Hepatology Research

Hepatology Research 2011; 41: 126–132 doi: 10.1111/j.1872-034X.2010.00750.x

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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Received 8 September 2010; revision 24 September 2010; accepted 9 November 2010.

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

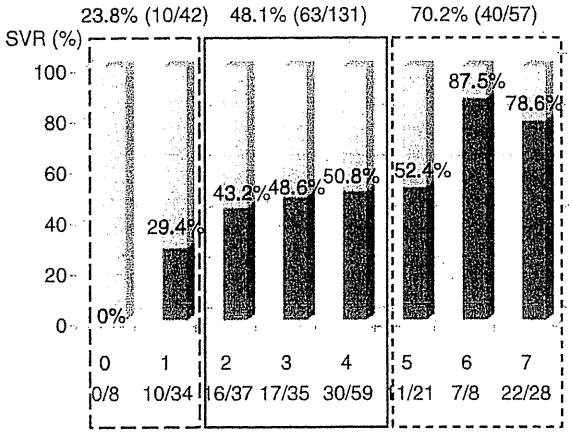

Figure 1 Scoring data according to formula A and the SVR rate in the 230 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group A). Patients were classified into a poorly responsive group (score 0 to 1), a moderately responsive group (score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

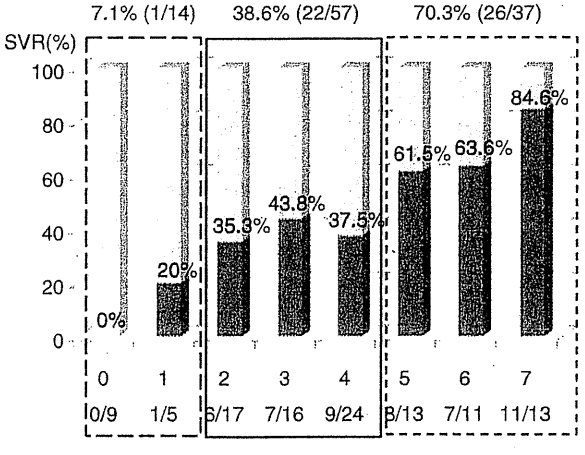

Figure 2 Scoring data according to formula A and the SVR rate in the 108 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group B). Score 0 to 1 represents a poorly responsive group, score 2 to 4 a moderately responsive group and score 5 to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

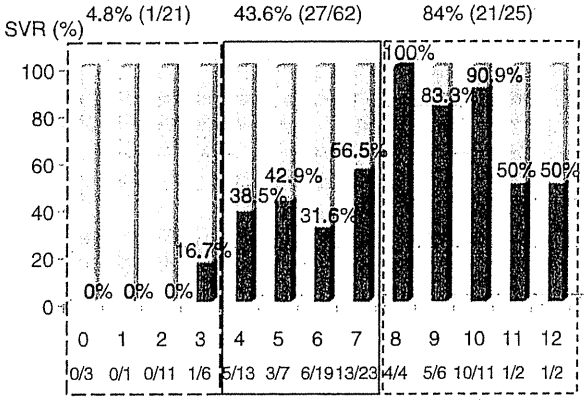

Figure 3 Scoring data according to formula B and the SVR rate in the 108 patients with chronic hepatitis C with high viral loads and treated with PEG-IFN and RBV combination therapy (Group B). Score 0 to 3 represents a poorly responsive group, score 4 to 7 a moderately responsive group and score 8 to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

ACKNOWLEDGEMENTS AND DISCLOSURE

THIS WORK WAS supported by a Grant-in-Aid from Ministry of Health Labour and Welfare of Japan.

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doi:10.1111/j.1440-1746.2010.06568.x

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

Accepted for publication 22 October 2010.

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Financial support: None.

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.

