MU/week), and 21.9 MU/week (range, 3.7-43.9 MU/week), respectively.

Clinical Features of Patients and Cumulative Hepatocarcinogenesis Rates According to Study Groups. The clinical features of patients in groups A, B, and C, at the start of the first IFN monotherapy are summarized in Table 1. The age of patients of group B was significantly higher than those of group A ($P = 0.009$; Bonferroni test) and group C ($P = 0.007$; Bonferroni test). Viremia levels in group A were significantly lower than those in group B ($P < 0.001$; Bonferroni test) and group C ($P < 0.001$; Bonferroni test). Fibrosis stage of group A was significantly milder than those of group B ($P = 0.006$; Bonferroni test) and group C ($P = 0.009$; Bonferroni test). There were no other significant differences in clinical features at the start of IFN therapy among the 3 groups.

During follow-up, 1 (1.5%), 17 (15.2%), and 15 (11.0%) patients developed HCC in groups A, B, and C, respectively. In groups A, B, and C, the cumulative hepatocarcinogenesis rates were 2.3%, 11.5%, and 0.8%, respectively, at the end of 5 years; 2.3%, 25.3%, and 7.2%, respectively, at the end of 10 years; and 2.3%, 33.0%, and 25.6%, respectively, at the end of 15 years. The rates were significantly different among the 3 groups ($P < 0.001$; Log-rank test) (Figure 1). In particular, the rates in group B were significantly higher than in group C ($P < 0.001$; Log-rank test) and group A ($P < 0.001$; Log-rank test), and the rates in group C were significantly higher than group A ($P = 0.037$; Log-rank test).

Hepatocarcinogenesis Rates According to aa Substitutions of HCV-CR. During follow-up, 5 of 82 patients (6.1%) and 18 of 130 patients (13.8%) developed HCC in double wild-type and nondouble wild-type, respectively. In double wild-type and nondouble wild-type, the cumulative hepatocarcinogenesis rates were, respectively, 1.6% and 2.6% at the end of 5 years; 3.4% and 12.3% at the end of 10 years; and 11.3% and 23.5% at the end of 15 years. The rates in double wild-type of HCV-CR were significantly lower than those in nondouble wild-type ($P = 0.036$; log-rank test) (Fig. 2).

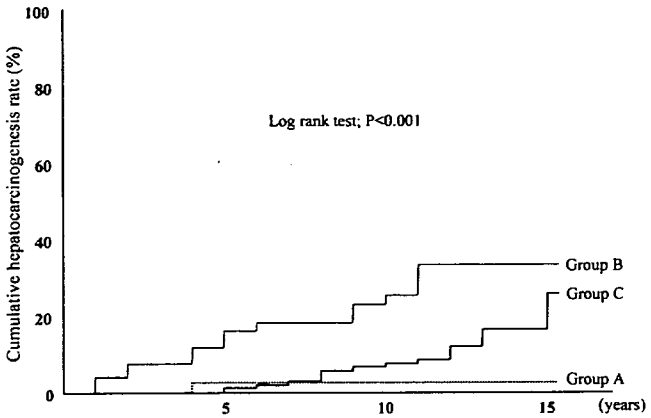

Fig. 1. Cumulative hepatocarcinogenesis rates were significantly different among the 3 study groups ($P < 0.001$; Log-rank test). In particular, the rates in group B were significantly higher than in group C ($P < 0.001$; Log-rank test) and group A ($P < 0.001$; log-rank test), and the rates in group C were significantly higher than in group A ($P = 0.037$; log-rank test).

Predictive Factors Associated with Hepatocarcinogenesis via Multivariate Analysis. We then analyzed the data for the whole population sample to determine those factors that could predict hepatocarcinogenesis. Univariate analysis identified 6 parameters that tended to or significantly correlated with carcinogenesis: age ($P < 0.001$), fibrosis stage ($P < 0.001$), platelet count ($P < 0.001$), group ($P < 0.001$), viremia level ($P = 0.018$), and aa substitution in HCV-CR ($P = 0.036$). These factors were entered into multivariate analysis, which identified 3 parameters that tended to or significantly influenced carcinogenesis independently: fibrosis stage ($P < 0.001$), aa substitutions in HCV-CR ($P = 0.008$), and group ($P = 0.056$) (Table 2).

