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).

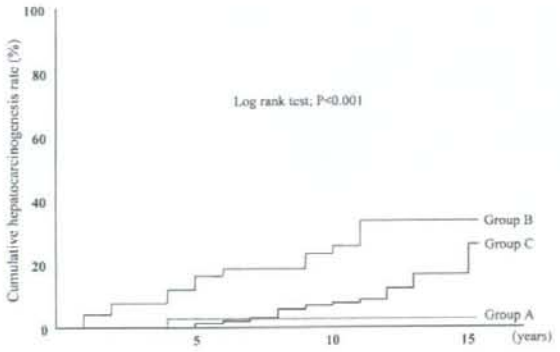


Fig. 1. Cumulative hepatocarcinogenesis rates were significantly different among the 3 study groups ($P < 0.001$; Log-rank test). In particular, the rates in group B were significantly higher than in group C ($P < 0.001$; Log-rank test) and group A ($P < 0.001$; log-rank test), and the rates in group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

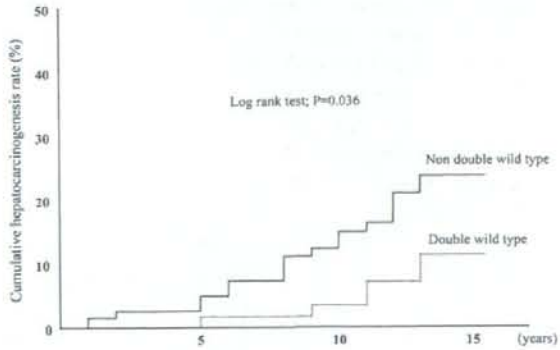


Fig. 2. Cumulative hepatocarcinogenesis rates according to aa substitutions of HCV-CR. The rates in double wild-type (arginine at aa 70/leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type ($P = 0.036$; log-rank test).

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;50:16–23
DOI: 10.1159/000096308

Received: December 14, 2005
Accepted: December 21, 2005
Published online: November 24, 2006

Long-Term Outcome after Interferon Therapy in Elderly Patients with Chronic Hepatitis C

Yasuji Arase Kenji Ikeda Fumitaka Suzuki Yoshiyuki Suzuki Satoshi Saitoh Masahiro Kobayashi Norio Akuta Takashi Someya Rikako Koyama Tetsuya Hosaka Hitomi Sezaki Mariko Kobayashi Hiromitsu Kumada

Department of Gastroenterology, and Hepatic Research Unit, Toranomon Hospital, Toranomon, Tokyo, Japan

Key Words

Chronic hepatitis C · Elderly patients · Interferon · Hepatocellular carcinoma · IFN therapy in elderly patients, survival

Abstract

Objective: The purpose of this study was to elucidate the long-term outcome after interferon (IFN) therapy in chronic hepatitis C elderly patients. ***Methods:*** We studied the incidence of hepatocellular carcinoma (HCC) and survival probability after the initiation of IFN therapy in 500 Japanese chronic hepatitis C patients >60 years. The mean age of initiation of IFN was 63 years and the mean follow-up period was 7.4 years. Cox proportional hazard regression analysis was used to evaluate the long-term outcome after initiation of IFN therapy. Sustained virological response (SVR) was defined as negative HCV-RNA by RT-nested PCR 6 months after the completion of long-term IFN therapy. Non-response (NR) was applied to patients who did not show SVR. Hepatic fibrosis was defined as the fibrosis score (score 0–4) according to Knodell et al. ***Results:*** 140 patients (28%) had an SVR and 360 patients (72%) had an NR. 71 of 500 patients developed HCC during follow-up. The cumulative incidence of HCC was 9.6% at the 5th year, 17.4% at the 10th year, and 31.3% at the 15th year. HCC developed with significance when: (1) HCV was not cleared after IFN therapy ($p < 0.0001$), (2) sex was male ($p < 0.0001$), and (3) staging of liver fibrosis was >2 ($p = 0.008$). 53 of the patients died. The cumulative survival probability was 95.7% at the 5th year, 86.4% at the 10th year, and 78% at the 15th year. Patients achieved a long survival with significance when: (1) staging of liver fibrosis was 1 ($p < 0.0001$), (2) HCV was cleared after IFN therapy ($p = 0.034$), and (3) sex was female ($p = 0.015$). ***Conclusion:*** Chronic hepatitis C patients with clearance of HCV after IFN therapy had a significantly reduced risk of HCC appearance and achieved prolonged survival even if they are ≥60 years.

Introduction

Hepatocellular carcinoma (HCC) often occurs in patients with hepatitis C virus (HCV)-RNA-positive chronic liver disease [1]. The majority of deaths due to HCC are ascribed to hepatitis viruses, of which 70–80% corresponding to approximately 30,000 per year is attributed to the persistent infection with HCV in Japan [2, 3]. It is important to eradicate HCV or decrease levels of

KARGER

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

0300–5526/07/0501–0016$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

alanine aminotransferase (ALT) for preventing HCC with interferon (IFN) therapy [4, 5].

Nowadays, patients with HCV in Japan tend to be aged. Also, HCV-related HCC patients have been shown to become old with a peak around the age of 70 [6]. When such aged chronic hepatitis C patients with abnormal ALT levels consult a doctor, the first problem is whether or not therapy should be used for chronic hepatitis C. Moreover, when treatment for chronic hepatitis C is decided in such aged patients, whether IFN therapy should be used or not is the second problem. However, a few studies have targeted IFN therapy and prolonged prognosis in elderly patients with chronic hepatitis C [7, 8]. Until now, IFN treatment for chronic hepatitis C has mainly been introduced when patients are less than 60–65 years of age because of IFN-related side effects and safety standards in Japan. Owing to IFN-related side effects or various complicated diseases, there is a tendency not to give IFN to aged patients. Thus, IFN therapy for chronic hepatitis C has been conventionally limited to patients aged less than 60–65 years. We therefore assessed the long-term efficacy of IFN therapy in elderly patients with chronic hepatitis C by a retrospective cohort study.

Patients and Methods

Patients

The number of chronic hepatitis C patients treated with IFN therapy in our hospital between 1989 and 2004 was 3,320. Of these, 500 patients had the following criteria: (1) ≥ 60 years of age; (2) ALT elevation greater than double the upper limits (ALT normal range 12–50 IU/l) within 6 months; (3) no corticosteroid immunosuppressive agents, or antiviral agents used within 6 months; (4) no hepatitis B surface antigens, antinuclear antibodies, or antimitochondrial antibodies detectable in serum, determined by radioimmunoassay; (5) leukocytes >3,000/mm^3, platelet count >80,000/mm^3, and bilirubin <2.0 mg/ml, and (6) IFN therapy >4 weeks. Next, we excluded those patients from the study with a history of alcohol abuse or advanced liver cirrhosis of encephalopathy, bleeding esophageal varices, or ascites. Our study was approved by the institutional Ethics Review Board of our hospital. The physician in charge explained the purpose and method of this clinical trial, as well as the potential adverse reactions to each patient, who later gave his/her informed consent for participation.

IFN Therapy

For the first IFN treatment regimen, the IFN treatment consisted of 3–12 million units (MU) of IFN-α or IFN-β. For the IFN treatment regimen, one group of 245 patients was assigned to receive IFN intramuscularly every day for the first 2–8 weeks and then 2–3 times/week for the following 16–96 weeks. Another group of 116 cases was assigned to receive IFN 3 times/week for 24–104 weeks. A third group of 108 patients was assigned to be treated with IFN by intravenous injection daily for 4–8 weeks. The fourth group of 31 patients was given combination therapy of IFN and ribavirin. The median total dose was 624 MU (range 168–2,430) and median administration period was 165 days (range 28–730).