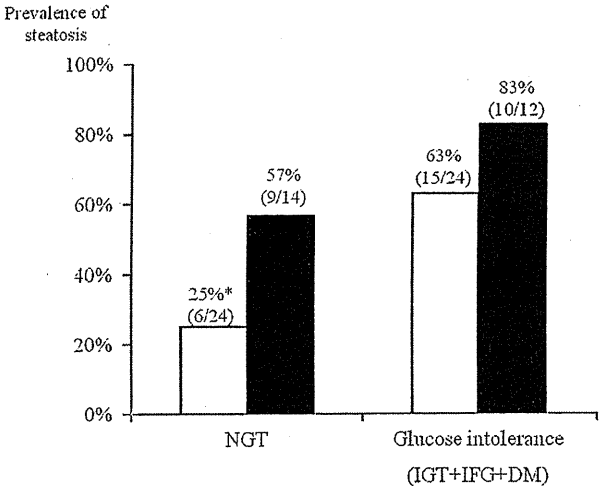

Figure 1 Proportion of patients with steatosis and Gln70 (His70), and glucose tolerance. *$P = 0.037$ versus Gln70 (his70) with normal glucose tolerance (NGT), $P = 0.019$ versus Arg70 with glucose intolerance, and $P = 0.001$ versus Gln70 (his70) with glucose intolerance. DM, diabetes mellitus; IFG,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

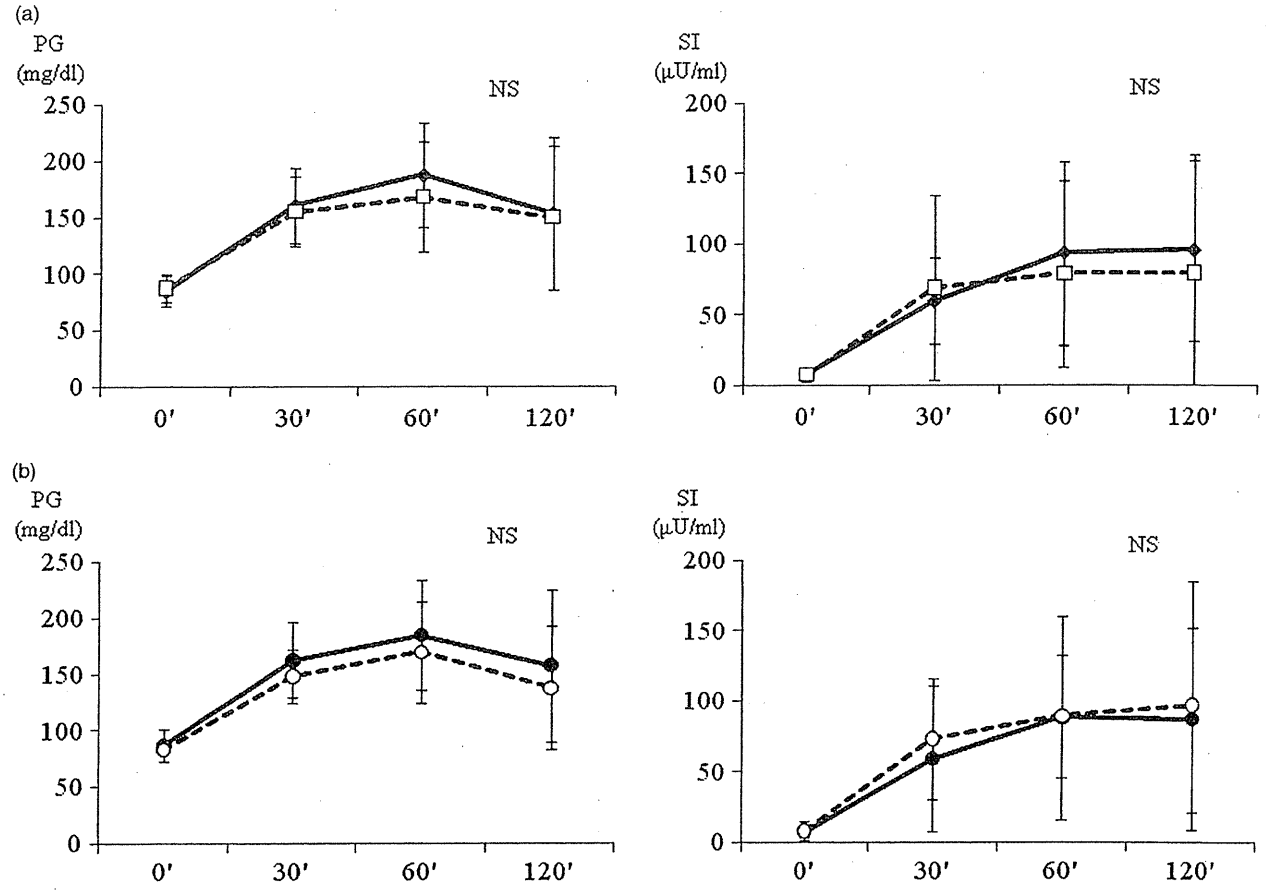

Figure 2 Results of plasma glucose (PG) and serum insulin (SI) with 75-g oral glucose tolerance test (OGTT) in patients with and without core amino acid substitutions. Data are expressed as means ± standard deviation. (a) Core aa 70; (b) core aa 91. IRI, immunoreactive insulin; NS, not significant. (a)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

between the two studies. The number of patients who had severe IR who were enrolled in our study (four of 74, 5.4%) was smaller than that in Akuta's study (13 of 34, 38.2%), although three of our four patients with severe IR (HOMA-IR $\geq$ 3.5) showed core aa 70 substitutions. Akuta *et al.* have also reported that substitutions of the HCV-1b core region are not an important predictor of IR (HOMA-IR $\geq$ 2.5). Third, the method to assess IR was different between the two studies. Most studies have reported on the correlations between HCV infection and hepatic IR using the HOMA-IR index, as in the study of Akuta *et al.* Recently, some researchers[25,41–43] have reported that some indices obtained from OGTT could be useful for the evaluation of systemic glucose metabolism. These indices include PG-AUC, SI-AUC and ISI composite.[25,41] In particular, the ISI composite is highly correlated with the rate of whole-body glucose disposal during euglycemic insulin clamping. As IR develops simultaneously in multiple organs and cells, including the liver, skeletal muscle and adipocytes, it is necessary to investigate the association between HCV infection and systemic glucose metabolism using these indices. Unexpectedly, in our study, evaluation of IR or glucose tolerance by carrying out the 75-g OGTT, PG-AUC, SI-AUC and ISI composite did not differ between patients with and without core amino acid substitutions (Fig. 2). To the best of our knowledge, our study is the first to examine the correlations between substitutions of core amino acids and glucose tolerance evaluated with the 75-g OGTT. Thus, further studies based on a large number of patients should be carried out in the future.

The other factor that was related to liver steatosis was glucose intolerance rather than IR. Controversial results concerning the correlations between IR and hepatic steatosis exist. Fartoux *et al.* have revealed an association of IR with hepatic steatosis in non-diabetic, non-cirrhotic patients with HCV genotype 1.[44] In contrast, according to Tachi *et al.*,[39] IR that was evaluated by HOMA-IR was not selected as a parameter to predict hepatic steatosis. In transgenic mice that express HCV core protein, Shintani *et al.* has shown that IR precedes the occurrence of steatosis, which suggests that IR is not a consequence but a cause of hepatic steatosis.[9] The mechanisms of development of IR in patients with chronic HCV infection are not well understood. It is plausible that HCV itself has a direct role in the development of IR, as recently reported by clinical and experimental studies.[9–11,13] HCV seems to lead to IR through interference of intracellular insulin signaling by HCV proteins, mainly the serine phosphorylation of insulin receptor-1 (IRS-1) and impairment of the downstream Akt signaling pathway. A high level of tumor necrosis factor α, which has been observed in CHC patients, is considered to act by disturbing tyrosine phosphorylation of IRS-1. Our results suggest that hyperglycemia has an important role in hepatic steatosis. Glucose activates the nuclear transcription factor carbohydrate response element-binding protein (ChREBP), by upregulating the conversion of glucose into pyruvate by increasing the expression of L-pyruvate, a glycolysis rate-limiting enzyme. ChREBP also increases transcription of the lipogenic enzyme acetyl CoA carboxylase and fatty acid synthase genes. High blood glucose levels further enhance hepatic lipogenesis by ChREBP activation, hence increasing the hepatic expression of all hepatic lipogenic genes.[45]

In conclusion, the results of the present study indicated that substitutions of HCV-1b core region besides glucose intolerance were an important predictor of steatosis in patients without cirrhosis and overt diabetes mellitus. The cause of steatosis in CHC with HCV genotype 1 can be split into viral factors (substitutions of core aa 70), and host factors (glucose tolerance). Substitution of core amino acids does not seem to influence glucose tolerance or IR.

Acknowledgments

This study was presented at the 61st annual meeting of the American Association for the Study of Liver Diseases (AASLD) on 31 October 2010.[46]

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Journal of Medical Virology 83:871–878 (2011)

Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

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Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology-Japan; Grant sponsor: Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan; Grant sponsor: Japan Health Sciences Foundation; Grant sponsor: Miyakawa Memorial Research Foundation; Grant sponsor: National Institute of Biomedical Innovation.

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Accepted 10 January 2011

DOI 10.1002/jmv.22038
Published online in Wiley Online Library (wileyonlinelibrary.com).

2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. ***J. Med. Virol. 83:871–878, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon-λ3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon-λ3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN-α2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μg/kg subcutaneously once a week) or PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μg subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w: 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the *IL28B* locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years)[a]	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m^2)[a] (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl)[b] (N = 94)	5,115 ± 1,630
Neutrophils (/μl)[b] (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl)[b] (N = 95)	14.2 ± 1.3
Platelet count (×10^{-3}/μl)[b] (N = 98)	187 ± 95
ALT (IU/L)[b] (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml))[a,c]	6.2 (3.6–7.4)
Treatment duration (>16, ≦24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.
[a]Data are shown as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log(IU/ml)).

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a ($P = 0.036$) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≧80% and <80% of RBV dose, $P = 0.021$, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, $P = 0.019$).

TABLE II. Response Rates to Therapy

Character			Number/total number (%)
Overall			
RVR			68/112 (61)
ETR			125/129 (97)
SVR			98/129 (76)
Genotype	2a	2b	*P*-value
RVR	46/67 (69)	22/45 (49)	**0.036**
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.
Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≧80%	<80%	*P*-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	**0.021**

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin.
The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy.
Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	*P*-value
Genotype (2a/2b)		56/42	21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	**0.024**
Age (years)[a]	56 (20–73)	61 (40–72)	**0.002**
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m^2)[a]	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/μl)[b]	5,318 ± 1,617	4,489 ± 1,540	**0.032**
Neutrophils (/μl)[b]	2,913 ± 1,139	2,278 ± 983	**0.021**
Hemoglobin (g/dl)[b]	14.2 ± 1.4	14.1 ± 1.1	NS
Platelet count (×10^{-3}/μl)[b]	193 ± 105	171 ± 54	NS
ALT (IU/ml)[b]	79 ± 73	94 ± 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml))[a,c]	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≧80%/<80%)	75/23	21/10	NS
RBV adherence (≧80%/<80%)	61/37	12/19	**0.024**
RVR/non-RVR	57/24	11/20	**0.001**

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.
[a]Data are show as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log (IU/ml)).
Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	*P*-value
Regression analysis			
RVR	RVR	1	**0.019**
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≧80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
Bold indicated *P*-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II), In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	*P*-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

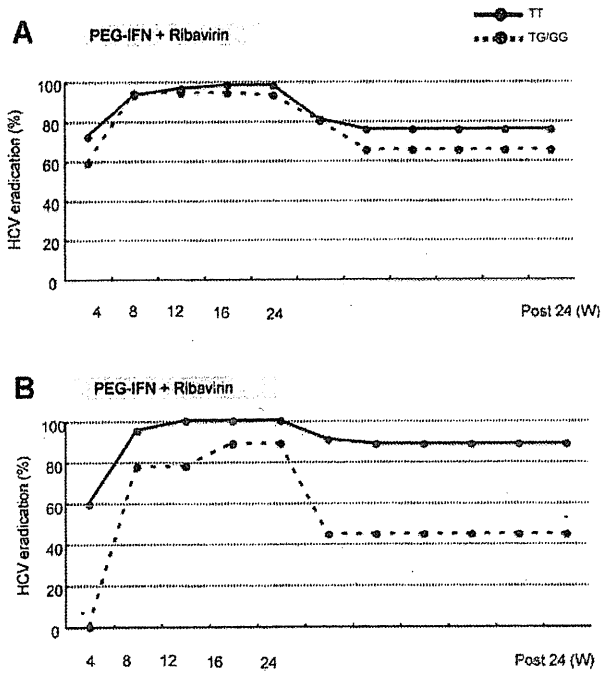

Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. $^{*}P < 0.01$.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment (n = 2), general fatigue (n = 2), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent in vitro study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate