multivariate stepwise Cox analysis, older age, male gender, advanced fibrosis, severe steatosis, lower serum albumin levels, non-SVR, and higher post-IFN treatment ALT and AFP levels, but not pre-IFN treatment ALT and AFP levels, were identified as independent factors that were significantly associated with HCC development (Table 2).

Association of Post-IFN Treatment ALT and AFP Levels With HCC Development in SVRs and Non-SVRs. Because our multivariate analysis identified post-IFN treatment ALT and AFP levels as independent factors associated with HCC risk, we determined the cutoff values of these factors for predicting the development of HCC by receiver operator characteristics (ROC) analysis. The area under the ROC curve for post-IFN treatment ALT and AFP levels were higher than that for pre-IFN treatment ALT and AFP levels, suggesting that quantification of post-IFN treatment ALT and AFP levels rather than pre-IFN treatment levels of these values is useful for predicting HCC (Fig. 1A). From this ROC analysis, ALT <40 IU/L and AFP <6.0 ng/mL were identified as cutoff values. Negative predictive values were extremely high at 0.960 in each value, suggesting patients with ALT and/or AFP levels below these cutoff values are at a lower risk for HCC.

As shown in Fig. 1B, the hazard ratio determined by Cox proportional hazard analysis after adjustment for age, sex, stage of liver fibrosis, degree of liver steatosis, serum albumin levels, and virological response to therapy demonstrated that the hazard ratio for HCC was dependent on post-IFN treatment ALT and AFP levels. These hazard ratios increased predominantly when post-IFN treatment ALT and AFP levels were more than the cutoff values.

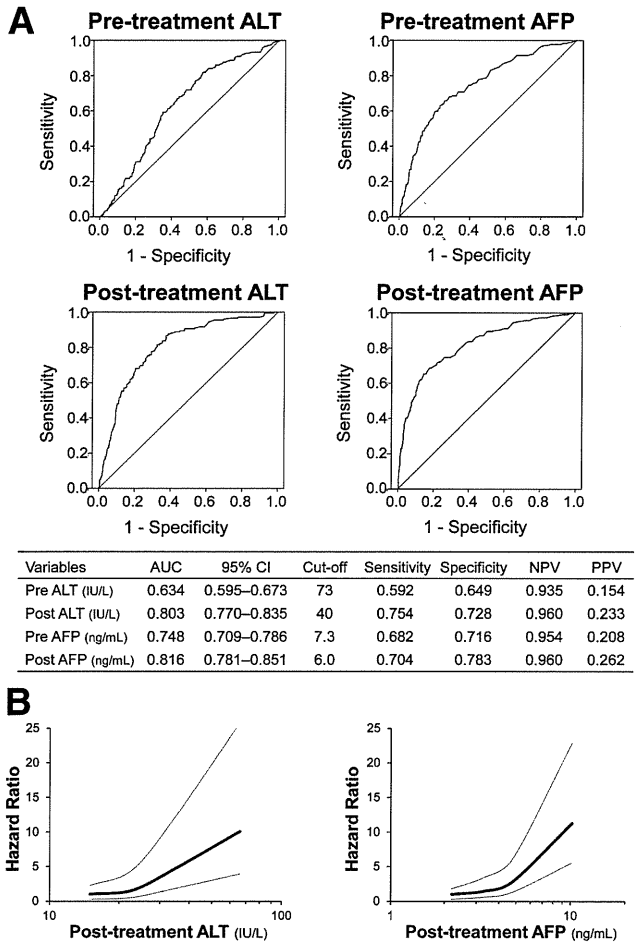
As shown in Fig. 2, the cumulative incidence of HCC was closely related to post-IFN treatment ALT and AST levels and was significantly lower in patients whose post-IFN treatment ALT and AFP levels was suppressed to <40 IU/L and 6.0 ng/mL, respectively. This suppressive effect was also notable in non-SVRs (Fig. 2C,D). Moreover, the cumulative incidence of HCC was significantly higher even in SVRs whose post-IFN treatment ALT and AFP levels were not <40 IU/L and 10 ng/mL, respectively (Fig. 2E,F).

Changes in ALT and AFP Levels With IFN Therapy and HCC Development. In the entire cohort, the mean ALT and AFP levels significantly decreased after IFN therapy (ALT = 78.4 to 36.6 IU/L, 95% confidence interval [CI] = 38.6-45.0, $P < 0.0001$; AFP = 11.3 to 6.9 ng/mL, 95% CI = 3.25-5.69, $P < 0.0001$; paired Student t test), and this significant decrease was found not only in SVRs, but also non-SVRs (Fig. 3A). Because post-IFN treatment ALT and AFP levels rather than pre-IFN treatment levels were

Table 2. Factors Associated With Hepatocellular Carcinoma

Risk Factor	Hazard Ratio (95% CI)	*P* Value
Univariate analysis		
Age (by every 10 year)	1.82 (1.52-2.20)	<0.0001
Sex		
Female	1	
Male	1.61 (1.20-2.17)	<0.0001
Fibrosis stage		
F1/F2	1	
F3/F4	4.90 (3.64-6.61)	<0.0001
Activity grade		
A0/A1	1	
A2/A3	3.38 (2.41-4.74)	<0.0001
Degree of steatosis		
<10%	1	
≥10%	3.84 (2.62-5.63)	<0.0001
Albumin (by every 1 g/dL)	0.18 (0.22-0.25)	<0.0001
Pre-ALT (by every 40 IU/L)	1.04 (0.96-1.08)	0.525
Post-ALT (by every 40 IU/L)	1.68 (1.55-1.81)	<0.0001
γ-GTP (by every 40 IU/L)	1.17 (1.08-1.27)	<0.0001
Fasting blood sugar (by every 100 mg/dL)	1.82 (1.35-2.45)	<0.0001
Pre-AFP (by every 10 ng/mL)	1.07 (1.05-1.09)	<0.0001
Post-AFP (by every 10 ng/mL)	1.08 (1.06-1.12)	<0.0001
Platelet counts (by every $10^4/\mu L$)	0.88 (0.85-0.90)	<0.0001
Genotype		
Non-1	1	
1	2.27 (1.51-3.45)	<0.0001
Core 70 mutation		
Wild	1	
Mutant	2.79 (1.19-6.53)	0.018
ISDR		
More than 1 mutation	1	
Wild or 1 mutation	1.27 (0.87-1.85)	0.216
Virological response		
SVR	1	
Non-SVR	3.66 (2.51-5.35)	<0.0001
Multivariate analysis		
Age (by every 10 year)	2.18 (1.71-2.81)	<0.0001
Sex		
Female	1	
Male	2.66 (1.86-3.80)	<0.0001
Fibrosis stage		
F1/F2	1	
F3/F4	2.27 (1.58-3.27)	<0.0001
Degree of steatosis		
<10%	1	
≥10%	2.29 (1.49-3.50)	<0.0001
Albumin (by every 1 g/dL)	0.35 (0.23-0.55)	<0.0001
Post-ALT (by every 40 IU/L)	1.81 (1.55-2.12)	<0.0001
Post-AFP (by every 10 ng/mL)	1.06 (1.02-1.10)	0.007
Virological response		
SVR	1	
Non-SVR	1.58 (1.01-2.48)	0.044

Hazard ratios for development of hepatocellular carcinoma were calculated by the Cox proportional hazards analysis. ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; AFP, alpha-fetoprotein; ISDR, interferon sensitivity determining region; SVR, sustained virological responder. As covariates in the multivariate stepwise Cox model, age, sex, stage of liver fibrosis, grade of histological activity, presence of hepatic steatosis, serum albumin levels, γ-GTP level, fasting blood sugar levels, platelet counts, pre-IFN ALT levels, pre-IFN AFP levels, post-IFN ALT levels, post-IFN AFP levels, and virological response were included.



Variables	AUC	95% CI	Cut-off	Sensitivity	Specificity	NPV	PPV
Pre ALT (IU/L)	0.634	0.595–0.673	73	0.592	0.649	0.935	0.154
Post ALT (IU/L)	0.803	0.770–0.835	40	0.754	0.728	0.960	0.233
Pre AFP (ng/mL)	0.748	0.709–0.786	7.3	0.682	0.716	0.954	0.208
Post AFP (ng/mL)	0.816	0.781–0.851	6.0	0.704	0.783	0.960	0.262

Fig. 1. Predictive values for ALT and AFP, and hazard ratios (HRs) according to post-IFN treatment ALT and AFP levels. (A) ROC curve for prediction of HCC. Area under the ROC curve, 95% CI, cutoff value, sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) are shown in the bottom of the figure. (B) Spline curves of HR (solid line) and 95% CI (dotted line) for HCC development according to post-IFN treatment ALT and AFP levels. Curves were fitted using polynomial regression.

significantly associated with the development of HCC in non-SVRs, we determined the effects of changes in ALT and AFP levels by IFN therapy on hepatocarcinogenesis. Even in non-SVRs with equal or higher pre-IFN treatment ALT or AFP levels than the cutoff values, the cumulative incidence of HCC was significantly suppressed in patients whose post-IFN treatment ALT or AFP level was reduced to less than the cutoff values (Fig. 3B). In contrast, persistence of post-IFN treatment ALT or AFP levels to more than the cutoff values after IFN therapy was associated with a significantly higher incidence of HCC (Fig. 3B).

Relation Between AFP and ALT or Histological Findings. Univariate analysis using logistic regression determined factors that were associated with post-IFN treatment ALT or AFP levels (Supporting Table). Although many clinical factors were associated with post-IFN ALT and/or AFP levels, post-IFN ALT and AFP levels were not correlated with each other ($r^2 = 0.050$). Therefore, the cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels, even when patients were stratified by post-IFN treatment ALT levels (Fig. 4A,B).