Definition of Response of IFN Efficacy

Patients treated with IFN were divided into the following two groups based on the serum HCV-RNA after the termination of IFN. Sustained virological response (SVR) was defined as negative HCV-RNA by RT-nested PCR 6 months after the completion of long-term IFN therapy. Non-response (NR) was applied to patients who did not show SVR.

Blood and Urine Tests

Blood samples were obtained just before and 6 months after IFN treatment. The samples were stored at –80° until analyzed. Using these blood samples, HCV-RNA levels before IFN therapy were analyzed by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0, Roche Molecular Systems) [9]. On the other hand, HCV-RNA 6 months after the termination of IFN therapy was analyzed by qualitative PCR assay. The lower detection limit of the qualitative assay is 100 copies/ml [10]. HCV genotype was examined by PCR assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported previously [11].

Follow-Up Protocol

The start of the follow-up period was defined as the first day of IFN treatment. Clinical evaluation and biochemical and hematologic tests were performed at 1–3 monthly intervals. Thirty-four patients were lost to follow-up. Because the appearance of HCC and death was not identified in these 34 patients, they were considered as censored data in statistical analyses [12]. Moreover, patients retreated with IFN in order to eradicate HCV-RNA were regarded as withdrawals at the start of IFN retreatment.

Diagnosis of HCC was based on the presence of typical hypervascular characteristics on angiography, in addition to the findings on computed tomography and ultrasonography. Microscopic examination of fine-needle biopsy material was performed in patients whose angiograms did not demonstrate a typical image of HCC. Histopathological confirmation using surgically resected specimens was made in 21 patients.

Cause of death was divided into liver-related and liver-unrelated. The former included HCC, liver failure, and esophagogastric variceal bleeding, and the latter included extrahepatic malignancies, heart disease, cerebrovascular accidents, pulmonary disease, and others.

Liver Histology before IFN Therapy

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), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The biopsy specimens were scored according to the system of Knodell et al. [13]. Histologic index score: 0–10 for periportal bridging necrosis and 0–4 for interlobular degeneration and focal necrosis, portal inflammation, and fibrosis.

Table 1. Clinical characteristics before IFN treatment according to efficacy of IFN therapy in chronic hepatitis C elderly patients

Characteristics	SVR (n = 140)	NR (n = 360)	p
Age, years*	63 ± 3.2	64 ± 3.2	0.070
Male/female	83/57	168/192	0.011
Liver histology (fibrosis: 1/2/3/4)**	59/47/6/12	120/109/29/58	0.009
Liver histology (activity)*, **	9.3 ± 3.4	9.8 ± 2.7	0.223
HCV genotype (1b/2a/2b/others)	47/74/12/17	255/48/38/23	<0.0001
HCV load, kIU/ml*	172 ± 204	661 ± 506	<0.0001
AST, IU/l*	83 ± 70	87 ± 51	0.143
ALT, IU/l*	113 ± 102	118 ± 82	0.200
Hb, g/dl	14.2 ± 1.4	14.1 ± 1.3	0.547
Platelets, $\times 10^4/mm^3$*	15.0 ± 4.6	14.9 ± 4.8	0.768
WBC, $\times 10^3/mm^3$*	4.6 ± 1.4	4.6 ± 1.3	0.751
Period of observation, years	7.0 ± 3.3	7.7 ± 3.6	0.011

Activity was defined as sum score of periportal bridging necrosis, interlobular degeneration and focal necrosis, and portal inflammation.

ALT = Alanine aminotransferase; AST = aspartate aminotransferase; NR = non-response; SVR = sustained virological response; WBC = white blood cells.

* Data are number of patients or mean ± SD.

** Histologic index score: 0–10 for periportal bridging necrosis and 0–4 for interlobular degeneration and focal necrosis, portal inflammation, and fibrosis.

Activity was defined as sum score of periportal bridging necrosis (score 0–10), interlobular degeneration and focal necrosis (score 0–4), and portal inflammation (score 0–4). Fibrosis was defined as fibrosis score (score 0–4).

Statistical Analysis

Baseline characteristics and treatment differences among groups based on efficacy of IFN treatment were analyzed using Kruskal-Wallis test. HCC appearance rates were analyzed by the log-rank test. A Cox proportional hazards model was used to analyze the factors contributing to the HCC appearance rate and death: factors examined included age, gender, histologic findings, HCV genotype, HCV load, aspartate aminotransferase (AST), ALT, and efficacy of IFN administration. A p value <0.05 was considered statistically significant. The SPSS Software Package (SPSS Inc., Chicago, Ill., USA) was used for analyses.

Results

Characteristics of the Patients and the Efficacy of the IFN Therapy

500 patients were enrolled in the present study. 140 patients (28%) had a SVR and 360 patients (72%) had a NR. Table 1 shows the baseline characteristics of the patients based on the efficacy of IFN therapy. The frequency distributions of the HCV genotype, the stage of liver fibrosis and HCV load differed between the two groups.

Development of HCC and Risk Factors for Appearance of HCC

During follow-up, HCC developed in 71 patients. The cumulative incidence as shown in figure 1 was based on efficacy of IFN therapy. The cumulative incidence of HCC was 9.6% at the 5th year, 17.4% at 10th year, and 31.3% at 15th year.

Cox regression analysis was performed using nine variables, including age, sex, histopathological severity (staging), viral load, HCV genotype, serum AST, serum ALT, and efficacy of IFN therapy. Univariate analysis showed that the following five factors significantly affected the cumulative HCC appearance rate in all patients as shown in table 2. Because the variables were mutually correlated, multivariate Cox regression analysis was performed with the statistically significant variables in the model (table 3). HCC developed with significance when: (1) HCV was not cleared ($p < 0.0001$), (2) sex was male ($p < 0.0001$), and (3) staging of liver fibrosis was >2 ($p = 0.008$).

