data, virological data, histological data, and treatment details were collected at enrollment. Age was determined at primary liver biopsy. Patients were examined for HCC with abdominal ultrasonography, dynamic computed tomography, and/or magnetic resonance imaging every 3-6 months. Serum alpha-fetoprotein (AFP) levels were measured every 1-2 months. This screening program constitutes the standard of care in Japan. To evaluate the effect of interferon-induced AFP reduction on hepatocarcinogenesis, the average AFP level after interferon treatment was calculated in each patient. HCC diagnosis was confirmed with needle biopsy, surgically resected specimens, or typical radiological findings diagnosed by board-certified radiologists. Figure 1 shows the schema for patient follow-up and clinical outcomes.

The start date of follow-up was the date of primary liver biopsy and the endpoint of follow-up was the development of HCC or the latest medical attendance until January 2009. The mean follow-up period was 7.5 years (range 0.5-17 years). The factors associated with development of HCC were retrospectively analyzed.

Change in Fibrosis Staging Over Time. To evaluate change in fibrosis staging over time, 271 patients who had not achieved a sustained virological response (SVR) with interferon therapy underwent a sequential biopsy after the initial biopsy. The interval between the paired biopsies was on average 4.8 years (range 0.7-14 years). The yearly rate of progression of fibrosis was calculated as the change in fibrosis staging divided by the time between paired biopsies.

Statistical Analysis. Categorical data were compared by the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed with Student's t test or the Mann-Whitney U test for two groups. All tests of significance were two-tailed and a P value of <0.05 was considered statistically significant. The cumulative incidence curve was determined with the Kaplan-Meier method and differences among groups were assessed using the log-rank test. Factors associated with HCC risk and virological response to interferon therapy were determined by the Cox proportional hazard model and logistic regression analysis, respectively. To depict the role of aging in developing risk for HCC, the multivariate Cox proportional hazard model was used after adjusting for stage of liver fibrosis, steatosis, and virological response to interferon. A polynomial regression was used to fit risk ratios for segments of the age distribution. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL).

Results

Patient Characteristics. Patient characteristics at the time of enrollment are shown in Table 1. The distribution of stages of liver fibrosis differed between younger and older patients, indicating the need to adjust for stage of liver fibrosis when comparing the two subgroups.

Response to Interferon Therapy. The response to interferon therapy was determined in 2042 (97.2%) of the interferon-treated patients, excluding those who received prolonged interferon treatment at the endpoint. SVR rates are shown in Table 1. The percentage of patients showing SVR was significantly lower in older patients (≥65 years) than in younger patients (<65 years) ($P < 0.001$). Overall response rates to the different types of interferon therapy were as follows: interferon monotherapy, 31.5% (312/992); interferon-alpha and ribavirin combination therapy, 28.6% (108/378); pegylated interferon-alpha monotherapy, 37.9% (108/285); and pegylated interferon-alpha and ribavirin combination therapy, 41.1% (159/387). Response rates in genotype-1 patients (n = 1347) were 20.6% (114/554), 17.9% (29/162), 18.9% (56/297), and 36.8% (123/334), and those in nongenotype-1 patients (n = 565) were 52.2% (163/312), 63.1% (77/122), 65.0% (52/80), and 70.6% (36/51). Overall response rates of interferon and pegylated interferon monotherapy seem to be high because of the high response rates in the nongenotype-1 patients treated with these regimens.

Overall Cumulative Incidence of HCC. During follow-up, HCC developed in 177 interferon-treated patients (Fig. 1). The cumulative incidence of HCC 5, 10, and 15 years after interferon therapy was 4.7%, 11.6%, and 15.5%, respectively. The cumulative incidence in SVR patients was 2.1%, 4.3%, and 4.3%, respectively, which was significantly lower than that in non-SVR patients (5.8%, 14.9%, and 20.2%, respectively; log-rank test, $P < 0.001$).

Effect of Aging on Risk for HCC. The risk ratio determined by multivariate Cox proportional hazards analysis after adjustment for stage of liver fibrosis, degree of liver steatosis, and virological response to interferon demonstrated that the risk for HCC after interferon treatment was age-dependent and increased predominantly when the age at primary liver biopsy was >65 years (Fig. 2A). Hence, we defined older patients as those ≥65 years of age at primary liver biopsy and younger patients as those aged <65 years. As shown in Fig. 2B, the cumulative incidence of HCC was significantly higher in older patients than in younger patients (log-rank test, $P < 0.001$).

Table 1. Characteristics of Patients Enrolled in the Present Study

Characteristics	Total	<65 year	≥65 year	*P* Value*
Patients, n	2166	1614	552	
Sex, n (%)				<0.001†
Male	1080 (49.9)	840 (52.0)	240 (43.6)	
Female	1086 (50.1)	774 (48.0)	312 (56.4)	
Age (SD), year	55.4 (12.1)	51.1 (10.8)	68.4 (2.9)	<0.001‡
BMI (SD), kg/m^2	23.3 (3.1)	23.4 (3.0)	23.3 (3.1)	0.9‡
Fibrosis stage, n (%)				<0.001†
F0	27 (1.3)	24 (1.5)	3 (0.5)	
F1	860 (39.7)	704 (43.6)	156 (28.2)	
F2	733 (33.8)	515 (31.9)	218 (39.5)	
F3	444 (20.5)	301 (18.6)	143 (25.9)	
F4	102 (4.7)	70 (4.3)	32 (5.8)	
%Severe steatosis (≥10%)	27.6	27.1	29.3	0.4†
ALT level (SD), IU/L	95 (18)	101 (119)	76 (58)	<0.001‡
HCV load (SD), KIU/mL	880 (1046)	861 (1016)	924 (1116)	0.2‡
HCV genotype, n (%)				<0.001†
1a	7 (0.3)	5 (0.3)	2 (0.4)	
1b	1414 (69.6)	1036 (68.9)	378 (71.3)	
2a	373 (18.3)	273 (18.2)	100 (18.9)	
2b	211 (10.4)	164 (10.9)	47 (8.9)	
Others	28 (1.4)	25 (1.7)	3 (0.6)	
Duration (SD), year	7.5 (4.4)	8.1 (4.4)	5.8 (3.7)	<0.001‡
IFN regimen, n (%)				<0.001†
IFN mono	1062 (49.0)	833 (51.6)	229 (41.5)	
PEG-IFN mono	306 (14.1)	200 (12.4)	106 (19.2)	
IFN + RBV	386 (17.8)	291 (18.0)	95 (17.2)	
PEG-IFN + RBV	412 (19.0)	290 (18.0)	122 (22.1)	
SVR, n (%)	686 (33.6)§	565 (36.6)‖	121 (24.3)¶	<0.001‡

Unless otherwise indicated, data are given as the mean (SD).

ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; N/A, not applicable; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

*Comparison between <65 years and ≥65 years.

†Chi-squared test.

‡Student *t* test.

§Virological responses were determined in 2042 patients.

‖Virological responses were determined in 1545 patients.

¶Virological responses were determined in 497 patients.

As shown in Fig. 2C-E, even when stratified by stage of fibrosis the cumulative incidences among patients at stages F0/F1, F2, and F3 were significantly greater in older patients than in younger patients (log-rank test, $P < 0.001$). These differences were not significant among patients with cirrhosis (Fig. 2F, log-rank test, $P = 0.7$).

The annual incidence of HCC after interferon treatment was calculated by the person-years method (Table 2); it increased with the degree of liver fibrosis from 0.2% (F0 or F1) to 4.6% (F4) and was higher among older patients at the same stage of liver fibrosis.

Among the 177 patients with HCC, 92 showed evidence of a single blood transfusion. We analyzed the relationship between duration of infection and age in these 92 patients. A significant and strong negative correlation was found between the interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion ($r = -0.74$, $P < 0.001$) (Fig. 3A). The mean duration of chronic infection was 22.0 years in patients who had received blood transfusion at >40 years of age, which was significantly shorter than that in patients who received it at ≤40 years of age (40.6 years, $P < 0.001$).

The presence of cirrhosis at the time of development of HCC, which was defined as having any of the following criteria, was evaluated: (1) histological evidence for cirrhosis, (2) findings of cirrhosis in any radiological study, or (3) presence of marked portal hypertension (i.e., presence of esophagogastric varices). Following this, 142 of the 177 with HCC (80.2%) were diagnosed as having cirrhosis, of which 42 were diagnosed histologically, 69 radiologically, and 31 based on the presence of marked portal hypertension. No significant difference was found in the proportion of patients with cirrhosis between older and younger patients, at the rate of 78.3% (94/120) in older

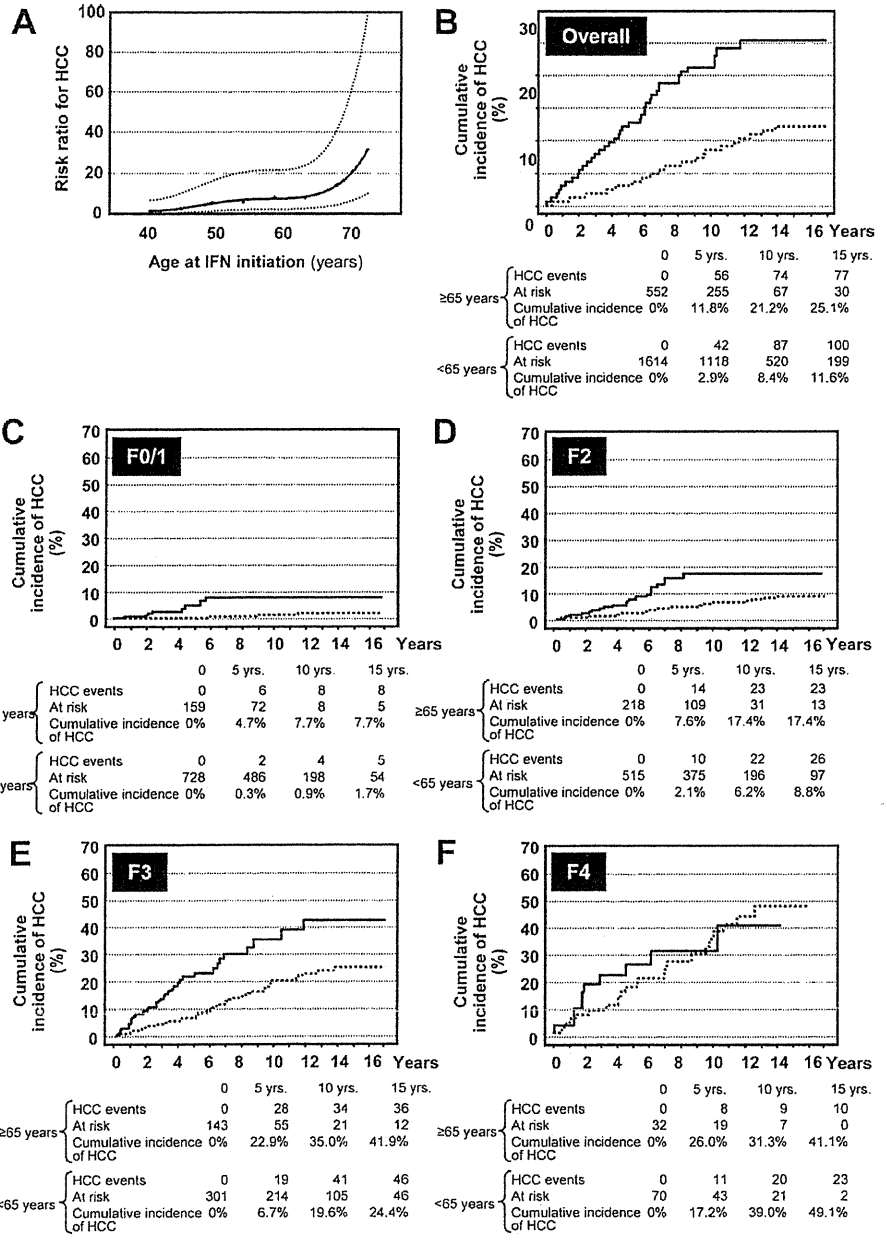

Fig. 2. Effect of aging on the risk for HCC. (A) Risk ratio (solid line) and 95% CI (dotted lines) for the risk of HCC according to age. To show the age-dependent relationship, a multivariate Cox proportional hazard model was used after adjustment for gender, stage of liver fibrosis, body mass index, and virological response to interferon therapy. Curves were fitted using polynomial regression. (B-F) Cumulative incidence of HCC after interferon therapy among younger (<65 years, n = 552, dotted line) and older patients (≥65 years, n = 1614, solid line). (B) Overall data, $P < 0.001$. (C) Patients with stage F0 or F1 liver fibrosis (no or mild fibrosis with portal expansion), $P < 0.001$. (D) Patients with stage F2 liver fibrosis (bridging fibrosis without architectural distortion), $P < 0.001$. (E) Patients with stage F3 liver fibrosis (bridging fibrosis with architectural distortion), $P < 0.001$. (F) Patients with stage F4 liver fibrosis (cirrhosis), $P = 0.7$. All P values were obtained by the log-rank test. The numbers of HCC events and patients at risk at each timepoint are shown below the graphs.

patients and 84.2% (48/57) in younger patients ($P = 0.36$, comparison at the age of HCC development).

Influence of Aging on Progression in Fibrosis Staging Over Time. In 271 patients who underwent paired biopsies, fibrosis staging progressed in 69 patients (25.5%), remained unchanged in 154 (56.8%), and regressed in 48 patients (17.7%). The overall rate of progression of fibrosis in these patients was 0.06 ± 0.02 fibrosis stages per year. Progression of fibrosis over time was significantly accelerated in older patients than in younger patients (0.21 ± 0.10 versus 0.03 ± 0.21 fibrosis stages per year, $P = 0.03$, Mann-Whitney U test) (Fig. 3B).

Effect of Viral Eradication on Risk for HCC in Older Patients. As shown in Fig. 4, the effect of viral eradication on the prevention of HCC was less significant in older patients than in younger patients. The annual incidence was higher among older patients than among younger patients with the same virological response (Table 2).

Influence of Liver Steatosis on Risk for HCC. The cumulative incidence of HCC after interferon therapy was significantly higher in patients with severe steatosis (≥10%) than in those with milder steatosis (at 5, 10, and 15 years: 8.6%, 19.1%, 32.0% versus 1.8%, 4.8%, 7.0%, respectively, log-rank test, $P < 0.001$).

Table 2. Annual Incidence of HCC After IFN Treatment

Factors	Total	<65 Years	≥65 Years
Fibrosis stage			
F0/F1	0.2%	0.1%	0.9%
F2	0.8%	0.6%	1.7%
F3	2.5%	1.8%	4.6%
F4	4.6%	4.4%	5.1%
Total	1.1%	0.8%	2.4%
Degree of liver steatosis			
<10%	0.5%	0.2%	1.4%
≥10%	2.0%	1.8%	3.0%
Virological response			
SVR	0.4%	0.2%	1.3%
Non-SVR	1.4%	1.0%	2.9%

Data were calculated by the person-years method. IFN, interferon; SVR, sustained virological response.

The annual incidence was higher in older patients than in younger patients with the same degree of liver steatosis (Table 2). In patients with severe steatosis (≥10%), superimposed NASH was diagnosed in 6.0% (26/435). Overall, superimposed NASH was significantly associated with hepatocarcinogenesis on univariate analysis (risk ratio, 4.1; 95% confidence interval [CI], 1.8-9.4; $P < 0.001$), but not on multivariate analysis. Superimposed NASH was significantly associated with high body mass index (27.2 ± 4.6 kg/m^2 versus 23.0 ± 3.1 kg/m^2, $P < 0.001$), hyperglycemia (186 ± 67 mg/dL versus 115 ± 39 mg/dL, $P < 0.001$), and advanced fibrosis (F3) (risk ratio, 2.9; 95% CI, 1.4-6.0; $P = 0.005$).

Factors Associated with Hepatocarcinogenesis After Interferon Therapy. Univariate analysis demonstrated factors that increase the risk ratio for the development of HCC (Table 3). Multivariate analysis using Cox proportional hazards regression confirmed that aging was one of the most significant independent factors associated with the development of HCC after interferon therapy. In this analysis, advanced fibrosis, presence of steatosis, male gender, lower total cholesterol level, higher fasting blood sugar level, higher baseline AFP level, insignificant improvement of mean AFP level after interferon therapy, and nonresponse to interferon therapy were also significantly associated with risk for HCC (Table 3).

We identified 22 patients in whom HCC developed even after achieving SVR. Univariate and multivariate logistic regression analyses indicated that both liver steatosis and aging were independently associated with the development of HCC among patients who achieved SVR (n = 686) (Table 4). Anti-HBc was detected in only 4 out of 22 patients and the age distribution was similar among anti-HBc-positive and anti-HBc-negative patients.

Response to Interferon Therapy in Older Patients. Multivariate logistic regression analysis confirmed that aging, female gender, severe liver fibrosis, extremely severe liver steatosis, genotype-1, high HCV load, and nonuse of pegylated interferon and ribavirin were independent risk factors for non-SVR (Supporting Table 1). The odds ratio, determined by multivariate logistic regression analysis after adjustment for these factors, demonstrated that the risk for non-SVR was age-dependent (Supporting Fig. 1). It was also ≈2.5 times higher in patients aged ≥65 years than in those aged <35 years.

In patients with genotype-1b and a high viral load who were treated with pegylated interferon and ribavirin combination therapy, the SVR rate was significantly lower in older patients than in younger patients (<49 years, 59.3%; 50-59 years, 50.5%; 60-65 years, 27.3%; ≥65 years, 25.2%; intention-to-treat analysis). Multivariate logistic regression analysis showed that

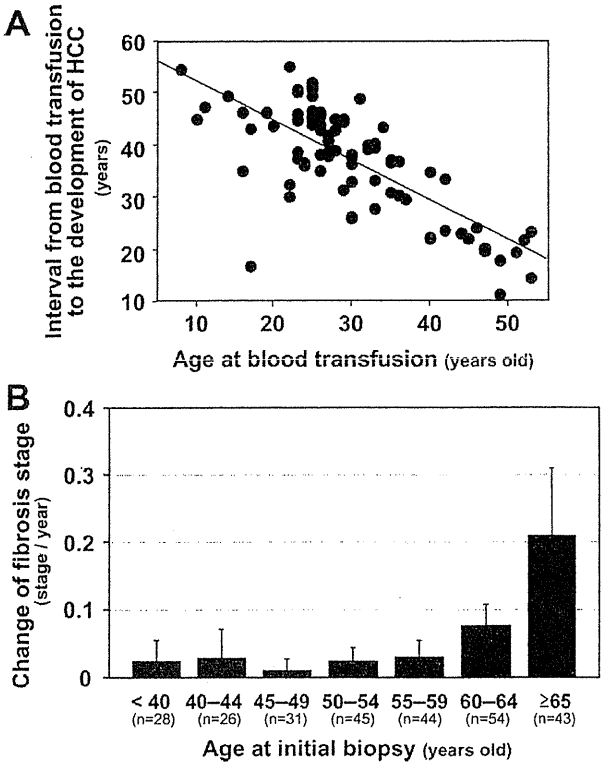

Fig. 3. (A) Relationship between the interval from blood transfusion to development of HCC and the age at blood transfusion (n = 92). A significant and strong negative correlation was observed ($r = -0.74$, $P < 0.001$). (B) Change in fibrosis staging over time. A total of 271 patients who had not achieved SVR by interferon therapy underwent a sequential biopsy after the initial biopsy. The yearly rate of progression of fibrosis was calculated as the change in fibrosis stage divided by the time between the paired biopsies. The yearly rate of progression of fibrosis was significantly higher in older patients (≥65 years) than in younger patients (<65 years) ($P = 0.03$, Mann-Whitney U test).

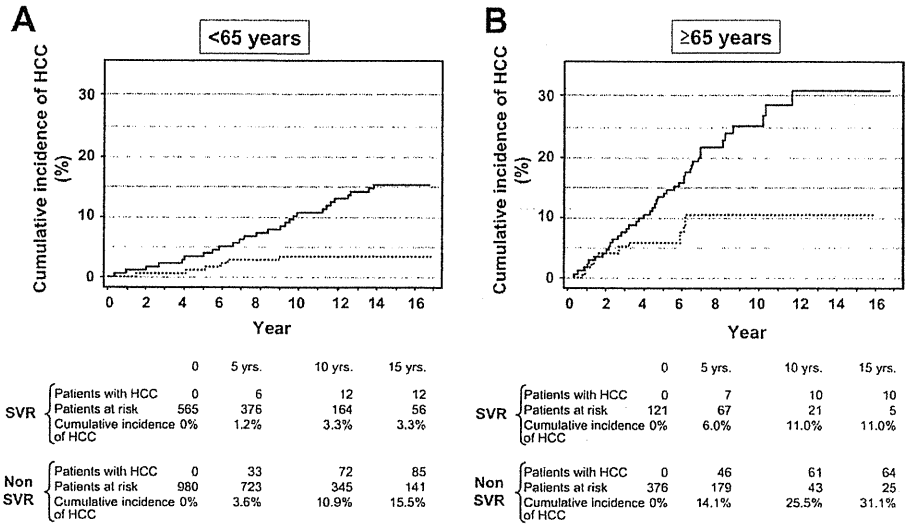

Fig. 4. Cumulative incidence of HCC after interferon therapy among SVRs (dotted lines) and non-SVRs (solid lines) according to age. (A) Younger patients (<65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P < 0.001$). (B) Older patients (≥65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P = 0.02$). However, the difference between SVR and non-SVR was less in older patients than in younger patients. The number of HCC events and patients at risk at each timepoint are shown below the graphs.

aging was the strongest independent factor contributing to SVR in these patients (data not shown). The odds ratio for the risk of non-SVR was 1.8 for each additional 10 years of age (95% CI, 1.5-2.3, $P < 0.001$).

Discussion

In this large cohort study we demonstrated that aging is significantly associated with the development of HCC in patients treated with interferon. The risk ratio increased predominantly in patients older than 65 years, which was more than 15 times that in patients in their 20s. Aging is becoming the most critical risk factor for the development of HCC. Although liver fibrosis was also an important risk factor, we clearly demonstrated that the risk for hepatocarcinogenesis after interferon treatment was significantly higher in older patients at each stage of liver fibrosis except for cirrhosis. Hence, physicians should be aware that older patients can develop HCC regardless of the stage of fibrosis.

Because the present study included a large cohort, it was difficult to determine the duration of infection in all patients, and this might have affected the risk determination for HCC development. Therefore, we analyzed the relationship between duration of chronic infection and HCC development in patients who underwent a single blood transfusion. We found a significant and strong negative correlation between the interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion. Consistent with our results, a previous report with posttransfusion HCV demonstrated that the age of patients, rather than the duration of HCV infection, was more significant for HCC development.[14-16] Therefore, older age and not duration of infection is more likely to influence hepatocarcinogenesis. Moreover, our analysis of sequential biopsy specimens demonstrated that the progression rate of liver fibrosis significantly accelerated in patients aged >65 years. Hence, the progression of fibrosis along with aging may also contribute to the increased risk for hepatocarcinogenesis in older patients.

We further demonstrated that liver steatosis was an independent risk factor for the development of HCC, which was not mentioned in previous reports.[8-11] The presence of steatosis is related to both viral (genotype-3 or HCV core protein) and host metabolic factors.[17,18] In our cohort, most superimposed NASH was associated with host metabolic factors such as high body mass index and hyperglycemia, whereas infection of genotype-3 was only noted in two patients. In vitro experiments have suggested an association between liver steatosis induced by HCV core protein and hepatocarcinogenesis,[19] and have proposed virus-associated steatohepatitis as a new aspect of chronic hepatitis C.[20,21] Because steatosis was likely to be related to hepatocarcinogenesis, patients with chronic hepatitis C, whose liver histology shows superimposed NASH,

Table 3. Factors Associated with HCC After IFN Therapy

	Univariate Analysis		Multivariate Analysis	
Risk Factor Value	Risk Ratio (95% CI)	*P* Value	Risk Ratio (95% CI)	*P* Value
Age (by every 10 year)	2.2 (1.8-2.7)	<0.001	3.0 (1.9-4.8)	<0.001
Sex				
Female	1		1	
Male	1.2 (0.9-1.6)	0.2	2.0 (1.0-3.8)	0.04
BMI (by every 10 kg/m^2)	2.0 (1.2-1.3)	0.005	1.1 (0.4-3.5)	0.8
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	5.4 (3.9-7.5)	<0.001	2.5 (1.2-4.9)	0.01
Degree of steatosis				
<10%	1		1	
≥10%	4.5 (3.0-6.9)	<0.001	3.5 (1.9-6.4)	<0.001
Esophagogastric varices				
No	1		1	
Yes	3.3 (2.0-5.3)	<0.001	1.6 (0.6-4.4)	0.3
Virological response				
SVR	1		1	
Non-SVR	3.3 (2.1-5.2)	<0.001	2.6 (1.2-5.5)	0.001
Genotype				
Non-1	1		1	
1	1.7 (1.2-2.5)	0.006	1.0 (0.5-2.3)	0.9
Albumin (by every 1 g/dL)	0.2 (0.1-0.3)	<0.001	0.6 (0.2-2.2)	0.3
ALT (by every 100 IU/L)	1.0 (0.9-1.0)	0.8	0.4 (0.1-1.8)	0.6
AST (by every 100 IU/L)	1.2 (1.1-1.3)	0.001	1.1 (0.6-1.8)	0.8
γ-GTP (by every 100 IU/L)	1.3 (1.1-1.6)	0.009	0.6 (0.3-1.6)	0.3
ALP (by every 100 IU/L)	1.3 (1.2-1.5)	<0.001	0.6 (0.3-1.2)	0.2
Total bilirubin (by every 1 mg/dL)	1.6 (1.3-2.1)	<0.001	1.2 (0.6-2.7)	0.6
Total cholesterol (by every 100 mg/dL)	0.3 (0.2-0.6)	<0.001	0.2 (0.1-0.6)	0.006
Triglyceride (by every 100 mg/dL)	0.8 (0.5-1.1)	0.2	0.1 (0.02-1.1)	0.08
Fasting blood sugar (by every 100 mg/dL)	1.8 (1.5-2.2)	<0.001	1.1 (1.0-1.1)	0.04
WBC (by every 100/μL)	0.1 (0.03-0.3)	<0.001	0.1 (0.01-2.2)	0.2
RBC (by every 10^6/μL)	0.5 (0.4-0.7)	<0.001	1.8 (0.7-4.4)	0.2
Platelet counts (by every 10^6/μL)	0.3 (0.2-0.4)	<0.001	0.6 (0.3-1.5)	0.3
Baseline AFP (by every 10 ng/mL)	1.0 (0.9-1.1)	0.2	1.3 (1.0-1.7)	0.04
Post IFN AFP (by every 10 ng/mL)	1.2 (1.1-1.3)	<0.001	1.9 (1.5-2.4)	<0.001
HCV load (by every 100 KIU/mL)	1.0 (0.9-1.0)	0.4	1.0 (1.0-1.1)	0.06
IFN regimen				
IFN monotherapy	1		1	
IFN + RBV (24 W)	1.2 (0.8-1.8)	0.4	1.5 (0.7-3.2)	0.3
PEG-IFN monotherapy (48 W)	1.1 (0.6-1.9)	0.8	1.5 (0.4-5.5)	0.6
PEG-IFN + RBV	0.4 (0.2-0.9)	0.03	1.0 (0.3-3.1)	0.9

Risk ratios for development of HCC were calculated by Cox proportional hazards regression analysis. AFP, alpha fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspirate aminotransferase; BMI, body mass index; γ-GTP, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; IFN, interferon; PEG, pegylated; RBC, red blood cell counts; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell count.

may be at a higher risk of developing HCC. Further study is necessary to confirm this association in a clinical situation. Because several developed countries are in the midst of a growing obesity epidemic, the risk related to obesity cannot be ignored in patients with chronic hepatitis C who are treated with interferon.

Several retrospective cohort studies have been conducted to evaluate the effect of interferon on the incidence of HCC among patients with chronic hepatitis C.[8-11] Our results, obtained from one of the largest cohort studies, confirm the efficacy of viral eradication in preventing HCC. In one study conducted in a Western population, no statistically significant reduction was found in the development of HCC among patients with SVR compared with those without SVR (adjusted hazard ratio, 0.46; 95% CI, 0.12-1.70; $P = 0.25$).[12] Because relatively few occurrences of HCC were observed in this cohort, and the duration of follow-up was shorter, the differences in HCC development between patients with and without SVR might be less pronounced.

Interestingly, our results demonstrated that the risk for HCC remains even after achieving SVR in older patients, confirming the findings of previous studies conducted with a smaller number of patients.[22,23] The cumulative incidence of HCC during the first 5 years

Table 4. Factors Associated with Development of HCC After Achieving SVR

Risk Factor	Odds Ratio (95% CI)	*P*-value
Univariate analysis		
Age (by every 10 year)	3.2 (1.8-5.5)	<0.001
Sex		
Female	1	
Male	3.0 (1.0-8.8)	0.04
Fibrosis stage		
F0/F1/F2	1	
F3/F4	5.9 (2.5-14.0)	<0.001
Degree of steatosis		
<10%	1	
≥10%	5.5 (2.0-15.2)	0.001
BMI (by every 10 kg/m^2)	3.2 (0.8-12.6)	0.09
ALT (by every 10 IU/L)	0.9 (0.7-1.3)	0.7
AST (by every 10 IU/L)	1.1 (0.9-1.4)	0.3
Genotype		
Non-1	1	
1	1.2 (0.6-3.0)	0.5
HCV load (by every 100 KIU/mL)	0.9 (0.8-1.0)	0.2
IFN regimen		
IFN monotherapy	1	
IFN + RBV (24 W)	0.7 (0.2-2.3)	0.5
PEG-IFN monotherapy (48 W)	0.8 (0.2-3.6)	0.8
PEG-IFN + RBV	0.3 (0.03-2.0)	0.2
Multivariate analysis		
Age (by every 10 year)	2.7 (1.5-5.1)	0.002
Sex		
Female	1	
Male	4.1 (0.9-18.9)	0.06
Fibrosis stage		
F0/F1/F2	1	
F3/F4	2.6 (0.9-7.5)	0.08
Degree of steatosis		
<10%	1	
≥10%	5.6 (1.9-16.5)	0.002

Odds ratios for SVR were calculated by logistic regression analysis.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

after completion of interferon therapy was similar between SVR and non-SVR patients in the older age group, and the risk for HCC remained for 9 years after eradication of HCV in our patients. Therefore, HCC patients with SVR who have a risk factor should be screened for at least 5-10 years after the completion of interferon therapy.

It has been reported that coffee consumption has a protective effect against hepatocarcinogenesis[24,25] and liver disease progression in patients with chronic HCV infection.[26] Because we could not review coffee consumption in all the patients and fewer data were available in the previous literature as to whether a habitual change of reducing coffee consumption occurs in older patients, it is unclear whether increased risk for HCC in older patients is an effect of this habitual change in older patients. However, the majority (68%) of Japanese patients who have HCV (n = 1058) drink less than 1 cup of coffee per day, and only 7.6% consume more than 3 cups of coffee per day.[27] Therefore, it is unlikely that a habitual change in older patients affects the increased risk for hepatocarcinogenesis in older patients.

Recently, it was reported that interferon therapy might be less effective in preventing HCC among patients with chronic hepatitis C who are positive for anti-HBc antibody,[28] but this finding is still controversial.[29,30] In the present study, anti-HBc was only detected in 4 of 22 patients in whom HCC developed after viral eradication, and age distribution was similar among anti-HBc-positive and anti-HBc-negative patients. Because no significant difference in mean age was found between anti-HBc-positive and anti-HBc-negative patients in the recent study conducted in Japan,[28] it is unlikely that previous exposure to hepatitis B virus or occult hepatitis B virus infection is responsible for the difference in risk for HCC between younger and elderly patients found in the present study.

In conclusion, aging has become one of the most important risk factors for HCC. Even after stratification by stage of fibrosis, the risk for HCC after antiviral treatment was significantly higher in older patients, and HCV eradication had a smaller effect on HCC-free survival in older patients. Patients with HCV should therefore be identified at an earlier age and antiviral treatment should be initiated. The present results have potentially important clinical implications for physicians that may influence their decisions about the treatment strategy in individual patients.

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Original Article

Simple formula to predict response to peginterferon alpha2b and ribavirin combination therapy in genotype 1 chronic hepatitis C patients with high viral loads

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Aim: We advocate a simple formula which can conveniently predict the outcome of Peg-interferon (IFN) alpha2b and ribavirin (RBV) combination therapy for genotype 1 chronic hepatitis C (CH-C) with high viral load.

Methods: A total of 338 (group A: 230, Group B: 108) genotype 1 CH-C patients treated with Peg-IFN alfa-2b and RBV were enrolled. Clinical parameters differing significantly between sustained virological responders (SVRs) and non-SVRs in group A were categorized, then a simple formula to predict SVR was constructed and re-evaluated in group B. Another formula containing hepatitis C virus amino acid mutations/substitutions also was constructed.

Results: In group A, gender and HCV RNA load <1000 KIU were significant predictors of SVR by multivariate logistic regression analysis. A simple formula was constructed (formula A): male gender (point 2) + HCV RNA load <1000 KIU (3) + platelet counts $\geq 15 \times 10^4/mm^3$ (1) + age <60 (1). In group A, score (0–1) predicted SVR rate 23.8% (2–4): 48.1% and (5–7): 70.2%. According to this formula, score (0–1) predicted SVR rate 7.1% (2–4): 38.6%, and (5–7): 70.3% in group B. Information on HCV amino acid mutations/substitutions seemed to add some accuracy.

Conclusions: This simple formula can be used to roughly determine, at the patients' first/second visit, the probability of response to Peg-IFN alpha2b and RBV combination therapy for genotype 1 CH-C with high viral load.

Key words: chronic hepatitis C, genotype 1b, peginterferon and ribavirin combination therapy, predictive formula.

INTRODUCTION

WITH THE ADVANCES in antiviral therapy for chronic hepatitis C (CH-C), around 50% of genotype 1 patients with high hepatitis C virus (HCV) RNA loads can now be cured by peginterferon (Peg-IFN)/ribavirin (RBV) combination therapy.[1,2] However, in Japan the majority of patients with CH-C are relatively old[3,4] and IFN based antiviral therapy sometimes cannot be completed because of adverse effects,[5] which suggests to us the need to identify before treatment the patients highly likely or unlikely to be cured by the combination therapy.

A simple and convenient formula to predict the likelihood of cure before starting treatment is recommended to establish effective Peg-IFN and RBV combination therapy for genotype 1 CH-C, because a substantial proportion of CH-C patients are not followed by experts in clinical hepatology without antiviral therapy.

A previous paper reported that a logistic regression model, including mutations in the interferon sensitivity determining region (ISDR) in the nonstructural protein

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5A (NS5A) region of HCV, T helper type 1/T helper type 2 balance, body weight and neutrophil count, is useful for predicting accurately the likelihood of SVR before starting therapy.[6] Recently, prediction of SVR has been achieved using another formula containing the on-treatment laboratory data.[7] Although these formulae are superior in accuracy, the necessity for laboratory work or complicated calculations hamper their use in clinical practice.

In this study, we set out to construct a simple formula, based on pretreatment clinical data, which can be used to determine conveniently at the patients' first visit the probability of response to Peg-IFN and RBV combination therapy for genotype 1 CH-C with high viral load.

SUBJECTS AND METHODS

Patients

THIS STUDY WAS conducted at University Hospital of Kyoto Prefectural University of Medicine, Kyoto, Osaka University, Osaka, Ehime University, Ehime, Japan and related hospitals. Enrollment of the patients was started in January 2006 and ended in July 2008, and the follow up study was completed in January 2009. Among the patients with genotype 1 CH-C who had high viral loads (Amplicor HCV RNA kit, version 2.0; Roche Diagnostics, Tokyo) and completed the course of Peg-IFN alpha2b and RBV combination therapy for 48 weeks, 370 patients, aged 23 to 73 years, were enrolled. Two hundred and thirty patients were randomly assigned to group A. Among the remaining140 patients, 108 patients whose amino acid substitution of HCV core 70 and mutations in the ISDR were determined were assigned to group B.

Patients with decompensated liver disease, co-infection with hepatitis B virus or human immunodeficiency virus, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis and Wilson's disease were excluded by liver biopsy before treatment or by appropriate serological/biochemical data. Patients with uncontrollable hypertension or diabetes mellitus and those with a history of heavy alcohol drinking also were excluded.

Study design

All patients received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg.bw and oral administration of RBV (Rebetol; Shering-Plough) of 600 to 1000 mg/day. The amount of RBV was adjusted based on the body weight; (600 mg for <60 kg.bw, 800 mg for ≥60 kg.bw and <80 kg.bw, 1000 mg for ≥80 kg.bw. The dose of PEG-IFN-α-2b was decreased by 50% when platelet counts was below 8×10^4 /mm^3 or the neutrophil counts was below 750 /mm^3. The dose of RBV was lowered by 200 mg/day when the hemoglobin concentration fell below 10 g/dL. The full dose regimen was re-started when the adverse events improved. Written informed consent was obtained from all patients before treatment and this study was approved in 2005 by the ethical committee of the university.

Determination of HCV core amino acids 70 and the interferon sensitivity determining region (ISDR)

Frozen serum samples obtained before the commencement of therapy were stored at −80 °C for the analyses of amino acid substitutions in HCV core 70 and mutations in ISDR in NS5A. The sequences corresponding to amino acids 1–191 (HCV core) and amino acids 2209–2248 (ISDR) were analyzed by direct sequencing, as described by Akuta *et al.*[8,9] and Enomoto *et al.*[10] Briefly, after total RNA was extracted from the sera and converted into cDNA, first and second round polymerase chain reactions (PCRs) were performed. Primers used in the PCR were as follows. (i) For the core region: the first-round PCR was performed with CC11 (sense, 5′-GCC ATA DTD GTC TGC GGA ATG-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (ii) For the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers (hemi-nested PCR). The amplicons were sequenced and the sequences were compared with the consensus sequence of genotype 1b (HCV-J).[11] Amino acids 70 were arginine in the wild type and glutamine/histidine in the mutant.

Statistical analysis

All data analyses were conducted using the Statistical Package (SPSS). Individual characteristics between groups were evaluated by means of the Mann-Whitney *U*-test. Variables exhibiting statistical significance ($P < 0.05$) in the univariate analysis were subjected to multivariate logistic regression analysis. Multivariate logistic regression analysis with stepwise method was used to investigate the multivariate association of SVR

Table 1 Clinical background of the 230 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (group A). Data are compared between SVR and non-SVR patients by Mann-Whitney *U*-test

	SVR	Non-SVR	*P* value
Gender (male/female)	75/38	56/61	0.005
Age	54 (25–73)	57 (27–73)	0.010
HCV RNA (KIU/mL)	1 500 (100–>5 000)	2 000 (139–>5 000)	0.015
Hb (g/dL)	14.5 (10.7–18.1)	14.1 (11.9–20.2)	0.011
PLT($\times 10^4$/μL)	18.1 (6.2–36.6)	16.1 (7.1–30.1)	0.001
WBC (/μL)	5 200 (2 300–11 000)	4 900 (2 600–11 000)	0.077
Neutrophil (/μL)	2 652 (1 071–7 040)	2 511 (524–6 457)	0.424
ALT (IU/L)	71 (15–740)	60.5 (17–298)	0.143
LDH (IU/L)	194.5 (122–425)	193.5 (113–472)	0.956
ALP (IU/L)	248 (82–620)	261 (55–897)	0.575
γGTP (IU/L)	43 (5–282)	45 (10–501)	0.151
T-Chol (mg/dL)	170 (82–294)	174 (101–249)	0.422
TG (mg/dL)	89 (45–296)	96 (37–395)	0.260
Ferritin (mg/dL)	140.0 (7.3–1491.1)	164.3 (19.0–949.8)	0.161
Hyaluronate (ng/dL)	55 (9–555)	63 (9–694)	0.197

P-value <0.05 was considered to be statistically significant.

with clinical background. All *P*-values of $P < 0.05$ by the two-tailed test were considered statistically significant.

RESULTS

Baseline laboratory data of the patients and construction of a simple and convenient formula to predict the response to peginterferon alpha2b and ribavirin combination therapy

THE BASELINE CHARACTERISTICS of 230 group A patients with genotype 1 CH-C were compared between those with SVR and non-SVR (Table 1). The SVR patients were significantly more often male ($P = 0.005$), younger ($P = 0.010$), had less HCV RNA at baseline ($P = 0.015$), higher hemoglobin concentrations ($P = 0.011$) and higher platelet counts ($P = 0.001$). The other parameters did not differ significantly between the two groups.

Multivariate logistic regression analysis was performed with five items (gender, age, HCV RNA load at baseline, platelet counts and hemoglobin concentration) and the *P*-values were calculated as 0.036, 0.206, 0.101, 0.009 and 0.959, respectively. Because the *P*-value of hemoglobin concentration was 0.959, this item was omitted. Then, four items (gender, age, HCV RNA load at baseline, platelet counts) were analyzed by receiver operating characteristic (ROC) analysis for categorization. The appropriate categories were as follows: gender (male, female), HCV RNA load at baseline (≥1000, <1000 KIU/mL), platelet counts ($\geq 15 \times 10^4$, $< 15 \times 10^4$ /mm^3) and age (≥60, <60 years old).

After categorization, the data were subjected to multivariate logistic regression analysis to investigate the association of SVR with clinical background. As shown in Table 2, the *P*-values were 0.004 and 0.002 for gender and HCV RNA load at baseline, 0.110 and 0.175 for platelet counts and age. Because the *P*-value of HCV

Table 2 Multivariate logistic regression analysis of categorized clinical background, based on SVR and non-SVR, in the 230 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group A). Based on this result, a simple formula (formula A) was constructed

	Odds ratio	(95% CI)	*P* value
Gender (female/male)	2.277	(1.288–4.025)	0.004
HCV RNA			
(1000 KIU/mL ≥/<)	2.579	(1.417–4.693)	0.002
PLT (15 × 104/mL ≥/<)	1.624	(0.895–2.944)	0.110
Age (60 years old ≥/<)	1.510	(0.831–2.743)	0.175

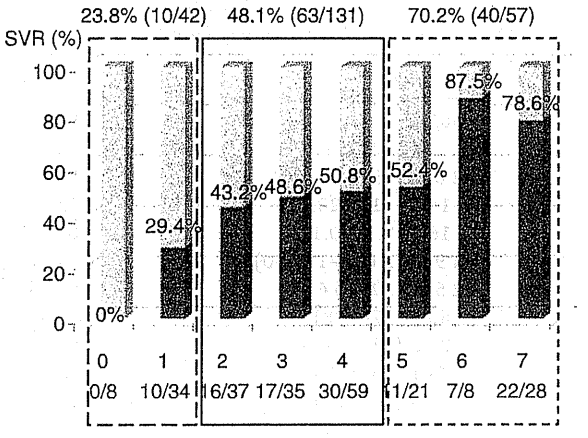

Figure 1 Scoring data according to formula A and the SVR rate in the 230 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group A). Patients were classified into a poorly responsive group (score 0 to 1), a moderately responsive group (score 2 to 4) and a moderately to highly responsive group (score 5 to 7).

RNA load at baseline was 0.002 with the highest Odds ratio (2.579), we set point 3 to HCV RNA load <1000 KIU. Similarly, because the *P*-value of gender was 0.004 with higher Odds ratio (2.277), we set point 2 to male gender. The *P*-values of platelet counts and age were not statistically significant. However, because the Odds ratios of these two items were relatively high (1.624 and 1.510), we set point 1 to platelet counts $\geq 15 \times 10^4$ /mm^3 and age <60. Based on these data, a simple formula was constructed: male gender (point 2) + HCV RNA load <1000 KIU (point 3) + platelet counts $\geq 15 \times 10^4$ /mm^3 (point 1) + age <60 (point 1). This formula was referred to as formula A.

For easy use of formula A in clinical practice, patients in group A could be classified into three groups depending on their response to therapy, that is, poorly responsive (point 0 to 1), moderately responsive (point 2 to 4) and moderately to highly responsive (point 5 to 7) groups (Fig. 1). The SVR rate in the poorly responsive group was 23.8% (10/42), that in moderately responsive group was 48.1% (63/131) and that in moderately to highly responsive group was 70.2% (40/57). To determine the efficacy of formula A, we applied it to group B (Fig. 2). The poorly responsive group (point 0 to 1) showed an SVR rate of 7.1% (1/14), the moderately responsive group (point 2 to 4) 38.6% (22/57) and the moderately to highly responsive group (point 5 to 7) 70.3% (26/37).

Impact of information on amino acid sequences in the ISDR and HCV core on the accuracy of formula A

Because amino acid mutations in the ISDR and substitutions in core region of HCV affect the responsiveness to Peg-IFN/RBV combination therapy,[8–10] we constructed another formula by adding this information, but without liver histology. Because patients with ≥2 amino acid mutations in the ISDR and HCV core amino acid 70 wild type have higher probability to attain SVR,[8–10] we performed multivariate logistic regression analysis with six items (gender, HCV RNA load at baseline, platelet counts, age, amino acid substitutions in ISDR and HCV core amino acid 70) and the *P*-values were calculated to be 0.009, 0.008, 0.143, 0.204, 0.051 and 0.023, respectively (Table 3).

Because the *P*-values of gender and HCV RNA load at baseline were 0.009 and 0.008 with high Odds ratios (3.357 and 3.471), we set point 3 to male gender and HCV RNA load at baseline <1000 KIU. Similarly, because the *P*-values of ISDR mutation and Core 70 mutant/wild type were 0.051 and 0.023 with relatively high Odds ratios (2976 and 3.139), we set point 2 to ≥2 amino acid substitutions in ISDR and HCV core amino acid 70 wild type. The *P*-values of platelet counts and age were not statistically significant. However, because the Odds ratios of these two items were relatively high (2.021 and

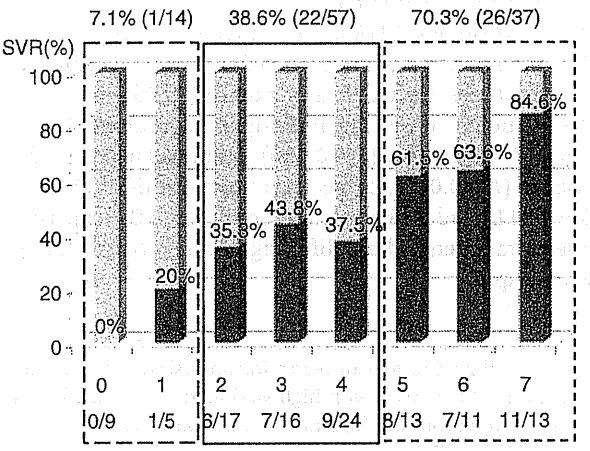

Figure 2 Scoring data according to formula A and the SVR rate in the 108 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group B). Score 0 to 1 represents a poorly responsive group, score 2 to 4 a moderately responsive group and score 5 to 7 a moderately to highly responsive group, which is similar to the data presented in Figure 1.

Table 3 Multivariate logistic regression analysis, based on SVR and non-SVR in the 108 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group B). Based on this result, formula B was constructed

	Odds ratio	(95% CI)	*P* value
Gender (female/male)	3.357	(1.346–8.375)	0.009
HCV RNA			
(1000 KIU/mL ≥/<)	3.471	(1.390–8.666)	0.008
PLT ($15 \times 10^4/\mu L$ ≥/<)	2.021	(0.895–2.944)	0.143
Age (60 years old ≥/<)	1.929	(0.700–5.316)	0.204
ISDR mutation (0.1/≥2)	2.976	(0.995–8.904)	0.051
Core 70 mutant/wild type	3.139	(1.172–8.406)	0.023

1.929), we set point 1 to platelet counts $\geq 15 \times 10^4$ /mm^3 and age <60. Based on these data, formula B was constructed: male gender (point 3) + HCV RNA load at baseline <1000 KIU (point 3) + platelet counts $\geq 15 \times 10^4$ /mm^3 (point 1) + age <60 (point 1) + ≥2 amino acid substitutions in ISDR (point 2) + HCV core amino acid 70 wild type (point 2). In group B, a total score of 0 to 3 could be categorized as the poorly responsive group (SVR ratio: 4.8% [1/21]), that of 4 to 7 the moderately responsive group (SVR ratio: 43.6% [27/62]) and that of 8 to 12 the moderately to highly responsive group (SVR ratio: 84% [21/25]) (Fig. 3).

DISCUSSION

IN THIS STUDY, we constructed a formula to predict the efficacy of Peg-IFN/RBV combination therapy:

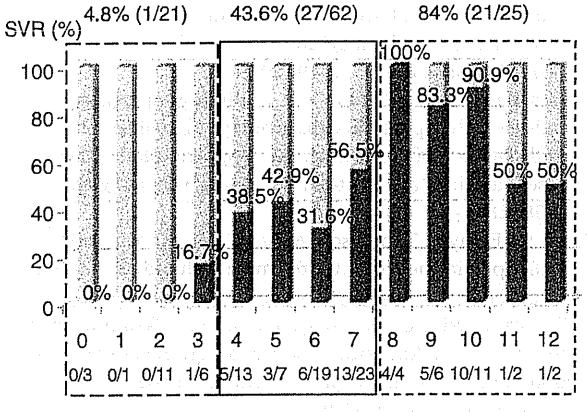

Figure 3 Scoring data according to formula B and the SVR rate in the 108 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group B). Score 0 to 3 represents a poorly responsive group, score 4 to 7 a moderately responsive group and score 8 to 12 a highly responsive group.

male gender (point 2) + HCV RNA load at baseline <1000 KIU (point 3) + platelet counts $\geq 15 \times 10^4$ /mm^3 (point 1) + age <60 (1 point). This simple formula (formula A) could distinguish a poorly responsive group (score [0–1]), a moderately responsive group (score [2–4]) and a moderately to highly responsive group (score [5–7]) (Fig. 1). Thus, formula A may be used by general physicians easily to roughly guess the probability of response to Peg-IFN and RBV combination therapy at the patient's first visit. Another formula (formula B) was constructed by adding the information of amino acid substitutions in the HCV genome. Although examination of amino acid substitutions in the HCV genome is not covered by the public health insurance in Japan, formula B distinguished a poorly responsive group (score [0–3]), a moderately responsive group (score [4–7]) and a highly responsive group (score [8–12]) (Fig. 3).

In Peg-IFN and RBV combination therapy for CH-C with a high viral load, the interval between the start of therapy and disappearance of HCV RNA from the serum is widely accepted as the most reliable marker to predict outcome,[12] and response-guided therapy is recommended. According to nationwide registration trials in Japan, in patients with a rapid virological response (RVR), demonstrating disappearance of HCV RNA within the first four weeks, the SVR rate was expected to be 76% to 100%, and in patients with an early virological response (EVR), showing the disappearance of HCV RNA in the first 5 to 12 weeks, the SVR rate was expected to be 71% to 73%.[13,14] In contrast, in patients with a late virological response (LVR), demonstrating clearance of HCV RNA between weeks 13 to 24, the expected SVR rate was as low as 29 to 36%. However, in clinical practice, most patients are happy to know the probability of SVR at the first or second visit, or at least before starting therapy. In this regard, formula A we advocate may be useful for a wide range of physicians.

According to formula B which included the substitutions of amino acids in the ISDR and HCV core, the predicted SVR rate also was classified into three groups, and with increased accuracy (Fig. 3). Recently, a strong association between interleukin 28B (IL28B) gene polymorphism and the response to PEG-IFN and RBV combination therapy was reported for CH-C patients.[15–17] Because determination of IL28B gene polymorphism as well as the amino acid sequences of the ISDR or HCV core is not covered by the public health insurance in Japan, it is difficult to advocate a formula containing these factors for a wide range of Japanese general physicians.

In patients with CH-C, liver biopsy is recommended to determine the treatment.[12] Because liver biopsy is not required for IFN-based antiviral therapy in Japanese public health insurance, a proportion of the patients refuse liver biopsy but are willing to be treated by Peg-IFN and RBV combination therapy. In this regard, formula A is useful in providing information concerning the likely efficacy of treatment at the first or second visit.

We constructed a simple formula to predict the outcome of treatment of genotype 1 CH-C with high viral load with Peg-IFN and RBV for 48 weeks. Recently, response-guided therapy recommended prolonged therapy up to 72 weeks for patients with LVR.[18–21] A larger study is required to establish a better formula to be utilized readily by the general physicians.

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HEPATOLOGY

Impact of amino acid substitutions in hepatitis C virus genotype 1b core region on liver steatosis and glucose tolerance in non-cirrhotic patients without overt diabetes

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glucose tolerance, hepatitis C virus, insulin resistance, steatosis.

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Abstract

Background and Aim: The hepatitis C virus (HCV) core protein induces hepatic steatosis and glucose intolerance in transgenic mice. The aim of this study was to clarify the impact of mutations in the HCV core region on hepatic steatosis and glucose tolerance in patients with chronic hepatitis C.

Methods: Seventy-four Japanese patients (27 men, 47 women; mean age, 61.9 years) infected with HCV 1b with high viral load (>5 log IU/ml), without cirrhosis and overt diabetes, were enrolled. Substitutions in amino acids 70 and 91 of the HCV genotype 1b core region, the percentage of hepatic steatosis by liver histology, and glucose tolerance evaluated by the oral glucose tolerance test were investigated in all patients.

Results: Steatosis was observed in 40 patients (54%). Transaminase activities, γ-glutamyl-transpeptidase, serum ferritin levels, homeostasis model assessment of insulin resistance index, and substitutions of amino acid 70 were significantly associated with the presence of steatosis, upon univariate analysis. Glucose intolerance was more prevalent in patients with steatosis (63%) than in those without steatosis (32%, $P = 0.012$). Multivariate analysis showed that substitution of amino acid 70 (odds ratio: 4.924; 95% confidence interval: 1.442–16.815; $P = 0.014$) and glucose intolerance (odds ratio: 3.369; 95% confidence interval: 1.076–10.544; $P = 0.040$) were independent factors related to liver steatosis. Levels of plasma glucose and serum insulin after glucose load were similar between patients with and without substitutions of amino acids 70 and 91.

Conclusions: Amino acid substitutions in the HCV genotype 1b core region are associated with hepatic steatosis in patients with chronic hepatitis C, independent of glucose intolerance.

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease, with approximately 170 million people infected worldwide. The severity of liver disease ranges from mild fibrosis to cirrhosis and hepatocellular carcinoma. Hepatic steatosis is a frequent histological finding in chronic hepatitis C (CHC).[1] Viral factors such as HCV genotype 3 and core protein contribute to the development of hepatic steatosis.[2,3] Several host factors, such as metabolic syndrome, obesity and a high body mass index (BMI) have an influence on liver steatosis in CHC patients.[4] We have previously reported that BMI and serum ferritin levels were also independent predictors of hepatis steatosis in 184 Japanese patients with CHC.[5] The prevalence of type 2 diabetes mellitus or glucose intolerance in liver diseases associated with chronic HCV infection is higher than in other liver diseases.[6–8] Experimental and clinical studies support a role of HCV infection in the development of insulin resistance (IR).[9–12] Patients with mild chronic hepatitis have a higher homeostasis model of assessment insulin resistance index (HOMA-IR) than healthy controls matched for age and BMI.[13] The mechanisms of development of IR in patients with chronic HCV infection are not well understood. Previous studies have shown that hepatic steatosis and IR[14–18] might be predictors of poor virological response to pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy.

Amino acid substitutions at position 70 and/or 91 in the HCV core region of genotype 1b (HCV-1b core region) are predictors of poor virological response to 48 weeks combination therapy with PEG-IFN plus RBV,[19–21] and are also risk factors for severe IR.[22] HOMA-IR has been used widely as an indicator of IR in most studies of the correlations between CHC and IR. The 75-g oral glucose tolerance test (OGTT), which is frequently used to assess

glucose tolerance, identifies patients at the early stage of glucose intolerance, such as impaired glucose tolerance (IGT).

We speculated that these mutations in the HCV core region might be related to hepatic steatosis or glucose tolerance in CHC patients. To investigate this hypothesis, we analyzed the impact of amino acid substitutions in the HCV core region on histological hepatic steatosis and glucose tolerance evaluated using the 75-g OGTT.

Methods

Patient enrollment and entry criteria

Between January 2009 and April 2010, 135 consecutive CHC patients with HCV genotype 1b underwent liver biopsy in our liver unit. All were positive for serum HCV antibody and HCV RNA. Among these, 74 patients were selected according to the following criteria: (i) HCV RNA levels of 5.0 log IU/mL or more determined using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan); (ii) no personal history of diabetes or fasting plasma glucose (FPG) < 126 mg/dL; (iii) absence of serum hepatitis B surface antigen; (iv) absence of cirrhosis and hepatocellular carcinoma, based on biopsy examination, laboratory tests, and imaging studies at baseline; and (v) absence of co-existing chronic liver diseases, such as autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α1 antitrypsin deficiency. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the human research committee of our institution. Some patients were treated with hepatoprotective drugs, but no drugs that might influence plasma glucose (PG) or serum insulin (SI) were prescribed.

Laboratory determinations

Serum and plasma were obtained from venous blood in the fasting state before breakfast. Laboratory evaluation of all patients included a blood cell count and determinations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), cholinesterase, cholesterol, triglyceride, high-density lipoprotein–cholesterol (HDL-C) and serum ferritin levels. These parameters were measured by standard techniques used in clinical chemistry laboratories.

For the OGTT, patients ingested a solution that contained 75-g glucose and venous blood samples were collected at 0, 30, 60 and 120 min for the measurement of PG and SI concentrations. PG levels were determined by a glucokinase method and serum immunoreactive insulin (IRI) levels were measured using a chemiluminescent enzyme immunoassay kit (Abbott Japan, Tokyo, Japan). Glucose tolerance was evaluated according to the criteria of the World Health Organization[23]: normal glucose tolerance (NGT) (FPG level < 110 and 2-h PG level < 140 mg/dL); impaired fasting glycemia (IFG) (110 ≤ FPG level < 126 and 2-h PG level < 140 mg/dL); IGT (FPG level < 126 and 140 ≤ 2-h PG level < 200 mg/dL); diabetes mellitus (FPG level ≥ 126 or 2-h PG level ≥ 200 mg/dL). Although this study included patients who met the criteria for diabetes mellitus, their FPG levels were < 126 mg/dL. The HOMA-IR was calculated on the basis of fasting values of PG and SI according to the HOMA model formula: HOMA-IR = fasting SI (FSI) (μU/ml) × FPG (mg/dl)/405. β-Cell function (HOMA-β) = FSI (μU/ml) × 360/(FPG (mg/dl)—63). The insulinogenic index (II), a marker of early-phase insulin secretion, was calculated as (SI 30—FSI) / (PG 30—FPG).[24] The composite insulin sensitivity index (ISI composite)[25] was calculated as $10\,000/(\text{FPG} \times \text{FSI} \times \text{mean PG } 0\text{–}120 \times \text{mean SI } 0\text{–}120)^{0.5}$. The PG response and total insulin secretion were estimated by the area under the curve of PG (PG–area under the curve [AUC]) and SI (SI-AUC), calculated from the four points of the OGTT using the trapezoid rule. BMI was calculated as weight (kg) divided by height (m) squared.

Nucleotide sequencing of core and NS5A mutation

The nucleotide sequences that encode aa 1–191 (HCV core) were analyzed by direct sequencing as described by Akuta *et al.*[19,26] RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows. Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers, and the second-round PCR with CC9 (sense) and e14 (antisense) primers.[19,26] The nucleotide sequences that encode aa 2209–2248 (interferon sensitivity determining region [ISDR]) were analyzed by direct sequencing as described by Enomoto *et al.*[27] Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers, and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers. These sequences were compared with the consensus sequence of genotype 1b (HCV-J).[28] Wild-type virus encoded Arg and Leu at aa 70 and 91, respectively, and the amino acid substitutions were Gln or His at aa 70 and Met at aa 91.

Histological evaluation

The liver specimens were embedded in paraffin and stained with hematoxylin–eosin, Masson–trichrome, and reticulin silver stain. The histological findings were interpreted and scored according to the new Inuyama classification[29] by a histopathologist (S.I.) who was blind to the laboratory data. The new Inuyama classification, which uses simpler criteria for diagnosing chronic hepatitis than the histological activity index scoring system proposed by Knodell *et al.*[30] has been used by many Japanese hepatologists. The activity of hepatitis (grading) was defined as follows: A0, no necroinflammatory reaction; A1, mild necroinflammatory reaction; A2, moderate necroinflammatory reaction; and A3, severe necroinflammatory reaction. The severity of hepatic fibrosis (staging) was defined as follows: F0, no fibrosis; F1, fibrous portal expansion; F2, fibrous bridging fibrosis (portal–portal or portal–central linkage); F3, bridging fibrosis with lobular distortion (disorganization); and F4, cirrhosis. Subjects were considered to have steatosis in the presence of fat droplets in ≥5% of hepatocytes. The degree of hepatic steatosis was graded as follows: 1, 5–33% of hepatocytes affected; 2, 33–66% of hepatocytes affected; and 3, >66% of hepatocytes affected.[31]

Table 1 Clinical characteristics of patients according to prevalence of steatosis

Parameters	Steatosis absent ($n = 34$)	Steatosis present ($n = 40$)	*P*-value
Sex (female)	23 (68%)	24 (60%)	0.6289
Age (years)	61.4 (6.4)	62.3 (8.0)	0.6369
BMI (kg/m^2)	22.9 (2.8)	23.8 (2.5)	0.1430
obesity (BMI > 25 kg/m^2)	8 (24%)	15 (38%)	0.2184
Hemoglobin (g/dl)	13.5 (1.2)	14.0 (1.6)	0.1230
Platelet (10^4/μL)	16.2 (4.6)	16.1 (5.5)	0.9574
AST (IU/l)	44.7 (24.1)	69.3 (41.3)	0.0037
ALT (IU/l)	43.0 (20.6)	59.7 (40.2)	0.0348
GGT (IU/l)	34.9 (22.5)	69.5 (73.9)	0.0119
Cholinesterase (IU/l)	273.2 (63.7)	293.7 (103.5)	0.3403
Cholesterol (mg/dl)	178.6 (29.0)	176.1 (38.7)	0.7682
Triglyceride (mg/dl)	91.7 (27.5)	120.2 (83.0)	0.0644
HDL-C (mg/dl)	60.2 (18.5)	54.8 (13.8)	0.1777
Ferritin (ng/mL)	100.6 (82.3)	194.4 (153.4)	0.0033
FPG (mg/dl)	82.6 (12.0)	90.0 (15.2)	0.0245
IRI (μU/ml)	6.09 (2.94)	9.36 (6.34)	0.0078
HOMA-IR	1.27 (0.71)	2.14 (1.58)	0.0038
Amino acid mutation of ISDR			
0–1/2≦	26/8	30/10	1.000
Substitutions of core aa			
Arg70/Gln70(His70)	27/7	21/19	0.0270
Leu91/Met91	24/10	30/10	0.7941
Activity (A1/A2/A3)	13/17/4	15/15/10	0.2397
Fibrosis (F0/F1/F2/F3)	1/17/11/5	2/12/9/17	0.0178
Glucose tolerance			
NGT/IGT (IFG)/DM	23/8/3	15/13/12	0.0052

P-values were calculated by the *t*-test or χ^2 analysis.
Results are presented as numbers with percentages in parenthesis for qualitative data or as means with standard deviation in parenthesis for quantitative data.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus; FPG, fasting plasma glucose; GGT, gamma-glutamyl-transpeptidase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; IRI, immunoreactive insulin; ISDR, interferon sensitivity determining region; NGT, normal glucose tolerance.

Statistical analysis

Results are presented as the means and standard deviation (SD) for quantitative data or as numbers with percentages in parentheses for qualitative data. Statistical differences in quantitative data were determined using the *t*-test (Tables 1,3). Fisher's exact probability test or χ^2 analysis was used for qualitative data (Tables 1,3). Multivariate analysis was performed by logistic regression analysis to identify variables independently associated with the presence of steatosis (Table 2). Differences were considered statistically significant at all *P*-values < 0.05.

Results

Hepatic steatosis was detected in 54% (40/74) of patients: 36 had grade 1, four had grade 2, and none had grade 3 steatosis. Among all patients, Gln70 (His70) was found in 35% (26/74) and Met91 was in 50% (20/40). Based on the results of the 75-g OGTT, 38 (51%) had NGT, 21 (28%) had IGT/IFG, and 15 (20%) had diabetes mellitus. Comparisons of clinical characteristics according to the presence of steatosis are shown in Table 1. There were significant positive associations between the presence of steatosis and AST, ALT, GGT and serum ferritin concentrations, HOMA-IR and substitutions of aa 70. Patients with steatosis had more advanced stages of fibrosis than those without steatosis. Severe fibrosis (F3) was more frequently found in patients with steatosis (43%, 17/40) than in those without (15%, 5/34). Glucose intolerance (IGT + IFG + DM) was more prevalent in patients with steatosis (63%, 25/40) than in those without (32%, 11/34, $P = 0.0115$). On multivariate logistic regression analysis, factors associated with the presence of steatosis were substitutions of aa 70 and glucose intolerance (Table 2).

The prevalence of steatosis was shown according to the substitutions of aa 70 and glucose intolerance (Fig. 1). The prevalence of steatosis in patients with Arg70 and NGT is lower (25%) than in those with Gln70 (His70) and NGT (57%, $P = 0.037$), with Arg70 and glucose intolerance (63%, $P = 0.019$), and with Gln70 (His70) and glucose intolerance (83%, $P = 0.001$).

According to analysis of glucose tolerance with the 75-g OGTT, FPG, FSI, PG and SI levels at 30, 60 and 120 min after glucose loading did not differ between the presence and absence of core aa 70 or 90 substitutions (Fig. 2). HOMA-IR, HOMA-β, II, ISI composite, PG-AUC and SI-AUC did not differ between the two groups (Table 3).

Table 2 Associations with steatosis found by multivariate logistic regression ($n = 74$)

Characteristics	Category	Odds ratio (95%CI)	*P*-value
Core amino acid substitution	1: Arg70	1	
	2: Gln70(His70)	4.924 (1.442–16.815)	0.011
Glucose tolerance	1: NGT	1	
	2: glucose intolerance (IGT+IFG+DM)	3.369 (1.076–10.544)	0.037
HOMA-IR	1: <2.0	1	
	2: ≧2.0	3.888 (0.820–18.434)	0.087
AST	1: <50 IU/L	1	
	2: ≧50 IU/L	1.895 (0.373–9.628)	0.441
ALT	1: <60 IU/L	1	
	2: ≧60 IU/L	1.507 (0.275–8.262)	0.637
GGT	1: <55 IU/L	1	
	2: ≧55 IU/L	0.898 (0.214–3.764)	0.883

CI, confidence interval; DM, diabetes mellitus; HOMA-IR, homeostasis model assessment for insulin resistance; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; NGT, normal glucose toerance.

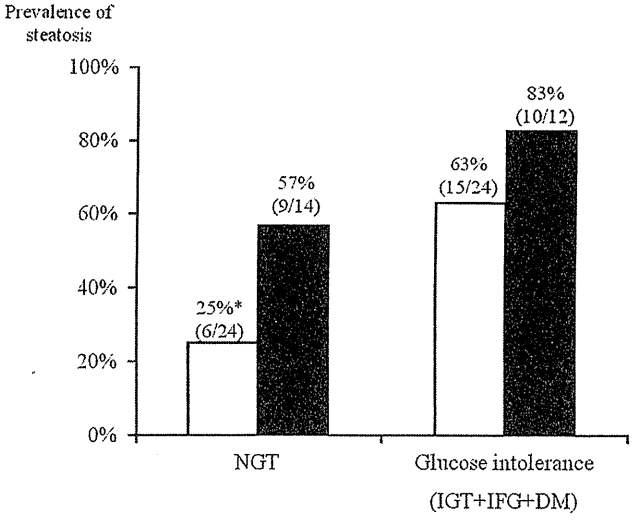

Figure 1 Proportion of patients with steatosis and Gln70 (His70), and glucose tolerance. *$P = 0.037$ versus Gln70 (his70) with normal glucose tolerance (NGT), $P = 0.019$ versus Arg70 with glucose intolerance, and $P = 0.001$ versus Gln70 (his70) with glucose intolerance. DM, diabetes mellitus; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; NGT, normal glucose tolerance. □, Arg70; ■, Gln70(His70).

Discussion

Steatosis was observed in 40 of 74 patients (54%). This result is similar to those of previous studies that have reported rates of between 31% and 72%.[5,32,33] Consistent with previous studies, the grade of steatosis was mild in most cases. Steatosis has been viewed as a characteristic feature of chronic HCV-infected liver,[1] but whether the steatosis is directly related to the presence of HCV or results from a host-related factor remains uncertain. HCV core protein has been demonstrated to inhibit microsomal transfer protein activity and very low-density lipoprotein secretion,[34] and to upregulate the promoter activity of sterol-regulatory element-binding protein 1c, a transcription factor involved in lipid synthesis.[35] Yasui *et al.* have reported that levels of messenger RNA and protein of peroxisome proliferator-activated receptor α, which regulates β-oxidation of fatty acid, were lower in patients with steatosis than in those without.[32] Several investigators have reported a higher prevalence of steatosis among patients infected with HCV genotype 3 compared with genotype 1,[2] but all patients in the present study had HCV genotype 1. As we have previously reported, multivariate analysis clearly shows that BMI and serum ferritin level are related to steatosis in HCV-infected Japanese patients with HCV genotype 1 or 2,[5] although we did not evaluate core amino acid substitutions or glucose intolerance. Several lines of evidence suggest that host factors are important for the development of hepatic steatosis in patients with non-3 genotype HCV. In the present study, substitution of HCV-1b core region 70 was shown to be an independent predictor in the development of steatosis in CHC patients with HCV genotype 1. However, the mechanisms of substitutions of the core aa70 that lead to hepatic steatosis remain unclear. Sequence analysis to identify the mutations that are associated with steatosis in patients with HCV genotype 3 has been performed, and it has been reported that aa 164, 182 and 186 of the HCV genotype 3 core region might have important roles in lipid metabolism.[36–38] Consistent with our results, Tachi *et al.* have reported that substitution of HCV-1b core region 70 and triglyceride levels are independently associated with the presence of steatosis in the liver.[39] That study examined correlations between hepatic/urinary 8-hydroxydeoxyguanine (8-OHdG) levels, an indicator of oxidative stress, and amino acid substitutions at positions 70, 75 and 91 in the HCV core region. Hepatic/urinary 8-OHdG levels were significantly lower in patients with Leu90 than in those with Met91, but patients with Arg70 had similar levels of hepatic/urinary 8-OHdG to those with Gln70 (His70). It is unknown whether oxidative stress is responsible for hepatic steatosis in patients with Gln70 (His70). In contrast with these studies including our own, Fukuhara *et al.*[40] have reported that the substitution rates of aa70 and aa91 are not associated with steatosis. Although the mechanisms that underlie these controversial results remain unknown, 33 of 69 patients enrolled in their study had stage 4 fibrosis. The reason why diabetic or cirrhotic patients were excluded from our study was that we wanted to determine solely the influencing factor on steatosis in CHC patients.

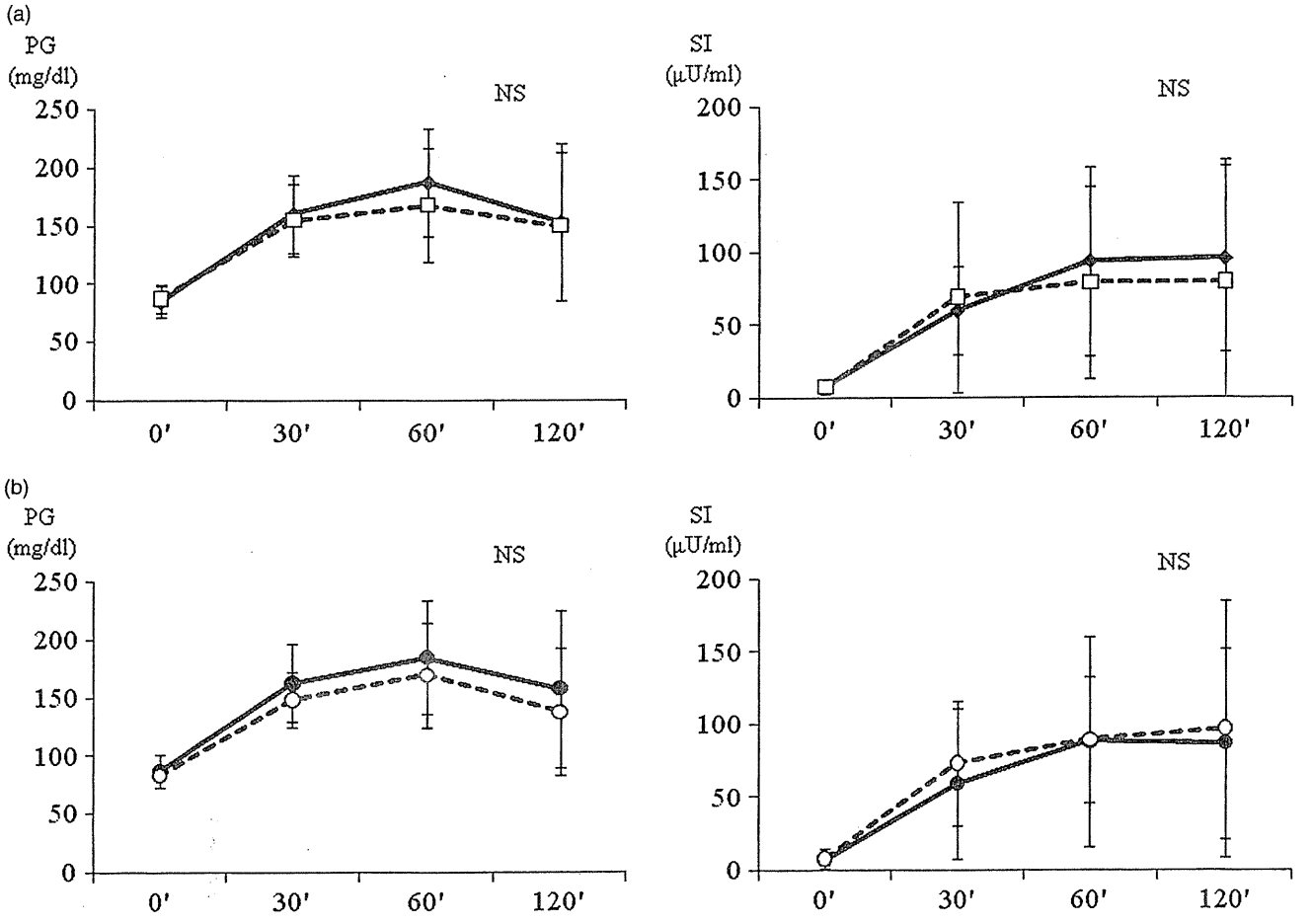

Figure 2 Results of plasma glucose (PG) and serum insulin (SI) with 75-g oral glucose tolerance test (OGTT) in patients with and without core amino acid substitutions. Data are expressed as means ± standard deviation. (a) Core aa 70; (b) core aa 91. IRI, immunoreactive insulin; NS, not significant. (a) -◆-, Arg70; -□-, Gln70 (His70); (b) -●-, Leu91; -○-, Met91.

Table 3 Several parameters determined by 75-g oral glucose tolerance test according to amino acid substitutions in core region

	Amino acid 70			Amino acid 91		
	Arg70 ($n = 48$)	Gln70(His70) ($n = 26$)	*P*-value	Leu91 ($n = 54$)	Met91 ($n = 20$)	*P*-value
HOMA-IR	1.74 (1.33)	1.62 (1.11)	0.7101	1.69 (1.17)	1.70 (1.47)	0.9734
HOMA-β	142 (82)	197 (366)	0.3350	166 (265)	152 (103)	0.8243
Insulinogenic index	0.76 (0.66)	0.87 (0.60)	0.4790	0.73 (0.66)	1.00 (0.51)	0.1003
PG-AUC (mg/dl·h)	318 (76)	300 (74)	0.3221	320 (79)	291 (61)	0.1348
SI-AUC (μU/ml·h)	150 (85)	135 (98)	0.4790	141 (95)	154 (81)	0.6036
ISI composite	5.27 (2.66)	6.37 (3.88)	0.1567	5.74 (3.44)	5.47 (2.36)	0.7434
Glucose tolerance						
NGT/IGT(IFG) /DM	24/14/10	14/7/5	0.7644	25/18/11	13/3/4	0.2740

Results are presented as numbers with percentages in parenthesis for qualitative data or as means with standard deviation in parenthesis for quantitative data.

DM, diabetes mellitus; HOMA, homeostasis model assessment; IGT, impaired glucose tolerance; IR, insulin resistance; ISI, insulin sensitivity index; NGT, normal glucose tolerance; PG-AUC, plasma glucose area under the curve; SI-AUC, serum insulin area under the curve.

In the present study, we did not detect any correlations between substitutions of HCV-1b core region and IR or glucose tolerance. Akuta *et al.* have reported that substitutions of HCV-1b core region are an important predictor of severe IR (HOMA-IR ≥ 3.5) in CHC patients without cirrhosis and overt diabetes.[22] There are some plausible reasons to explain this discrepancy. First, the number of our patients was too small to detect significant differences. Second, the characteristics of the patients were different