As shown in Fig. 4C-F, the cumulative incidence of HCC development was significantly lower in patients whose post-IFN treatment AFP level was <6.0 ng/mL in all subgroups stratified by stage of fibrosis and grade of activity. Therefore, reduction in post-IFN treatment AFP levels reduces HCC risk even in patients with advanced fibrosis. Although pre-IFN treatment AFP levels correlated with the advance of histological fibrosis and grade of activity, such correlations became less significant with post-IFN treatment AFP levels (data not shown).

Platelet Counts and Aspartate Aminotransferase-to-Platelet Ratio Index (APRI) in Patients Without Advanced Fibrosis. Because a substantial amount of HCC cases developed in the patients without histologically advanced fibrosis (Fig. 4C), we characterized these individuals using platelet counts and APRI,[25] which are the readily available surrogate markers for liver fibrosis. We first determined the cutoff values of platelet counts and APRI for predicting HCC development by ROC analyses. Accordingly, platelet counts $<150 \times 10^3/\mu L$ and APRI >0.96 were identified as cutoff values, and the areas under the ROC curve for platelet counts and APRI were 0.715 (95% CI: 0.675-0.755) and 0.740 (95% CI: 0.701-0.779), respectively (Supporting Figure). Even in individuals without advanced fibrosis (F1 and F2 patients), the proportion of patients with platelet counts $<150 \times 10^3/\mu L$ or APRI >0.96 was significantly higher in patients with HCC than in those without HCC (platelet counts, 53.0% [35/66] versus 31.3% [387/1238], $P = 0.0002$; APRI, 53.0% [35/66] versus 26.4% [325/1229], $P < 0.0001$). Moreover, the cumulative incidence of HCC development was significantly higher in patients with platelet counts $<150 \times 10^3/\mu L$ or APRI >0.96 in the subgroups without advanced fibrosis (Supporting Figure). Therefore, patients with low platelet counts or high APRI still have a substantial risk for HCC development even though they were diagnosed with mild fibrosis by liver biopsy.

Hepatic Steatosis and Post-IFN ALT and AFP Levels in SVRs. To characterize SVRs without ALT and AFP normalization after IFN therapy, we evaluated the percentage of severe hepatic steatosis in these patients. Accordingly, the percentages of severe hepatic steatosis were significantly higher in SVRs without ALT and AFP normalization than in those with normal

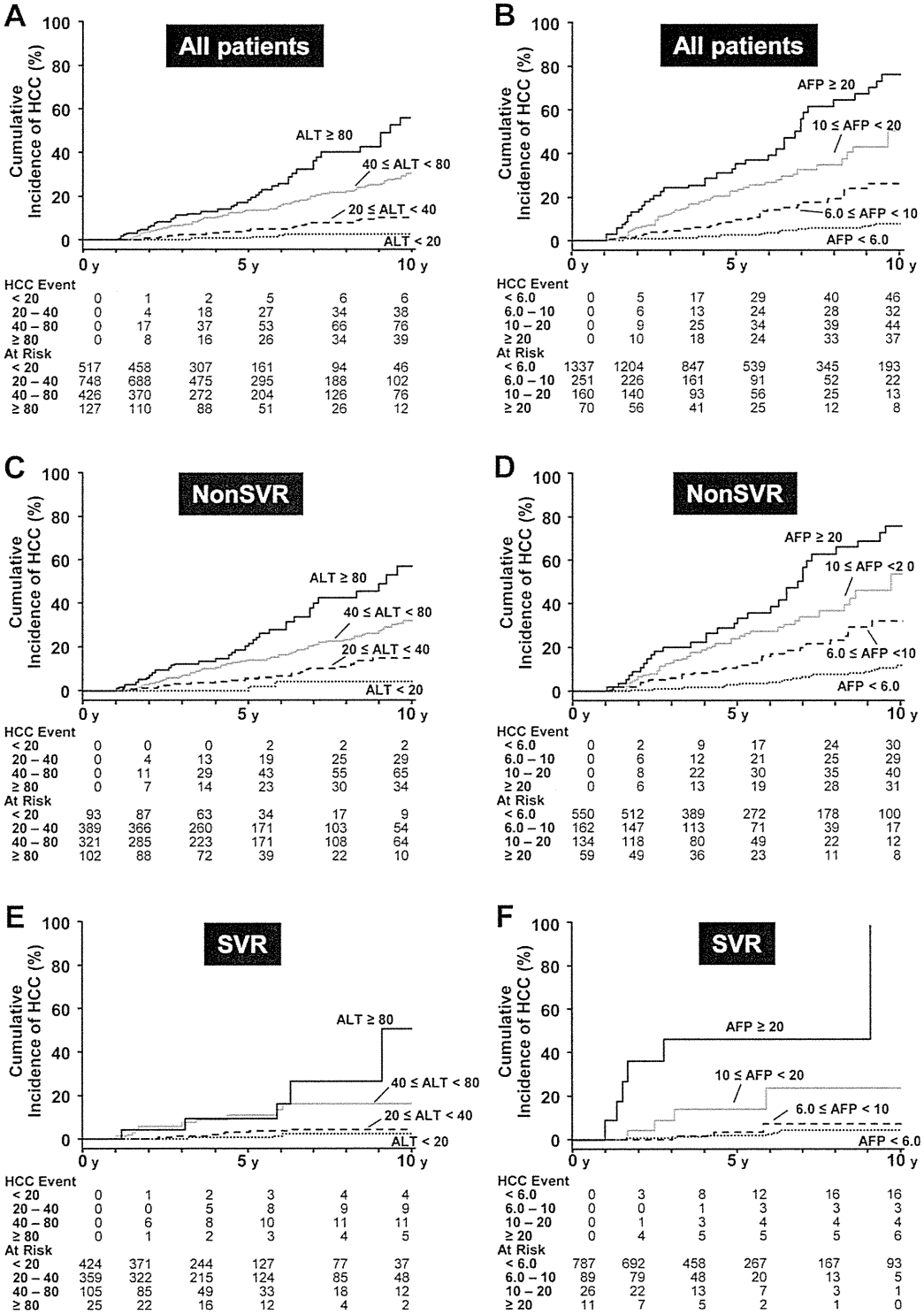
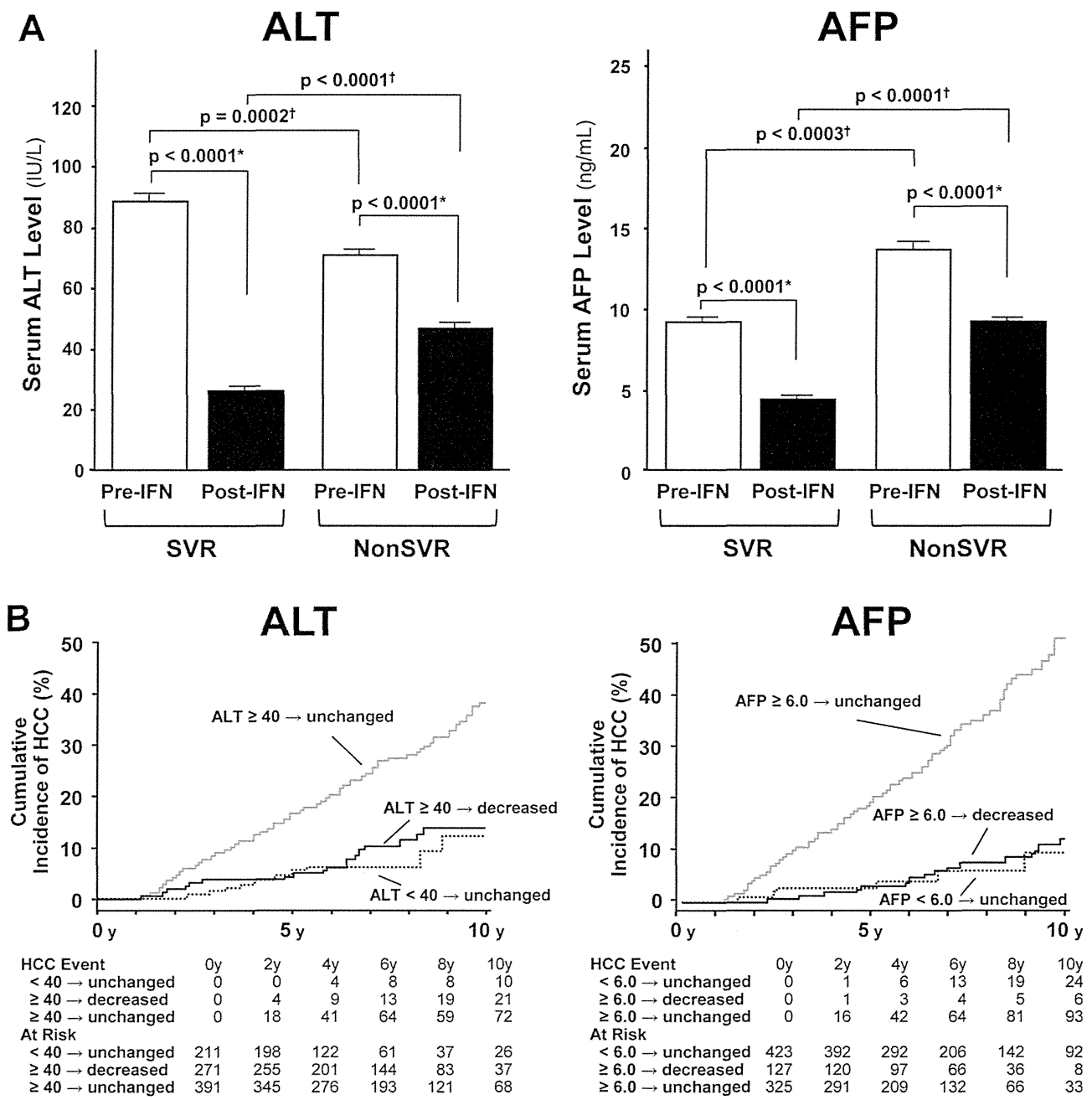


Fig. 2. Cumulative incidence of HCC according to post-IFN treatment ALT and AFP levels. (A) Entire cohort stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (B) Entire cohort stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). (C) Non-SVRs stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (D) Non-SVRs stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). (E) SVRs stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (F) SVRs stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). The number of HCC events and patients at risk at each timepoint are shown below the graphs.