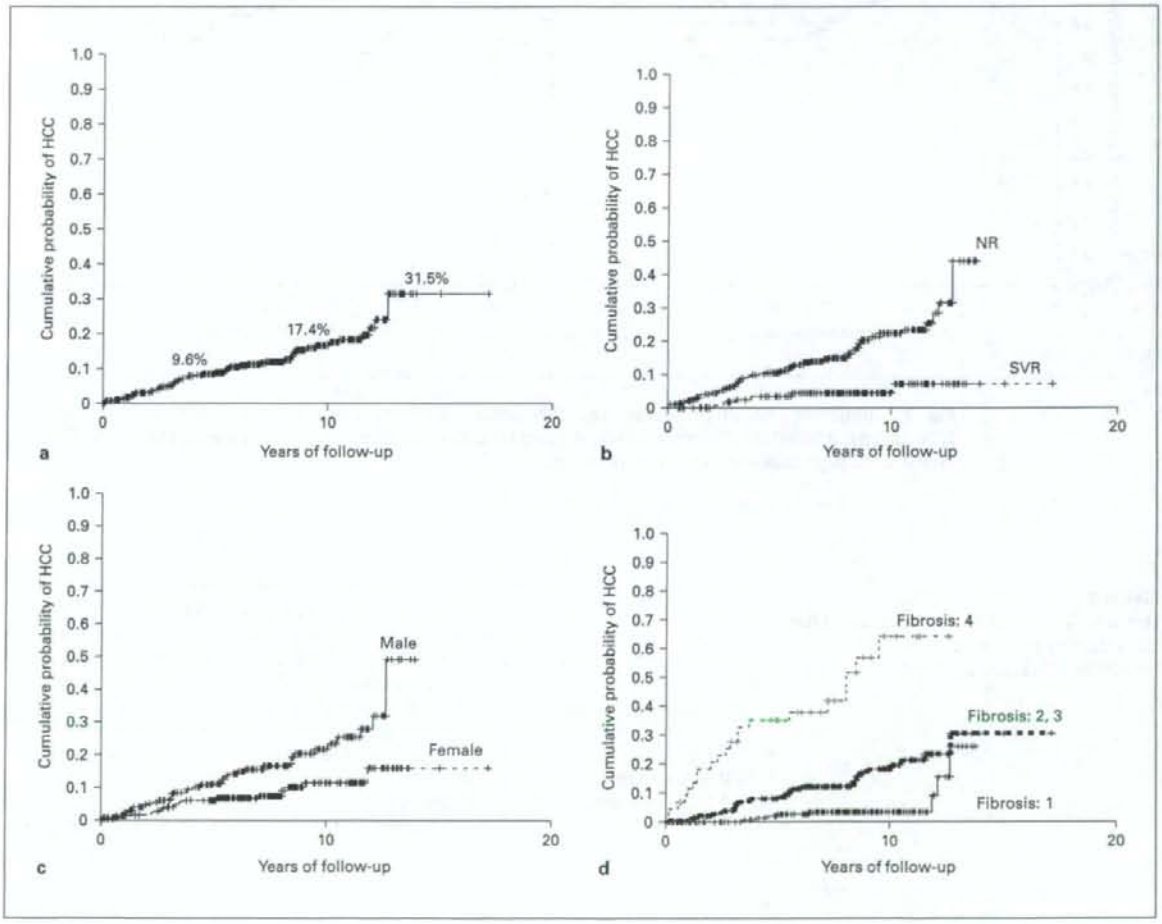
Fig. 1. Cumulative appearance probability of HCC. **a** In total patients; **b** based on difference of efficacy of IFN therapy; **c** based on difference of sex; **d** based on difference of histological fibrosis. SVR = Sustained virological response; NR = non-response.

Table 2. Predictive factors for hepatocellular carcinoma appearance after IFN therapy by Cox proportional hazards model (univariate analysis)

Factor	Category	Risk ratio*	95% CI	p
Liver histology (fibrosis)	1/2,3,4	1/4.22	2.81–6.34	<0.0001
Sex	Male/female	1/0.44	0.26–0.75	0.002
Age, years	<65/≥65	1/1.95	1.14–3.32	0.015
HCV genotype	1/2	1/0.55	0.31–0.97	0.046
AST, IU/l	<76/≥76	1/1.75	0.83–3.67	0.141
ALT, IU/l	<100/≥100	1/1.64	0.79–3.40	0.184
HCV-RNA, kIU/ml	<100/≥100	1/1.47	0.79–2.76	0.224
Liver histology (activity)	<10/≥10	1/1.55	0.79–3.07	0.206
Efficacy of IFN therapy	NR/SVR	1/0.22	0.096–0.52	<0.0001

ALT = Alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; NR = non-response; SVR = sustained virological response.

* Risk ratio for development of HCC (71 events among all 500 patients) were calculated by using Cox proportional hazards regression analysis.



1

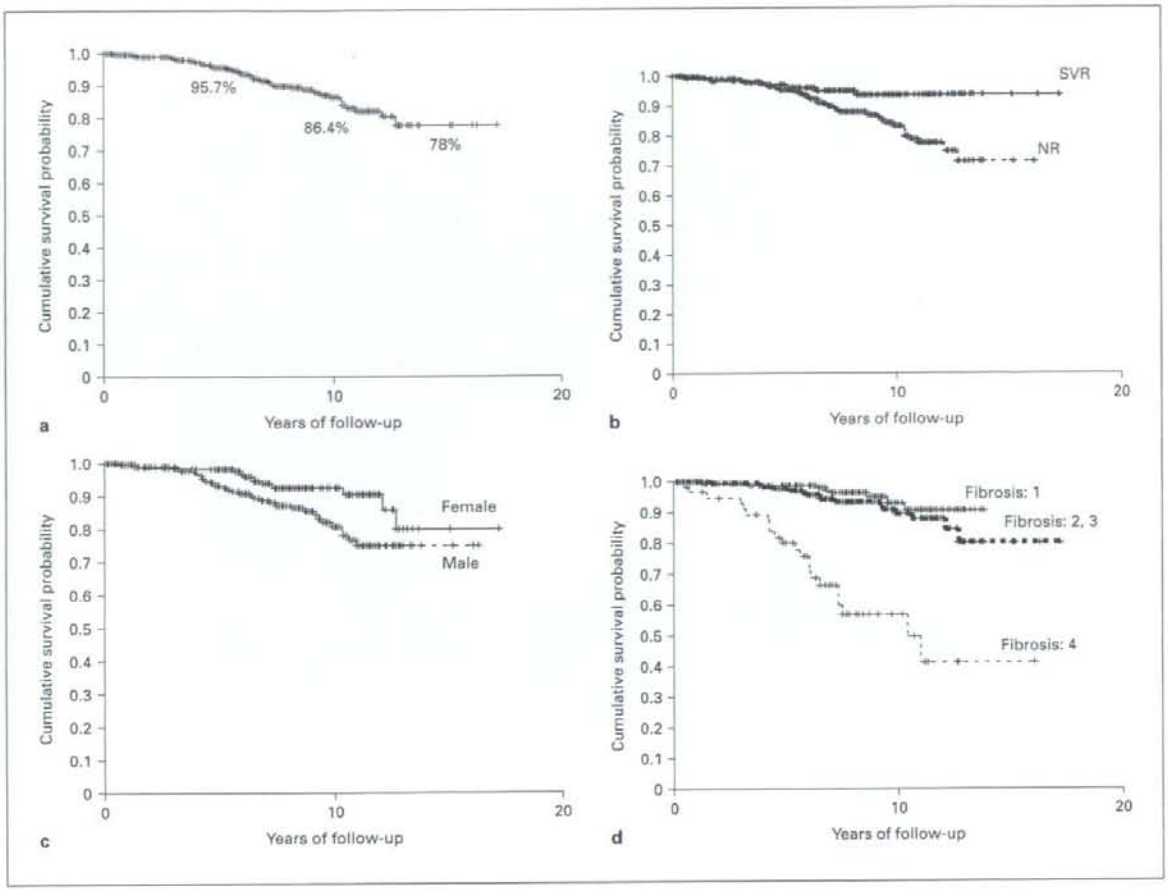


Fig. 2. Cumulative survival probability after IFN therapy. **a** In all patients; **b** based on difference of efficacy of IFN therapy; **c** based on difference of sex; **d** based on difference of efficacy of histological fibrosis. SVR = Sustained virological response; NR = non-response.

Table 3. Predictive factors for hepatocellular carcinoma appearance after IFN therapy by Cox proportional hazards model (multivariate analysis)

Factor	Category	Risk ratio	95% CI	p
Efficacy of IFN therapy	NR/SVR	1/0.193	0.083–0.45	<0.0001
Sex	Male/female	1/0.36	0.21–0.62	<0.0001
Liver histology (fibrosis)*	1/2,3,4	1/2.08	1.22–3.57	0.008

ALT = Alanine aminotransferase; CI = confidence interval; NR = non-response; SVR = sustained virological response.

* Histologic index score: 0–4 for fibrosis.

Cause of Death and Cumulative Survival Probability

Fifty-three of 500 patients died during an average follow-up of 7.4 years. The cumulative survival probability was 95.7% at the 5th year, 86.4% at the 10th year, and 78% at the 15th year (fig. 2). The number of liver-related and liver-unrelated deaths is shown in table 4. Liver-related death corresponded to 64.2% (34/53) of all deaths. HCC was the major cause of liver-related deaths. Univariate analysis showed that the following four factors significantly affected the cumulative survival probability in all patients as shown in table 5. Multivariate analysis revealed that patients achieved a significant survival when: (1) staging of liver fibrosis was 1 ($p<0.0001$), (2) HCV was cleared ($p = 0.034$), and (3) sex was female ($p = 0.015$).

Table 4. Cause of death

	Efficacy of IFN	
	SVR (n = 140)	NR (n = 360)
Deaths	9	44
Liver related	2 (22%)	32 (73%)
Hepatocellular carcinoma	2	26
Liver failure	0	5
Gastrointestinal bleeding	0	1
Liver unrelated	7 (78%)	12 (27%)
Malignancies	3	7
Heart disease	2	2
Cerebrovascular disease	2	1
Pulmonary disease	1	2

SVR = Sustained virological response; NR = non-response.

Discussion

In order to protect hepatocarcinogenesis, patients with chronic hepatitis C are often treated with antivirus drugs and/or anti-inflammatory drugs. Antivirus drugs, such as IFN, are given to eradicate HCV-RNA for chronic hepatitis C. Moreover, it has recently been reported that hepatitis C viral clearance or normalization of serum ALT after IFN therapy contribute to the notably suppressed incidence of HCC caused by chronic HCV infection [14–17]. Yoshida et al. [14] reported the efficacy of IFN for HCV-positive patients with chronic hepatitis or cirrhosis in a retrospective surveillance study in Japan, which focused on the HCC appearance rate and survival in a total of 2,890 patients, 2,400 treated with IFN and 490 not treated with IFN. Their conclusion was that IFN therapy improved survival of chronic hepatitis C patients by preventing liver-related deaths. Ikeda et al. [15] reported similar results from 1,643 patients of whom 1,191 had received IFN monotherapy. The incidence of HCC in treated patients was 7.6% after 10 years of follow-up evaluation, compared with 12.4% in untreated patients. Imazeki et al. [16] presented a follow-up study of 459 patients with chronic hepatitis C over 8.2 years to examine the survival rate relative to IFN therapy. Cirrhotic patients with a SVR showed a reduction in mortality (relative risk 0.219). Serfaty et al. [17] showed a beneficial effect of IFN on the development of HCC and on survival.

In the present study we found that the clearance of HCV after IFN therapy reduced HCC appearance and liver-related death in elderly patients (table 6). Horiike et al. [7] reported that elderly patients with a low HCV-RNA

Table 5. Predictive factors for survival probability after IFN therapy by Cox proportional hazards model (univariate analysis)

Factor	Category	Risk ratio*	95% CI	p
Liver histology (fibrosis)	1/2,3/4	1/3.90	2.49–6.10	<0.0001
Sex	Male/female	1/0.47	0.26–0.86	0.014
Efficacy of IFN therapy	NR/SVR	1/0.37	0.17–0.83	0.015
Age, years	<65/≥65	1/2.08	1.14–3.78	0.016
HCV genotype	1/2	1/0.55	0.31–0.97	0.046
AST, IU/l	<76/≥76	1/0.80	0.37–1.74	0.571
ALT, IU/l	<100/≥100	1/0.81	0.37–1.76	0.588
HCV-RNA, kIU/ml	<100/≥100	1/0.92	0.49–1.71	0.781
Liver histology (activity)	<10/≥10	1/1.62	0.73–3.62	0.206

ALT = Alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; NR = non-response; SVR = sustained virological response.

* Risk ratio for development of HCC (71 events among all 500 patients) were calculated by using Cox proportional hazards regression analysis.

Table 6. Predictive factors for death after IFN therapy

Factors (category)	Overall deaths		Liver-related death		Liver-unrelated death	
	risk ratio* (95% CI)	p	risk ratio* (95% CI)	p	risk ratio* (95% CI)	p
Liver histology (fibrosis: 1/2,3,4)	1/3.55 (2.28–5.51)	<0.0001	1/6.18 (3.37–11.35)	<0.0001	1/1.10 (0.52–2.36)	0.799
Efficacy after IFN therapy (NR/SVR)	1/0.39 (0.16–0.93)	0.034	1/0.13 (0.03–0.59)	0.007	1/0.79 (0.27–2.32)	0.668
Male/female	1/0.46 (0.25–0.86)	0.015	1/0.40 (0.18–0.88)	0.022	1/0.71 (0.25–2.00)	0.799

CI = Confidence interval; IFN = interferon; NR = non-response; SVR = sustained virological response.
* Risk ratio for development of death (53 events among all 500 patients) were calculated by using Cox proportional hazards regression analysis.

level might benefit from IFN therapy, although they should decide the indications for IFN very carefully in this age group. Imai et al. [8] reported that some aged patients with chronic hepatitis C might be recommended IFN therapy.

Next, IFN therapy can be associated with various side effects and is costly, therefore the selection of aged patients for IFN therapy is extremely important. Factors predictive of a SVR to IFN have been extensively studied, i.e. short duration of disease, young age, absence of cirrhosis, low HCV-RNA levels and HCV genotype 2a [18]. Chronic hepatitis C patients with genotype 2a/2b or genotype 1b and lower virus load show a particularly good response to IFN therapy. Even if small amounts of IFN are given to patients with these factors, a biochemical response as well as a SVR can be expected. Therefore, when aged patients with chronic hepatitis C have various factors that would respond well to IFN treatment, IFN therapy can be recommended to aim for prolonged survival after screening for diseases other than chronic hepatitis C. On the other hand, when aged patients with chronic hepatitis C do not have factors that would respond well to IFN treatment, IFN therapy should not be recommended.

In conclusion, our results suggest that clearance of HCV after IFN therapy significantly reduces the risk of HCC appearance and death in aged chronic hepatitis C patients.

Acknowledgement

The present work was supported in part by grants-in-aid from Okinaka Memorial Institute for Medical Research and the Japanese Ministry of Health, Labor and Welfare.

References

1 Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino L, Fiorentino G, Craxi A, Ciccaglione A, Giuseppetti R: Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. A case-control study. Ann Intern Med 1992; 116:97–102.

2 Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, Maeda Y, Shirai Y, Fukuzaki T, Kaji I, Ishikawa H, Matsuda Y, Nishikawa M, Seki K, Matsuzawa Y: Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. Ann Intern Med 1998;129:94–99.

3 Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, Nishioji K, Murakami Y, Kashima K: 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 1,148 patients. Viral Hepatitis Therapy Study Group. J Hepatol 1999;30:653–659.

4 Kasahara A, Hayashi N, Mochizuki K, Takayanagi M, Yoshioka K, Kakumu S, Iijima A, Urushihara A, Kiyosawa K, Okuda M, Hino K, Okita K: Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology 1998;27:1394–1402.

5 Ikeda K, Saitoh S, Kobayashi M, Suzuki Y, Tsubota A, Arase Y, Murashima N, Chayama K, Kumada H: Long-term interferon therapy for 1 year or longer reduces the hepatocellular carcinogenesis rate in patients with liver cirrhosis caused by hepatitis C virus: a pilot study. J Gastroenterol Hepatol 2001;16:406–415.

6 Tanaka H, Tsukuma H, Yamano H, Oshima A, Shibata H: Prospective study on the risk of hepatocellular carcinoma among hepatitis C virus positive blood donors focusing on demographic factors. Alanine aminotransferase level at donation and interaction with hepatitis B virus. Int J Cancer 2004;112:1075–1080.

7 Horiike N, MasumotoT, Nakanishi K, Michitaka K, Kurose K, Ohkura I, Onji M: Interferon therapy for patients more than 60 years of age with chronic hepatitis C. J Gastroenterol Hepatol, 1995;10:246–249.
8 Imai Y, Kasahara A, Tanaka H, Okanoue T, Hiramatsu N, Tsubouchi H, Yoshioka K, Kawata S, Tanaka F, Hino K, Hayashi K, Tamura S, Itoh Y, Sasaki Y, Kiyosawa K, Kakumu S, Okita K, Hayashi N: Interferon therapy for aged patients with chronic hepatitis C; improved survival in patients exhibiting a biochemical response. J Gastroenterol 2004;39: 1069–1077.
9 Albadalejo J, Alonso R, Antinozzi R, Bogard M, Bourgault AM, Colucci G, Fenner T, Petersen H, Sala F, Vincelette J, Young C: Multicenter evaluation of the COBAS Amplicor HCV assay, an integrated PCR system for rapid detection of hepatitis C virus RNA in the diagnostic laboratory. J Clin Microbiol 1998; 36:862–865.
10 Doglio A, Laffont C, Caroli-Bose FX, Rochet P, Lefebvre J: Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. J Clin Microbiol 1999;37:1567–1569.
11 Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, McIntyre N, Simmonds P: Hepatitis C virus genotypes; an investigation of type-specific differences in geographic origin and disease. Hepatology 1994;19:13–18.
12 Harrington DP, Fleming TR: A class of rank test procedures for censored survival data. Biomedica 1983;62:205–209.
13 Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J: Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431–435.
14 Yoshida H, Arakawa Y, Sata M, Nishiguchi S, Yano M, Fujiyama S, Yamada G, Yokosuka O, Shiratori Y, Omata M: Interferon therapy prolonged life expectancy among chronic hepatitis C patients. Gastroenterology 2002;123: 483–491.
15 Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M: Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999;29:1124–1130.
16 Imazeki F, Yokosuka O, Fukai K, Saisho H: Favorable prognosis of chronic hepatitis C after interferon therapy by long-term cohort study. Hepatology 2003;38:493–502.
17 Serfaty L, Aumaitre H, Chazouilleres O, Bonnand AM, Rosmorduc O, Poupon RE, Poupon R: Determinants of outcome of compensated hepatitis C virus-related cirrhosis. Hepatology 1998;27:1435–1440.
18 Shiratori Y, Kato N, Yokosuka O, Imazeki F, Hashimoto E, Hayashi N, Nakamura A, Asada M, Kuroda H, Tanaka N, Arakawa Y, Omata M: Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Tokyo-Chiba Hepatitis Research Group. Gastroenterology 1997;113:558–566.

ELSEVIER

Journal of Hepatology 46 (2007) 403–410

Journal of Hepatology

www.elsevier.com/locate/jhep

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

Norio Akuta[1,*], Fumitaka Suzuki[2], 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], Hiromitsu Kumada[1]

[1] *Department of Hepatology, Toranomon Hospital, Tokyo, Japan*
[2] *Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

Background/Aims: **We showed previously that amino acid (aa) substitutions in the HCV core region (HCV-CR) are predictors of non-virological response (NVR) to peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the predictive factors of sustained virological response (SVR) and early virologic response (EVR) to this treatment.**

Methods: **We evaluated the response to 48-week PEG-IFN-RBV therapy in 114 Japanese adults infected with HCV genotype 1b and determined the predictors of EVR and SVR.**

Results: **EVR was achieved by 70% and SVR by 45% of patients. 64% of patients who achieved EVR also showed SVR, while none of non-EVR achieved SVR. Multivariate analysis identified low-density lipoprotein cholesterol (LDL-C) ($\geqslant$86 mg/dl), aa substitutions in HCV-CR (double-wild-type; arginine at aa 70/leucine at aa 91), gamma-glutamyl transpeptidase (GGT) (<109 IU/l), RBV dose ($\geqslant$11.0 mg/kg), and leukocyte count ($\geqslant$4500/mm^3) as significant determinants of EVR, and aa substitutions in HCV-CR (double-wild-type), LDL-C ($\geqslant$86 mg/dl), male gender, ICG R15 (<10%), GGT (<109 IU/l), and RBV dose ($\geqslant$11.0 mg/kg) as determinants of SVR. Prediction of response to therapy based on combination of these factors had high sensitivity, specificity, positive, and negative predictive values.**

Conclusions: **Our study identified aa substitutions in the core region and serum LDL-C as predictors of response to PEG-IFN-RBV therapy in Japanese patients infected with HCV genotype 1b.**

Keywords: **HCV; Core region; LDL cholesterol; Peginterferon; Ribavirin; Early virologic response; Sustained virological response; Mutation-specific primer; Double-wild type; ICG R15**

1. Introduction

For chronic hepatitis C virus (HCV) infection, the early virologic response (EVR) at 12 weeks after the completion of 48-week treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) is an important predictor of the sustained virological response (SVR) [1]. The observation that patients lacking EVR following PEG-IFN-α-2a-RBV combination therapy are highly unlikely to develop SVR was adopted as an assessment criterion by the National Institutes of Health Consensus Development Conference [2]. The predictive potential of EVR was also confirmed in patients treated with PEG-IFN-α-2b-RBV [3]. The underlying mechanisms of the

Received 23 July 2006; received in revised form 18 September 2006; accepted 19 September 2006; available online 7 November 2006

* Corresponding author. Tel.: +81 44 877 5111; fax: +81 44 860 1623.
E-mail address: akuta-gi@umin.ac.jp (N. Akuta).

0168-8278/$32.00
doi:10.1016/j.jhep.2006.09.019

different virological responses to treatment are still unclear.

We studied previously determinants of the response to the IFN-RBV therapy in patients with high titers of genotype 1b (≥100 kiloIU (KIU)/ml), which is dominant in Japan [4,5]. Our results identified substitutions of amino acid (aa) 70 and/or 91 in the HCV core region as an independent and significant pretreatment factor associated with non-virologic response (NVR), i.e., patients who do not achieve HCV-RNA negativity, as determined by PCR. Especially, substitutions of arginine by glutamine at aa 70 and/or leucine by methionine at aa 91 were significantly more common in NVR patients. Furthermore, we also showed that the falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region (HCV-CR) were significantly less than in those without such substitutions [4,5]. Whether aa substitutions in HCV core region are also useful as a predictor of EVR and SVR await, further investigation.

Recent studies have shown that various host factors, such as body mass index (BMI), fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), and hepatocyte steatosis, are significant predictors of efficacy of IFN monotherapy and PEG-IFN-RBV dual therapy [6–9]. However, more studies that implement multivariate analysis are required to confirm the predictive values of these factors for the efficacy of PEG-IFN-RBV dual therapy, especially where these factors are analyzed with other factors, including viral and host factors.

The aims of the present study were to analyze the response to 48-week PEG-IFN-RBV therapy in Japanese patients with HCV genotype 1b. Specifically, the study was designed to (1) identify the pretreatment predictive factors associated with EVR and SVR and (2) determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the EVR and SVR predictive factors.

2. Patients and methods

2.1. Study population

A total of 201 HCV-infected Japanese patients were consecutively recruited into the study protocol between December of 2001 and June of 2005 at Toranomon Hospital, Tokyo. Among these, 114 patients were selected based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, CA). (2) Infection with HCV genotype 1b alone. (3) A high viral load (≥100 KIU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) within the preceding 2 months of enrolment. (4) No HCC. (5) Body weight >40 kg. 6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of hepatitis, such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (10) None of the females was pregnant or lactating mother. (11) All accepted treatment for ≥24 weeks as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (12) All patients have completed 24 weeks after cessation of treatment, and SVR could be evaluated. (13) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

Patients received PEG-IFNα-2b at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week-oral RBV at a median dose of 10.9 mg/kg (range, 3.4–14.2 mg/kg) daily for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for ≤60 kg, 800 mg for >60 kg and ≤80 kg, and 1000 mg for >80 kg), except for 27 patients who started at a reduction dose according to low pretreatment levels of hemoglobin (Hb). In 35 patients, the dose of RBV was reduced during treatment due to falls in Hb concentration.

Table 1 summarizes the profiles of the patients. They included 75 men and 39 women. The median duration of treatment was 48 weeks (range, 24–48 weeks). Patients who achieved HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 24 weeks after cessation of combination therapy were defined as SVR. Patients who achieved >2 $\log_{10}$ falls in HCV-RNA level compared with baseline based on HCV-RNA quantitative PCR analysis or HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 12 weeks of combination therapy were defined as EVR.

2.2. Laboratory tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80 °C within 4 h 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 [10]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after therapy. The lower limit of the assay was 5 KIU/ml. Samples collected during and after therapy that had undetectable levels of HCV-RNA (<5 KIU/ml) were checked also by qualitative PCR (Amplicor, Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml. For evaluation of EVR, we used the $\log_{10}$ of the cut-off value (5 KIU/ml) for HCV-RNA values below the limit of detection.

2.3. Histopathological examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens 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 was diagnosed based on the histological scoring system of Desmet et al. [11]. Hepatocyte steatosis was graded as none (absent), mild (<33% of hepatocytes involved), moderate (>33% but <66% of hepatocytes involved), or severe (>67% of hepatocytes involved) [12].

2.4. 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

Table 1
Profile and laboratory data of participating patients infected with HCV genotype 1b at commencement of 48-week peginterferon-ribavirin combination therapy

Demographic data	
Number	114
Gender (M/F)	75 / 39
Age (years)*	54 (30–70)
History of blood transfusion	38 (33.3%)
Family history of liver disease	35 (30.7%)
Body mass index (kg/m^2)*	23.2 (17.6–30.3)
Laboratory data*	
Serum aspartate aminotransferase (IU/l)	60 (17–266)
Serum alanine aminotransferase (IU/l)	81 (25–504)
Serum albumin (g/dl)	3.7 (3.0–4.5)
γ-Glutamyl transpeptidase (IU/l)	67 (15–393)
Leukocytes (/mm^3)	4800 (2300–8800)
Hemoglobin (g/dl)	14.6 (10.6–17.6)
Platelets (× 10^4/mm^3)	17.6 (6.6–30.9)
ICG R15 (%)	15 (4–49)
Serum iron (μg/dl)	147 (18–308)
Serum ferritin (μg/l)	150 (<10–927)
Creatinine clearance (ml/min)	100 (53–146)
Viremia level (KIU/ml)	2000 (67–>5000)
Total cholesterol (mg/dl)	169 (100–236)
High-density lipoprotein cholesterol (mg/dl)	45 (10–83)
Low-density lipoprotein cholesterol (mg/dl)	100 (53–162)
Triglycerides (mg/dl)	100 (33–362)
Uric acid (mg/dl)	5.6 (2.3–8.8)
Fasting blood sugar (mg/dl)	96 (75–257)
Histological findings	
Stage (F1/F2/F3/F4/ND)	51/28/16/1/18
Hepatocyte steatosis (none to mild/moderate to severe/ND)	86/8/20
Treatment	
PEG-IFNα-2b dose (μg/kg)	1.5 (0.8–1.8)
Ribavirin dose (mg/kg)	10.9 (3.4–14.2)
Amino acid substitutions in the core region**	
aa 70 (wild/non-wild/ND)	54/38/8
aa 91 (wild/non-wild/ND)	58/40/2
aa 70 and aa 91 (double-wild/non-double-wild/ND)	35/61/4

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

** Amino acid substitutions were evaluated in pretreatment serum samples of 100 patients by PCR with mutation-specific primers.

Two patterns of mutant and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, while the other patterns were considered non-double-wild-type. ND, not determined.

(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, the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases [13]. 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 non-double-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 [4,14]. In the present study, the PCR genotyping could be performed in 100 patients; the remaining 14 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

HCV-RNA was extracted from the serum samples and cDNA was prepared by reverse transcription using MMLV Superscript II reverse transcriptase. The obtained cDNA was amplified by PCR using the following primers: the first PCR was performed using cc11 (sense, 5′-GCC ATG GTG GTC TGC GGA AC-3′: 125–144) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′: 933–953) primers. In the second PCR, for aa 70 the wild-type-specific reaction was performed using 70W2 (sense, 5′-TAT CCC CAA GGC TCG CCG-3′: 521–538) and e14, and the mutant-specific reaction was performed using 70M2 (sense, 5′-TAT CCC CAA GGC TCG CCA-3′: 521–538) and e14. For aa 91, the wild-type-specific reaction was performed using cc9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′: 237–253) and 91W (antisense, 5′-CAT CCT GCC CAC CCC AR-3′, R = A or G: 600–616), and the mutant-specific reaction was performed using cc9 and 91M (5′-CAT CCT GCC CAC CCC AT-3′: 600–616) [13].

The cycle conditions were 94 °C for 4 min + (94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min) × 20 cycles + 72 °C for 7 min in the first PCR; and 94 °C for 1 min + (94 °C for 30 s and 72 °C for 1.5 min) × 23 cycles + 72 °C for 7 min for aa 70, and 94 °C for 1 min + (94 °C for 30 s and 68 °C for 1.5 min) × 21 cycles + 72 °C for 7 min for aa 91 in the second PCR. Two microliters of cDNA was used in the first PCR and 1 μl of the first PCR product was used in the second PCR. For detection, 5 μl of the second PCR product was electrophoresed for 30 min on 3.0% agarose gel. The final concentration of all primers was 0.2 pmol/μl [13].

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [15] to prevent contamination were strictly applied to these PCR assays. No false positive results were observed in this study.

2.5. Statistical analysis

SVR was analyzed on an intention to treat basis. Non-parametric tests were used to compare variables between groups (Mann–Whitney U test, χ^2 test and Fisher's exact probability test). Univariate and multivariate logistic regression analyses were used to determine the predictors of SVR and EVR. We also calculated the odds ratios and 95% confidence intervals (95%CI). 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 SVR and EVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, BMI, aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase (GGT), leukocyte count, Hb, platelets, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), TG, uric acid (UA), FBS, hepatocyte steatosis, pathological staging, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitutions in HCV-CR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Sensitivity, specificity, PPV, and NPV were also calculated to determine the reliability of predictors of the response to therapy.

3. Results

3.1. Response to therapy

EVR and SVR were evaluated in all 114 patients. EVR was achieved by 80 of 114 (70.2%) patients, and SVR by 51 of 114 (44.7%) patients. 44.7% (51/114 patients) achieved both EVR and SVR, 29.8% (34/114) were considered non-EVR and non-SVR, 25.4% (29/

Table 2
Factors associated with early virologic response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	*P*
LDL cholesterol (mg/dl)	1: <86	1	
	2: ⩾86	30.29 (4.855–189.0)	<0.001
Amino acid substitution in core region	1: double-wild-type[a]	1	
	2: non-double wild-type	0.046 (0.006–0.346)	0.003
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	
	2: ⩾109	0.166 (0.035–0.782)	0.023
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ⩾11.0	4.341 (1.075–17.53)	0.039
Leukocyte count (/mm^3)	1: <4500	1	
	2: ⩾4500	4.209 (1.061–16.70)	0.041

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal range for LDL cholesterol: 86–135 mg/dl.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

114) were EVR and non-SVR, and 0% (0/114) as non-EVR and SVR. Thus, 63.8% (51/80) of those who achieved EVR also achieved SVR, and none of non-EVR could achieve SVR.

3.2. Predictors of EVR as determined by univariate and multivariate analyses

Univariate analysis identified nine parameters that influenced the EVR: LDL-C (⩾86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.001$), leukocyte count (⩾4500/mm^3; $P = 0.003$), GGT (<109 IU/l; $P = 0.008$), TC (⩾170 mg/dl; $P = 0.038$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.042$), PEG-IFN dose/body weight (⩾1.25 μg/kg; $P = 0.055$), TG (<100 mg/dl; $P = 0.059$), and AST (<60 IU/l; $P = 0.065$). Multivariate analysis that included the above variables identified five parameters that independently influenced the EVR: LDL-C (⩾86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.003$), GGT (<109 IU/l; $P = 0.023$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.039$), and leukocyte count (⩾4500/mm^3; $P = 0.041$). Especially, LDL-C (⩾86 mg/dl) and aa substitutions of HCV-CR (double-wild-type) of five parameters increased chances for EVR 20-fold or more (Table 2).

3.3. Assessment of amino acid substitutions and LDL-cholesterol as predictors of EVR

EVR rates of patients with double-wild-type of HCV-CR or high-serum LDL-C levels (⩾86 mg/dl) were defined as PPV (prediction of EVR). Non-EVR rates of patients with non-double-wild-type of HCV-CR or low-serum LDL-C levels (<86 mg/dl) were defined as NPV (prediction of non-EVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for prediction of EVR were 46.4%, 88.9%, 91.4%, and 39.6%, respectively. In patients with high serum LDL-C levels, the sensitivity, specificity, PPV, and NPV were 81.0%, 56.3%, 82.1%, and 54.5%, respectively. Thus, evaluation of aa substitutions in HCV-CR indicated high specificity and PPV, while that of serum LDL-C level indicated high sensitivity and PPV for prediction of EVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 32.4%, 100%, 100%, and 37.0%, respectively. When one or two predictors

Table 3
Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of early virologic response (EVR) to dual therapy, based on the combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) level

	Sensitivity	Specificity	PPV[b]	NPV[c]
(A) Double-wild-type of core region[a]	46.4 (32/69)	88.9 (24/27)	91.4 (32/35)	39.3 (24/61)
(B) High level of LDL-C	81.0 (64/79)	56.3 (18/32)	82.1 (64/78)	54.5 (18/33)
(A) and (B)	32.4 (22/68)	100 (27/27)	100 (22/22)	37.0 (27/73)
(A) and/or (B)	97.4 (74/76)	39.3 (11/28)	81.3 (74/91)	84.6 (11/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV and NPV.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.
[b] PPV; EVR rates for patients with a combination of double-wild-type of the core region, or high levels (⩾86 mg/dl) of LDL-C (prediction of EVR).
[c] NPV; non-EVR rates for patients with non-double-wild-type of the core region, or low levels (<86 mg/dl) of LDL-C (prediction of non-EVR).

Table 4
Factors associated with sustained virological response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	*P*
Amino acid substitution in core region	1: double-wild-type[a]	1	
	2: non-double wild-type	0.102 (0.022–0.474)	0.004
LDL cholesterol (mg/dl)	1: <86	1	
	2: ⩾86	12.87 (2.177–76.09)	0.005
Gender	1: male	1	
	2: female	0.091 (0.017–0.486)	0.005
ICG R15 (%)	1: <10	1	
	2: ⩾10	0.107 (0.017–0.678)	0.018
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	
	2: ⩾109	0.096 (0.011–0.819)	0.032
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ⩾11.0	5.173 (1.152–23.22)	0.032

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal range for LDL cholesterol: 86–135 mg/dl.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

were used, the sensitivity, specificity, PPV, and NPV were 97.4%, 39.3%, 81.3%, and 84.6%, respectively. Thus, prediction of EVR by the combination of aa substitutions in HCV-CR and serum LDL-C level had high sensitivity, specificity, PPV, and NPV (Table 3).

3.4. Predictors of SVR as determined by univariate and multivariate analyses

Univariate analysis identified 12 parameters that influenced SVR: histopathological staging of liver fibrosis (F1; $P = 0.002$), leukocyte count (⩾4500/mm^3; $P = 0.004$), aa substitutions of HCV-CR (double-wild-type; $P = 0.005$), PEG-IFN dose/body weight (⩾1.25 μg/kg; $P = 0.006$), gender (male; $P = 0.007$), age (<55 years; $P = 0.009$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.009$), GGT (<109 IU/l; $P = 0.019$), ICG R15 (<10%; $P = 0.029$), LDL-C (⩾86 mg/dl; $P = 0.063$), Hb (⩾14.0 g/dl; $P = 0.064$), and AST (<60 IU/l; $P = 0.064$). Multivariate analysis identified six parameters that independently influenced the SVR: aa substitutions of HCV-CR (double-wild-type; $P = 0.004$), LDL-C (⩾86 mg/dl; $P = 0.005$), gender (male; $P = 0.005$), ICG R15 (<10%; $P = 0.018$), GGT (<109 IU/l; $P = 0.032$), and RBV dose/body weight (⩾11.0 mg/kg; $P = 0.032$) (Table 4). These results indicate that aa substitutions of HCV-CR and LDL-C levels are significant and independent predictors of both EVR and SVR, especially.

3.5. Assessment of amino acid substitutions and LDL cholesterol as predictors of SVR

Finally, we evaluated the ability to predict SVR by aa substitutions of HCV-CR and serum LDL-C level (each, $P < 0.01$). The SVR rates of patients with double-wild-type of HCV-CR or high serum levels of LDL-C were defined as PPV (prediction of SVR). The non-SVR rates of patients with non-double-wild-type of HCV-CR or low serum levels of LDL-C were defined as NPV (prediction of non-SVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for SVR were 52.4%, 75.9%, 62.9%, and 67.2%, respectively. Thus, aa substitutions in HCV-CR have a high specificity for prediction of SVR. In patients with high-serum levels

Table 5
Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of sustained virological response (SVR), based on a combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) levels

	Sensitivity	Specificity	PPV[b]	NPV[c]
(A) Double-wild-type of core region[a]	52.4 (22/42)	75.9 (41/54)	62.9 (22/35)	67.2 (41/61)
(B) High level of LDL-C	80.0 (40/50)	37.7 (23/61)	51.3 (40/78)	69.7 (23/33)
(A) and (B)	33.3 (15/45)	87.9 (51/58)	68.2 (15/22)	63.0 (51/81)
(A) and/or (B)	100 (47/47)	22.8 (13/57)	51.6 (47/91)	100 (13/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV, and NPV.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.
[b] PPV, EVR rates for patients with a combination of double-wild-type of the core region or high levels (⩾86 mg/dl) of LDL-C (prediction of EVR).
[c] NPV, Non-EVR rates for patients with non-double-wild-type of the core region or low levels (<86 mg/dl) of LDL-C (prediction of non-EVR).

of LDL-C, the sensitivity, specificity, PPV, and NPV were 80.0%, 37.7%, 51.3%, and 69.7%, respectively. Thus, serum LDL-C level has high sensitivity in predicting SVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 33.3%, 87.9%, 68.2%, and 63.0%, respectively. When one or more of the two predictors were used, the sensitivity, specificity, PPV, and NPV were 100%, 22.8%, 51.6%, and 100%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, and NPV for prediction of SVR (Table 5).

4. Discussion

We reported previously that substitutions of aa 70 and/or 91 in the HCV core region are an independent and significant predictor of NVR [4,5]. Based on a larger number of patients, the present study also identified aa substitutions in HCV-CR as a predictor of EVR and SVR in patients on 48-week PEG-IFN-RBV dual therapy. Previous studies reported that the HCV core region might be associated with resistance to IFN monotherapy involving the Jak-STAT signaling cascade [16–19]. Our result could be also interpreted to mean that aa substitutions in HCV-CR are associated with those proteins involved in resistance to IFN monotherapy, such as SOCS proteins known to inhibit IFN-α-induced activation of the Jak-STAT pathway and expression of the antiviral proteins 2′,5′-OAS and MxA [20]. Furthermore, our result also indicates that aa substitutions in HCV-CR might serve as a surrogate marker for other proteins associated with resistance to the antiviral actions of IFN. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Importantly, our study also identified serum LDL-C levels as a predictor of the response to PEG-IFN-RBV therapy, and we agree with the recent findings of Gopal et al. [21]. Previous studies reported that endocytosis of HCV via the LDL receptor(s) is mediated by the formation of a complex between HCV and VLDL or LDL [22,23]. Furthermore, there is evidence that intracellular cholesterol level modulates LDLr expression, and thus a high LDL-C could downregulate LDLr and diminish the spread of hepatocyte HCV infection. Thus, the correlation between treatment efficacy and LDL-C may be explained by the role of LDL-C in transporting the HCV-LDL complex into the hepatocyte. It should be noted, however, that other *in vitro* studies also showed that statins, which upregulate LDLr, might decrease HCV replication [24–26]. Other mechanisms could also explain the role of LDL-C and the response to PEG-IFN-RBV therapy. For example, high-LDL-C levels could act by modulating cytokine release [27] and antiviral cellular immune response [28,29]. On the other hand, it is also reported that apolipoprotein E4 allele is associated with high LDL-C levels [30], and with poor response to treatment in patients with genotype 1 HCV [31]. The discrepancy between our results and such findings may be explained by the small number of patients in our study, differences in host factors including race [32–34], and/or differences in vial factors, such as the distribution of genotype 1a or 1b, and geographic diversities of genotype 1b [35]. Further studies of large number of patients matched for race and HCV genotype are required to explore the relationship between serum LDL-C level and the response to PEG-IFN-RBV therapy.

Our results also showed that a high ICG R15 value was a negative predictor of SVR to PEG-IFN-RBV therapy. Previous data indicated that absence of advanced liver fibrosis is a predictor of SVR to IFN monotherapy and IFN-RBV dual therapy [36–38], and that advanced liver fibrosis is usually associated with high rates of ICG R15 [39]. However, our study showed that a milder form of liver fibrosis was not a predictor of response to dual treatment, whereas a low level of ICG R15 was. This discrepant finding may be due to the fact that estimates of liver dysfunction assessed by the degree of liver fibrosis (which is evaluated using only four stages (F1, F2, F3, F4), in contrast to ICG R15), are less sensitive to those by ICG R15. It is also possible that the above discrepancy is related to our exclusion of patients with cirrhosis (F4) (the exclusion was because the Japanese Government Health Insurance system does not provide cover for combination therapy for patients with cirrhosis). Further studies are required to explore the relationship between the severity of histopathological changes in the liver and response to dual therapy especially in patients with cirrhosis.

Our results should be interpreted with caution since we did not include patients of other races or other HCV genotypes. Any generalization of the results should await confirmation by studies of patients of other races infected with other HCV genotypes.

Pretreatment prediction of the response to PEG-IFN-RBV therapy is still incomplete. So far, viral factors (e.g., aa substitutions in HCV-CR), host factors (e.g., LDL-C [21], gender [40], ICG R15, and GGT [41]), and treatment-related factors (e.g., RBV dose [5,42]) have been confirmed to influence the response to such treatment in Japanese patients infected with HCV genotype 1b. Furthermore, evaluation using a combination of predictors indicates the high-sensitivity, specificity, PPV, and NPV of such prediction. We conclude that the response to PEG-IFN-RBV therapy 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.

Acknowledgement

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

References

[1] Fried MW, Shiffman ML, Reddy R, Smith C, Marinos G, Gonçales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347: 975–982.
[2] National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002-June 10-12, 2002. Hepatology 2002; 36: S3-20.
[3] Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 2003;38:645–652.
[4] 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 genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–380.
[5] 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 genotype1b and high viral load. J Med Virol 2006;78:83–90.
[6] D'Souza R, Sabin CA, Foster GR. Insulin resistance plays a significant role in liver fibrosis in chronic hepatitis C and in the response to antiviral therapy. Am J Gastroenterol 2005;100: 1509–1515.
[7] Martinez-Bauer E, Crespo J, Romero-Gomez M, Moreno-Otero R, Sola R, Tesei N, et al. Development and validation of two models for early prediction of response to therapy in genotype 1 chronic hepatitis C. Hepatology 2006;43:72–80.
[8] Poynard T, Ratziu V, McHutchison J, Manns M, Goodman Z, Zeuzem S, et al. Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. Hepatology 2003;38:75–85.
[9] Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, et al. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 2002;37:831–836.
[10] 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.
[11] Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.
[12] D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, et al. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 1991;51: 157–163.
[13] Okamoto K, Akuta N, Kumada H, Kobayashi M, Matsuo Y, Tazawa H. A nucleotide sequence variation detection system for the core region of hepatitis C virus-1b. J Virol Methods (in press).
[14] 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 USA 1990;87:9524–9528.
[15] Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989;339:237–238.
[16] Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, et al. Expression of hepatitis C virus proteins inhibits interferon α signaling in the liver of transgenic mice. Gastroenterology 2003;124:1465–1475.
[17] Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.
[18] Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 2004;73:536–547.
[19] de Lucas S, Bartolome J, Carreno V. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. J Infect Dis 2005;191:93–99.
[20] Voltides G, Sorensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, et al. SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Commun 2004;30:1007–1014.
[21] Gopal K, Johnson TC, Gopal S, Walfish A, Bang CT, Suwandhi P, et al. Correlation between beta-lipoprotein levels and outcome of hepatitis C treatment. Hepatology 2006;44:335–340.
[22] Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci USA 1999;96:12766–12771.
[23] Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919–6928.
[24] Ye J, Wang C, Sumpter R, Brown MS, Goldstein JL, Gale M. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc Natl Acad Sci USA 2003;100:15865–15870.
[25] Kapadia SB, Chisari FV. Hepatitis C virus replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci USA 2005;102:2561–2566.
[26] Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology 2006;44:117–125.
[27] Netea MG, Demacker PNM, Kullberg BJ, Boerman OC, Verschueren I, Stalenhoef AFH, et al. Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. J Clin Invest 1996;97:1366–1372.
[28] Ludewig B, Jaggi M, Dumrese T, Brduscha-Riem K, Odermatt B, Hengartner H, et al. Hypercholesterolemia exacerbates virus-induced immunopathologic liver disease via suppression of antiviral cytotoxic T cell responses. J Immunol 2001;166:3369–3376.
[29] Muldoon MF, Marsland A, Flory JD, Rabin BS, Whiteside TL, Manuck SB. Immune system differences in men with hypo- or hypercholesterolemia. Clin Immunol Immunopathol 1997;84:145–149.
[30] Schaefer EJ, Lamon-Fava S, Johnson S, Ordovas JM, Schaefer MM, Castelli WP, et al. Effects of gender and menopausal status on the association of apolipoprotein E phenotype with plasma lipoprotein levels. Results from the Framingham Offspring Study. Arterioscler Thromb 1994;14:1105–1113.
[31] Mueller T, Gessner R, Sarrazin C, Graf C, Halangk J, Witt H, et al. Apolipoprotein E4 allele is associated with poor treatment response in hepatitis C virus (HCV) genotype 1. Hepatology 2003:1592 (Correspondence).