We also analyzed the data for 219 patients, except for 94 patients who achieved SVR, to determine those factors

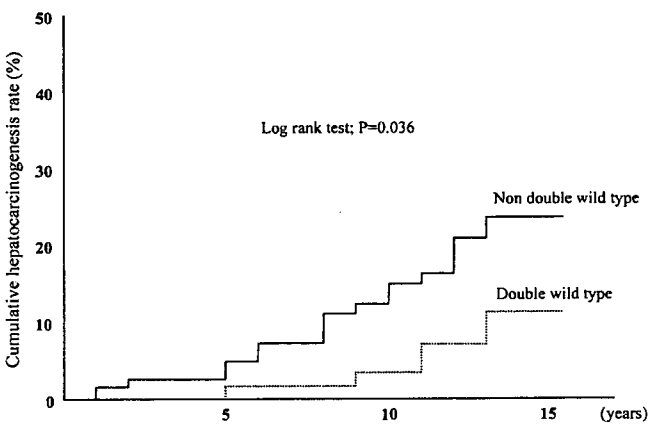

Fig. 2. Cumulative hepatocarcinogenesis rates according to aa substitutions of HCV-CR. The rates in double wild-type (arginine at aa 70/leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type ($P = 0.036$; log-rank test).

Table 2. Factors Associated With Hepatocareinogenesis in 313 Patients Infected with HCV Genotype 1b, Identified via Multivariate Analysis

Factors	Category	Odds Ratio (95% CI)	P Value
Fibrosis stage	1: F1, F2	1	
	2: F3	10.2 (3.65-28.5)	<0.001
Amino acid substitutions in the core region	1: double-wild	1	
	2: nondouble-wild	5.92 (1.58-22.2)	0.008
Group	1: A, C	1	
	2: B	2.75 (0.98-7.76)	0.056

NOTE. Cox proportional hazard model.

that could predict hepatocarcinogenesis. Univariate analysis identified 5 parameters that tended to or significantly correlated with carcinogenesis: fibrosis stage ($P < 0.001$), platelet count ($P < 0.001$), age ($P = 0.001$), group ($P = 0.008$), and aa substitution in HCV-CR ($P = 0.028$). These factors were entered into multivariate analysis, which identified 2 parameters that significantly influenced carcinogenesis independently: fibrosis stage ($P < 0.001$) and aa substitution in HCV-CR ($P = 0.017$) (Table 3).

Hepatocarcinogenesis Rates in Group C According to HCV-CR and ALT Levels. In group C, the hepatocarcinogenesis rates were evaluated according to the ALT levels at the start of IFN. For this purpose, we selected 112 patients (82.4%) from group C in whom HCV-CR could be evaluated. In double wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 (<75 IU/L) and above 1.5 (>75 IU/L) times the upper limit of normal (6-50 IU/L) were 0% (0/6 patients) and 8.6% (3/35 patients), respectively. In nondouble wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 and above 1.5 times the upper limit of normal was 0% (0/7 patients), and 15.6% (10/64 patients), respectively (Table 4). In conclusion, regardless of whether aa substitutions in HCV-CR are present or not, lower hepatocarcinogenesis rates were noted in patients with ALT levels below 1.5 the upper limit of normal (0%) than in other patients (13.1%), but they did not achieve statistical significance on univariate analysis.

Table 3. Factors Associated with Hepatocarcinogenesis in 219 Patients of Non-SVR Infected with HCV Genotype 1b, Identified via Multivariate Analysis

Factors	Category	Odds Ratio (95% CI)	P Value
Fibrosis stage	1: F1, F2	1	
	2: F3	6.50 (2.39-17.6)	<0.001
Amino acid substitutions in the core region	1: double-wild type	1	
	2: nondouble wild-type	4.65 (1.32-16.4)	0.017

NOTE. Cox proportional hazard model.

Table 4. Hepatocarcinogenesis Rates in Group C According HCV Core Region and ALT Levels at the Start of IFN

	ALT Level (IU/L)*			
	<75	75–100	100–200	>200
Nondouble wild-type	0% (0/7)	14.3% (2/14)	13.3% (4/30)	20.0% (4/20)
Double wild-type	0% (0/6)	16.7% (1/6)	5.6% (1/18)	9.1% (1/11)

* Normal level of ALT: 6–50 IU/L

In group C, the hepatocarcinogenesis rates were also evaluated according to the mean ALT levels at the IFN-free period. For this purpose, we selected 76 consecutive patients (55.9%) from group C in whom ALT levels were closely monitored. In double wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 4 (<200 IU/L) and above 4 (>200 IU/L) times the upper limit of normal were 0% (0/26 patients) and 50% (1/2 patients), respectively. In nondouble wild-type, the hepatocarcinogenesis rates in patients with ALT levels below 1.5 (<75 IU/L), from 1.5 to 2 (75-100 IU/L), from 2 to 4 (100-200 IU/L), and above 4 (>200 IU/L) times the upper limit of normal were 0% (0/13 patients), 33.3% (3/9 patients), 22.7% (5/22 patients), and 25.0% (1/4 patients), respectively (Table 5). In conclusion, regardless of whether aa substitutions in HCV-CR are present or not, significantly lower hepatocarcinogenesis rates were noted in patients with ALT levels below 1.5 times the upper limit of normal (0%) than in other patients (18.9%) ($P = 0.027$). In particular, significantly higher hepatocarcinogenesis rates were noted in patients of nondouble–wild-type with ALT levels above 1.5 times the upper limit of normal (25.7%) than in other patients (2.4%) ($P = 0.004$).

Discussion

Despite numerous lines of epidemiological evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC.[12] It is evident that the HCV-CR has oncogenic potential through the use of transgenic mice,[13] but its clinical impact on hepatocarcinogenesis is still unclear. Our study identified that cumulative hepatocarcinogenesis rates of double wild-type HCV-CR, as a predictor of virological response for PEG-IFN plus RBV therapy, were significantly lower than those of nondouble wild-type. We speculate that the resistant cases for treatment might reasonably lead to HCC. To our knowledge, this is the first report to support the findings of oncogenic potential via HCV-CR from the clinical aspect. Previous reports identified PA28γ-dependent pathway as one of the mechanisms of HCV-associated hepatocarcinogenesis. Morishi and colleagues showed that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts development of both hepatic steatosis and HCC.[18,19] Furthermore, HCV core protein also enhanced the binding of liver X receptor α/retinoid X receptor α to liver X receptor response element in the presence of PA28γ.[19] Thus, it is reported that PA28γ plays a crucial role in the development of HCV-associated steatogenesis and hepatocarcinogenesis. Further studies should be performed to connect evidence from animal model studies and the clinical impact of aa substitution in HCV-CR on hepatocarcinogenesis.

Viral factors associated with hepatocarcinogenesis in patients infected with HCV are still incompletely investigated. Ogata et al. reported that HCV genotype 1b strains might be associated with HCC on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein.[20] Giménez-Barcons et al. reported that high aa variability within the NS5A of HCV might be associated with HCC in patients with HCV-1b–related cirrhosis.[21] In the present study, we could not investigate the clinical impact of the other region on hepatocarcinogenesis, except for the HCV-CR. Further studies should be performed to investigate the clinical impact of the other region of HCV on hepatocarcinogenesis.

Patients who fail to achieve SVR after single-course IFN should receive multicourse IFN at the time of ALT relapse at certain intervals. Based on previous reports showing increased incidence of HCC in 5 years or more

Table 5. Hepatocarcinogenesis Rates in Group C According HCV Core Region and ALT Levels at the IFN-Free Period

	ALT level (IU/L)*			
	<75	75–100	100–200	>200
Nondouble wild-type	0% (0/13)	33.3% (3/9)	22.7% (5/22)	25.0% (1/4)
Double wild-type	0% (0/10)	0% (0/4)	0% (0/12)	50.0% (1/2)

* Normal level of ALT: 6–50 IU/L

after IFN therapy in transient biochemical responders, it is important to normalize ALT levels via multicourse IFN monotherapy at certain intervals.[11,22] We reported previously that results of multicourse IFN showed a 0% hepatocarcinogenesis rate in patients with ALT levels below 75 IU/L at the IFN-free periods, emphasizing the importance of keeping low ALT levels at such periods with respect to suppression of hepatocarcinogenesis.[11] Furthermore, hepatocarcinogenesis rates according to HCV-CR and ALT levels during the IFN-free period were also evaluated in this study. In double wild-type, the rates in patients with ALT levels below 200 IU/L and above 200 IU/L were 0% and 50%, respectively. In nondouble wild-type, the rates in patients with ALT levels below 75 IU/L and above 75 IU/L were 0% and 25.7%, respectively. Thus, significantly higher hepatocarcinogenesis rates were noted in patients of nondouble wild-type with ALT levels above 75 IU/L than in other patients. In particular, in multicourse IFN therapy in nondouble wild-type, we emphasize the importance of reducing the risk of hepatocarcinogenesis by the mean ALT during the IFN-free period below 1.5 times the upper limit of normal.

It is unclear whether ALT levels during the IFN-free period might be more important than those at the start of IFN. In the present study, at the start of IFN, lower hepatocarcinogenesis rates were noted in patients with ALT levels below 1.5 the upper limit of normal compared with other patients, but they did not achieve statistical significance on univariate analysis. During the IFN-free period, significantly lower hepatocarcinogenesis rates were noted in patients with mean ALT levels below 1.5 times the upper limit of normal compared with other patients. Thus, in multicourse IFN therapy, especially in nondouble wild-type, we emphasize the importance of reducing the risk of hepatocarcinogenesis via ALT levels below 1.5 times the upper limit of normal during the IFN-free period rather than at the start of IFN. Further studies should be conducted in the future to confirm this finding.

To our knowledge, our study is the first to report the hepatocarcinogenesis rates for a long-term follow-up period of 15 years in IFN monotherapy. Previous studies have shown that sex, age, fibrosis stage, and IFN regimen are important pretreatment predictors of hepatocarcinogenesis.[11,23-25] In the present study, a more progressive fibrosis stage as host factor, nondouble wild-type of HCV-CR as viral factor, and group B (non-SVR after single-course IFN) as treatment-related factor were associated with higher hepatocarcinogenesis rates in the whole population sample. Even if we also analyzed non-SVR patients, multivariate analyses similarly identified more progressive fibrosis stage and nondouble wild-type of HCV-CR that significantly influenced hepatocarcinogenesis independently. Hence, we assess that the risk of HCC is not necessarily secondary to the lack of response to IFN therapy rather than aa substitution. We conclude that hepatocarcinogenesis 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 Japan, only 5 years had elapsed since the induction of IFN-α2b plus RBV combination therapy (especially, only 2 years in PEG-IFN-α2b plus RBV) based on the Japanese Government Health Insurance system, so we could not exactly evaluate the long-term efficacy of combination therapy as a treatment-related factor of hepatocarcinogenesis in this study. Further studies that include patients treated not only with IFN monotherapy but also with RBV combination therapy should be performed in the future.

The relationship between the development of cirrhosis and HCC is still unclear. We investigated liver fibrosis stage of 13 patients who underwent partial hepatectomy for HCC in this study. Interestingly, 8 of 13 patients (61.5%) developed HCC in the absence of cirrhosis (5 patients of fibrosis stage 2, 3 patients of fibrosis stage 3). As a whole, it is regrettable that we could not exactly evaluate how frequently HCC occurs in the absence of cirrhosis. Further studies based on all patients, whether or not they develop HCC, should be performed to investigate the relationship between the development of cirrhosis and HCC.

In conclusion, aa substitutions in the HCV-CR are the primary predictor of hepatocarcinogenesis. In particular, in multicourse IFN therapy in nondouble wild-type as a pretreatment negative predictor of SVR for PEG-IFN plus RBV combination therapy, we emphasize the importance of reducing the risk of hepatocarcinogenesis via mean ALT levels below 1.5 times the upper limit of normal during the IFN-free period. Furthermore, IFN monotherapy should be recommended as a therapeutic regimen to reduce the risk of hepatocarcinogenesis in patients unsuitable for PEG-IFN plus RBV combination therapy. Large-scale prospective studies should be conducted in the future to confirm this finding.

References

1. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, et al. Progress of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.
2. Dusheiko GM. The natural course of chronic hepatitis C: implications for clinical practice. J Viral Hepatol 1998;5(Suppl 1):9-12.
3. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930-938.

4. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228-1233.
5. Akuta N, Chayama K, Suzuki F, Someya T, Kobayashi M, Tsubota A, et al. Risk factors of hepatitis C virus-related liver cirrhosis in young adults: positive family history of liver disease and transporter associated with antigen processing 2 (TAP2) *0201 allele. J Med Virol 2001;64:109-116.
6. Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC Jr, Perrillo RP, et al. Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Group. N Engl J Med 1989;321:1501-1506.
7. Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggoner J, et al. Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. N Engl J Med 1989;321:1506-1510.
8. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372-380.
9. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 2006;78:83-90.
10. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403-410.
11. Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, et al. Long-term follow-up of interferon monotherapy in 454 consecutive naive patients infected with hepatitis C virus: multi-course interferon therapy may reduce the risk of hepatocellular carcinoma and increase survival. Scand J Gastroenterol 2005;40:688-696.
12. Koike K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. Clin Gastroenterol Hepatol 2005; 3(10 Suppl 2):S132-S135.
13. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
14. Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, et al. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 1993;8:150-156.
15. Okamoto K, Akuta N, Kumada H, Kobayashi M, Matsuo Y, Tazawa H. An amino-acid substitution detection system for the HCV-1b core region. J Virol Method 2007;141:1-6.
16. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
17. Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
18. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237-10249.
19. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661-1666.
20. Ogata S, Florese RH, Nagano-Fujii M, Hidajat R, Deng L, Ku Y, et al. Identification of hepatitis C virus subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. J Clin Microbiol 2003;41:2835-2841.
21. Giménez-Barcons M, Franco S, Suárez Y, Forns X, Ampurdanès S, Puig-Basagoiti F, et al. High amino acid variability within the NS5A of hepatitis C virus is associated with hepatocellular carcinoma in patients with HCV-1b-related cirrhosis. HEPATOLOGY 2001;34:158-167.
22. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. J Hepatol 1999;30:653-659.
23. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
24. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk of hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. Ann Intern Med 1999;131:174-181.
25. Hino K, Kitase A, Satoh Y, Fujiwara D, Yamaguchi Y, Korenaga M, et al. Interferon retreatment reduces or delays the incidence of hepatocellular carcinoma in patients with chronic hepatitis C. J Viral Hepat 2002;9:370-376.

Original Paper

Intervirology

Intervirology 2007;50:161–165
DOI: 10.1159/000098958

Received: February 17, 2006
Accepted after revision: April 10, 2006
Published online: January 26, 2007

Long-Term Presence of HBV in the Sera of Chronic Hepatitis B Patients with HBsAg Seroclearance

Yasuji Arase[a] Fumitaka Suzuki[a] Yoshiyuki Suzuki[a] Satoshi Saitoh[a] Masahiro Kobayashi[a] Norio Akuta[a] Takashi Someya[a] Tetsuya Hosaka[a] Hitomi Sezaki[a] Junko Sato[b] Mariko Kobayashi[b] Kenji Ikeda[a] Hiromitsu Kumada[a]

[a]Department of Gastroenterology and [b]Hepatic Research Unit, Toranomon Hospital, Tokyo, Japan

Key Words
Chronic hepatitis B · Hepatitis B virus DNA · Seroclearance, hepatitis B surface antigen

Abstract
Objects: The aim of this study was to elucidate the presence of serum hepatitis B virus (HBV) DNA at a prolonged time after seroclearance of hepatitis B surface antigen (HBsAg). ***Methods:*** Seventy Japanese patients who had been observed for >5 years after HBsAg seroclearance were included in this study. Anti-HBs, anti-HBe and anti-HBc antibodies were measured 0, 5 and 10 years after HBsAg seroclearance. Serum HBV DNA was measured using nested polymerase chain reaction (PCR) at 0, 5 and 10 years after HBsAg seroclearance. The PCR detection of serum HBV DNA using the X gene and core gene primers was done. The HBV DNA was regarded as positive when PCR detection of HBV DNA using either or both the X gene and core gene primers was positive. A multivariate regression analysis was used to assess the factors contributing to the positivity of serum HBV DNA 5 years after HBsAg seroclearance: the factors examined included age, gender, histological findings, HBV genotype, aminotransferase, total protein and interferon administration. ***Results:*** The titers of 200-fold diluted serum anti-HBc were 6.5 ± 4.0 at 0 year after HBsAg seroclearance, 1.8 ± 1.4 at 5 years and 0.9 ± 0.7 at 10 years. The titers of 200-fold diluted serum anti-HBc decreased 5 and 10 years after HBsAg seroclearance with statistical significance. The positive rate of HBV DNA by the nested PCR was 71.4% (50/70) at 0 year after HBsAg seroclearance, 21.4% (15/70) at 5 years and 14.3% (3/21) at 10 years. However, there were no significant factors contributing to the positivity of serum HBV DNA 5 years after HBsAg seroclearance. ***Conclusion:*** Our results suggest that serum HBV DNA disappears with an incidence of 10–20% 5 and 10 years after HBsAg seroclearance.

Introduction

Chronic hepatitis B is a serious liver disease with significant mortality. In patients with chronic hepatitis B virus (HBV) infection, persistent viral replication is associated with ongoing necroinflammation in the liver and progressive liver damage [1–3]. However, in patients with seroclearance of hepatitis B envelope antigen (HBeAg) and marked reduction of HBV DNA, the prognosis of the disease is generally improved [4–6]. Moreover, hepatitis B surface antigen (HBsAg) seroclearance has probably been associated with a good prognosis [7–12].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/07/0503–0161$23.50/0

Accessible online at:
www.karger.com/int

Yasuji Arase, MD
Department of Gastroenterology, Toranomon Hospital
2-2-2 Toranomon, Minato-ku
Tokyo 105-8470 (Japan)
Tel. +81 3 3588 1111, Fax +81 3 3582 7068, E-Mail es9y-ars@asahi-net.or.jp

An explosion of papers argue that some patients with seroclearance of HBsAg showed positive HBV DNA at the time of HBsAg seroclearance or within 1 year of HBsAg seroclearance [13–17]. However, it is not clear how long serum HBV DNA could be detected after prolonged observation after HBsAg seroclearance. Moreover, it is still a question whether the patients with seroclearance of HBsAg could be really cleared of serum HBV DNA or not. To further investigate these issues, we performed the present study on the long-term virological outcome after HBsAg seroclearance in Japanese patients.

Materials and Methods

Patients

From 1972 to 2002, a total of 5,055 chronic HBsAg carriers, who were known to be seropositive for HBsAg for at least 6 months, were studied in Toranomon Hospital in Tokyo, Japan. After a mean follow-up period of 4 years (range 0.5–30 years), 231 patients were noted to have delayed HBsAg seroclearance, which is defined as persistent absence of HBsAg antigenemia by radioimmunoassay for at least 1 year until the last examination. Of these 70 patients had the following criteria: (1) laparoscopy and liver biopsy taken before HBsAg seroclearance showed histological features of chronic active hepatitis or liver cirrhosis; (2) the follow-up period was more than 5 years after seroclearance of HBsAg.

We excluded from the study all the patients: (1) with concurrent hepatitis C virus and hepatitis D virus; (2) with a history of alcohol abuse or autoimmune liver disease; (3) with clinical evidence of hepatocellular carcinoma at entry into the study on the basis of ultrasonography, α-fetoprotein levels (<200 ng/ml) and/or histology; (4) with a history or clinical evidence of complications of decompensated cirrhosis at enrollment (that is ascites, encephalopathy or icterus).

Thirty-seven of 70 patients had spontaneous seroclearance of HBsAg, 20 patients had been given interferon (IFN) therapy for 1–16 months, 9 had been given steroid withdrawal monotherapy and 4 had been treated with combination therapy of steroid + IFN. The total median dose of IFN therapy was 336 mega units (range, 168–624 mega units). The patients treated with steroids were generally given prednisolone for 4 weeks, in a single dose of 40 mg/day for 1 week, 30 mg/day for 1 week, 20 mg/day for 1 week and then 10 mg/day for 1 week until it was abruptly withdrawn (total dose 700 mg).

Methods

The serums were stored at −80° until enzyme assays and measurement of HBV DNA level by the nested PCR method could be performed on all the samples for 70 patients at one time. Serum samples had been conserved at 0, 5 and 10 years after seroclearance of serum HBsAg. Serum HBV DNA was determined using the nested PCR independently by an experienced technician (J.S.), who had no clinical information or knowledge of each patient. The sensitivity of HBV DNA according to the manufacturer is about 50–100 copies/ml in the nested PCR method. Two kinds of primers in the core and X gene of HBV were used in the nested PCR method. First of all, primers used for the detection of HBV were Cof1 (sense, 5′-CTGCCTTACTTTTGGAGAGA-3′) and Cer1 (antisense, 5′-ACTTTACTGGGCTTTATTA-3′) for the first PCR and core sense (sense, 5′-GAGTGTGGATTCGCACTCC-TC-3′) and anticore (5′-GATTGAGATCTTCTGCGACGC-3′) for the second PCR in the core gene. Second, primers used for detection of HBV were P2 (sense, 5′-GTCCCGTCGGCGCTGAAT-CCC-3′) and Br102 (antisense, 5′-GCAGATGAGAAGGCACA-GAC-3′) for the first PCR and X sense (sense, 5′-CTGGATCCT-GCGCGG GACGTCCTT-3′) and anti-X (5′-GTTCACGGTGGT-CTCCAT-3′) for the second PCR in the X gene. In the first PCR and the second PCR, amplification was performed over 35 cycles (94 for 1 s; 55° for 1 s; 72° for 1 s) after initial denaturing at 94° for 4 min and a final extension at 72° for 7 min. Negative and positive controls confirmed the HBV DNA band in parallel. Ten healthy volunteers without HBsAg and anti-HCV were selected for negative HBV DNA controls. Ten patients with chronic hepatitis B and with HBsAg were selected for positive controls. The HBV DNA was considered positive when PCR detection of HBV DNA using either or both the X gene and core gene primers showed positivity. On the other hand, the HBV DNA was considered negative when PCR detection of HBV DNA using both the X gene and core primers showed negativity.

When serum samples showed positive HBV DNA by the nested PCR, we also examined the serum HBV DNA level. It was measured by a transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per milliliter. The lower detection limit of this assay is 3.7 LGE/ml, which is equivalent to 5,000 copies/ml.

HBsAg, anti-HBs, HBeAg, anti-HBe and antibody to HDV were all assayed using commercially available radioimmunoassay kits. Anti-HBc was assayed by chemiluminescent enzyme immunoassay. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo). The HBV genotype was determined using a previously reported method [18]. Biochemical tests were made using routine automated techniques and carried out in the laboratories of Toranomon Hospital. This study was approved by the institutional review board of our hospital. The physicians in charge explained the purpose and method of this clinical trial to each patient, who gave their informed consent for participation.

Liver Histology

Liver biopsy specimens were obtained percutaneously under the observation by laparoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan).

Statistical Analysis

We used Fisher's exact test (two-tailed) or the Wilcoxon rank sum test to compare differences between groups. Moreover, we used univariate analysis and multivariate analysis (multiple logistic regression analysis) to establish which factors contributed to the positivity of HBV DNA 5 years after HBsAg seroclearance. Results for each variable were transformed into categorical data consisting of two simple original numbers for univariate and multivariate analyses. Variables that achieved statistical significance

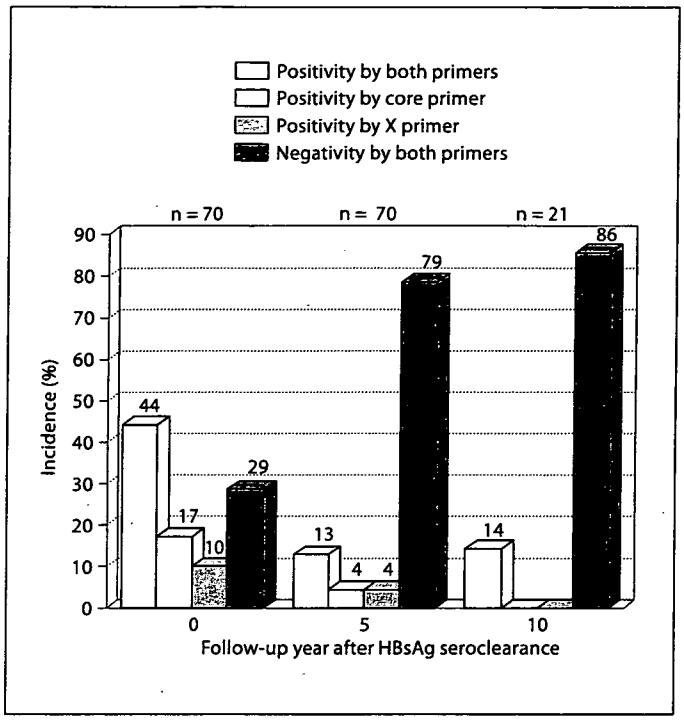

Fig. 1. Changes of detection pattern of serum HBV DNA after seroclearance of HBsAg. Negative controls: 10 healthy volunteers; positive controls: 10 patients with chronic hepatitis B; core (X): positivity indicates positive HBV DNA by nested PCR using core (X) gene primers, negativity indicates negative HBV DNA by nested PCR using core (X) gene primers.

Table 1. Characteristics of subjects at the time of seroclearance of HBsAg

Number	70
Sex (male/female)	55/15
Age, years	53 (30–82)
HBV genotype (A/B/C/D/F)	3/7/45/2/6
US (non-LC/LC)	40/30
Total protein, g/dl	7.4 (6.6–8.8)
Albumin, g/dl	4.2 (3.4–5.1)
Total bilirubin, g/dl	0.7 (0.1–1.7)
AST, IU/l	21 (11–71)
ALT, IU/l	16 (6–101)
Hb, g/dl	15.2 (12.9–17.1)
Platelets, $\times 10^4/mm^3$	17.3 (8.4–32.5)
Follow-up period after disappearance of HBs antigen, years	8.3 (5.3–23.6)

Data are numbers of patients or medians, with ranges in parentheses. ALT = Alanine aminotransferase; AST = aspartate aminotransferase; Hb = hemoglobin; US = ultrasonographic findings; LC = liver cirrhosis.

Table 2. Change of anti-HBc antibody after HBsAg seroclearance

	Follow-up year of HBsAg seroclearance		
	0	5	10
Anti-HBc antibody	14.2 ± 2.7	13.9 ± 2.2	13.3 ± 3.6
Anti-HBc antibody (200-fold dilution)	6.5 ± 4.0	1.8 ± 1.4	0.9 ± 0.7

The serum was diluted 1:200 with saline. The titer of anti-HBc antibody was determined by the chemiluminescent immunoassay method.

($p < 0.05$) were subjected to multiple logistic regressions to identify significant independent predictors. The SPSS software package (SPSS 10.0 for Windows; SPSS Inc., Chicago, Ill., USA) was used for analyses.

Results

Clinical Profiles

Table 1 shows the characteristics of the 70 patients who had seroclearance of HBsAg. The median age of the 70 patients (male 55, female 15) was 53 years. Thirty-seven patients had spontaneously cleared HBsAg. At the time of HBsAg seroclearance, 30 patients showed liver cirrhosis.

Sixty-three of 70 (87.9%) patients had normal alanine aminotransferase levels after HBsAg seroclearance. Seven patients with elevated alanine aminotransferase had 4 fatty infiltrations of the liver and 3 cases of alcohol abuse.

Changes of Anti-HBs, Anti-HBe and Anti-HBc

Table 2 shows the titers of serum anti-HBc. As regards the titer of nondiluted anti-HBc, there was no difference between the time of HBsAg seroclearance, 5 years and 10 years after HBsAg seroclearance. The titers of 200-fold diluted serum anti-HBc decreased 5 and 10 years after HBsAg seroclearance with statistical significance.

Serum HBV DNA after HBsAg Seroclearance

The detection pattern of serum HBV DNA based on the difference of HBV primers by the nested PCR is shown in figure 1. The negative controls of healthy volunteers showed negative HBV DNA with both primers. On the