ALT and AFP (ALT, 37.9% [36/95] versus 13.8% [77/557], $P < 0.0001$; AFP, 31.6% [31/98] versus 14.8% [82/554], $P < 0.0001$). Therefore, it is likely that presence of hepatic steatosis is associated with ALT and/or AFP elevation, and it is one of the risks for HCC development even after achieving SVR.



HCC Event	0y	2y	4y	6y	8y	10y
< 40 → unchanged	0	0	4	8	8	10
≥ 40 → decreased	0	4	9	13	19	21
≥ 40 → unchanged	0	18	41	64	59	72
At Risk						
< 40 → unchanged	211	198	122	61	37	26
≥ 40 → decreased	271	255	201	144	83	37
≥ 40 → unchanged	391	345	276	193	121	68

HCC Event	0y	2y	4y	6y	8y	10y
< 6.0 → unchanged	0	1	6	13	19	24
≥ 6.0 → decreased	0	1	3	4	5	6
≥ 6.0 → unchanged	0	16	42	64	81	93
At Risk						
< 6.0 → unchanged	423	392	292	206	142	92
≥ 6.0 → decreased	127	120	97	66	36	8
≥ 6.0 → unchanged	325	291	209	132	66	33

Fig. 3. Changes in pre- and post-IFN treatment ALT and AFP levels, and their effects on HCC development. (A) Mean serum pre- (open columns) and post-IFN treatment (solid columns) ALT and AFP levels in SVRs and non-SVRs. Error bars indicate the standard error. *Paired Student *t* test. †Unpaired Student *t* test. (B) Cumulative incidence of HCC stratified by changes in pre- and post-IFN treatment ALT and AFP levels (log-rank test: $P < 0.0001$). ALT <40 → unchanged, patients with ALT <40 IU/L before IFN therapy unchanged after IFN therapy; ALT ≥40 → decreased, patients with ALT ≥40 IU/L before IFN therapy decreased to ALT <40 IU/L after IFN therapy; ALT ≥40 → unchanged, patients with ALT ≥40 IU/L before IFN therapy unchanged at ALT not <40 IU/L after IFN therapy. AFP <6.0 → unchanged, patients with AFP <6.0 ng/mL before IFN therapy unchanged at AFP <6.0 ng/mL after IFN therapy; AFP ≥6.0 → decreased, patients with AFP ≥6.0 ng/mL before IFN therapy decreased to AFP <6.0 ng/mL after IFN therapy; AFP ≥6.0 → unchanged, patients with AFP ≥6.0 ng/mL before IFN therapy unchanged at AFP ≥6.0 ng/mL after IFN therapy.

Discussion

This large-scale, long-term cohort study establishes important findings, which demonstrate a strict association between hepatocarcinogenesis and post-IFN treatment ALT and AFP levels in patients with CHC. This association was notable in both SVR and non-SVR subgroups, and suppression of these values by IFN therapy reduced the hepatocarcinogenesis risk despite failure of HCV eradication. These data, which

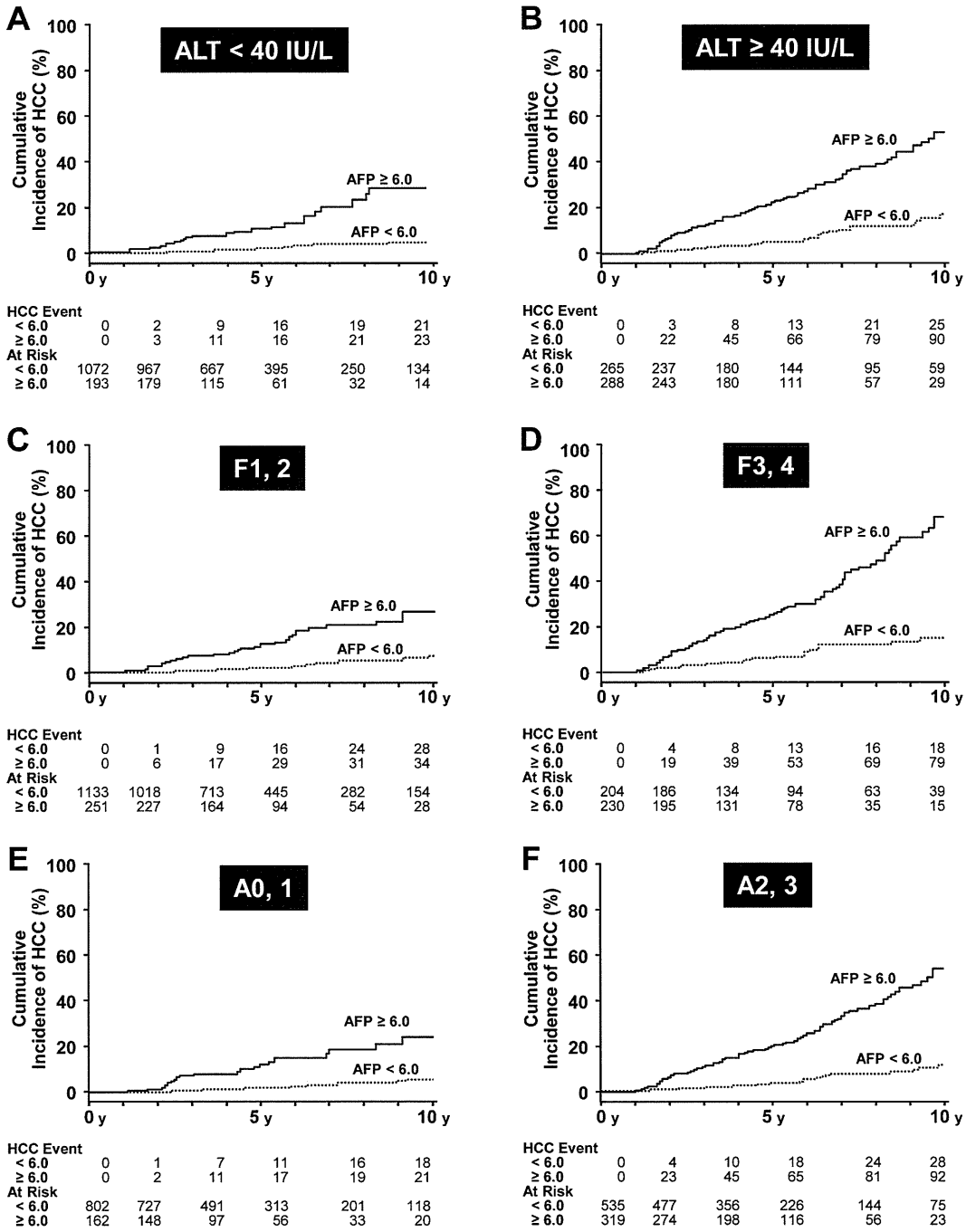


Fig. 4. Relationship between AFP and ALT or histological findings. (A) The cumulative incidence of HCC stratified by post-IFN treatment AFP levels in subgroups according to the post-IFN treatment ALT (log-rank test: $P < 0.0001$ in both subgroups). (B) Cumulative incidence of HCC stratified by post-IFN treatment AFP levels in subgroups according to the histological stage of fibrosis (log-rank test: $P < 0.0001$ in both subgroups). (C) Cumulative incidence of HCC stratified by post-IFN treatment levels of AFP in subgroups according to the histological grade of activity (log-rank test: $P < 0.0001$ in both subgroups).

demonstrate the efficacy of IFN against HCC development associated with suppression of AFP, have clinically important implications for physicians.

Although there have been reports on the association between baseline pretreatment AFP levels and HCC risk,[26-35] little is known regarding the effects of IFN therapy on change in post-IFN treatment AFP and its relation to HCC risk.[36] Although a previous report demonstrated that a decrease in AFP levels in patients receiving IFN therapy reduced the incidence of HCC,[37] this study was performed in a small number of patients (n = 382), and cutoff values, relation to ALT, or histological findings were not determined. Our study, performed in a large well-characterized

cohort, had a greater advantage in that it allowed determination of cutoff values for post-IFN treatment ALT and AFP levels useful for predicting HCC development. Although a higher cutoff value of 20 ng/mL was used to determine the incidence of HCC in the previous study,[36] we propose a lower value for negatively predicting HCC. From our results, those with AFP levels ≥6.0 ng/mL have a substantial HCC risk, even if it is <20 ng/mL. Therefore, post-IFN treatment AFP levels should be <6.0 ng/mL to suppress HCC risk in patients with CHC.

It should be noted that AFP produced by HCC itself was carefully excluded in our study. Serum AFP elevation is frequently observed in patients with advanced CHC in the absence of HCC.[19-23] Although the precise mechanisms accounting for this observation are unknown, Hu et al.[38] found a correlation between AFP and measures of liver disease activity, suggesting that AFP production is enhanced in the presence of necroinflammatory injury of the liver. However, in our study post-IFN treatment ALT and AFP levels were not correlated, and the cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels, even when patients were stratified by post-IFN treatment ALT levels. Moreover, multivariate analysis confirmed that AFP and ALT are independently associated with HCC risk. Therefore, observed elevation in AFP levels in patients with subsequent HCC development is not necessarily caused by necroinflammation of the liver. Alternatively, increased AFP levels have been reported during liver regeneration following hepatic resection and during recovery from massive hepatic necrosis,[39-41] suggesting that elevated AFP levels are a surrogate for proliferative activity of liver cells, which may cause hepatocarcinogenesis in patients with CHC.

Other possible reasons accounting for HCC risk related to AFP are the close association between AFP levels and the stage of liver fibrosis, which is consistent with a previous report.[35] However, we further clarified the fact that correlation between post-IFN treatment AFP levels and liver fibrosis was less notable in patients without subsequent development of HCC (data not shown). Cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels at each stage when patients were stratified by the histological stage of fibrosis (Fig. 4). Therefore, post-IFN treatment AFP is not just a surrogate marker for liver fibrosis, and elevation of post-IFN treatment AFP as a potential risk for hepatocarcinogenesis is not only the result of advanced liver fibrosis. Conversely, suppression of post-IFN treatment AFP levels may reduce HCC risk even in patients with advanced fibrosis.

This study has a few limitations, the first being the heterogeneity of our cohort, which included various treatment regimens with different treatment responses. However, we obtained similar results in a more uniform subgroup of HCV genotype 1b patients treated with PEG-IFNα/RBV (data not shown). The second limitation is the ethnic homogeneity of the Japanese population. Because the baseline incidence of HCC development differs among population groups, longer-term longitudinal studies in larger cohorts with various population subgroups are required to verify the generality of our results.

With the development of potent direct-acting antiviral agents combinations, IFN-free therapy is likely to be approved in the near future. This raises the question of whether posttreatment ALT and/or AFP levels will remain a significant predictor of HCC risk. Moreover, it is uncertain whether the suppressive effect of viral eradication by IFN-free regimens on hepatocarcinogenesis will be identical to that obtained by IFN-based regimens. Therefore, it is extremely interesting to prove these issues in future studies.

In conclusion, post-IFN treatment ALT and AFP levels are strictly associated with hepatocarcinogenesis risk in patients with CHC. Measurement of these values is useful for predicting future HCC risk in IFN-treated patients. Suppression of these values after IFN therapy reduces HCC risk even in patients without HCV eradication, while SVRs with increased ALT and/or AFP levels are at risk for HCC development. The present results have potentially important clinical implications for physicians and may influence their decisions regarding treatment strategy and HCC surveillance for individual patients.

References

1. Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. Semin Liver Dis 1995;15:64-69.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907-1917.
3. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533-543.
4. Kiyosawa K, Sodeyama T, Tanaka E, Gigo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. HEPATOLOGY 1990;12:671-675.
5. Asahina Y, Tsuchiya K, Tamaki T, Hirayama I, Tanaka T, Sato M, et al. Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. HEPATOLOGY 2010;52:518-527.
6. Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, et al. Relation of IFN therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Ann Intern Med 1998;129:94-99.
7. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of IFN 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.
8. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. IFN therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. Ann Intern Med 1999;131:174-181.
9. Bruno S, Stroffolini T, Colombo M, Bollani S, Benvegnù L, Mazzella G, et al. SVR to IFN-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. HEPATOLOGY 2007;45:579-587.
10. Lazarevich NL. Molecular mechanisms of alpha-fetoprotein gene expression. Biochemistry (Mosc) 2000;65:117-133.
11. Ball D, Rose E, Alpert E. Alpha-fetoprotein levels in normal adults. Am J Med Sci 1992;303:157-159.
12. Matsui H, Rimal N, Kamakura K, Uesugi S, Yamamoto H, Ikeda S, et al. Serum alpha-fetoprotein levels in healthy Japanese adults. Acta Med Okayama 1998;52:149-154.
13. Sherman M. Hepatocellular carcinoma: epidemiology, risk factors, and screening. Semin Liver Dis 2005;25:143-154.
14. Trevisani F, D'Intino PE, Morselli-Labate AM, Mazzella G, Accogli E, Caraceni P, et al. Serum alpha-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HBsAg and anti-HCV status. J Hepatol 2001;34:570-575.
15. Cedrone A, Covino M, Caturelli E, Pompili M, Lorenzelli G, Villani MR, et al. Utility of alpha-fetoprotein (AFP) in the screening of patients with virus-related chronic liver disease: does different viral etiology influence AFP levels in HCC? A study in 350 Western patients. Hepatogastroenterology 2000;47:1654-1658.
16. Gebo KA, Chander G, Jenckes MW, Ghanem KG, Herlong HF, Torbenson MS, et al. Screening tests for hepatocellular carcinoma in patients with chronic hepatitis C: a systematic review. HEPATOLOGY 2002;36:S84-S92.
17. Arguedas MR, Chen VK, Eloubeidi MA, Fallon MB. Screening for hepatocellular carcinoma in patients with hepatitis C cirrhosis: a cost utility analysis. Am J Gastroenterol 2003;98:679-690.
18. Nguyen MH, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Racial differences in effectiveness of alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. HEPATOLOGY 2002;36:410-417.
19. Di Bisceglie AM, Hoofnagle JH. Elevations in serum alphafetoprotein levels in patients with chronic hepatitis B. Cancer 1989;64:2117-2120.
20. Liaw YF, Tai DI, Chen TJ, Chu CM, Huang MJ. Alpha-fetoprotein changes in the course of chronic hepatitis: relation to bridging hepatic necrosis and hepatocellular carcinoma. Liver 1986;6:133-137.
21. Bayati N, Silverman AL, Gordon SC. Serum alpha-fetoprotein levels and liver histology in patients with chronic hepatitis C. Am J Gastroenterol 1998;93:2452-2456.
22. Goldstein NS, Blue DE, Hankin R, Hunter S, Bayati N, Silverman AL, et al. Serum alpha-fetoprotein levels in patients with chronic hepatitis C. Relationships with serum alanine aminotransferase values, histologic activity index, and hepatocyte MIB-1 scores. Am J Clin Pathol 1999;111:811-816.
23. Chu CW, Hwang SJ, Luo JC, Lai CR, Tsay SH, Li CP, et al. Clinical, virologic, and pathologic significance of elevated serum alphafetoprotein levels in patients with chronic hepatitis C. J Clin Gastroenterol 2001;32:240-244.
24. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
25. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. HEPATOLOGY 2003;38:518-526.
26. Rodriguez-Diaz JL, Rosas-Camargo V, Vega-Vega O, Morales-Espinosa D, Mendez-Reguera A, Martinez-Tlahuel JL, et al. Clinical and pathological factors associated with the development of hepatocellular carcinoma in patients with hepatitis virus-related cirrhosis: a long-term follow-up study. Clin Oncol 2007;19:197-203.
27. Bruix J, Sherman M. Management of hepatocellular carcinoma. HEPATOLOGY 2005;42:1208-1236.
28. Colombo M, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, et al. Hepatocellular carcinoma in Italian patients with cirrhosis. N Engl J Med 1991;325:675-680.
29. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797-1801.
30. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. HEPATOLOGY 1994;19:61-66.
31. Ganne-Carrie N, Chastang C, Chapel F, Munz C, Pateron D, Sibony M, et al. Predictive score for the development of hepatocellular carcinoma and additional value of liver large cell dysplasia in Western patients with cirrhosis. HEPATOLOGY 1996;23:1112-1118.
32. Sangiovanni A, Colombo E, Radaelli F, Bortoli A, Bovo G, Casiraghi MA, et al. Hepatocyte proliferation and risk of hepatocellular carcinoma in cirrhotic patients. Am J Gastroenterol 2001;96:1575-1580.
33. Ikeda K, Arase Y, Saitoh S, Kobayashi M, Someya T, Hosaka T, et al. Prediction model of hepatocarcinogenesis for patients with hepatitis C virus-related cirrhosis. Validation with internal and external cohorts. J Hepatol 2006;44:1089-1097.
34. Bruce MG, Bruden D, McMahon BJ, Christensen C, Homan C, Sullivan D, et al. Clinical significance of elevated alpha-fetoprotein in Alaskan Native patients with chronic hepatitis C. J Viral Hepat 2008;15:179-187.
35. Tateyama, M, Yatsuhashi H, Taura N, Motoyoshi Y, Nagaoka S, Yanagi K, et al. Alpha-fetoprotein above normal levels as a risk factor for the development of hepatocellular carcinoma in patients infected with hepatitis C virus. J Gastroenterol 2010;46:92-100.
36. Di Bisceglie1 AM, Sterling RK, Chung RT, Everhart JE, Dienstag JL, Bonkovsky HL, et al. Serum alpha-fetoprotein levels in patients with advanced hepatitis C: Results from the HALT-C Trial. J Hepatol 2005;43:434-441.
37. Osaki Y, Ueda Y, Marusawa H, Nakajima J, Kimura T, Kita R, et al. Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study. J Gastroenterol 2012;47:444-451.
38. Hu K-Q, Kyulo NL, Lim N, Elhazin B, Hillebrand DJ, Bock T. Clinical significance of elevated alpha-fetoprotein (AFP) in patients with chronic hepatitis C, but not hepatocellular carcinoma. Am J Gastroenterol 2004;99:860-865.
39. Watanabe A, Shiota T, Hayashi S, Nagashima H. Serum alphafetoprotein in fulminant hepatitis and hepatic regeneration following partial hepatectomy. Biochem Med 1984;32:132-137.
40. Yang SS, Cheng KS, Lai YC, Wu CH, Chen TK, Lee CL, Chen DS. Decreasing serum alpha-fetoprotein levels in predicting poor prognosis of acute hepatic failure in patients with chronic hepatitis B. J Gastroenterol 2002;37:626-632.
41. Horn KD, Wax P, Schneider SM, Martin TG, Nine JS, Moraca MA, et al. Biomarkers of liver regeneration allow early prediction of hepatic recovery after acute necrosis. Am J Clin Pathol 1999;112:351-357.

Hepatology Research

Hepatology Research 2013; **43**: 865–875 doi: 10.1111/hepr.12032

Original Article

Serum RANTES level influences the response to pegylated interferon and ribavirin therapy in chronic hepatitis C

Kazuki Komase,[1] Shinya Maekawa,[1,2] Mika Miura,[1] Ryota Sueki,[1] Makoto Kadokura,[1] Hiroko Shindo,[1] Kuniaki Shindo,[1] Fumitake Amemiya,[1] Yasuhiro Nakayama,[1] Taisuke Inoue,[1,2] Minoru Sakamoto,[1] Atsuya Yamashita,[3] Kohji Moriishi[3] and Nobuyuki Enomoto[1]

[1]*First Department of Medicine, Faculty of Medicine,* [2]*Department of Advanced Medicine for Liver Diseases, Faculty of Medicine, and* [3]*Department of Microbiology, University of Yamanashi, Yamanashi, Japan*

Aim: Prediction of treatment responses to pegylated interferon (PEG IFN) plus ribavirin (RBV) therapy is uncertain for genotype 1b chronic hepatitis C.

Methods: In this study, 96 patients were investigated for the correlation between 36 pretreatment serum chemokine/cytokine levels and PEG IFN/RBV treatment efficacy by a sandwich enzyme-linked immunoassay (ELISA) and a bead array.

Results: First, chemokines/cytokines were measured semi-quantitatively by sandwich ELISA in 31 randomly-selected patients and the serum regulated on activation normal T-cell expressed and secreted (RANTES) level was found to be significantly higher in the sustained virological response (SVR) group than the non-SVR group ($P = 0.048$). Precise RANTES measurement in all 96 patients using a bead array confirmed this correlation ($P = 0.002$). However, the genetic RANTES haplotype was not significantly related to the serum level. The serum RANTES level was extracted by multivariate analysis (odds ratio = 4.09, 95% confidence interval = 1.02–16.5, $P = 0.048$) as an independent variable contributing to SVR.

Conclusion: The serum RANTES level is an important determinant influencing the virological response to PEG IFN/RBV therapy in chronic hepatitis C.

Key words: hepatitis C virus, pegylated interferon plus ribavirin therapy, RANTES

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease worldwide and persistent infection may lead to liver cirrhosis and hepatocellular carcinoma.[1] Therapy leading to HCV eradication is the only treatment with proven efficacy in decreasing the occurrence of hepatocellular carcinoma.[2] Recently, treatment with telaprevir, a non-structural (NS)3/4A protease inhibitor, combined with pegylated interferon (PEG IFN) and ribavirin (RBV), increased the rates of sustained viral response (SVR) up to 64–75%[3,4] compared to the SVR rate of approximately 50% for the previous PEG IFN/RBV therapy. However, it has become evident that genotype 1-infected patients with a null response to previous PEG IFN/RBV therapy have poor responses to PEG IFN/RBV/telaprevir,[5] with an SVR rate as low as approximately 30%, illustrating the difficulty in treating patients infected with genotype 1 HCV. Therefore, precise and accurate prediction of the viral response to PEG IFN/RBV therapy remains an important issue.

Treatment resistance is attributed to various factors associated with the virus and host. Viral factors, such as amino acid (a.a.) sequence variation in the core and NS5A regions, have been investigated extensively for their contribution to the outcome of IFN-based therapy,[6,7] including PEG IFN/RBV therapy. On the other hand, host factors such as African-American race, older age, being obese, the presence of cirrhosis and

Correspondence: Dr Shinya Maekawa, First Department of Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Email: maekawa@yamanashi.ac.jp

Conflict of interest: Shinya Maekawa and Taisuke Inoue belong to a donation-funded department that is funded by MSD (Tokyo, Japan). Nobuyuki Enomoto received research funding from MSD (Tokyo, Japan) and Roche (Tokyo, Japan).

Received 26 September 2012; revision 18 November 2012; accepted 26 November 2012.

steatosis, and insulin resistance have been reported to be associated with treatment resistance.[8–11] Especially, single nucleotide polymorphisms (SNP) near the interleukin (*IL*)-28B gene, including rs12979860 and rs8099917, have been reported to have a significant correlation with the response to IFN-based therapy.[12,13] However, even with inclusion of these factors, prediction of the treatment response in chronic HCV infection remains uncertain at present.

Chemokines are a group of small, exogenously secreted cytokines that modulate the migration of leukocytes to sites of tissue damage and inflammation in a variety of infectious and autoimmune diseases.[14] In chronic HCV infection, chemokines such as *RANTES* (regulated on activation normal T-cell expressed and secreted), macrophage inflammatory protein (*MIP*)-1α, *MIP-1β* and interferon-γ inducible protein 10 kDa (*IP-10*) are elevated and considered to play crucial roles in inflammatory processes and viral elimination, as well as the transition from innate to adaptive immunity.[14,15] Upregulation of several serum chemokines, such as eotaxin, *IP-10* and *RANTES* also has been reported in HCV infection, possibly reflecting hepatic inflammation.[16] Considering the roles of chemokines/cytokines in establishing chronic hepatitis, it is possible that these chemokines also affect the response to antiviral therapy, and actually several chemokines as interleukin (*IL*)-8, *IL-10*, *MIP-1β*, *RANTES* or *IP-10* have been investigated previously for their association with the treatment response.[16–20] However, the importance of those chemokines has not been established yet and, moreover, these studies did not characterize in detail these chemokines in association with other factors, including *IL-28B* influencing the response to therapy.

In this study, we explored extensively the association of 36 serum cytokines/chemokines and the treatment response, with detailed information of host and virus, to predict better the treatment response to PEG IFN and RBV therapy in genotype 1b HCV infection. Because the pretreatment serum *RANTES* level was found to be correlated significantly with the response, we analyzed further the association between the serum level of *RANTES* and the genomic SNP.

METHODS

Patients

NINETY-SIX CONSECUTIVE PATIENTS with genotype 1b HCV and receiving PEG IFN/RBV therapy between 2004 and 2010 at Yamanashi University Hospital were recruited retrospectively into the study. All patients received the standard therapy according to the treatment protocol of PEG IFN/RBV therapy for Japanese patients, established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan (PEG IFN-α-2b 1.5 μg/kg bodyweight, once weekly s.c., and RBV 600–800 mg daily p.o. for 48 weeks).[21] All patients enrolled fulfilled the following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) not co-infected with HIV; and (iv) a signed consent was obtained for the study protocol that had been approved by the Human Ethics Review Committee of Yamanashi University Hospital. The study was approved by the ethics committees of University of Yamanashi, and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

Definition of treatment outcome

An SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Relapse was defined as reappearance of detectable HCV RNA levels following discontinuation of treatment. Null response was defined as less than 2 log decrease of the baseline HCV RNA levels after 12 weeks of treatment. Based on this definition, when patients were classified according to the achievement of SVR, patients with relapse or null response were classified as non-SVR.

Serum cytokine measurement

Sandwich enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected before initiation of treatment and were stored at −80°C until use. Semiquantitation of serum cytokines was performed using the Proteome Profiler Human Cytokine Array Kit Panel A (R&D Systems, Minneapolis, CA, USA) according to the manufacturer's instructions. The kit consists of a nitrocellulose membrane containing 36 different anti-cytokine antibodies (anti-C5a, anti-CD154, anti-G-CSF, anti-GM-CSF, anti-CXCL1, anti-CCL1, anti-sICAM-1, anti-IFN-γ, anti-IL-1α, anti-IL-1β, anti-IL-1ra, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-8, anti-IL-10, anti-IL-12p70, anti-IL-13, anti-IL-16, anti-IL-17, anti-IL-17E, anti-IL-23, anti-IL-27, anti-IL-32α, anti-IP-10, anti-CXCL11, anti-CCL2, anti-MIF, anti-CCL3, anti-CCL4, anti-PAI-1, anti-RANTES, anti-CXCL12, anti-TNF-α, anti-sTREM-1), spotted in duplicate. Serum samples were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture

was then incubated with the membrane. Any cytokine/detection antibody complex present was bound to the membrane by its cognate immobilized capture antibody. Following washing to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescent detection reagents (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, UK) were added sequentially. Arrays were scanned using a LAS-3000 mini-luminescent image analyzer (Fujifilm, Tokyo, Japan) and were quantified for the densities using Multi Gauge ver. 3.0 software (Fujifilm). Concentrations of cytokines and chemokines were expressed as their signal intensity ratios relative to that of the positive control spotted on the same membrane.

Bead array

Precise serum concentrations of regulated on *RANTES* were measured using the Luminex Bio-Plex system (Bio-Rad, Hercules, CA, USA) and the Procarta Cytokine Assay Kit (Panomics, Fremont, CA, USA) in a 96-well plate ELISA-based format according to the manufacturers' recommendations. The sensitivity of the assays is greater than 10 pg/mL cytokine. Serum and standards were incubated with a mixture of the Luminex antibody-conjugated beads for 30 min with constant shaking. After washing, the detection antibodies and substrates were added and incubated for another 30 min. Fluorescent signals were collected and data expressed, using internal standards, in pg/mL as the mean of two individual experiments carried out in duplicate.

Viral core and interferon sensitivity-determining region (ISDR) sequence determination by direct sequencing

Hepatitis C virus RNA extraction from serum samples, complementary DNA synthesis and amplification by two-step nested polymerase chain reaction (PCR) were carried out using specific primers for the HCV core and ISDR. PCR amplicons were sequenced directly by Big Dye Terminator ver. 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

SNP typing of the *RANTES* and *IL-28B* genes

Genomic DNA of the patients was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 (ABI) using FAM-labeled SNP primers for the loci rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 (ABI) for *RANTES* and the locus rs8099917 (ABI) for IL-28B.

Statistical analysis

Student's *t*-test and Mann–Whitney *U*-test were used to analyze continuous variables, as appropriate. Fisher's exact test was used for the analysis of categorical variables. Receiver–operator curve (ROC) analyses were performed to establish cut-off values for serum cytokine concentration. The optimum cut-off was defined as the value that maximized the area under the ROC. Spearman's correlation coefficient (R) was calculated to clarify the strength of relationship between the pretreatment serum cytokine concentrations and clinical parameters. Variables that achieved statistical significance ($P < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The odds ratios and 95% confidence intervals also were calculated. Data were analyzed using Ekuseru-Toukei 2008 (SSRI, Tokyo, Japan). The haplotype block among rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 variants was analyzed using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan). $P < 0.05$ was considered significant.

RESULTS

Semiquantitative measurement of pretreatment serum cytokines in 31 randomly-selected patients

AT FIRST, TO identify cytokines/chemokines related to the treatment responses to PEG IFN/RBV therapy, semiquantitative measurement of the serum concentrations of 36 comprehensive cytokines/chemokines was performed by sandwich ELISA method by randomly selected patients. Next, to further confirm the result, cytokines showing the associations with the response were measured more precisely by bead array method in all patients.

In the first analysis, 31 patients were randomly selected from the 96 patients. The clinical characteristics of these 31 patients at the start of the therapy are shown in Table 1. Significant differences in the clinical backgrounds between those who did and those who did not

Table 1 Baseline characteristics of the 31 patients analyzed using the sandwich ELISA method

Factor	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
Age (years)	52 ± 11†	57 ± 10	0.25‡
Sex (male : female)	11:9	6:5	0.64§
Bodyweight (kg)	60.9 ± 9.6†	61.9 ± 13.9	0.81‡
Body mass index (kg/m^2)	22.6 (18.9–31.3)¶	22.7 (17.5–26.8)	0.87††
History of IFN therapy (%)	30	36	0.78§
ALT (IU/L)	130 ± 100†	75 ± 35	0.09‡
AST (IU/L)	76 (22–331)¶	64 (24–178)	0.73††
γ-GTP (IU/L)	40 (12–289)	52 (24–137)	0.17††
Albumin (g/dL)	4.1 (3.7–4.5)	4.0 (3.0–4.7)	0.46††
Total cholesterol (mg/dL)	170 ± 24†	149 ± 33	0.06‡
HbA1c (%)	5.3 ± 0.5	5.3 ± 0.6	0.95‡
Creatinine (mg/dL)	0.71 ± 0.15	0.68 ± 0.15	0.54‡
WBC count (/μL)	4561 ± 1631	4056 ± 1277	0.38‡
Neutrophil count (/μL)	2130 (820–4200)¶	1500 (800–2700)	0.02††
Hemoglobin (g/dL)	14.5 ± 1.0†	13.8 ± 1.6	0.15‡
Platelet count (×10^{-4}/μL)	16.4 ± 5.4	12.2 ± 3.9	0.03‡
α-Fetoprotein (ng/mL)	4.6 (1.4–28.9)¶	22.3 (11.4–79.7)	0.00005††
HCV RNA (KIU/mL)	1520 ± 1079†	2146 ± 899	0.11‡
Fibrosis (F1/F2/F3/F4)‡‡	14/1/1/2	3/2/2/3	0.02††
Activity (A1/A2/A3)‡‡	12/5/1	3/5/2	0.06††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney *U*-test.
‡‡SVR, $n = 18$; non-SVR, $n = 10$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunoassay; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

achieve SVR were neutrophil counts, platelet counts, serum α-fetoprotein levels and the score of fibrosis in liver biopsies. Table 2 shows the difference in the cytokine/chemokine expression between the SVR and the non-SVR group. Because some cytokines/chemokines were below the measurement limit of the ELISA kit, as shown in Table 1, those cytokine/chemokines were not studied further. As shown here, the *RANTES* level was significantly higher in the SVR group than the non-SVR group ($P = 0.048$).

Precise measurement of serum *RANTES* in all 96 patients

Because the semiquantitative measurement of pretreatment serum *RANTES* levels in 31 randomly selected patients demonstrated their significant correlation with the SVR, we determined the precise serum RANTES levels in all 96 patients using the bead array method and investigated the correlation between those concentrations and the treatment outcome. The clinical characteristics of the 96 patients are shown in Table 3. Significant differences were seen between those with and without SVR in platelet count, viral loads and the liver fibrosis score, but there was no apparent difference in the total doses of PEG IFN and RBV. As shown in Figure 1, the distribution of serum *RANTES* levels in each treatment response differed significantly; the median serum *RANTES* level in the SVR group was significantly higher than that in the non-SVR group. Successive ROC analysis confirmed a significant association of the serum *RANTES* level with SVR, and the cut-off value of 3400 pg/mL to be most appropriate (Table 4). Using the cut-off value of 3400 pg/mL, 50.9% sensitivity, 79.5% specificity, 78.4% positive predictive value and 52.5% negative predictive value (area under the ROC, 0.643) were obtained for the prediction of SVR by serum *RANTES* level.

Table 2 Difference in cytokine and chemokine expression between the SVR group and the non-SVR group in the 31 patients

Cytokine/chemokine	SVR ($n = 20$)	Non-SVR ($n = 11$)	*P*-value
RANTES	4.99 (0.25–8.32)†	1.24 (0.17–8.01)	0.048‡
MIF	1.31 (0.06–3.31)†	0.45 (0.08–2.67)	0.0630
IL-1ra	0.09 (0.00–3.30)†	0.07 (0.00–2.05)	0.2300
PAI-1	3.10 (0.35–7.34)†	2.73 (0.46–8.42)	0.3900
sICAM-1	3.18 (0.37–8.33)†	2.78 (0.74–10.3)	0.4800
IL-23	0.08 (0.01–0.78)†	0.07 (0.00–0.38)	0.5900
IL-27	0.05 (0.02–0.18)†	0.05 (0.00–0.23)	0.6500
IL-6	0.08 (0.01–3.22)†	0.10 (0.00–1.36)	0.7100
C5a	0.21 (0.01–2.72)†	0.12 (0.00–1.67)	0.7700
IFN-γ	0.07 (0.02–0.31)†	0.08 (0.00–0.40)	0.8000
CCL4	0.04 (0.01–3.08)†	0.05 (0.00–0.69)	0.8400
IL-32α	0.04 (0.00–0.71)†	0.07 (0.00–0.20)	0.9000
IL-8	0.16 (0.05–2.61)†	0.17 (0.03–2.21)	0.9300
IL-1α			N.A.
IL-1β			N.A.
IL-2			N.A.
IL-4			N.A.
IL-5			N.A.
IL-10			N.A.
IL-12 p70			N.A.
IL-13			N.A.
IL-16			N.A.
IL-17			N.A.
IL-17E			N.A.
CCL1			N.A.
CCL2			N.A.
CCL3			N.A.
CXCL1			N.A.
CXCL11			N.A.
CXCL12			N.A.
CD154			N.A.
G-CSF			N.A.
GM-CSF			N.A.
IP-10			N.A.
TNF-α			N.A.
sTREM-1			N.A.

†Median (range).
‡Mann–Whitney *U*-test.
N.A., not available; SVR, sustained virological response.

Correlation between serum *RANTES* level and clinical parameters

Spearman's correlation coefficients between the pre-treatment serum *RANTES* level and clinical parameters in all 96 patients are shown in Table 5. As a result, a significant negative correlation with aspartate aminotransferase level and a significant positive correlation with platelet count were found, while no significant correlation was observed in other clinical parameters.

Univariate and multivariate analysis of factors related to SVR

Univariate and multivariate analyses were performed successively in order to clarify the factors related to SVR. The viral factors included in the analysis were the ISDR and core a.a. 70 and 91, along with the host factor, IL-28B SNP. Those factors, conventional clinical background factors and serum *RANTES* levels were subjected to univariate and multivariate analysis. In the univariate

Table 3 Baseline characteristics of all patients analyzed using the bead array method ($n = 96$)

Factor	SVR ($n = 57$)	Non SVR ($n = 39$)	*P*-value
Age (years)	53 ± 10†	57 ± 8	0.08‡
Sex (male : female)	34:23	23:16	0.56§
Bodyweight (kg)	60.6 ± 10.5†	57.8 ± 7.8	0.17‡
Body- mass index (kg/m^2)	22.9 ± 2.8	22.1 ± 2.2	0.15‡
History of IFN therapy (%)	25	28	0.74§
ALT (IU/L)	68 (19–413)¶	64 (20–215)	0.25††
AST (IU/L)	58 (21–331)	62 (21–178)	0.80††
γ-GTP (IU/L)	37 (11–289)	50 (13–167)	0.12††
Albumin (g/dL)	4.1 ± 0.3†	4.1 ± 0.4	0.93‡
Total cholesterol (mg/dL)	166 ± 30	158 ± 31	0.25‡
HbA1c (%)	5.2 (4.7–6.6)¶	5.3 (4.5–7.4)	0.47††
Creatinine (mg/dL)	0.72 ± 0.15†	0.69 ± 0.16	0.39††
WBC count (/μL)	4497 ± 1247	4501 ± 1281	0.99‡
Neutrophil count (/μL)	2243 ± 857	2144 ± 825	0.57‡
Hemoglobin (g/dL)	14.1 ± 1.2	14.2 ± 1.2	0.87‡
Platelet count (×10^{-4}/μL)	15.1 (7–29)¶	13.2 (6.9–19.7)	0.03††
α-Fetoprotein (ng/mL)	4.8 (1.3–137.1)	9.0 (1.4–79.7)	0.05††
HCV RNA (KIU/mL)	1300 (100–5000)	2400 (620–5000)	0.0002‡
Fibrosis (F1/F2/F3/F4)‡‡	35/6/5/6	11/13/5/6	0.006††
Activity (A1/A2/A3)‡‡	27/18/7	12/20/3	0.26††
PEG IFN dose (%)	92 (40–113)¶	73 (27–147)	0.23††
RBV dose (%)	97 (44–147)	100 (33–135)	0.38††

†Mean ± standard deviation.
‡Student's *t*-test.
§Fisher's exact probability test.
¶Median (range).
††Mann–Whitney's *U*-test.
‡‡SVR, $n = 52$; non-SVR, $n = 35$.
Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

analysis, significant differences were observed for the ISDR mutation, core a.a. 70, viral loads, platelet counts, IL-28B SNP and serum *RANTES* levels. When multivariate analysis was carried out with these factors, the serum RANTES level was extracted as an independent factor related to SVR (Table 6).

RANTES haplotyping and serum *RANTES* level

Because a high serum *RANTES* level was an independent factor predicting SVR, we sought to examine further the role of the RANTES gene and tried to clarify the association of the SNP of the gene with the serum levels. First, we determined how many and which SNP in the *RANTES* gene should be investigated to represent all *RANTES* haplotypes found in the Japanese population. Reference to the HapMap project database (http://snp.cshl.org) made it clear that the information from five unique SNP was required to determine the majority of haplotypes found in the Japanese population. Therefore, to determine the *RANTES* haplotype of each patient, we investigated these five SNP in the 65 of the 96 patients available for the haplotype analysis. The *RANTES* haplotypes were finally divided into three types (named R1, R2 and R3 for convenience), as shown in Figure 2(a). However, the *RANTES* gene haplotype and serum RANTES level did not show any clear correlation (Fig. 2b).

DISCUSSION

FROM THE ANALYSIS of 36 cytokine and chemokine species, we discovered that a high pretreatment serum *RANTES* level was significantly related to SVR

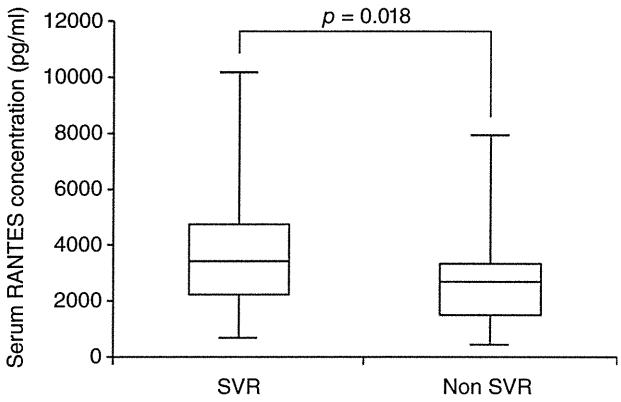


Figure 1 Difference in serum *RANTES* levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum *RANTES* level using the bead array method. They were divided into the SVR ($n = 57$) and non-SVR groups ($n = 39$) and their serum *RANTES* levels compared. Box and whisker plots show the distributions of serum *RANTES* levels for the SVR and non-SVR groups. The boxes represent the 25th to 75th percentile and horizontal lines within the box show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test.

following PEG IFN/RBV combination therapy of patients infected with genotype 1b HCV. In particular, a high serum *RANTES* level was an independent factor contributing to SVR in the multivariate analysis, even among other treatment-restricting factors as the HCV ISDR, core a.a. 70, viral loads, platelets or IL-28B SNP. On the other hand, a systematic haplotyping study did not reveal any correlation between the *RANTES* haplotype and serum *RANTES* level.

RANTES, also known as CC-chemokine ligand (CCL)5, is classified as a chemotactic T-helper (Th)1-type chemokine.[23] In chronic hepatitis C, *RANTES* is significantly upregulated in the infected liver, and considered to play a role in recruiting T cells to portal and periportal regions, regulating liver inflammation and innate and adaptive immunity through interactions with CC-chemokine receptor (*CCR*)5, *CCR1* and *CCR3* expressed on activated T cells.[24] The serum *RANTES* level is significantly upregulated in the early stages of fibrosis in chronic hepatitis and its upregulation becomes weaker in advanced chronic disease.[14] HCV-encoded proteins are considered to affect *RANTES* production, for example, exposure of peripheral blood mononuclear cells to the HCV envelope 2 (E2) protein induces the release of *RANTES*,[25] the HCV NS3/4A proteins suppress *RANTES* promoter activity[26] and the HCV core protein may either induce or inhibit the expression of *RANTES* in various cell types.[27] A recent *in vitro* study has shown that human hepatoma cells secrete *RANTES* via the Toll-like receptor (*TLR*)3-mediated recognition of HCV dsRNA and activation of the nuclear factor (*NF*)-*kB* pathway, suggesting that the hepatocytes themselves may serve as the source of *RANTES*.[15]

In this study, we showed the close association between the serum *RANTES* level and SVR in the PEG IFN/RBV combination therapy by analyzing 31 randomly selected, primary test patients and then all 96 patients. In addition to the association with SVR, we also searched the association between *RANTES* and the initial viral response because SVR could be influenced by the initial viral dynamics, and revealed that complete early viral response (HCV RNA negative at 12 weeks

Table 4 Association between the serum *RANTES* level and SVR rate in all 96 patients analyzed using the bead array method

Cytokine/ chemokine	Serum concentration	SVR rate	*P*-value
RANTES	≥3400 pg/mL†	78% (29/37)	0.002‡
	<3400 pg/mL†	47% (28/59)	

†A cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
‡Fisher's exact probability test.
SVR, sustained virological response.

Table 5 Spearman's correlation coefficient (*R*) between the pretreatment serum *RANTES* level and clinical parameters ($n = 96$)

Clinical parameters	Serum *RANTES* level	
	R	*P*-value
Platelet count	−0.30	0.0025
Aspartate aminotransferase	−0.24	0.0200
White blood cell	−0.15	0.1600
Total cholesterol	−0.11	0.2700
Alanine aminotransferase	−0.088	0.3900
α-Fetoprotein	−0.088	0.4100
Neutrophil count	−0.064	0.5400
Hemoglobin A1c	−0.056	0.6300
γ-Glutamyl transpeptidase	−0.047	0.6500
Albumin	−0.021	0.7900
Hemoglobin	−0.025	0.8000
Creatinine	−0.00098	0.9900

Table 6 Factors associated with SVR analyzed by univariate and multivariate analysis

Characteristic	Subcategory	Univariate analysis			Multivariate analysis		
		Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Platelet count		1.13	1.03–1.25	0.012	1.20	1.00–1.41	0.042
IL-28B SNP	T/T or not	16.0	3.37–76.2	0.0005	9.48	1.40–64.3	0.02
RANTES	≥3400†	4.01	1.58–10.2	0.0036	4.09	1.02–16.5	0.048
Viral loads		0.99	0.99–0.99	0.0012	0.99	0.99–1.00	0.51
ISDR mutation	≥2	21.7	2.76–170	0.0034	28.2	2.05–388	0.013
Core a.a. 70	R or not	2.52	1.03–6.20	0.044	3.19	0.73–13.9	0.12

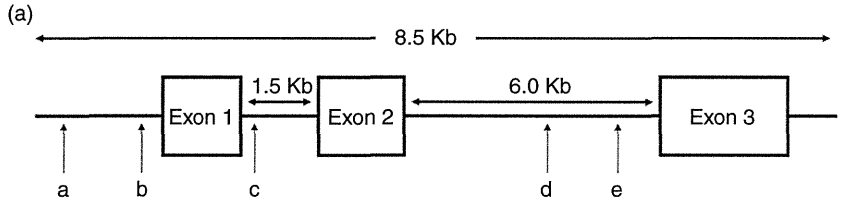
†The cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
a.a., amino acids; CI, confidence interval; IL, interleukin; ISDR, interferon sensitivity-determining region; R, arginine; SNP, single nucleotide polymorphisms.

after commencement of therapy) was also significantly correlated with high pretreatment serum *RANTES* level ($P = 0.015$, data not shown). Moreover, we could also show that high serum *RANTES* levels correlated with the clinical background factors low alanine aminotransferase values and high platelet counts, suggesting that the patients with high *RANTES* levels have less severe hepatitis. A previous study also showed a tendency for correlation between the serum *RANTES* level and SVR in PEG IFN/RBV therapy, but this correlation did not reach significance.[16] Although the reason for this discrepancy is not known, we speculate that a difference in drug dosage may have contributed. In our study, most of the patients received a sufficient dose of both PEG IFN and RBV, as shown in Table 3. However, the previous study lacks information regarding drug dosage, suggesting that the study group comprised a heterogeneous population.

Then, what is the mechanism of the association between high serum *RANTES* levels and high SVR? Because *RANTES* is a chemotactic Th1-type chemokine, it may be speculated that a high serum *RANTES* level reflects activation and preservation of the Th1-type immune responses needed to suppress viral replication and so enhances viral elimination by PEG IFN/RBV therapy. Although it is also possible that a high *RANTES* level could be simply a reflection of early stages of the disease, we suggest that it could have a more direct role in achieving SVR, because multivariate analysis extracted a high serum *RANTES* level as a variable contributing to SVR independently of the platelet count, which reflects the stage of disease. Importantly, our result also demonstrated that the serum *RANTES* level was a factor contributing to SVR independently of other treatment-restricting factors, including the *IL-28B* SNP and the viral factors of NS5A and core. This independent contribution of a high serum *RANTES* level among other variables indicates its importance and potency in improving the prediction of the treatment efficacy.

Concerning the association between the serum *RANTES* level and *RANTES* haplotype, we could not find a significant correlation in the HCV-infected patients, although there was a tendency that patients with the R3 haplotype had higher serum *RANTES* levels. In patients with coronary artery disease and type 1 diabetes mellitus, and in healthy volunteers, the serum *RANTES* level has been reported to correlate with the *RANTES* gene SNP. Specifically, those patients and healthy volunteers with the A allele in the *RANTES* promoter polymorphism at position –403 (rs2107538) had lower serum *RANTES* levels than those with the G allele.[28,29] On the other hand, in previous reports of chronic hepatitis C, no evident correlation was reported between the *RANTES* SNP at position –403 (rs2107538) and serum *RANTES* level.[30] In this study, through more systematic haplotyping analysis based upon the HapMap Database, we tried to determine the correlation between the serum *RANTES* level and the *RANTES* gene SNP in chronic hepatitis C in more detail. However, we could not find any association and the result shows that the serum *RANTES* level is not primarily determined by the *RANTES* haplotype in chronic hepatitis C. The result seems strange at first, however, it is understandable considering that *RANTES* expression is modulated by multiple factors in chronic hepatitis C, including viral components and the stage of liver disease, as described before.

However, there are some limitations in our study. Namely, the number of investigated patients was rather small, and included patients for the analysis were limited to those with genotype 1b HCV infection. Therefore, it is considered that additional independent studies including the analysis of other genotypes would



RANTES haplotype	a	b	c	d	e	Haplotype frequency
	rs2107538	rs2280788	rs2280789	rs4796120	rs3817655	
R1	G	C	T	A	A	0.599
R2	A	C	C	G	T	0.197
R3	A	G	C	A	T	0.189

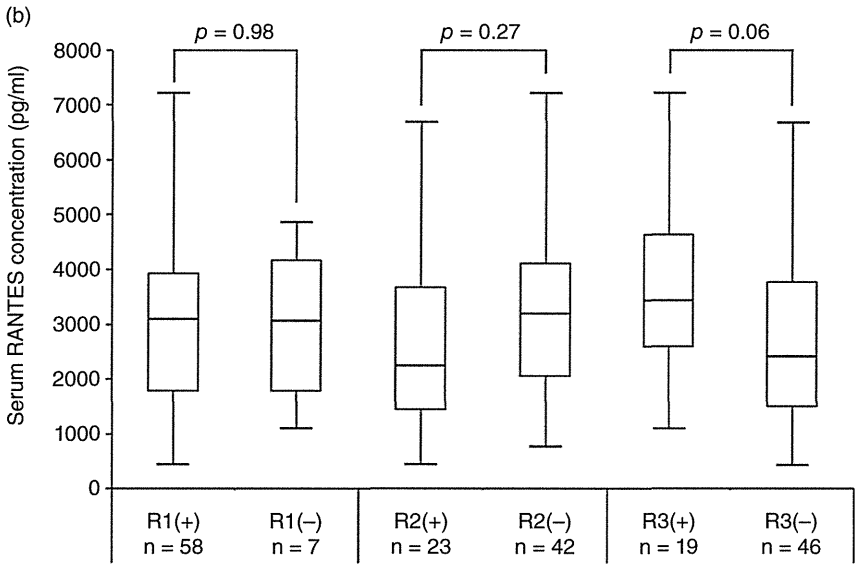


Figure 2 *RANTES* haplotypes and serum *RANTES* level. (a) *RANTES* haplotypes in the patients studied. The human *RANTES* gene spans 8.5 kb on chromosome 17q11-q12 and has the characteristic three exon and two intron organization of the CC chemokine family.[22] Exons are shown as open boxes while introns are shown as solid lines. Five single nucleotide polymorphisms (SNP) (rs2107538/rs2280788/rs2280789/rs4796120/rs3817655) were selected on the basis of data from the HapMap project (http://snp.cshl.org) to obtain complete coverage of the *RANTES* gene in the Japanese population. The locations of SNP variants are indicated by arrows. After the analysis of five *RANTES* SNP in 65 hepatitis C virus patients, haplotypes were determined using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan) and divided into three groups on the basis of linkage disequilibrium. These were designated R1, R2 and R3 on the basis of haplotype frequency. (b) Serum *RANTES* level and *RANTES* haplotype. The correlation between serum the *RANTES* level and *RANTES* haplotype was investigated. Box and whisker plots shows distributions of serum *RANTES* levels for the haplotypes R1(+), R1(−), R2(+), R2(−), R3(+) and R3(−). The boxes represent the 25th to 75th percentile and horizontal lines within the boxes show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann–Whitney's *U*-test. R1(+), the patients with the R1 haplotype; R1(−), the patients with a non-R1 haplotype; R2(+), the patients with the R2 haplotype; R2(−), the patients with a non-R2 haplotype; R3(+), the patients with the R3 haplotype; R3(−), the patients with a non-R3 haplotype.

further clarify the correlation. On the other hand, we could not show an association of pretreatment cytokines/chemokine concentrations with the treatment response to PEG IFN/RBV therapy for the other 35 cytokine and chemokine species investigated in this study. Recently, the serum level of *IP-10* was reported to be strongly associated with the response to PEG IFN/RBV therapy and baseline *IP-10* levels were elevated in patients infected with HCV genotype 1 or 4 who did not achieve an SVR after completion of interferon therapy.[19,20] In our study, however, *IP-10* was not extracted as a molecule associated with treatment responses. Actually, due to the measurement limit of the ELISA kit used, several cytokines and chemokines, including *IP-10*, were undetectable in this study, as shown in Table 2, raising the possibility that some cytokines and chemokines associated with SVR were not extracted. Therefore, our study cannot exclude the possibility of other cytokine/chemokines making a contribution to treatment efficacy.

In conclusion, we found that a high pretreatment serum *RANTES* level was related to the efficacy of PEG IFN/RBV therapy in genotype 1b HCV, independent of other treatment-restricting factors, and prediction of treatment outcome could be improved with the measurement of the pretreatment serum *RANTES* level.

ACKNOWLEDGMENTS

THIS WORK WAS supported by Grants-in-Aid from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture (21590836, 21590837, 23390195); and a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan (H22-kanen-006).

REFERENCES

1 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–5.
2 Singal AK, Singh A, Jaganmohan S *et al.* Antiviral therapy reduces risk of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *Clin Gastroenterol Hepatol* 2010; **8**: 192–9.
3 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405–16.
4 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; **364**: 2417–28.
5 Fried MW. The role of triple therapy in HCV genotype 1-experienced patients. *Liver Int* 2011; **31** (Suppl 1): 58–61.
6 Akuta N, Suzuki F, Sezaki H *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**: 372–80.
7 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77–81.
8 Conjeevaram HS, Kleiner DE, Everhart JE *et al.* Race, insulin resistance and hepatic steatosis in chronic hepatitis C. *Hepatology* 2007; **45**: 80–7.
9 Hickman IJ, Powell EE, Prins JB *et al.* In overweight patients with chronic hepatitis C, circulating insulin is associated with hepatic fibrosis: implications for therapy. *J Hepatol* 2003; **39**: 1042–8.
10 Shiffman ML, Mihas AA, Millwala F *et al.* Treatment of chronic hepatitis C virus in African Americans with genotypes 2 and 3. *Am J Gastroenterol* 2007; **102**: 761–6.
11 Shiffman ML, Suter F, Bacon BR *et al.* Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; **357**: 124–34.
12 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41**: 1100–4.
13 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105–9.
14 Katsounas A, Schlaak JF, Lempicki RA. CCL5: a double-edged sword in host defense against the hepatitis C virus. *Int Rev Immunol* 2011; **30**: 366–78.
15 Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology* 2012; **55**: 666–75.
16 Yoneda S, Umemura T, Joshita S *et al.* Serum chemokine levels are associated with the outcome of pegylated interferon and ribavirin therapy in patients with chronic hepatitis C. *Hepatol Res* 2011; **41**: 587–93.
17 Polyak SJ, Khabar KS, Rezeiq M, Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* 2001; **75**: 6209–11.
18 Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; **33**: 708–12.
19 Butera D, Marukian S, Iwamaye AE *et al.* Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 2005; **106**: 1175–82.

20 Romero AI, Lagging M, Westin J *et al.* Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J Infect Dis* 2006; **194**: 895–903.
21 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 8–13.
22 Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM. Genomic organization and transcriptional regulation of the RANTES chemokine gene. *J Immunol* 1993; **151**: 2601–12.
23 Schall TJ, Jongstra J, Dyer BJ *et al.* A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988; **141**: 1018–25.
24 Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000; **12**: 121–7.
25 Nattermann J, Nischalke HD, Feldmann G, Ahlenstiel G, Sauerbruch T, Spengler U. Binding of HCV E2 to CD81 induces RANTES secretion and internalization of CC chemokine receptor 5. *J Viral Hepat* 2004; **11**: 519–26.
26 Kaukinen P, Sillanpaa M, Kotenko S *et al.* Hepatitis C virus NS2 and NS3/4A proteins are potent inhibitors of host cell cytokine/chemokine gene expression. *Virol J* 2006; **3**: 66.
27 Ruggieri A, Franco M, Gatto I, Kumar A, Rapicetta M. Modulation of RANTES expression by HCV core protein in liver derived cell lines. *BMC Gastroenterol* 2007; **7**: 21.
28 Jang Y, Chae JS, Hyun YJ *et al.* The RANTES −403G > A promoter polymorphism in Korean men: association with serum RANTES concentration and coronary artery disease. *Clin Sci (Lond)* 2007; **113**: 349–56.
29 Zhernakova A, Alizadeh BZ, Eerligh P *et al.* Genetic variants of RANTES are associated with serum RANTES level and protection for type 1 diabetes. *Genes Immun* 2006; **7**: 544–9.
30 Promrat K, McDermott DH, Gonzalez CM *et al.* Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* 2003; **124**: 352–60.

Journal of Medical Virology 85:449–458 (2013)

Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

Masayuki Kurosaki,[1] Yasuhito Tanaka,[2] Nao Nishida,[3] Naoya Sakamoto,[4] Nobuyuki Enomoto,[5] Kentaro Matsuura,[2] Yasuhiro Asahina,[6] Mina Nakagawa,[6] Mamoru Watanabe,[6] Minoru Sakamoto,[5] Shinya Maekawa,[5] Katsushi Tokunaga,[3] Masashi Mizokami,[7] and Namiki Izumi[1]*

[1]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[2]*Department of Virology, Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[4]*Department of Gastroenterology and Hematology, Hokkaido University, Sapporo, Japan*
[5]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan*
[6]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
[7]*Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan*

This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.
Conflicts of interest and financial disclosures: None reported.

*Correspondence to: Namiki Izumi, MD, PhD, Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. E-mail: nizumi@musashino.jrc.or.jp

Accepted 19 November 2012
DOI 10.1002/jmv.23497
Published online 7 January 2013 in Wiley Online Library (wileyonlinelibrary.com).

have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: for males, CLcr = [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl) and for females, CLcr = 0.85 × [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis